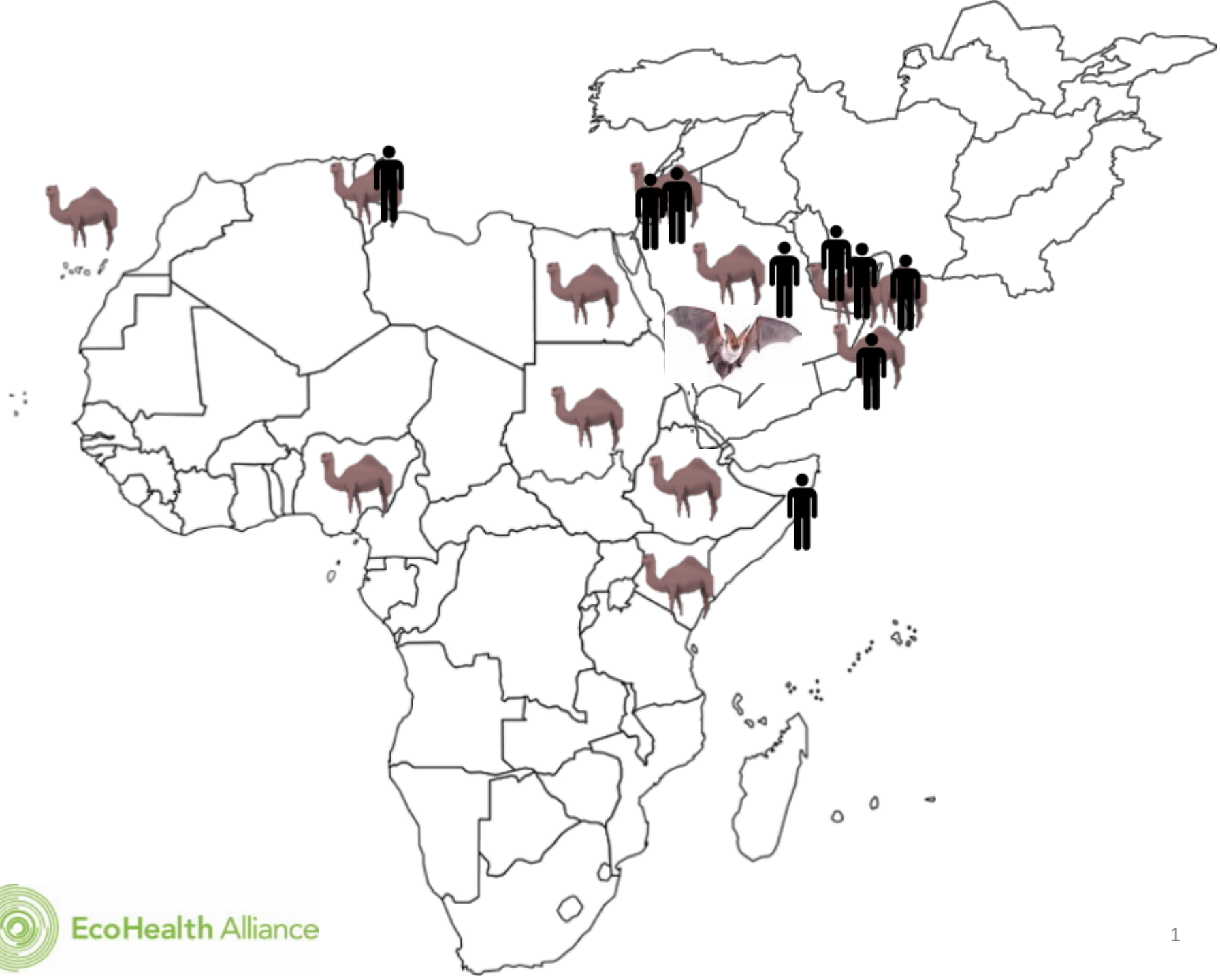
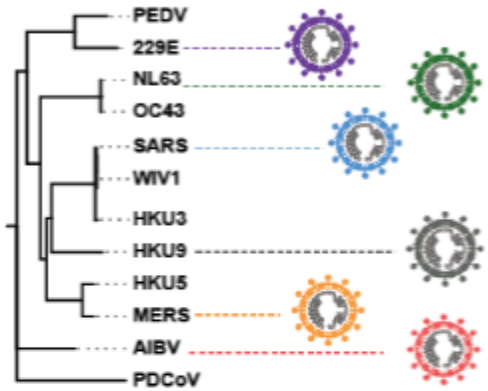


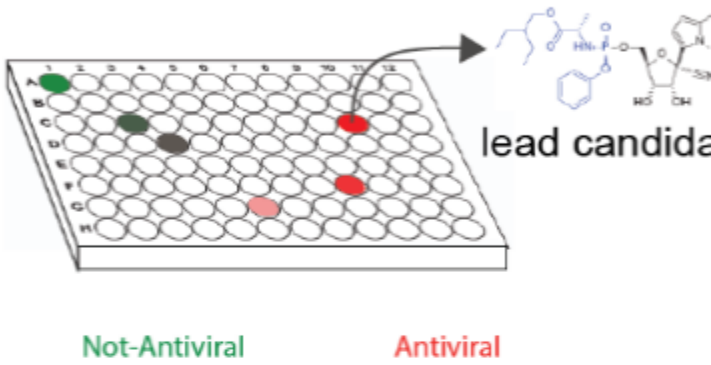
EcoHealth Alliance

Virus discovery in humans and nature

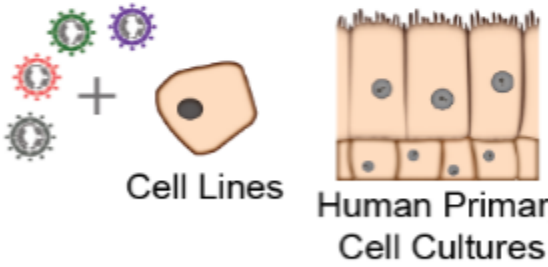
Synthetically reconstruct virus from metagenomic data

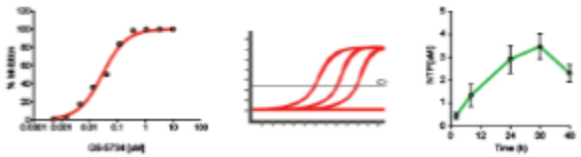


Compound Screening and Candidate Selection



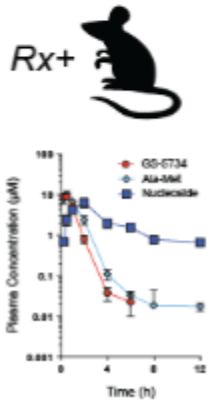
In vitro efficacy



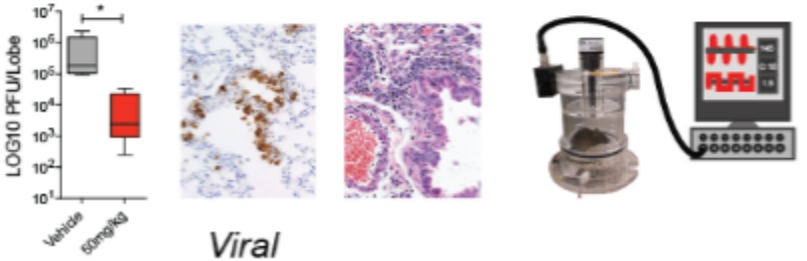


EC50 and toxicity | *Virus growth* | *Rx metabolism*

In vivo pharmacokinetics and pharmacodynamics



In vivo PK



Virus growth | *Viral Antigen Staining* | *Tissue Pathology* | *Physiological measures of diseas*

Sheahan T et al., Science Translational Medicine, 2017

U.S. FOOD & DRUG

ADMINISTRATION

FDA U.S. FOOD & DRUG
ADMINISTRATION

U.S. FOOD & DRUG

ADMINISTRATION

CHEMICAL REVIEWS

Review

pubs.acs.org/CR

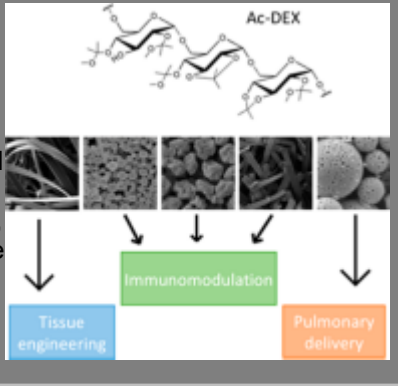
Acetalated Dextran: A Tunable and Acid-Labile Biopolymer with Facile Synthesis and a Range of Applications

Eric M. Bachelder*, Erica N. Pino, and Kristy M. Ainslie

Division of Pharmacoengineering and Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599, United States

* Supporting Information

Acetalated dextran (Ac-DEX) is a tunable acid-labile biopolymer with facile synthesis, aptly designed for the formulation of microparticles for vaccines and immunemodulation. Tunability of degradation is achieved based on the kinetics of reaction and the molecular weight of the parent dextran polymer. This tunability translated to differential rates of activation of CD8+ T cells in an in vitro ovalbumin model and illustrated that acid-labile polymer can activate CD8+ T cells at an increased rate compared to acid-insensitive polymers. In addition, Ac-DEX has been used to encapsulate small molecules, deliver nucleotides, transport inorganic molecules, formulate immune modulating therapies and vaccines, and trigger pH responsive constructs for therapy. Here we highlight the properties and results of Ac-DEX nano-/microparticles as well as the use of the polymer in other constructs and chemistries.



CONTENTS

1. Introduction 1915
2. Adjuvant and Vaccine Delivery 1917
2.1. Adjuvant Encapsulation 1917
2.2. Vaccine Formulations 1917
3. Delivery to Macrophages 1920
4. Controlled Release 1920
5. Devices 1922
6. Copolymer and Other Acetalated Polymers 1922
7. Conclusion 1923
Associated Content 1923
Supporting Information 1923
Author Information 1923
Corresponding Author 1923
ORCID 1923
Notes 1923
Biographies 1923
Acknowledgments 1924
References 1924

1. INTRODUCTION

Dextran is an FDA approved polysaccharide that has been used for decades as a plasma expander. The acetalation of dextran was first reported by Bachelder et al. wherein 2-methoxypropene was used to modify dextran, resulting in a biopolymer with dextran, methanol, and acetone as degradation products (Figure 1).[1] The acetalation of the dextran pendant hydroxyl group results in the polymer being hydrophobic, which allows the encapsulation of drugs through emulsion chemistry, similar to what is done with polyester polymers. This acetalized polymer (Ac-DEX) facilitated the encapsulation of hydrophobic and other drugs that would typically be difficult to deliver. Additionally, acetalated dextran (Ac-DEX) is an acid-sensitive polymer that degrades more rapidly at lower pH, such as in the endosome of phagocytic cells, tumors, or areas of inflammation, allowing for site-specific degradation of the polymer and subsequent release of the encapsulated therapeutic.

In the reaction of dextran with 2-methoxypropene, the kinetically favored acyclic acetals will initially form, followed by the thermodynamically stable cyclic acetals. Acyclic acetals degrade rapidly during hydrolysis and result in the generation of methanol and acetone. In contrast, cyclic acetals degrade more slowly through hydrolysis and result in the generation of acetone only. This differential acetal formation was shown to be dependent on the reaction time of the polymer and to result in varying degradation rates based on the ratio of cyclic to acyclic acetals. With the differential tunability of Ac-DEX came a marked difference in T cell activation using β-galactosidase reporter cells and the model antigen protein ovalbumin. Most notably, faster degrading materials at lower pHs resulted in the most significant major histocompatibility complex class I (MHC I) and MHC II presentation. When comparing fast and slow degrading Ac-DEX to other acid-sensitive (polyacrylamide), degradable (poly(lactic-*co*-glycolic acid) (PLGA)), and nondegradable (iron oxide) carriers,[2] fast degrading Ac-DEX displayed enhanced MHC I presentation and the best MHC II presentation over other carriers. CD8+ T cells are cytotoxic T cells that can kill infected cells indirectly by inducing apoptosis in the target cell, or can kill cells directly through the secretion of perforin. Therefore, CD8+ T cell

Received: August 9, 2016
Published: December 29, 2016

© 2016 American Chemical Society

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

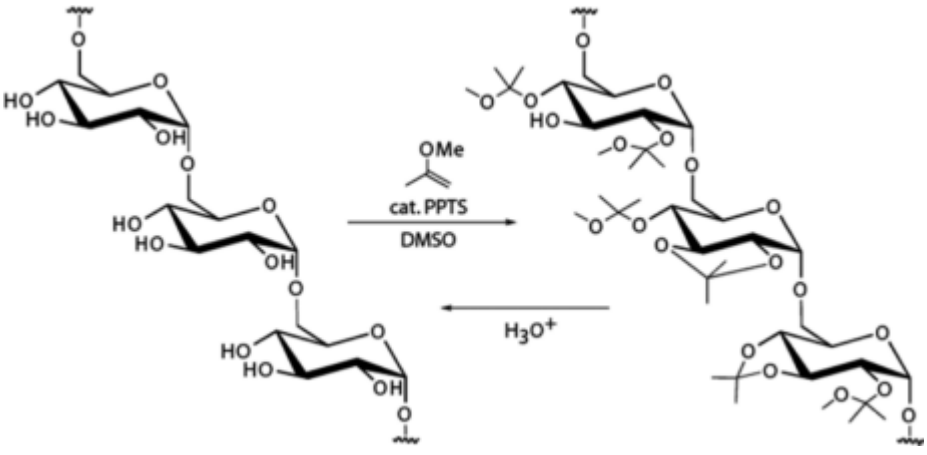


Figure 1. Reaction scheme for the formation of Ac-DEX with 2-methoxypropene. Reprinted with permission from ref 1. Copyright 2008 American Chemical Society.

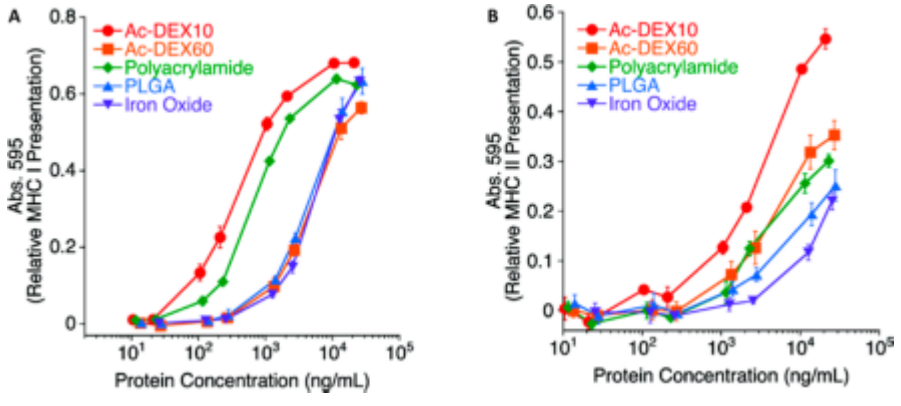


Figure 2. MHC I and MHC II class presentation of OVA with biopolymers of different degradation rates. Ac-DEX10 and Ac-DEX60 correspond to degradation half-lives of 1.7 and 16 h, respectively. Reprinted with permission from ref 2. Copyright 2009 National Academy of Sciences.

activation, via MHC I, is desirable to generate protective vaccine mediated immune responses against intracellular pathogens such as viruses, as well as some bacteria, parasites, and fungi. Moreover, MHC I presentation is required for the development of a therapeutic cancer vaccine. This tunability and enhanced MHC I class presentation illustrates Ac-DEX is a well-designed polymer for vaccine applications (Figure 2). Moreover, Ac-DEX particles displayed significantly greater CD4+ (MHC II) presentation in comparison to other biomaterials, exhibiting that Ac-DEX particles cross-presented to both CD4+ and CD8+ T cells, arming both the cellular and humoral responses that would be needed for many vaccines.[2]

Apart from enhanced activation of cellular immune responses, Ac-DEX has several other benefits when compared to other commonly used biopolymers. A primary benefit is its facile synthesis. Ac-DEX synthesis is a single vessel reaction with subsequent purification through precipitation.[3] Moreover, the degradation products of Ac-DEX are pH-neutral, unlike commonly used polyesters (e.g., poly beta amino esters (PBAEs), poly(lactic-co-glycolic) acid (PLGA), polycaprolactone (PCL)) and several polyanhydrides. This allows for minimal degradation of the payload due to low pH, without the addition of excipients.[4−6]

Other acid-sensitive polymers have been developed for drug delivery applications, including PBAEs, poly(ortho esters) (POEs), and polyketals. PBAEs, while associated with increased cellular internalization and transfection efficiencies over PLGA particles, have illustrated increased cytotoxicity, likely due to their highly positive zeta potentials from their cationic amines in the backbone.[7] In contrast to PBAEs, POEs are very biocompatible but have relatively slow degradation at pH 5.[8] Polyketals, another acid-sensitive polymer class, offer great polymer degradation control, but their difficult synthesis is less appealing than that of Ac-DEX.[9] In comparison to these polymers, Ac-DEX's inherent acid-sensitivity allows for tunable degradation profiles based on reaction time, thus allowing precise control over payload release. Since both sustained and burst release can be selectively achieved, Ac-DEX is an ideal carrier for a wide range of therapeutics. Ac-DEX is a unique biopolymer in that where other commonly used polymers degrade into acidic byproducts, all byproducts of Ac-DEX degradation are pH-neutral. This property gives Ac-DEX the following two advantages: first, a neutral pH should not adversely affect the encapsulated payloads; second, the environment surrounding Ac-DEX should not be harmed upon degradation as it could by the degradation of polymers with acidic byproducts, such as PLGA.

Ac-DEX stands alone as an easily made, tunable, and acid-sensitive polymer, which makes it understandable why it has been used in over 30 publications since 2008 and has been mentioned in five reviews.[10−14] Here we report its application to deliver a variety of compounds, including small molecules, proteins, peptides, nucleotides, and inorganic molecules. Moreover, the most common formulation of Ac-DEX, nano-/

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

microparticles, has been studied extensively in vitro and in vivo and has been delivered through parenteral and needle-free routes. In addition to the particulate constructs, Ac-DEX has been incorporated into a variety of copolymers and advanced designed delivery devices which rely on the polymer's acid sensitivity and tunable degradation rates (Supporting Information Table 1). The many publications that use Ac-DEX, as well as its broad range of applications, make it an emerging biomaterial for drug delivery.

2. ADJUVANT AND VACCINE DELIVERY

The original proposed application of Ac-DEX was the formation of nano-/microparticles as a subunit vaccine. Since subunit vaccines often rely on adjuvant activity for innate immune activation,[15] it goes hand-in-hand that adjuvant encapsulation in Ac-DEX would also be studied. If Ac-DEX microparticles (MPs) are of a certain size, they can passively target antigen presenting cells (APCs). Unlike most cell types in vivo, phagocytes (e.g., dendritic cells (DCs), macrophages) can internalize particles larger than 100 nm, thereby allowing uptake only by phagocytic cells if particles are greater than 100 nm.[16,17] Furthermore, passive targeting of professional APCs (DCs) is possible with further sizing. Studies have shown that MPs in the range of 1−2 μm are four times more likely to be phagocytosed by CD11c+/CD11b+ cells (DCs, professional APCs) than CD11c-/CD11b+ cells (e.g., macrophages). Also, in this same size range, when observing cells extracted from the local draining lymph nodes, approximately 50 times more CD11c+CD11b+ or CD11c+CD11b+ cells (DCs) phagocytosed the MPs, compared to CD11c-CD11b+ cells (macrophages). These results indicate that large particles (1−2 μm) are predominately transported by DCs from the injection site to the lymph nodes, whereas smaller particles are cleared by resident macrophages.[18] Moreover, once the MPs are taken up, the acid-sensitivity of particles leads to an enhanced release of cargo through more rapid degradation of the polymer, compared to degradation at neutral pH. This combination of acid-sensitivity and passive targeting makes Ac-DEX MPs apt carriers for adjuvants and vaccines.

2.1. Adjuvant Encapsulation

Primarily, toll-like receptor (TLR) agonists are the type of adjuvants that have been reported to be encapsulated in Ac-DEX particles. Of the TLRs studied, TLR 3 and TLR 7/8 are the most common, since these are intracellular Pathogen-Associated Molecular Patterns (PAMPs) found within the cell's phagolysosome, which is where Ac-DEX particles would reside after phagocytosis. Imiquimod, the active ingredient in Aldera cream and a TLR 7/8 agonist was first encapsulated into Ac-DEX particles by Bachelder et al.[19] A dose sparing of the hydrophobic adjuvant was observed in vitro in both macrophages and bone-marrow derived dendritic cells (BMDCs). A statistically significant difference in both mRNA and soluble cytokine responses was observed when BMDCs and macrophages were cultured with Ac-DEX MPs encapsulating imiquimod in comparison to empty Ac-DEX MPs and soluble imiquimod, at the same concentrations (Figure 3).[19] Resiquimod, a more hydrophilic imiquimod derivative, was later encapsulated into Ac-DEX MPs, and also displayed a dose sparing response in cytokine production in macrophages.[20] Moreover, by creating a library of polymers with unique cyclic acetal coverage and molecular weight, it has been illustrated that the loading of resiquimod as well as its release and bioactivity in macrophages can be controlled and illustrate dose sparing.[21]

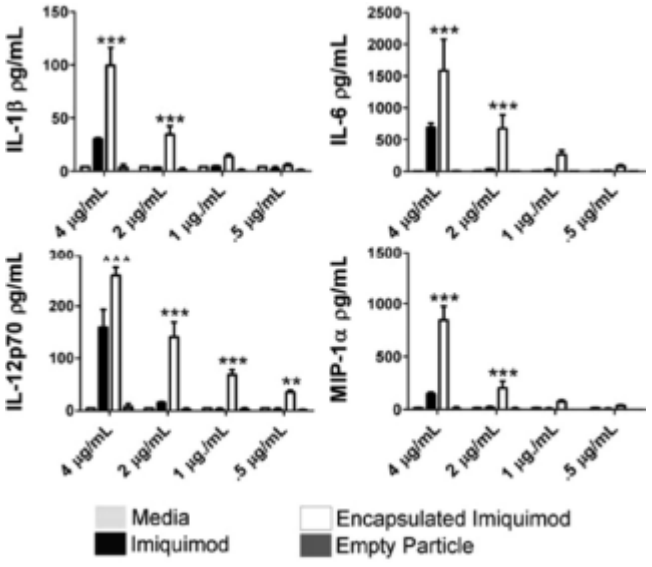


Figure 3. Pro-inflammatory cytokine response of a bone-marrow derived dendritic cell after 24 h of culturing with varying concentrations of media, soluble imiquimod, Ac-DEX particles encapsulating imiquimod, or empty Ac-DEX particles. Reprinted with permission from ref 19. Copyright 2010 American Chemical Society.

In addition to the encapsulation of small molecule adjuvants, nucleotide adjuvants have been formulated into Ac-DEX MPs. Two adjuvant-based nucleotides, in particular, have been used: Poly I:C, which is an agonist for TLR 3, and CpG, which activates TLR 9. In contrast to TLR 8 expression, which is uniform across species, TLR 9 activity differs between mice and humans, where in it is significantly reduced in humans because it is limited to only plasmacytoid DCs and B cells.[22] Despite this, CpG is heavily studied and therefore a practical adjuvant for vaccine formulation. In the comparison of poly I:C encapsulation with Ac-DEX versus PLGA, there was a significant increase in adjuvant encapsulation efficacy (EE) at intermediate (71K) dextran molecular weights. Also, the more quickly degrading poly I:C loaded Ac-DEX MPs resulted in increased cellular activation compared to more slowly degrading Ac-DEX and PLGA. One or both degradation rates of Ac-DEX resulted in a dose sparing compared to soluble drug.[23] Increased EE in comparison to PLGA and dose sparing compared to soluble drug was also observed with the encapsulation of CpG in Ac-DEX MPs. Moreover, the viability of Ac-DEX MPs was shown to be comparable to that of PLGA MPs, indicating that increased cytotoxicity is not the cause of the increased adjuvant activity observed with Ac-DEX MPs. The encapsulation of adjuvant in Ac-DEX particles paves the way for formulation of subunit vaccines using the biopolymer.

2.2. Vaccine Formulations

The model antigen ovalbumin (OVA) was encapsulated into Ac-DEX to evaluate the effect of Ac-DEX encapsulation[1] and tunability[2] on MHC I and MHC II presentation using model cell lines (B3Z for MHC I presentation and KZO for MHC II presentation). Both cell lines will express β-galactosidase downstream of CD3 signaling when the immunodominant peptide of OVA is presented on APCs. The enhanced MHC I presentation (indicator of cellular activity) observed by

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

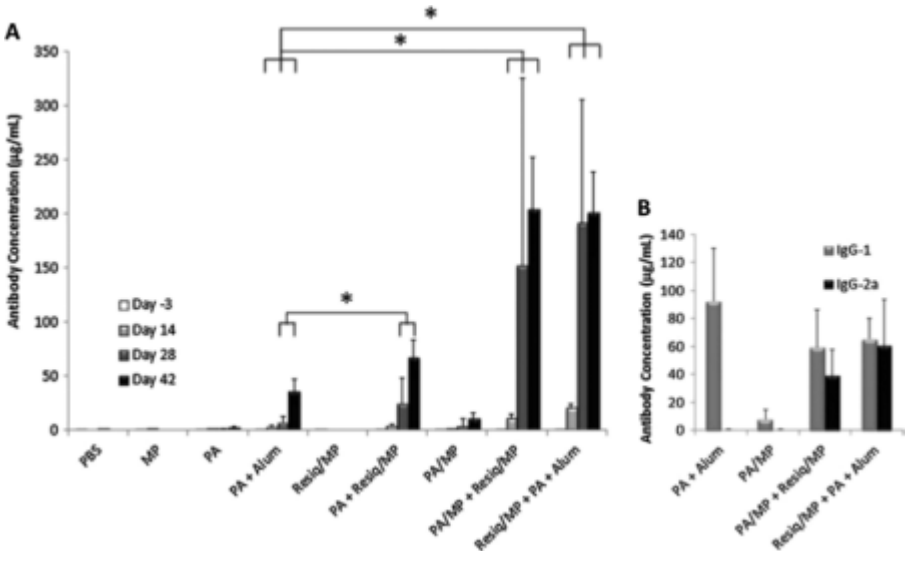


Figure 4. (A) Total IgG antibody and (B) antibody isotype generated when AJ mice were vaccinated at day 0 and 7 with Ac-DEX microparticles (MP) containing recombinant protective antigen (PA) and/or resiquimod (Resiq). A backslash (/) indicates encapsulation, whereas a plus sign (+) indicates addition of component. Statistically significant differences between groups are indicated by *. Reprinted with permission from ref 25. Copyright 2013 Springer.

Bachelder et al. in the initial reporting of Ac-DEX is due in part because particle cross-presentation of protein antigen has resulted in both MHC II and MHC I expression of antigen.[24] Broaders et al. then illustrated that the acid-sensitivity of Ac-DEX generates a greater cellular response at more rapid degradation rates and in comparison to non-acid-sensitive formulations. It is this foundation in enhanced cellular responses compared to free protein as well as other biomaterials that leads to the formation of vaccines with Ac-DEX MPs.[2]

Using separate particles, a complete subunit vaccine comprised of Ac-DEX was formulated for protection against anthrax. Separate emulsion based particles were used for adjuvant and antigen because the adjuvant had poor EE using homogenization compared to sonication, but homogenization was less damaging to the protein antigen. For the study, ~400 nm homogenized particles encapsulated recombinant protective antigen (rPA) and ~200 nm sonicated particles loaded with resiquimod were used. Ac-DEX MPs were shown to be an efficacious vaccine carrier for both antigen and adjuvant over unencapsulated delivery. The study also indicated that high levels of total IgG antibody, significantly greater than Alum and rPA, were generated in the particle vaccine group; however, these antibodies were non-neutralizing, likely due to the denaturation of the protein during the emulsion fabrication process. Despite the poor neutralizing response, a Type 1 helper T-cell (Th1) response was observed in both the antigen recall study as well as by the generation of IgG2 antibodies (Figure 4). This illustrated that the protection observed with the lethal challenge could be the result of a cellular immunity,[25] which corresponds with the results of both Bachelder et al.[1] and Broaders et al.[2]

The work with the anthrax vaccine paved the way for the evaluation of a Burkholderia pseudomallei vaccine for the prevention of melioidosis. Using cell lysate as a crude antigen and resiquimod, again in separate particles, vaccination with Ac-DEX particles was shown to significantly delay time to death, after lethal challenge, in both a rapid and more traditional vaccine schedule. The particle formulation generated humoral and cellular immune responses greater than those of alum and lysate. Additionally, approximately 10% of mice were sterile (blood, liver, spleen) after challenge when treated with encapsulated resiquimod Ac-DEX particles, with and without antigen. This indicates a potential post exposure treatment for this bioterrorism agent.[26]

With the use of emulsion for both the anthrax and B. pseudomallei vaccine work, there were several inherent manufacturing limitations that needed to be addressed prior to additional development of the Ac-DEX vaccine platform. These limitations included: (1) batch processing, (2) high energy methods (e.g., sonication, homogenization) which could denature the protein antigen, (3) extended solvent and protein antigen contact times during the solvent evaporation process which can also cause denaturation of the protein; (4) limited control over the amount of drug and efficacy at which it is encapsulated, and (5) broad polydispersity of particles. The effects of protein denaturation can result in poor B cell activation, which was illustrated by the lack of neutralizing antibodies observed with the emulsion anthrax work.[25] To address these limitations, an Ac-DEX MP anthrax vaccine was formulated using scalable electrospray.[27] In contrast to emulsion, electrospray can be scaled by multiplexing the electrospray heads. Electrospray can have limited interaction with solvents, offers a broader range of control of EE of the payload, produces fairly monodisperse MPs, and uses slow flow rates, which translates to reduced shear stress (Figure 5). When electrospray was used to encapsulate PA, decreased protein denaturation was observed compared to emulsion, which resulted in increased toxin neutralization of generated antibodies (Figure 5). Also due to the flexibility of particle fabrication via electrospray, we were able to encapsulate both protein and adjuvant into a single MP. In comparison to separately encapsulated adjuvant and antigen, the coencapsulated group provided decreased survival in response to a lethal challenge (Figure 5), although both groups outperformed the FDA approved anthrax vaccine formulation, BioThrax. This work illustrates the feasibility of manufacturing an Ac-DEX MP

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

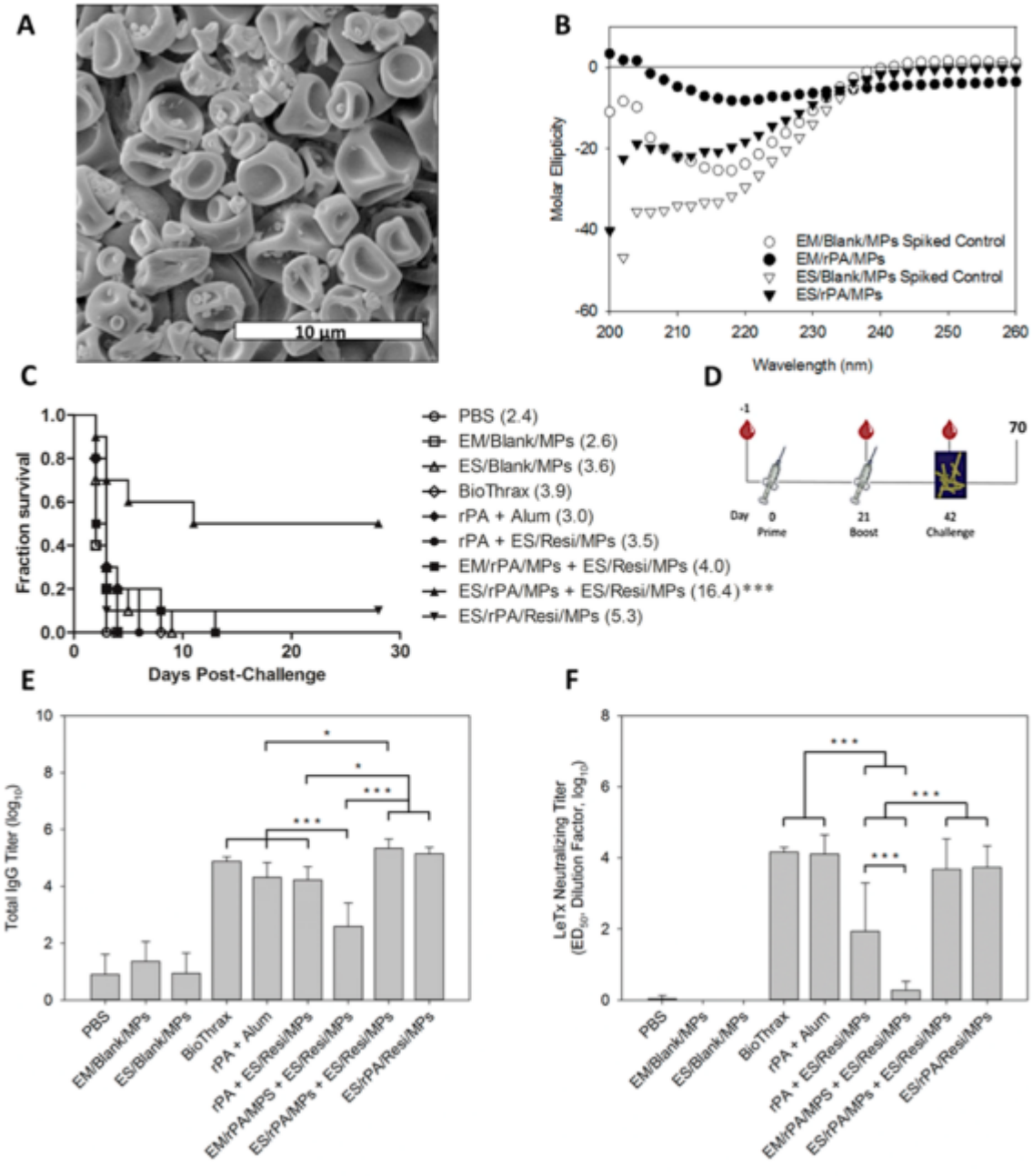


Figure 5. (A) Scanning electron micrograph of electrosprayed particles encapsulating recombinant protective antigen (rPA). (B) Circular dichroism spectra for rPA microparticles (MPs) fabricated by emulsion (EM/rPA/MPs) and electrospray (ES/rPA/MPs). (C) Survival curves of mice (n = 10) intratracheally challenged with 12x LD_{50} of B. anthracis Ames spores. ES = electrospray, EM = emulsion, Resi = Resiquimod (R848); /MPs indicates encapsulation in Ac-DEX. (D) Vaccination schedule of BALB/c mice. (E) Total IgG anti-PA antibodies in mouse sera collected on Day 42. Data are displayed as the mean logarithmic transformation (base 10) of the titers (ng/mL) + 95% confidence interval (n = 10). (F) Lethal toxin (LeTx) neutralizing titers for Day 42 mouse sera. Data are reported as the mean of the log-transformed (base 10) reciprocals of the sera dilution that inhibits LeTx cytotoxicity by 50% (ED_{50}) + 95% confidence interval (n = 5−10). Reprinted with permission from ref 27. Copyright 2016 Wiley.

vaccine platform, and the platform's ability to protect better than the current standard vaccine.

Ac-DEX particles have not only been used to stimulate an effector immune response, but they have also been used to attenuate immune responses in an antigen-specific manner. By using a tolerizing agent (e.g., dexamethasone) with an antigen (Myelin Oligodendrocyte Glycoprotein (MOG)), the costimulatory signals required for generation of an effector response are absent in the education of the T cell by the DC. This can result in antigen-specific attenuation of the immune response via mechanisms such as the formation of T-regulatory cells, anergy, or T cell deletion. Using Ac-DEX particles to encapsulate MOG and dexamethasone into one particle, via emulsion, it was shown using a Multiple Sclerosis (MS) model (experimental autoimmune encephalomyelitis (EAE)) that mice could be taken, on average, from full hind and partial front paralysis to only exhibiting a limp tail. The application of Ac-DEX for these autoimmune therapies is reviewed by Chen et al.[10] and, later, by Northrup et al.[14] The application of Ac-DEX particles has gone beyond the model antigen to illustrate protective responses in

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

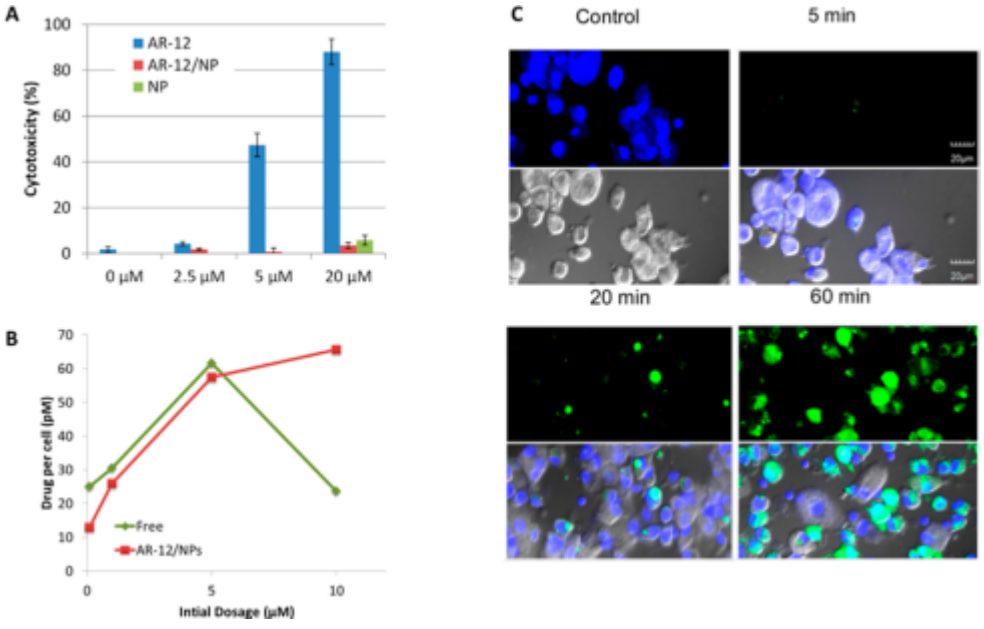


Figure 6. (A) Cytotoxicity, as measured with an LDH, of varying doses of empty Ac-DEX NPs, soluble AR-12, or AR-12 loaded Ac-DEX nanoparticles (NPs) when cultured with human monocyte derived macrophages. (B) Intracellular drug concentration of AR-12 in macrophages as measured with HPLC. (C) Uptake of Ac-DEX NPs when cultured with macrophages. Reprinted with permission from ref 33. Copyright 2014 Elsevier.

disease models, further illustrating the strong application of this biopolymer in a vaccine formulation.

3. DELIVERY TO MACROPHAGES

Like DCs, macrophages can be targeted by particles that are too large to be taken up by nonphagocytic cells. Moreover, since smaller particles are taken up and cleared by macrophages, one can also have a concentrated uptake in macrophages by sizing the particles between 100 nm and 1,000 nm.[18] Since macrophages serve as host cells to a variety of pathogens, including viral, bacterial, parasitic, and fungal, they can act as interdiction points for therapy. By disrupting the mechanisms the pathogen usurps to reside intracellularly, pathogen growth and survival can be decreased. These host-directed therapies (HDT) are thought to mitigate the emergence of drug resistance, since they are not targeting the pathogen directly. Ac-DEX has been used to formulate one HDT, AR-12 (also known as OSU-03012). AR-12 has illustrated broad spectrum activity against multiple bacteria, viruses, and the parasite Leishmania donovani,[12,28−33] and its activity has been reviewed by Collier et al.[31] Compared to unencapsulated drug, AR-12 in Ac-DEX particles had significantly reduced cytotoxicity and higher intracellular drug loading. Additionally, the particles were taken up freely by human monocyte derived macrophages (Figure 6).[33] In vivo, AR-12 in Ac-DEX particles displayed enhanced clearance of Francisella tularensis infection compared to the unencapsulated compound. When AR-12 in Ac-DEX particles were co-delivered with a suboptimum dose of antibiotic intranasally, there was a significant increase in mouse survival compared to free drug and free suboptimum antibiotic. In the host-directed treatment of intracellular parasite, L. donovani used intravenously delivered AR-12 in Ac-DEX particles and displayed a significant reduction in parasite burden over free AR-12, both alone and when co-delivered with amphotericin B. Overall, this work displays that Ac-DEX particles can more aptly deliver HDTs that target macrophages to reduce drug toxicity and increase drug efficacy.

4. CONTROLLED RELEASE

Probably the broadest application of Ac-DEX is its use as a controlled release drug delivery vehicle. Similar to the payloads seen with vaccine formulations, small molecules, proteins, peptides, and nucleotides have all been encapsulated or adsorbed to Ac-DEX to achieve a desired effect. In addition, inorganic based silver carbine has been encapsulated into Ac-DEX to serve as an antibacterial agent.[24] Many of these formulations are the traditional emulsion based nano-/microparticles that are used for both vaccine and macrophage targeting applications. By varying emulsion parameters with Ac-DEX, it has been shown that the encapsulation of rapamycin is dependent on the molecular weight of the base dextran,[35] which corresponds to what was observed with the encapsulation of poly I:C.[23] Also using traditional emulsion chemistry, it was shown that a model protein enzyme (horseradish peroxidase), encapsulated in Ac-DEX particles, had enhanced stability compared to unencapsulated protein. Moreover, Ac-DEX protected the enzyme and its activity from −20 to 45 °C, while PLGA melted at these higher temperatures. As expected, homogenized particles maintained better activity of the enzyme over sonicated particles (Figure 7).[36]

Using Ac-DEX to encapsulate proteins has advantages over polyesters and polyanhydrides because the degradation products are pH-neutral and can therefore be less damaging to the encapsulated protein. In contrast to Ac-DEX, when PLGA degrades, the core of the PLGA particle becomes extremely acidic.[4] It is then not surprising that Ac-DEX has been used to deliver proteins and peptides for therapeutic treatment. Henniget al. compared the EE of a peptide in Ac-DEX particles versus PLGA. They observed a decreased EE with Ac-DEX over PLGA for positively charged peptides for PLGA terminated with a carboxylic acid. The higher

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

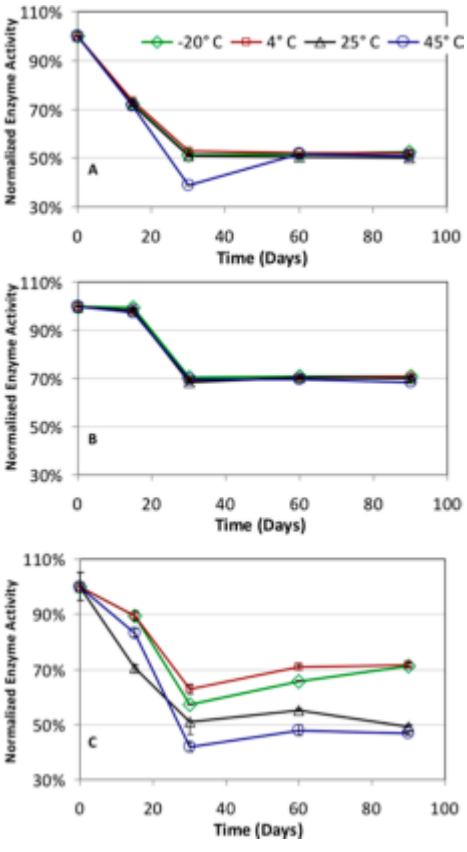


Figure 7. Activity of horseradish peroxidase when (A) encapsulated in sonicated Ac-DEX particles, (B) encapsulated in homogenized Ac-DEX particles, or (C) unencapsulated and stored at different temperatures as a dry powder. Reprinted with permission from ref 36. Copyright 2012 Elsevier.

encapsulation for PLGA is probably due to the interaction between the positive charge of the peptide and the negative charge of the carboxylic acid.[37] For neutral charged peptides and small molecules, Ac-DEX had higher EE. Other studies have encapsulated peptide into Ac-DEX particles[38] but have not compared their encapsulation to PLGA. Despite differences in EE, another advantage of Ac-DEX over polyesters is the tunable delivery. Suarez et al. illustrate this as an advantage in the delivery of basic fibroblast growth factor (bFGF) and myoglobin for treatment after myocardial infarction (MI). The tunability of release of these proteins afforded optimization of the release rates for the best therapeutic effect.[39] The temporal control of Ac-DEX was also illustrated to deliver a hepatocyte growth factor fragment for cardioprotective therapy.[40] As with the MI treatment, an optimum therapeutic effect was determined by varying the degradation rate of the polymer, underlining the importance of this Ac-DEX property.

For the delivery of nucleotides, polycations have been covalently attached to Ac-DEX to facilitate ionic absorption of siRNA,[41] resulting in increased knock-down compared to the free siRNA control. The increased intracellular trafficking over soluble siRNA of the Ac-DEX particles likely contributed to the increased siRNA efficacy.[41] Similarly, poly beta amino esters (PBAEs) were blended with Ac-DEX to deliver plasmid. Plasmid transfection in both HeLa and RAW macrophages was found to be dependent on the weight fraction of PBAE to Ac-DEX.[42]

Other constructs of Ac-DEX have been generated to offer controlled release, including porous microparticles, composite spray dried particles, electrosprayed microparticles, electrospun mats, and electrospun mat fragments called microconfetti (Figure 8). To mimic the large porous PLGA microparticles originally developed by Edwards et al.[43] Ac-DEX was made into porous microparticles, which display a significantly reduced density to volume ratio, compared to traditional emulsion particles. When modeled in a cascade impactor, the porous Ac-DEX particles had aerosol properties for deep lung penetration. The porous particles displayed a controlled release of the chemotherapeutic camptothecin as well as degradation-dependent release.[44] To advance lung delivery using Ac-DEX, nanoparticles made of the biopolymer were loaded with either tobramycin or paclitaxel[45,46] and then spray dried in a secondary matrix to form composite microparticles. Ac-DEX adjuvant microparticles, albeit not composite, have also been made through electrospray. Compared to emulsion, electrospray

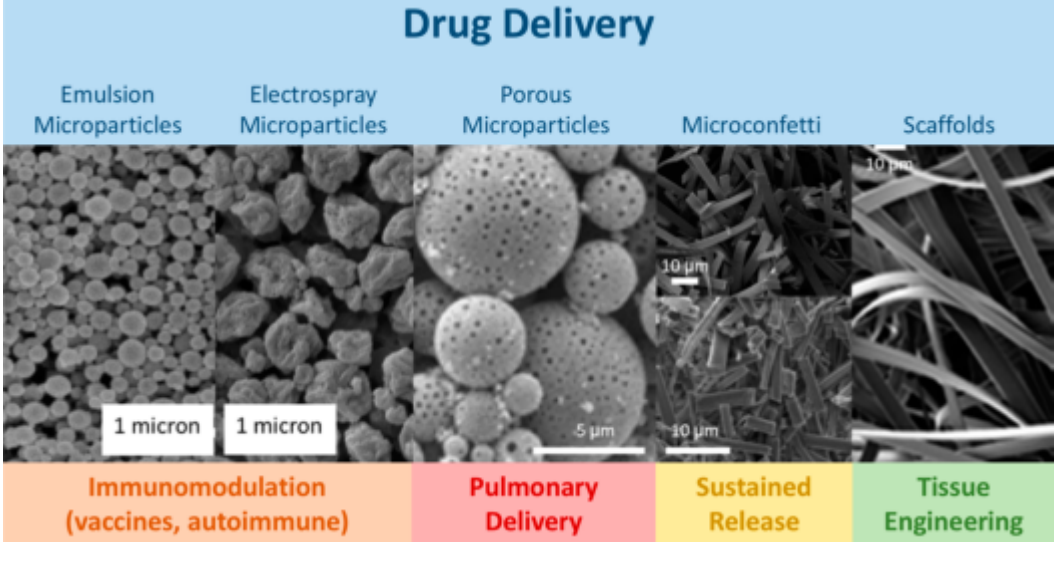


Figure 8. Various constructs of Ac-DEX and their general applications.[22,41−43,60]

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

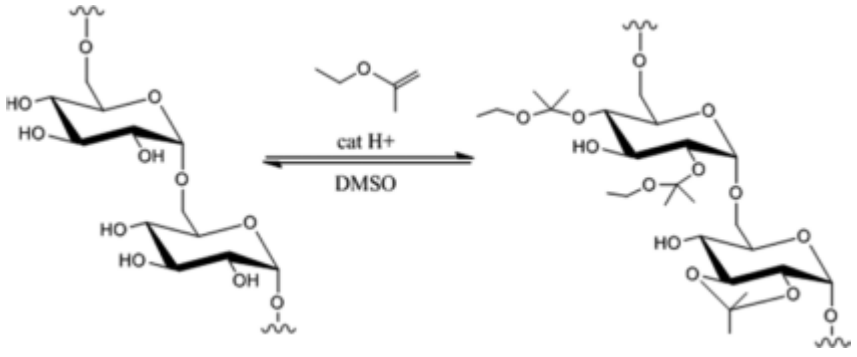


Figure 9. Ace-DEX made from 2-ethoxypropene. Reprinted with permission from ref 63. Copyright 2012 American Chemical Society.

affords a more scalable method that uses air as a continuous phase, in place of an aqueous or organic solution, resulting in increased EEs of compounds. The EE of resiquimod was increased almost 10-fold over emulsion with electrospray to form microparticles that could be delivered intravenously to treat L. donovani infection.[47] A method similar to electrospray, electrospinning, has also been used to generate controlled release nanofibrous scaffolds for Ac-DEX. By electrospinning Ac-DEX with different degradation rates, controlled release of resiquimod was observed, as well as a differential effect of resiquimod on macrophages.[47] The differential effect of Ac-DEX degradation rates aligns with the cardioprotective effects with the MI treatment with bFGF and myoglobin and also the release of saquinavir from electrospun fibers ground into injectable fragments called microconfetti.[48] Collier et al. illustrated that fragments of nanofibrous Ac-DEX scaffolds that are milled can afford nearly week-long release of the protease inhibitor saquinavir when injected subcutaneously. Microconfetti could not be fabricated from PLGA or PCL, even when milled under liquid nitrogen. The high glass transition temperature of Ac-DEX (~160−190 °C)[36] allowed electrospun nanofibrous scaffolds, loaded up to 50 w/w % with saquinavir, to be milled into small injectable pieces.[48]

Ac-DEX has also been used in the formation of Janus particles with PLGA.[49] By using electrohydrodynamic (EHD; electrospray) cojetting, Rahman et al. formed microparticles where one-half of the particle consists of PLGA, and the other half consists of a PLGA/Ac-DEX blend. They loaded the chemotherapeutic irinotecan into the part of the particle that consisted of Ac-DEX, and they were capable of releasing in a pH-sensitive manner.[50] Expanding on their previous work, Ross et al. then proceeded to use their Janus particles for the delivery of drugs to the cochlea in an animal model of cochlear implants. By using piribedil loaded microparticles, they were able to show extended release in vivo.[51] These controlled release studies illustrate that the range of degradation and processability afforded with Ac-DEX is much greater than that which is observed with PLGA, leading to better controlled release systems, as included in reviews by Miladi et al.[13] and Chifiriuc et al.[11]

5. DEVICES

The acid-sensitive acceleration in degradation as well as Ac-DEX's broadly tunable degradation make it an appealing candidate for incorporation into highly engineered devices. Ac-DEX has been used to more rapidly release insulin in response to changes in glucose,[52] as valves in mesoporous silicon,[53,54] and as vehicles to trap water for laser-assisted release of payload.[55] Gu et al. coated Ac-DEX microparticles with chitosan or alginate to give alternating charges. By creating a low acid environment with gluconic acid, one product of glucose and glucose oxidase, insulin encapsulated in the Ac-DEX particles is released at an accelerated rate in the presence of glucose. This device was shown to attenuate blood sugar in diabetic mice.[52] This is similar to the work that was done in the 1990s using poly ortho-esters as the pH-sensitive material.[8] Additionally, Wang et al. has incorporated anti-PD1 antibodies into Ac-DEX for microneedle applications.[56] By incorporating anti-PD1 into a polymeric microneedle, they show enhanced antibody efficacy. Also taking advantage of Ac-DEX's acid-sensitivity, two laboratories have reported the incorporation of the biopolymer into mesoporous silicon to work as valves to control the release of drug that has been absorbed into the inorganic material. Either by using different degradation rates of polymer or by exposing the composite particles to an acid-environment, drug can be released in a more controlled manner, compared to drug absorbed on mesoporous silicon alone.[53] Furthermore, Ac-DEX was used to functionalize the silicon, by attaching cell penetrating peptide to the biopolymer incorporated in the mesoporous silicon.[54] Beyond mesoporous silicon, Viger et al. trapped water in Ac-DEX microparticles fabricated via electrospray. They then heated the water into vapor using a near-IR laser, which results in destabilization of the Ac-DEX matrix and more triggered drug release.[55] These devices display the broad applicability of Ac-DEX.

6. COPOLYMER AND OTHER ACETALATED POLYMERS

To further enhance Ac-DEX, functional groups have been covalently attached to the polymer and copolymers have been formed to create new micelles and other drug delivery vehicles. Initially Ac-DEX was modified with oxime chemistry to covalently link cell penetrating peptide to the particle surface. This chemistry was used to attach the peptide with a fluorophore that then displayed strong covalent attachment to the peptide.[57] Also for enhanced targeting of Ac-DEX particles, mannose was ligated to the biopolymer to actively target the mannose receptor, which is highly expressed in immune cells. Mannose targeting resulted in increased MHC I presentation of OVA over conventional Ac-DEX particles.[58] To cationically charge the Ac-DEX particles, spermine[59] and diethylenetriamine (DET) grated poly(L-aspartic acid)[60] have been covalently bound to Ac-DEX. The spermine functionalized Ac-DEX resulted in better knock-down than sham siRNA, without a marked difference in cell viability.[59] For the DET grated poly(L-aspartic acid) functionalized to Ac-DEX particles,

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

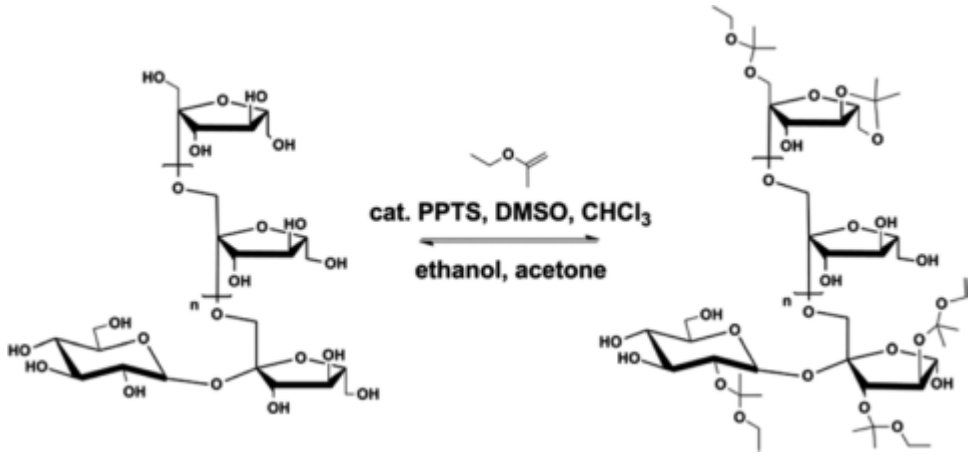


Figure 10. Ace-Inulin made from 2-ethoxypropene and inulin from dahlia. Reprinted with permission from ref 64. Copyright 2016 Royal Society of Chemistry.

both doxorubicin and plasmid were delivered to cancer cells with polymeric particles formed through emulsion and micelles. These carriers resulted in significant uptake of doxorubicin in cancer cells.[60] In addition to the peptide-DET-Ac-DEX micelles formed, poly(ethylene glycol) (PEG) has been covalently attached in two ways to Ac-DEX to form copolymer micelles with pH-sensitive triggers. With the addition of PEG to Ac-DEX with varying degradation rates, micelles were formed to deliver doxorubicin. The Ac-DEX-PEG micelles released drug in a pH-sensitive manner and illustrated increased cell death compared to PEG-b-polylactide (PLA) pH-insensitive micelles at some concentrations when cultured with HeLa cells.[61] PEG was also attached to Ac-DEX by adenine termination of the biopolymer and thymine termination of PEG to form a triblock polymer. Using Ac-DEX at different degradation rates resulted in differential pH-sensitive release of doxorubicin. All triblock micelles loaded with doxorubicin resulted in a significant decrease in cell viability when cultured with HeLa cells.[62] In each of these cases, the acid-sensitivity and/or degradation tunability of Ac-DEX was used to help facilitate better drug delivery.

In addition to copolymers, two acetalated polymers have been produced that display similar properties as the original Ac-DEX. Ace-DEX, which was used in several of the previously mentioned results,[21,31,33,52] is formed by the reaction of dextran and 2-ethoxypropene (leading to the use of the e in Ace over Ac), resulting in ethanol, in place of methanol, as a degradation product. Ace-DEX displays similar degradation tunability as Ac-DEX as well as similar cytocompatibility (Figure 9).[63] Using similar chemistry, Ace-Inulin was formed (Figure 10). Whereas dextran is immunologically inert, inulin has naturally occurring immune stimulating properties, allowing Ace-Inulin to serve as its own adjuvant in vaccine formulations. Blending Ace-Inulin with Ace-DEX results in better encapsulation of protein, but decreased immune stimulatory activity, as measured through tumor necrosis factor-alpha (TNF-α) production by RAW macrophages. Overall, an Ace-Inulin blended OVA vaccine did elicit strong total IgG antibodies on order with OVA and alum.[64] Similarly, silylated dextran and inulin have shown tunable properties and generation of IgG.[65] These second generation Ac-DEX polymers have the same beneficial properties of Ac-DEX with some added features, including more benign degradation products and immune stimulating capabilities.

7. CONCLUSION

In conclusion, we have reviewed the emerging biopolymer acetalated dextran (Ac-DEX). Ac-DEX has several features which make it a unique biodegradable polymer, including facile synthesis, acid-sensitivity, and tunable degradation rates. For these reasons it has emerged as a top candidate for applications in vaccines, targeted host-directed therapies to macrophages, controlled release of drug, chemotherapeutic delivery, engineered drug delivery devices, and as copolymers in drug and vaccine carriers. Ac-DEX has displayed delivery of small molecules, proteins, peptides, nucleotides, and inorganic compounds, illustrating the broad applicability of this material. Moreover, it has been used to modify inorganic mesoporous silicon, spun into nanofibrous mats, made into microconfetti, and covalently bound to other polymers to form micelles. Current uses of this biopolymer are merely the tip of the iceberg, the applications of Ac-DEX will certainly expand in the coming years.

ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrev.6b00532

Uses of Ac-DEX currently in the literature (PDF)

AUTHOR INFORMATION

Corresponding Author

* Eric M. Bachelder, Ph.D., 4210 Marsico Hall, 125 Mason Farm Road, Chapel Hill, NC 27599. E-mail, ebacheld@email.unc.edu; phone, 919-962-4857.

ORCID

Eric M. Bachelder 0000-0002-8572-888X

Notes

The authors declare no competing financial Interest.

Biographies

Eric M. Bachelder gained extensive immunological experience while completing his graduate studies in Dr. Polly Matzinger's lab at the National Institutes of Health (NIH), National Institute of Allergy and Infectious Diseases (NIAID). During his graduate studies, he studied

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

the interaction of T-cells and dendritic cells. After completing his Ph.D. in Chemical Engineering, he joined Prof. Jean Fréchet's lab at the University of California, Berkeley's chemistry department for a postdoc. While there, Dr. Bachelder invented various biodegradable polymers for vaccine and drug delivery applications, including Acetalated Dextran (Ac-DEX). Dr. Bachelder continued his work on Ac-DEX as an Assistant Professor at the Ohio State University and currently at the University of North Carolina at Chapel Hill. The focus of Dr. Bachelder's research program is to develop novel drug delivery moieties for the formulation of vaccines, infectious disease treatments, and autoimmune therapies.

Erica N. Pino received her B.S. in Biology from the Massachusetts Institute of Technology in 2013. Her undergraduate thesis work in the laboratories of Dr. Robert Desimone and Dr. Edward Boyden centered on characterizing heat and light propagation through rodent cortex to establish safe guidelines for optogenetic studies in nonhuman primates. Following graduation, she continued research in the Desimone lab, focusing on optogenetic inactivation of frontal eye field neurons during memory-guided saccade tasks. She is now a graduate student in the Ainslie lab, working toward her Ph.D. in the Division of Pharmacoengineering and Molecular Pharmaceutics at the University of North Carolina at Chapel Hill.

Kristy M. Ainslie is an Associate Professor with appointments in the Division of Pharmacoengineering and Molecular Pharmaceutics, within the Eshelman School of Pharmacy, and the Department of Biomedical Engineering at the University of North Carolina at Chapel Hill. Her research focuses on the use of novel micro- and nanoscaled carriers for the modulation of immune responses to create vaccines for and therapies against infectious diseases. Previously, she was an Assistant Professor in the Division of Pharmaceutics and Pharmaceutical Chemistry at The Ohio State University. As a postdoctoral scholar at the University of California, San Francisco, she researched the microfabrication of oral drug delivery vehicles and the immune response of nanobiomaterials. Previously, she studied biosensors at the Naval Research Laboratory in Washington, DC, and completed a Ph.D. in Chemical Engineering at The Pennsylvania State University in the application of nanobiomaterials for antifouling implant surfaces.

ACKNOWLEDGMENTS

This work was supported by internal funds from the Eshelman School of Pharmacy at UNC-Chapel Hill.

REFERENCES

(1) Bachelder, E. M.; Beaudette, T. T.; Broaders, K. E.; Dashe, J.; Frechet, J. M. Acetal-Derivatized Dextran: An Acid-Responsive Biodegradable Material for Therapeutic Applications. J. Am. Chem. Soc. 2008, 130, 10494–10495.
(2) Broaders, K. E.; Cohen, J. A.; Beaudette, T. T.; Bachelder, E. M.; Frechet, J. M. J. Acetalated Dextran Is a Chemically and Biologically Tunable Material for Particulate Immunotherapy. Proc. Natl. Acad. Sci. U. S. A. 2009, 106, 5497–5502.
(3) Helaly, F. M.; Hashem, M. S. Preparation and Characterization of Poly(B-Amino Ester) Capsules for Slow Release of Bioactive Material. J. Encapsulation Adsorpt. Sci. 2013, 2013, 65.
(4) Ding, A. G.; Schwendeman, S. P. Acidic Microclimate pH Distribution in Plga Microspheres Monitored by Confocal Laser Scanning Microscopy. Pharm. Res. 2008, 25, 2041–2052.
(5) Li, L.; Schwendeman, S. P. Mapping Neutral Microclimate pH in Plga Microspheres. J. Controlled Release 2005, 101, 163–173.
(6) Schwendeman, S. P.; Tobio, M.; Joworowicz, M.; Alonso, M. J.; Langer, R. New Strategies for the Microencapsulation of Tetanus Vaccine. J. Microencapsulation 1998, 15, 299–318.
(7) Fields, R. J.; Cheng, C. J.; Quijano, E.; Weller, C.; Kristofik, N.; Duong, N.; Hoimes, C.; Egan, M. E.; Saltzman, W. M. Surface Modified Poly(B Amino Ester)-Containing Nanoparticles for Plasmid DNA Delivery. J. Controlled Release 2012, 164, 41–48.
(8) Heller, J.; Barr, J. Poly(Ortho Esters) from Concept to Reality. Biomacromolecules 2004, 5, 1625–1632.
(9) Paramonov, S. E.; Bachelder, E. M.; Beaudette, T. T.; Standley, S. M.; Lee, C. C.; Dashe, J.; Fréchet, J. M. Fully Acid-Degradable Biocompatible Polyacetal Microparticles for Drug Delivery. Bioconjugate Chem. 2008, 19, 911–919.
(10) Chen, N.; Peine, K.; Bachelder, E.; Ainslie, K. Micro- and Nano-Particulate Strategies for Antigen Specific Immune Tolerance to Treat Autoimmune Diseases. Pharm. Nanotechnol. 2015, 3, 85–100.
(11) Chifiriuc, M. C.; Grumezescu, A. M.; Grumezescu, V.; Bezirtzoglou, E.; Lazar, V.; Bolocan, A. Biomedical Applications of Natural Polymers for Drug Delivery. Curr. Org. Chem. 2014, 18, 152–164.
(12) Collier, M. A.; Gallovic, M. D.; Peine, K. J.; Duong, A. D.; Bachelder, E. M.; Gunn, J. S.; Schlesinger, L. S.; Ainslie, K. M. Delivery of Host Cell-Directed Therapeutics for Intracellular Pathogen Clearance. Expert Rev. Anti-Infect. Ther. 2013, 11, 1225–1235.
(13) Miladi, K.; Ibraheem, D.; Iqbal, M.; Sfar, S.; Fessi, H.; Elaissari, A. Particles from Preformed Polymers as Carriers for Drug Delivery. EXCLI J. 2014, 13, 28.
(14) Northrup, L.; Christopher, M. A.; Sullivan, B. P.; Berlind, C. Combining Antigen and Immunomodulators: Emerging Trends in Antigen-Specific Immunotherapy for Autoimmunity. Adv. Drug Delivery Rev. 2016, 98, 86–98.
(15) Janeway, C.; Travers, P.; Walport, M.; Shlomchik, M. Immunobiology, 5th ed.; Garland Science: New York, 2001.
(16) Foged, C.; Brodin, B.; Frokjaer, S.; Sundblad, A. Particle Size and Surface Charge Affect Particle Uptake by Human Dendritic Cells in an in Vitro Model. Int. J. Pharm. 2005, 298, 315–322.
(17) Hirota, K.; Hasegawa, T.; Hinata, H.; Ito, F.; Inagawa, H.; Kochi, C.; Soma, G.; Makino, K.; Terada, H. Optimum Conditions for Efficient Phagocytosis of Rifampicin-Loaded Plga Microspheres by Alveolar Macrophages. J. Controlled Release 2007, 119, 69–76.
(18) Manolova, V.; Flace, A.; Bauer, M.; Schwarz, K.; Saudan, P.; Bachmann, M. F. Nanoparticles Target Distinct Dendritic Cell Populations According to Their Size. Eur. J. Immunol. 2008, 38, 1404–1413.
(19) Bachelder, E. M.; Beaudette, T. T.; Broaders, K. E.; Frechet, J. M. J.; Albrecht, M. T.; Mateczun, A. J.; Ainslie, K. M.; Pesce, J. T.; Keane-Myers, A. M. In Vitro Analysis of Acetalated Dextran Microparticles as a Potent Delivery Platform for Vaccine Adjuvants. Mol. Pharmaceutics 2010, 7, 826–835.
(20) Duong, A. D.; Collier, M. A.; Bachelder, E. M.; Wyslouzil, B. E.; Ainslie, K. M. One Step Encapsulation of Small Molecule Drugs in Liposomes Via Electrospray-Remote Loading. Mol. Pharmaceutics 2015, 13, 92.
(21) Chen, N.; Collier, M. A.; Gallovic, M. D.; Collins, G. C.; Sanchez, C. C.; Fernandes, E. Q.; Bachelder, E. M.; Ainslie, K. M. Degradation of Acetalated Dextran Can Be Broadly Tuned Based on Cyclic Acetal Coverage and Molecular Weight. Int. J. Pharm. 2016, 512, 147–157.
(22) Hochrein, H.; Wagner, H. Of Men, Mice and Pigs: Looking at Their Plasmacytoid Dendritic Cells [Corrected]. Immunology 2004, 112, 26–27.
(23) Peine, K. J.; Bachelder, E. M.; Vangundy, Z.; Papenfuss, T.; Brackman, D. J.; Gallovic, M. D.; Schully, K.; Pesce, J.; Keane-Myers, A.; Ainslie, K. M. Efficient Delivery of the Toll-Like Receptor Agonists Polyinosinic:Polycytidylic Acid and Cpg to Macrophages by Acetalated Dextran Microparticles. Mol. Pharmaceutics 2013, 10, 2849–2857.
(24) Kovacsovics-Bankowski, M.; Clark, K.; Benacerraf, B.; Rock, K. L. Efficient Major Histocompatibility Complex Class I Presentation of Exogenous Antigen Upon Phagocytosis by Macrophages. Proc. Natl. Acad. Sci. U. S. A. 1993, 90, 4942–4946.
(25) Schully, K. L.; Sharma, S.; Peine, K. J.; Pesce, J.; Elberson, M. A.; Fonseca, M. E.; Prouty, A. M.; Bell, M. G.; Borteh, H.; Gallovic, M.;

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915–1926

et al. Rapid Vaccination Using an Acetalated Dextran Microparticulate Subunit Vaccine Confers Protection against Triplicate Challenge by Bacillus Anthracis. Pharm Res 2013, 30, 1349−1361.
(26) Schully, K. L.; Bell, M. G.; Prouty, A. M.; Gallovic, M. D.; Gautam, S.; Peine, K. J.; Sharma, S.; Bachelder, E. M.; Pesce, J. T.; Elberson, M. A.; et al. Evaluation of a Biodegradable Microparticulate Polymer as a Carrier for Burkholderia Pseudomallei Subunit Vaccines in a Mouse Model of Melioidosis. Int. J. Pharm. 2015, 495, 849−861.
(27) Gallovic, M. D.; Schully, K. L.; Bell, M. G.; Elberson, M. A.; Palmer, J. R.; Darko, C. A.; Bachelder, E. M.; Wyslouzil, B. E.; Keane-Myers, A. M.; Ainslie, K. M. Acetalated Dextran Microparticulate Vaccine Formulated Via Coaxial Electrospray Preserves Toxin Neutralization and Enhances Murine Survival Following Inhalational Bacillus Anthracis Exposure. Adv. Healthcare Mater. 2016, 5, 2617.
(28) Booth, L.; Roberts, J. L.; Cash, D. R.; Tavallai, S.; Jean, S.; Fidanza, A.; Cruz-Luna, T.; Siembiba, P.; Cycon, K. A.; Cornelissen, C. N.; et al. Grp78/Bip/Hspa5/DnaK Is a Universal Therapeutic Target for Human Disease. J. Cell. Physiol. 2015, 230, 1661−1676.
(29) Chiu, H. C.; Soni, S.; Kulp, S. K.; Curry, H.; Wang, D.; Gunn, J. S.; Schlesinger, L. S.; Chen, C. S. Eradication of Intracellular Francisella Tularensis in Thp-1 Human Macrophages with a Novel Autophagy Inducing Agent. J. Biomed. Sci. 2009, 16, 110.
(30) Chiu, H. C.; Kulp, S. K.; Soni, S.; Wang, D.; Gunn, J. S.; Schlesinger, L. S.; Chen, C. S. Eradication of Intracellular Salmonella Enterica Serovar Typhimurium with a Small-Molecule, Host Cell-Directed Agent. Antimicrob. Agents Chemother. 2009, 53, 5236−5244.
(31) Collier, M. A.; Peine, K. J.; Gautam, S.; Oghumu, S.; Varikuti, S.; Borteh, H.; Papenfuss, T. L.; Sataoskar, A. R.; Bachelder, E. M.; Ainslie, K. M. Host-Mediated Leishmania Donovani Treatment Using Ar-12 Encapsulated in Acetalated Dextran Microparticles. Int. J. Pharm. 2016, 499, 186−194.
(32) Hoang, K. V.; Curry, H.; Collier, M. A.; Borteh, H.; Bachelder, E. M.; Schlesinger, L. S.; Gunn, J. S.; Ainslie, K. M. Needle-Free Delivery of Acetalated Dextran-Encapsulated Ar-12 Protects Mice from Francisella Tularensis Lethal Challenge. Antimicrob. Agents Chemother. 2016, 60, 2052−2062.
(33) Hoang, K. V.; Borteh, H. M.; Rajaram, M. V. S.; Peine, K. J.; Curry, H.; Collier, M. A.; Homsy, M. L.; Bachelder, E. M.; Gunn, J. S.; Schlesinger, L. S.; et al. Acetalated Dextran Encapsulated Ar-12 as a Host-Directed Therapy to Control Salmonella Infection. Int. J. Pharm. 2014, 477, 334−343.
(34) Ornelas-Megiatto, C.; Shah, P. N.; Wich, P. R.; Cohen, J. L.; Tagaev, J. A.; Smolen, J. A.; Wright, B. D.; Panzner, M. J.; Youngs, W. J.; Frechet, J. M.; et al. Aerosolized Antimicrobial Agents Based on Degradable Dextran Nanoparticles Loaded with Silver Carbene Complexes. Mol. Pharmaceutics 2012, 9, 3012−3022.
(35) Kauffman, K. J.; Kanthamneni, N.; Meenach, S. A.; Pierson, B. C.; Bachelder, E. M.; Ainslie, K. M. Optimization of Rapamycin-Loaded Acetalated Dextran Microparticles for Immunosuppression. Int. J. Pharm. 2012, 422, 356−363.
(36) Kanthamneni, N.; Sharma, S.; Meenach, S. A.; Billet, B.; Zhao, J. C.; Bachelder, E. M.; Ainslie, K. M. Enhanced Stability of Horseradish Peroxidase Encapsulated in Acetalated Dextran Microparticles Stored Outside Cold Chain Conditions. Int. J. Pharm. 2012, 431, 101−110.
(37) Hennig, D.; Schubert, S.; Dargatz, H.; Kostenis, E.; Fahr, A.; Schubert, U. S.; Heinzel, T.; Imhof, D. Novel Insights into Appropriate Encapsulation Methods for Bioactive Compounds into Polymers: A Study with Peptides and Hdac Inhibitors. Macromol. Biosci. 2014, 14, 69−80.
(38) Peine, K. J.; Guerau-de-Arellano, M.; Lee, P.; Kanthamneni, N.; Severin, M.; Probst, G. D.; Peng, H. Y.; Yang, Y. H.; Vangundy, Z.; Papenfuss, T. L.; et al. Treatment of Experimental Autoimmune Encephalomyelitis by Codelivery of Disease Associated Peptide and Dexamethasone in Acetalated Dextran Microparticles. Mol. Pharmaceutics 2014, 11, 828−835.
(39) Suarez, S.; Grover, G. N.; Braden, R. L.; Christman, K. L.; Almutairi, A. Tunable Protein Release from Acetalated Dextran Microparticles: A Platform for Delivery of Protein Therapeutics to the Heart Post-Mi. Biomacromolecules 2013, 14, 3927−3935.
(40) Suarez, S. L.; Munoz, A.; Mitchell, A. C.; Braden, R. L.; Luo, C. L.; Cochran, J. R.; Almutairi, A.; Christman, K. L. Degradable Acetalated Dextran Microparticles for Tunable Release of an Engineered Hepatocyte Growth Factor Fragment. ACS Biomater. Sci. Eng. 2016, 2, 197−204.
(41) Wich, P. R.; Fréchet, J. M. J. Degradable Dextran Particles for Gene Delivery Applications. Aust. J. Chem. 2012, 65, 15−19.
(42) Cohen, J. A.; Beaudette, T. T.; Cohen, J. L.; Broaders, K. E.; Bachelder, E. M.; Frechet, J. M. Acetal-Modified Dextran Microparticles with Controlled Degradation Kinetics and Surface Functionality for Gene Delivery in Phagocytic and Non-Phagocytic Cells. Adv. Mater. 2010, 22, 3593−3597.
(43) Edwards, D. A.; Hanes, J.; Caponetti, G.; Hrkach, J.; Ben-Jebria, A.; Eskew, M. L.; Mintzes, J.; Deaver, D.; Lotan, N.; Langer, R. Large Porous Particles for Pulmonary Drug Delivery. Science 1997, 276, 1868−1871.
(44) Meenach, S. A.; Kim, Y. J.; Kauffman, K. J.; Kanthamneni, N.; Bachelder, E. M.; Ainslie, K. M. Synthesis, Optimization, and Characterization of Camptothecin-Loaded Acetalated Dextran Porous Microparticles for Pulmonary Delivery. Mol. Pharmaceutics 2012, 9, 290−298.
(45) Wang, Z. M.; Meenach, S. A. Ieee Dry Powders Based on Mucus-Penetrating Nanoparticle Entrapped Microparticles for Pulmonary Delivery of Tobramycin. 2014 40th Annual Northeast Bioengineering Conference (Nebec) 2014.
(46) Torrico-Guzman, E. A.; Meenach, S. Ieee In 2015 41st Annual Northeast Biomedical Engineering Conference, 2015.
(47) Duong, A. D.; Sharma, S.; Peine, K. J.; Gupta, G.; Satoskar, A. R.; Bachelder, E. M.; Wyslouzil, B. E.; Ainsie, K. M. Electrospray Encapsulation of Toll-Like Receptor Agonist Resiquimod in Polymer Microparticles for the Treatment of Visceral Leishmaniasis. Mol. Pharmaceutics 2013, 10, 1045−1055.
(48) Collier, M. A.; Gallovic, M. D.; Bachelder, E. M.; Sykes, C. D.; Kashuba, A.; Ainslie, K. M. Saquinavir Loaded Acetalated Dextran Microconfetti − a Long Acting Protease Inhibitor Injectable. Pharm. Res. 2016, 33, 1998−2009.
(49) Rahmani, S.; Park, T.-H.; Dishman, A. F.; Lahann, J. Multimodal Delivery of Irinotecan from Microparticles with Two Distinct Compartments. J. Controlled Release 2013, 172, 239−245.
(50) Rahmani, S.; Ross, A. M.; Park, T. H.; Durmaz, H.; Dishman, A. F.; Prieskorn, D. M.; Jones, N.; Altschuler, R. A.; Lahann, J. Dual Release Carriers for Cochlea Delivery. Adv. Healthcare Mater. 2016, 5, 94−100.
(51) Ross, A. M.; Rahmani, S.; Prieskorn, D. M.; Dishman, A. F.; Miller, J. M.; Lahann, J.; Altschuler, R. A. Persistence, Distribution, and Impact of Distinctly Segmented Microparticles on Cochlear Health Following in Vivo Infusion. J. Biomed. Mater. Res., Part A 2016, 104, 1510−1522.
(52) Gu, Z.; Aimetti, A. A.; Wang, Q.; Dang, T. T.; Zhang, Y.; Veiseh, O.; Cheng, H.; Langer, R. S.; Anderson, D. G. Injectable Nano-Network for Glucose-Mediated Insulin Delivery. ACS Nano 2013, 7, 4194−4201.
(53) Lin, Z.; Li, J. Z.; He, H. Y.; Kuang, H. H.; Chen, X. S.; Xie, Z. G.; Jing, X. B.; Huang, Y. B. Acetalated-Dextran as Valves of Mesoporous Silica Particles for Ph Responsive Intracellular Drug Delivery. RSC Adv. 2015, 5, 9546−9555.
(54) Liu, D.; Zhang, H.; Makila, E.; Fan, J.; Herranz-Blanco, B.; Wang, C. F.; Rosa, R.; Ribeiro, A. J.; Salonen, J.; Hirvonen, J.; et al. Microfluidic Assisted One-Step Fabrication of Porous Silicon@Acetalated Dextran Nanocomposites for Precisely Controlled Combination Chemotherapy. Biomaterials 2015, 39, 249−259.
(55) Viger, M. L.; Sheng, W. Z.; Dore, K.; Alhasan, A. H.; Carling, C. J.; Lux, J.; Lux, C. D.; Grossman, M.; Malinow, R.; Almutairi, A. Near-Infrared-Induced Heating of Confined Water in Polymeric Particles for Efficient Payload Release. ACS Nano 2014, 8, 4815−4826.
(56) Wang, C.; Ye, Y.; Hochu, G. M.; Sadeghifar, H.; Gu, Z. Enhanced Cancer Immunotherapy by Microneedle Patch-Assisted Delivery of Anti-Pd1 Antibody. Nano Lett. 2016, 16, 2334−2340.

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

(57) Beaudette, T. T.; Cohen, J. A.; Bachelder, E. M.; Broaders, K. E.; Cohen, J. L.; Engleman, E. G.; Frechet, J. M. Chemoselective Ligation in the Functionalization of Polysaccharide-Based Particles. J. Am. Chem. Soc. 2009, 131, 10360−10361.
(58) Cui, L. N.; Cohen, J. A.; Broaders, K. E.; Beaudette, T. T.; Frechet, J. M. J. Mannosylated Dextran Nanoparticles: A Ph-Sensitive System Engineered for Immunomodulation through Mannose Targeting. Bioconjugate Chem. 2011, 22, 949−957.
(59) Cohen, J. L.; Schubert, S.; Wich, P. R.; Cui, L.; Cohen, J. A.; Mynar, J. L.; Frechet, J. M. J. Acid-Degradable Cationic Dextran Particles for the Delivery of Sirna Therapeutics. Bioconjugate Chem. 2011, 22, 1056−1065.
(60) Li, Q.; Liu, W.; Dai, J.; Zhang, C. Synthesis of Polysaccharide-Block-Polypeptide Copolymer for Potential Co-Delivery of Drug and Plasmid DNA. Macromol. Biosci. 2015, 15, 756−764.
(61) Zhang, Z.; Chen, X.; Chen, L.; Yu, S.; Cao, Y.; He, C.; Chen, X. Intracellular Ph-Sensitive Peg-Block-Acetalated-Dextran as Efficient Drug Delivery Platforms. ACS Appl. Mater. Interfaces 2013, 5, 10760−10766.
(62) Kuang, H. H.; Wu, Y. J.; Zhang, Z. Y.; Li, J. Z.; Chen, X. S.; Xie, Z. G.; Jing, X. B.; Huang, Y. B. Double Ph-Responsive Supramolecular Copolymer Micelles Based on the Complementary Multiple Hydrogen Bonds of Nucleobases and Acetalated Dextran for Drug Delivery. Polym. Chem. 2015, 6, 3625−3633.
(63) Kauffman, K. J.; Do, C.; Sharma, S.; Gallovic, M. D.; Bachelder, E. M.; Ainslie, K. M. Synthesis and Characterization of Acetalated Dextran Polymer and Microparticles with Ethanol as a Degradation Product. ACS Appl. Mater. Interfaces 2012, 4, 4149−4155.
(64) Gallovic, M. D.; Montjoy, D. G.; Collier, M. A.; Do, C.; Wyslouzil, B. E.; Bachelder, E. M.; Ainslie, K. M. Chemically Modified Inulin Microparticles Serving Dual Function as a Protein Antigen Delivery Vehicle and Immunostimulatory Adjuvant. Biomater. Sci. 2016, 4, 483−493.
(65) Gallovic, M. D.; Bandyopadhyay, S.; Borteh, H.; Montjoy, D. G.; Collier, M. A.; Peine, K. J.; Wyslouzil, B. E.; Bachelder, E. M.; Ainslie, K. M. Microparticles Formulated from a Family of Novel Silylated Polysaccharides Exhibit Tunable Hydrolytic Degradability. J. Mater. Chem. B 2016, 4, 4302.

DOI:10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

WORKSPACE FORM

1-800-518-4726
SUPPORT@GRANTS.GOV

This Workspace form is one of the forms you need to complete prior to submitting your Application Package. This form can be completed in its entirety offline using Adobe Reader. You can save your form by clicking the "Save" button and see any errors by clicking the “Check For Errors” button. In-progress and completed forms can be uploaded at any time to Grants.gov using the Workspace feature.

When you open a form, required fields are highlighted in yellow with a red border. Optional fields and completed fields are displayed in white. If you enter invalid or incomplete information in a field, you will receive an error message. Additional instructions and FAQs about the Application Package can be found in the Grants.gov Applicants tab.

OPPORTUNITY & PACKAGE DETAILS:

Opportunity Number:	HR001118S0017
Opportunity Title:	PREventing EMerging Pathogenic Threats
Opportunity Package ID:	PKG00237724
CFDA Number:	12.910
CFDA Description:	Research and Technology Development
Competition ID:	
Competition Title:	
Opening Date:	01/19/2018
Closing Date:	03/27/2018
Agency:	DARPA - Biological Technologies Office
Contact Information:	BAA Coordinator PREEMPT@darpa.mil

APPLICANT & WORKSPACE DETAILS:

Workspace ID:	WS00094394
Application Filing Name:	Project DEFUSE
DUNS:	0770900660000
Organization:	ECOHEALTH ALLIANCE INC.
Form Name:	R & R Subaward Budget 10 YR Subform
Form Version:	1.4
Subform Name:	UNC Medical School
Requirement:	Optional
Download Date/Time:	Mar 06, 2018 05:29:59 PM EST
Form State:	

FORM ACTIONS:

RESEARCH & RELATED BUDGET - Budget Period 1

OMB Number: 4040-0001
Expiration Date: 10/31/2019

ORGANIZATIONAL DUNS: 6081952770000 **Enter name of Organization:** University of North Carolina Medical School

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 1** **Start Date:** **End Date:**

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
Prof.	Ralph		Baric								

Project Role: Co-Investigator

Additional Senior Key Persons: Add Attachment | Delete Attachment | View Attachment

Total Funds requested for all Senior Key Persons in the attached file

Total Senior/Key Person

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Post Doctoral Associates						
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						

Total Number Other Personnel

Total Other Personnel

Total Salary, Wages and Fringe Benefits (A+B)

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment item	Funds Requested ($)

Additional Equipment: Add Attachment | Delete Attachment | View Attachment

Total funds requested for all equipment listed in the attached file	
Total Equipment	

D. Travel

	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	
2. Foreign Travel Costs	
Total Travel Cost	

E. Participant/Trainee Support Costs

	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other	
Number of Participants/Trainees / **Total Participant/Trainee Support Costs**	

F. Other Direct Costs

		Funds Requested ($)
1.	Materials and Supplies	
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.		
9.		
10.		
	Total Other Direct Costs	

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)

Total Indirect Costs	

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	

J. Fee

Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	

L. Budget Justification

(Only attach one file.) Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Cumulative Budget

		Totals ($)
Section A, Senior/Key Person		
Section B, Other Personnel		
Total Number Other Personnel		
Total Salary, Wages and Fringe Benefits (A+B)		
Section C, Equipment		
Section D, Travel		
1. Domestic		
2. Foreign		
Section E, Participant/Trainee Support Costs		
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other		
6. Number of Participants/Trainees		
Section F, Other Direct Costs		
1. Materials and Supplies		
2. Publication Costs		
3. Consultant Services		
4. ADP/Computer Services		
5. Subawards/Consortium/Contractual Costs		
6. Equipment or Facility Rental/User Fees		
7. Alterations and Renovations		
8. Other 1		
9. Other 2		
10. Other 3		
Section G, Direct Costs (A thru F)		
Section H, Indirect Costs		
Section I, Total Direct and Indirect Costs (G + H)		
Section J, Fee		
Section K, Total Costs and Fee (I + J)		

Choose flights

« New search

Your trip summary

Main Cabin

Round trip (Non-refundable)

$343 per person

Total $342.60 (all passengers)
Price and tax information

Good value with benefits

- Carry-on & personal item (access to overhead bin)
- Choose your seat (fee may apply)
- Eligible for upgrades on American flights
- Flight changes allowed (fee applies)
- General boarding

Includes taxes and carrier imposed fees.
Baggage and optional service fees

Depart Raleigh/ Durham, NC to Washington, DC

Monday, October 1, 2018

5:42 AM → 7:04 AM 1h 22m Nonstop Main Cabin

AA 4230 ■ ERJ-Embraer ERJ-145 ■ Operated by Trans States As American Eagle

Details | Change

Return Washington, DC to Raleigh/ Durham, NC

Wednesday, October 3, 2018

5:05 PM → 6:26 PM 1h 21m Nonstop Main Cabin

AA 5185 ■ CR7-Canadair RJ 700 ■ Operated by PSA Airlines As American Eagle

Details | Change

Earn up to a $200 statement credit

Plus, receive 40,000 bonus miles after qualifying purchases with this credit card offer.

Learn more

Log in and continue | Continue as guest

Choose flights

« New search

Your trip summary

Main Cabin

Round trip (Non-refundable)

$282 per person

Total $281.59 (all passengers)
Price and tax information

Good value with benefits

- Carry-on & personal item (access to overhead bin)
- Choose your seat (fee may apply)
- Eligible for upgrades on American flights
- Flight changes allowed (fee applies)
- General boarding

Includes taxes and carrier imposed fees.
Baggage and optional service fees

Depart Raleigh/ Durham, NC to New York, NY

Monday, September 17, 2018

7:45 AM → 9:22 AM 1h 37m Nonstop Main Cabin

AA 4050 ■ ERD-Embraer ERJ-140 ■ Operated by Envoy Air As American Eagle

Details | Change

Return New York, NY to Raleigh/ Durham, NC

Friday, September 21, 2018

5:45 PM → 7:33 PM 1h 48m Nonstop Main Cabin

AA 3482 ■ ERD-Embraer ERJ-140 ■ Operated by Envoy Air As American Eagle

Details | Change

Earn up to a $200 statement credit

Plus. receive 40,000 bonus miles after qualifying purchases with this credit card offer.

Learn more

Log in and continue | Continue as guest

MY TRIPS BOOK A TRIP FLIGHT STATUS CHECK IN SIGN UP LOG IN

BOOK A TRIP

New Search Flights Passengers Payment

FLIGHTS

MON 17 SEP

RDU - WUH
12:55 PM 11:00 PM ARRIVES NEXT DAY

DL 725, DL 583, DL 6504 [1]
22h 5m | MULTI-STOP

Main Cabin (L) |
Main Cabin (L) |
Economy (L)
Changeable / Nonrefundable

Delta Air Lines Baggage Information

Change Flight

▸ Details View Seats + More

FRI 21 SEP

WUH - RDU
07:25 AM 5:53 PM

DL 6505 [1], DL 186, DL 1774
22h 28m | MULTI-STOP

Economy (K) |
Main Cabin (K) |
Main Cabin (K)
Changeable / Nonrefundable

Delta Air Lines Baggage Information

Change Flight

▸ Details View Seats

Price per Passenger $1,256.00

Taxes, Fees and Charges $108.01

GET MORE WITH
FLEX MAIN CABIN

$2,158.50
Per Person Each Way

UPGRADE

LUXURY AT A HIGHER ALTITUDE
DELTA ONE®

$7,843.50
Per Person Each Way

UPGRADE See Details

Total Price (USD) $1,364.01

Earn a $150 Statement Credit and 50,000 Bonus Miles

Apply for the Gold Delta SkyMiles Credit Card from American Express

- **$150 Statement Credit** after you use your new Card on a Delta purchase in your first 3 months*
- **50,000 bonus miles** after you make $2,000 in purchases on your new Card within your first 3 months. Special Offer Expires 4/11/2018
- **First checked bag free** and **Priority Boarding** on Delta flights
- **$0 introductory annual fee** for the first year, then $95 Rates & Fees | Offer Terms | Benefit Terms

Terms and Conditions Apply

☐ **Yes! I'd like to apply now for a Card and then complete my booking**

**Statement credit will be issued approximately 8-12 weeks after making your first Delta purchase.*

Statement Credit* -$150.00

Total After Statement Credit (USD) $1,214.01

CONTINUE

TERMS AND CONDITIONS

GENERAL CONDITIONS OF PURCHASE

You agree to accept all **Fare Rules** for each flight, all **Trip Extras Terms & Conditions**, and all terms in Delta's applicable **Contract of Carriage**. Once your ticket is purchased, **Risk-Free Cancellation** may apply. No contract exists until you receive confirmation that payment was received and processed.

View **Fare Rules, Change & Cancellation Policies**. This ticket is **changeable/ nonrefundable**. Fees may apply.

The advertised price is not an offer and is subject to change. All prices are (USD) unless otherwise noted. You may see separate transactions on your credit card based on the vendors and the countries you are purchasing. Amounts may vary dependent on the rate of exchange at time of transaction.

In-flight services and amenities may vary and are subject to change.

Final baggage fees will be assessed and charged at time of check-in. **Baggage fees** may change based on the date of service or frequent flyer status. CURRENCY CALCULATOR

JOURNAL OF VIROLOGY , Mar. 2008, p. 2274–2285 Vol. 82, No. 5
0022-538X/08/$08.00ff 0 doi:10.1128/JVI.02041-07
Copyright © 2008, American Society for Microbiology. All Rights Reserved.

Mechanisms of Zoonotic Severe Acute Respiratory Syndrome Coronavirus Host Range Expansion in Human Airway Epithelium ff

Timothy Sheahan, [1] Barry Rockx, [2] Eric Donaldson, [1] Amy Sims, [2] Raymond Pickles, [1,3] Davide Corti, [4] and Ralph Baric [1,2] *

Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina [1]; Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina [2]; Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina [3]; and Institute for Research in Biomedicine, Bellinzona, Switzerland [4]

Received 14 September 2007/Accepted 10 December 2007

In 2003, severe acute respiratory syndrome coronavirus (SARS-CoV) emerged and caused over 8,000 human cases of infection and more than 700 deaths worldwide. Zoonotic SARS-CoV likely evolved to infect humans by a series of transmission events between humans and animals for sale in China. Using synthetic biology, we engineered the spike protein (S) from a civet strain, SZ16, into our epidemic strain infectious clone, creating the chimeric virus icSZ16-S, which was infectious but yielded progeny viruses incapable of propagating in vitro. After introducing a K479N mutation within the S receptor binding domain (RBD) of SZ16, the recombinant virus (icSZ16-SK479N) replicated in Vero cells but was severely debilitated in growth. The in vitro evolution of icSZ16-SK479N on human airway epithelial (HAE) cells produced two viruses (icSZ16-SK479N D8 and D22) with enhanced growth on HAE cells and on delayed brain tumor cells expressing the SARS-CoV receptor, human angiotensin I converting enzyme 2 (hACE2). The icSZ16-SK479N D8 and D22 virus RBDs contained mutations in ACE2 contact residues, Y442F and L472F, that remodeled S interactions with hACE2. Further, these viruses were neutralized by a human monoclonal antibody (MAb), S230.15, but the parent icSZ16-SK479N strain was eight times more resistant than the mutants. These data suggest that the human adaptation of zoonotic SARS-CoV strains may select for some variants that are highly susceptible to select MAbs that bind to RBDs. The epidemic, icSZ16-SK479N, and icSZ16-SK479N D22 viruses replicate similarly in the BALB/c mouse lung, highlighting the potential use of these zoonotic spike SARS-CoVs to assess vaccine or serotherapy efficacy in vivo.

Diseases caused by emerging viruses such as human immunodeficiency virus, Ebola virus, influenza virus H5N1, West Nile virus, and dengue virus have had a profound impact on global public health (28). In 2003, a novel coronavirus, severe acute respiratory syndrome coronavirus (SARS-CoV), emerged suddenly as the causative agent of SARS and spread worldwide, causing about 8,000 cases and ffi700 deaths (3, 17, 41). SARS-CoV most likely evolved from viruses circulating within the Chinese horseshoe bat and other bat species that are believed to be the natural animal reservoirs (18). Within live-animal markets in the Guangdong region of China, it is hypothesized that the close cohabitation of bats and palm civets allowed for subsequent cross-species transmission and amplification of bat strains in civets (9, 18). Palm civets then served as an intermediate host for the subsequent viral evolution of strains that could infect and transmit between humans (18). Clinical data suggest that the sporadic early human SARS-CoV infections were significantly less pathogenic than later ones and that a progressive series of adaptive mutations was necessary for increased human-to-human transmission and the expanding phases of the epidemic (2, 9, 41). Despite initial reports that civet strains SZ16 and SZ3 could be propagated in cell culture, subsequent studies have indicated that these viruses could not be successfully maintained in culture, thereby hampering our understanding of their pathogenicities and mechanisms of cross-species transmission in humans (19).

Within the past 4 years, multiple newly emerging coronaviruses of human relevance have been identified, highlighting the emerging disease potential of the coronavirus family (35, 41, 50, 53). SARS-CoV and human coronavirus HKU1 are newly emerging members of coronavirus genogroup II, and both cause pneumonic disease in humans, with SARS-CoV being the most pathogenic of the known human coronaviruses (7, 17, 41, 52, 53). Viruses related to the SARS-CoV epidemic strain have recently been found in Chinese horseshoe bats during surveillance of wild animals in Hong Kong (18). Since viruses similar to the epidemic strain have been found circulating within zoonotic pools, there is the potential for yet another reemergence (18). Moreover, a promiscuous RNA-dependent RNA polymerase coupled with high-frequency recombination rates makes the evolution of future human coronavirus pathogens a real possibility (6, 36, 57).

The coronavirus spike glycoprotein (S) is a key determinant of host specificity, and elucidating the molecular mechanisms of viral host expansion may help us understand the events that rendered SARS-CoV pathogenic to humans (16, 24). Virus sequence data isolated throughout the course of the epidemic suggest that the S gene was under heavy positive selection

* Corresponding author. Mailing address: Department of Epidemiology, 2107 McGavran-Greenberg, CB#7435, University of North Carolina, Chapel Hill, NC 27699-7435. Phone: (919) 966-3895. Fax: (919) 966-0584. E-mail: rbaric@email.unc.edu.

ff Published ahead of print on 19 December 2007.

Downloaded from jvi.asm.org at UNIVERSITY OF NORTH CAROLINA on December 20, 2008

during the early phase of the epidemic but eventually stabilized as the epidemic progressed (2). The S protein is 1,225 amino acids (aa) in length and can be divided into two main functional domains, S1 and S2. The S1 region (aa 17 to 756) contains the receptor binding domain (RBD; aa 318 to 510), while the S2 region (aa 757 to 1225) contains the two heptad repeat regions responsible for viral fusion and a transmembrane domain (aa 1189 to 1227) that anchors S to the viral envelope. SARS-CoV entry into the host cell is mediated by a receptor, angiotensin I converting enzyme 2 (ACE2), and perhaps other coreceptors (15, 22). ACE2 has been detected on the apical surfaces of ciliated cells within the lung epithelia as well as in the kidneys and colon (12). After S and receptor binding, the virus enters the cell by receptor-mediated endocytosis. Cleavage of the SARS-CoV S by cathepsin L within the endosome is required for SARS-CoV infection and precedes fusion (42).

Sequences isolated throughout the initial epidemic from 2002 to 2003 and during the reemergence from 2003 to 2004 chronicle the mutations in S that may have allowed for host expansion. During the epidemic and the reemergence, the early 2003 SARS-CoV animal isolate, SZ16, and the 2004 reemergent human isolate, GD03, were among the most divergent viruses isolated from civets and humans, respectively. SARS-CoV SZ16 was identified in palm civets in live-animal markets within the Guangdong region of China during the epidemic, and its S protein differs from that of the epidemic strain, SARS Urbani, in 18 amino acids, 16 of which reside in the S1 domain. Using pseudotyped viruses, recent work has shown that the mutation of residue 479 within the SZ16 RBD was sufficient to allow for the host range expansion of zoonotic SARS-CoV spike-bearing pseudovirus to infect human cells (21, 24).

In this study, we describe the synthetic construction and characterization of a SARS-CoV chimera bearing a zoonotic SZ16 S protein. Importantly, we find that the mutations acquired during the in vitro evolution of a zoonotic, S-bearing recombinant virus in human airway epithelial (HAE) cells can vary from those that occurred during the epidemic, highlighting the adaptive plasticity of the zoonotic S protein. Also, these data suggest that HAE cells can be used as a model to evaluate the possible avenues of SARS-CoV zoonotic S evolution toward the efficient infection of human cells. Lastly, recombinant viruses bearing zoonotic S proteins replicated efficiently in mice and will serve as a valuable tool in assessing the efficacies of vaccines and therapies.

MATERIALS AND METHODS

Viruses and cells. The recombinant epidemic virus strain "icSARS" (GenBank accession no. AY278741), icSZ16-S (GenBank accession no. AY304488), and the icSZ16-S K479N, icSZ16-S K479N D8, and icSZ16-S K479N D22 mutant strains were propagated on Vero E6 cells as described previously (58). Virus stocks used throughout this study were grown and titers were determined by plaque assays with Vero E6 cells as described previously (58). Vero E6 and delayed brain tumor (DBT) cells were grown in minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% FCII (HyClone, South Logan, UT) and gentamicin/kanamycin (UNC Tissue Culture Facility). Mink lung epithelial cells (Mv1lu) were grown in Dulbecco's minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% FCII (HyClone, South Logan, UT) and gentamicin/kanamycin (UNC Tissue Culture Facility). HAE cells were obtained from airway tissues harvested from patients undergoing elective surgery according to the UNC Institutional Review Board-approved protocols of the UNC Cystic Fibrosis Center Tissue Culture Core. Briefly, primary cells were expanded on plastic to generate passage 1 cells and plated at a density of 250,000 cells per well on permeable Transwell-Col (12-mm-diameter) supports (8, 33). HAE cell cultures were generated by the provision of an air-liquid interface for 4 to 6 weeks to form well-differentiated, polarized cultures that resemble in vivo pseudostratified mucociliary epithelia (33). All virus work was performed in a class II biological safety cabinet in a certified biosafety level 3 laboratory containing redundant exhaust fans and by laboratorians wearing Tyvek suits and powered air-purifying respirators as described previously (56).

Construction of chimeric SARS-CoVs bearing the SZ16 spike protein icSZ16-S. The infectious clone of the epidemic strain from R. Baric's laboratory is divided into six subgenomic cDNA clones (A to F) that span the SARS-CoV genome. The first two-thirds of the S gene is located within the icSARS E fragment (nucleotides [nt] 21492 to 24056), and the last third is located within the icSARS F fragment (nt 24057 to 25259). A synthetic DNA containing SZ16 nt 21541 to 24056 was purchased from Blue Heron Technology (Bothell, WA). The fragment was digested with AgeI and XbaI (NEB, Ipswich, MA) and cloned into the existing icSARS-E pSMART plasmid, replacing the Urbani S glycoprotein with the SZ16 variant sequence to create icSZ16-E pSMART. Within the SARS F fragment, the two remaining coding changes of the SZ16 S were introduced via PCR and the class II restriction enzyme AarI. Briefly, three amplicons, A, B, and C, were generated by PCR using Expand high-fidelity polymerase (Roche, Indianapolis, IN). The primer pairs used are as follows: amplicon A, PasIF (5′-CTGTTTTCCCTGGGATCGC-3′) and AarIM1NR (5′-NNNNNNCACCTGCTTTTGGGCAACTCCAATGCC-3′); amplicon B, AarIM1NF (5′-NNNNNNCACCTGCAGTTGCCCAAAATGTTCTCTATGA GAAC-3′) and AarIM2NR (5′-NNNNNNCACCTGCATTTCTTCTTGAATG TTGACGACAGAAG-3′); and amplicon C, AarIM2NF (5′-NNNNNNCACCT GCTCAAGAAGAAATTGACCGCCTC-3′) and BamHIR (5′-CATAAATTG GATCCATTGCTGG-3′). Amplicons A, B, and C were digested with AarI for 1.5 h at 37°C (Fermentas, Burlington, Ontario, Canada), gel purified, and ligated to create the fragment ABC. The ABC ligation products were gel purified, and TA was cloned into TOPO-XL (Invitrogen, Carlsbad, CA) to create pTOPO-XL ABC. The pTOPO-XL ABC subclones were confirmed by sequencing. pTOPO-XL ABC was BamHI digested at 37°C for 1 h, which released a 2,059-nt fragment containing the icSZ16 F fragment S sequence. icSARS F-pSMART was digested with BamHI at 37°C for 1 h, which excised the epidemic strain S sequence and released a 5,749-bp vector fragment. The 2,059-bp SZ16 insert and the 5,749-bp vector fragments were gel purified using a QIAquick gel purification kit (Qiagen, Valencia, CA) and ligated (NEB, Ipswich, MA) to create icSZ16 F-pSMART.

Construction of the chimeric icSZ16-S K479N mutant. The icSZ16-E pSMART sequence for S residue 479 was mutated from lysine (AAA) to an asparagine (AAT) using overlap PCR. Amplicon A (NcoIF [5′-TGTTTCTAA ACCCATGGGTACACAG-3′] and SZ16479R [5′-CCATAATCATTTAATGG CCAATAAC-3′]) and amplicon B (SZ16479NF [5′-GTTATTGGCCATTAAA TGATTATGG-3′] and XbaIR [5′-GGGCCCTCTAGAGATCGAGC-3′]) were generated using Expand high-fidelity polymerase (Roche, Indianapolis, IN). To join the fragments, amplicons A (1,010 bp) and B (1,153 bp) were used as templates in overlapping PCR with primers NcoIF and XbaIR. The final AB amplicon was purified using a Qiagen PCR purification kit (Qiagen, Valencia, CA) and digested with NcoI and XbaI (NEB, Ipswich, MA) for 1 h at 37°C. The digested amplicon (2.2 kb) was gel purified using a QIAquick gel purification kit (Qiagen, Valencia, CA), ligated to the 5.3-kb NcoI/XbaI fragment of icSARS SZ16-E pSMART, and sequence verified.

Isolation of recombinant viruses. From the ligation of appropriate infectious clone fragments, icSZ16-S or icSZ16-S K479N full-length chimeric cDNA was generated, and full-length transcripts were synthesized, mixed with polyadenylated N gene transcripts, and then electroporated into cells as previously described (56). Clarified supernatants from the icSZ16-S or icSZ16-K479N electroporations were passaged onto naive Vero E6 cells to confirm the presence of replicating virus. If a cytopathic effect (CPE) was not detected, viruses were passaged every 48 h onto Vero E6 cells until cytopathology was observed. With icSZ16-S K479N, a mild CPE was observed at passage 3 (p3), and a more robust CPE was seen by p6. The icSZ16-S K479N p3 and p6 viruses were plaque purified in Vero E6 cells. The S, 3a, E, and M genes of the recombinant viruses were sequence verified, and stocks were grown in T75 flasks and stored at −80°C for future use.

RT-PCR to detect the subgenomic leader containing transcripts. Reverse transcriptase (RT)-PCR to detect the subgenomic leader containing transcripts was performed to demonstrate viral replication. RNA from virus-infected cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), according to protocol. One to five micrograms of total RNA was used to generate cDNA by

Downloaded from jvi.asm.org at UNIVERSITY OF NORTH CAROLINA on December 20, 2008

Superscript II (Invitrogen, Carlsbad, CA), using random hexamer primers (Invitrogen, Carlsbad, CA). cDNA was then used as a template for the PCR using *Taq* polymerase (NEB, Ipswich, MA) with a leader forward primer (CTCTTG TAGATCTGTTCTCTAAACGAAC) and a reverse primer in the M gene (TT ACTGTACTAGCAAAGCAATATTGTCG). The detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (primer GAPDHF [5′-CATGGGGAA GGTGAAGGTCG-3′] and primer GAPDHR [5′-TTGATGGTACATGACAA GGTGC-3′]) messages by RT-PCR was done as a positive control. The PCR products were separated by gel electrophoresis on 1.8% agarose–Tris-acetate-EDTA gels and visualized by ethidium bromide staining.

In vitro evolution of icSARS SZ16 K479N in HAE cell cultures for 22 days. HAE cell cultures were created as described previously (43). The cultures were infected with 10^5 PFU of icSARS K479N in a 200-μl volume. After a 2-h infection, the inoculum was removed, and the cells were washed briefly with Dulbecco's phosphate-buffered saline (DPBS) and then maintained for 4 days. After 4 days of infection, the apical surfaces of the cultures were rinsed, and supernatants were placed onto the apical surfaces of fresh cultures for 1 h, washed, and maintained for another 4 days. Apical washes were then plaque purified to isolate the icSZ16-S K479N D8 virus. A total of 200 μl of day 8 supernatants was used to infect naive HAE cell cultures as described above, and the cultures were passaged two additional times at 4-day intervals and then three times every 48 h to preferentially enrich for more efficiently replicating virus variants. After 22 days of selection for efficient growth in HAE cells, the apical surfaces were rinsed and the supernatants were plaque purified to isolate the icSZ16-S K479N D22 virus. Plaques were expanded on 60-mm dishes, and RNA was isolated using TRIzol (Invitrogen Carlsbad, CA). Amplicons spanning from the S, 3a, E, and M genes were generated by RT-PCR and directly sequenced.

Generation of hACE2-expressing DBT cells. A plasmid carrying an N-terminal myc-tagged human ACE2 (hACE2) was kindly donated by M. Farzan of Harvard Medical School. The hACE2 gene was amplified by PCR using Expand high-fidelity polymerase (Roche, Indianapolis, IN) (ACE2For [5′-CACCATGTCAA GCTCTTCCTGGCTCC-3′] and ACE2Rev [5′-CTAAAAGGAGGTCTGAAC ATCATCAGTG-3′]). Amplicons were gel purified using the QIAquick gel purification kit (Qiagen, Valencia, CA) and cloned into pcDNA3.1/V5-His (Invitrogen, Carlsbad, CA) according to protocol to create pcDNA3.1/V5-His hACE2. The ACE2 gene plasmid was sequence verified. DBT cells were transfected with 4 μg of pcDNA3.1/V5-His hACE2 using Fugene reagent (Roche, Indianapolis, IN). After 24 h, the cells were placed under drug selection using 700 μg/ml of G418 (Invitrogen, Carlsbad, CA) in complete growth medium and remained under drug selection throughout all experiments. The cells were passaged four times, sorted for high ACE2 expression by flow cytometry, and then expanded under drug selection before use.

Growth curve analysis of HAE, Vero E6, DBT hACE2, or DBT cells. HAE cells were infected with 4.4×10^4 PFU of the recombinant epidemic strain icSARS, icSZ16-S K479N p3, icSZ16-S K479N D8, or icSZ16-S K479N D22 for 2 h at 37°C, after which the inoculum was removed and the apical surface rinsed with DPBS. At 0, 6, 12, 24, 36, 48, and 72 h postinfection (hpi), the apical surfaces of the HAE cells were rinsed with 200 μl DPBS and the samples stored at −80°C until titration by plaque assay. Vero E6 and DBT hACE2 cells were infected with icSARS, icSZ16-S K479N p3, icSZ16-S K479N p6, icSZ16-S K479N D8, or icSZ16-S K479N D22 at a multiplicity of infection (MOI) of 0.01 for 1 h at 37°C, after which the inoculum was removed, the monolayer was rinsed with DPBS, and growth medium was added. DBT cells were infected in a similar manner and with the same panel of viruses as the Vero E6 and DBT hACE2 cells, except that icSZ16-S K479N p3 was excluded from the experiment. The cell media were sampled at 0, 6, 12, 24, and 36 hpi, and the samples were stored at −80°C until titers were determined by plaque assay (56). The growth curve data presented are representative of two separate experiments.

Immunohistochemistry of infected HAE cells. To detect SARS-CoV antigens in HAE cells, infected or mock-infected cultures were fixed in 4% paraformaldehyde (PFA) for 24 h, transferred to 70% ethanol, and prepared as paraffin-embedded histological sections by the UNC Cystic Fibrosis Center Morphology and Morphometrics Core. After deparaffinization, histological sections were incubated for 1 h in PBS containing 3% bovine serum albumin. Primary antibodies directed against SARS N or tubulin were applied at a 1:100 dilution in PBS with 1% bovine serum albumin overnight and detected with fluorescein isothiocyanate or Texas Red secondary antibodies (anti-mouse antibodies conjugated to Texas Red; Jackson ImmunoResearch). Immunofluorescence was visualized with a Leica Leitz DMIRB inverted fluorescence microscope equipped with a cooled-color, charge-coupled digital camera (MicroPublisher, Q-Imaging). A tricolor filter cube set (green fluorescent protein-Texas Red-DAPI [4′,6-diamidino-2-phenylindole]) was used to show the morphology of the tissue section (by combining low autofluorescence levels across the three filters), thus aiding the determination of fluorescent-antibody localization to specific regions of the cells.

Plaque reduction neutralization titer assay. Neutralizing titers were determined by plaque reduction neutralization titer assay (PRNT) (40). Twenty-four hours prior to infection, six-well plates were seeded with 5×10^5 Vero E6 cells/well. A human monoclonal antibody (MAb), S230.15, directed against the SARS-CoV RBD and an isotype control antibody, D2.2, directed against cholera toxin were kindly provided by A. Lanzavecchia. The MAbs were serially diluted twofold and incubated with 100 PFU of either icSARS, icSZ16-S K479N p6, icSZ16-S K479N D8, or icSZ16-S K479N D22 for 1 h at 37°C. Virus and antibodies were then added to six-well plates of Vero E6 cells in duplicate and incubated at 37°C for 1 h, after which the cells were overlaid with 3 ml of 0.8% agarose in medium. The plates were incubated for 48 h at 37°C and stained with neutral red for 3 h, and the plaques were counted. The percentages of neutralization were calculated as follows: 1 − (number of plaques with antibody/number of plaques without antibody) × 100%.

Infection of 6-week-old and senescent BALB/c mice. Six-week-old female BALB/c mice (Charles River, Wilmington, MA) or 12-month-old female BALB/c mice were anesthetized with a ketamine and xylazine mixture administered intraperitoneally in a 50-μl volume. Each mouse was inoculated with 1×10^5 PFU/50 μl of icSARS, icSZ16-S K479N p6, or icSZ16-S K479N D22 intranasally (10 mice for each virus for the 6-week-old animals; 12 mice for each virus for the 12-month-old animals), and weight was monitored daily for 4 days postinfection. On days 2 and 4, three mice per group were sacrificed by isoflurane overdose, and the lungs were removed for virus titer determination. One-half of each lung was frozen at −80°C until titration by plaque assay. The lungs were weighed and homogenized, and PBS was added to generate a 20% solution. The solution was then clarified by centrifugation and serially diluted for use in a standard plaque assay with Vero E6 cells (56).

Phylogenetic analysis of SARS-CoV spikes and computer modeling. Nucleotide and protein sequence alignments of Urbani (GenBank accession no. AY27841), HKU3 (GenBank accession no. DQ022305), HC/SZ/61/03 (GenBank accession no. AY515512), SZ16 (GenBank accession no. AY304488), GD03 (GenBank accession no. AY525636), GZ02 (GenBank accession no. AY390556), and CUHK-W1 (GenBank accession no. AY278554) were created using ClustalX v1.83 (37). A neighbor joining tree was generated from the ClustalX nucleotide sequence alignment, bootstrapped 1,000 times, and exported into TreeView to create neighbor joining tree graphics (31). The numbers at each node represent the corresponding bootstrap values.

The crystal structure coordinates of the SARS-CoV RBD interacting with the hACE2 receptor (PDB code 2AJF) were used as a template to generate each set of mutations using the Rosetta Design web server (http://rosettadesign.med.unc.edu/) (21). In each case, the SARS-CoV RBD structure was analyzed using the molecular modeling tool MacPyMol (DeLano Scientific) to determine which amino acids were proximal to the amino acid(s) being targeted for replacement. Briefly, each amino acid to be altered was highlighted, and all other amino acids within an interaction distance of 5 angstroms were identified. Using the Rosetta Design website, the amino acid replacements were incorporated, and all amino acids within the 5-angstrom interaction distance were relaxed to allow the program to repack the side chains to an optimal energetic state. This process was repeated for each mutation and series of mutations. Ten models were generated for each set of mutations, and the best model, based on the lowest energy score, was selected and further evaluated using MacPyMol.

RESULTS

Construction and characterization of icSZ16-S and icSZ16-S K479N viruses. Public genetic databases and synthetic biology were harnessed to rapidly translate electronic S gene sequence information into S gene DNA fragments. The SZ16 virus was isolated in live-animal markets in China, and its sequence was published in 2003. When nucleotide sequences of S proteins isolated over the course of the epidemic are compared, the SZ16 S is similar to viruses isolated from Chinese horseshoe bats (HKU3) and to the evolved human-adapted strains (Fig. 1A). The SZ16 S differs from that of the epidemic strain in 18 amino acid positions, 16 of which reside in the S1 region and may well represent the progenitor strains for the 2003 human epi-

Downloaded from jvi.asm.org at UNIVERSITY OF NORTH CAROLINA on December 20, 2008

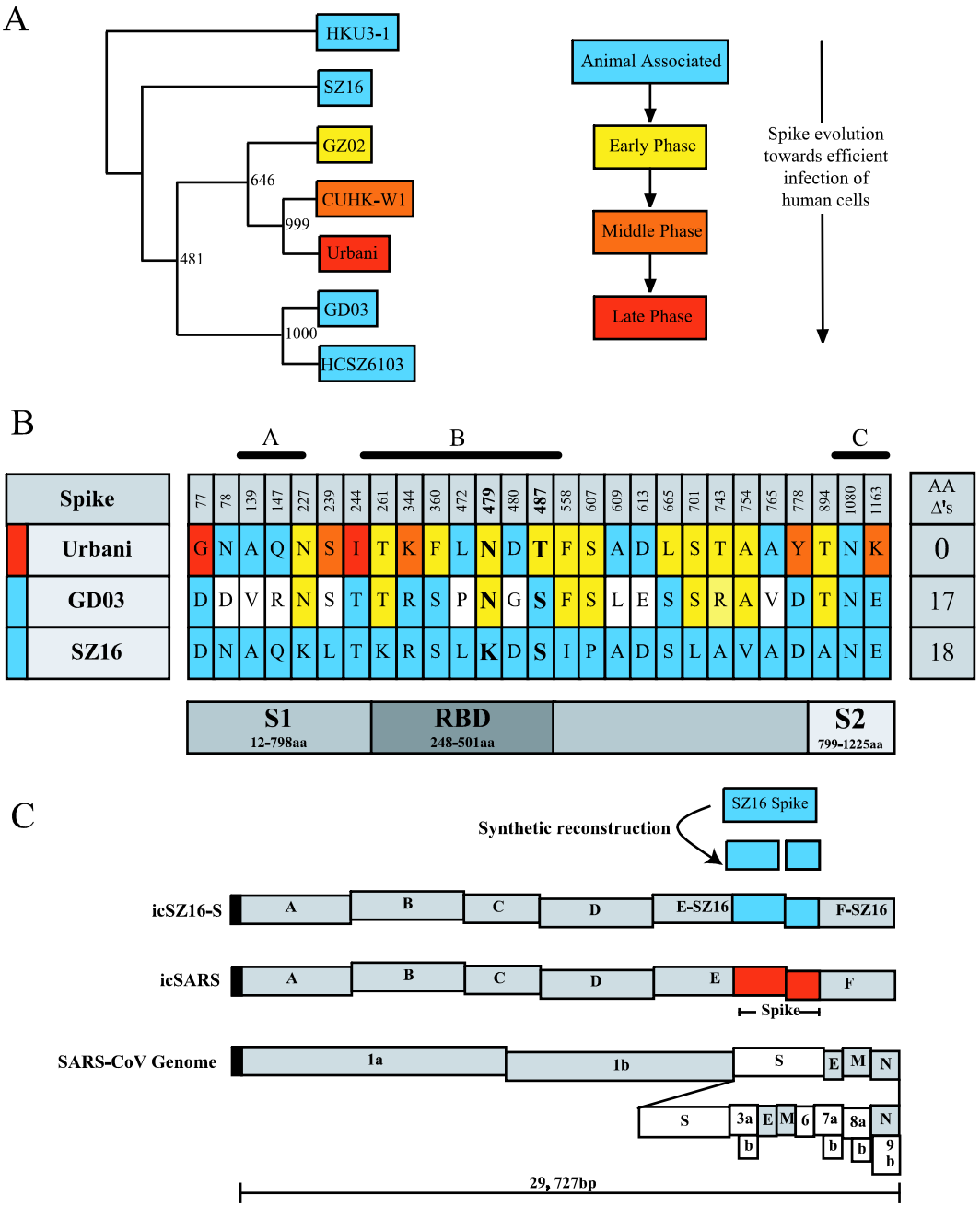


FIG. 1. Phylogenetic relationships of zoonotic SARS-CoV and construction of zoonotic spike protein chimeras within the SARS-CoV infectious clone. The colors in panels A, B, and C denote the phase of the SARS-CoV epidemic at which the viruses or amino acid residues evolved: blue, animal associated; yellow, early phase; orange, middle phase; and red, late phase. (A) Neighbor joining tree constructed from nucleotide sequences of various SARS-CoV S genes. The numbers represent bootstrap values based on 1,000 bootstrap replicates. (B) Spike protein amino acid differences between Urbani, GD03, and SZ16. Known neutralizing epitopes are denoted A, B, and C. (C) Schematic of infectious clone fragments A to F and the SARS-CoV genes contained therein. Using synthetic biology and site-directed mutagenesis, we reconstructed the SZ16 S that was then inserted into our infectious clone, replacing the epidemic strain S.

demic. Much of the SZ16 sequence variability maps in and around the three known neutralizing epitopes marked as regions A, B, and C in Fig. 1B, suggesting that human MAbs derived from human SARS-CoV infections might be less efficacious against SZ16 early civet isolates. As the SZ16 virus could not be successfully maintained in culture, we used synthetic biology and reverse genetics to molecularly resurrect a recombinant and genetically modifiable SARS-CoV bearing the SZ16 S glycoprotein (icSZ16-S) (Fig. 1C).

Although icSZ16-S recombinant virus infection was not evident by cytopathology-based assays or by plaque assay, RT-PCR confirmed the presence of virus replication within the electroporated cell culture by detecting the subgenomic leaders containing transcripts for open reading frames 3a, E, and M

Downloaded from jvi.asm.org at UNIVERSITY OF NORTH CAROLINA on December 20, 2008

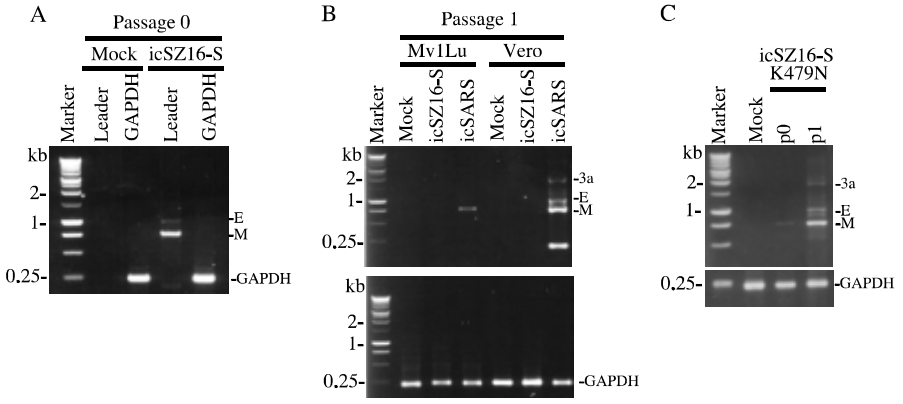


FIG. 2. SARS-CoV SZ16 spike protein chimera icSZ16-S replicates in Vero E6 cells, but infection cannot be passed in culture until a point mutation (K479N) is introduced within the RBD. The expected sizes of the target RT-PCR products are as follows: for 3a, 1,796 bp; E, 949 bp; M, 666 bp; and GAPDH, 235 bp. (A) RT-PCR for the leader containing transcripts detects actively replicating genomic RNA. RT-PCR using RNA extracted from Vero E6 cells transfected with genomic icSZ16-S RNA (passage 0) detects transcripts for the E and M genes of icSZ16-S. (B) The transfer of icSZ16-S supernatants from passage 0 to naive Vero E6 or mink lung epithelial Mv1Lu cells does not result in a productive infection. As a positive control, Vero E6 and Mv1Lu cells were successfully infected with epidemic strain supernatants, as replication was detected by the presence of the leader containing transcripts. (C) In contrast to wild-type icSZ16-S, a point mutation (K479N) in the icSZ16-S spike protein allows for passage of icSZ16-S K479N virus into Vero E6 cells.

(Fig. 2A). The transfer of the icSZ16-S supernatants from the electroporated cell culture to a naive Vero E6 or a mink lung epithelial (Mv1Lu) cell culture did not result in a productive infection, as the leader containing transcripts was not detected (Fig. 2B). As a positive control, we passaged icSARS supernatants to Vero E6 and Mv1Lu cell cultures, and both infections resulted in detectable wild-type virus replication by RT-PCR (Fig. 2B).

Since the icSZ16-S genomic RNA was capable of replicating in Vero E6 cells but the progeny virions were incapable of reinfection and amplification, we hypothesized that the infection was blocked due to inefficient S glycoprotein and ACE2 receptor interaction. In 2005, Li et al. crystallized the RBD of SARS-CoV bound to ACE2 and proposed that residues 479 and 487 of the civet S inhibited efficient usage of the hACE2 receptor (21). Using site-directed mutagenesis, we changed residue 479 of the SZ16 S from lysine to the more human tropic asparagine (K479N) and constructed and recovered the recombinant virus (icSZ16-S K479N). Unlike icSZ16-S virus infection, icSZ16-S K479N infection could be passed on to Vero E6 cell cultures by the transfer of supernatants to naive cell cultures (Fig. 2C), providing direct support for the hypothesis that SZ16 infection of human and/or primate cells is blocked by the civet S residue at position 479.

In vitro evolution of icSZ16-S K479N virus in primary HAE cell cultures. Zoonotic reintroductions represent the most likely mechanism for the reemergence of epidemic SARS-CoV strains in human populations. The SZ16 S K479N mutation permitted binding and entry into primate cells, but our preliminary data suggested that infections of HAE were inefficient (data not shown). To select for civet viruses with enhanced growth in HAE cells, cultures were apically infected with icSZ16-S K479N and maintained and passaged for a total of 22 days. The virus isolated after 8 days in culture, icSZ16-S K479N D8, contained one mutation from tyrosine to phenylalanine at position 442 within the RBD (Y442F). The virus recovered after 22 days in culture, icSZ16-S K479N D22, contained the Y442F mutation as well as an additional mutation within the RBD from leucine to phenylalanine at position 472 (L472F). No other coding mutations were found in the S, 3a, E, or M genes. Interestingly, both 442 and 472 are contact residues between S and ACE2 according to the reported crystal structure (21).

The evolved icSZ16-S K479N D8 and D22 viruses exhibit growth kinetics similar to those of the wild type in various cell types. The growth fitness of icSARS, icSZ16-S K479N p3, and the HAE-cell-evolved icSZ16-S K479N D8 and D22 viruses was assessed with HAE cells, Vero E6 cells, murine DBT cells stably expressing hACE2, and nonpermissive murine DBT cells. HAE cell cultures were infected with 4.4 # 10^4 PFU of virus for 2 h and then rinsed, and apical wash samples were taken at various intervals over 72 hpi to monitor virus growth. The epidemic strain, icSARS, grew to peak titers approaching 10^7 PFU/ml by 72 hpi (Fig. 3A). icSZ16-S K479N p3 grew poorly in the HAE cell cultures, with peak titers reaching only 10^3 PFU/ml (Fig. 3A). Compared to the epidemic strain, the icSZ16-S K479N D22 virus grew with similar growth kinetics at early time points, but peak titers were reduced by about 1 log and approached $ 10^6 PFU/ml by 72 hpi (Fig. 3A). The growth of the icSZ16-S K479N D8 virus lagged at early time points but recovered to peak titers similar to that of the icSZ16-S K479N D22 virus (Fig. 3A). In Vero E6 cells, the epidemic strain and the icSZ16-S K479N D22 virus reached peak titers of 10^7 PFU/ml 24 hpi, while icSZ16-S K479N D8, icSZ16-S K479N p3, and icSZ16-S K479N p6 grew to 10^5 PFU/ml (Fig. 3B).

To model the SARS S and hACE interactions within the primary HAE cells and to prove that these mutations were influencing docking and entry via hACE2, we constructed DBT cells that stably express hACE2. In comparison to those of HAE cells, viral growth kinetics were more rapid in DBT-hACE2, although the overall trends in growth among viruses were more or less conserved. In DBT-hACE2 cells, icSARS replicated to peak titers of 10^7 PFU/ml by 24 hpi and then diminished over time to 10^6 PFU/ml by 36 hpi (Fig. 3C).

Downloaded from jvi.asm.org at UNIVERSITY OF NORTH CAROLINA on December 20, 2008

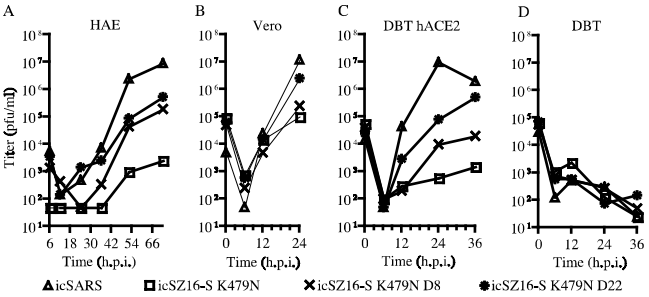


FIG. 3. Growth curve analysis of the mutant virus panel in HAE, Vero E6, DBT-hACE2, and DBT cells. (A) HAE cell cultures were infected with 4.4 # 10^4 PFU/200 " l of the indicated viruses for 2 h at 37°C. The inoculum was removed, and the apical surfaces were rinsed with DPBS. Apical-surface washes were performed at 0, 6, 12, 24, 36, 48, and 72 hpi. Virus titers were assessed for Vero E6 cells by a standard plaque assay. (B to D) Vero E6, DBT-hACE2, and DBT cells (respectively) were infected with the indicated viruses at an MOI of 0.01 for 1 h at 37°C. The inoculum was removed, cultures were rinsed with DPBS, and growth medium was added. The medium was sampled at 0, 6, 12, 24, and 36 hpi, and virus titer was assessed by plaque assay on Vero E6 cells. The data presented are representative of two separate experiments.

Similarly to the HAE model, early growth of the icSZ16-S K479N D22 virus was more rapid than that of the icSZ16-S K479N D8 virus in DBT-hACE2, but the growth of icSZ16-S K479N D8 virus never matched the icSZ16-S K479N D22 virus growth even after 36 hpi (Fig. 3C). Similarly to the HAE model, the icSZ16-S K479N p3 and the icSZ16-S K479N p6 viruses replicated poorly in DBT-hACE2 cells, reaching titers of only 10^3 PFU/ml by 36 hpi (Fig. 3C). DBT cells did not support the replication of any of the viruses within our panel, which supports the hypothesis that the expression of ACE2 within DBT-hACE2 cells is required for the growth of all viruses within our panel (Fig. 3D).

Immunohistochemistry of infected HAE cells. Host range expansion of other coronaviruses resulted in a change in cell tropism that was oftentimes associated with a change in receptor usage (4, 27). To determine the types of cells infected by the panel viruses in HAE, infected cultures were fixed and sectioned for the histological staining of SARS-CoV nucleocapsid antigen and tubulin for the staining of cilia. SARS N antigen was not detected in mock-infected cultures, though cilia on the apical surface of ciliated epithelial cells show bright staining (Fig. 4A). SARS N antigen is readily detected within the ACE2-expressing ciliated cells of icSARS (Fig. 4B)- and icSZ16-S K479N D22 (Fig. 4D)-infected cell cultures but is only sporadically detected in icSZ16-S K479N p6 (Fig. 4C)-infected cell cultures. Nevertheless, the HAE-evolved icSZ16-S K479N D22 virus and the parent zoonotic icSZ16-D K479N virus encode ciliated cell tropisms that are similar to those seen in late-phase epidemic strains.

PRNT using human MAb S230.15. Future outbreaks of SARS-CoV would most likely emerge from pools of zoonotic virus, and it is imperative that vaccinations or serotherapies be effective against both past and future emergent zoonotic strains. S230.15 is a human MAb that is broadly neutralizing through its binding to the RBD, though the specific location of the epitope is unknown (48, 59). The epidemic strain and the evolved icSZ16-S K479N D8 and icSZ16-S K479N D22 viruses are equally neutralized by S230.15 (50% neutralization at a concentration between 0.0625 and 0.125 " g/ml), but icSZ16-S K479N is eight times more resistant (50% neutralization at 0.5 " g/ml) (Fig. 5). Since icSZ16-S K479N and the evolved

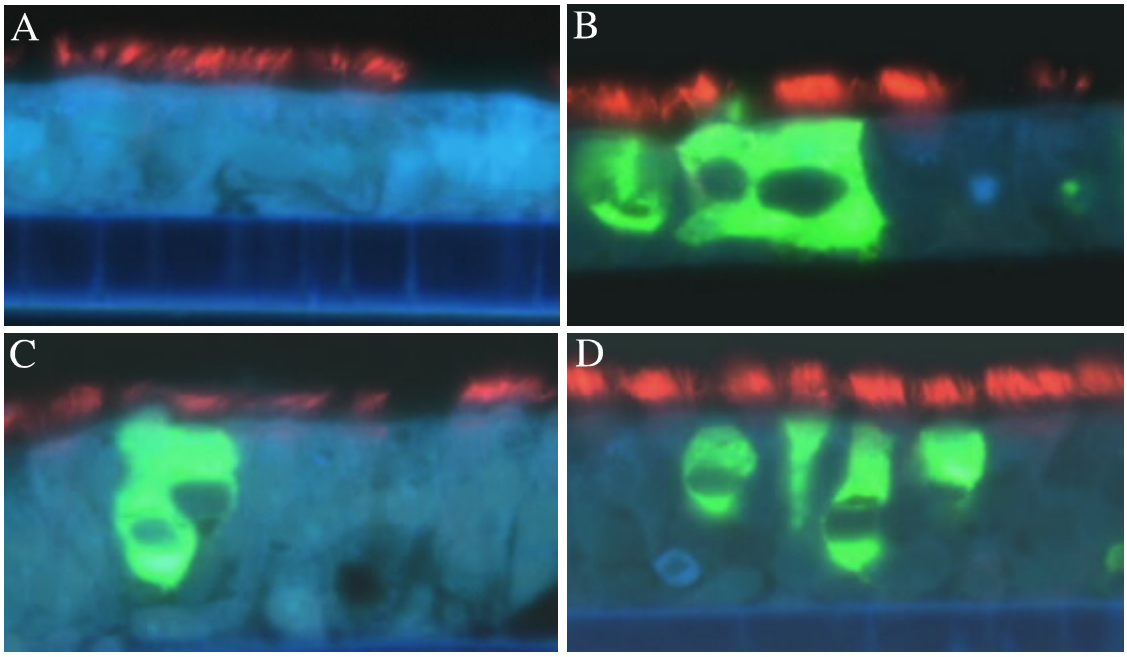


FIG. 4. Immunofluorescence staining of HAE cell cultures infected with the mutant virus panel. At 72 hpi, mock and infected HAE cell cultures were PFA fixed and paraffin embedded for tissue sectioning. Sections were stained for SARS-CoV N (fluorescein isothiocyanate) and tubulin within cilia (Texas Red) and viewed by fluorescence microscopy. (A) Mock; (B) icSARS; (C) icSZ16-S K479N; (D) icSZ16-S K479N D22.

Downloaded from jvi.asm.org at UNIVERSITY OF NORTH CAROLINA on December 20, 2008

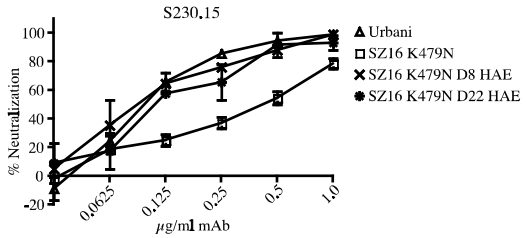


FIG. 5. PRNT assay of the mutant panel viruses using human MAb S230.15. One hundred PFU of the indicated virus was incubated at 37°C for 1 h with twofold dilutions of antibody or DPBS in duplicate. After the incubation, the virus-antibody cocktails were used to infect Vero E6 cell monolayers for 1 h, after which cultures were overlaid with growth medium containing agarose. After 48 h, plaques were enumerated. The percentages of neutralization were calculated as follows: 1 ! (number of plaques with antibody/number of plaques without antibody) # 100%. Error bars represent the standard errors of the means.

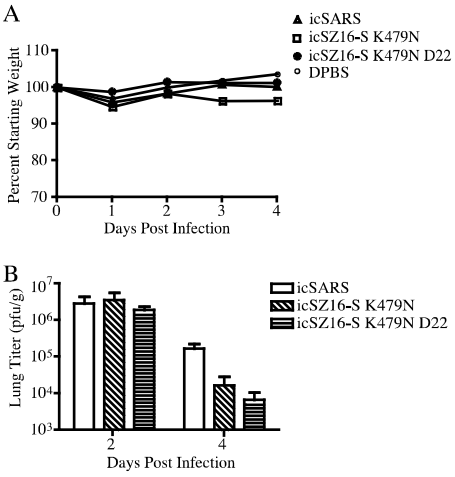


FIG. 6. Clinical signs and lung virus titers of 6-week-old BALB/c mice infected with DPBS, icSARS, icSZ16-S K479N, or icSZ16-S K479N D22. (A) Mice were infected with 10^5 PFU/50 " l intranasally (10 mice per virus), and weight was monitored daily. (B) Lungs were removed on days 2 and 4 postinfection (3 mice per group per day), homogenized, and centrifuged to pellet debris. Supernatants were used in a standard plaque assay to determine lung virus titers (PFU/g).

icSZ16-S K479N D8 virus only differ in 1 amino acid within the S protein, these data suggest that the S230.15 epitope resides near residue 442. Moreover, these data suggest that as SZ16-like zoonotic SARS-CoVs adapt to the hACE2 receptor in the human airway, they may become more susceptible to S230.15 neutralization, especially if they evolve with mutations at residue 442. As an isotype control, an irrelevant cholera-toxin-specific antibody (D2.2) was used in a PRNT assay with the mutant virus panel, and none of the viruses were neutralized at any concentration of the antibody (data not shown).

The icSZ16-SK479N and icSZ16-SK479N D22 viruses replicate in the lungs of young BALB/c mice but do not cause clinical signs in young or senescent BALB/c mice. Since the epidemic strain may not exist in nature and future epidemic SARS-CoV strains would most likely evolve from zoonotic pools of virus, it is prudent to employ an antigenically divergent panel of zoonotic challenge viruses to assess their therapeutic efficacies and cross-reactivities. To this end, it is imperative that the challenge viruses behave similarly in current animal models used to assess vaccine efficacy. Infection of young BALB/c mice with SARS Urbani typically does not cause clinical symptoms (5, 39). Young mice that were either mock infected or infected with the icSARS, icK479N-S K479N, or icSZ16-S K479N D22 viruses did not lose weight or present with other clinical signs (Fig. 6A). Clinical outcomes in senescent mice infected with icSZ16-S K479N or icSZ16-S K479N D22 mirrored those of young mice, with senescent mice losing little to no weight following infection (data not shown). The icSZ16-S K479N and icSZ16-S K479N D22 viruses replicated in the lungs of young BALB/c mice to titers similar to the epidemic strain on day 2 postinfection ($ 3 # 10^6 PFU/g) (Fig. 6B). By day 4 postinfection, the titers of the civet viruses were about 1 log lower than the epidemic strain, suggesting that the civet viruses might be cleared more rapidly (Fig. 6B).

Rosetta Design modeling of S RBD and ACE2 interactions. Rosetta Design was used to model hACE2's interactions with the RBDs of the SZ16, SZ16 K479N, icSZ16-S K479N D8, and icSZ16-S K479N D22 viruses. In contrast to the epidemic strain N479 residue, the SZ16 K479 residue appears to electrostatically clash with the ACE2 binding partners K31 and H34. After a point mutation (K479N) is introduced in the civet S RBD, the repulsive forces of the civet K479 are eradicated, allowing for S and ACE2 binding (Fig. 7A, B, and C). The Urbani RBD residues 442 and 479 are predicted to have single hACE2 binding partners (residue 442 interacts with H34; residue 479 interacts with K31) (Fig. 7A). Though residues 442 and 479 of the SZ16 K479N RBD are identical to Urbani, residues 442 and 479 are predicted to compete for hACE2 binding partner H34, and residue 442 is also predicted to interact with H34 and K31 of hACE2 (Fig. 7C). The difference in binding efficiencies of the seemingly similar Urbani and K479N RBDs is likely due to subtle alterations of the RBD hydrogen bonding network created by mutations at the peripheral RBD residue 487. Residue S487 of the SZ16 K479N RBD binds to one ACE2 residue, but T487 of Urbani binds to three residues of ACE2, thereby enhancing S and ACE2 binding interactions (Fig. 7C). The Rosetta Design model predicts that the Y442F mutation within the icSZ16-S K479N D8 S protein creates an RBD architecture similar to that of SARS Urbani, where S residues F442 and N479 have singular and unique hACE2 binding partners (residue 442 binds K31 of hACE2, and residue 479 binds H34 of hACE2) while retaining the S487 and Y41 interaction and the hydrogen bonding network (Fig. 7C and D). Finally, while the icSZ16-S K479N D8 L472 residue is predicted to have only two potential ACE2 binding partners (L79 and M82), the L472F mutation of the icSZ16-S K479N D22 S protein is predicted to increase the numbers of possible binding partners of ACE2 to three residues (L79, M82, and Y83), thereby strengthening S binding (Fig. 7D and E).

DISCUSSION

The past and present sporadic reemergences of the Chikungunya, Ebola, and Nipah viruses highlight the severe pathogenic and epidemic potential of zoonotic viruses, and the identification of the animal reservoir host is often elusive (13, 20, 25, 47, 51). Live-animal markets in China and elsewhere have

Downloaded from jvi.asm.org at UNIVERSITY OF NORTH CAROLINA on December 20, 2008

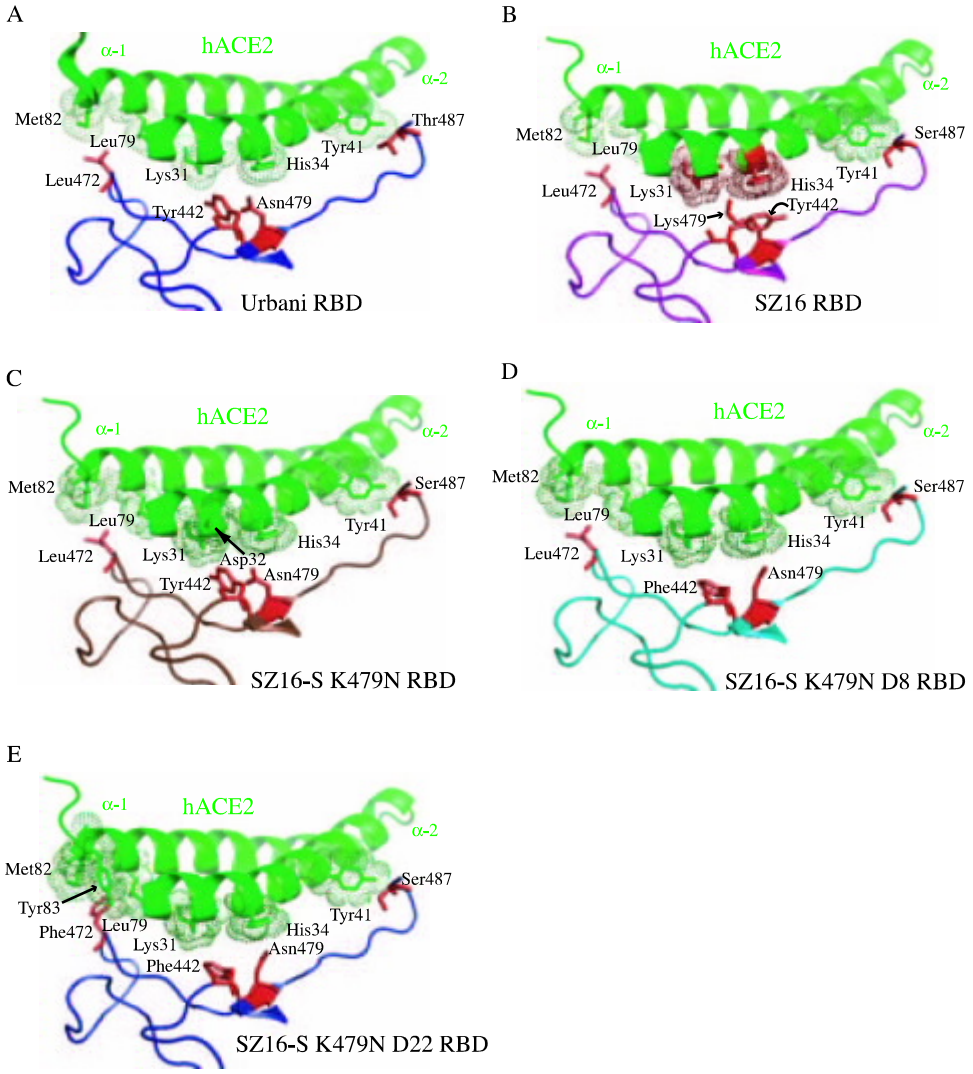


FIG. 7. Rosetta Design modeling of "evolved" mutations that enhance spike protein binding to ACE2. Rosetta Design was used to generate structural models of SZ16 and mutant RBDs that were then superimposed onto the existing crystal structure of the SARS Urbani RBD bound to ACE2. (A) Epidemic strain and hACE2 RBD architecture. (B) SZ16 and hACE2 interaction is inhibited by steric clashing, shown as red dots, of K479 of S and residues K31 and H34 of hACE2. (C) Electrostatic repulsion at residue 479 is eradicated, allowing S and ACE2 binding, but local remodeling within the RBD due to hydrogen bonding differences at residue 487 creates cross-reactions whereby residues 442 and 479 of K479N compete with each other for interaction partners H34, K31, and D32 of hACE2. (D) The Y442F mutation of icSZ16-S K479N D8 restores an optimal RBD, allowing for favorable packing to create an architecture similar to that of the wild type. (E) Leucine 472 of the icSZ16-S K479N and icSZ16-S K479N D8 S interacts with L79 and M82 of ACE2. The icSZ16-S K479N D22 L472F mutation is predicted to have hydrophobic interactions with three potential partners, L79, M82, and Y83, of hACE2 that will increase the stability of the binding. Green dots on hACE2 indicate residues which are within 4 angstroms and thus are predicted to interact with the S residues shown in red.

been proven to be an important factor in cultivating food-borne illnesses and cross-species transmission of SARS-CoV, avian influenza viruses, and other pathogens (29, 54, 55). Within the live-animal markets of China, dense human and animal populations mix, creating an environment ripe for the exchange of viruses from animals to humans and from humans to animals. During the SARS-CoV epidemic, the Himalayan palm civet was identified as the principal source of the SARS-CoV zoonosis even though 1,107 civets on farms that supplied the markets tested negative, suggesting that the civets were infected within the markets (16). Moreover, sequence analysis of bat- and civet-associated SARS-CoV has provided more definitive evidence that bat-associated SARS-CoV jumped the species barrier to infect civets and that bat and civet viruses are similar yet distinct from late-phase epidemic strain isolates (2, 9, 18, 26, 34, 41).

After the identification of ACE2 as the cellular receptor for SARS-CoV, several investigators have defined the molecular determinants of the virus and receptor interactions that allowed for the epidemic SARS-CoV host expansion. Prior to this report, key residues in the civet S gene responsible for changes in the host range were identified using SARS-CoV S pseudotyped retroviruses, but these results had not yet been confirmed in the context of wild-type SARS-CoV infection in vivo or in vitro. In support of data from Li et al., we demonstrate that the civet-spiked icSZ16-S virus was capable of a

Downloaded from jvi.asm.org at UNIVERSITY OF NORTH CAROLINA on December 20, 2008

single round of replication following transfection of full-length genomic RNA but was incapable of infecting naive Vero E6 cells in subsequent rounds of infection (21). However, the introduction of a single amino acid mutation (K479N) in the icSZ16-S virus S protein allowed for low-level spread and passage in Vero E6 cells. Both icSZ16-S K479N p3 and p6 viruses grew similarly in Vero and DBT-hACE2 cells, suggesting that the slight differences in their abilities to cause CPE did not affect virus binding, entry, or replication fitness during the time observed and at the MOI used in our assays. Though we did not use icSZ16-S K479N p6 in our HAE cell growth studies, we would expect both p3 and p6 isolates to perform similarly, since their growth kinetics in Vero and DBT-hACE2 cells were similar. Importantly, the K479N mutation did not result in a virus capable of efficient replication in HAE or DBT-hACE2 cells, supporting the argument that additional mutations are required to enhance the SZ16 S interaction with the human ACE2 receptor. The differences seen in icSZ16-S K479N growth in HAE and Vero E6 cells highlight the strength and relevance of the HAE model of the human airway. Even though the contact residues of primate and human ACE2 are predicted to be similar, the differences in icSZ16-S K479N growth in HAE and Vero E6 cells suggest that differences in primate and human ACE2 proteins (89% similar on the amino acid level) subtly influence binding efficiency or that the abundance of mucus and a unique extracellular matrix on the surfaces of HAE cells differentially antagonize virus entry. Thus, in order to study and model S protein and hACE2 interactions, HAE or DBT-hACE2 cells should be used as more relevant alternatives to Vero E6 cells. Importantly, the expression of hACE2 in a nonpermissive DBT cell line rendered these cells permissive for icSARS, icSZ16-S K479N, icSZ16-S K479N D8, and icSZ16-S K479N D22 virus infection, which strongly supports earlier hypotheses that ACE2 is the principal receptor required for the docking and entry of both human and zoonotic strains (24).

For both the canine parvovirus (CPV) and the 1918 Spanish influenza virus H1N1, point mutations in the viral structural proteins responsible for binding to the host cell were sufficient to catalyze host range expansion (14, 46). In 1978, two mutations in the capsid protein of feline parvovirus that were sufficient to catalyze host expansion evolved, causing severe canine infection and a worldwide epidemic of CPV-naive canine populations (14, 32). In 1918, the zoonotic H1N1 influenza virus acquired two mutations in hemagglutinin, changing the preferential binding from the avian to the human sialic acid receptor. When the hemagglutinin mutations were coupled with a few additional mutations in the genome, a human pandemic ensued with estimated deaths of 20 million to 40 million (32, 46). Similarly to those of the CPV and H1N1 viruses, analyses of the zoonotic and human SARS-CoV S sequences show a logical progression of S protein mutations that most likely fostered host range expansion and increased human pathogenesis (16). Of the 14 amino acids of S that contact 18 amino acids of ACE2, the K479N and S487T mutations have been shown to be equally essential for civet S adaptation to human ACE2 (21). The K479N mutation removed an electrostatic clash, allowing the S protein RBD to interact with human ACE2, while S487T appears to have subtly remodeled the RBD through hydrogen bonding differences that optimized hACE2 binding. Considering our in vitro evolution data and sequence data from the SARS epidemic, we hypothesize that at least two independent mutational pathways can optimize hACE2 binding. The first was observed during the epidemic, where the S487T mutation optimized binding as described above. The second route occurred during our in vitro evolution, whereby the Y442F mutation optimized hACE2 binding in the presence of the S487 variant. ACE2-expressing ciliated epithelial cells in the airway are targets for SARS-CoV infection in humans, primate and rodent models making the HAE model an important in vitro system for studying the molecular mechanisms of animal S adaptation to the human host (10, 11, 30, 38, 39). Since the icSZ16-S virus could not be propagated in Vero E6 cells, we introduced the K479N point mutation in the virus S that was sufficient to allow multiple rounds of replication of the civet-spiked icSZ16-S K479N virus in cell culture. Passage of the icSZ16-S K479N recombinant virus in HAE cells resulted in the emergence of S variants that could replicate more efficiently. Surprisingly, S487T was not selected with the passage of SZ16 K479N, but new mutations evolved within contact residues of the icSZ16-S K479N D8 and icSZ16-S K479N D22 virus RBDs (Y442F and L472F) that enhanced virus infection of HAE, Vero E6, and DBT-hACE2 cells. To rule out non-receptor-specific cell culture adaptations that enhance entry into cells in vitro, we infected DBT cells and did not see evidence of virus growth with any of the viruses tested. Moreover, our immunohistochemistry of infected HAE cell cultures (Fig. 4) suggests that in vitro-evolved viruses and the epidemic strain infect similar cell types, providing further support that non-receptor-specific entry adaptations had not occurred in our model. Recent passage of a related coronavirus, mouse hepatitis virus, in cell lines resulted in variants with an expanded host range due to the acquisition of a common non-receptor-specific cell culture adaptation using heparin sulfate for virus entry into cells (4). Most recently, McRoy and Baric demonstrated that the passage of mouse hepatitis virus in cell lines resulted in an expanded host range through mutations within the fusion peptide and heptad repeats, demonstrating that host range can be governed by changes in spike fusogenicity (27). From sequence analysis, we concluded that the in vitro evolved mutations of icSZ16-S K479N had not created a heparin consensus binding site (XBXBBX) and that our adaptive mutations were constrained to the RBD.

To determine the potential impact of the mutations acquired during HAE passage, we used Rosetta Design to generate structural models of the SZ16, icSZ16-S K479N, icSZ16-S K479N D8, and icSZ16-S K479N D22 RBDs and then superimposed these models onto the existing crystal structure of the SARS Urbani RBD bound to hACE2 (PDB code 2AJF) (Fig. 7A). Similarly to Li et al., we observed that residue K479 of the SZ16 S inhibited binding to hACE2 by sterically clashing with residues K31 and H34 (Fig. 7B). After the K479N mutation, the electrostatic repulsion at residue 479 is eradicated, but Rosetta Design predicts that local remodeling within the RBD creates cross-reactions whereby residues 442 and 479 of the K479N RBD compete with each other for interaction with partners H34, K31, and D32 of ACE2 (Fig. 7C). It appears that Y442 could interact with all three hACE2 residues, but N479 is predicted to interact only with H34 of hACE2. We predict that this network of competing interactions reduces the avidity be-

Downloaded from jvi.asm.org at UNIVERSITY OF NORTH CAROLINA on December 20, 2008

tween the K479N RBD and the hACE2 receptor by misaligning other key residues in the RBD. Of note, the only difference in the RBDs of SZ16 K47N and Urbani occurs at position 487. The fact that SZ16 K479N is compromised in growth compared to Urbani suggests that the hydrogen bonding differences between S487 of SZ16 K47N and Thr487 of Urbani influence the receptor interaction. During the 2003 epidemic, S487T likely evolved to eliminate this putative inhibitory effect. Interestingly, the icSZ16-S K479N D8 Y442F mutation also restores an optimal RBD by allowing for favorable amino acid packing that recreated an RBD architecture similar to that of the wild type (Fig. 7A and D). In this case, the icSZ16-S K479N D8 RBD residues 442 and 479 have distinct and unique interaction partners in hACE2, which likely restores avidity to near wild-type levels and may explain why the icSZ16-S K479N D8 virus grows more efficiently than the icSZ16-S K479N virus in cells expressing hACE2 (Fig. 3C and 7D). Thus, at least two independent routes of evolution could increase S RBD and human ACE2 interactions. Leucine 472 of the icSZ16-S K479N RBD and the icSZ16-S K479N D8 RBD interacts with L79 and M82 of ACE2. The icSZ16-S K479N D22 virus RBD is mutated at position 472 from leucine to phenylalanine, which Rosetta Design predicts will induce strong hydrophobic interactions between F472 and partners L79, M82, and Y83 of ACE2 (Fig. 7E). The icSZ16-S K479N D22 virus F472 mutation likely strengthens the binding interface of the icSZ16-S K479N D22 virus over the icSZ16-S K479N D8 strain, which may explain why the icSZ16-S K479N D22 virus has a growth advantage over the icSZ16-S K479N D8 virus in HAE, Vero E6, and DBT-hACE2 cells. Of note, it is possible that these viruses acquired replication-enhancing mutations in structural or nonstructural genes that we did not sequence. Nevertheless, these data demonstrate the plasticity of the RBD-ACE2 interface site and its ability to subtly remodel the RBD and hACE2 binding interface to promote efficient entry and growth. Clearly, multiple genetic pathways likely exist to allow for zoonotic SARS-CoV host range expansion, and it will be interesting to determine if other contact interface point mutations can enhance zoonotic virus infection of HAE cell cultures.

Since viruses similar to the epidemic strain are currently circulating in bats in China, we may see yet another emergence of human SARS-CoV from zoonotic pools of virus. Therefore, it is imperative that current vaccinations and passive serotherapies be effective against all known SARS-CoV zoonotic strains and their human-adapted progeny. Previous work by Deming et al. has highlighted the value of using zoonotic strains as challenge viruses to assess vaccine efficacy (5). Deming et al. demonstrated that sera from animals vaccinated with epidemic strain antigens were not as effective in neutralizing the zoonotic icGD03-S virus as they were in neutralizing the epidemic strain (5). Within this study, we demonstrated that two zoonotic spiked viruses, icSZ16-S K479N and icSZ16-K479N D22, grew to peak titers in the mouse lung similar to those of the epidemic strain 2 days postinfection. In contrast to recombinant SARS-CoV bearing early human (GZ02) or civet S glycoprotein (HC/SZ/61/05) S proteins that were extremely pathogenic in aged animals, icSZ16-S K479N and icSZ16-S K479N D22 caused little to no disease in either young or senescent BALB/c mice (40). Although the SZ16 and HC/SZ/61/03 viruses are both of civet origin, their S proteins differ by 18 amino acids (9; G. Yi, SARS coronavirus HC/SZ/61/03 complete genome). The 7-month period of time between the isolation of SZ16 (May 2003) and the isolation of HC/SZ/61/03 (December 2003) may help explain their sequence differences (9; G. Yi, SARS coronavirus HC/SZ/61/03 complete genome [http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db %nuccore&id %42556132]). Zoonotic SARS-CoV infection of humans is typically less severe than infection with human strains, but it is possible that the sequence differences between the SZ16 and the HC/SZ/61/03 S proteins alter the structure of the S proteins such that the viruses bearing the HC/SZ/61/03 S are lethal in mice, while our SZ16 lineage viruses are attenuated (2, 9, 19, 23, 40, 45). Nevertheless, the icSZ16-S K479N and icSZ16-S K479N D22 viruses grow to levels similar to those of the epidemic strain in mice and could be useful as challenge viruses to assess the efficacies of vaccines or serotherapies. The human MAb S230.15 binds within the RBD, although the specific epitope has yet to be defined (48, 59). Recent data from Traggiai et al. and Zhu et al. have shown MAbs S230.15 and, to a lesser extent, m396 to be potent and cross-neutralizing antibodies against human and zoonotic SARS-CoV isolates (48, 59). We have demonstrated that the "evolved" icSZ16-S K479N D8 and D22 viruses were equally susceptible to neutralization by S230.15 but that icSZ16-S K479N was eight times more resistant. In this instance, these data suggest that zoonotic virus adaptation to the human ACE2 receptor may enhance susceptibility to neutralization by S230.15. Since there was a dramatic difference in neutralization between icSZ16-S K479N and icSZ16-S K479N D8, these data suggested that the epitope of S230.15 is in close proximity to or was affected by structural changes associated with residue 442 of the S protein. Future efforts to revive the divergent wild-type icSZ16-S virus in vitro will be especially helpful with mapping the S230.15 epitope, since it is possible that the K479N mutation may contribute to enhanced S230.15 binding. Even though S230.15 is less effective at neutralizing icSZ16-S K479N in vitro, recently published passive transfer studies in mice have shown that the S230.15 antibody is extremely potent and broadly neutralizing in vivo where replication of SARS Urbani, icGD03-S, and icSZ16-S K479N were not detected in the mouse lung (59). These data highlight the power and utility of zoonotic strains in assessing the efficacy of passive serotherapy both in vitro and in vivo.

In conclusion, synthetic biology has been employed to rapidly construct and assemble a completely synthetic and infectious 5.3-kb bacteriophage, poliovirus, 1918 influenza virus, and a panel of SARS viruses bearing animal and human S glycoproteins (1, 5, 40, 44, 49). We provide yet another example of the utility of synthetic biology by resurrecting the civet SZ16 S. We have shown that a single point mutation (K479N) in the SZ16 S facilitates host range expansion, but additional mutations must occur for robust infection of human cells. Mutations of residues 479 (K479N) and 487 (S487T) occurred during the early phase of the SARS-CoV epidemic to help facilitate host range expansion. Though we did not generate a mutant SZ16 S containing S487T, previous studies have shown that the inclusion of a mutation at either position 479 or 487 in pseudotype virus bearing the civet S allowed for the infection of human cells, but this approach provides little information on

Downloaded from jvi.asm.org at UNIVERSITY OF NORTH CAROLINA on December 20, 2008

growth efficiency and spread. Through our in vitro evolution of the icSZ16-S K479N virus, we discovered a novel evolutionary path by which SARS-CoV S can interface with hACE2 to allow for the efficient infection of human cells. When S mutations acquired during our in vitro evolution are compared to mutations that occurred during the evolution of the epidemic strain, the plasticity of the SARS-CoV S protein becomes starkly apparent. Through the use of HAE cell cultures, synthetic biology, reverse genetics, and animal models, we have created a system to isolate and characterize novel genetic pathways of zoonotic SARS-CoV S adaptation to the human ACE2 receptor. We have also generated antigenically divergent SARS-CoV zoonotic strains that might be useful in assessing the efficacies of SARS-CoV vaccines or serotherapies. Since the future emergence of SARS-CoV will most likely evolve from zoonotic pools of virus, it is imperative that current vaccinations and passive serotherapies be effective against the zoonotic virus, the epidemic virus strain, and all of the evolutionary permutations in between.

ACKNOWLEDGMENTS

We thank Michael Farzan at Harvard Medical School for kindly providing the hACE2 expression plasmid. We also thank Antonio Lanzevecchia at the Institute for Research in Biomedicine, Bellinzona, Switzerland, for graciously providing MAbs S230.15 and D2.2 and Boyd Yount for superb technical assistance and expert cloning advice.

This work was supported by research grants (R01 AI059136 and AI059443) to R.B. from the National Institutes of Health, Division of Allergy and Infectious Diseases.

REFERENCES

1. **Cello, J., A. V. Paul, and E. Wimmer.** 2002. Chemical synthesis of poliovirus cDNA: generation of infectious virus in the absence of natural template. Science **297:**1016–1018.
2. **Chinese SARS Molecular Epidemiology Consortium.** 2004. Molecular evolution of the SARS coronavirus during the course of the SARS epidemic in China. Science **303:**1666–1669.
3. **Christian, M. D., S. M. Poutanen, M. R. Loutfy, M. P. Muller, and D. E. Low.** 2004. Severe acute respiratory syndrome. Clin. Infect. Dis. **38:**1420–1427.
4. **de Haan, C. A. M., Z. Li, E. te Lintelo, B. J. Bosch, B. J. Haijema, and P. J. M. Rottier.** 2005. Murine coronavirus with an extended host range uses heparan sulfate as an entry receptor. J. Virol. **79:**14451–14456.
5. **Deming, D., T. Sheahan, M. Heise, B. Yount, N. Davis, A. Sims, M. Suthar, J. Harkema, A. Whitmore, R. Pickles, A. West, E. Donaldson, K. Curtis, R. Johnston, and R. Baric.** 2006. Vaccine efficacy in senescent mice challenged with recombinant SARS-CoV bearing epidemic and zoonotic spike variants. PLoS Med. **3:**e525.
6. **Domingo, E., and J. J. Holland.** 1997. RNA virus mutations and fitness for survival. Annu. Rev. Microbiol. **51:**151–178.
7. **Drosten, C., S. Gunther, W. Preiser, S. van der Werf, H. R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. Fouchier, A. Berger, A. M. Burguiere, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J. C. Manuguerra, S. Muller, V. Rickerts, M. Sturmer, S. Vieth, H. D. Klenk, A. D. Osterhaus, H. Schmitz, and H. W. Doerr.** 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1967–1976.
8. **Fulcher, M. L., S. Gabriel, K. A. Burns, J. R. Yankaskas, and S. H. Randell.** 2005. Well-differentiated human airway epithelial cell cultures. Methods Mol. Med. **107:**183–206.
9. **Guan, Y., B. J. Zheng, Y. Q. He, X. L. Liu, Z. X. Zhuang, C. L. Cheung, S. W. Luo, P. H. Li, L. J. Zhang, Y. J. Guan, K. M. Butt, K. L. Wong, K. W. Chan, W. Lim, K. F. Shortridge, K. Y. Yuen, J. S. Peiris, and L. L. Poon.** 2003. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. Science **302:**276–278.
10. **Haagmans, B. L., T. Kuiken, B. E. Martina, R. A. Fouchier, G. F. Rimmelzwaan, G. van Amerongen, D. van Riel, T. de Jong, S. Itamura, K. H. Chan, M. Tashiro, and A. D. Osterhaus.** 2004. Pegylated interferon-alpha protects type 1 pneumocytes against SARS coronavirus infection in macaques. Nat. Med. **10:**290–293.
11. **Haagmans, B. L., and A. D. Osterhaus.** 2006. Nonhuman primate models for SARS. PLoS Med. **3:**e194.
12. **Hamming, I., W. Timens, M. L. Bulthuis, A. T. Lely, G. J. Navis, and H. van Goor.** 2004. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J. Pathol. **203:**631–637.
13. **Hsu, V. P., M. J. Hossain, U. D. Parashar, M. M. Ali, T. G. Ksiazek, I. Kuzmin, M. Niezgoda, C. Rupprecht, J. Bresee, and R. F. Breiman.** 2004. Nipah virus encephalitis reemergence, Bangladesh. Emerg. Infect. Dis. **10:**2082–2087.
14. **Hueffer, K., J. S. L. Parker, W. S. Weichert, R. E. Geisel, J.-Y. Sgro, and C. R. Parrish.** 2003. The natural host range shift and subsequent evolution of canine parvovirus resulted from virus-specific binding to the canine transferrin receptor. J. Virol. **77:**1718–1726.
15. **Jeffers, S. A., S. M. Tusell, L. Gillim-Ross, E. M. Hemmila, J. E. Achenbach, G. J. Babcock, W. D. Thomas, Jr., L. B. Thackray, M. D. Young, R. J. Mason, D. M. Ambrosino, D. E. Wentworth, J. C. Demartini, and K. V. Holmes.** 2004. CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. Proc. Natl. Acad. Sci. USA **101:**15748–15753.
16. **Kan, B., M. Wang, H. Jing, H. Xu, X. Jiang, M. Yan, W. Liang, H. Zheng, K. Wan, Q. Liu, B. Cui, Y. Xu, E. Zhang, H. Wang, J. Ye, G. Li, M. Li, Z. Cui, X. Qi, K. Chen, L. Du, K. Gao, Y.-T. Zhao, X.-Z. Zou, Y.-J. Feng, Y.-F. Gao, R. Hai, D. Yu, Y. Guan, and J. Xu.** 2005. Molecular evolution analysis and geographic investigation of severe acute respiratory syndrome coronavirus-like virus in palm civets at an animal market and on farms. J. Virol. **79:**11892–11900.
17. **Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A. E. Ling, C. D. Humphrey, W. J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Y. Yang, N. Cox, J. M. Hughes, J. W. LeDuc, W. J. Bellini, and L. J. Anderson.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1953–1966.
18. **Lau, S. K., P. C. Woo, K. S. Li, Y. Huang, H. W. Tsoi, B. H. Wong, S. S. Wong, S. Y. Leung, K. H. Chan, and K. Y. Yuen.** 2005. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc. Natl. Acad. Sci. USA **102:**14040–14045.
19. **Lau, Y. L., and J. S. Peiris.** 2005. Pathogenesis of severe acute respiratory syndrome. Curr. Opin. Immunol. **17:**404–410.
20. **Leroy, E. M., B. Kumulungui, X. Pourrut, P. Rouquet, A. Hassanin, P. Yaba, A. Delicat, J. T. Paweska, J. P. Gonzalez, and R. Swanepoel.** 2005. Fruit bats as reservoirs of Ebola virus. Nature **438:**575–576.
21. **Li, F., W. Li, M. Farzan, and S. C. Harrison.** 2005. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. Science **309:**1864–1868.
22. **Li, W., M. J. Moore, N. Vasilieva, J. Sui, S. K. Wong, M. A. Berne, M. Somasundaran, J. L. Sullivan, K. Luzuriaga, T. C. Greenough, H. Choe, and M. Farzan.** 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature **426:**450–454.
23. **Li, W., S.-K. Wong, F. Li, J. H. Kuhn, I.-C. Huang, H. Choe, and M. Farzan.** 2006. Animal origins of the severe acute respiratory syndrome coronavirus: insight from ACE2–S-protein interactions. J. Virol. **80:**4211–4219.
24. **Li, W., C. Zhang, J. Sui, J. H. Kuhn, M. J. Moore, S. Luo, S. K. Wong, I. C. Huang, K. Xu, N. Vasilieva, A. Murakami, Y. He, W. A. Marasco, Y. Guan, H. Choe, and M. Farzan.** 2005. Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2. EMBO J. **24:**1634–1643.
25. **Ligon, B. L.** 2006. Reemergence of an unusual disease: the chikungunya epidemic. Semin. Pediatr. Infect. Dis. **17:**99–104.
26. **Marra, M. A., S. J. Jones, C. R. Astell, R. A. Holt, A. Brooks-Wilson, Y. S. Butterfield, J. Khattra, J. K. Asano, S. A. Barber, S. Y. Chan, A. Cloutier, S. M. Coughlin, D. Freeman, N. Girn, O. L. Griffith, S. R. Leach, M. Mayo, H. McDonald, S. B. Montgomery, P. K. Pandoh, A. S. Petrescu, A. G. Robertson, J. E. Schein, A. Siddiqui, D. E. Smailus, J. M. Stott, G. S. Yang, F. Plummer, A. Andonov, H. Artsob, N. Bastien, K. Bernard, T. F. Booth, D. Bowness, M. Czub, M. Drebot, L. Fernando, R. Flick, M. Garbutt, M. Gray, A. Grolla, S. Jones, H. Feldmann, A. Meyers, A. Kabani, Y. Li, S. Normand, U. Stroher, G. A. Tipples, S. Tyler, R. Vogrig, D. Ward, B. Watson, R. C. Brunham, M. Krajden, M. Petric, D. M. Skowronski, C. Upton, and R. L. Roper.** 2003. The Genome sequence of the SARS-associated coronavirus. Science **300:**1399–1404.
27. **McRoy, W. C., and R. S. Baric.** 2008. Amino acid substitutions in the S2 subunit of mouse hepatitis virus variant V51 encode determinants of host range expansion. J. Virol. **82:**1414–1424.
28. **Morens, D. M., G. K. Folkers, and A. S. Fauci.** 2004. The challenge of emerging and re-emerging infectious diseases. Nature **430:**242–249.
29. **Nguyen, D. C., T. M. Uyeki, S. Jadhao, T. Maines, M. Shaw, Y. Matsuoka, C. Smith, T. Rowe, X. Lu, H. Hall, X. Xu, A. Balish, A. Klimov, T. M. Tumpey, D. E. Swayne, L. P. T. Huynh, H. K. Nghiem, H. H. T. Nguyen, L. T. Hoang, N. J. Cox, and J. M. Katz.** 2005. Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. J. Virol. **79:**4201–4212.
30. **Nicholls, J. M., J. Butany, L. L. Poon, K. H. Chan, S. L. Beh, S. Poutanen, J. S. Peiris, and M. Wong.** 2006. Time course and cellular localization of SARS-CoV nucleoprotein and RNA in lungs from fatal cases of SARS. PLoS Med. **3:**e27.

Downloaded from jvi.asm.org at UNIVERSITY OF NORTH CAROLINA on December 20, 2008

31. **Page, R. D.** 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12**:357–358.
32. **Parrish, C. R., and Y. Kawaoka.** 2005. The origins of new pandemic viruses: the acquisition of new host ranges by canine parvovirus and influenza A viruses. Annu. Rev. Microbiol. **59**:553–586.
33. **Pickles, R. J., D. McCarty, H. Matsui, P. J. Hart, S. H. Randell, and R. C. Boucher.** 1998. Limited entry of adenovirus vectors into well-differentiated airway epithelium is responsible for inefficient gene transfer. J. Virol. **72**: 6014–6023.
34. **Poon, L. L. M., D. K. W. Chu, K. H. Chan, O. K. Wong, T. M. Ellis, Y. H. C. Leung, S. K. P. Lau, P. C. Y. Woo, K. Y. Suen, K. Y. Yuen, Y. Guan, and J. S. M. Peiris.** 2005. Identification of a novel coronavirus in bats. J. Virol. **79**:2001–2009.
35. **Pyrc, K., B. Berkhout, and L. van der Hoek.** 2007. The novel human coronaviruses NL63 and HKU1. J. Virol. **81**:3051–3057.
36. **Pyrc, K., R. Dijkman, L. Deng, M. F. Jebbink, H. A. Ross, B. Berkhout, and L. van der Hoek.** 2006. Mosaic structure of human coronavirus NL63, one thousand years of evolution. J. Mol. Biol. **364**:964–973.
37. **Ramu, C.** 2003. SIRW: a web server for the Simple Indexing and Retrieval System that combines sequence motif searches with keyword searches. Nucleic Acids Res. **31**:3771–3774.
38. **Roberts, A., C. Paddock, L. Vogel, E. Butler, S. Zaki, and K. Subbarao.** 2005. Aged BALB/c mice as a model for increased severity of severe acute respiratory syndrome in elderly humans. J. Virol. **79**:5833–5838.
39. **Roberts, A., and K. Subbarao.** 2006. Animal models for SARS. Adv. Exp. Med. Biol. **581**:463–471.
40. **Rockx, B., T. Sheahan, E. Donaldson, J. Harkema, A. Sims, M. Heise, R. Pickles, M. Cameron, D. Kelvin, and R. Baric.** 2007. Synthetic reconstruction of zoonotic and early human severe acute respiratory syndrome coronavirus isolates that produce fatal disease in aged mice. J. Virol. **81**:7410–7423.
41. **Rota, P. A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P. Icenogle, S. Penaranda, B. Bankamp, K. Maher, M. H. Chen, S. Tong, A. Tamin, L. Lowe, M. Frace, J. L. DeRisi, Q. Chen, D. Wang, D. D. Erdman, T. C. Peret, C. Burns, T. G. Ksiazek, P. E. Rollin, A. Sanchez, S. Liffick, B. Holloway, J. Limor, K. McCaustland, M. Olsen-Rasmussen, R. Fouchier, S. Gunther, A. D. Osterhaus, C. Drosten, M. A. Pallansch, L. J. Anderson, and W. J. Bellini.** 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science **300**:1394–1399.
42. **Simmons, G., D. N. Gosalia, A. J. Rennekamp, J. D. Reeves, S. L. Diamond, and P. Bates.** 2005. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proc. Natl. Acad. Sci. USA **102**:11876–11881.
43. **Sims, A. C., R. S. Baric, B. Yount, S. E. Burkett, P. L. Collins, and R. J. Pickles.** 2005. Severe acute respiratory syndrome coronavirus infection of human ciliated airway epithelia: role of ciliated cells in viral spread in the conducting airways of the lungs. J. Virol. **79**:15511–15524.
44. **Smith, H. O., C. A. Hutchison III, C. Pfannkoch, and J. C. Venter.** 2003. Generating a synthetic genome by whole genome assembly: phiX174 bacteriophage from synthetic oligonucleotides. Proc. Natl. Acad. Sci. USA **100**: 15440–15445.
45. **Song, H. D., C. C. Tu, G. W. Zhang, S. Y. Wang, K. Zheng, L. C. Lei, Q. X. Chen, Y. W. Gao, H. Q. Zhou, H. Xiang, H. J. Zheng, S. W. Chern, F. Cheng, C. M. Pan, H. Xuan, S. J. Chen, H. M. Luo, D. H. Zhou, Y. F. Liu, J. F. He, P. Z. Qin, L. H. Li, Y. Q. Ren, W. J. Liang, Y. D. Yu, L. Anderson, M. Wang, R. H. Xu, X. W. Wu, H. Y. Zheng, J. D. Chen, G. Liang, Y. Gao, M. Liao, L. Fang, L. Y. Jiang, H. Li, F. Chen, B. Di, L. J. He, J. Y. Lin, S. Tong, X. Kong, L. Du, P. Hao, H. Tang, A. Bernini, X. J. Yu, O. Spiga, Z. M. Guo, H. Y. Pan, W. Z. He, J. C. Manuguerra, A. Fontanet, A. Danchin, N. Niccolai, Y. X. Li, C. I. Wu, and G. P. Zhao.** 2005. Cross-host evolution of severe acute respiratory syndrome coronavirus in palm civet and human. Proc. Natl. Acad. Sci. USA **102**:2430–2435.
46. **Stevens, J., O. Blixt, T. M. Tumpey, J. K. Taubenberger, J. C. Paulson, and I. A. Wilson.** 2006. Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. Science **312**:404–410.
47. **Towner, J. S., X. Pourrut, C. G. Albarino, C. N. Nkogue, B. H. Bird, G. Grard, T. G. Ksiazek, J. P. Gonzalez, S. T. Nichol, and E. M. Leroy.** 2007. Marburg virus infection detected in a common African bat. PLoS ONE **2**:e764.
48. **Traggiai, E., S. Becker, K. Subbarao, L. Kolesnikova, Y. Uematsu, M. R. Gismondo, B. R. Murphy, R. Rappuoli, and A. Lanzavecchia.** 2004. An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. Nat. Med. **10**:871–875.
49. **Tumpey, T. M., C. F. Basler, P. V. Aguilar, H. Zeng, A. Solorzano, D. E. Swayne, N. J. Cox, J. M. Katz, J. K. Taubenberger, P. Palese, and A. Garcia-Sastre.** 2005. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science **310**:77–80.
50. **van der Hoek, L., K. Pyrc, M. F. Jebbink, W. Vermeulen-Oost, R. J. Berkhout, K. C. Wolthers, P. M. Wertheim-van Dillen, J. Kaandorp, J. Spaargaren, and B. Berkhout.** 2004. Identification of a new human coronavirus. Nat. Med. **10**:368–373.
51. **Walsh, P. D., R. Biek, and L. A. Real.** 2005. Wave-like spread of Ebola Zaire. PLoS Biol. **3**:e371.
52. **Woo, P. C. Y., S. K. P. Lau, C.-M. Chu, K.-H. Chan, H.-W. Tsoi, Y. Huang, B. H. L. Wong, R. W. S. Poon, J. J. Cai, W.-K. Luk, L. L. M. Poon, S. S. Y. Wong, Y. Guan, J. S. M. Peiris, and K.-Y. Yuen.** 2005. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J. Virol. **79**:884–895.
53. **Woo, P. C. Y., S. K. P. Lau, C. C. Y. Yip, Y. Huang, H.-W. Tsoi, K.-H. Chan, and K.-Y. Yuen.** 2006. Comparative analysis of 22 coronavirus HKU1 genomes reveals a novel genotype and evidence of natural recombination in coronavirus HKU1. J. Virol. **80**:7136–7145.
54. **Yano, Y., M. Kaneniwa, M. Satomi, H. Oikawa, and S. S. Chen.** 2006. Occurrence and density of Vibrio parahaemolyticus in live edible crustaceans from markets in China. J. Food Prot. **69**:2742–2746.
55. **Yano, Y., M. Yokoyama, M. Satomi, H. Oikawa, and S. S. Chen.** 2004. Occurrence of Vibrio vulnificus in fish and shellfish available from markets in China. J. Food Prot. **67**:1617–1623.
56. **Yount, B., K. M. Curtis, E. A. Fritz, L. E. Hensley, P. B. Jahrling, E. Prentice, M. R. Denison, T. W. Geisbert, and R. S. Baric.** 2003. Reverse genetics with a full-length infectious cDNA of severe acute respiratory syndrome coronavirus. Proc. Natl. Acad. Sci. USA **100**:12995–13000.
57. **Yount, B., R. S. Roberts, L. Lindesmith, and R. S. Baric.** 2006. Rewiring the severe acute respiratory syndrome coronavirus (SARS-CoV) transcription circuit: engineering a recombination-resistant genome. Proc. Natl. Acad. Sci. USA **103**:12546–12551.
58. **Yount, B., R. S. Roberts, A. C. Sims, D. Deming, M. B. Frieman, J. Sparks, M. R. Denison, N. Davis, and R. S. Baric.** 2005. Severe acute respiratory syndrome coronavirus group-specific open reading frames encode nonessential functions for replication in cell cultures and mice. J. Virol. **79**:14909–14922.
59. **Zhu, Z., S. Chakraborti, Y. He, A. Roberts, T. Sheahan, X. Xiao, L. E. Hensley, P. Prabakaran, B. Rockx, I. A. Sidorov, D. Corti, L. Vogel, Y. Feng, J. O. Kim, L. F. Wang, R. Baric, A. Lanzavecchia, K. M. Curtis, G. J. Nabel, K. Subbarao, S. Jiang, and D. S. Dimitrov.** 2007. Potent cross-reactive neutralization of SARS coronavirus isolates by human monoclonal antibodies. Proc. Natl. Acad. Sci. USA **104**:12123–12128.

Downloaded from jvi.asm.org at UNIVERSITY OF NORTH CAROLINA on December 20, 2008

JOURNAL OF VIROLOGY , Sept. 2008, p. 8721–8732 Vol. 82, No. 17
0022-538X/08/$08.00ff 0 doi:10.1128/JVI.00818-08
Copyright © 2008, American Society for Microbiology. All Rights Reserved.

Pathways of Cross-Species Transmission of Synthetically Reconstructed Zoonotic Severe Acute Respiratory Syndrome Coronavirus ff

Timothy Sheahan, [1] Barry Rockx, [2] Eric Donaldson, [1] Davide Corti, [3] and Ralph Baric [1,2] *

Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina [1]; Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina [2]; and Institute for Research in Biomedicine, Bellinzona, Switzerland [3]

Received 16 April 2008/Accepted 16 June 2008

Zoonotic severe acute respiratory syndrome coronavirus (SARS-CoV) likely evolved to infect humans by a series of transmission events between humans and animals in markets in China. Virus sequence data suggest that the palm civet served as an amplification host in which civet and human interaction fostered the evolution of the epidemic SARS Urbani strain. The prototypic civet strain of SARS-CoV, SZ16, was isolated from a palm civet but has not been successfully cultured in vitro. To propagate a chimeric recombinant SARS-CoV bearing an SZ16 spike (S) glycoprotein (ic SZ16-S), we constructed cell lines expressing the civet ortholog (DBT-cACE2) of the SARS-CoV receptor (hACE2). Zoonotic SARS-CoV was completely dependent on ACE2 for entry. Urbani grew with similar kinetics in both the DBT-cACE2 and the DBT-hACE2 cells, while ic SZ16-S only grew in DBT-cACE2 cells. The SZ16-S mutant viruses adapted to human airway epithelial cells and displayed enhanced affinity for hACE2 but exhibited severe growth defects in the DBT-cACE2 cells, suggesting that the evolutionary pathway that promoted efficient hACE2 interactions simultaneously abolished efficient cACE2 interactions. Structural modeling predicted two distinct biochemical interaction networks by which zoonotic receptor binding domain architecture can productively engage hACE2, but only the Urbani mutational repertoire promoted efficient usage of both hACE2 and cACE2 binding interfaces. Since dual species tropism was preserved in Urbani, it is likely that the virus evolved a high affinity for cACE2/hACE2 receptors through adaptation via repeated passages between human and civet hosts. Furthermore, zoonotic SARS-CoV was variably neutralized by antibodies that were effective against the epidemic strain, highlighting their utility for evaluating passive immunization efficacy.

Within the wet markets in China, a wide range of common and exotic food animals like bats and civets are sold live or as fresh meat for human consumption, in part to improve health and for sexual performance (2, 8, 32, 34). In southern China, the consumption of exotic animals is especially common during the winter months when most respiratory tract infections are highly prevalent (32). Since many zoonotic viral pathogens are shed in stool, densely housed birds and mammals shedding excreta in the wet marketplace create a rich atmosphere for zoonotic virus transmission to human consumer populations (32). Most recently, wet markets in Southeast Asia have been associated with cultivating the cross-species transmission of avian influenza H5N1 and the newly emerged severe acute respiratory syndrome coronavirus (SARS-CoV) (8, 13, 32).

In 2002, a novel coronavirus, SARS-CoV, emerged as the causative agent of severe acute respiratory syndrome and spread worldwide, causing about 8,000 cases and more than 700 deaths (3, 23, 32). Viruses similar to the epidemic strain SARS-CoV Urbani were isolated from animals for sale within wet markets in China during the epidemic in 2003 and the reemergence in 2004 (2, 8). Genome sequences of viruses isolated from bats, civets, and humans suggest that viruses circulating in bats crossed the species barrier to infect civets, which then served as an amplification host for yet another host range shift to generate a human-tropic virus (2, 8, 13, 19). Recent surveys of animal populations in North America, Africa, and China implicate bats as reservoirs of a diverse array of coronaviruses, including SARS-CoV-like progenitors (5, 6, 13, 14, 20, 31–33). As some bat species were found to be coinfected with more than one coronavirus lineage, the recombination between and within genogroups and the subsequent emergence of novel coronaviruses are real possibilities (14). In 2007, surveys of wild Asian leopard cats and Chinese ferret badgers revealed the presence of yet more novel and highly divergent coronaviruses circulating in animals typically sold in wet markets in China (6). Since viruses similar to the epidemic strain of SARS-CoV are currently circulating in zoonotic pools, the future emergence of another SARS-CoV-like virus may occur. Therefore, it is imperative that current vaccination and passive immunization therapies are effective in protecting humans from infection by a wide range of epidemic and zoonotic SARS-CoV. Unfortunately, bat SARS-CoV and the prototypical civet SARS-CoV strain SZ16 have not been successfully cultured in vitro, hampering our ability to evaluate their epidemic potential, pathogenesis, and susceptibility to current therapeutic interventions within in vitro and in vivo models (13, 15).

The SARS-CoV spike (S) gene sequences isolated from human cases during the early phase of the epidemic in 2002 to 2003 and during the reemergence from 2003 to 2004 are very similar to those of the SZ16 strain (2, 8, 12). SZ16 was isolated from the palm civet in wet markets within the Guangdong

* Corresponding author. Mailing address: Department of Epidemiology, 2107 McGavran-Greenberg, CB no. 7435, University of North Carolina, Chapel Hill, NC 27699-7435. Phone: (919) 966-3895. Fax: (919) 966-2089. E-mail: rbaric@email.unc.edu.

ff Published ahead of print on 25 June 2008.

Downloaded from jvi.asm.org at University of North Carolina - Chapel Hill on December 20, 2008

region of China during the epidemic, and its S protein differs from the that of the epidemic strain by 18 amino acids, 16 of which reside in the S1 domain containing the receptor binding domain (RBD) (8). The SARS-CoV strain GD03 sequence was isolated from a sporadic human case during the epidemic's reemergence in 2003 to 2004 and closely resembles those viruses isolated from civets and raccoon dogs from the wet markets (2). The SARS-CoV GD03 S protein differs from that of the epidemic strain by 17 amino acids, 16 of which reside in the S1 domain (2). Recently, we have shown that the recombinant SARS-CoV bearing the SZ16 S gene (icSZ16-S) was capable of replication within Vero E6 cells, but progeny virions were unable to infect naïve cell cultures, supporting evidence that the binding of the civet S protein to the human SARS-CoV receptor (human angiotensin-converting enzyme II [hACE2]) or a very closely related primate ortholog was inefficient (19). Since clinical SZ16 isolates and our recombinant chimeric icSZ16-S virus strains could not be successfully cultured in vitro, the biology of the SZ16 S protein and its effect on virus growth and pathogenesis remain elusive.

In this study, we describe the recovery and growth of the icSZ16-S strain in culture using delayed brain tumor (DBT) cells stably expressing the civet ortholog (cACE2) of hACE2. Like the epidemic strain, and in contrast to an earlier report, both icSZ16-S and icGD03-S are completely dependent on ACE2 for entry (36). In DBT-cACE2 cells, icSZ16-S grows with kinetics similar to that of the epidemic strain, but point mutations in the SZ16 S glycoprotein that enhance growth in hACE2-expressing cells abrogate growth in cACE2-expressing cells. The epidemic strain grows equally well in cACE2- and hACE2-expressing cells, suggesting that the epidemic strain probably evolved through repeated exchanges of virus between civet and human, thereby providing the evolutionary pressure to retain dual host tropism. icSZ16-S and icGD03-S are moderately susceptible to neutralization by the human monoclonal antibody (hu-MAb) S230.15. Unlike the epidemic strain, zoonotic viruses are resistant to hu-MAb S3.1, suggesting that therapeutics successfully directed against the epidemic strain may not work as well against the zoonotic viruses. These data highlight the utility of employing an antigenically diverse zoonotic S panel of SARS-CoV in the study of host range expansion and antiviral therapy efficacy.

MATERIALS AND METHODS

Viruses and cells. The recombinant epidemic virus strain "icSARS" (GenBank accession no. AY278741) and icSZ16-S (GenBank accession no. AY304488), icGD03-S (GenBank accession no. AY525636), icCUHK-W1 (GenBank accession no. AY278554), icGZ02 (GenBank accession no. AY390556), icSZ16-S K479N, icSZ16-S K479N D8, and icSZ16-S K479N D22 were propagated on Vero E6 or DBT cells stably expressing cACE2 (DBT-cACE2) as described previously (4, 22, 25, 26, 37). The icSZ16-S virus supernatant from the electroporated cell culture (passage 0) was passaged on DBT-cACE2 cells four times at 48-h intervals, after which a robust cytopathic effect was observed. icSZ16-S was then plaque purified on DBT-cACE2 cells, and plaques were expanded in six-well dishes of DBT-cACE2 cells. Six-well-dish supernatant was used to infect a T175 flask of DBT-cACE2 cells to generate a virus stock that was harvested 20 h postinfection (hpi). Viral RNA from the icSZ16-S virus stock was isolated using TRIzol (Invitrogen, Carlsbad, CA), cDNA was synthesized using Superscript II reverse transcriptase (SSII; Invitrogen, Carlsbad, CA), and amplicons spanning the S, 3a, E, and M genes were generated by reverse transcription-PCR (RT-PCR) and sequenced directly. The virus stocks used throughout this study were grown and titers were determined by plaque assay with Vero E6 and/or DBT-cACE2 cells as described previously (38). Vero E6 and DBT cells were grown in minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Clone II (HyClone, South Logan, UT), kanamycin (0.25 ffg/ml), and gentamycin (0.05 ffg/ml) (UNC tissue culture facility). DBT-cACE2 and DBT-hACE2 cells were grown in Vero/DBT growth media supplemented with 700 ffg/ml G418 (Invitrogen, Carlsbad, CA). All work with viruses was performed in a class II biological safety cabinet in a certified biosafety level 3 laboratory containing redundant exhaust fans while wearing Tyvek suits and powered air-purifying respirators as described previously (38).

Generation of cACE2-expressing DBT cells. Plasmids encoding N-terminal Myc-tagged hACE2 and cACE2 were kindly provided by M. Farzan, New England Primate Research Center. Construction of the DBT-hACE2 cell line was carried out as described by Sheahan et al. (26). The cACE2 gene was amplified by PCR using Expand High Fidelity polymerase (cACE2F primer, 5ffCACCAT GTCAGGCTCTTTCTGGCTCC-3 ffland cACE2R primer, 5ffAAATGAAGTC TGAACGTCATCAG-3 ffl. Amplicon was gel purified and cloned into pcDNA3.1/V5-His according to the protocol. The cACE2 reverse primer did not contain a stop codon, allowing for a read through and the inclusion of a six-His tag on the 3ffl end. The pcDNA cACE2-His plasmids were sequenced and found to have two amino acid changes, G354D and R736Q, compared to those of the published sequence (GenBank accession no. AY881174), one of which (G354D) resides at a proposed interaction site with SARS-CoV S residues Y491 and G488, but these changes were also found in the parent gifted plasmid. cACE2 is polymorphic at residue 354, where sequences containing D354 and G354 can be found in civet tissue samples (personal communication with Michael Farzan) (18). Murine DBT cells were transfected with 4 ffg of pcDNA3.1/V5-His expressing cACE2-His or green fluorescent protein (GFP)-His, using Fugene reagent (Roche, Indianapolis, IN). After 24 h, cells were placed under continuous drug selection using 700 ffg/ml G418 (Invitrogen, Carlsbad, CA). DBT-cACE2-His, DBT-hACE2, and DBT-GFP-His cells were passaged four times under drug selection and then sorted for high ACE2 or GFP expression levels and expanded for future use.

Cell sorting and fluorescence-activated cell sorter analysis of ACE2-expressing DBT stable cell lines. A total of 10^6 DBT (control), DBT-hACE2, and DBT-cACE2-His cells were blocked with Dulbecco's phosphate-buffered saline (DPBS)-5% fetal bovine serum and stained with polyclonal goat anti-hACE2 (R&D Systems, Minneapolis, MN), followed by a secondary anti-goat immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC; Invitrogen, Carlsbad, CA) antibody. Stained DBT, DBT-hACE2, and DBT-cACE2-His and unstained DBT-GFP-His cells were sorted on a Cytomation Inc. MoFlo cell sorter (Dako, Denmark) for FITC or GFP expression, and sorted cells were expanded by four passages, after which stocks of DBT, DBT-hACE2, DBT-cACE2-His, and DBT-GFP-His cells were cryopreserved for future experiments. To assess transgene expression in postsorted cell stocks, 5 ! 10^5 DBT, DBT-hACE2, DBT-cACE2-His, DBT-GFP-His, or Vero E6 cells were seeded into six-well dishes. Twenty-four hours later, medium was removed, and the cells were removed from the plate, with versene (UNC tissue culture facility). Cells were stained for fluorescence-activated cell sorter analysis as described above and analyzed for FITC/GFP expression by FACScan (BD Biosciences, San Jose, CA).

Western blot analysis of ACE2-expressing DBT stable cell lines. To assess transgene expression in postsorted cell stocks by Western blot analysis, DBT, DBT-hACE2, DBT-cACE2-His, DBT-GFP-His, and Vero E6 cells were seeded in six-well dishes at 5 ! 10^5 cells/well. Twenty-four hours later, cells were lysed with protein lysis buffer (1% Triton X-100, 0.5% sodium dodecyl sulfate [SDS], in 1! Tris-buffered saline [TBS]). Twenty microliters of cell lysate was heated at 70°C in 1! NuPage buffer-4% beta-mercaptoethanol, loaded on a NuPage 12% bis-Tris SDS polyacrylamide gel (Invitrogen, Carlsbad, CA) and separated at 150 V for 2 h. Proteins were transferred to a nylon membrane, using the NuPage transfer apparatus according to the manufacturer's protocol. Membranes were blocked with 5% milk and probed with either polyclonal goat anti-human ACE2 (R&D Systems, Minneapolis, MN) or mouse anti-penta-His antibody (Qiagen, Valencia, CA). Membranes were washed with 1! TBS-0.1% Tween 20 and then probed with either rabbit anti-goat horseradish peroxidase (HRP) (Invitrogen, Carlsbad, CA) or anti-mouse IgG-HRP antibody (Amersham, Piscataway, NJ). After membranes were rinsed, they were treated with ECL Plus reagent (Amersham) and then exposed to radiographic film (Amersham).

RT-PCR to detect viral subgenomic leader-containing transcripts, ACE2, and GAPDH transcripts. RT-PCR to detect subgenomic leader-containing transcripts was performed to detect viral replication as described by Sheahan et al. (26). DBT, DBT-GFP-His, DBT-cACE2, DBT-hACE2, or Vero E6 cells were seeded at 5 ! 10^5 cells/well in a 24-well dish. Twenty-four hours later, cells were infected with 100 ffl of viral supernatant from the initial icSZ16-S transfection or icSARS virus supernatants or were mock infected. At 24 hpi, total RNA was

Downloaded from jvi.asm.org at University of North Carolina - Chapel Hill on December 20, 2008

isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. One microgram of total RNA was used to generate cDNA by Superscript II (Invitrogen, Carlsbad, CA) using random hexamer primers (Invitrogen, Carlsbad, CA). cDNA was then used as the template for PCR using *Taq* polymerase (New England Biolabs, Ipswich, MA). Evidence of SARS-CoV replication and subgenomic mRNA 3a, E, and M (amplicon sizes, 1,796, 947, and 666 bp, respectively) was detected by using a leader forward primer (CTCTTG TAGATCTGTTCTCTAAACGAAC) and a reverse primer in the M gene (TT ACTGTACTAGCAAAGCAATATTGTCG). Detection of ACE2 transcripts was performed with a conserved primer set capable of detecting primate, human, and civet ACE2 (258 bp) (ACE2RTF2 primer, 5′-AGCCTAAAAT CAGCTCTTGG-3′ and ACE2RTR2 primer, 5′-CCGGGACATCCTGATG GC-3′). Detection of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (235 bp) (GAPDHF primer, 5′-CATGGGGAAGGTGAAGGTCG-3′ and GAPDHR primer, 5′-TTGATGGTACATGACAAGGTGC-3′) signals detected by RT-PCR was used as a positive control. PCRs were separated by gel electrophoresis on 1.8% agarose Tris-acetate-EDTA (TAE) gels and visualized by ethidium bromide staining.

Virus growth curve analysis within Vero E6, DBT-cACE2, DBT-hACE2, or DBT cells. Vero E6, DBT-cACE2, DBT-hACE2 or DBT cells were infected with the icCUHK-W1, icGD03-S, icGZ02, icSZ16-S K479N p6, icSZ16-S K479N D22, icSARS, or icSZ16-S strain, at a multiplicity of infection (MOI) of 0.01 for 1 h at 37°C, after which the inoculum was removed, the monolayer was rinsed with DPBS, and the growth medium was added. Virus stock titers generated by plaque assay in DBT-cACE2 cells were used to calculate the MOI for the DBT-cACE2 cell growth curve. Virus stock titers generated by plaque assay in Vero E6 cells were used to calculate the MOI for the Vero E6 and DBT-hACE2 growth curves. Twenty-five microliters of cell medium was removed at 0, 6, 12, 24, and 36 hpi, and samples were stored at " 80°C until titers were determined by plaque assay. For all growth curves except those performed within DBT cells, the cell type used for growth curve analysis was also utilized for the titration of growth curve samples by plaque assay. For example, titers of samples generated from growth curves performed within DBT-cACE2 cells were determined with DBT-cACE2 cells. All DBT cell growth curve titers were determined with DBT-cACE2 cells. Since previous experiments suggested that Vero E6 and DBT-hACE2 cells were not permissive for icSZ16-S infection, icSZ16-S samples from DBT-hACE2 and Vero E6 growth curve titers were determined with DBT-cACE2 cells. Growth curves were performed in duplicate on two independent occasions, and the data shown are from one representative experiment.

ACE2 blockade experiments with DBT-cACE2 cells. DBT-cACE2 cells were seeded at 5 ! 10^5 cells/well in six-well dishes. On the following day, cell medium was removed, and cells were incubated with 10, 5, 2.5, 1.25, or 0.625 μg/ml polyclonal anti-ACE2 or anti-ACE antibody (R&D Systems, Minneapolis, MN) or with DBPS for 1 h at 37°C. After the 1-h pretreatment with antibody, 100 PFU/50 μl of icSARS, icSZ16-S, or icGD03-S was added to the antibody mixture and incubated for 1 h at 37°C. After the infection, the inoculum was removed, and the monolayer was rinsed twice with 1 ml of DPBS and then overlaid with 0.9% agarose in complete growth medium. After 48 hpi, plates were stained with neutral red, and plaques were counted. The average percentage of blockade was calculated by dividing the average number of plaques per Ab dilution by the average number of plaques in the DPBS no-Ab controls. Virus stock titers generated by plaque assay in DBT-cACE2 cells were used to calculate virus concentrations for the blockade experiment. Blockade experiments were performed in duplicate on two separate occasions.

PRNT assay. Neutralizing titers were determined by plaque reduction neutralization titer (PRNT) assay as described by Rockx et al. (21, 22) and Sheahan et al. (26). Briefly, 24 h prior to infection, six-well plates were seeded with 5 ! 10^5 DBT-cACE2 cells/well. hu-MAbs S230.15 and S3.1 directed against the SARS-CoV S protein and an isotype control antibody directed against cholera toxin D2.2 were kindly provided by A. Lanzavecchia. hu-MAbs were serially diluted twofold and incubated with 100 PFU of either icSARS, icSZ16-S, or icGD03-S for 1 h at 37°C. Virus and antibodies were then added to six-well plates of DBT-cACE2 cells in duplicate and incubated at 37°C for 1 h, after which the cells were overlaid with 0.8% agarose in medium. Plates were incubated for 48 h at 37°C and stained with neutral red for 3 h, and plaques were counted. The percentage of neutralization was calculated as 1 " (number of plaques with antibody/number of plaques without antibody) ! 100%.

Computer modeling of RBD interactions with cACE2 and hACE2. Structural models for SZ16, SZ16-K479N, and SZ16 K479N D22 RBD were based on the crystal coordinates reported for SARS-CoV Urbani RBD interacting with the hACE2 receptor (Protein Data Bank [PDB] code 2AJF) as previously reported (17, 26). Using the RosettaDesign website, we incorporated the amino acid replacements, and all amino acids within the 5-Å interaction distance were relaxed to allow the program to repack the side chains to an optimal energetic state. This process was repeated with each mutation and series of mutations. Ten models were generated for each set of mutations, and the best model, based on the lowest energy score, was selected and evaluated further using Macintosh PyMOL software. In addition, the crystal structure coordinates for the SARS-CoV RBD interacting with the hACE2 receptor (PDB code 2AJF, chains A and E) were used as a template to generate a homology model for the cACE2, using Modeler software (version 8.2). Briefly, chain E of the 2AJF template was aligned to the cACE2 sequence, and five models were generated under the automodel class. The model with the lowest objective function score was selected for further analyses. The PDB files generated by this program were visualized with PyMOL for Macintosh molecular modeling (DeLano Scientific) and Chimera software.

RESULTS

Construction and characterization of ACE2-expressing stable cell lines. At the amino acid level, primate ACE2 (Vero E6) and cACE2 are 89% and 84% similar to hACE2, the human SARS-CoV receptor, respectively. In comparison to the contact interface of hACE2 with the SARS-CoV S glycoprotein, that of cACE2 differs greatly, but primate ACE2's contact interface is identical (Fig. 3E). Since previous efforts to culture either the SARS-CoV SZ16 wild type or the recombinant icSZ16-S strain were unsuccessful, we used a cell-based approach to resurrect icSZ16-S (15, 26). We constructed stable cell lines expressing cACE2-His, hACE2, and GFP-His in the DBT cell background. The gifted cACE2 gene utilized to construct our stable cells varied by two amino acids (G354D and R736Q) compared to that of the published sequence, but both G354 and D354 polymorphisms in ACE2 can be found in civet tissues (personal communication with M. Farzan) (18). Stable cells were sorted for ACE2 or GFP expression, and cultivated master cell stocks were analyzed by flow cytometry and Western blotting (Fig. 1). ACE2 expression was detected by Western blotting in cell lysates generated from DBT-hACE2, DBT-cACE2, and Vero cell cultures but was not detected in wild-type DBT cells or in DBT-GFP-His control cells (Fig. 1B). To generate quantitative data for ACE2 expression levels within our stable cells, the cell cultures were analyzed by flow cytometry. ACE2 expression in DBT-hACE2 cells appears to be greater than the expression within Vero E6 cells, while expression in DBT-cACE2 appears to be the lowest of the three ACE2-expressing cell types (Fig. 1A and C). Since the polyclonal primary antibody used to stain the cells for flow cytometry was raised against the ectodomain of hACE2, the differences we see in expression levels may be mediated by less efficient interactions between the antibody and Vero E6 ACE2 (89% amino acid homology to hACE2) or cACE2 (84% amino acid homology to hACE2) in their natural conformations. Positive control DBT-GFP-His cells exhibited a high level of GFP expression by flow cytometry and Western blot analysis. ACE2 expression was not detected in untransfected DBT cells.

Resurrection of icSZ16-S in DBT-cACE2 cells. To assess virus replication and ACE2 transgene expression in our cell panel, we employed a highly sensitive RT-PCR-based assay. In the coronavirus model of discontinuous transcription, subgenomic messages all contain the same 5′-leader sequence (24). Thus, active SARS-CoV mRNA synthesis can be detected by RT-PCR for viral subgenomic leader-containing transcripts. In our assay, we used a forward primer directed

Downloaded from jvi.asm.org at University of North Carolina - Chapel Hill on December 20, 2008

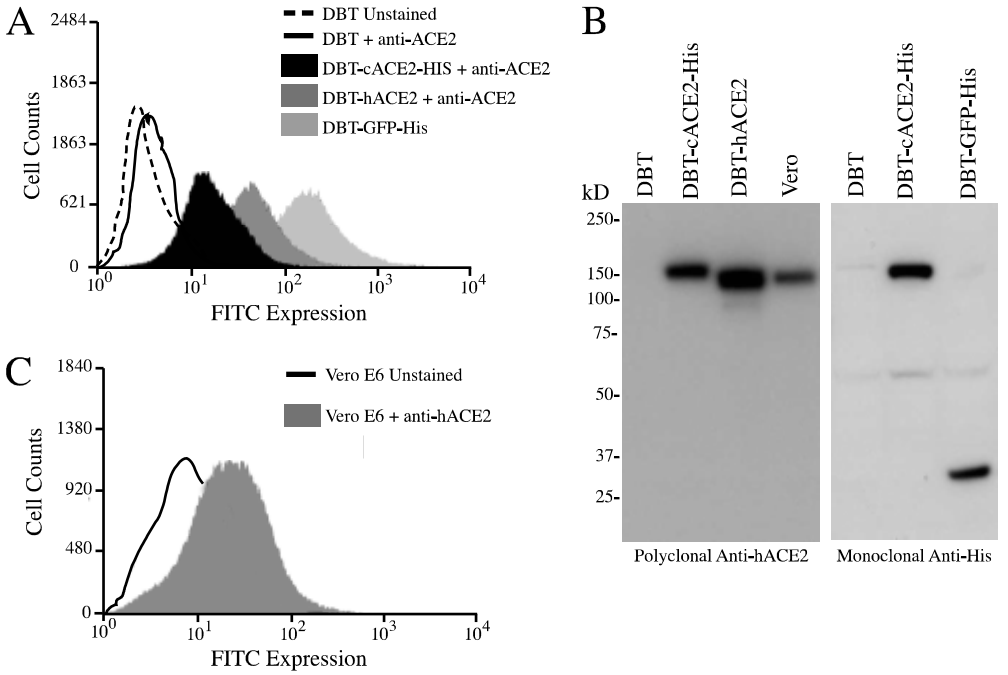


FIG. 1. Characterization of cACE2- and hACE2-expressing DBT cells by flow cytometry and Western blotting. ACE2 expression levels in Vero E6 cells or DBT cells stably expressing cACE2, hACE2, or GFP were assessed by flow cytometry and Western blotting. DBT cells stably transfected with plasmids carrying cACE2-His, hACE2, or GFP-His were passaged four times, after which cells were stained for ACE2 expression (primary antibody, polyclonal anti-hACE2; secondary antibody, anti-goat-FITC) and sorted for mid-to-high ACE2 expression (FITC). GFP control cells were also sorted for mid-to-high expression. (A and C) To assess ACE2 expression in expanded postsorted cell stocks and in Vero E6 cells, cells were stained as described above and analyzed for FITC/GFP expression by flow cytometry. (B) To assess ACE2 expression in Vero E6 cells and in postsorted cell stocks by Western blotting, similar numbers of Vero E6, DBT, DBT-hACE2, DBT-cACE2-His, and DBT-GFP-His cells were lysed and separated on a NuPage 12% bis-Tris SDS-polyacrylamide gel. After membrane transfer, blots were probed with either polyclonal goat anti-human ACE2 or mouse anti-penta-His antibody. After membranes were washed, they were probed with either rabbit anti-goat HRP or anti-mouse IgG-HRP antibody. Membranes were rinsed and treated with ECL Plus reagent and exposed to radiographic film.

against the leader sequence and a reverse primer in the M gene, allowing us to preferentially detect subgenomic transcripts encoding the open reading frame (ORF) of the 3a, E, and M genes. ACE2 transcripts were detected by RT-PCR in mock-infected cell cultures of DBT-hACE2, DBT-cACE2, and Vero E6 cells (Fig. 2A). ACE2 transcripts were not detected in DBT cells, corroborating our flow cytometry and Western blot results. As expected, GAPDH-positive control transcripts were

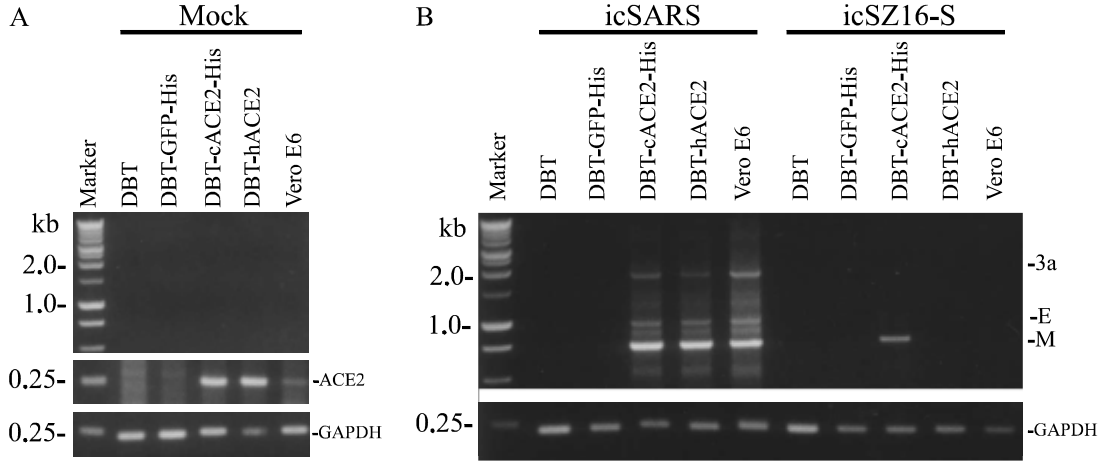


FIG. 2. Resurrection of icSZ16-S with DBT-cACE2 cells. RT-PCR to detect subgenomic leader-containing transcripts was performed to detect viral replication. DBT, DBT-GFP-His, DBT-cACE2, DBT-hACE2, or Vero E6 cells were infected with 100 ffil of viral supernatant from the initial icSZ16-S transfection, or the icSARS supernatants (B), or they were mock infected (A). At 24 hpi, total RNA was isolated, and cDNA was generated and then used as template for PCR. Evidence of SARS-CoV replication and subgenomic transcription, ACE2 gene expression, and control GAPDH gene expression was detected by the production of amplicons (SARS-CoV, 3a [1,796 bp], E [947 bp], M [666 bp]; control, 235 bp GAPDH; ACE2, 258 bp) visualized by electrophoresis using a 1.8% agarose TAE gel.

Downloaded from jvi.asm.org at University of North Carolina - Chapel Hill on December 20, 2008

detected at similar levels in all mock-infected cell cultures, while none of the mock-infected cultures was positive for SARS-CoV replication (Fig. 2A). In the icSARS virus-infected cell panel, DBT-cACE2, DBT-hACE2, and Vero E6 cells were clearly capable of supporting efficient SARS-CoV replication. Under identical conditions, SARS-CoV leader-containing transcripts were not detected in control DBT and DBT-GFP-His cells (Fig. 2B). Interestingly, only cACE2-expressing cells efficiently supported the replication of icSZ16-S (Fig. 2B). Importantly, icSZ16-S was successfully passaged and maintained in DBT-cACE2 cells in vitro, producing a robust cytopathology after four passages at 48-h intervals. Passage-four virus was plaque purified, stocks were grown, and the S, 3a, E and M genes were sequenced. Only one coding mutation (Y107H) was found in the 3a gene.

Assessment of virus growth in DBT, DBT-cACE2, DBT-hACE2, and Vero cells. Prior to this report, zoonotic SARS S virus and h/cACE2 interactions were evaluated by using pseudovirus bearing the GD03 and SZ16 S proteins, but these assays were unable to address virus growth fitness (19, 36). Moreover, one study suggested that the GD03 S protein was not dependent on ACE2 for docking and entry (36). Consequently, we assessed the growth fitness of recombinant virus bearing various S glycoproteins derived from the early, middle, and late phases of the SARS epidemic in China. We employed recombinant SARS-CoV bearing the S genes isolated from humans during the early (icGZ02), middle (icCUHK-W1), and late (icSARS) phases of the epidemic and one isolate from a sporadic human case in early 2004 (icGD03-S). The GD03, GZ02, and CUHK-W1 S glycoproteins differ from the those of the epidemic strain by 17, 8, and 2 amino acids, respectively (Fig. 3D). We also evaluated the growth of the icSZ16 parent virus and two SZ16 mutants, previously described by Sheahan et al., which were selected for efficient growth in HAE cells (26). The SZ16, SZ16-K479N, and SZ16-K479N D22 S glycoproteins differ from those of the epidemic strain by 18, 17, and 19 amino acids, respectively (26). The growth fitness of the virus panel was assessed in DBT, DBT-cACE2, DBT-hACE2, and Vero E6 cell cultures (Fig. 3A, B, and C). Cultures were infected at an MOI of 0.01 for 1 h and washed to remove input virus, and growth medium was added; then, cultures were sampled at various times postinfection. DBT cells that were not transfected with the SARS receptor ACE2 were not permissive to infection by any of the viruses tested (data not shown). In DBT-cACE2 cells, icSARS, icSZ16-S, icGZ02, and icGD03-S grew to similar peak titers by 36 hpi (Fig. 3A). In DBT-cACE2 cells at 12 hpi, the icSZ16-S titers were approximately one log higher than those of all of the other viruses tested (Fig. 3A). Interestingly, CUHK-W1 had severely delayed growth kinetics within the cACE2-expressing cells. Similarly, icSZ16-S K479N and icSZ16-S K479N D22 exhibited little to no growth in the DBT-cACE2 cells, though their S protein sequences differ from that of SZ16 by one (K479N) and three (D22) amino acids (Fig. 3A). Within DBT cells expressing hACE2, the epidemic strain exhibited superior growth fitness compared to those of the other viruses tested, with a 1 to 2 log titer advantage at 12 hpi, but icSARS, icCUHK-W1, icGZ02, icGD03-S, and icSZ16-K479N D22 grew to similar peak titers by 36 hpi. As we had seen previously, icSZ16-K479N grew poorly in the DBT-hACE2 cells, reaching titers of less than 10^4 PFU/ml, and icSZ16-S did not grow at all (Fig. 3B). Vero E6 cell growth kinetics for icSARS, icCUHK-W1, icGZ02, and icSZ16-S K479N D22 were quite similar, reaching titers of around 10^7 PFU/ml by 36 hpi, while icSZ16-S K479N and icGD03-S grew much less efficiently (# 10^5 PFU/ml at 36 hpi) (Fig. 3C). As we had seen by RT-PCR, the Vero E6 cell was not permissive to infection by icSZ16-S (Fig. 3C).

icGD03-S and icSZ16-S are dependent on ACE2 for entry. Using pseudotyped viruses, previous reports have suggested that some zoonotic SARS-CoV do not use ACE2 for entry (36). Since the SARS S pseudotyped viruses lack the other SARS-CoV structural proteins found in the SARS-CoV envelope (E, M, 3a, and 7a) and the amounts of S protein present on the surface of pseudovirus may differ from those found on the surface of a natural SARS-CoV particle, the use of SARS S pseudotyped viruses to study binding and entry might not faithfully recapitulate natural virus-cell interactions. To assess the receptor dependence of the recombinant SARS-CoV strains bearing zoonotic S proteins, we utilized a receptor antibody blockade assay (Fig. 4). Twofold dilutions of antibody (polyclonal anti-ACE2) or control antibody (polyclonal anti-ACE) were used to block ACE2 within DBT-cACE2 cells. The blocked cells were subsequently infected with 100 PFU of icSARS, icGD03-S, or icSZ16-S, rinsed and overlaid with medium containing agarose. All three viruses tested were equally dependent on ACE2 for entry, exhibiting a dose-dependent response to anti-ACE2 receptor blocking antibody (Fig. 4). Control antibody (polyclonal anti-ACE) was not capable of blocking entry of any of the viruses tested in a dose-dependent manner, firmly establishing the fact that ACE2 is a receptor for both the epidemic and the civet strains of SARS-CoV (Fig. 4).

hu-MAb S230.15 and S3.1 neutralization profiles differ between epidemic, in vitro-evolved, and zoonotic strains of SARS-CoV. hu-MAbs S230.15 and S3.1 were isolated from a convalescent SARS patient's B cells (21, 30). To assess the neutralization efficacy of these hu-MAbs, we employed a PRNT assay with the following challenge viruses: icSARS (epidemic strain S protein), icGD03-S (divergent human strain S protein), icSZ16-S (civet strain S protein), icSZ16-S K479N (SZ16 S protein mutated at residue 479 from lysine to asparagine), and icSZ16-S K479N D22 (in vitro-evolved mutant with enhanced growth in human cells whose S protein is mutated at Y442F and L472F). The broadly neutralizing hu-MAb S230.15 was able to neutralize 50% ($PRNT_{50}$) of icSARS virus and the evolved icSZ16-S K479N D22 virus, using less than 0.03125 ffig/ml of antibody, while icGD03-S, icSZ16-S K479N and icSZ16-S were approximately four times more resistant ($PRNT_{50}$ $ 0.125 ffig/ml) (Fig. 5A). The S3.1 hu-MAb was not as effective at neutralizing the viruses tested ($PRNT_{50}$ for icSARS and icSZ16-S K479N was 0.25 ffig/ml and for icSZ16-S K479N D22 was 0.625 ffig/ml) (Fig. 5B). Unlike hu-MAb S230.15, hu-MAb S3.1 was weakly efficacious against icGD03-S ($PRNT_{50}$ % 1 ffig/ml) and completely ineffective against icSZ16-S (Fig. 5B).

Structural models of SARS and ACE2 interactions. To gain a better understanding of the structural changes associated with the mutation of both the S and the ACE2 proteins, we developed predictive structural models of S and cACE2/hACE2 interactions using RosettaDesign and Modeler software. Our models suggest that additional methyl groups in E30 and Y34 of cACE2 likely

Downloaded from jvi.asm.org at University of North Carolina - Chapel Hill on December 20, 2008

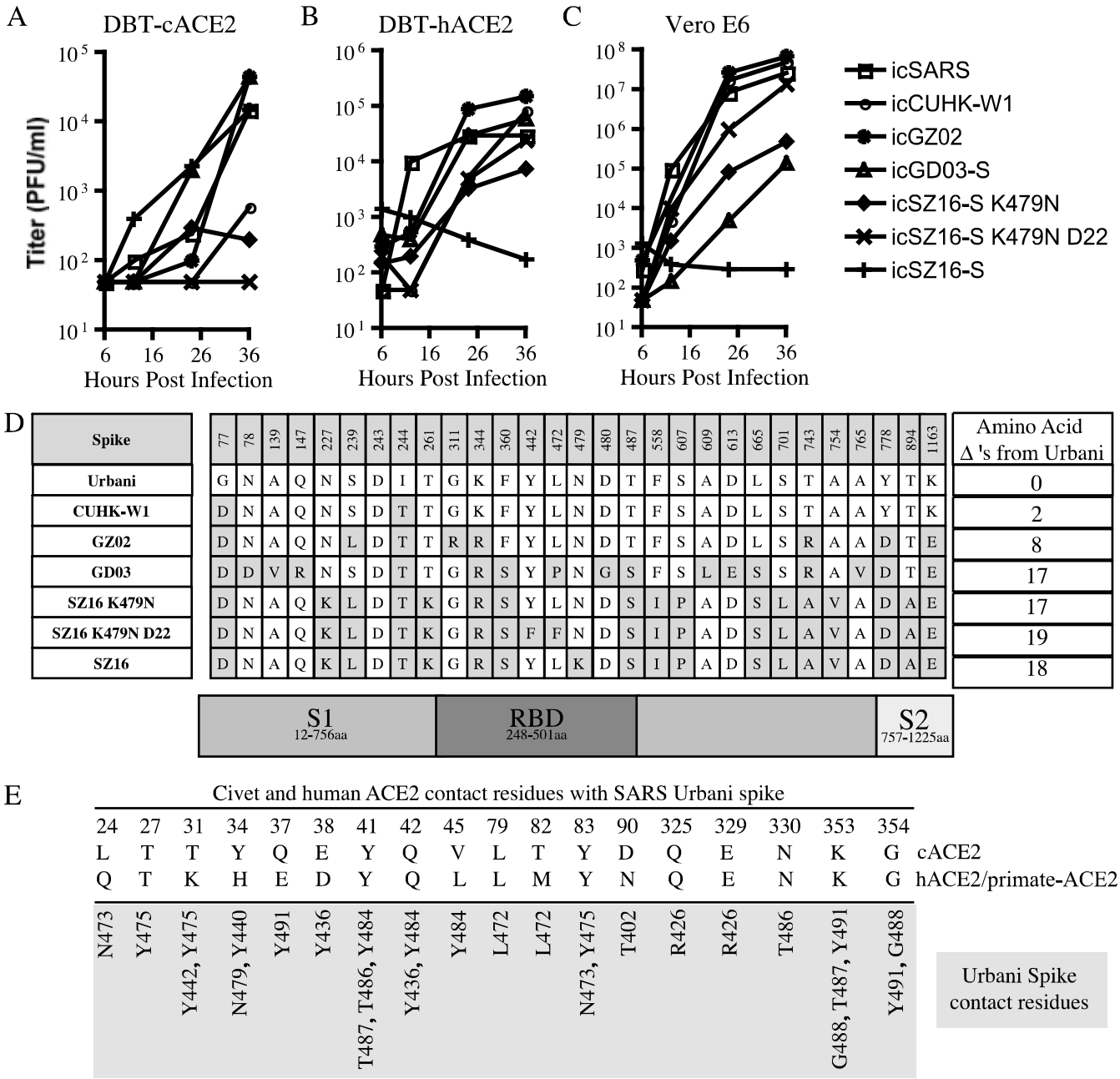


FIG. 3. Assessment of virus growth in DBT, DBT-cACE2, DBT-hACE2, and Vero cells, the spike amino acid variation in our recombinant virus panel, and the ACE2 contact residues with the Urbani spike. DBT-cACE2 (A), DBT-hACE2 (B), Vero E6 (C), or DBT (data not shown) cells were infected with icCUHK-W1, icGD03-S, icGZ02, icSZ16-S K479N p6, icSZ16-S K479N D22, icSARS, or icSZ16-S at an MOI of 0.01 for 1 h at 37°C. Cell medium (25 ffil) was removed at 0, 6, 12, 24, and 36 hpi, and samples were stored at " 80°C until titers were determine by plaque assay. Growth curves were performed in duplicate on two separate occasions, and the data shown are representative of one experiment. (D) The location of spike amino acid differences among the recombinant virus panels. (E) Urbani, cACE2, primate-ACE2, and hACE2 contact residues, adapted from the crystal structure published by Li et al. (17).

create a protrusion from the binding interface that is enhanced compared to that of hACE2. The architecture of the Urbani RBD can accommodate this protrusion on the cACE2 binding interface, thus retaining efficient binding to both cACE2 and hACE2 (Fig. 6A and B). We and others have previously published structural models that suggest that K479 of the SZ16 S protein inhibits binding to hACE2 because of electrostatic clashes with the hACE2 binding partners K31 and H34, but the SZ16 binding partners within cACE2 are different (Y34 and E30) and allow for efficient binding of SZ16 S (Fig. 6C) (17, 19, 26). Interestingly, the human ACE2 adaptive mutations in SZ16 K479N and SZ16 K479N D22 that progressively remodel the RBD binding interface to enhance binding to hACE2 (Fig. 6D and F) create a steric clash between the RBDs V404/Y440 and E30/Y34 of cACE2

Downloaded from jvi.asm.org at University of North Carolina - Chapel Hill on December 20, 2008

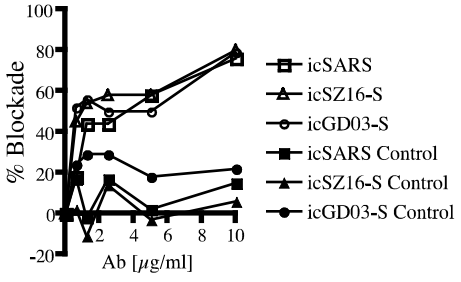


FIG. 4. icGD03-S and icSZ16-S are dependent on ACE2 for entry. DBT-cACE2 cells were seeded at 5 ! 10^5 cells/well in six-well dishes. The following day, cell medium was removed, and cells were incubated with 10, 5, 2.5, 1.25, or 0.625 ffg/ml polyclonal anti-ACE2 or anti-ACE or DBPS for 1 h at 37°C. After the 1-h pretreatment with antibody, 100 PFU/50 ffil of icSARS, icSZ16-S, or icGD03-S was added to the monolayer and incubated for 1 h at 37°C. After the infection, the inoculum was removed and the monolayer was rinsed with DPBS and then overlaid with 0.9% agarose in complete growth medium. At 48 hpi, plates were stained with neutral red, and plaques were counted. The average percentage of blockade was calculated by dividing the average number of plaques per Ab dilution by the average number of plaques in the DPBS no-Ab controls. Blockade experiments were performed in duplicate on two separate occasions.

(Fig. 6E and G). Similar to that which we had seen with in vitro growth analysis, the SZ16 K479N and D22 binding interfaces cannot accommodate the enhanced protrusion of cACE2 and are unable to bind efficiently.

DISCUSSION

The recent emergence of viruses like SARS-CoV, Ebola, influenza H5N1, Marburg, and Nipah virus highlights the pathogenic and epidemic potential of zoonoses in human populations (7, 10, 16, 29, 32). The identification of zoonotic virus animal reservoirs is often unsuccessful, hampering our ability to understand the epidemiology of zoonotic diseases and the conditions required for zoonotic host range expansion. Extensive virus sequencing efforts allowed the identification of palm civets as a potential amplification host for SARS-CoV and Chinese horseshoe bats as the potential SARS-CoV animal reservoir within a short span of 2 years (2, 8). Though sequencing efforts have revealed much about the biology of zoonotic SARS-CoV, bat and civet isolates have not yet been cultured in vitro (13, 15). Using synthetic biology and reverse genetics, we constructed a SARS-CoV bearing a civet SZ16 S glycoprotein. In corroboration with Lau and Peiris, we have shown that icSZ16-S could not be maintained by passage in cell cultures that normally support SARS-CoV infection (15). Without the successful cultivation of zoonotic SARS-CoV, their mechanisms of host range expansion and pathogenic potential remain elusive, and the efficacy of current therapies against zoonotic strains remains unknown.

Rather than mutate the SZ16 S glycoprotein to promote growth in cell culture, we constructed a stable cell line expressing the cACE2 orthologue of the human SARS-CoV receptor (hACE2). Compared to the published gene sequence, the cACE2 gene utilized to construct our stable cells differed by two residues, G354D and R736Q, but only the residue G354 resides at a proposed interaction site with the SARS S RBD (Y491 and G488) (17). Residue 354 is polymorphic in nature, where some civet samples contain the G354 ACE2 mutation, while others contain the D354 ACE2 mutation (personal communication, M. Farzan) (18). All of the viruses used within this study have the same amino acid at residues 491 (tyrosine) and 488 (glycine) (Fig. 3E). In turn, if the G354D mutation in our cACE2 protein has a deleterious effect on binding and entry, we predict that all viruses would be affected equally. As our data regarding Urbani and SZ16 binding to cACE2 and hACE2 are concordant with previously published data by Li et al., it is unlikely that the G354D mutation in cACE2 is altering our virus growth phenotypes, and this hypothesis is supported by our predictive structural modeling which suggests that the G354D mutation does not affect the conformation of the cACE2 binding interface (data not shown) (19).

Using DBT-cACE2 cells, we have demonstrated that icSZ16-S can be grown, maintained, and plaque purified. In support of Li et al., we have shown that civet SARS-CoV utilizes cACE2 but cannot utilize hACE2 or Vero E6 ACE2,

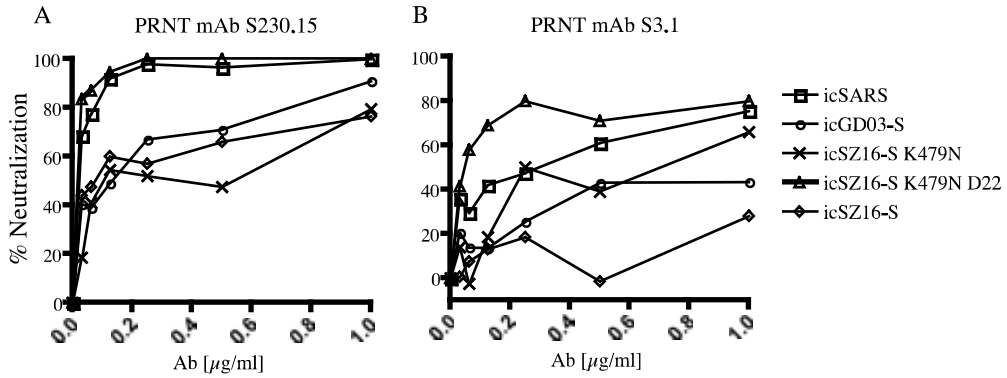


FIG. 5. hu-MAb S230.15 and S3.1 neutralization profiles differ between the epidemic, the in vitro-evolved, and the zoonotic strains of SARS-CoV. Neutralizing titers were determined by PRNT assay. Twenty-four hours before cells were infected, six-well plates were seeded with 5 ! 10^5 DBT-cACE2 cells/well. hu-MAb S230.15 and S3.1 and an isotype control antibody directed against cholera toxin, D2.2, were serially diluted twofold and incubated with 100 PFU of either icSARS, icSZ16-S, or icGD03-S for 1 h at 37°C. Virus and antibodies were then added to six-well plates of DBT-cACE2 cells in duplicate and incubated at 37°C for 1 h, after which the cells were overlaid with 3 ml of 0.8% agarose in medium. After 48 h, plates were stained with neutral red, and plaques were counted. The percentage of neutralization was calculated as 1 " (number of plaques with antibody/number of plaques without antibody) ! 100%.

Downloaded from jvi.asm.org at University of North Carolina - Chapel Hill on December 20, 2008

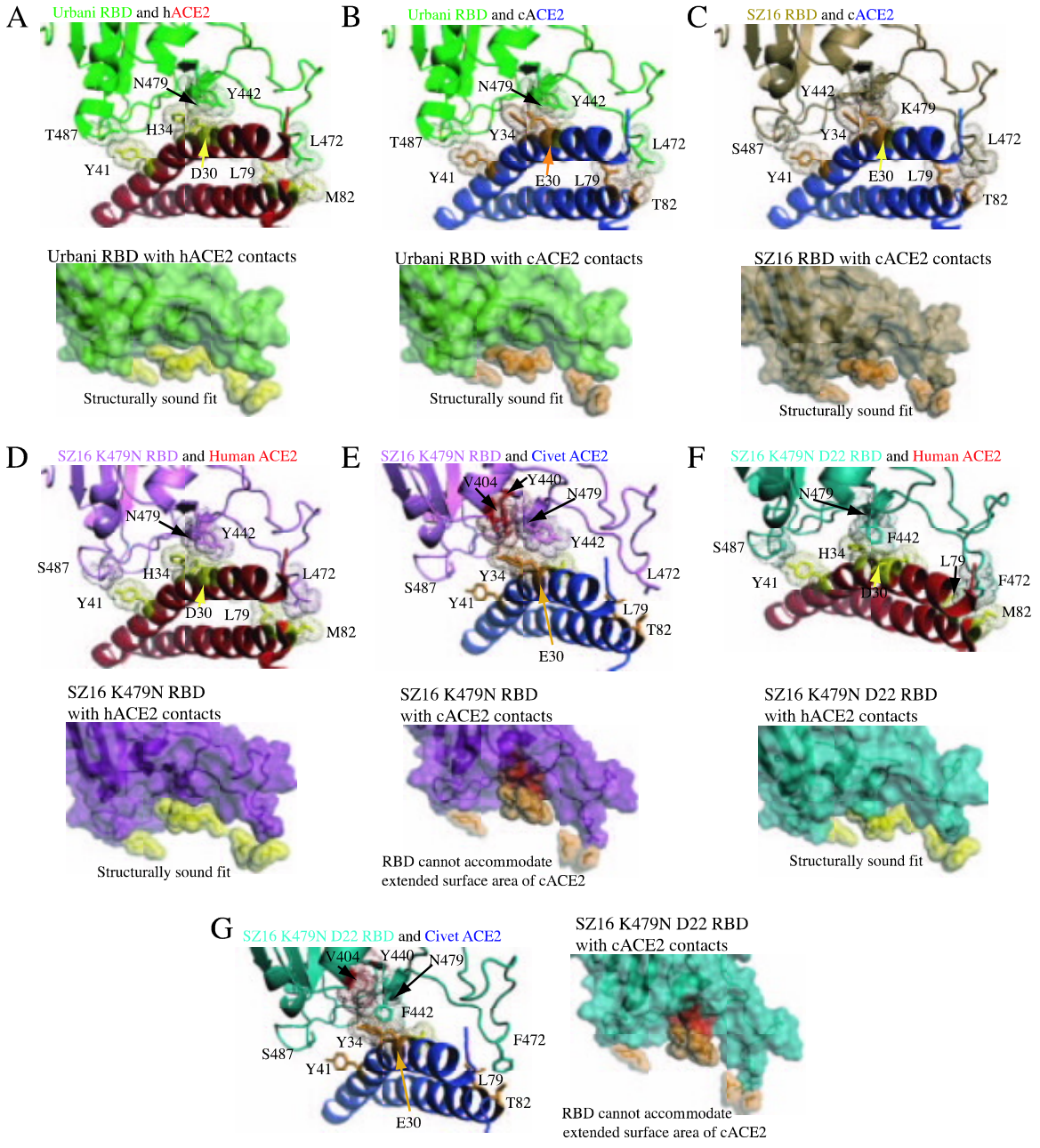


FIG. 6. Molecular modeling demonstrating structural mechanisms of ACE2 tropism. Based on the reported crystal coordinates of SARS Urbani RBD interacting with the hACE2 receptor, we generated models of Urbani, SZ16, SZ16-K479N, and SZ16 K479N D22 RBD interactions with either cACE2 or hACE2, using RosettaDesign and Modeler software. Ribbon structures and "space filling" schematics of each RBD and ACE2 combination are shown. Dotted spheres around the RBD and ACE2 residues indicate they are within 4 Å and thus are predicted to interact. Red spheres around the RBD and ACE2 residues indicate a steric clash. (A) Urbani RBD and hACE2 architecture. (B) Additional methyl groups of the cACE2 E30 and Y34 mutations add a surface protrusion to the contact interface. The Urbani RBD can accommodate the increased surface protrusion of cACE2, thereby retaining an efficient binding interface. (C) Similar to the Urbani RBD, the SZ16 RBD can accommodate the increased surface protrusion of cACE2 for efficient binding. (D) The N479 mutation in SZ16 K479N remodels the SZ16 binding interface to promote binding to hACE2. (E) The remodeling of the SZ16 K479N binding interface by the N479 mutation creates a clash between S residues (V404 and Y440) and cACE2 residues (E30 and Y34), blocking S and ACE2 binding. The SZ16 K479N RBD cannot accommodate the extended surface protrusion of the cACE2 RBD. (F) In addition to the N479 mutation, the F442 and F472 mutations further remodel the SZ16 K479N D22 RBD, further enhancing the binding efficiency to hACE2. (G) Similar to the SZ16 K479N RBD interaction with cACE2, the interaction of the SZ16 K479N D22 RBD cannot accommodate the protrusion of cACE2, abrogating binding.

confirming that the civet SZ16 virus is restricted in its host range (Fig. 2B). Using pseudotyped lentivirus, Li et al. showed that the SARS Urbani S protein was capable of utilizing both cACE2 and hACE2, and we confirmed these data by showing that icSARS is capable of infecting both cACE2- and hACE2-expressing cells and replicating to a high titer (Fig. 2B and 3A and B). In support of our RT-PCR data suggesting that icSZ16-S was restricted in its host range (Fig. 2B), icSZ16-S

Downloaded from jvi.asm.org at University of North Carolina - Chapel Hill on December 20, 2008

grew only within cACE2-expressing cells and grew to titers similar to those of the epidemic strains icGD03-S and icGZ02 (Fig. 3 A, B, and C). Interestingly, icSZ16-S exhibited a clear growth advantage at 12 hpi, suggesting that it might gain entry into cACE2-expressing cells more rapidly than the other viruses tested. The icSZ16-S K479N and D22 viruses were adapted for efficient growth on a primary HAE cell culture, which is known to be a robust and relevant model of the complex human airway epithelium (27). Importantly, these viruses did not grow efficiently within DBT-cACE2 cells, suggesting that HAE cell growth-enhancing mutations in the civet S (K479N, Y442F and L472F) proteins diminished binding of the icSZ16-S K479N/D22 S protein to cACE2. To conclusively demonstrate that differences in the cACE2 binding affinities of the icSZ16-S, icSZ16-S K479N, and D22 viruses account for the observed differences in growth kinetics, binding and entry assays will be performed in the future. In hACE2-expressing cells, the epidemic strain displayed a clear growth advantage at 12 hpi, but most all of the viruses tested grew to similar peak titers by 36 hpi. In Vero E6 cells, the viruses bearing S proteins that were more similar to that of the epidemic strain (CUHK-W1, GZ02) grew to peak titers that were far greater than those of viruses bearing the S proteins that were more civet-like (SZ16, GD03, and SZ16 K479N). As we had expected, icSZ16-S was not capable of growth within DBT-hACE2 or Vero E6 cells, but mutations in the SZ16 S protein at select interaction sites with ACE2 (K479N, Y442F, L472F) rescued and promoted growth in hACE2 or Vero E6 cells. These data suggest that the SARS-CoV S and receptor interactions are quite specific and capable of restricting the host range or limiting infection efficiency. Moreover, these data also suggest that very few mutations in the civet S protein can enhance infection of human cells (K479N, Y442F, L472F) and simultaneously abrogate infection of cACE2-expressing cells. A corollary is seen in the more human-tropic strains. icCUHK-W1, a middle-phase epidemic isolate whose S protein differs from that of Urbani by two amino acids, is quite capable of infecting cells expressing hACE2 while it grows very poorly in cACE2-expressing cells compared to the epidemic strain. These data suggest that G77 and/or I244 of the epidemic strain enhances growth in cACE2-expressing cells. Though G77 and I244 reside outside of the RBD (amino acids 248 to 501), their close proximity to the RBD may either promote a conformational rearrangement of the S protein, subtly altering the S binding interface through long-range protein interactions, or somehow contribute to binding or entry by an unknown mechanism. Through the passage of zoonotic S-bearing SARS-CoV on Vero E6 cells, Rockx et al. described mutations just outside of the RBD (HC/SZ/61/03; amino acid positions 578; and A031G, amino acid position 577) that seemed to enhance growth, suggesting that mutations outside of the RBD may influence the architecture of the binding interface or enhance binding and entry by an unknown mechanism (22). All together, the growth curve data highlight the plasticity of both zoonotic and human-tropic SARS-CoV S proteins, where the subtle remodeling RBD can both enhance and abrogate binding to ACE2.

Serological evaluation of workers in wet markets suggests that zoonotic SARS-CoV was transmitted to humans, though these seropositive individuals did not present clinically with SARS (1). These data support the hypothesis that repeated transfer of zoonotic SARS-CoV from civet to human and/or from human to civet fostered the evolution of the epidemic strain. Alternatively, repeated single introductions of civet SARS-CoV into the human population may eventually have resulted in the generation of a human-tropic SARS-CoV that coincidently retained affinity for cACE2. Our growth curve analyses demonstrating that the epidemic strain is equally fit at growth within cACE2- or hACE2-expressing cells supports both evolutionary hypotheses. In fact, recent data suggest that civets infected with human-tropic SARS-CoV develop symptoms and a pathology similar to those seen in human cases of SARS-CoV infection (35). Our predictive RBD and ACE2 structure model data provide a possible explanation of how the dual host tropism of SARS Urbani is retained. The cACE2 molecule differs from that of hACE2 by two key residues (E30 and Y34), which likely creates a pronounced protrusion from the cACE2 binding interface. The Urbani RBD is able to accommodate the various binding interfaces of both cACE2 and hACE2, thus retaining dual host tropism. We also demonstrate that the SZ16 lineage viruses passaged with HAE cells, in the absence of cACE2, evolved a mutant RBD that allowed for more efficient binding of hACE2 (icSZ16-S K479N, D22) but simultaneously lost the ability to efficiently infect cACE2-expressing cells. Our predictive structural models suggest that the human adaptive RBD mutations in icSZ16-S K479N and D22 abrogate their abilities to accommodate the protrusive cACE2 binding interfaces. Therefore, we hypothesize that in the absence of evolutionary pressure to retain the ability to grow within both cACE2- and hACE2-expressing cells, a series of mutations are acquired to enhance binding to only one of the two receptors. We might have been able to generate a strain more similar to the dual tropic epidemic strain had we done repeated passages of the icSZ16-S virus, alternating between cACE2- and hACE2-expressing cells. Nevertheless, these data suggest that the natural evolution of the epidemic strain probably occurred through repeated transfer of virus from civet to human and from human to civet over a long period of time, providing the evolutionary pressure to retain dual ACE2 tropism. This hypothesis is supported by three serological surveys performed to determine the prevalence of SARS-CoV-specific IgG in healthy subjects in China. First, samples collected from volunteers in Guangdong province in China, in May 2003, demonstrated that SARS virus-specific IgG was found in animal traders (13%; n = 508), hospital workers (2.9%; n = 137), Guangdong CDC workers (1.6%; n = 63), and healthy adults at the clinic (1.2%; n = 84) (1). The second study was a retrospective serological survey performed with 1,621 serum samples from March 2002, isolated from healthy 18-year-old male Chinese soldiers from 17 provinces, including Guangdong (39). Of these 1,621 samples, 0.68% tested positive for SARS-CoV by enzyme-linked immunosorbent assay, though the provinces that the SARS-CoV IgG-positive soldiers inhabited were not mentioned. A third study retrospectively examined 938 serum samples collected in May of 2001 from healthy adults in Hong Kong. Shockingly, 1.8% of these samples collected 2 years prior to the epidemic tested positive for SARS-CoV by immunofluorescence assay (40). These data suggest that less serious or asymptomatic cases of SARS-CoV existed at least 2 years prior to the beginning of the epidemic in various geographical locations in

Downloaded from jvi.asm.org at University of North Carolina - Chapel Hill on December 20, 2008

China. If the populations sampled in the May 2003 cohort are representative of the Guangdong province population (estimated population of 100,000,000), there were probably many more unreported or less serious cases (%1,000,000) of SARS-CoV infection prior to the evolution of the highly pathogenic epidemic strain of 2002 to 2003.

Though molecular evolution studies of SARS-CoV genomes have demonstrated that the S gene had undergone a significant amount of mutation and positive selection during the early phases of the epidemic, the nonstructural ORF1a demonstrated a similar pattern (2). We have chosen to focus on the importance of the S mutation and ACE2 interaction for viral evolution and host range expansion, but the molecular genetic data clearly suggest that mutation of nonstructural genes may also have been important for the efficient adaptation to new hosts.

In 2005, Yang et al. reported that pseudoviruses bearing the zoonotic S (GD03 and SZ3) protein were much less dependent on ACE2 for entry in 786-O cells (36). Using 786-O cells and similar pseudoviruses bearing the Tor2, GD03, or SZ3 S proteins, both Li et al. and He et al. were not able to infect 786-O cells with pseudoviruses (9, 19). Since SARS S pseudotyped viruses lack SARS-CoV membrane-bound proteins (E, M, 3a, 7a) and may contain unnatural amounts of surface SARS S glycoprotein, pseudovirus may produce false-positive or false-negative results for binding and entry assays. Moreover, the pseudovirus systems are limited to the evaluation of binding and entry and are unable to evaluate the effects of zoonotic glycoproteins on replication and growth. To reevaluate the pseudovirus findings in the context of natural SARS-CoV infection, we constructed SARS-CoV strains bearing zoonotic S proteins. The SZ3 S protein differs from that of SZ16 by one amino acid outside of the RBD (SZ3 F558, SZ16 I558). Since our molecular and growth curve data suggested that icSZ16-S efficiently infects only cACE2-expressing cells, we performed receptor blockade experiments with DBT-cACE2 cells. We found that icSARS, icSZ16-S, and icGD03-S were similarly blocked from entry in a dose-dependent manner, using an antibody directed against ACE2, while the control antibody had little effect. Though we did not achieve 100% blockade with the highest concentration of antibody tested, we believe that polyclonal anti-hACE2 may interact less efficiently with native cACE2. Alternatively, we may not have completely saturated the available surface cACE2, which would result in an incomplete blockade. At this time, we believe it is more likely that our inability to block 100% of infection was due the technical issues discussed above and less likely that zoonotic viruses gain entry into cells via alternative receptors. Since the viruses we evaluated could not productively infect nontransfected DBT cells, it is unlikely that the incomplete block in entry with the Ab was due to entry via alternative receptors. However, coreceptors (L-SIGN) have been reported for SARS-CoV, but to our knowledge, the ability of zoonotic SARS-CoV to engage these receptors has not been rigorously examined (11). Therefore, we believe we most likely did not block 20% of the surface ACE2 with 10 ffig/ml Ab.

Since it is impossible to predict the antigenic identity of future emergent SARS viruses, the development of highly cross-reactive antibody, drug, or vaccination therapies would provide the greatest potential benefit to public health. Recently, we and others have shown that hu-MAb S230.15 was less effective at neutralizing icSZ16-S K479N in vitro but was very effective in passive transfer studies with mice, lowering viral titers of both icGD03-S and icSZ16-S K479N below the detectable limit (41). In in vitro PRNT assays, icSZ16-S and icGD03-S were also more resistant to neutralization by S230.15 than the epidemic strain. Since icSZ16-S and icGD03-S are similarly neutralized by S230.15 in vitro, passive transfer studies by Zhu et al. suggest that S230.15 would be effective against icSZ16-S in vivo as well (41). Yang et al. described an antibody-dependent enhancement phenomenon using hu-MAb S3.1 in neutralization assays with 786-O cells, using the GD03 and SZ16 pseudoviruses (36). We performed PRNT assays with cACE2 cells, using icSARS, icSZ16-S, and icGD03-S, and found that S3.1 is moderately effective against icSARS, weakly effective against icGD03-S, and ineffective against icSZ16-S. Since there was such a dynamic range of neutralization efficacies using S3.1 against a panel of epidemic and zoonotic SARS-CoV, these data highlight the pitfalls of using only the epidemic strain when passive immunization or vaccination therapies are evaluated. Of note, ter Meulen et al. explored the possibility of antibody-dependent enhancement in human macrophages using SARS-CoV and subneutralizing concentrations of monoclonal antibodies and found that SARS-CoV is taken up by macrophages but does not productively infect the cells (28).

Coronaviruses similar to SARS Urbani are currently circulating within bat species in China. Constant surveillance and biological evaluation of zoonotic pools of coronavirus are impractical, making it quite difficult to predict the antigenic identity of emergent SARS-CoV-like viruses in the future. Therefore, it is imperative that current antiviral therapies be broadly cross-reactive against all known SARS-CoV, thereby maximizing the potential public health benefit. We have constructed a SARS-CoV bearing the prototypic civet S protein from the SZ16 strain. Prior to this publication, SARS-CoVs bearing the SZ16 S protein were not able to be propagated in vitro, hampering the study of the SZ16 biology and its pathogenic potential. We have demonstrated that icSZ16-S can be propagated in vitro in DBT-cACE2 cell cultures, that it is dependent on ACE2 for entry, and that it is incapable of using hACE2 for entry. We have also demonstrated that point mutations in the SZ16 S protein that enhance virus growth in primary HAE or DBT-hACE2 cells severely diminish growth in DBT-cACE2 cells, while the epidemic strain retains dual ACE2 tropism. These data and retrospective serological surveys in China suggest that the natural evolution of the epidemic strain probably occurred over a long period of time through the repeated transfer of virus from civet to human and from human to civet and that this dual host evolutionary scenario provided the evolutionary pressure to retain dual ACE2 tropism. We have also demonstrated that both icGD03-S and icSZ16-S are neutralized by hu-MAb S230.15 but are more resistant to S3.1, highlighting the utility of using an antigenically diverse SARS-CoV panel to assess serotherapy efficacy. The icSZ16-S virus is yet another antigenically divergent zoonotic S protein-bearing SARS-CoV that will prove useful in evaluating future serotherapies or vaccination therapies.

Downloaded from jvi.asm.org at University of North Carolina - Chapel Hill on December 20, 2008

ACKNOWLEDGMENTS

We thank Michael Farzan, Harvard Medical School, Boston, MA, for kindly providing the cACE2 and hACE2 expression plasmids. We also thank Antonio Lanzevecchia, Institute for Research in Biomedicine, Bellinzona, Switzerland, for graciously providing hu-MAbs S230.15, S3.1, and D2.2. We also thank Amy Sims and Boyd Yount for world-class technical assistance and superb cloning advice.

This work was supported by research grants (R01 AI059136 and AI059443) to R.B. from the National Institutes of Health, Division of Allergy and Infectious Diseases.

REFERENCES

1. **Centers for Disease Control and Prevention.** 2003. Prevalence of IgG antibody to SARS-associated coronavirus in animal traders—Guangdong Province, China, 2003. MMWR Morb. Mortal. Wkly. Rep. **52:**986–987.
2. **Chinese SARS Molecular Epidemiology Consortium.** 2004. Molecular evolution of the SARS coronavirus during the course of the SARS epidemic in China. Science **303:**1666–1669.
3. **Christian, M. D., S. M. Poutanen, M. R. Loutfy, M. P. Muller, and D. E. Low.** 2004. Severe acute respiratory syndrome. Clin. Infect. Dis. **38:**1420–1427.
4. **Deming, D., T. Sheahan, M. Heise, B. Yount, N. Davis, A. Sims, M. Suthar, J. Harkema, A. Whitmore, R. Pickles, A. West, E. Donaldson, K. Curtis, R. Johnston, and R. Baric.** 2006. Vaccine efficacy in senescent mice challenged with recombinant SARS-CoV bearing epidemic and zoonotic spike variants. PLoS Med. **3:**e525.
5. **Dominguez, S. R., T. J. O'Shea, L. M. Oko, and K. V. Holmes.** 2007. Detection of group 1 coronaviruses in bats in North America. Emerg Infect. Dis. **13:**1295–1300.
6. **Dong, B. Q., W. Liu, X. H. Fan, D. Vijaykrishna, X. C. Tang, F. Gao, L. F. Li, G. J. Li, J. X. Zhang, L. Q. Yang, L. L. Poon, S. Y. Zhang, J. S. Peiris, G. J. Smith, H. Chen, and Y. Guan.** 2007. Detection of a novel and highly divergent coronavirus from Asian leopard cats and Chinese ferret badgers in Southern China. J. Virol. **81:**6920–6926.
7. **Gonzalez, J. P., X. Pourrut, and E. Leroy.** 2007. Ebola virus and other filoviruses. Curr. Top. Microbiol. Immunol. **315:**363–387.
8. **Guan, Y., B. J. Zheng, Y. Q. He, X. L. Liu, Z. X. Zhuang, C. L. Cheung, S. W. Luo, P. H. Li, L. J. Zhang, Y. J. Guan, K. M. Butt, K. L. Wong, K. W. Chan, W. Lim, K. F. Shortridge, K. Y. Yuen, J. S. Peiris, and L. L. Poon.** 2003. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. Science **302:**276–278.
9. **He, Y., J. Li, W. Li, S. Lustigman, M. Farzan, and S. Jiang.** 2006. Cross-neutralization of human and palm civet severe acute respiratory syndrome coronaviruses by antibodies targeting the receptor-binding domain of spike protein. J. Immunol. **176:**6085–6092.
10. **Hsu, V. P., M. J. Hossain, U. D. Parashar, M. M. Ali, T. G. Ksiazek, I. Kuzmin, M. Niezgoda, C. Rupprecht, J. Bresee, and R. F. Breiman.** 2004. Nipah virus encephalitis reemergence, Bangladesh. Emerg. Infect. Dis. **10:**2082–2087.
11. **Jeffers, S. A., S. M. Tusell, L. Gillim-Ross, E. M. Hemmila, J. E. Achenbach, G. J. Babcock, W. D. Thomas, Jr., L. B. Thackray, M. D. Young, R. J. Mason, D. M. Ambrosino, D. E. Wentworth, J. C. Demartini, and K. V. Holmes.** 2004. CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. Proc. Natl. Acad. Sci. USA **101:**15748–15753.
12. **Kan, B., M. Wang, H. Jing, H. Xu, X. Jiang, M. Yan, W. Liang, H. Zheng, K. Wan, Q. Liu, B. Cui, Y. Xu, E. Zhang, H. Wang, J. Ye, G. Li, M. Li, Z. Cui, X. Qi, K. Chen, L. Du, K. Gao, Y. T. Zhao, X. Z. Zou, Y. J. Feng, Y. F. Gao, R. Hai, D. Yu, Y. Guan, and J. Xu.** 2005. Molecular evolution analysis and geographic investigation of severe acute respiratory syndrome coronavirus-like virus in palm civets at an animal market and on farms. J. Virol. **79:**11892–11900.
13. **Lau, S. K., P. C. Woo, K. S. Li, Y. Huang, H. W. Tsoi, B. H. Wong, S. S. Wong, S. Y. Leung, K. H. Chan, and K. Y. Yuen.** 2005. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc. Natl. Acad. Sci. USA **102:**14040–14045.
14. **Lau, S. K., P. C. Woo, K. S. Li, Y. Huang, M. Wang, C. S. Lam, H. Xu, R. Guo, K. H. Chan, B. J. Zheng, and K. Y. Yuen.** 2007. Complete genome sequence of bat coronavirus HKU2 from Chinese horseshoe bats revealed a much smaller spike gene with a different evolutionary lineage from the rest of the genome. Virology **367:**428–439.
15. **Lau, Y. L., and J. S. Peiris.** 2005. Pathogenesis of severe acute respiratory syndrome. Curr. Opin. Immunol. **17:**404–410.
16. **Leroy, E. M., B. Kumulungui, X. Pourrut, P. Rouquet, A. Hassanin, P. Yaba, A. Delicat, J. T. Paweska, J. P. Gonzalez, and R. Swanepoel.** 2005. Fruit bats as reservoirs of Ebola virus. Nature **438:**575–576.
17. **Li, F., W. Li, M. Farzan, and S. C. Harrison.** 2005. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. Science **309:**1864–1868.
18. **Li, W., J. Sui, I. C. Huang, J. H. Kuhn, S. R. Radoshitzky, W. A. Marasco, H. Choe, and M. Farzan.** 2007. The S proteins of human coronavirus NL63 and severe acute respiratory syndrome coronavirus bind overlapping regions of ACE2. Virology **367:**367–374.
19. **Li, W., C. Zhang, J. Sui, J. H. Kuhn, M. J. Moore, S. Luo, S. K. Wong, I. C. Huang, K. Xu, N. Vasilieva, A. Murakami, Y. He, W. A. Marasco, Y. Guan, H. Choe, and M. Farzan.** 2005. Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2. EMBO J. **24:**1634–1643.
20. **Muller, M. A., J. T. Paweska, P. A. Leman, C. Drosten, K. Grywna, A. Kemp, L. Braack, K. Sonnenberg, M. Niedrig, and R. Swanepoel.** 2007. Coronavirus antibodies in African bat species. Emerg. Infect. Dis. **13:**1367–1370.
21. **Rockx, B., D. Corti, E. Donaldson, T. Sheahan, K. Stadler, A. Lanzavecchia, and R. Baric.** 2008. Structural basis for potent cross-neutralizing human monoclonal antibody protection against lethal human and zoonotic SARS-CoV challenge. J. Virol. **82:**3220–3235.
22. **Rockx, B., T. Sheahan, E. Donaldson, J. Harkema, A. Sims, M. Heise, R. Pickles, M. Cameron, D. Kelvin, and R. Baric.** 2007. Synthetic reconstruction of zoonotic and early human severe acute respiratory syndrome coronavirus isolates that produce fatal disease in aged mice. J. Virol. **81:**7410–7423.
23. **Rota, P. A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P. Icenogle, S. Penaranda, B. Bankamp, K. Maher, M. H. Chen, S. Tong, A. Tamin, L. Lowe, M. Frace, J. L. DeRisi, Q. Chen, D. Wang, D. D. Erdman, T. C. Peret, C. Burns, T. G. Ksiazek, P. E. Rollin, A. Sanchez, S. Liffick, B. Holloway, J. Limor, K. McCaustland, M. Olsen-Rasmussen, R. Fouchier, S. Gunther, A. D. Osterhaus, C. Drosten, M. A. Pallansch, L. J. Anderson, and W. J. Bellini.** 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science **300:**1394–1399.
24. **Sawicki, S. G., D. L. Sawicki, and S. G. Siddell.** 2007. A contemporary view of coronavirus transcription. J. Virol. **81:**20–29.
25. **Sheahan, T., D. Deming, E. Donaldson, R. Pickles, and R. Baric.** 2006. Resurrection of an "extinct" SARS-CoV isolate GD03 from late 2003. Adv. Exp. Med. Biol. **581:**547–550.
26. **Sheahan, T., B. Rockx, E. Donaldson, A. Sims, R. Pickles, D. Corti, and R. Baric.** 2008. Mechanisms of zoonotic severe acute respiratory syndrome coronavirus host range expansion in human airway epithelium. J. Virol. **82:**2274–2285.
27. **Sims, A. C., R. S. Baric, B. Yount, S. E. Burkett, P. L. Collins, and R. J. Pickles.** 2005. Severe acute respiratory syndrome coronavirus infection of human ciliated airway epithelia: role of ciliated cells in viral spread in the conducting airways of the lungs. J. Virol. **79:**15511–15524.
28. **ter Meulen, J., E. N. van den Brink, L. L. Poon, W. E. Marissen, C. S. Leung, F. Cox, C. Y. Cheung, A. Q. Bakker, J. A. Bogaards, E. van Deventer, W. Preiser, H. W. Doerr, V. T. Chow, J. de Kruif, J. S. Peiris, and J. Goudsmit.** 2006. Human monoclonal antibody combination against SARS coronavirus: synergy and coverage of escape mutants. PLoS Med. **3:**e237.
29. **Towner, J. S., X. Pourrut, C. G. Albarino, C. N. Nkogue, B. H. Bird, G. Grard, T. G. Ksiazek, J. P. Gonzalez, S. T. Nichol, and E. M. Leroy.** 2007. Marburg virus infection detected in a common African bat. PLoS ONE **2:**e764.
30. **Traggiai, E., S. Becker, K. Subbarao, L. Kolesnikova, Y. Uematsu, M. R. Gismondo, B. R. Murphy, R. Rappuoli, and A. Lanzavecchia.** 2004. An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. Nat. Med. **10:**871–875.
31. **Woo, P. C., S. K. Lau, K. S. Li, R. W. Poon, B. H. Wong, H. W. Tsoi, B. C. Yip, Y. Huang, K. H. Chan, and K. Y. Yuen.** 2006. Molecular diversity of coronaviruses in bats. Virology **351:**180–187.
32. **Woo, P. C., S. K. Lau, and K. Y. Yuen.** 2006. Infectious diseases emerging from Chinese wet-markets: zoonotic origins of severe respiratory viral infections. Curr. Opin. Infect. Dis. **19:**401–407.
33. **Woo, P. C., M. Wang, S. K. Lau, H. Xu, R. W. Poon, R. Guo, B. H. Wong, K. Gao, H. W. Tsoi, Y. Huang, K. S. Li, C. S. Lam, K. H. Chan, B. J. Zheng, and K. Y. Yuen.** 2006. Comparative analysis of 12 genomes of three novel group 2c and group 2d coronaviruses reveals unique group and subgroup features. J. Virol. **81:**1574–1585.
34. **Wu, D., C. Tu, C. Xin, H. Xuan, Q. Meng, Y. Liu, Y. Yu, Y. Guan, Y. Jiang, X. Yin, G. Crameri, M. Wang, C. Li, S. Liu, M. Liao, L. Feng, H. Xiang, J. Sun, J. Chen, Y. Sun, S. Gu, N. Liu, D. Fu, B. T. Eaton, L. F. Wang, and X. Kong.** 2005. Civets are equally susceptible to experimental infection by two different severe acute respiratory syndrome coronavirus isolates. J. Virol. **79:**2620–2625.
35. **Xiao, Y., Q. Meng, X. Yin, Y. Guan, Y. Liu, C. Li, M. Wang, G. Liu, T. Tong, L. F. Wang, X. Kong, and D. Wu.** 2008. Pathological changes in masked palm civets experimentally infected by severe acute respiratory syndrome (SARS) coronavirus. J. Comp. Pathol. **138:**171–179.
36. **Yang, Z. Y., H. C. Werner, W. P. Kong, K. Leung, E. Traggiai, A. Lanzavecchia, and G. J. Nabel.** 2005. Evasion of antibody neutralization in emerging severe acute respiratory syndrome coronaviruses. Proc. Natl. Acad. Sci. USA **102:**797–801.
37. **Yount, B., K. M. Curtis, E. A. Fritz, L. E. Hensley, P. B. Jahrling, E. Prentice, M. R. Denison, T. W. Geisbert, and R. S. Baric.** 2003. Reverse genetics with a full-length infectious cDNA of severe acute respiratory syndrome coronavirus. Proc. Natl. Acad. Sci. USA **100:**12995–13000.
38. **Yount, B., R. S. Roberts, A. C. Sims, D. Deming, M. B. Frieman, J. Sparks, M. R. Denison, N. Davis, and R. S. Baric.** 2005. Severe acute respiratory

Downloaded from jvi.asm.org at University of North Carolina - Chapel Hill on December 20, 2008

syndrome coronavirus group-specific open reading frames encode nonessential functions for replication in cell cultures and mice. J. Virol. **79:**14909–14922.

39. **Yu, S., M. Qiu, Z. Chen, X. Ye, Y. Gao, A. Wei, X. Wang, L. Yang, J. Wang, J. Wen, Y. Song, D. Pei, E. Dai, Z. Guo, C. Cao, J. Wang, and R. Yang.** 2005. Retrospective serological investigation of severe acute respiratory syndrome coronavirus antibodies in recruits from mainland China. Clin. Diagn. Lab. Immunol. **12:**552–554.
40. **Zheng, B. J., K. H. Wong, J. Zhou, K. L. Wong, B. W. Young, L. W. Lu, and S. S. Lee.** 2004. SARS-related virus predating SARS outbreak, Hong Kong. Emerg. Infect. Dis. **10:**176–178.
41. **Zhu, Z., S. Chakraborti, Y. He, A. Roberts, T. Sheahan, X. Xiao, L. E. Hensley, P. Prabakaran, B. Rockx, I. A. Sidorov, D. Corti, L. Vogel, Y. Feng, J. O. Kim, L. F. Wang, R. Baric, A. Lanzavecchia, K. M. Curtis, G. J. Nabel, K. Subbarao, S. Jiang, and D. S. Dimitrov.** 2007. Potent cross-reactive neutralization of SARS coronavirus isolates by human monoclonal antibodies. Proc. Natl. Acad. Sci. USA **104:**12123–12128.

Downloaded from jvi.asm.org at University of North Carolina - Chapel Hill on December 20, 2008

DEFENSE ADVANCED RESEARCH PROJECTS AGENCY
675 NORTH RANDOLPH STREET
ARLINGTON, VA 22203-2114

Dr. Peter Daszak
EcoHealth Alliance
460 West 34th Street, 17th Floor
New York, NY 10001

Ref. #: HR001118S0017-PREEMPT-BAA-FP-019, Project DEFUSE: Defusing the Threat of Bat-borne Coronaviruses

Dear Dr. Daszak:

This letter is in response to the proposal referenced above, submitted in response to the Defense Advanced Research Projects Agency (DARPA) Broad Agency Announcement (BAA) HR001118S0017, PREventing EMerging Pathogenic Threats (PREEMPT). Your submission has been reviewed in accordance with the criteria set forth in that announcement.

Based on that review, your proposal was not selected for funding. We understand the time and effort it takes to put together a proposal, but unfortunately, we are unable to select all concepts that we receive. If you would like an informal feedback session to discuss your proposal, we request that you e-mail the BAA Mailbox (PREEMPT@darpa.mil) within three weeks of the date of this letter. Thank you for your interest in DARPA and the Biological Technologies Office.

Sincerely,

GIMLETT.JAMES.L.1470429883 Digitally signed by GIMLETT.JAMES.L.1470429883 Date: 2018.06.12 20:29:17 -04'00'

James Gimlett, Ph.D.
Program Manager
Biological Technologies Office

cc: Mr. Luke Hamel

Program of
The 8th International Symposium on Emerging Viral Diseases

Date 日期	Time 时间	Content 议程
Saturday 星期六 **Oct. 20, 2018** **10** 月 **20** 日	**Venue** 地点 09:00-21:00	**Registration**/报到注册 Ground Floor, Optics Valley Kingdom Plaza Hotel/光谷金盾大酒店一楼大厅
Sunday 星期日 **Oct. 21, 2018** **10** 月 **21** 日	**Day 1, Morning Session /第一天上午**	
	Venue 地点	Banquet Hall of Optics Valley Kingdom Plaza 3nd floor of the hotel 光谷金盾大酒店三楼宴会厅
	08:30-08:40	**Opening Address**/开幕式致词
	08:40-11:50	**Session 1: Antiviral Immunity** **Session Chairs: Peng ZHOU, Linfa WANG**
	08:40-09:10 Keynote Speech S-01	**Title:** Holy immune balance, batman! **Speaker: Linfa Wang** Programme in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore
	09:10-09:30 S-02	**Title: To be determined** **Speaker: Yanyi Wang** Wuhan Institute of Virology, Chinese Academy of Sciences
	09:30-09:50	Group Photo of Symposium Participants/与会代表合影 Coffee Break /茶歇
	09:50-10:10 S-03	**Title:** Recent advances in developing therapeutics monoclonal antibodies Against Ebola Virus Infection **Speaker: Xiangguo Qiu** Special Pathogens Program, National Microbiology laboratory, Public Health Agency of Canada
	10:10-10:30 S-04	**Title:** Nipah virus and Hendra Virus: Basic Science to Global Countermeasures **Speaker: Christopher Broder** Department of Microbiology, Uniformed Services University, Bethesda, MD, USA
	10:30-10:50 S-05	**Title:** Immunopathogenesis of Nipah virus infection **Speaker: Branka Horvat** International Center for Infectiology Research - CIRI, INSERM U1111, University Lyon 1, France
	10:50-11:10 S-06	**Title:** Incorporation of NS1 and PrM/M confer more effective protection for ZIKA virus vaccine **Speaker: Ling Chen** Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China

11:10-11:30 S-07	**Title:** Antiviral RNAi immunity – from basic to translation **Speaker: Xi Zhou** Wuhan Institute of Virology, Chinese Academy of Sciences
11:30-11:50 S-08	**Title:** The role of MVP in viral infection **Speaker: Shi Liu** College of Life Sciences, Wuhan University, China
11:50-12:05 Sponsor Presentation	Newly Technology development of Cryo TEM by JEOL **By Jianzhong Yuan** TEM product manager of JEOL in China
12:05-14:00	Lunch/午餐
Day 1, Afternoon Session /第一天下午	
14:00-17:30	**Session 2: Emerging viral pathogens** **Session Chairs: Zhengli SHI, Peter DASZAK**
14:00-:14:30 Keynote Speech S-09	**Title:** To be determined **Speaker: Hualan Chen** Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China
14:30-14:50 S-10	**Title:** Forecasting future viral pandemics and the Global Virome Project **Speaker: Peter Daszak** EcoHealth Alliance, New York, USA
14:50-15:10 S-11	**Title:** Infection and Immune Responses of Jamaican Fruit Bats (*Artibeus jamaicensis)* Experimentally Challenged with a Bat HL18NL11 Influenza A Virus **Speaker: Tony Schountz** Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University
15:10-15:30 S-12	**Title:** To be determined **Speaker: Di Liu** Wuhan Institute of Virology, Chinese Academy of Sciences
15:30-15:50	Coffee Break and Poster Presentation/茶歇和展板
15:50-16:10 S-13	**Title:** Risks of MERS-cluster coronaviruses in China **Speaker: Peng Zhou** Wuhan Institute of Virology, Chinese Academy of Sciences
16:10-16:30 S-14	**Title:** Molecular mechanisms for cross-species transmissions of SARS and MERS coronaviruses **Speaker: Fang Li** Department of Veterinary and Biomedical Sciences, University of Minnesota
16:30-16:50 S-15	**Title:** Human coronavirus OC43 (HCoV-OC43) and bovine coronavirus (BCoV) **Speaker: Astrid Vabret** Laboratory of Virology, University Hospital of Caen, France

	16:50-17:10 S-16	**Title:** Origin and cross-species transmission of bat coronaviruses in China **Speaker: Alice Latine** EcoHealth Alliance, New York, USA
	17:10-17:30 S-17	**Title:** Coronaviruses phylogenetics and intra-colony evolution of the SARS-CoV sister-clade Betacoronavirus in bats in western Palearctics **Speaker: Meriadeg Ar Gouilh** Groupe de Recherche sur l'Adaptation Microbienne, Normandy University, France
	18:00-20:00	Banquet 会议晚宴

Program of
The 8th International Symposium on Emerging Viral Diseases

Date 日期	Time 时间	Content 议程
Monday 星期一 **Oct. 22, 2018** **10** 月 **22** 日	**Day 2, Morning Session /第二天上午**	
	08:30-12:10	**Session 3: Virus-Host Interaction** **Session Chairs: Xi ZHOU, Ralph BARIC**
	08:30-09:00 Keynote Speech S-18	**Title:** To be determined **Speaker: Ralph Baric** Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
	09:00-09:30 Keynote Speech S-19	**Title:** Peptide-based Virus Entry Inhibitors against Class I and II Enveloped Viruses **Speaker: Shibo Jiang** Basic Medical College, Fudan University, Shanghai, China
	09:30-09:50 S-20	**Title:** Small molecules as filoviral entry inhibitors and chemical probes **Speaker: Lijun Rong** Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, Chicago, IL, USA
	09:50-10:10 S-21	**Title:** Entry mechanisms of highly pathogenic coronaviruses: MERS-CoV and SARS-CoV **Speaker: Yi Shi** CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China
	10:10-10:30	Coffee Break and Poster Presentation/茶歇和展板
	10:30-10:50 S-22	**Title:** Pathology of and development of antiviral therapy with favipiravir for severe fever with thrombocytopenia syndrome **Speaker: Masayuki Saijo** Department of Virology, National Institute of Infectious Diseases, Tokyo, Japan
	10:50-11:10 S-23	**Title:** Epistasis and complementation contribute to the evolution of the Rabies virus phosphoprotein in the face of severe functional constraints within the replication complex **Speaker: Hervé Bourhy** Institut Pasteur, Unit of Lyssavirus Dynamics and Host Adaptation, Paris, France
	11:10-11:30 S-24	**Title:** Influenza A virus-derived siRNAs increase in the absence of NS1 yet fail to inhibit virus replication **Speaker: Kevin Tsai** Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina, USA

	11:30-11:50 S-25	**Title:** Mechanisms of Herpesvirus capsid assembly and maturation **Speaker: Xiangxi Wang** Institute of Biophysics, Chines Academy of Sciences, Beijing, China
	11:50-12:10 S-26	**Title:** To be determined **Speaker: Yu Chen** College of Life Sciences, Wuhan University, China
	12:10-13:40	Lunch/午餐
	Day 2, Afternoon Session /第二天下午	
	13:40-17:30	**Session 4: Arbovirus** **Session Chairs: Zhihong HU, Pei-Yong SHI**
	13:40-14:10 Keynote Speech S-27	**Title:** Zika Virus: Emergence and Vaccine Development **Speaker: Pei-Yong Shi** University of Texas Medical Branch, Galveston, Texas, USA
	14:10-14:30 S-28	**Title:** Replicase Proteins of Alphaviruses as Determinants of Viral Pathogenesis and Vector Transmission **Speaker: Andres Merits** Institute of Technology, University of Tartu, Estonia
	14:30-14:50 S-29	**Title:** Identification of prognostic biomarkers for Dengue disease severity through an integrated 'omics analysis of patient serum **Speaker: Andrew Davidson** University of Bristol, Bristol, United Kingdom
	14:50-15:10 S-30	**Title:** Zika virus tropism for neural stem cells: the bad and the good **Speaker: Cheng-Feng Qin** Beijing Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, Beijing, China
	15:10-15:30 S-31	**Title:** A gut commensal bacterium promotes mosquito permissiveness to arboviruses **Speaker: Gong Cheng** Tsinghua-Peking Center for Life Sciences, School of Medicine, Tsinghua University, Beijing, China
	15:30-15:50	Coffee Break 茶歇
	15:50-16:10 S-32	**Title:** The fabulous NSs protein of Rift Valley fever virus **Speaker: Pierre-Yves Lozach** Cluster of Excellence and Center for Integrative Infectious Disease Research, University Hospital Heidelberg, Germany
	16:10-16:30 S-33	**Title:** Novel delivery of a live-attenuated chikungunya virus vaccine candidate **Speaker: Adam Taylor** Griffith University, Southport, Queensland, Australia

	16:30-16:50 S-34	**Title:** Viromes of ticks reveals highly potential risks of tick-borne viral diseases in Xinjiang, China **Speaker: Fei Deng** Wuhan Institute of Virology, Chinese Academy of Sciences
	16:50-17:10 S-35	**Title:** Species-specific disruption of STING-dependent antiviral cellular defenses by the Zika virus NS2B3 protease **Speaker: Qiang Ding** School of Medicine, Tsinghua University, Beijing, China
	17:10-17:30 S-36	**Title:** ISG15 regulates Zika Virus Replication through Jak/STAT Signaling pathway and its ISGylation **Speaker: Yancui Wang** Institute of Blood Transfusion, Chinese Academy of Medical Sciences and Peking Union Medical College, Chengdu, China
	17:30-17:40	**Closing Remarks**
	18:00-19:00	Dinner/晚餐

From: Peter Daszak[daszak@ecohealthalliance.org]
Sent: Fri 11/2/2018 3:00:14 PM (UTC-04:00)
To: Baric, Ralph S[rbaric@email.unc.edu]
Subject: latest draft
Attachment: R01 renewal text 6.docx

Cheers,

Peter

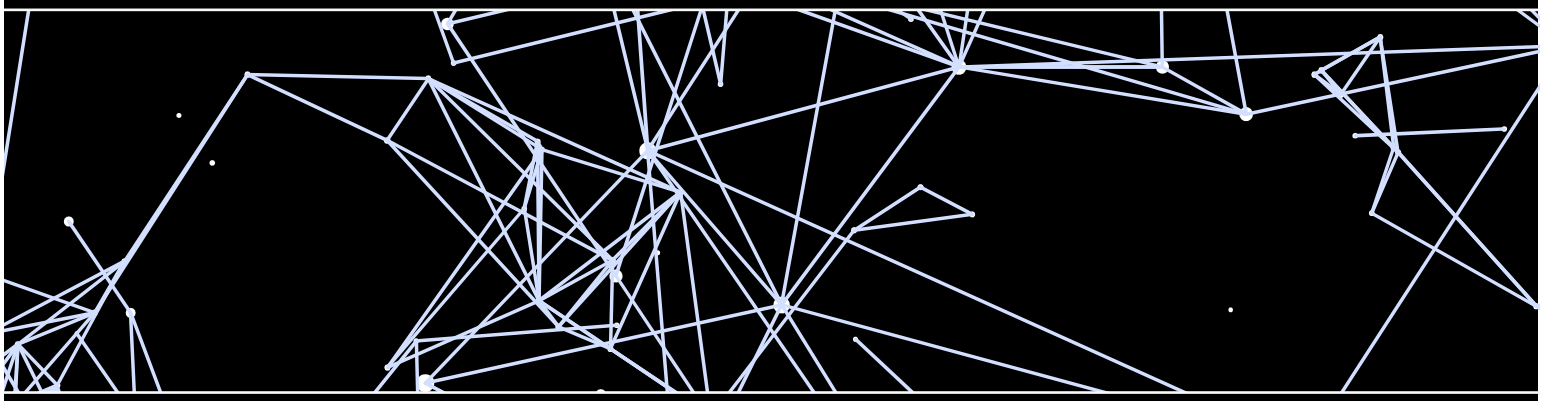
Peter Daszak
President

EcoHealth Alliance
460 West 34th Street – 17th Floor
New York, NY 10001

Tel. +1 212-380-4474
Website: www.ecohealthalliance.org
Twitter: @PeterDaszak

EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

THE NATIONAL SCIENCE FOUNDATION



PROPOSAL & AWARD POLICIES AND PROCEDURES GUIDE

Effective January 29, 2018
NSF 18-1
OMB Control Number 3145-0058

Significant Changes and Clarifications to the *Proposal & Award Policies & Procedures Guide* (PAPPG)

Effective January 29, 2018

Overall Document

Editorial changes have been made to either clarify or enhance the intended meaning of a sentence or section. The document has been updated to ensure consistency with data contained in NSF systems or guidance located in other NSF or Federal policy documents. Throughout the PAPPG, website, address and document references and organizational names have been updated to reflect current information.

Significant Changes

- **Chapter I.E, Who May Submit Proposals,** incorporates new coverage on the eligibility of international branch campuses of US institutions of higher education. The definition of colleges and universities in Chapter I.E.1 has been updated to now refer to institutions of higher education, for consistency with 2 CFR § 200. In addition, changes have been made to the eligibility requirements for foreign organizations.

- **Chapter II.C.1.e, Collaborators & Other Affiliations Information,** has been significantly revised to request information regarding collaborators and other affiliations (COA) be provided through use of a standard NSF COA template. Footnotes also have been added to address frequently asked questions relating to the new COA template.

- **Chapter II.C.2.d, Project Description**, has been modified to reflect that the Project Description must now contain a separate section specifically identified as “Intellectual Merit”.

- **Chapter II.C.2.g, Budget and Budget Justification,** has been revised to increase the number of pages allowed for the budget justification to no more than five pages per proposal. This change applies to budget justifications for both proposers and subawardees.

- **Chapter VII.A.2, Grantee Notifications to NSF**, has been restructured to remove information on requests for NSF approval. In addition, Exhibit VII-1 has been deleted, as coverage on grantee requests for approval from NSF is contained in the *Research Terms and Conditions* Appendix A and Chapter X.A.3.

- **Chapter X.A.3, Prior Written Approvals,** has been updated to reference the *Research Terms and Conditions* Appendix A, which is the authoritative source of NSF prior approval requirements.

Clarifications and Other Changes

- **Section B, Foreword,** has been modified to refer to the applicable standard grant conditions, instead of solely the NSF *Grant General Conditions*, now that the *Research Terms and Conditions* have been implemented.

- **Section D, Definitions & NSF-Grantee Relationships,** provides additional guidance on the types of cooperative agreements awarded by NSF.

- **Section E, NSF Organizations,** has been revised to reflect the current responsibilities of the organizations that are normally of most direct interest to the NSF proposer and grantee community.

- **Chapter II.C.1.f, Submission of Proposals by Former NSF Staff,** incorporates new coverage to address submission of proposals from former NSF staff and the procedures that must be followed in such circumstances.

- **Chapter II.C.2.d(iii), Results from Prior NSF Support**, clarifies the timeframe during which any PI or co-PI that has received NSF support must report on such funding. Chapter II.E.7 on conference proposals, II.E.8 on equipment proposals, II.E.9 on travel proposals and Exhibit II-1, the Proposal Preparation Checklist, also have been updated with this guidance.

- **Chapter II.C.2.g(i)(a), Senior Personnel Salaries & Wages Policy**, has been supplemented with guidance that reflects it is the proposing organization's responsibility to define and apply the term "year" and include the definition in the budget justification.

- **Chapter II.C.2.g(viii), Indirect Costs**, has been updated to state that amounts for indirect costs should be specified in the budget justification.

- **Chapter II.C.2.j, Special Information and Supplementary Documentation,** includes additional guidance on the content for data management plans that involve collaborative activities.

- **Chapter II.D.4, Proposals Involving Vertebrate Animals,** has been revised to enhance the clarity of guidance on the use of vertebrate animals for research or education on NSF supported projects. For projects at an international organization that involve the use of vertebrate animals, a statement from the international organization will need to be provided.

- **Chapter II.D.5, Proposals Involving Human Subjects**, has been supplemented with additional language regarding international projects.

- **Chapters II.D.6 and XI.B.5, Life Sciences Dual Use Research of Concern (DURC)**, include new coverage regarding NSF's funding of research that would be considered to lead to a gain of function of agents. The title of these sections also has been changed for consistency with the *US Government Policy for Institutional Oversight of Life Sciences Dual Use Research of Concern.*

- **Chapter II.E.11, Research Infrastructure Proposal**, has been revised to reflect current practices and provide greater clarity in the description of these types of proposals.

- **Exhibit II-1, Proposal Preparation Checklist**, has been updated to reflect relevant changes made to Chapter II of the PAPPG. Additional checklist components also have been added to assist proposers in the pre-submission administrative review of proposals to NSF.

- **Chapter V, Renewal Proposals,** has been modified to update reference information regarding recompetition of expiring awards. Section B on Accomplishment-Based Renewals has been updated to provide greater clarity regarding the submission of reprints.

- **Chapter VII.B.2.e, Substitute (Change) PI/PD or co-PI/co-PD**, has been supplemented with guidance on the reappointment of prior NSF staff as PI.

- **Chapter VII.B,3, Subawarding or Transferring Part of an NSF Award (Subaward)**, has been modified for consistency with terminology in 2 CFR § 200.

- **Chapter VII.D.2, Final Project Report**, has been updated to reflect that when PIs submit the report, they are indicating that the scope of work is complete and no further administrative actions are anticipated on the grant.

- **Chapter VIII.E.6, Award Financial Reporting Requirements and Final Disbursements**, has been supplemented to clarify the intent of NSF notifications regarding canceling appropriations.

- **Chapter X.B, Direct Costs and X.C, Other Direct Costs,** have been modified to remove coverage that is redundant with 2 CFR § 200 and other sections of the PAPPG. Terminology on rearrangement and reconversion costs has been updated for consistency with 2 CFR § 200.462.

- **Chapter XI.A, Non-Discrimination Statutes and Regulations,** has been revised to provide current information on NSF grantee obligations to comply with civil rights laws and regulations. These changes provide NSF grantees and applicants for NSF grants with an overview of relevant civil rights regulatory obligations and compliance mechanisms. Information also has been included on how grantee program participants can file complaints with NSF alleging discrimination in an NSF grantee's programs.

- **Chapter XI.B.1, Human Subjects and XI.B.3, Vertebrate Animals,** include the relevant new award-specific condition on organizational responsibilities. In addition, language has been added on post-award responsibilities.

- **Chapter XI.D.1.d, Intellectual Property**, has been updated to specify that grantees are required to use iEdison to disclose NSF subject inventions. In addition, NSF now reserves the option to request an Annual Utilization Report or a Final Invention Statement and Certification.

- **Chapter XI.M.4, Executive Order 13788, Buy American and Hire American,** is a new section which serves as NSF's implementation of Executive Order 13788.

PROPOSAL & AWARD POLICIES & PROCEDURES GUIDE (PAPPG)
Table of Contents

Introduction:

A. About the National Science Foundation
B. Foreword
C. Acronym List
D. Definitions & NSF-Grantee Relationships
E. NSF Organizations
F. NSF Electronic Capabilities Modernization Status

Part I: Proposal Preparation and Submission Guidelines

Chapter I: Pre-Submission Information I-1

A. NSF Proposal Preparation and Submission I-1
B. NSF Programs and Funding Opportunities I-1
C. Categories of Funding Opportunities I-1
1. Program Descriptions I-1
2. Program Announcements I-2
3. Program Solicitations I-2
4. Dear Colleague Letters I-2
D. Types of Submissions I-3
1. Letters of Intent I-3
2. Preliminary Proposals I-3
a. Invite/Not Invite Decisions I-3
b. Encourage/Discourage Decisions I-4
3. Full Proposals I-4
E. Who May Submit Proposals I-4
Categories of Proposers I-5
1. Institutions of Higher Education (IHEs) I-5
Special Instructions for International Branch Campuses of US IHEs I-5
2. Non-profit, Non-academic Organizations I-5
3. For-profit Organizations I-5
4. State and Local Governments I-5
5. Unaffiliated Individuals I-5
6. Foreign organizations I-5
7. Other Federal Agencies I-6
F. When to Submit Proposals I-6
1. Target Dates I-6
2. Deadline Dates I-6
3. Submission Windows I-6
Special Exceptions to NSF's Deadline Date Policy I-7
Natural or Anthropogenic Disasters I-7
Closure of NSF I-7
G. How to Submit Proposals I-7
1. Electronic Requirements I-7
2. Submission Instructions I-7
3. Requirements Relating to Data Universal Numbering System (DUNS) Numbers and Registration in the System for Award Management (SAM) I-8
4. NSF ID I-8
5. Proposal Receipt I-8
H. Proposal Processing I-9

Chapter II: Proposal Preparation Instructions II-1

A. Conformance with Instructions for Proposal Preparation II-1
B. Format of the Proposal II-2
1. Proposal Pagination Instructions II-2
2. Proposal Margin and Spacing Requirements II-3
3. Page Formatting II-3
C. Proposal Contents II-3
1. Single-Copy Documents II-3
a. Authorization to Deviate from NSF Proposal Preparation Requirements II-3
b. List of Suggested Reviewers or Reviewers Not to Include II-4
c. Proprietary or Privileged Information II-4
d. Proposal Certifications II-4
e. Collaborators & Other Affiliations Information II-6
f. Submission of Proposals by Former NSF Staff II-7
2. Sections of the Proposal II-8
a. Cover Sheet II-8
b. Project Summary II-11
c. Table of Contents II-11
d. Project Description II-11
(i) Content II-11
(ii) Page Limitations and Inclusion of Uniform Resource Locators (URLs) within the Project Description II-12
(iii) Results from Prior NSF Support II-12
(iv) Unfunded Collaborations II-13
(v) Group Proposals II-13
(vi) Proposals for Renewed Support II-13
e. References Cited II-13
f. Biographical Sketch(es) II-14
(i) Senior Personnel II-14
(a) Professional Preparation II-14
(b) Appointments II-14
(c) Products II-14
(d) Synergistic Activities II-15
(ii) Other Personnel II-15
(iii) Equipment Proposals II-15
g. Budget and Budget Justification II-15
(i) Salaries and Wages II-16
(a) Senior Personnel Salaries & Wages Policy II-16
(b) Administrative and Clerical Salaries & Wages Policy II-16
(c) Procedures II-16
(d) Confidential Budgetary Information II-17
(ii) Fringe Benefits II-17
(iii) Equipment II-17
(iv) Travel II-18
(a) General II-18
(b) Domestic Travel II-18
(c) Foreign Travel II-18
(v) Participant Support II-18
(vi) Other Direct Costs II-19
(a) Materials and Supplies (including Costs of Computing Devices) II-19
(b) Publication/Documentation/Dissemination II-19
(c) Consultant Services (also referred to as Professional Service Costs) II-19
(d) Computer Services II-20
(e) Subawards II-20
(f) Other II-20
(vii) Total Direct Costs II-21

(viii) Indirect Costs II-21
(ix) Total Direct and Indirect Costs II-21
(x) Fees II-21
(xi) Amount of This Request II-21
(xii) Cost Sharing II-21
(xiii) Allowable and Unallowable Costs II-23
(a) Entertainment II-23
(b) Meals and Coffee Breaks II-23
(c) Alcoholic Beverages II-23
h. Current and Pending Support II-23
i. Facilities, Equipment and Other Resources II-23
j. Special Information and Supplementary Documentation II-24
k. Appendices II-26
D. Special Processing Instructions II-27
1. Proprietary or Privileged Information II-27
2. Beginning Investigators (applies to proposals submitted to the Biological Sciences Directorate only) II-27
3. Collaborative Proposals II-27
4. Proposals Involving Vertebrate Animals II-29
5. Proposals Involving Human Subjects II-30
6. Life Sciences Dual Use Research of Concern (DURC) II-32
7. Projects Requiring High-Performance Computing Resources, Large Amounts of Data Storage, or Advanced Visualization Resources II-32
E. Types of Proposals II-33
1. Rapid Response Research (RAPID) II-33
2. EArly-concept Grants for Exploratory Research (EAGER) II-33
3. Research Advanced by Interdisciplinary Science and Engineering (RAISE) II-34
4. Grant Opportunities for Academic Liaison with Industry (GOALI) II-35
5. Ideas Lab II-36
6. Facilitation Awards for Scientists and Engineers with Disabilities (FASED) II-38
7. Conference Proposal II-39
8. Equipment Proposal II-41
9. Travel Proposal II-42
10. Center Proposal II-44
11. Research Infrastructure Proposal II-44
Exhibit II-1: Proposal Preparation Checklist II-45
Exhibit II-2 Potentially Disqualifying Conflicts of Interest II-49
Exhibit II-3: Drug-Free Workplace Certification II-50
Exhibit II-4 Debarment and Suspension Certification II-52
Exhibit II-5 Lobbying Certification II-54
Exhibit II-6 Nondiscrimination Certification II-55
Exhibit II-7: Definitions of Categories of Personnel II-56

Chapter III: NSF Proposal Processing and Review III-1

A. Merit Review Principles and Criteria III-1
Intellectual Merit III-2
Broader Impacts III-2
B. Selection of Reviewers III-3
C. Proposal File Updates III-3
D. Revisions to Proposals Made During the Review Process III-4
E. Funding Recommendation III-4
F. NSF's Risk Management Framework and the Decision to Award or Decline Proposals III-5
G. Review Information Provided to PI III-7
H. Release of Grantee Proposal Information III-7
Exhibit III-1: NSF Proposal & Award Process & Timeline III-8

Chapter IV: Non-Award Decisions and Transactions IV-1

A. Proposal Withdrawal IV-1
B. Proposals Not Accepted or Returned Without Review IV-2
C. Declinations IV-2
D. Reconsideration IV-2
E. Resubmission IV-5

Chapter V: Renewal Proposals V-1

A. Traditional Renewal V-1
B. Accomplishment-Based Renewal V-1

Part II: Award, Administration and Monitoring of Grants and Cooperative Agreements

Chapter VI: NSF Awards VI-1

A. Acceptance of Assistance Agreements VI-1
B. Award Instrument VI-1
C. NSF Award Conditions VI-2
D. NSF Grant Periods VI-2
1. Definitions VI-2
2. Significance of Grant Period VI-2
3. Changes in a Grant Period VI-2
a. Start Date VI-2
b. End Date VI-2
c. No-Cost Extension VI-2
d. Two-Year Extensions for Special Creativity VI-3
E. Additional Funding Support VI-3
1. Types of Additional Funding Support VI-3
2. Renewed Support VI-4
3. Support under Continuing Grants VI-4
4. Supplemental Support VI-4

Chapter VII: Grant Administration VII-1

A. Monitoring Project Performance VII-1
1. Grantee Responsibilities VII-1
2. Grantee Notifications to NSF and Requests for NSF Approval VII-1
B. Changes in Project Direction or Management VII-2
1. Changes in Objectives, Scope or Methods/Procedures VII-2
a. Changes in Objectives or Scope VII-2
b. Significant Changes in Methods or Procedures VII-2
c. Significant Changes, Delays or Events of Unusual Interest VII-2
2. Changes in PI/PD, co-PI/co-PD or Person-Months Devoted to the Project VII-2
a. Long-Term Disengagement of PI/PD or co-PI/co-PD VII-2
b. Change in Person-Months Devoted to the Project VII-3
c. Addition of co-PI/co-PD VII-3
d. Withdrawal of PI/PD or co-PI/co-PD VII-3
e. Substitute (Change) PI/PD or co-PI/co-PD VII-3
f. Disposition of a Grant When a PI/PD Transfers from One Organization to Another Organization VII-4
3. Subawarding or Transferring Part of an NSF Award (Subaward) VII-5
4. Postaward Addition of Postdoctoral Scholars. VII-5
C. Cost Sharing VII-6
1. General VII-6
2. Mandatory Cost Sharing Commitments VII-6

3. Mandatory Cost Sharing Records and Reports VII-6
D. Technical Reporting Requirements VII-7
1. Annual Project Reports VII-7
2. Final Project Report VII-7
3. Project Outcomes Report for the General Public VII-8
Section Contents VII-8
4. Compliance with Technical Reporting Requirements VII-8
5. Grant Closeout VII-8
E. Record Retention and Audit VII-9

Chapter VIII: Financial Requirements and Payments VIII-1

A. Standards for Financial Management VIII-1
B. Definitions VIII-1
C. Payment Requirements VIII-2
1. Requesting Payments VIII-2
2. Payment Policies VIII-3
3. Request for Payment VIII-3
4. Special Payment Grantees VIII-4
5. Working Capital Advance VIII-4
6. Grantee Banking Information for Payments VIII-4
D. Cash Refunds and Credits To NSF VIII-4
1. Final Unobligated Balance VIII-4
2. Erroneous Payments VIII-4
3. Interest Earned on Advance Payments VIII-4
4. Program Income VIII-6
5. Other Cost Credits VIII-7
E. Award Financial Reporting Requirements and Final Disbursement VIII-7

Chapter IX: Grantee Standards IX-1

A. Conflict of Interest Policies IX-1
B. Responsible Conduct of Research (RCR) IX-3
1. Background IX-3
2. Institutional Responsibilities IX-3
C. Financial Management Systems Standards IX-3
D. Property Management Standards IX-3
1. Title to Equipment IX-4
a. Title to Equipment – Non-Profit Organizations IX-4
b. Title to Equipment – For-Profit Organizations IX-4
2. Conditions for Acquisition and Use of Equipment IX-4
3. Principles Relating to Use of NSF-Supported Research Instrumentation & Facilities IX-5
4. Property Management Standards When Title Retained by NSF IX-5
5. Excess Government Personal Property IX-5
a. Policy IX-5
b. Eligibility IX-6
c. Procedures IX-6
d. Visiting Holding Agency Facilities IX-7
e. Dollar Limitation IX-7
f. Restrictions IX-8
g. Costs IX-8
h. Title IX-9
i. Accountability and Recordkeeping IX-9
E. Procurement Standards IX-9

Chapter X: Allowability of Costs X-1

A. Basic Considerations X-1
1. Conflicting Guidelines X-1
2. Other Considerations X-1
a. Maximum Obligation X-1
b. Pre-Award Costs X-1
c. Post-End Date Costs X-2
3. Prior Written Approvals X-2
B. Direct Costs X-3
1. Compensation – Personal Services X-3
a. Salaries and Wages X-3
b. Fringe Benefits X-3
2. Intra-University (IHE) Consulting X-3
3. Federal Employees X-3
C. Other Direct Costs X-3
1. Rearrangement and Reconversion Costs X-3
2. News Release Costs X-4
3. Travel and Temporary Dependent Care Costs X-4
D. Indirect Costs X-4
1. NSF Policy X-4
2. NSF Cognizant Organizations X-6
E. Fee Payments under NSF Grants X-6

Chapter XI: Other Post Award Requirements and Considerations XI-1

A. Non-Discrimination Statutes and Regulations XI-1
1. General XI-1
2. Certification of Compliance/Civil Rights Certifications XI-2
3. Title VI of the Civil Rights Act of 1964 ..... XI-2
4. Section 504 of the Rehabilitation Act of 1973 ..... XI-2
5. Title IX of the Education Amendments of 1972 ..... XI-3
6. Age Discrimination Act of 1975 ..... XI-3
7. Equal Employment Opportunity under E.O. 11246 ..... XI-4
8. Limited English Proficiency under E.O. 13166 ..... XI-4
B. Protection of Living Organisms XI-4
1. Human Subjects XI-4
2. Research Involving Recombinant or Synthetic Nucleic Acid Molecules XI-5
3. Vertebrate Animals XI-6
4. Government Permits and Activities Abroad XI-7
5. Life Sciences Dual Use Research of Concern (DURC) XI-7
C. Construction, Rearrangements and Alterations XI-8
1. Davis-Bacon Act XI-8
2. Bonding and Insurance XI-8
3. Seismic Safety of Buildings XI-8
D. Intellectual Property XI-8
1. Patents and Inventions XI-8
2. Copyright XI-10
3. Special Patent and Copyright Situations XI-11
4. Dissemination and Sharing of Research Results XI-12
5. Tangible Property XI-12
E. Publication/Distribution of Grant Materials XI-13
1. NSF Policy XI-13
2. Costs XI-13
3. Responsibilities XI-13
4. Grantee Obligations XI-13

F. International Considerations XI-14
1. Travel to Foreign Countries XI-14
a. Policy XI-14
b. Use of US-Flag Air Carriers XI-14
c. Use of Foreign-Flag Air Carriers XI-15
2. Charter Flights XI-16
3. Projects in a Foreign Country XI-16
4. Passports and Visas XI-16
G. Intergovernmental Review of Federal Programs XI-16
H. Handling of Information XI-17
1. Questionnaires: Data Collection Under NSF Grants XI-17
2. Release of Information by NSF XI-17
I. Tax Status XI-18
J. Protection of Properties in the National Register of Historic Places XI-18
K. Environmental Compliance XI-18
L. National Security XI-18
M. Miscellaneous XI-18
1. Liabilities and Losses XI-18
2. Pre-College Students and Experimental Curriculum Development Projects XI-18
3. Use of Metric Measurements XI-19
4. Executive Order 13788, Buy American and Hire American XI-19

Chapter XII: Grant Administration Disputes and Misconduct XII-1

A. Suspension and Termination Procedures XII-1
1. Definitions XII-1
2. Suspension and Termination XII-1
3. Termination by Mutual Agreement XII-2
4. NSF Suspension or Termination Review Procedure XII-3
B. Informal Resolution of Grant Administration Disputes XII-3
C. Research Misconduct XII-4
1. NSF Policies and Responsibilities XII-4
2. Role of Grantees XII-5
3. Reporting Possible Misconduct XII-6

Subject Index .....

Privacy Act and Public Burden Statements .....

Introduction:

A. About the National Science Foundation

The National Science Foundation (NSF) is an independent Federal agency created by Congress in 1950 to "promote the progress of science; [and] to advance the national health, prosperity and welfare" by supporting research and education in all fields of science and engineering.

From those first days, NSF has had a unique place in the Federal Government: it is responsible for the overall health of science and engineering across all disciplines. In contrast, other Federal agencies support research focused on specific missions such as health or defense. The Foundation also is committed to ensuring the nation's supply of scientists, engineers, and science and engineering educators.

NSF funds research and education in most fields of science and engineering. It does this through grants and cooperative agreements to approximately 2,000 colleges, universities, K-12 school systems, businesses, informal science organizations and other research organizations throughout the US. The Foundation accounts for about one-fourth of Federal support to academic institutions for basic research.

The Foundation considers proposals[1] submitted by organizations on behalf of individuals or groups for support in most fields of research. Interdisciplinary proposals also are eligible for consideration.

NSF does not normally support technical assistance, pilot plant efforts, research requiring security classification, the development of products for commercial marketing, or market research for a particular project or invention. Research with disease-related goals, including work on the etiology, diagnosis or treatment of physical or mental disease, abnormality, or malfunction in human beings or animals, is normally not supported. Animal models of such conditions or the development or testing of drugs or other procedures for their treatment also are not eligible for support. However, research in bioengineering, with diagnosis- or treatment-related goals, that applies engineering principles to problems in biology and medicine while advancing engineering knowledge is eligible for support. Bioengineering research to aid persons with disabilities also is eligible.

NSF does not have any programs involving the construction of public works in metropolitan areas, no development assistance programs, no programs requiring State plans as a condition of assistance, none involving coordination of planning in multi-jurisdictional areas and no programs of grants to State and local governments as defined in Section 6501(4) of Title 31 of the United States Code (USC).

NSF receives over 50,000 proposals each year for research, education and training projects, of which approximately 11,000 are funded. In addition, the Foundation receives over 15,000 applications for graduate and postdoctoral fellowships. One of NSF's flagship programs, the NSF Graduate Research Fellowship Program (GRFP) helps ensure the vitality and diversity of the scientific and engineering workforce; the program awards about 2,000 fellowships each year. Guidance regarding the GRFP program may be found in the program solicitation, as well as in the GRFP Administrative Guide. The agency operates no laboratories itself but does support National Research Centers, user facilities, certain oceanographic vessels and Antarctic research stations. The Foundation also supports cooperative research between universities and industry, US participation in international scientific and engineering efforts, and educational activities at every academic level.

NSF is structured much like a university, with divisions/offices for the various disciplines and fields of science and engineering and for science, technology, engineering and mathematics education. NSF also uses a variety of management mechanisms to coordinate research in areas that cross traditional disciplinary boundaries. The Foundation is assisted by advisors from the scientific and engineering communities who serve on formal committees or as *ad hoc* reviewers of proposals. This advisory system, which focuses on both program directions and specific proposals, involves approximately 50,000 scientists and engineers each year. NSF staff members who are experts in a certain field or area make award recommendations; Principal Investigators (PIs) receive unattributed verbatim copies of peer reviews.

[1] For purposes of this Guide, the term "proposal" is interchangeable with the term "application."

Grantees are wholly responsible for conducting their project activities and preparing the results for publication. Thus, the Foundation does not assume responsibility for such findings or their interpretation.

Facilitation Awards for Scientists and Engineers with Disabilities provide funding for special assistance or equipment to enable persons with disabilities to work on NSF-supported projects. See Chapter II.E.6. for instructions regarding preparation of these types of proposals.

NSF has Text Telephone (TTY) and Federal Information Relay Service (FIRS) capabilities that enable individuals with hearing or speech impairments to communicate with the Foundation about NSF programs, employment or general information. TTY may be accessed at (703) 292-5090 and (800) 281-8749, FIRS at (800) 877-8339.

The NSF Information Center may be reached at (703) 292-5111.

B. Foreword

The *Proposal & Award Policies & Procedures Guide (PAPPG)* is comprised of documents relating to the Foundation's proposal and award process for the assistance programs of NSF. The PAPPG, in conjunction with the applicable standard award conditions incorporated by reference in the award[2], serve as the Foundation's implementation of 2 CFR § 200, *Uniform Administrative Requirements, Cost Principles, and Audit Requirements for Federal Awards*. If the PAPPG and the award conditions are silent on a specific area covered by 2 CFR § 200, the requirements specified in 2 CFR § 200 must be followed.

It has been designed for use by both our customer community and NSF staff and consists of two parts:

- Part I sets forth NSF's proposal preparation and submission guidelines. The coverage provides guidance for the preparation and submission of proposals to NSF. Some NSF programs have program solicitations that modify the general provisions of the PAPPG, and, in such cases, the guidelines provided in the solicitation must be followed.

  The policy and procedural guidance contained in the *NSF Grants.gov Application Guide* should be followed when preparing and submitting proposals to NSF via Grants.gov.

- Part II of the NSF PAPPG sets forth NSF policies and procedures regarding the award, administration, and monitoring of grants and cooperative agreements. Coverage includes the NSF award process, from issuance and administration of an NSF award through closeout. Guidance is provided regarding other grant requirements or considerations that either are not universally applicable or do not follow the award cycle. Part II also implements other Public Laws, Executive Orders (E.O.) and other directives insofar as they apply to grants, and is issued pursuant to the authority of Section 11(a) of the NSF Act (42 USC § 1870). When NSF Grant General Conditions or an award notice reference a particular section of the PAPPG, then that section becomes part of the award requirements through incorporation by reference.

The PAPPG does not apply to NSF contracts. For information relating to NSF contracts, consult the Guide to the NSF Contracting Process.

General information about NSF programs may be found on the NSF website at: https://www.nsf.gov/funding/. Additional information about special requirements of individual NSF programs may be obtained from the appropriate Foundation program office. Information about most program deadlines and target dates for proposals are available on the NSF website at: http://www.nsf.gov/funding/pgm_list.jsp?org=NSF&ord=date. Program deadline and target date information also appears in individual program announcements and solicitations and on relevant NSF Divisional/Office websites.

Catalog of Federal Domestic Assistance

NSF programs fall under the following categories in the latest Catalog of Federal Domestic Assistance (CFDA) issued by the Office of Management and Budget and the General Services Administration:

47.041 -- Engineering
47.049 -- Mathematical and Physical Sciences
47.050 -- Geosciences
47.070 -- Computer and Information Science and Engineering
47.074 -- Biological Sciences
47.075 -- Social, Behavioral and Economic Sciences
47.076 -- Education and Human Resources
47.079 -- Office of International Science & Engineering
47.083 -- Office of Integrative Activities

Any questions or comments regarding the NSF PAPPG should be addressed to the Policy Office, Division of Institution & Award Support, at (703) 292-8243 or by e-mail to policy@nsf.gov.

[2] See Chapter VI.C. for additional information on NSF award conditions.

C. Acronym List

ABR	Accomplishment-Based Renewal
ACH	Automated Clearing House (US Treasury)
ACM$	Award Cash Management Service
AD	Assistant Director
ADPE	Automatic Data Processing Equipment
APHIS	Animal and Plant Health Inspection Service
AOR	Authorized Organizational Representative
BFA	Budget, Finance & Award Management
CA	Cooperative Agreement
CA/FATC	Cooperative Agreement Financial/Administrative Terms and Conditions
CAP	Cost Analysis and Pre-award Branch
CA/PTC	Cooperative Agreement Programmatic Terms and Conditions
CEQ	Council on Environmental Quality
CFR	Code of Federal Regulations
CGI	Continuing Grant Increment
CMIA	Cash Management Improvement Act
COA	Collaborators and Other Affiliations
COI	Conflict of Interest
CSA	Cooperative Support Agreement
co-PD	co-Project Director
co-PI	co-Principal Investigator
DACS	Division of Acquisition and Cooperative Support
DAS	Division of Administrative Services
DCL	Dear Colleague Letter
DD	Division Director
DFM	Division of Financial Management
DGA	Division of Grants and Agreements
DHHS	Department of Health and Human Services
DIAS	Division of Institution and Award Support
DOC	Department of Commerce
DUNS	Data Universal Numbering System
DURC	Dual Use Research of Concern
EAGER	EArly-Concept Grants for Exploratory Research
EFT	Electronic Funds Transfer
E.O.	Executive Order
F&A	Facilities & Administrative Costs
FAPIIS	Federal Awardee Performance and Integrity Information System
FAQs	Frequently Asked Questions
FAR	Federal Acquisition Regulation
FASED	Facilitation Awards for Scientists and Engineers with Disabilities
FDP	Federal Demonstration Partnership
FEMA	Federal Emergency Management Agency
FIRS	Federal Information Relay Service
FOIA	Freedom of Information Act
FWA	FederalWide Assurance
GC-1	Grant General Conditions
GOALI	Grant Opportunities for Academic Liaison with Industry
GOE	Government-Owned Equipment
GPO	Government Publishing Office
GSA	General Services Administration
IACUC	Institutional Animal Care and Use Committee
IBC	Institutional Biosafety Committee
IPA	Intergovernmental Personnel Act
IRB	Institutional Review Board
IRS	Internal Revenue Service
ISE	International Science & Engineering
LEP	Limited English Proficiency
LFM	Large Facilities Manual
LFO	Large Facilities Office
LOI	Letters of Intent
MREFC	Major Research Equipment and Facilities Construction Account
NASEM	National Academies of Science, Engineering and Medicine
NEPA	National Environmental Policy Act
NIH	National Institutes of Health
NSB	National Science Board
NSF	National Science Foundation
ODI	Office of Diversity and Inclusion
OGC	Office of the General Counsel
OHRP	Office for Human Research Protections
OIA	Office of Integrative Activities
OIG	Office of Inspector General
OLAW	Office for Laboratory Animal Welfare
OMB	Office of Management and Budget
ONR	Office of Naval Research
PAPPG	Proposal & Award Policies & Procedures Guide
PD	Project Director
PHS	Public Health Service
PI	Principal Investigator
PNAG	Prospective New Awardee Guide
PO	Program Officer
RAISE	Research Advanced by Interdisciplinary Science and Engineering
RAM	Resolution and Advance Monitoring Branch
RAPID	Rapid Response Research
REU	Research Experiences for Undergraduates
ROA	Research Opportunity Awards
RTC	Research Terms and Conditions
RUI	Research in Undergraduate Institutions
SAM	System for Award Management
SBA	Small Business Administration
SBIR	Small Business Innovation Research
SF	Standard Form
SF LLL	Disclosure of Lobbying Activities

SPO	Sponsored Projects Office
SSN	Social Security Number
STTR	Small Business Technology Transfer
TDD	Telephonic Device for the Deaf
TTY	Text Telephone
URL	Uniform Resource Locator
USC	United States Code
USDA	US Department of Agriculture
VSEE	Visiting Scientist, Engineer or Educator

D. Definitions & NSF-Grantee Relationships

1. Definitions

a. An AUTHORIZED ORGANIZATIONAL REPRESENTATIVE (AOR)/AUTHORIZED REPRESENTATIVE means the administrative official who, on behalf of the proposing organization is empowered to make certifications and assurances and can commit the organization to the conduct of a project that NSF is being asked to support as well as adhere to various NSF policies and grant requirements.

b. A GRANT AGREEMENT[3] means a legal instrument of financial assistance between NSF and a grantee that, consistent with 31 USC 6302, 6304:

(1) Is used to enter into a relationship the principal purpose of which is to transfer anything of value from NSF to the grantee to carry out a public purpose authorized by a law of the United States (see 31 USC 6101(3)); and not to acquire property or services for NSF's direct benefit or use;

(2) Is distinguished from a cooperative agreement in that it does not provide for substantial involvement between NSF and the grantee in carrying out the activity contemplated by the NSF award.

NSF awards the following two types of grants:

(a) A STANDARD GRANT means a type of grant in which NSF agrees to provide a specific level of support for a specified period of time with no statement of NSF intent to provide additional future support without submission of another proposal.

(b) A CONTINUING GRANT means a type of grant in which NSF agrees to provide a specific level of support for an initial specified period of time, usually a year, with a statement of intent to provide additional support for the project for additional periods, provided funds are available and the results achieved warrant further support.

c. A COST REIMBURSEMENT AWARD means a type of grant under which NSF agrees to reimburse the grantee for work performed and/or costs incurred by the grantee up to the total amount specified in the grant. Such costs must be allowable in accordance with the applicable cost principles. Accountability is based primarily on technical progress, financial accounting and fiscal reporting. Except under certain programs and under special circumstances, NSF grants and cooperative agreements are normally cost reimbursement type awards.

d. A FIXED AMOUNT AWARD means a type of award in which NSF provides a specific level of support without regard to actual costs incurred under the award. This type of NSF award reduces some of the administrative burden and recordkeeping requirements for both the grantee and NSF. Accountability is based primarily on performance and results.

e. A COOPERATIVE AGREEMENT means a legal instrument of financial assistance between NSF and an awardee that, consistent with 31 USC 6302–6305:

(1) Is used to enter into a relationship the principal purpose of which is to transfer anything of value from NSF to the grantee to carry out a public purpose authorized by a law of the United States (see 31 USC 6101(3)); and not to acquire property or services for NSF's direct benefit or use;

[3] For purposes of this Guide, except where explicitly noted, the term "grant" is interchangeable with the term "cooperative agreement", and the term "grantee" is interchangeable with the "awardee" of a cooperative agreement.

(2) Is distinguished from a grant in that it provides for substantial involvement between NSF and the grantee in carrying out the activity contemplated by the NSF award.

In the case of NSF, assistance awards involve the support or stimulation of scientific and engineering research, science and engineering education or other related activities. NSF is authorized to use grants or cooperative agreements for this purpose. Grants, however, are the primary mechanism of NSF support.

f. A GRANTEE means the organization or other entity that receives a grant and assumes legal and financial responsibility and accountability both for the awarded funds and for the performance of the grant-supported activity. NSF grants are normally made to organizations rather than to individual Principal Investigator/Project Director(s). Categories of eligible proposers may be found in Chapter I.E.

g. PRINCIPAL INVESTIGATOR/PROJECT DIRECTOR (PI/PD) means the individual(s) designated by the proposer, and approved by NSF, who will be responsible for the scientific or technical direction of the project. NSF does not infer any distinction in scientific stature among multiple PIs, whether referred to as PI or co-PI. If more than one, the first one listed will serve as the contact PI, with whom all communications between NSF program officials and the project relating to the scientific, technical, and budgetary aspects of the project should take place. The PI and any identified co-PIs, however, will be jointly responsible for submission of the requisite project reports. The term "Principal Investigator" generally is used in research projects, while the term "Project Director" generally is used in centers, large facilities, and other projects. For purposes of this Guide, PI/co-PI is interchangeable with PD/co-PD.

2. NSF-Grantee Relationships

a. Grants will be used by NSF when the accomplishment of the project objectives requires minimal NSF involvement during performance of the activities. Grants establish a relationship between NSF and the grantee in which:

(1) NSF agrees to provide up to a specified amount of financial support for the project to be performed under the conditions and requirements of the grant. NSF will monitor grant progress and assure compliance with applicable standards.

(2) The grantee agrees to: perform the project as proposed; the prudent management of the funds provided; and carry out the supported activities in accordance with the provisions of the grant. (See Chapter VI.B for the documents that comprise an NSF grant.)

b. Cooperative agreements will be used by NSF when the accomplishment of the project objectives requires substantial ongoing Foundation involvement during the project performance period. Substantial agency involvement may be necessary when an activity is technically and/or managerially complex and would require extensive or close coordination between NSF and the awardee. This, however, does not affect NSF's right to unilaterally suspend or terminate support for cause or consider termination in accordance with Chapter XII, if it is in the best interest of NSF or the Government. The doctrine of substantial involvement is set forth in the Federal Grant and Cooperative Agreement Act of 1977 (31 USC 6301-6308).

NSF utilizes two types of cooperative agreements:

- **Standalone Cooperative Agreement (CA)**, which consists of a cooperative agreement for a single, unified award where there is no need to provide separate, discrete funding and oversight for the projects or programs under that award.

- **Master Cooperative Agreement/Cooperative Support Agreement (CA/CSA),** which consists of a master or overall agreement having separate and specific awards (CSAs) that are funded individually under the umbrella of the master agreement. CSAs have their own terms and conditions in addition to those of the master agreement. The scope of CSAs falls within the scope

of the master agreement, but each CSA has its own distinct award number and funding based on its approved budget; no funding is attached to the master CA.

Examples of projects suitable for cooperative agreements include: management of research centers, large curriculum projects, multi-user facilities, projects which involve complex subcontracting, construction or operations of major in-house university facilities and major instrumentation development, and projects in which NSF participates with other stakeholder agencies or organizations that have influence over project direction and/or development.

Under a cooperative agreement, the awardee has primary responsibility for the conduct of the project. To the extent that NSF does not reserve responsibility for coordinating or integrating the project activities with other related activities or does not assume a degree of shared responsibility for certain aspects of the project, all such responsibilities remain with the awardee. While NSF will monitor the cooperative agreement in accordance with the terms and conditions of the award, the Foundation will not assume overall control of a project or unilaterally change or direct the project activities.

The cooperative agreement will specify the extent to which NSF will advise, review, approve or otherwise be involved with project activities, as well as NSF's right to require more clearly defined deliverables. NSF may provide advice, guidance or assistance of a technical, management, or coordinating nature and may require that the awardee obtain NSF prior approval of specific decisions, milestones, or project activities. Substantial involvement is incorporated in key areas of accountability in both financial and programmatic award terms; examples include prior agency approval requirements, type and frequency of project plans, special reporting requirements, and project and awardee reviews that NSF will conduct during the term of the award.

Cooperative agreements for construction are generally funded through a separate appropriation from Congress for Major Research Equipment and Facilities Construction (MREFC). NSF maintains the MREFC appropriations in a separate budget account, for major construction projects that successfully undergo a rigorous selection process. MREFC funds cannot be co-mingled with funds for activities other than construction; therefore, NSF issues a separate award for operations and other activities related to commissioning and management of the facility or major instrument. The awardee is required to maintain an accounting system capable of segregating MREFC and operating costs, and to ensure that such costs are applied accordingly.

Many large facility awards, including those for NSF-supported Federally Funded Research and Development Centers (FFRDCs), consist of a cooperative agreement as an umbrella award, establishing the overall basic provisions of the agreements, and separate cooperative support agreements with specific terms and conditions for construction activities, management and operations, research activities that are co-sponsored by other agencies, and any other focused activities that NSF needs to monitor separately from the overall objectives of the cooperative agreement.

E. NSF Organizations

The NSF organizations/offices described below are normally of most direct interest to the NSF proposer and awardee community. Consult the NSF website at: http://www.nsf.gov/staff/orglist.jsp for the most current listing of NSF offices/directorates and an organizational chart.

1. National Science Board (NSB)

The NSB was established by Congress in 1950, and along with the Director, constitutes the National Science Foundation. The Board provides oversight for, and establishes the policies of, the agency within the framework of applicable national policies set forth by the President and Congress. In this capacity, the Board identifies issues that are critical to NSF's future, approves NSF's strategic budget directions, approves annual budget submissions to the Office of Management and Budget (OMB), approves major awards and consults on certain programs, analyzes NSF's budget to ensure progress and consistency along the strategic direction set for NSF, and ensures balance between initiatives and core programs. In addition, the Board serves as an independent body of advisors to both the President and Congress on broad national policy issues and, together with the Director, recommends and encourages the pursuit of national policies related to science and engineering research and education. The Board is comprised of 24 members appointed by the President. The NSF Director also serves as an *ex officio* voting member of the Board. Members are selected on the basis of their distinguished service in science and engineering research and education, and are representative of scientific, engineering, and educational leadership throughout the Nation.

2. Program Divisions/Offices

Program Divisions/Offices are responsible for the scientific, technical and programmatic review and evaluation of proposals and for recommending that proposals be declined or awarded. The scientific, engineering and/or educational aspects of an award will be monitored by the NSF Program Officer identified in the award notice. (See Chapter III for a detailed description of the NSF Merit Review Process.) Integral staff in the program division/office relative to the NSF proposal and award process are:

a. NSF Program Officers. Program Officers are considered subject matter experts in both technical and programmatic areas. They conduct merit review of proposals and recommend which projects should be funded by the Foundation.

b. NSF Division Directors. Division Directors are NSF executives whose responsibilities include long-range planning, contributing to the achievement of the Foundation's strategic goals and objectives, and providing stewardship for budgetary and other resources. They are responsible for ensuring the integrity of the merit review and award process.

3. Division of Grants & Agreements (DGA)

DGA is responsible for the award and administration of the majority of NSF's assistance awards – i.e., grants, cooperative agreements, and fellowship awards, recommended for support by NSF program offices. From pre-award through closeout, DGA conducts a variety of business, financial, and administrative reviews to ensure compliance with award terms and conditions and consistency with applicable NSF policies and Federal rules and regulations. DGA provides stewardship and support to a diverse set of stakeholders including awardees, NSF Directorates/Offices, and others within the scientific research and education communities. DGA also maintains a leadership role in the Federal grants arena. In carrying out NSF's primary mission of promoting the progress of science, DGA continually interacts with academic and non-academic institutions, private industry, State and local governments, and other Federal agencies.

Grants & Agreements Officers (Grants Officers) have delegated warrant authority to issue awards on behalf of the Foundation and their approval constitutes a legal obligation of Federal funds for awardees to expend

to fulfill the scope of the approved proposal. In addition, Grants Officers are responsible for issuing all award amendments and certain post-award prior approvals, for monitoring awardee compliance with award terms and conditions, and for the administration and closeout of these awards.

4. Division of Institution & Award Support (DIAS)

DIAS is responsible for the development and implementation of proposal and award policies and procedures, clearance of NSF funding opportunities, advanced monitoring activities, cost analysis and award support, audit resolution, electronic award systems administration, and outreach to the external community. DIAS also advocates for the needs of the research community in NSF electronic research administration activities, including the modernization of NSF's externally-facing FastLane system via Research.gov. DIAS organizational units are as follows:

a. The Policy Office is responsible for the development, coordination, and issuance of NSF pre- and post-award policies for the assistance programs of NSF. The Policy Office provides guidance on the interpretation of NSF policies and procedures within NSF's electronic proposal and award systems, as well as official clearance for NSF funding opportunities.

The Policy Office coordinates outreach programs for external stakeholders across the broad research community and NSF staff. Through a variety of mechanisms, it releases timely information regarding NSF policies and procedures, proposal preparation, and award management. In addition to working closely with professional research administration associations, the Policy Office coordinates two major grants conferences held at various locations throughout the US, typically in the spring and fall of each year.

b. The Cost Analysis and Pre-award (CAP) Branch specializes in determinations with regard to the allowability, allocability and reasonableness of costs either budgeted or claimed under NSF awards. CAP evaluates accounting systems, internal controls, and policies and procedures of prospective and current NSF grantees. Its major functions include: pre-award reviews of new grantees, Phase II research projects involving small businesses; budgets of large-scale awards; and indirect cost rate negotiation. CAP provides guidance to NSF Program and Grants Officers, as well as grantees for questions related to its areas of responsibility.

c. The Resolution and Advanced Monitoring (RAM) Branch specializes in determining the allowability, allocability and reasonableness of costs claimed under NSF awards. RAM also evaluates the accounting systems, internal controls and policies and procedures of current NSF grantees. RAM's primary responsibilities include: audit resolution, advanced monitoring to assess grantees' administrative capability, performance, and compliance with award terms and conditions; and review of certain post-award adjustments to expenditures. RAM provides guidance to NSF program staff and grantees for questions related to its areas of responsibility.

d. The Systems Office plays a major role in the design, implementation, administration, and oversight of business rules for assistance awards across NSF electronic corporate systems. It is also responsible for systems analysis and requirements development necessary for the implementation of award business rules.

5. Division of Acquisition and Cooperative Support (DACS)

DACS provides acquisition and cooperative agreement award support for the Foundation. The Contracts Branch is responsible for planning, solicitation, negotiation, award and administration of professional, administrative, and research support contracts for NSF. The Cooperative Support Branch (CSB) is responsible for planning, solicitation, negotiation, award and administration of cooperative agreements for FFRDCs and major research facilities in various stages of construction and operations, including multi-institutional and international programs. This includes participation and input on NSF project advisory teams, business process reviews and redesign, risk assessments, and administrative assistance. The CSB Grants & Agreements Officers (Grants Officers) have delegated warrant authority to issue awards on behalf

of the Foundation and their approval constitutes a legal obligation of Federal funds for awardees to expend to fulfill the scope of the approved proposal. In addition, Grants Officers are responsible for issuing all award amendments and certain post-award prior approvals, for monitoring awardee compliance with award terms and conditions, and for the administration and closeout of these awards.

6. Division of Financial Management (DFM)

DFM is responsible for the financial policy and financial management of NSF. The Division is responsible for NSF's financial reporting, grantee business office relationships and payment of vendors. The Cash Management Branch of DFM is available to assist grantee financial and business officials in matters of payment and financial reporting discussed in Chapter VIII of the PAPPG.

7. Large Facilities Office (LFO)

LFO is the Foundation's primary resource for all oversight practices related to major facility projects, and is the NSF-wide resource on project management best practices. LFO has the institutional authority and resources to effectively develop mandatory policies, practices and procedures, which are approved by senior management, for all stages of the facility life-cycle. The Office provides: (1) expert assistance on non-scientific and non-technical aspects of project planning, budgeting, and implementation for major facilities; (2) assurance that all applicable requirements are followed in order to give credence to NSF's oversight capabilities; and (3) facilitates the use of best practices by fostering coordination and collaboration throughout NSF to share application of lessons learned from prior major facility projects.

8. Office of the General Counsel (OGC)

OGC is the legal advisor and advocate for the Foundation, providing legal advice and counsel on all aspects of the Foundation's programs, policies, and operations, as well as areas affecting science and technology more broadly. Advice is provided in a wide variety of areas, such as: grants and contracts; ethics and conflicts of interest; Freedom of Information Act; Privacy Act; labor and personnel law; environmental law; Federal fiscal and administrative law and procedure; and international law and agreements.

9. Office of Diversity & Inclusion (ODI)

ODI is responsible for responding to all civil rights matters pertaining to NSF programs or activities receiving Federal financial assistance. (See Chapter XI.A for additional information.)

10. Office of the Inspector General (OIG)

OIG is an independent oversight office that reports directly to the NSB and Congress. It is responsible for conducting audits, reviews, and investigations of NSF programs, and of organizations and individuals that apply for or receive NSF funding. OIG also investigates allegations of research misconduct, such as plagiarism, falsification, or fabrication, involving researchers who request or receive NSF funding (see Chapter XII.C.1). The OIG staff includes scientists, attorneys, auditors, law enforcement personnel, evaluators, and information technology specialists. OIG audits focus on NSF's internal agency programs, as well as grants, cooperative agreements and contracts funded by NSF. Their purpose is to ensure that the financial, administrative, and program activities of NSF are conducted efficiently and effectively, and that the NSF awardee organization's claimed costs are allowable, reasonable and properly allocated.

OIG investigations focus on program integrity and financial or nonfinancial wrongdoing by organizations and individuals who submit proposals to, receive awards from, conduct business with, or work for NSF, and can result in criminal, civil, or administrative sanctions. Statutory law enforcement authority was granted to OIG by the US Attorney General.

Anyone, including grantees, administrators, and NSF personnel, should contact the OIG (1-800-428-2189 or oig@nsf.gov) to report instances of possible misconduct, fraud, waste, or abuse.

F. NSF Electronic Capabilities Modernization Status

NSF has undertaken a multi-year effort to modernize and transition grantee electronic capabilities from FastLane to Research.gov. During this time, electronic capabilities will be developed and launched in Research.gov. These capabilities may simultaneously still be available in FastLane for a period of time prior to being ultimately retired and the functionality available exclusively through Research.gov. In order to assist grantees, NSF has developed a matrix that lists NSF's grantee electronic capabilities, and whether they can be found in FastLane, Research.gov or both. This matrix will be updated as appropriate, independent of the PAPPG revision cycle. The Electronic Capabilities Modernization Status website is available at: http://www.nsf.gov/bfa/dias/policy/estatus_matrix.jsp.

PART I: PROPOSAL PREPARATION AND SUBMISSION GUIDELINES

Part I sets forth NSF's proposal preparation and submission guidelines. The coverage provides guidance for the preparation and submission of proposals to NSF. Some NSF programs have program solicitations that modify the general provisions of the PAPPG, and, in such cases, the guidelines provided in the solicitation must be followed.

Chapter I: Pre-Submission Information

A. NSF Proposal Preparation and Submission

Unless specified in an NSF program solicitation, proposals submitted to NSF must be submitted via use of either the NSF FastLane System or Grants.gov. Further information on each system is provided below.

- **Proposal Preparation and Submission via the NSF FastLane System.** The policy and procedural guidance contained in Part I of the PAPPG relates specifically to proposals submitted via the NSF FastLane System. FastLane may be used for proposal preparation, submission, file updates, and select postaward administrative activities. (See Introduction, Section F.) Detailed information about the FastLane System is available from the FastLane website at: https://www.fastlane.nsf.gov.

- **Proposal Preparation and Submission via Grants.gov.** The policy and procedural guidance contained in the NSF *Grants.gov Application Guide* relates specifically to proposals submitted via Grants.gov. Grants.gov was established as a government-wide resource to electronically find grant opportunities, as well as to apply for Federal awards. Grants.gov is a central storehouse for information on over 1,000 grant programs from the 26 Federal grant-making agencies and provides access to approximately $550 billion annually in awards. Detailed information about Grants.gov is available from the Grants.gov website at: http://www.grants.gov.

B. NSF Programs and Funding Opportunities

The NSF website provides the most comprehensive source of information on NSF Directorates/Offices (including contact information), programs and funding opportunities. Use of this website by potential proposers is strongly encouraged. In addition, "NSF Update" is an information-delivery system designed to keep potential proposers and other interested parties apprised of new NSF funding opportunities and publications, important changes in proposal and award policies and procedures, and upcoming NSF Grants Conferences. Subscribers are informed each time new publications are issued that match their identified interests. "NSF Update" is available on NSF's website at: https://public.govdelivery.com/accounts/USNSF/subscriber/new?qsp=823.

C. Categories of Funding Opportunities

NSF utilizes a variety of mechanisms to communicate opportunities for research and education support, as well as to generate proposals. A brief description of each category of funding opportunity follows.

1. Program Descriptions

The term "program description" includes broad, general descriptions of programs and activities in NSF Directorates/Offices and Divisions. Program descriptions are posted on Directorate/Division websites to encourage the submission of proposals in specific program areas of interest to NSF.

Program descriptions utilize the generic eligibility and proposal preparation instructions specified in Part I of the PAPPG, as well as the NSB-approved merit review criteria. See Chapter III for additional information.

2. Program Announcements

The term "program announcement" refers to formal NSF publications that announce NSF programs. Program announcements utilize the generic eligibility and proposal preparation guidelines specified in Part I of the PAPPG and incorporate the NSB-approved merit review criteria.

3. Program Solicitations

The term "program solicitation" refers to formal NSF publications that encourage the submission of proposals in specific program areas of interest to NSF. They generally are more focused than program announcements, and normally apply for a limited period of time. Competition among proposals is more precisely defined than with program announcements, and proposals received compete directly with each other for NSF funding. Program solicitations are issued when the funding opportunity has one or more of the following features:

- provides supplemental proposal preparation guidance or deviates from the guidelines established in Part I of the PAPPG;
- contains additional review criteria relevant to the program;
- requires submission of a letter of intent (see Chapter I.D.1) or preliminary proposal (see Chapter I.D.2);
- deviates from (or restricts) the standard categories of proposers specified in Section E. below;
- limits the number of proposals that may be submitted by an organization and/or researcher/educator;[4]
- requires cost sharing;
- requires a limitation in indirect (Facilities & Administrative (F&A)) costs;
- specifies additional award conditions and/or reporting requirements;
- anticipates use of a cooperative agreement.

4. Dear Colleague Letters (DCLs)

DCLs are intended to provide general information to the community, clarify or amend an existing policy or document, or inform the NSF proposer community about upcoming opportunities or special competitions for supplements to existing awards. In addition, DCLs are often used to draw attention to an impending change in NSF policies or programs.

[4]Unless otherwise specified, the term “organization” refers to all categories of proposers.

D. Types of Submissions

1. Letters of Intent (LOI)

Some NSF program solicitations require or request submission of an LOI in advance of submission of a full proposal. An LOI is not a binding document. The predominant reason for its use is to help NSF program staff gauge the size and range of the competition, enabling earlier selection and better management of reviewers and panelists. In addition, the information contained in an LOI is used to help avoid potential conflicts of interest in the review process.

An LOI normally contains the Principal Investigator's (PI's) and co-PI's names, a proposed title, a list of possible participating organizations (if applicable), and a synopsis that describes the work in sufficient detail to permit an appropriate selection of reviewers. Proposers that plan to submit a collaborative proposal from multiple organizations should submit a single LOI for the entire project, given that NSF considers a collaborative proposal to be a unified research project. An LOI is not externally evaluated or used to decide on funding. The requirement to submit an LOI will be identified in the program solicitation, and such letters are submitted electronically to NSF. Failure to submit a required LOI identified in a program solicitation will result in a full proposal not being accepted or returned without review.

2. Preliminary Proposals

Some NSF program solicitations require or request submission of a preliminary proposal in advance of submission of a full proposal. The three predominant reasons for requiring submission of a preliminary proposal are to:

- reduce the proposers' unnecessary effort in proposal preparation when the chance of success is very small. This is particularly true of exploratory initiatives when the community senses that a major new direction is being identified, or competitions that will result in a small number of awards;
- increase the overall quality of the full submission; and
- assist NSF program staff in managing the review process and in the selection of reviewers.

The NSF program solicitation will specify content and submission requirements when preliminary proposals are to be used. Preliminary proposals are prepared by the PI using the Proposal Preparation Module in FastLane. On the Cover Sheet, the PI clicks on the "Preliminary Proposal" check box. The PI completes only the sections appropriate to the preliminary proposal. The PI then forwards the proposal to the appropriate office at his/her organization, and the Authorized Organizational Representative (AOR) signs and submits the preliminary proposal via use of NSF's electronic systems.

One of the following two types of decisions may be received from NSF upon submission of a preliminary proposal. The program solicitation will specify the type of decision to be rendered for a particular program.

a. Invite/Not Invite Decisions

This type of mechanism is used when the NSF decision made on the preliminary proposal is final, affecting the organization's eligibility to submit a full proposal. Only submitters of favorably reviewed preliminary proposals are invited and eligible to submit full proposals. The PI and the organization's Sponsored Projects Office (SPO) (or equivalent) will be electronically notified of NSF's decision to either invite submission of a full proposal or decline NSF support.

b. Encourage/Discourage Decisions

This type of mechanism is used when the NSF decision made on the preliminary proposal is advisory only. This means that submitters of both favorably and unfavorably reviewed preliminary proposals are eligible to submit full proposals. The PI and the organization's SPO will be notified of NSF's decision to either encourage or discourage submission of a full proposal.

3. Full Proposals

The full proposal should present the (1) objectives and scientific, engineering, or educational significance of the proposed work; (2) suitability of the methods to be employed; (3) qualifications of the investigator and the grantee organization; (4) effect of the activity on the infrastructure of science, engineering and education, if applicable; and (5) amount of funding required. It should present the intellectual merit and broader impacts of the proposed project clearly and should be prepared with the care and thoroughness of a paper submitted for publication. The requisite proposal preparation instructions are contained in Chapter II. Sufficient information should be provided to enable reviewers to evaluate the proposal in accordance with the two merit review criteria established by the NSB. (See Chapter III for additional information on NSF processing and review of proposals.)

NSF expects strict adherence to the rules of proper scholarship and attribution. The responsibility for proper scholarship and attribution rests with the authors of a proposal; all parts of the proposal should be prepared with equal care for this concern. Authors other than the PI (or any co-PI) should be named and acknowledged. Serious failure to adhere to such standards can result in findings of research misconduct. NSF policies and rules on research misconduct[5] are discussed in the Chapter XII.C, as well as 45 CFR Part 689.

The Metric Conversion Act of 1975, as amended, and E.O. 12770 of 1991 encourage Federal agencies to use the Metric System (SI) in procurement, grants and other business-related activities. Proposers are encouraged to use the Metric System of weights and measures in proposals submitted to the Foundation. Grantees also are encouraged to use metric units in reports, publications and correspondence relating to proposals and awards.

E. Who May Submit Proposals

NSF welcomes proposals on behalf of all qualified scientists, engineers and educators. The Foundation strongly encourages women, minorities and persons with disabilities to participate fully in its programs. In accordance with Federal statutes, regulations and NSF policies, no person on grounds of race, color, age, sex, national origin or disability shall be excluded from participation in, be denied the benefits of, or be subjected to discrimination under, any program or activity receiving financial assistance from NSF, although some programs may have special requirements that limit eligibility.

Scientists, engineers and educators usually initiate proposals that are officially submitted by their employing organization. Before formal submission, the proposal may be discussed with appropriate NSF program staff. Graduate students are not encouraged to submit research proposals, but should arrange to serve as research assistants to faculty members. Some NSF divisions accept proposals for Doctoral Dissertation Research Grants when submitted by a faculty member on behalf of the graduate student.

[5] Research misconduct refers to fabrication, falsification, or plagiarism in proposing or performing research funded by NSF, reviewing research proposals submitted to NSF, or in reporting research results funded by NSF. Organizations involved in international collaborations might find the following document provided by the Organisation for Economic Co-operation and Development (OECD) to be useful: "Best Practices for Ensuring Scientific Integrity and Preventing Misconduct"

Categories of Proposers

Except where a program solicitation establishes more restrictive eligibility criteria, individuals and organizations in the following categories may submit proposals:

1. Institutions of Higher Education (IHEs) — Two- and four-year IHEs (including community colleges) accredited in, and having a campus located in the US, acting on behalf of their faculty members. IHEs located outside the US fall under paragraph 6. below.

Special Instructions for International Branch Campuses of US IHEs

If the proposal includes funding to be provided to an international branch campus of a US institution of higher education (including through use of subawards and consultant arrangements), the proposer must explain the benefit(s) to the project of performance at the international branch campus, and justify why the project activities cannot be performed at the US campus.

2. Non-profit, Non-academic Organizations — Independent museums, observatories, research laboratories, professional societies and similar organizations located in the US that are directly associated with educational or research activities.

3. For-profit Organizations — US commercial organizations, especially small businesses with strong capabilities in scientific or engineering research or education. An unsolicited proposal from a commercial organization may be funded when the project is of special concern from a national point of view, special resources are available for the work, or the proposed project is especially meritorious. NSF is interested in supporting projects that couple industrial research resources and perspectives with those of universities; therefore, it especially welcomes proposals for cooperative projects involving both universities and the private commercial sector.

4. State and Local Governments — State educational offices or organizations and local school districts may submit proposals intended to broaden the impact, accelerate the pace, and increase the effectiveness of improvements in science, mathematics and engineering education in both K-12 and post-secondary levels.

5. Unaffiliated Individuals — Unaffiliated individuals in the US and US citizens rarely receive direct funding support from NSF. Recipients of Federal funds must be able to demonstrate their ability to fully comply with the requirements specified in 2 CFR § 200, *Uniform Administrative Requirements, Cost Principles, and Audit Requirements for Federal Awards.* As such, unaffiliated individuals are strongly encouraged to affiliate with an organization that is able to meet the requirements specified in 2 CFR § 200.

Unaffiliated individuals must contact the cognizant Program Officer prior to preparing and submitting a proposal to NSF.

6. Foreign Organizations — NSF rarely provides funding support to foreign organizations. NSF will consider proposals for cooperative projects involving US and foreign organizations, provided support is requested only for the US portion of the collaborative effort. In cases however, where the proposer considers the foreign organization's involvement to be essential to the project (e.g., through subawards or consultant arrangements), the proposer must explain why local support is not feasible and why the foreign organization can carry out the activity more effectively. In addition, the proposed activity must demonstrate how one or more of the following conditions have been met:

- The foreign organization contributes a unique organization, facilities, geographic location and/or access to unique data resources not generally available to US investigators (or which would require significant effort or time to duplicate) or other resources that are essential to the success of the proposed project; and/or

- The foreign organization to be supported offers significant science and engineering education, training or research opportunities to the US.

7. Other Federal Agencies — NSF does not normally support research or education activities by scientists, engineers or educators employed by Federal agencies or FFRDCs. Under unusual circumstances, other Federal agencies and FFRDCs may submit proposals directly to NSF. A proposed project is only eligible for support if it meets one or more of the following exceptions, as determined by a cognizant NSF Program Officer:

- Special Projects. Under exceptional circumstances, research or education projects at other Federal agencies or FFRDCs that can make unique contributions to the needs of researchers elsewhere or to other specific NSF objectives may receive NSF support.

- National and International Programs. The Foundation may fund research and logistical support activities of other Government agencies or FFRDCs directed at meeting the goals of special national and international research programs for which the Foundation bears special responsibility, such as the US Antarctic Research Program.

- International Travel Awards. In order to ensure appropriate representation or availability of a particular expertise at an international conference, staff researchers of other Federal agencies may receive NSF international travel awards.

Proposers who think their project may meet one of the exceptions listed above must contact a cognizant NSF Program Officer before preparing a proposal for submission. In addition, a scientist, engineer or educator who has a joint appointment with a university and a Federal agency (such as a Veterans Administration Hospital, or with a university and a FFRDC) may submit proposals through the university and may receive support if he/she is a faculty member (or equivalent) of the university, although part of his/her salary may be provided by the Federal agency. Preliminary inquiry must be made to the appropriate program before preparing a proposal for submission.

F. When to Submit Proposals

Proposers should allow adequate time for processing of proposals (see Chapter I.H for further information). Many NSF programs accept proposals at any time. Other programs, however, establish due dates for submission of proposals. The following types of due dates are utilized by NSF:

1. **Target Dates:** dates after which proposals will still be accepted, although they may miss a particular panel or committee meeting.

2. **Deadline Dates:** dates after which proposals will not be accepted or will be returned without review by NSF. The deadline date will be waived only in extenuating circumstances. Such a deviation may be authorized only in accordance with Chapter II.A.

3. **Submission Windows:** designated periods of time during which proposals will be accepted for review by NSF. It is NSF's policy that the end date of a submission window converts to, and is subject to, the same policies as a deadline date.

These target dates, deadlines, and submission windows are published in specific program descriptions, program announcements and solicitations that can be obtained through the NSF website.[6] Unless otherwise stated in a program announcement or solicitation, proposals must be received by the specified date. If the deadline date falls on a weekend, it will be extended to the following Monday; if the date falls

[6]A listing of upcoming target dates and deadlines, sorted by date and by program area is available on the NSF website at: http://nsf.gov/funding/pgm_list.jsp?org=NSF&ord=date.

on a Federal holiday, it will be extended to the following business day. Proposals must be received by 5 p.m. submitter's local time on the established deadline date. Failure to submit by 5.p.m. submitter's local time will result in the proposal not being accepted. See Chapter IV.B for additional information. Letters of intent or preliminary proposals also follow the 5 p.m. submitter's local time standard.

Special Exceptions to NSF's Deadline Date Policy

In the event of a natural or anthropogenic disaster, or when NSF is closed due to inclement weather or other reason that interferes with an organization's ability to meet a proposal submission deadline, NSF has developed the following guidelines for use by impacted organizations.

Natural or Anthropogenic Disasters - Flexibility in meeting announced deadline dates because of a natural or anthropogenic disaster that impacts a proposer's ability to submit a proposal to NSF may be granted with the approval of the cognizant NSF Program Officer. Where possible, such requests should be submitted in advance of the proposal deadline. Proposers should contact the cognizant NSF Program Officer in the Division/Office to which they intend to submit their proposal and request authorization to submit a proposal after the deadline date. Proposers should then follow the written or verbal guidance provided by the cognizant NSF Program Officer. The Foundation will work with each impacted organization on a case-by-case basis to address its specific issue(s). Generally, NSF permits extension of the deadline by up to five business days.

To submit the proposal after the deadline date, proposers must check the "Special Exception to the Deadline Date Policy" box on the NSF Cover Sheet, indicating NSF approval has been obtained. A statement identifying the nature of the event that impacted the ability to submit the proposal on time should be uploaded under Nature of Natural or Anthropogenic event in the Single Copy Document section in FastLane. If available, written approval from the cognizant NSF Program Officer also should be uploaded under the Additional Single Copy Documents in the Single Copy Document section in FastLane.

Closure of NSF – When NSF is closed due to inclement weather or other reason[7], deadline(s) that occurred during the closure automatically will be extended to the following business day after the closure ends.

G. How to Submit Proposals

1. Electronic Requirements

Proposals to NSF must be submitted via the NSF FastLane System or via use of Grants.gov. For proposals prepared and submitted via Grants.gov, the guidelines specified in the NSF *Grants.gov Application Guide* apply.

2. Submission Instructions

The same work/proposal cannot be funded twice. If the proposer envisions review by multiple programs, more than one program may be designated on the Cover Sheet. The submission of duplicate or substantially similar proposals concurrently for review by more than one program without prior NSF approval will result in the return of the redundant proposals. (See Chapter IV.B for further information.)

In submission of a proposal for funding, the AOR is required to provide certain proposal certifications. (See Chapter II.C.1.d for a listing.) This certification process will occur concurrently with submission of the proposal.

[7] This policy is not intended to cover NSF closures due to lapses in appropriation. In such cases, specific guidance will be issued, as appropriate.

3. Requirements Relating to Data Universal Numbering System (DUNS) Numbers and Registration in the System for Award Management (SAM)

Proposers must provide a Dun and Bradstreet (D&B) DUNS number when applying for a new or renewal award. NSF requires that organizations registering to use NSF's electronic systems have a valid and active SAM registration and have a valid DUNS number. NSF will validate that each proposer's DUNS number and SAM registration are active and valid prior to allowing submission of a proposal to NSF. Any subrecipients named in the proposal also are required to obtain a DUNS number and register in FastLane. Subrecipients named in the proposal, however, do not need to be registered in SAM.

The SAM is the primary registrant database for the US Government. SAM collects, validates, stores, and disseminates data in support of agency acquisition missions, including Federal agency contract and assistance awards. This SAM registration must be maintained with current information at all times during which the organization has an active award or a proposal under consideration by NSF. Failure to comply with the SAM registration requirement prior to proposal submission may impact the processing of the proposal. To register in the SAM, go to: https://www.sam.gov. Proposers are advised that entity registration will become active after three to five days when the Internal Revenue Service (IRS) validates the Tax ID Number.

The organization also is responsible for updating all SAM registration information as it changes. Once an award is made, failure to maintain current and complete information within SAM will impact receipt of funding. To maintain an active status in SAM, an organization's registration must be renewed and revalidated at least every 12 months from the date of the previous registration. If the registration is not renewed, it will expire. An expired registration will impact the organization's ability to submit proposals or receive grant payments.

SAM is the NSF system of record for organizational information, including financial and address information. The Legal Business Name and Physical Address information are automatically input into the proposer's SAM registration from D&B, and it is the organization's responsibility to keep this information updated.

4. NSF ID

The NSF ID is a unique numerical identifier assigned to users by NSF. The NSF ID may be used throughout NSF's electronic systems as a login ID and identification verification. Each individual user of NSF systems, (e.g., Fastlane and Research.gov) should not have more than one NSF ID. Users with more than one NSF ID should contact the Help Desk at (800) 673-6188 or (703) 292-8142 or by e-mail to fastlane@nsf.gov for assistance.

Submission of Social Security Numbers (SSNs) only will be requested where it is necessary for business purposes, e.g., financial reimbursement. An SSN is solicited under the NSF Act of 1950, as amended.

5. Proposal Receipt

Once the proposal is submitted, PIs can check the status of the proposal via use of NSF's electronic systems. If a proposal number is not reflected in the electronic systems, contact the Help Desk at (800) 673-6188 or (703) 292-8142 or by e-mail to fastlane@nsf.gov.

Communications about the proposal should be addressed to the cognizant NSF Program Officer with reference to the proposal number. Proposers are strongly encouraged to use NSF's electronic systems to verify the status of their submission to NSF.

H. Proposal Processing

Proposers should allow up to six months for programmatic review and processing (see Chapter III for additional information on the NSF merit review process). In addition, proposers should be aware that the NSF Division of Grants and Agreements generally makes awards within 30 days after the program division/office makes its recommendation. Grants being made to organizations that have not received an NSF award within the preceding five years, or involving special situations (such as coordination with another Federal agency or a private funding source), cooperative agreements, and other unusual arrangements may require additional review and processing time. Proposals that are time-sensitive (e.g., conference and group travel) will be accepted for review only if, in the opinion of the cognizant Program Officer, they are received in sufficient time to permit appropriate NSF review and processing to support an award in advance of the activity to be supported. Every effort is made to reach a decision and inform the proposer promptly. Until an award is made, NSF is not responsible for any costs incurred by the proposing organization.

Chapter II: Proposal Preparation Instructions

Each proposing organization that is new to NSF or has not had an active NSF assistance award within the previous five years should be prepared to submit basic organization and management information and certifications, when requested, to the applicable award-making division within the Office of Budget, Finance & Award Management (BFA). The requisite information is described in the *NSF Prospective New Awardee Guide*. The information contained in this Guide will assist the organization in preparing documents which NSF requires to conduct administrative and financial reviews of the organization. This Guide also serves as a means of highlighting the accountability requirements associated with Federal awards.

To facilitate proposal preparation, Frequently Asked Questions (FAQs) regarding proposal preparation and submission are available on the NSF website.[8]

Proposers should also be aware of core strategies that are essential to the fulfillment of NSF's mission, as articulated in *Investing in Science, Engineering, and Education for the Nation's Future: NSF Strategic Plan, 2014-2018*. These strategies are integrated in the program planning and implementation process, of which proposal review is one part. NSF's mission is particularly well-implemented through the integration of research and education and broadening participation in NSF programs, projects, and activities.

One of the strategic objectives in support of NSF's mission is to foster integration of research and education through the programs, projects, and activities it supports at NSF grantee organizations. These organizations recruit, train, and prepare a diverse science, technology, engineering, and mathematics (STEM) workforce to advance the frontiers of science and participate in the US technology-based economy. NSF's contribution to the national innovation ecosystem is to provide cutting-edge research under the guidance of the Nation's most creative scientists and engineers. NSF also supports development of a strong STEM workforce by investing in building the knowledge that informs improvements in STEM teaching and learning.

NSF's mission calls for the broadening of opportunities and expanding participation of groups, organizations, and geographic regions that are underrepresented in STEM disciplines, which is essential to the health and vitality of science and engineering. NSF is committed to this principle of diversity and deems it central to the programs, projects, and activities it considers and supports.

A. Conformance with Instructions for Proposal Preparation

It is important that all proposals conform to the proposal preparation instructions contained in Part I of the PAPPG or the NSF *Grants.gov Application Guide*. Conformance is required and will be strictly enforced unless an authorization to deviate from standard proposal preparation requirements has been approved. NSF will not accept or will return without review proposals that are not consistent with these instructions. See Chapter IV.B for additional information. NSF authorization to deviate from these instructions must be received prior to proposal submission. Deviations may be authorized in one of two ways:

1. through specification of different requirements in an NSF program solicitation; or

2. by the written approval of the cognizant NSF Assistant Director/Office Head or designee. These approvals to deviate from NSF proposal preparation instructions may cover a particular program or programs or, in rare instances, an individual deviation for a particular proposal.

Proposers may deviate from these instructions only to the extent authorized. Proposals must include an authorization to deviate from standard NSF proposal preparation instructions in one of the following ways, as appropriate: (a) by identifying the solicitation number that authorized the deviation in the appropriate block on the Cover Sheet; or (b) for individual deviations, by identifying the name, date and title of the NSF official authorizing the deviation.

[8]FAQs regarding FastLane proposal preparation and submission also are available in FastLane.

B. Format of the Proposal

Prior to submission, it is strongly recommended that proposers conduct an administrative review to ensure that proposals comply with the guidelines established in Part I of the PAPPG or the NSF *Grants.gov Application Guide*. The Proposal Preparation Checklist (Exhibit II-1) may be used to assist in this review. The checklist is not intended to be an all-inclusive repetition of the required proposal contents and associated proposal preparation guidelines. It is, however, meant to highlight certain critical items so they will not be overlooked when the proposal is prepared.

During completion of the proposal Cover Sheet (See Chapter II.C.2.a.), the PI will be prompted to select the applicable response that describes the nature and type of proposal being developed:

The type of proposal being developed:

- Research (see Chapter II, Sections A through C);
- Rapid Response Research (RAPID) (see Chapter II.E.1);
- Early-concept Grants for Exploratory Research (EAGER) (see Chapter II.E.2);
- Research Advanced by Interdisciplinary Science and Engineering (RAISE)(see Chapter II.E.3);
- Grant Opportunities for Academic Liaison with Industry (GOALI) (see Chapter II.E.4);
- Ideas Lab (see Chapter II.E.5);
- Facilitation Awards for Scientists and Engineers with Disabilities (FASED) (see Chapter II.E.6);
- Conference (see Chapter II.D.7);
- Equipment (see Chapter II.D.8);
- Travel (see Chapter II.D.9);
- Center/Research Infrastructure (see relevant funding opportunity); or
- Fellowship (see relevant funding opportunity).

Whether the proposal is:

- A collaborative proposal from one organization (see Chapter II.D.3.a);
- A collaborative proposal from multiple organizations (see Chapter II.D.3.b); or
- Not a collaborative proposal.

The requested proposal information noted above will be used to determine the applicable proposal preparation business rules that must be followed. Proposers are strongly advised to review the applicable sections of Part I of the PAPPG pertinent to the type of proposal being developed **PRIOR** to submission. Additional information on NSF auto-compliance checks run during the proposal preparation and submission process can be found at: http://www.nsf.gov/bfa/dias/policy/autocompliance.jsp.

1. Proposal Pagination Instructions

Proposers are advised that FastLane does not automatically paginate a proposal. Each section of the proposal that is uploaded as a file must be individually paginated prior to being uploaded to the electronic system.

2. Proposal Margin and Spacing Requirements

The proposal must conform to the following requirements:

a. Use one of the following typefaces identified below:

- Arial[9], Courier New, or Palatino Linotype at a font size of 10 points or larger;
- Times New Roman at a font size of 11 points or larger; or
- Computer Modern family of fonts at a font size of 11 points or larger.

A font size of less than 10 points may be used for mathematical formulas or equations, figures, table or diagram captions and when using a Symbol font to insert Greek letters or special characters. PIs are cautioned, however, that the text must still be readable.

b. No more than six lines of text within a vertical space of one inch.

c. Margins, in all directions, must be at least an inch.

These requirements apply to all uploaded sections of a proposal, including supplementary documentation.

3. Page Formatting

Proposers are strongly encouraged to use only a standard, single-column format for the text.

The guidelines specified above establish the minimum type size requirements; however, PIs are advised that readability is of paramount importance and should take precedence in selection of an appropriate font for use in the proposal. **Small type size makes it difficult for reviewers to read the proposal; consequently, the use of small type not in compliance with the above guidelines may be grounds for NSF to return the proposal without review.** Adherence to type size and line spacing requirements also is necessary to ensure that no proposer will have an unfair advantage, by using smaller type or line spacing to provide more text in the proposal.

C. Proposal Contents

1. Single-Copy Documents

Certain categories of information that are submitted in conjunction with a proposal are for "NSF Use Only." As such, the information is not provided to reviewers for use in the review of the proposal. With the exception of proposal certifications (which are submitted via the Authorized Organizational Representative function[10]), these documents should be submitted via the FastLane Proposal Preparation Module. A summary of each of these categories follows:

a. Authorization to Deviate from NSF Proposal Preparation Requirements (if applicable)

Instructions for obtaining authorization to deviate from NSF proposal preparation instructions are provided in Chapter II.A.

[9] Macintosh users also may use Helvetica and Palatino typefaces.
[10] Further instructions for this process are available in FastLane.

b. List of Suggested Reviewers or Reviewers Not to Include (optional)

Proposers may include a list of suggested reviewers (including email address and organizational affiliation) who they believe are especially well qualified to review the proposal. Proposers also may designate persons they would prefer not review the proposal, indicating why. These suggestions are optional. Exhibit II-2 contains information on conflicts of interest that may be useful in preparation of this list.

The cognizant Program Officer handling the proposal considers the suggestions and may contact the proposer for further information. The decision whether or not to use the suggestions, however, remains with the Program Officer.

c. Proprietary or Privileged Information (if applicable)

Instructions for submission of proprietary or privileged information are provided in Chapter II.D.1.

d. Proposal Certifications

With the exception of the *Disclosure of Lobbying Activities (SF LLL)* identified below, the procedures for submission of the proposal certifications differ from those used with other single-copy documents. The AOR must use the "Authorized Organizational Representative function" in FastLane to sign and submit the proposal, including the proposal certifications. It is the proposing organization's responsibility to assure that only properly authorized individuals perform this function.[11]

The required proposal certifications are as follows:

- **Certification for Authorized Organizational Representative (AOR) or Individual Proposer:** The AOR is required to complete certifications regarding the accuracy and completeness of statements contained in the proposal, as well as to certify that the organization (or individual) agrees to accept the obligation to comply with grant terms and conditions.

- **Certification Regarding Conflict of Interest:** The AOR is required to complete certifications stating that the organization has implemented and is enforcing a written policy on conflicts of interest (COI), consistent with the provisions of Chapter IX.A: that, to the best of his/her knowledge, all financial disclosures required by the conflict of interest policy were made; and that conflicts of interest, if any, were, or prior to the organization's expenditure of any funds under the grant, will be, satisfactorily managed, reduced or eliminated in accordance with the organization's conflict of interest policy. Conflicts that cannot be satisfactorily managed, reduced or eliminated, and research that proceeds without the imposition of conditions or restrictions when a conflict of interest exists, must be disclosed to NSF via use of NSF's electronic systems.

- **Drug-Free Workplace:** The AOR is required to complete a certification regarding the Drug-Free Workplace Act. See Exhibit II-3 for the full text of the Drug-Free Workplace Certification.

- **Debarment and Suspension:** The AOR is required to complete a certification regarding Debarment and Suspension. See Exhibit II-4for the full text of the Debarment and Suspension Certification.

- **Certification Regarding Lobbying:** When the proposal exceeds $100,000, the AOR is required to complete a certification regarding lobbying restrictions. The *Certification for Contracts, Grants, Loans and Cooperative Agreements* is included in full text on the FastLane submission screen as well as in Exhibit II-5. The box for "Disclosure of Lobbying Activities" must be checked on the Cover Sheet only if, pursuant to paragraph 2 of the certification, submission of the SF LLL is required.[12]

[11]Detailed instructions for completion of this process are available in FastLane.
[12]Detailed instructions for submission of the SF LLL are available in FastLane.

- **Certification Regarding Nondiscrimination:** The AOR is required to complete a certification regarding compliance with NSF nondiscrimination regulations and policies. See Exhibit II-6 for the full text of the Nondiscrimination Certification. This certification sets forth the nondiscrimination obligations with which all grantees must comply. These obligations also apply to subrecipients or contractors under the grant. The proposer, therefore, shall obtain the NSF Nondiscrimination Certification from each organization that applies to be, or serves as a subrecipient or contractor under the award (for other than the provision of commercially available supplies, materials, equipment or general support services) prior to entering into the subaward or contract arrangement.

- **Certification Regarding Flood Hazard Insurance:** Two sections of the National Flood Insurance Act of 1968 (42 USC § 4012a and § 4106) bar Federal agencies from giving financial assistance for acquisition or construction purposes in any area identified by the Federal Emergency Management Agency (FEMA) as having special flood hazards unless the:

  (1) community in which that area is located participates in the national flood insurance program; and

  (2) building (and any related equipment) is covered by adequate flood insurance.

  By signing the certification pages, AORs for prospective grantees located in FEMA-designated special flood hazard areas are certifying that adequate flood insurance has been or will be obtained in the following situations:

  (1) for NSF grants for the construction of a building or facility, regardless of the dollar amount of the grant; and

  (2) for other NSF grants when more than $25,000 has been budgeted in the proposal for repair, alteration or improvement (construction) of a building or facility.

  Prospective grantees should contact their local government or a Federally-insured financial institution to determine what areas are identified as having special flood hazards and the availability of flood insurance in their community.

- **Certification Regarding Responsible Conduct of Research (RCR)**[13]**:** The AOR is required to complete a certification that the institution has a plan to provide appropriate training and oversight in the responsible and ethical conduct of research to undergraduates, graduate students, and postdoctoral researchers who will be supported by NSF to conduct research.

  Additional information on NSF's RCR policy is available in the Chapter IX.B. While training plans are not required to be included in proposals submitted to NSF, institutions are advised that they are subject to review upon request.

- **Certification Regarding Organizational Support:** The AOR is required to complete a certification that there is organizational support for the proposal as required by Section 526 of the *America COMPETES Reauthorization Act of 2010.* This support extends to the portion of the proposal developed to satisfy the broader impacts review criterion as well as the intellectual merit review criterion, and any additional review criteria specified in the solicitation. Organizational support will be made available, as described in the proposal, in order to address the broader impacts and intellectual merit activities to be undertaken.

[13] A wide array of information exists to help inform development of an institution's RCR training plan. For example, many professional societies as well as governmental licensing authorities for professional scientists and engineers have adopted policies or best practices that might be usefully considered. In addition, research is illuminating existing practices surrounding ethical issues, and providing an evaluation of pedagogical innovations in ethics education.

- **Certification Regarding Federal Tax Obligations:** When the proposal exceeds $5,000,000, the AOR is required to complete a certification regarding Federal tax obligations. By signing the certification pages, the AOR certifies that, to the best of his/her knowledge and belief, the proposing organization:

  (1) has filed all Federal tax returns required during the three years preceding this certification;

  (2) has not been convicted of a criminal offense under the Internal Revenue Code of 1986; and

  (3) has not, more than 90 days prior to this certification, been notified of any unpaid Federal tax assessment for which the liability remains unsatisfied, unless the assessment is the subject of an installment agreement or offer in compromise that has been approved by the IRS and is not in default, or the assessment is the subject of a non-frivolous administrative or judicial proceeding.

- **Certification Regarding Unpaid Federal Tax Liability:** If the proposer is a corporation, the AOR (or equivalent) is required to complete a certification that the corporation has no unpaid Federal tax liability that has been assessed, for which all judicial and administrative remedies have been exhausted or lapsed, and that is not being paid in a timely manner pursuant to an agreement with the authority responsible for collecting the tax liability.

- **Certification Regarding Criminal Convictions:** If the proposer is a corporation, the AOR (or equivalent) is required to complete a certification that the corporation has not been convicted of a felony criminal violation under any Federal law within the 24 months preceding the date on which the certification is signed.

- **Certification Regarding Dual Use Research of Concern:** The AOR is required to complete a certification that the organization will be or is in compliance with all aspects of the *United States Government Policy for Institutional Oversight of Life Sciences Dual Use Research of Concern.*

e. Collaborators & Other Affiliations Information

The following information regarding collaborators and other affiliations (COA) must be separately provided for each individual identified as senior project personnel. The COA information must be provided through use of the COA template. If there are more than 10 individuals designated as senior project personnel on the proposal, or if there are print preview issues, each *completed* template must be saved as a .txt file [select the Text (Tab Delimited) option] rather than as an .xlsx or .xls file. This format will still enable preservation of searchable text and avoid delays in processing and review of the proposal.

Please note that some information requested in prior versions of the PAPPG is no longer requested. THIS IS PURPOSEFUL AND WE NO LONGER REQUIRE THIS INFORMATION TO BE REPORTED. Certain relationships will be reported in other sections (i.e., the names of postdoctoral scholar sponsors should not be reported, however if the individual collaborated on research with their postdoctoral scholar sponsor, then they would be reported as a collaborator). The information in the tables is not required to be sorted, alphabetically or otherwise.

There are five separate categories of information which correspond to the five tables in the COA template:

COA template Table 1:

List the individual's last name, first name, middle initial, and organizational affiliation (including considered affiliation) in the last 12 months.

COA template Table 2:

List names as last name, first name, middle initial, for whom a personal, family, or business relationship would otherwise preclude their service as a reviewer.

COA template Table 3:

List names as last name, first name, middle initial, and provide organizational affiliations, if known, for the following:[14]

- The individual's Ph.D. advisors; and
- All of the individual's Ph.D. thesis advisees.

COA template Table 4:

List names as last name, first name, middle initial, and provide organizational affiliations, if known, for the following:

- Co-authors on any book, article, report, abstract or paper with collaboration in the last 48 months (publication date may be later); and
- Collaborators on projects, such as funded grants, graduate research or others in the last 48 months.

COA template Table 5:

List editorial board, editor-in chief and co-editors with whom the individual interacts. An editor-in-chief must list the entire editorial board.

- Editorial Board:[15] List name(s) of editor-in-chief and journal in the past 24 months; and
- Other co-Editors of journal or collections with whom the individual has directly interacted in the last 24 months.

The template has been developed to be fillable, however, the content and format requirements must not be altered by the user. This template must be saved in .xlsx or .xls format, and directly uploaded into FastLane as a Collaborators and Other Affiliations Single Copy Document. FastLane will convert the uploaded .xlsx or .xls files to PDF. Using the .xlsx or .xls format will enable preservation of searchable text that otherwise would be lost. It is therefore imperative that this document be uploaded in .xlsx or .xls **only**. Uploading a document in any format other than .xlsx or .xls may delay the timely processing and review of the proposal.

This information is used to manage reviewer selection. See Exhibit II-2 for additional information on potential reviewer conflicts.

f. Submission of Proposals by Former NSF Staff

For one year following separation from the Foundation, any communication with NSF by a former employee or IPA must be done through use of a "substitute negotiator." Unless a substitute negotiator has been designated by the proposer/grantee, the Division of Grants and Agreements (DGA) or the Division of Acquisition and Cooperative Support (DACS) will not process a new proposal with a former employee or IPA as PI or co-PI. If it has been less than a year since a former employee separated from NSF or an IPA ended their appointment and they submit a proposal, documentation from the Authorized Organizational

[14] Note that graduate advisors are no longer required to be reported.

[15] Editorial Board does not include Editorial Advisory Board, International Advisory Board, Scientific Editorial Board, or any other subcategory of Editorial Board. It is limited to those individuals who perform editing duties or manage the editing process (i.e., editor in chief).

Representative (AOR) needs to be included which designates a substitute negotiator for that proposal. A co-PI on a new proposal should designate the PI as the substitute negotiator. This information should be submitted as a single copy document and uploaded in the "Additional Single Copy Documents" category.

2. Sections of the Proposal

The sections described below represent the body of a research proposal submitted to NSF. Failure to submit the required sections will result in the proposal not being accepted[16], or being returned without review. See Chapter IV.B for additional information.

A full research proposal must contain the following sections[17]. Note that the *NSF Grants.gov Application Guide* may use different naming conventions, and sections may appear in a different order than in FastLane, however, the content is the same:

a. Cover Sheet
b. Project Summary
c. Table of Contents
d. Project Description
e. References Cited
f. Biographical Sketch(es)
g. Budget and Budget Justification
h. Current and Pending Support
i. Facilities, Equipment and Other Resources
j. Special Information and Supplementary Documentation
   - Data Management Plan
   - Postdoctoral Mentoring Plan (if applicable)
k. Single Copy Documents
   - Collaborators & Other Affiliations Information

Proposal preparation instructions for RAPID, EAGER, RAISE, GOALI, Ideas Lab, FASED, conference, equipment, travel, center, research infrastructure, and fellowships may deviate from the above content requirements.

All proposals to NSF will be reviewed using the two NSB-approved merit review criteria described in greater length in Chapter III.

a. Cover Sheet

There are four major components of the Cover Sheet. A number of the boxes contained on the Cover Sheet are pre-filled as part of the FastLane login process. The information requested on the Cover Sheet is as follows:

(1) Awardee Organization/Primary Place of Performance

The information on the Awardee Organization is prefilled on the Cover Sheet based on the login information entered. If the project will be performed at the awardee organization, check the designated box. If the project, however, will be performed at a location other than the awardee, provide the following information (where applicable):

- Organization Name (identify the organization name of the primary site where the work will be performed, if different than the awardee);
- Street;

[16] Proposal Not Accepted is defined as FastLane will not permit submission of the proposal.
[17] See Chapter II.E for the proposal preparation requirements for other types of proposals submitted to NSF.

- City;
- State;
- Country; and
- 9-digit Zip Code.

(2) Program Announcement/Solicitation/Program Description Number

Proposers are required to select the applicable funding opportunity (i.e., program announcement, solicitation or description.) If the proposal is not submitted in response to a specific funding opportunity, proposers should select "Proposal and Award Policies and Procedures Guide." Proposers are advised to select "No Closing Date" when the proposal is not submitted in response to any relevant NSF funding opportunity.

(3) NSF Unit of Consideration

Proposers must follow instructions for selection of an applicable NSF Division/Office and Program(s) to which the proposal should be directed.

(4) Remainder of the Cover Sheet

(a) Title of Proposed Project

The title of the project must be brief, scientifically or technically valid, and suitable for use in the public press. NSF may edit the title of a project prior to making an award.

(b) Budget and Duration Information

The proposed duration for which support is requested should be consistent with the nature and complexity of the proposed activity. The Foundation encourages proposers to request funding for durations of three to five years when such durations are necessary for completion of the proposed work and are technically and managerially advantageous. The requested start date should allow at least six months for NSF review, processing and decision. The PI should consult his/her organization's SPO for unusual situations (e.g., a long lead time for procurement) that create problems regarding the proposed start date. Specification of a desired start date for the project is important and helpful to NSF staff; however, requests for specific start dates may not be met.

(c) Announcement and Consideration Information

This information is prefilled based on previously entered information.

(d) PI Information and co-PI Information

Information (including address information) regarding the PI is derived from login information and is not entered when preparing the Cover Sheet. The proposal also may identify up to four additional co-PIs.

Each individual's name and either NSF ID or primary registered e-mail address, must be entered in the boxes provided.

(e) Previous NSF Award

If the proposal is a renewal proposal, or an accomplishment-based renewal proposal, the applicable box must be checked. If yes, the proposer will be requested to select the applicable previous award number.

Some NSF program solicitations require submission of both a preliminary and full proposal as part of the proposal process. In such cases, the following instructions apply:

(i) During the preliminary proposal stage, the proposing organization should identify the submission as a preliminary proposal by checking the block entitled, “Preliminary Proposal” on the Cover Sheet;

(ii) During the full proposal submission stage, the proposing organization should identify in the block entitled, “Show Related Preliminary Proposal Number”, the related preliminary proposal number assigned by NSF.

For projects that support research infrastructure and tools, such as vessels, facilities, and telescopes, the project/performance site should correspond to the physical location of the research asset. For research assets or projects that are geographically distributed, the proposer should report information for the primary site, as defined by the proposer. For example, proposals for the operations and maintenance of research vessels may list the project/performance site as the vessel’s home port.

(f) Consideration by Other Federal Agencies

If the proposal is being submitted for consideration by another Federal agency, the abbreviated name(s) of the Federal agency(ies) must be identified in the spaces provided.

(g) Awardee Organization Information

The awardee organization name, address, NSF organization code, DUNS number and Employer Identification Number/Taxpayer Identification Number are derived from the profile information provided by the organization or pulled by NSF from the SAM database and are not entered when preparing the Cover Sheet.

Profit-making organizations must identify their status by checking the appropriate boxes on the Cover Sheet, using the following guidelines:

- A small business must be organized for profit, independently owned and operated (not a subsidiary of, or controlled by, another firm), have no more than 500 employees, and not be dominant in its field.

- A minority business must be: (i) at least 51 percent owned by one or more minority or disadvantaged individuals or, in the case of a publicly owned business, have at least 51 percent of the voting stock owned by one or more minority or disadvantaged individuals; and (ii) one whose management and daily business operations are controlled by one or more such individuals.

- A woman-owned business must be at least 51 percent owned by a woman or women, who also control and operate it. "Control" in this context means exercising the power to make policy decisions. "Operate" in this context means being actively involved in the day-to-day management.

(h) Primary Place of Performance

This information is prefilled based on previously entered information.

(i) Other Information

Should any of the following items on the Cover Sheet apply to a proposal, the applicable box(es) must be checked.

- Beginning Investigator (See Chapter II.D.2) (Note: this box is applicable only to proposals submitted to the Biological Sciences Directorate.)
- Disclosure of Lobbying Activities (See Chapter II.C.1.d)
- Proprietary or Privileged Information (See Chapter II.C.1.c and II.D.1)

- Historic Places (See Chapter II.C.2.j)
- Vertebrate Animals[18] (See Chapter II.D.4)
- Human Subjects[19] (See Chapter II.D.5)
- International Activities Country Name(s) -- For each proposal that describes an international activity, proposers should list the primary countries involved. A maximum of five countries may be listed. An international activity is defined as research, training, and/or education carried out in cooperation with international counterparts either overseas or in the US using virtual technologies. Proposers also should enter the country/countries with which project participants will engage and/or travel to attend international conferences. If the specific location of the international conference is not known at the time of the proposal submission, proposers should enter "Worldwide". (See Chapter II.C.2.j)

b. Project Summary

Each proposal must contain a summary of the proposed project not more than one page in length. The Project Summary consists of an overview, a statement on the intellectual merit of the proposed activity, and a statement on the broader impacts of the proposed activity.

The overview includes a description of the activity that would result if the proposal were funded and a statement of objectives and methods to be employed. The statement on intellectual merit should describe the potential of the proposed activity to advance knowledge. The statement on broader impacts should describe the potential of the proposed activity to benefit society and contribute to the achievement of specific, desired societal outcomes.

The Project Summary should be informative to other persons working in the same or related fields, and, insofar as possible, understandable to a broad audience within the scientific domain. It should not be an abstract of the proposal.

The Project Summary may **ONLY** be uploaded as a Supplementary Document if use of special characters is necessary. Such Project Summaries must be formatted with separate headings for Overview, Intellectual Merit and Broader Impacts. Failure to include these headings will result in the proposal being returned without review.

c. Table of Contents

A Table of Contents is automatically generated for the proposal. The proposer cannot edit this form.

d. Project Description (including Results from Prior NSF Support)

(i) Content

The Project Description must contain, as a separate section within the narrative, a section labeled "Intellectual Merit". The Project Description should provide a clear statement of the work to be undertaken and must include the objectives for the period of the proposed work and expected significance; the relationship of this work to the present state of knowledge in the field, as well as to work in progress by the PI under other support.

The Project Description should outline the general plan of work, including the broad design of activities to be undertaken, and, where appropriate, provide a clear description of experimental methods and procedures. Proposers should address what they want to do, why they want to do it, how they plan to do

[18]If the proposal includes use of vertebrate animals, supplemental information is required. See Chapter II.D.4 for additional information.

[19] If the proposal includes use of human subjects, supplemental information is required. See Chapter II.D.5 for additional information.

it, how they will know if they succeed, and what benefits could accrue if the project is successful. The project activities may be based on previously established and/or innovative methods and approaches, but in either case must be well justified. These issues apply to both the technical aspects of the proposal and the way in which the project may make broader contributions.

The Project Description also must contain, as a separate section within the narrative, a section labeled "Broader Impacts". This section should provide a discussion of the broader impacts of the proposed activities. Broader impacts may be accomplished through the research itself, through the activities that are directly related to specific research projects, or through activities that are supported by, but are complementary to the project. NSF values the advancement of scientific knowledge and activities that contribute to the achievement of societally relevant outcomes. Such outcomes include, but are not limited to: full participation of women, persons with disabilities, and underrepresented minorities in science, technology, engineering, and mathematics (STEM); improved STEM education and educator development at any level; increased public scientific literacy and public engagement with science and technology; improved well-being of individuals in society; development of a diverse, globally competitive STEM workforce; increased partnerships between academia, industry, and others; improved national security; increased economic competitiveness of the US; and enhanced infrastructure for research and education.

Plans for data management and sharing of the products of research, including preservation, documentation, and sharing of data, samples, physical collections, curriculum materials and other related research and education products should be described in the Special Information and Supplementary Documentation section of the proposal (see Chapter II.C.2.j for additional instructions for preparation of this section).

(ii) Page Limitations and Inclusion of Uniform Resource Locators (URLs) within the Project Description

Brevity will assist reviewers and Foundation staff in dealing effectively with proposals. Therefore, the Project Description (including Results from Prior NSF Support, which is limited to five pages) **may not exceed 15 pages.** Visual materials, including charts, graphs, maps, photographs and other pictorial presentations are included in the 15-page limitation. PIs are cautioned that the Project Description must be self-contained and that URLs **must not** be used because: 1) the information could circumvent page limitations; 2) the reviewers are under no obligation to view the sites; and 3) the sites could be altered or deleted between the time of submission and the time of review.

Conformance to the 15-page limit will be strictly enforced and may not be exceeded unless a deviation has been specifically authorized. (Chapter II.A contains information on deviations.)

(iii) Results from Prior NSF Support

The purpose of this section is to assist reviewers in assessing the quality of prior work conducted with prior or current NSF funding. If **any PI or co-PI** identified on the proposal has received prior NSF support including:

- an award with an end date in the past five years; or
- any current funding, including any no cost extensions,

information on the award is required **for each PI and co-PI**, regardless of whether the support was directly related to the proposal or not. In cases where the PI or any co-PI has received more than one award (excluding amendments to existing awards), they need only report on the one award that is most closely related to the proposal. Support means salary support, as well as any other funding awarded by NSF, including research, Graduate Research Fellowship, Major Research Instrumentation, conference, equipment, travel, and center awards, etc.

The following information must be provided:

(a) the NSF award number, amount and period of support;

(b) the title of the project;

(c) a summary of the results of the completed work, including accomplishments, supported by the award. The results must be separately described under two distinct headings: Intellectual Merit and Broader Impacts;

(d) a listing of the publications resulting from the NSF award (a complete bibliographic citation for each publication must be provided either in this section or in the References Cited section of the proposal); if none, state "No publications were produced under this award."

(e) evidence of research products and their availability, including, but not limited to: data, publications, samples, physical collections, software, and models, as described in any Data Management Plan; and

(f) if the proposal is for renewed support, a description of the relation of the completed work to the proposed work.

If the project was recently awarded and therefore no new results exist, describe the major goals and broader impacts of the project. Note that the proposal may contain up to five pages to describe the results. Results may be summarized in fewer than five pages, which would give the balance of the 15 pages for the Project Description.

(iv) Unfunded Collaborations

Any substantial collaboration with individuals not included in the budget should be described in the Facilities, Equipment and Other Resources section of the proposal (see Chapter II.C.2.i) and documented in a letter of collaboration from each collaborator. Such letters should be provided in the supplementary documentation section of the FastLane Proposal Preparation Module and follow the format instructions specified in Chapter II.C.2.j. Collaborative activities that are identified in the budget should follow the instructions in Chapter II.D.3.

(v) Group Proposals

NSF encourages submission of proposals by groups of investigators; often these are submitted to carry out interdisciplinary projects. Unless stipulated in a specific program solicitation, however, such proposals will be subject to the 15-page Project Description limitation established in Section (ii) above. PIs who wish to exceed the established page limitations for the Project Description must request and receive a deviation in advance of proposal submission. (Chapter II.A contains information on deviations.)

(vi) Proposals for Renewed Support

See Chapter V for guidance on preparation of renewal proposals.

e. References Cited

Reference information is required. Each reference must include the names of all authors (in the same sequence in which they appear in the publication), the article and journal title, book title, volume number, page numbers, and year of publication. (See also Chapter II.C.2.d.(iii)(d)) If the proposer has a website address readily available, that information should be included in the citation. It is not NSF's intent, however, to place an undue burden on proposers to search for the URL of every referenced publication. Therefore, inclusion of a website address is optional. A proposal that includes reference citation(s) that do not specify

a URL is not considered to be in violation of NSF proposal preparation guidelines and the proposal will still be reviewed.

Proposers must be especially careful to follow accepted scholarly practices in providing citations for source materials relied upon when preparing any section of the proposal. While there is no established page limitation for the references, this section must include bibliographic citations only and must not be used to provide parenthetical information outside of the 15-page Project Description.

f. Biographical Sketch(es)

(i) Senior Personnel

A biographical sketch (limited to two pages) is required for each individual identified as senior personnel. (See Exhibit II-7 for the definitions of Senior Personnel.) Proposers may elect to use third-party solutions, such as NIH's SciENcv to develop and maintain their biographical sketch. However, proposers are advised that they are still responsible for ensuring that biographical sketches created using third-party solutions are compliant with NSF proposal preparation requirements.

The following information must be provided in the order and format specified below. Inclusion of additional information beyond that specified below may result in the proposal being returned without review.

Do not submit any personal information in the biographical sketch. This includes items such as: home address; home telephone, fax, or cell phone numbers; home e-mail address; driver's license number; marital status; personal hobbies; and the like. Such personal information is not appropriate for the biographical sketch and is not relevant to the merits of the proposal. NSF is not responsible or in any way liable for the release of such material. (See also Chapter III.H).

(a) Professional Preparation

A list of the individual's undergraduate and graduate education and postdoctoral training (including location) as indicated below:

Undergraduate Institution(s)	Location	Major	Degree & Year
Graduate Institution(s)	Location	Major	Degree & Year
Postdoctoral Institution(s)	Location	Area	Inclusive Dates (Years)

(b) Appointments

A list, in reverse chronological order, of all the individual's academic/professional appointments beginning with the current appointment.

(c) Products

A list of: (i) up to five products most closely related to the proposed project; and (ii) up to five other significant products, whether or not related to the proposed project. Acceptable products must be citable and accessible including but not limited to publications, data sets, software, patents, and copyrights. Unacceptable products are unpublished documents not yet submitted for publication, invited lectures, and additional lists of products. Only the list of ten will be used in the review of the proposal.

Each product must include full citation information including (where applicable and practicable) names of all authors, date of publication or release, title, title of enclosing work such as journal or book, volume, issue, pages, website and URL, or other Persistent Identifier.

If only publications are included, the heading "Publications" may be used for this section of the Biographical Sketch.

(d) Synergistic Activities

A list of up to five examples that demonstrate the broader impact of the individual's professional and scholarly activities that focuses on the integration and transfer of knowledge as well as its creation. Examples could include, among others: innovations in teaching and training (e.g., development of curricular materials and pedagogical methods); contributions to the science of learning; development and/or refinement of research tools; computation methodologies and algorithms for problem-solving; development of databases to support research and education; broadening the participation of groups underrepresented in STEM; and service to the scientific and engineering community outside of the individual's immediate organization.

In FastLane, Biographical sketches for all senior project personnel must be uploaded as a single PDF file associated with that individual.

(ii) Other Personnel

For the personnel categories listed below, the proposal also may include information on exceptional qualifications that merit consideration in the evaluation of the proposal. Such information should be clearly identified as "Other Personnel" biographical information and uploaded as a single PDF file in the Other Supplementary Documents section of the proposal.

(a) Postdoctoral associates

(b) Other professionals

(c) Students (research assistants)

(iii) Equipment Proposals

For equipment proposals, the following must be provided for each auxiliary user:

(a) Short biographical sketch; and

(b) List of up to five publications most closely related to the proposed acquisition.

Such information should be clearly identified as "Equipment Proposal" biographical information and uploaded as a single PDF file in the Other Supplementary Documents section of the proposal.

g. Budget and Budget Justification

Each proposal must contain a budget for each year of support requested. The budget justification must be no more than five pages per proposal. The amounts for each budget line item requested must be documented and justified in the budget justification as specified below. For proposals that contain a subaward(s), **each subaward** must include a separate budget justification of no more than five pages. See Chapter II.C.2.g.(vi)(e) for further instructions on proposals that contain subawards.

The proposal may request funds under any of the categories listed so long as the item and amount are considered necessary, reasonable, allocable, and allowable under 2 CFR § 200, Subpart E, NSF policy, and/or the program solicitation. For-profit entities are subject to the cost principles contained in the Federal Acquisition Regulation, Part 31. Amounts and expenses budgeted also must be consistent with the proposing organization's policies and procedures and cost accounting practices used in accumulating and reporting costs.

Proposals for major facilities also should consult NSF's Large Facilities Manual for additional budgetary preparation guidelines.

(i) Salaries and Wages (Lines A and B on the Proposal Budget)

(a) Senior Personnel Salaries & Wages Policy

NSF regards research as one of the normal functions of faculty members at institutions of higher education. Compensation for time normally spent on research within the term of appointment is deemed to be included within the faculty member's regular organizational salary.

As a general policy, NSF limits the salary compensation requested in the proposal budget for senior personnel to no more than two months of their regular salary in any one year. (See Exhibit II-7 for the definitions of Senior Personnel.) It is the organization's responsibility to define and consistently apply the term "year", and to specify this definition in the budget justification. This limit includes salary compensation received **from all NSF-funded grants**. This effort must be documented in accordance with 2 CFR § 200, Subpart E, including 2 CFR § 200.430(i). If anticipated, any compensation for such personnel in excess of two months must be disclosed in the proposal budget, justified in the budget justification, and must be specifically approved by NSF in the award notice budget.[20]

Under normal rebudgeting authority, as described in Chapters VII and X, a grantee can internally approve an increase or decrease in person months devoted to the project after an award is made, even if doing so results in salary support for senior personnel exceeding the two month salary policy. No prior approval from NSF is necessary as long as that change would not cause the objectives or scope of the project to change. NSF prior approval is necessary if the objectives or scope of the project change.

These same general principles apply to other types of non-academic organizations.

(b) Administrative and Clerical Salaries & Wages Policy

In accordance with 2 CFR § 200.413, the salaries of administrative and clerical staff should normally be treated as indirect costs (F&A). Direct charging of these costs may be appropriate only if all the conditions identified below are met:

(i) Administrative or clerical services are integral to a project or activity;

(ii) Individuals involved can be specifically identified with the project or activity;

(iii) Such costs are explicitly included in the approved budget or have the prior written approval of the cognizant NSF Grants Officer; and

(iv) The costs are not also recovered as indirect costs.

Conditions (i) (ii) and (iv) above are particularly relevant for consideration at the budget preparation stage.

(c) Procedures

The names of the PI(s), faculty, and other senior personnel and the estimated number of full-time-equivalent person-months for which NSF funding is requested, and the total amount of salaries requested per year, must be listed. For consistency with the NSF cost sharing policy, if person months will be requested for senior personnel, a corresponding salary amount must be entered on the budget. If no person months and

[20]NSF grantees remain subject to the provisions of OMB M-01-06, "*Clarification of OMB A-21 Treatment of Voluntary Uncommitted Cost Sharing and Tuition Remission Costs*," regarding requirements for committing and tracking "some level" of faculty (or senior researcher) effort as part of the organized research base.

no salary are being requested for senior personnel, they should be removed from Section A of the budget. Their name(s) will remain on the Cover Sheet and the individual(s) role on the project should be described in the Facilities, Equipment and Other Resources section of the proposal.
For postdoctoral associates and other professionals, the total number of persons for each position must be listed, with the number of full-time-equivalent person-months and total amount of salaries requested per year. For graduate and undergraduate students, secretarial, clerical, technical, etc., whose time will be charged directly to the project, only the total number of persons and total amount of salaries requested per year in each category is required. Compensation classified as salary payments must be requested in the salaries and wages category. Salaries requested must be consistent with the organization's regular practices. The budget justification should detail the rates of pay by individual for senior personnel, postdoctoral associates, and other professionals.

(d) Confidential Budgetary Information

The proposing organization may request that salary data on senior personnel not be released to persons outside the Government during the review process. In such cases, the item for senior personnel salaries in the proposal may appear as a single figure and the person-months represented by that amount omitted. If this option is exercised, senior personnel salaries and person-months must be itemized in a separate statement, and forwarded to NSF in accordance with the instructions specified in Chapter II.D.1. This statement must include all of the information requested on the proposal budget for each person involved. NSF will not forward the detailed information to reviewers and will hold it privileged to the extent permitted by law. The information on senior personnel salaries will be used as the basis for determining the salary amounts shown in the budget. The box for "Proprietary or Privileged Information" must be checked on the Cover Sheet when the proposal contains confidential budgetary information.[21]

(ii) Fringe Benefits (Line C on the Proposal Budget)

If the proposer's usual accounting practices provide that its contributions to employee benefits (leave, employee insurance, social security, retirement, other payroll-related taxes, etc.) be treated as direct costs, NSF grant funds may be requested to fund fringe benefits as a direct cost. These are typically determined by application of a calculated fringe benefit rate for a particular class of employee (full time or part-time) applied to the salaries and wages requested. They also may be paid based on actual costs for individual employees, if that institutional policy has been approved by the cognizant Federal agency. See 2 CFR § 200.431 for the definition and allowability of inclusion of fringe benefits on a proposal budget.

(iii) Equipment (Line D on the Proposal Budget)

Equipment is defined as tangible personal property (including information technology systems) having a useful life of more than one year and a per-unit acquisition cost which equals or exceeds the lesser of the capitalization level established by the proposer for financial statement purposes, or $5,000. It is important to note that the acquisition cost of equipment includes modifications, attachments, and accessories necessary to make the property usable for the purpose for which it will be purchased. Items of needed equipment must be adequately justified, listed individually by description and estimated cost.

Allowable items ordinarily will be limited to research equipment and apparatus not already available for the conduct of the work. General purpose equipment such as office equipment and furnishings, and information technology equipment and systems are typically not eligible for direct cost support. Special purpose or scientific use computers or associated hardware and software, however, may be requested as items of equipment when necessary to accomplish the project objectives and not otherwise reasonably available. Any request to support such items must be clearly disclosed in the proposal budget, justified in the budget justification, and be included in the NSF award budget. See 2 CFR § 200.313 for additional information.

[21] Detailed instructions for submission of confidential budgetary information are available in FastLane.

(iv) Travel (Line E on the Proposal Budget)

(a) General

When anticipated, travel and its relation to the proposed activities must be specified, itemized and justified by destination and cost. Funds may be requested for field work, attendance at meetings and conferences, and other travel associated with the proposed work, including subsistence. In order to qualify for support, however, attendance at meetings or conferences must be necessary to accomplish proposal objectives, or disseminate its results. Travel support for dependents of key project personnel may be requested only when the travel is for a duration of six months or more either by inclusion in the approved budget or with the prior written approval of the cognizant NSF Grants Officer. Temporary dependent care costs above and beyond regular dependent care that directly result from travel to conferences are allowable costs provided that the conditions established in 2 CFR § 200.474 are met.

Allowance for air travel normally will not exceed the cost of round-trip, economy airfares. Persons traveling under NSF grants must travel by US-Flag Air carriers, if available.

(b) Domestic Travel

Domestic travel includes travel within and between the US, its territories and possessions.[22] Travel, meal and hotel expenses of grantee employees who are not on travel status are unallowable. Costs of employees on travel status are limited to those specifically authorized by 2 CFR § 200.474.

(c) Foreign Travel

Travel outside the areas specified above is considered foreign travel. When anticipated, the proposer must enter the names of countries and dates of visit on the proposal budget, if known.

(v) Participant Support (Line F on the Proposal Budget)

This budget category refers to direct costs for items such as stipends or subsistence allowances, travel allowances, and registration fees paid to or on behalf of participants or trainees (but not employees) in connection with NSF-sponsored conferences or training projects. Any additional categories of participant support costs other than those described in 2 CFR § 200.75 (such as incentives, gifts, souvenirs, t-shirts and memorabilia), must be justified in the budget justification, and such costs will be closely scrutinized by NSF. (See also Chapter II.E.7) For some educational projects conducted at local school districts, however, the participants being trained are employees. In such cases, the costs must be classified as participant support if payment is made through a stipend or training allowance method. The school district must have an accounting mechanism in place (i.e., sub-account code) to differentiate between regular salary and stipend payments.

To help defray the costs of participating in a conference or training activity, funds may be proposed for payment of stipends, per diem or subsistence allowances, based on the type and duration of the activity. Such allowances must be reasonable, in conformance with the policy of the proposing organization and limited to the days of attendance at the conference plus the actual travel time required to reach the conference location. Where meals or lodgings are furnished without charge or at a nominal cost (e.g., as part of the registration fee), the per diem or subsistence allowance should be correspondingly reduced. Although local participants may participate in conference meals and coffee breaks, funds may not be proposed to pay per diem or similar expenses for local participants in the conference.

[22] According to the IRS, US territories and possessions are as follows: Puerto Rico, US Virgin Islands, Guam, American Samoa, the Commonwealth of the Northern Mariana Islands, Midway Island, Wake Island, Palmyra Island, Howland Island, Johnston Island, Baker Island, Kingman Reef, Jarvis Island, and other US islands, cays, and reefs that are not part of any of the fifty States.

Funds may be requested for the travel costs of participants. If so, the restrictions regarding class of accommodations and use of US-Flag air carriers are applicable.[23] In training activities that involve field trips, costs of transportation of participants are allowable. The number of participants to be supported must be entered in the parentheses on the proposal budget. Participant support costs must be specified, itemized and justified in the budget justification section of the proposal. Indirect costs (F&A) are not allowed on participant support costs. Participant support costs must be accounted for separately should an award be made.

(vi) Other Direct Costs (Lines G1 through G6 on the Proposal Budget)

Any costs proposed to an NSF project must be allowable, reasonable and directly allocable to the supported activity. When anticipated, the budget must identify and itemize other anticipated direct costs not included under the headings above, including materials and supplies, publication costs, computer services and consultant services. Examples include aircraft rental, space rental at research establishments away from the proposing organization, minor building alterations, payments to human subjects, and service charges. Reference books and periodicals only may be included on the proposal budget if they are specifically allocable to the project being supported by NSF.

(a) Materials and Supplies (including Costs of Computing Devices) (Line G1 on the Proposal Budget)

When anticipated, the proposal budget justification must indicate the general types of expendable materials and supplies required. Supplies are defined as all tangible personal property other than those described in paragraph (d)(iii) above. A computing device is considered a supply if the acquisition cost is less than the lesser of the capitalization level established by the proposer or $5,000, regardless of the length of its useful life. In the specific case of computing devices, charging as a direct cost is allowable for devices that are essential and allocable, but not solely dedicated, to the performance of the NSF project. Details and justification must be included for items requested to support the project.

(b) Publication/Documentation/Dissemination (Line G2 on the Proposal Budget)

The proposal budget may request funds for the costs of documenting, preparing, publishing or otherwise making available to others the findings and products of the work conducted under the grant. This generally includes the following types of activities: reports, reprints, page charges or other journal costs (except costs for prior or early publication); necessary illustrations; cleanup, documentation, storage and indexing of data and databases; development, documentation and debugging of software; and storage, preservation, documentation, indexing, etc., of physical specimens, collections or fabricated items.

(c) Consultant Services (also referred to as Professional Service Costs) (Line G3 on the Proposal Budget)

The proposal budget may request costs for professional and consultant services. Professional and consultant services are services rendered by persons who are members of a particular profession or possess a special skill, and who are not officers or employees of the proposing organization. Costs of professional and consultant services are allowable when reasonable in relation to the services rendered and when not contingent upon recovery of costs from the Federal government. Anticipated services must be justified and information furnished on each individual's expertise, primary organizational affiliation, normal daily compensation rate, and number of days of expected service. Consultants' travel costs, including subsistence, may be included. If requested, the proposer must be able to justify that the proposed rate of pay is reasonable. Additional information on the allowability of consultant or professional service costs is available in 2 CFR § 200.459. In addition to other provisions required by the proposing organization,

[23] See Chapter XI.F, Grant General Conditions (GC-1) Article 10, and Article 14 in the NSF Agency Specific Requirements to the Research Terms and Conditions, as applicable, for additional information on travel restrictions.

all contracts made under the NSF award must contain the applicable provisions identified in 2 CFR § 200 Appendix II.

(d) Computer Services (Line G4 on the Proposal Budget)

The cost of computer services, including computer-based retrieval of scientific, technical and educational information, may be requested only where it is institutional policy to charge such costs as direct charges. A justification based on the established computer service rates at the proposing organization must be included. The proposal budget also may request costs for leasing of computer equipment. As noted in Chapter II.C.2.g(iii), general purpose (such as word processing, spreadsheets, communication) computer equipment should not be requested. Note: See paragraph (a) above for guidance on acquisition of computing devices.

(e) Subawards[24] (Line G5 on the Proposal Budget)

Except for the purchase of materials and supplies, equipment or general support services allowable under the grant, no portion of the proposed activity may be subawarded or transferred to another organization without prior written NSF authorization. Such authorization must be provided either through inclusion of the subaward(s) on an NSF award budget or by receiving written prior approval from the cognizant NSF Grants Officer after an award is issued.

If known at the time of proposal submission, the intent to enter into such arrangements must be disclosed in the proposal. A separate budget and a budget justification of no more than five pages, must be provided for each subrecipient, if already identified, along with a description of the work to be performed.

All proposing organizations are required to make a case-by-case determination regarding the role of a subrecipient versus contractor for each agreement it makes. 2 CFR § 200.330 provides characteristics of each type of arrangement to assist proposing organizations in making that determination. However, inclusion of a subaward or contract in the proposal budget or submission of a request after issuance of an NSF award to add a subaward or contract will document the organizational determination required.

It is NSF's expectation that, consistent with 2 CFR § 200.414, NSF grantees will use the domestic subrecipient's applicable US Federally negotiated indirect cost rate(s). If no such rate exists, the NSF grantee may either negotiate a rate or will fund the subrecipient using the *de minimis* indirect cost rate recovery of 10% of modified total direct costs.

It is also NSF's expectation that NSF grantees will use the foreign subrecipient's applicable US Federally negotiated indirect cost rate(s). However, if no such rate exists, the NSF grantee will fund the foreign subrecipient using the *de minimis* indirect cost rate recovery of 10% of modified total direct costs.

(f) Other (Line G6 on the Proposal Budget)

Any other direct costs not specified in Lines G1 through G5 must be identified on Line G6. Such costs must be itemized and detailed in the budget justification.

Incentive payments, for example, payments to human subjects or incentives to promote completion of a survey, should be included on line G6 of the NSF budget. Incentive payments should be proposed in accordance with organizational policies and procedures. Indirect costs should be calculated on incentive payments in accordance with the organization's approved US Federally negotiated indirect cost rate(s). Performance based incentive payments to employees as described in 2 CFR §200.430(f) should not be included in this section of the budget.

[24]A subaward may be provided through any form of legal agreement, including an agreement that the proposing organization considers a contract. The substance of the relationship is more important than the form of the agreement.

(vii) Total Direct Costs (Line H on the Proposal Budget)

The total amount of direct costs requested in the budget, to include Lines A through G, must be entered on Line H.

(viii) Indirect Costs (also known as Facilities and Administrative Costs (F&A) for Colleges and Universities) (Line I on the Proposal Budget)

Except where specifically identified in an NSF program solicitation, the applicable US Federally negotiated indirect cost rate(s) must be used in computing indirect costs (F&A) for a proposal. Use of an indirect cost rate lower than the organization's current negotiated indirect cost rate is considered a violation of NSF's cost sharing policy. See section (xii) below. The amount for indirect costs should be calculated by applying the current negotiated indirect cost rate(s) to the approved base(s), and such amounts should be specified in the budget justification. Indirect cost recovery for IHEs are additionally restricted by 2 CFR § 200, Appendix III, paragraph C.7. which specifies Federal agencies are required to use the negotiated F&A rate that is in effect at the time of the initial award throughout the life of the sponsored agreement. Additional information on the charging of indirect costs to an NSF award is available in Chapter X.D.

Domestic proposing organizations that do not have a current negotiated rate agreement with a cognizant Federal agency, and who are requesting more than a *de minimis* 10% recovery of modified total direct costs should prepare an indirect cost proposal based on expenditures for its most recently ended fiscal year. Based on the information provided in the indirect cost proposal, NSF may negotiate an award-specific rate to be used only on the award currently being considered for funding. No supporting documentation is required for proposed rates of 10% or less of modified total direct costs. The contents and financial data included in indirect cost proposals vary according to the make-up of the proposing organization. Instructions for preparing an indirect cost rate proposal can be found at: http://www.nsf.gov/bfa/dias/caar/docs/idcsubmissions.pdf. NSF formally negotiates indirect cost rates for the organizations for which NSF has rate cognizance. NSF does not negotiate rates for organizations that are not direct recipients of NSF funding (e.g., subrecipients). The prime grantee is responsible for ensuring that proposed subrecipient costs, including indirect costs, are reasonable and appropriate.

Foreign organizations that do not have a current US Federally negotiated indirect cost rate(s) are limited to a *de minimis* indirect cost rate recovery of 10% of modified total direct costs. Foreign grantees that have a US Federally negotiated indirect cost rate(s) may recover indirect costs at the current negotiated rate.

(ix) Total Direct and Indirect Costs (F&A) (Line J on the Proposal Budget)

The total amount of direct and indirect costs (F&A) (sum of Lines H and I) must be entered on Line J.

(x) Fees (Line K on the Proposal Budget)

This line is available for use only by the SBIR/STTR programs.

(xi) Amount of This Request (Line L on the Proposal Budget)

The total amount of funds requested by the proposer.

(xii) Cost Sharing (Line M on the Proposal Budget)

The National Science Board issued a report entitled "*Investing in the Future: NSF Cost Sharing Policies for a Robust Federal Research Enterprise"* (NSB 09-20, August 3, 2009), which contained eight recommendations for NSF regarding cost sharing. In implementation of the Board's recommendation, NSF's guidance[25] is as follows:

[25] See NSF's Revised Cost Sharing Policy Statement for the Foundation's overarching policies on cost sharing.

Voluntary Committed and Uncommitted Cost Sharing

As stipulated in 2 CFR § 200.99, **"***Voluntary committed cost sharing* means cost sharing specifically pledged on a voluntary basis in the proposal's budget or the Federal award on the part of the non-Federal entity and that becomes a binding requirement of Federal award." As such, to be considered voluntary committed cost sharing, the amount must appear on the NSF proposal budget and be specifically identified in the approved NSF budget.[26] Unless required by NSF (see the section on Mandatory Cost Sharing below), inclusion of voluntary committed cost sharing is prohibited and Line M on the proposal budget will not be available for use by the proposer. NSF Program Officers are not authorized to impose or encourage mandatory cost sharing unless such requirements are explicitly included in the program solicitation.

In order for NSF, and its reviewers, to assess the scope of a proposed project, all organizational resources necessary for, and available to, a project must be described in the Facilities, Equipment and Other Resources section of the proposal (see Chapter II.C.2.i for further information). While not required by NSF, the grantee may, at its own discretion, continue to contribute voluntary uncommitted cost sharing to NSF-sponsored projects. **As noted above, however, these resources are not auditable by NSF and should not be included in the proposal budget or budget justification.**

Mandatory Cost Sharing

Mandatory cost sharing will only be required for NSF programs when explicitly authorized by the NSF Director, the NSB, or legislation. A complete listing of NSF programs that require cost sharing is available on the NSF website at: http://www.nsf.gov/bfa/dias/policy/. In these programs, cost sharing requirements will be clearly identified in the solicitation and must be included on Line M of the proposal budget. For purposes of budget preparation, the cumulative cost sharing amount must be entered on Line M of the first year's budget. Should an award be made, the organization's cost sharing commitment, as specified on the first year's approved budget, must be met prior to the award end date.

Such cost sharing will be considered as an eligibility, rather than a review criterion. Proposers are advised not to exceed the mandatory cost sharing level or amount specified in the solicitation.[27]

When mandatory cost sharing is included on Line M, and accepted by the Foundation, the commitment of funds becomes legally binding and is subject to audit. When applicable, the estimated value of any in-kind contributions also should be included on Line M. An explanation of the source, nature, amount and availability of any proposed cost sharing must be provided in the budget justification[28]. It should be noted that contributions derived from other Federal funds or counted as cost sharing toward projects of another Federal agency must not be counted towards meeting the specific cost sharing requirements of the NSF award.

Failure to provide the level of cost sharing required by the NSF solicitation and reflected in the NSF award budget may result in termination of the NSF award, disallowance of award costs and/or refund of award funds to NSF by the grantee.

[26] Inclusion in the Budget Justification also meets this definition.

[27] For further information on procedures for inclusion of programmatic cost sharing in an NSF solicitation, see: http://www.nsf.gov/bfa/dias/policy/csdocs/principles.pdf.

[28] 2 CFR § 200.306 describes criteria and procedures for the allowability of cash and in-kind contributions in satisfying cost sharing and matching requirements.

(xiii) Allowable and Unallowable Costs

2 CFR § 200, Subpart E provides comprehensive information regarding costs allowable under Federal awards. The following categories of unallowable costs are highlighted because of their sensitivity:

(a) Entertainment

Costs of entertainment, amusement, diversion and social activities, and any costs directly associated with such activities (such as tickets to shows or sporting events, meals, lodging, rentals, transportation and gratuities) are unallowable. Travel, meal and hotel expenses of grantee employees who are not on travel status are unallowable. See also 2 CFR § 200.438.

(b) Meals and Coffee Breaks

No funds may be requested for meals or coffee breaks for intramural meetings of an organization or any of its components, including, but not limited to, laboratories, departments and centers. (See 2 CFR § 200.432, for additional information on the charging of certain types of costs generally associated with conferences supported by NSF.) Meal expenses of grantee employees who are not on travel status are unallowable. See also 2 CFR § 200.438.

(c) Alcoholic Beverages

No NSF funds may be requested or spent for alcoholic beverages.

h. Current and Pending Support

This section of the proposal calls for required information on all current and pending support for ongoing projects and proposals, **including this project**, and any subsequent funding in the case of continuing grants. All current project support from whatever source (e.g., Federal, State, local or foreign government agencies, public or private foundations, industrial or other commercial organizations, or internal funds allocated toward specific projects) must be listed. The proposed project and all other projects or activities requiring a portion of time of the PI and any other senior personnel must be included, even if they receive no salary support from the project(s). The total award amount for the entire award period covered (including indirect costs) must be shown as well as the number of person-months per year to be devoted to the project, regardless of source of support. Similar information must be provided for all proposals already submitted or submitted concurrently to other possible sponsors, including NSF. Concurrent submission of a proposal to other organizations will not prejudice its review by NSF. The Biological Sciences Directorate exception to this policy is delineated in Chapter II.D.2.

If the project now being submitted has been funded previously by a source other than NSF, the information requested in the paragraph above must be furnished for the last period of funding.

In FastLane, Current and Pending Support for all senior project personnel must be uploaded as a single PDF file or inserted as text associated with that individual.

i. Facilities, Equipment and Other Resources

This section of the proposal is used to assess the adequacy of the resources available to perform the effort proposed to satisfy both the Intellectual Merit and Broader Impacts review criteria. Proposers should describe only those resources that are directly applicable. Proposers should include an aggregated description of the internal and external resources (both physical and personnel) that the organization and its collaborators will provide to the project, should it be funded. Such information must be provided in this section, in lieu of other parts of the proposal (e.g., Budget Justification, Project Description). The description should be narrative in nature and must not include any quantifiable financial information. Reviewers will

evaluate the information during the merit review process and the cognizant NSF Program Officer will review it for programmatic and technical sufficiency.

Although these resources are not considered voluntary committed cost sharing as defined in 2 CFR § 200.99, the Foundation **does expect** that the resources identified in the Facilities, Equipment and Other Resources section will be provided, or made available, should the proposal be funded. Chapter VII.B.1 specifies procedures for use by the grantee when there are postaward changes to objectives, scope or methods/procedures.

j. Special Information and Supplementary Documentation

Except as specified below, special information and supplementary documentation must be included as part of the Project Description (or part of the budget justification), if it is relevant to determining the quality of the proposed work. Information submitted in the following areas is not considered part of the 15-page Project Description limitation. This Special Information and Supplementary Documentation section also is not considered an appendix. Specific guidance on the need for additional documentation may be obtained from the organization's SPO or in the references cited below.

- Postdoctoral Researcher Mentoring Plan. Each proposal[29] that requests funding to support **postdoctoral researchers**[30] must upload under "Mentoring Plan" in the supplementary documentation section of FastLane, a description of the mentoring activities that will be provided for such individuals. In no more than one page, the mentoring plan must describe the mentoring that will be provided to all postdoctoral researchers supported by the project, regardless of whether they reside at the submitting organization, any subrecipient organization, or at any organization participating in a simultaneously submitted collaborative proposal. Proposers are advised that the mentoring plan must not be used to circumvent the 15-page Project Description limitation. See Chapter II.D.3 for additional information on collaborative proposals. Mentoring activities provided to postdoctoral researchers supported on the project will be evaluated under the Broader Impacts review criterion.

  Examples of mentoring activities include, but are not limited to: career counseling; training in preparation of grant proposals, publications and presentations; guidance on ways to improve teaching and mentoring skills; guidance on how to effectively collaborate with researchers from diverse backgrounds and disciplinary areas; and training in responsible professional practices.

- Plans for data management and sharing of the products of research. Proposals must include a document of no more than two pages uploaded under "Data Management Plan" in the supplementary documentation section of FastLane. This supplementary document should describe how the proposal will conform to NSF policy on the dissemination and sharing of research results (see Chapter XI.D.4), and may include:

  1. the types of data, samples, physical collections, software, curriculum materials, and other materials to be produced in the course of the project;

[29] For purposes of meeting the mentoring requirement, simultaneously submitted collaborative proposals, and collaborative proposals that include subawards, constitute a single unified project. Therefore, only one mentoring plan may be submitted for the entire project.

[30] In situations where a postdoctoral researcher is listed in Section A of the NSF Budget, and is functioning in a Senior Personnel capacity (i.e., responsible for the scientific or technical direction of the project), a mentoring plan is not required.

2. the standards to be used for data and metadata format and content (where existing standards are absent or deemed inadequate, this should be documented along with any proposed solutions or remedies);

3. policies for access and sharing including provisions for appropriate protection of privacy, confidentiality, security, intellectual property, or other rights or requirements;

4. policies and provisions for re-use, re-distribution, and the production of derivatives; and
5. plans for archiving data, samples, and other research products, and for preservation of access to them.

Data management requirements and plans specific to the Directorate, Office, Division, Program, or other NSF unit, relevant to a proposal are available at: http://www.nsf.gov/bfa/dias/policy/dmp.jsp. If guidance specific to the program is not available, then the requirements established in this section apply.

Simultaneously submitted collaborative proposals and proposals that include subawards are a single unified project and should include only one supplemental combined Data Management Plan, regardless of the number of non-lead collaborative proposals or subawards included. In such collaborative proposals, the data management plan should discuss the relevant data issues in the context of the collaboration.

A valid Data Management Plan may include only the statement that no detailed plan is needed, as long as the statement is accompanied by a clear justification. Proposers who feel that the plan cannot fit within the limit of two pages may use part of the 15-page Project Description for additional data management information. Proposers are advised that the Data Management Plan must not be used to circumvent the 15-page Project Description limitation. The Data Management Plan will be reviewed as an integral part of the proposal, considered under Intellectual Merit or Broader Impacts or both, as appropriate for the scientific community of relevance.

- Rationale for performance of all or part of the project off-campus or away from organizational headquarters.

- Documentation of collaborative arrangements of significance to the proposal through letters of collaboration. (See Chapter II.C.2.d.(iv).) Letters of collaboration should be limited to stating the intent to collaborate and should not contain endorsements or evaluation of the proposed project. The recommended format for letters of collaboration is as follows:

  "If the proposal submitted by Dr. [insert the full name of the Principal Investigator] entitled [insert the proposal title] is selected for funding by NSF, it is my intent to collaborate and/or commit resources as detailed in the Project Description or the Facilities, Equipment and Other Resources section of the proposal."

  While letters of collaboration are permitted, unless required by a specific program solicitation, letters of support should not be submitted as they are not a standard component of an NSF proposal. A letter of support is typically from a key stakeholder such as an organization, collaborator or Congressional Representative, and is used to convey a sense of enthusiasm for the project and/or to highlight the qualifications of the PI or co-PI. A letter of support submitted in response to a program solicitation requirement must be unique to the specific proposal submitted and cannot be altered without the author's explicit prior approval. Proposals that contain letters of support not authorized by the program solicitation may be returned without review.

- In order for NSF to comply with Federal environmental statutes (including, but not limited to, the National Environmental Policy Act (42 USC. §§ 4321, et seq.), the National Historic Preservation Act (54 USC § 306108 [previously codified at 16 USC § § 470, et seq.], and the Endangered

Species Act (16 USC. §§ 1531, et seq.), the proposer may be requested to submit supplemental post-proposal submission information to NSF in order that a reasonable and accurate assessment of environmental impacts by NSF may be made. The types of information that may be requested is shown in the Organization Environmental Impacts Checklist.

- International Activities

  1. International Research/Education/Training Activities. For each proposal that describes an international activity, PIs should list the primary countries involved on the Cover Sheet. An international activity is defined as research, training, and/or education carried out in cooperation with foreign counterparts either overseas or in the US using virtual technologies.

  2. International Conferences. Proposers also should enter on the Cover Sheet the country/countries with which project participants will engage and/or travel to attend international conferences. If the specific location of the international conference is not known at the time of the proposal submission, proposers should enter "Worldwide" on the Cover Sheet.

  3. Work in foreign countries. Some governments require nonresidents to obtain official approval to carry out investigations within their borders and coastal waters under their jurisdiction. PIs are responsible for obtaining the required authorizations. Advance coordination should minimize disruption of the research. (See Chapter XI.B.4.)

- Antarctic proposals to *any* NSF program require "Logistical Requirements and Field Plan" supplementary documents to be submitted with the proposal. See "proposal with fieldwork" in Chapter V.A of the Antarctic Research solicitation. Special budgetary considerations also apply. See also Chapter V.B of the Antarctic Research solicitation.

- Research in a location designated, or eligible to be designated, a registered historic place. (See Chapter XI.J). Where applicable, the box for "Historic Places" must be checked on the Cover Sheet.

- Research involving field experiments with genetically engineered organisms. (See Chapter XI.B.2)

- Documentation regarding research involving the use of human subjects, hazardous materials, vertebrate animals, or endangered species. (See Chapter XI.B, Chapter II.D.4 and II.D.5).

- Special components in new proposals or in requests for supplemental funding, such *as Facilitation Awards for Scientists and Engineers with Disabilities (FASED), Research Opportunity Awards (ROAs), Research Experiences for Undergraduates (REUs), and Facilitating Research at Primarily Undergraduate Institutions (RUIs).* See Chapter II.E.6 for information on FASED, and, for the other programs identified, consult the relevant solicitation).

In addition, the supplementary documentation section should alert NSF officials to unusual circumstances that require special handling, including, for example, proprietary or other privileged information in the proposal, matters affecting individual privacy, required intergovernmental review under E.O. 12372 (Intergovernmental Review of Federal Programs) for activities that directly affect State or local governments, or possible national security implications.

k. Appendices

All information necessary for the review of a proposal must be contained in Sections a through i of the proposal. **Appendices may not be included unless a deviation has been authorized.** Chapter II.A contains further information.

D. Special Processing Instructions

1. Proprietary or Privileged Information

Patentable ideas, trade secrets, privileged or confidential commercial or financial information, disclosure of which may harm the proposer, should be included in proposals only when such information is necessary to convey an understanding of the proposed project. Such information must be clearly marked in the proposal and be appropriately labeled with a legend such as,

> "The following is (proprietary or confidential) information that (name of proposing organization) requests not be released to persons outside the Government, except for purposes of review and evaluation."

Such information also may be included as a separate statement. If this method is used, the statement must be submitted as a single-copy document in the Proposal Preparation Module in FastLane. (See also Chapter II.C.1 for further information regarding submission of single-copy documents.)[31]

The box for "Proprietary or Privileged Information" must be checked on the Cover Sheet when the proposal contains such information. While NSF will make every effort to prevent unauthorized access to such material, the Foundation is not responsible or in any way liable for the release of such material.

2. Beginning Investigators (applies to proposals submitted to the Biological Sciences Directorate only)

Research proposals to the Biological Sciences Directorate ONLY (not proposals for conferences) cannot be duplicates of proposals to any other Federal agency for simultaneous consideration. The only exceptions to this rule are: (1) when the Program Officers at the relevant Federal agencies have previously agreed to joint review and possible joint funding of the proposal; or (2) proposals for PIs who are beginning investigators (individuals who have not been a PI or co-PI on a Federally funded award with the exception of doctoral dissertation, postdoctoral fellowship or research planning grants). For proposers who qualify under this latter exception, the box for "Beginning Investigator" must be checked on the Cover Sheet.

3. Collaborative Proposals

A collaborative proposal is one in which investigators from two or more organizations wish to collaborate on a unified research project. Collaborative proposals may be submitted to NSF in one of two methods: as a single proposal, in which a single award is being requested (with subawards administered by the lead organization); or by simultaneous submission of proposals from different organizations, with each organization requesting a separate award. In either case, the lead organization's proposal must contain all of the requisite sections as a single package to be provided to reviewers (that will happen automatically when procedures below are followed). All collaborative proposals must clearly describe the roles to be played by the other organizations, specify the managerial arrangements, and explain the advantages of the multi-organizational effort within the Project Description.

a. Submission of a collaborative proposal from one organization

The single proposal method allows investigators from two or more organizations who have developed an integrated research project to submit a single, focused proposal. A single investigator bears primary responsibility for the administration of the grant and discussions with NSF, and, at the discretion of the organizations involved, investigators from any of the participating organizations may be designated as co-PIs. Note, however, that if awarded, a single award would be made to the submitting organization, with

[31]Detailed instructions for submission of proprietary or privileged information are available in FastLane at: http://www.fastlane.nsf.gov/help/proprietary.htm.

any collaborators listed as subawards. (See Chapter II.C.2.g(vi)(e) for additional instructions on preparation of this type of proposal.)

If a proposed subaward includes funding to support postdoctoral researchers, the mentoring activities to be provided for such individuals must be incorporated in the supplemental mentoring plan outlined in Chapter II.C.2.j.

b. Submission of a collaborative proposal from multiple organizations

Simultaneous submission of proposals allows multiple organizations to submit a unified set of certain proposal sections, as well as information unique to each organization as specified below. All collaborative proposals arranged as separate submissions from multiple organizations must be submitted via FastLane. For these proposals, the project title must begin with the words "Collaborative Research:" If funded, each organization bears responsibility for a separate award.

Required sections of the proposal differ based on the organization's role. The following sections are required for a collaborative proposal submitted by:

Lead Organization

- Cover Sheet
- Project Summary
- Table of Contents (automatically generated)
- Project Description
- References Cited
- Biographical Sketch(es)
- Budget and Budget Justification
- Current and Pending Support
- Facilities, Equipment and Other Resources
- Data Management Plan
- Postdoctoral Mentoring Plan (if applicable)
- Collaborators & Other Affiliations Information

Non-Lead Organization

- Cover Sheet
- Table of Contents (automatically generated)
- Biographical Sketch(es)
- Budget and Budget Justification
- Current and Pending Support
- Facilities, Equipment and Other Resources
- Collaborators & Other Affiliations Information

See Chapter II.C.2.j for additional guidance on the mentoring and data management plan requirements for collaborative proposals. NSF will combine the proposal submission for printing or electronic viewing.

To submit the collaborative proposal, the following process must be completed:[32]

(i) Each non-lead organization must assign their proposal a proposal PIN. This proposal PIN and the temporary proposal ID generated by FastLane when the non-lead proposal is created must be provided to the lead organization before the lead organization submits its proposal to NSF.

[32]Detailed technical instructions for the preparation and submission of collaborative proposals are available in FastLane.

(ii) The lead organization must then enter each non-lead organization(s) proposal PIN and temporary proposal ID into the FastLane lead proposal by using the "Link Collaborative Proposals" option found on the FastLane "Form Preparation" screen.

(iii) **All components** of the collaborative proposal must meet any established deadline date, and failure to do so may result in the entire collaborative proposal being returned without review.

(iv) If funded, both lead and non-lead organizations are required to submit separate annual and final project reports. These reports should reference the work of the collaborative, while focusing on the distinct work conducted at each funded organization.

4. Proposals Involving Vertebrate Animals

a. Any project proposing use of vertebrate animals for research or education shall comply with the Animal Welfare Act (7 USC 2131, *et seq*.) and the regulations promulgated thereunder by the Secretary of Agriculture (9 CFR 1.1-4.11) pertaining to the humane care, handling, and treatment of vertebrate animals held or used for research, teaching, or other activities supported by Federal awards. In accordance with these requirements, proposed projects involving use of any vertebrate animal for research or education must be approved by the submitting organization's Institutional Animal Care and Use Committee (IACUC) before an award can be made. For this approval to be accepted by NSF, the organization must have a current Public Health Service (PHS) Approved Assurance. See also Chapter XI.B.3 for additional information on the administration of awards that utilize vertebrate animals. Note that for some types of vertebrate animals, additional review may be required.

Any project proposing use of vertebrate animals for research or education must comply with the provision in the PHS Assurance for Institutional Commitment (Section II) that requires the submitting organization to establish and maintain a program for activities involving animals in accordance with the *Guide for the Care and Use of Laboratory Animals* (Guide). Taxon-specific guidelines may be used as supplemental references.[33] Departures from the Guide must be approved by the IACUC and based on scientific, veterinary, medical, or animal welfare issues (for more information, see Office of Laboratory Animal Welfare (OLAW) Departures from the Guide).

b. Sufficient information must be provided within the 15-page Project Description to enable reviewers to evaluate the:

(i) rationale for involving animals;

(ii) choice of species and number of animals to be used;

(iii) description of the proposed use of the animals;

(iv) exposure of animals to discomfort, pain, or injury; and

(v) description of any euthanasia methods to be used.

c. Research facilities subject to the Animal Welfare Act using or intending to use live animals in research and who receive Federal funding are required to register the facility with the Animal and Plant Health Inspection Service (APHIS), US Department of Agriculture. A current listing of licensed animal dealers also may be obtained from APHIS. The location of the nearest APHIS Regional Office, as well as information concerning this and other APHIS activities, may be obtained at: http://www.aphis.usda.gov/.

[33] Guidelines to the Use of Wild Birds in Research; Guidelines of the American Society of Mammalogists for the Use of Wild Mammals in Research; Guidelines for the Use of Fishes in Research; and Guidelines for the Use of Live Amphibians and Reptiles in Field and Laboratory Research.

d. Projects involving the care or use of vertebrate animals at an international organization or international field site also require approval of research protocols by the US grantee's IACUC. If the project is to be funded through an award to an international organization or through an individual fellowship award that will support activities at an international organization, NSF will require a statement from the international organization explicitly listing the proposer's name and referencing the title of the award to confirm that the activities will be conducted in accordance with all applicable laws in the international country and that the *International Guiding Principles for Biomedical Research Involving Animals* (see: http://www.cioms.ch/) will be followed.

e. The following information regarding the organization's intention to utilize vertebrate animals as part of the project should be provided on the Cover Sheet:
(i) The box for "Vertebrate Animals" must be checked on the Cover Sheet if care and use of vertebrate animals is envisioned.

(ii) The date of IACUC approval of the animal-use protocol covering the proposed work, if such approval has been granted prior to proposal submission, must be identified in the space provided. If IACUC approval has not been obtained prior to submission, the proposer should indicate "Pending" in the space provided for the approval date. If a decision is made to fund the proposal, the organization must provide a copy of the approval letter from the IACUC. The approval letter must affirm that an animal-use protocol covering the proposed activities has been approved, and should explicitly list the organization's PHS Assurance Number, the proposer's name, the title and number of the NSF proposal, and the date of IACUC approval, as well as show an organizational signature. The approval letter must be provided to the cognizant NSF Program Officer before an award can be issued.

(iii) The PHS Approved Animal Welfare Assurance Number must be entered in the space provided.

f. For fellowship proposals submitted by individuals that involve the care and use of vertebrate animals, the proposal should contain the information specified in paragraph b. above. In addition, a copy of the approval letter from the IACUC of the organization that provides oversight of the proposed work should be included as an Other Supplementary Document in FastLane. The approval letter must affirm that an animal-use protocol covering the proposed activities has been approved, and should explicitly list the organization's PHS Assurance Number, the proposer's name, the title and number of the NSF proposal, and the date of IACUC approval, as well as show an organizational signature. If IACUC approval has not been obtained prior to submission, the individual should indicate "Pending" in the space provided for the approval date. If a decision is made to fund the proposal, the individual must provide a signed copy of the official IACUC approval letter (which includes the items specified above) to the cognizant NSF Program Officer before an award can be issued.

See also Chapter XI.B.3 for additional information on the administration of awards that include use of vertebrate animals.

5. Proposals Involving Human Subjects

a. Projects involving research with human subjects must ensure that subjects are protected from research risks in conformance with the relevant Federal policy known as the Common Rule (Federal Policy for the Protection of Human Subjects, 45 CFR § 690). All projects involving human subjects must either (1) have approval from an Institutional Review Board (IRB) before issuance of an NSF award; or, (2) must affirm that the IRB has declared the research exempt from IRB review, in accordance with the applicable subsection, as established in 45 CFR § 690.104(d) of the Common Rule. If certification of exemption is provided after submission of the proposal and before the award is issued, the exemption number corresponding to one or more of the exemption categories also must be included in the documentation provided to NSF.

NSF cannot accept any IRB document that requires continued monitoring of the award activities involving human subjects by NSF. For projects lacking definite plans for the use of human subjects, their data or their specimens, pursuant to 45 CFR § 690.118, NSF can accept a determination notice[34] that establishes a limited time period under which the PI may conduct preliminary or conceptual work that does not involve human subjects. Further instructions are provided in paragraph (iv) below.

If the project involves human subjects and is to be performed outside of the US, evidence of IRB approval also is required. If there is no IRB approval provided, nor is a Federal Wide Assurance (FWA) on file with the Department of Health and Human Services, Office for Human Research Protections (OHRP) (http://ohrp.cit.nih.gov/search/search.aspx?styp=bsc), NSF may decline to support the project. Pursuant to 45 CFR §690.101(g), the Common Rule is not intended to supersede any additional protections that may be afforded to human subjects under foreign laws or regulations. OHRP keeps a list of foreign laws, regulations, and guidelines governing human subjects research.

b. The following information regarding the organization's intention to use human subjects as part of the project should be provided on the Cover Sheet:

(i) The box for "Human Subjects" must be checked on the Cover Sheet if use of human subjects is envisioned.

(ii) If human subject activities are exempt from IRB review, provide the exemption number(s) corresponding to one or more of the exemption categories. The six categories of research that qualify for exemption from coverage by the regulations are defined in the *Common Rule for Protection of Human Subjects.*

(iii) If the research is not designated as exempt, the IRB approval date should be identified in the space provided. This date, at minimum, should cover the period at which the project is initiated. If IRB approval has not been obtained prior to submission, the proposer should indicate "Pending" in the space provided for the approval date. If a decision is made to fund the proposal, the organization must provide a signed copy of the IRB approval letter to the cognizant program prior to award. The letter should indicate approval of the proposed activities and must be submitted prior to an award being issued.

(iv) If the project lacks definite plans regarding use of human subjects, their data or their specimens, pursuant to 45 CFR §690.118, the proposer must check the box for "Human Subjects" on the Cover Sheet and enter "Pending" in the space provided for the approval date. If available at the time of proposal submission, the determination notice should be uploaded as an Other Supplementary Document in FastLane. If the determination notice is not available, and, the decision is made to fund the proposal, the organization must provide a signed copy of the determination notice to the cognizant program prior to award.

Prior to the expiration date specified in the determination notice, the AOR is required to provide to the cognizant NSF Program Officer:

1. verification that the project continues to lack definite plans for the involvement of human subjects, their data, or their specimens; or

2. documentation that demonstrates that IRB approval has been obtained.

The determination notice must indicate that no work with human subjects, including recruitment, will be conducted until full IRB approval is obtained. NSF will add conditions to the grant that prevent any research involving human subjects from being carried out, or otherwise restrict the drawing down of funds, until IRB approval has been obtained.

[34] An NSF-approved format for submission of these determination notices is available on the NSF website.

(v) The FWA Number that the proposer has on file with OHRP should be entered, if available.

See also Chapter XI.B.1 for additional information on the administration of awards that include use of human subjects.

6. Life Sciences Dual Use Research of Concern (DURC)

a. Applicability

This section applies to all research, for which NSF grant funds may be used, that potentially falls within the scope of the *US Government Policy for Institutional Oversight of Life Sciences Dual Use Research of Concern* (http://www.phe.gov/s3/dualuse/Pages/default.aspx) as published in September, 2014, hereafter referred to as the "Policy".

b. NSF Implementation of the Policy

NSF is committed to preserving the benefits of life sciences research while minimizing the risk of misuse of the knowledge, information, products, or technologies provided by such research. The purpose of NSF's implementation of the Policy is to clarify proposer expectations about NSF-funded research with certain high-consequence pathogens and toxins with potential to be considered DURC.

Proposing organizations are responsible for identifying NSF-funded life sciences proposals that could potentially be considered DURC as defined in the Policy and for compliance with the requirements established in that Policy therein. NSF will not fund research that would be considered to lead to a gain of function of agents associated with the US Government Policy on DURC (See also Chapter XI.B.5 for additional information.)

7. Projects Requiring High-Performance Computing Resources, Large Amounts of Data Storage, or Advanced Visualization Resources

Many research projects require access to computational, data storage or visualization resources in order to complete the work proposed. Typically such resources will be noted in the proposal under Facilities, Equipment and Other Resources. For those projects that require such resources at a scale that is beyond that available locally, NSF supports a number of national resources. For the most computationally massive projects, the Blue Waters system at University of Illinois, Urbana/Champaign, is the most suitable. Blue Waters is designed to support a small number (~50) of research teams involved with projects requiring the most advanced computational and data resources. The allocation review process is through proposal submission to the cognizant Program Officer in the Office of Advanced CyberInfrastructure within the Directorate for Computer & Information Science & Engineering. Proposals are reviewed for both their scientific and computational needs. The Blue Waters supercomputer provides sustained performance of 1 petaflop on a range of real-world science and engineering applications. It is one of the most powerful supercomputers in the world. Those interested should visit the Blue Waters portal (https://bluewaters.ncsa.illinois.edu/) for more details of the system's hardware and capabilities.

The National Center for Atmospheric Research (NCAR)-Wyoming Supercomputing Center (NWSC) is a significant resource, with current capabilities of 1.5 petaflops peak speed. The NWSC is designed to meet the needs of the atmospheric and related-sciences communities. NWSC operates its own allocations process and those interested in obtaining time and/or storage should consult the cognizant Program Officer in the Division of Atmospheric and Geospace Sciences within the Geosciences Directorate.

The most general set of large computational and data resources funded by NSF are accessible through the XSEDE project. XSEDE provides the integrating fabric for a collection of very powerful supercomputers, a high-throughput computing environment, high-volume data storage facilities, advanced visualization services, connected by a high-bandwidth private network, a training education and outreach program in how to use its services, and an extended collaborative support program to assist researchers in using the

advanced computational resources. The physical resources themselves are provided by Service Providers (SP) via separate awards from NSF. Allocations of those resources for large projects are determined by the XSEDE Resource Allocation Committee, which meets quarterly, using an external set of experts. Smaller requests do not require a proposal or review and a simple on-line process may be used to request such an allocation. NSF encourages prospective users to seek more information at: https://www.xsede.org/allocations.

E. Types of Proposals

In addition to standard research proposals that follow the proposal preparation instructions contained in sections A through C of this chapter, there are other types of proposals that may be submitted to NSF. Each of them is described below, along with instructions that may supplement or deviate from NSF's standard proposal preparation instructions.

1. Rapid Response Research (RAPID) Proposal

RAPID is a type of proposal used when there is a severe urgency with regard to availability of, or access to, data, facilities or specialized equipment, including quick-response research on natural or anthropogenic disasters and similar unanticipated events. PI(s) must contact the NSF Program Officer(s) whose expertise is most germane to the proposal topic before submitting a RAPID proposal. This will facilitate determining whether the proposed work is appropriate for RAPID funding.

- The Project Description is expected to be brief and must be no more than five pages. It must include clear statements as to why the proposed research is of an urgent nature and why RAPID is the most appropriate type of proposal for supporting the proposed work. Note this proposal preparation instruction deviates from the standard proposal preparation instructions contained in this Guide; RAPID proposals must otherwise be compliant with the proposal preparation requirements specified in Part I of the PAPPG.

- The "RAPID" proposal type must be selected in the proposal preparation module in FastLane.

- Only internal merit review is required for RAPID proposals. Under rare circumstances, Program Officers may elect to obtain external reviews to inform their decision. If external review is to be obtained, then the PI will be informed in the interest of maintaining the transparency of the review and recommendation process. The two standard NSB-approved merit review criteria will apply.

- Requests may be for up to $200K and up to one year in duration. The award size, however, will be consistent with the project scope and of a size comparable to grants in similar areas.

- No-cost extensions and requests for supplemental funding will be processed in accordance with standard NSF policies and procedures.

- Renewed funding of RAPID awards may be requested only through submission of a proposal that will be subject to full external merit review. Such proposals would be designated as "RAPID renewals."

2. EArly-concept Grants for Exploratory Research (EAGER) Proposal

EAGER is a type of proposal used to support exploratory work in its early stages on untested, but potentially transformative, research ideas or approaches. This work may be considered especially "high risk-high payoff" in the sense that it, for example, involves radically different approaches, applies new expertise, or engages novel disciplinary or interdisciplinary perspectives. These exploratory proposals also may be submitted directly to an NSF program, but the EAGER proposal type should not be used for projects that are appropriate for submission as "regular" (i.e., non-EAGER) NSF proposals. PI(s) must contact the NSF Program Officer(s) whose expertise is most germane to the proposal topic prior to submission of an EAGER

proposal. This will aid in determining the appropriateness of the work for consideration under the EAGER proposal type; this suitability must be assessed early in the process.

- The Project Description is expected to be brief and must be no more than eight pages. It must include clear statements as to why this project is appropriate for EAGER funding, including why it does not fit into existing programs and why it is a good fit for EAGER. Note this proposal preparation instruction deviates from the standard proposal preparation instructions contained in this Guide; EAGER proposals must otherwise be compliant with the proposal preparation requirements specified in Part I of the PAPPG.

- The "EAGER" proposal type must be selected in the proposal preparation module in FastLane.
- Only internal merit review is required for EAGER proposals. Under rare circumstances, Program Officers may elect to obtain external reviews to inform their decision. If external review is to be obtained, then the PI will be informed in the interest of maintaining the transparency of the review and recommendation process. The two standard NSB-approved merit review criteria will apply.

- Requests may be for up to $300K and up to two years in duration. The award size, however, will be consistent with the project scope and of a size comparable to grants in similar areas.

- No-cost extensions and requests for supplemental funding will be processed in accordance with standard NSF policies and procedures.

- Renewed funding of EAGER awards may be requested only through submission of a proposal that will be subject to full external merit review. Such proposals would be designated as "EAGER renewals."

3. Research Advanced by Interdisciplinary Science and Engineering (RAISE) Proposal

RAISE is a type of proposal that may be used to support bold, interdisciplinary projects whose:

- Scientific advances lie in great part outside the scope of a single program or discipline, such that substantial funding support from more than one program or discipline is necessary.

- Lines of research promise transformational advances.

- Prospective discoveries reside at the interfaces of disciplinary boundaries that may not be recognized through traditional review or co-review.

To receive funding as a RAISE-appropriate project, all three criteria must be met. RAISE is not intended to be used for projects that can be accommodated within other types of proposals or that continue well established practices. Prospective PIs must receive approval to submit a proposal from at least two NSF Program Officers, in intellectually distinct programs, whose expertise is most germane to the proposal topics.

Contingent on Program Officers' approval to submit a proposal:

- RAISE proposals must be compliant with Part I of the PAPPG unless a deviation from the standard proposal preparation instructions is indicated below.

- NSF will not accept collaborative RAISE proposals for a single project submitted separately from multiple organizations. A multi-organization RAISE project must be submitted as a single proposal requesting a single award with subawards administered by the lead organization.

- The RAISE proposal type must be selected in the proposal preparation module in FastLane.

- The project title will be preceded by the prefix "RAISE:"

- Email documentation from at least two NSF Program Officers confirming approval to submit a proposal must be uploaded under "RAISE – Program Officer Concurrence Emails" in the Supplementary Documentation section of FastLane.

- Requests may be for up to $1,000,000 and up to five years in duration. The award size and duration will be consistent with the project scope.

- The proposal must explicitly address how the project is better suited for RAISE than for a regular NSF review process.

- Only internal merit review is required for RAISE proposals. Program Officers may elect to obtain external reviews to inform their decision. If external review is to be obtained, then the PI will be informed in the interest of maintaining the transparency of the review and recommendation process.

- The two standard NSB-approved merit review criteria will apply. The interdisciplinary and transformative potential of the project will be evaluated within the intellectual merit of the proposal.

- On the basis of the review criteria, the cognizant Program Officers will decide whether to recommend a RAISE proposal for co-funding from their programs.

- No-cost extensions and requests for supplemental funding will be processed in accordance with standard NSF policies and procedures.

- There are no renewals for RAISE awards.

4. Grant Opportunities for Academic Liaison with Industry (GOALI) Proposal

GOALI is a type of proposal that seeks to stimulate collaboration between academic research institutions and industry. Under this proposal type, academic scientists and engineers request funding either in conjunction with a regular proposal submitted to a standing NSF program or as a supplemental funding request to an existing NSF-funded award. GOALI is not a separate program; GOALI proposals must be submitted to an active NSF funding opportunity and must be submitted in accordance with the deadlines specified therein. A proposer interested in submitting a GOALI proposal or a GOALI supplemental funding request to an existing NSF-funded award must contact the cognizant NSF Program Officer listed in the relevant funding opportunity prior to submission. Special interest is focused on affording opportunities for:

- Interdisciplinary university-industry teams to conduct collaborative research projects, in which the industry research participant provides critical research expertise, without which the likelihood for success of the project would be diminished;

- Faculty, postdoctoral fellows, and students to conduct research and gain experience in an industrial setting; and

- Industrial scientists and engineers to bring industry's perspective and integrative skills to academe.

GOALI proposals should focus on research that addresses shared interests by academic researchers and industrial partners. The research should further scientific and engineering foundations to enable future breakthrough technologies with the potential to address critical industry needs. Industry involvement assures that the research is industrially relevant. Principal Investigators are expected to integrate their research objectives with educational and industrial needs.

Interdisciplinary research and education projects that enable faculty from different academic departments or institutions to interact with one or more industrial partners in industry-university groups or networks are encouraged. Proposals may include the participation of a "third partner" such as a National Laboratory or a non-profit organization. NSF funding can be used for university research/education activities and may support activities of faculty and their students and research associates in the industrial setting. NSF funds are not permitted to be used to support the industrial research partner.

GOALI proposals and supplemental funding requests are reviewed by the program to which the proposal is submitted. In addition to any program-specific review criteria defined in the solicitation, reviewers may be asked to evaluate the degree and extent to which industry will be involved with the proposed research and the extent to which students and/or post-doctoral researchers will benefit from the interaction. The proposed research should be transformative, beneficial to industry, and further collaboration between the academic and industrial partners.

Specific instructions for each type of request are provided below.

a. Requests as part of a competitive proposal submission

(i) GOALI proposals must follow the deadlines applicable to an existing funding opportunity as well as the following GOALI-specific requirements: The title of a GOALI proposal should start with "GOALI:" (after any other title requirements specified by the funding opportunity to which the proposal is being submitted);

(ii) At least one industrial co-PI must be listed on the Cover Sheet at the time of submission although the industrial participant cannot use or receive any NSF funds;

(iii) The university-industry interaction should be described in the Project Description;

(iv) A GOALI-Industrial PI Confirmation Letter from the industrial partner that confirms the participation of a co-PI from industry **must be submitted with the proposal** (if applicable, the letter also must state the degree of industrial participation as well as detail any support that the industry is providing to the academic partner). All GOALI-related confirmation must be uploaded under “GOALI-Industrial PI Confirmation Letter” in the supplementary documentation section of FastLane. This supplementary documentation will not be counted towards the 15-page Project Description limitation; and

(v) Academic and industry partners should agree in advance as to how intellectual property (IP) rights will be handled. A signed university-industry agreement on IP (including publication and patent rights) must be submitted prior to issuance of an award. NSF will review this agreement to ensure that the graduation of students will not be unduly affected. NSF is responsible neither for the agreement reached nor the IP information exchanged between the academic institution and the industrial partner.

b. Supplemental funding requests to existing NSF awards

Supplemental funding requests to add GOALI elements to a currently funded NSF research project should be submitted by using the “Supplemental Funding Request” function in FastLane. Such requests should include a brief description of the proposed activity, a budget and a budget justification, in addition to items (iii)-(v) above. At least one industrial participant must be included in the GOALI activity and must be specified in the GOALI-Industrial PI Confirmation Letter. The industrial participant cannot use or receive any NSF funds.

5. Ideas Lab Proposal

"Ideas Lab" is a type of proposal to support the development and implementation of creative and innovative project ideas that have the potential to transform research paradigms and/or solve intractable problems. An Ideas Lab may be run independently, or in parallel, with the issuance of an NSF funding opportunity on the same topic. These project ideas typically will be high-risk/high-impact, as they represent new and

unproven ideas, approaches and/or technologies. This mechanism was developed collaboratively within NSF, modeled on the "sandpit" workshops that are a key component of the United Kingdom Research Council's "IDEAs Factory" program.

The Ideas Lab type of proposal is implemented using the four-stage process described below:

a. Stage 1: Selection of Panelists

There are two separate panels convened for an Ideas Lab: a selection panel and an Ideas Lab panel. The role of the selection panel is to provide advice on the selection of participants. The role of the Ideas Lab panel is to provide an assessment of the project ideas developed during the Ideas Lab. The individuals selected to participate in each of these panels are subject matter experts for the specific topic of the Ideas Lab. All panelists are barred from receiving any research funding through, or in any other way collaborating on, the particular Ideas Lab in which they are involved.

b. Stage 2: Selection of Participants

A "call for participants" solicitation that describes the specific focus of the Ideas Lab will be issued. The solicitation will specify the content and submission instructions for such applications.

The Project Description is limited to two pages and should include information regarding the applicant's specific expertise and interest in the topic area, as well as certain personal attributes that enhance the success of the Ideas Lab workshop (e.g., experience and interest in working in teams, communication skills, level of creativity, willingness to take risks). Applicants also must include a Biographical Sketch and Current and Pending Support information (both of which must be prepared in accordance with standard NSF formatting guidelines). All other elements of a "full proposal" are waived (i.e., Project Summary, References Cited, Budget and Budget Justification, Facilities, Equipment and Other Resources). The application must be submitted as a preliminary proposal in FastLane. No appendices or supplementary documents may be submitted.

Applicants are notified electronically of NSF's decision regarding whether they are invited or not invited to participate in the Ideas Lab. Applicants will be informed about the context of the review and the criteria that were used to assess the applications in the form of a panel summary, but will not receive individual reviews or other review-related feedback.

c. Stage 3: Ideas Lab

The agenda and duration[35] of the Ideas Lab are communicated to meeting participants by the cognizant NSF Program Officer. Typically, Anonymous real-time peer review involving the participants and the Ideas Lab panel is incorporated into the workshop format, providing iterative constructive feedback during the development of project ideas. The Ideas Lab concept incorporates a "guided creativity" process, thus the use of a facilitator(s) is included, both to guide the creation of interdisciplinary teams and the creative development of ideas, and to ensure that the workshop progresses in a productive manner. At the end of the Ideas Lab, the Ideas Lab panel will provide a consensus report summarizing their evaluation of each project idea. The recommendations of the Ideas Lab panel are advisory to NSF. Within seven to fourteen days following the Ideas Lab, the NSF Program Officers will determine which project ideas are meritorious and should be invited as full proposals. At the NSF Program Officers' discretion (subject to Division Director concurrence), they may invite none, some, or all of the project ideas as full proposals, with the final funding decision to occur after the full proposals have been received and reviewed. Invited full proposals (which are prepared in accordance with standard research proposal formatting guidelines) must be submitted within two months of receiving NSF notification after the Ideas Lab.

[35] Ideas Labs are generally one to five days in duration.

d. Stage 4: Review and recommendation of full proposals

Invited proposals will be reviewed internally by the cognizant NSF Program Officers, the Ideas Lab panelists, and other external reviewers, as appropriate. Resulting awards will be administered in accordance with standard NSF policies and procedures, including no-cost extensions and supplemental funding requests. Renewed funding of an Ideas Lab award may be requested only through submission of a full proposal that will be subject to external merit review. Such proposals would be designated as an "Ideas Lab renewal."

6. Facilitation Awards for Scientists and Engineers with Disabilities (FASED) Proposal

As part of its effort to promote full utilization of highly qualified scientists, mathematicians, and engineers, and to develop scientific and technical talent, the Foundation has the following goals:

- to reduce or remove barriers to participation in research and training by persons with physical disabilities by providing special equipment and assistance under awards made by NSF; and

- to encourage persons with disabilities to pursue careers in science and engineering by stimulating the development and demonstration of special equipment that facilitates their work performance.

Persons with disabilities eligible for facilitation awards include PIs, other senior personnel, and graduate and undergraduate students. The cognizant NSF Program Officer will make decisions regarding what constitutes appropriate support on a case-by-case basis. The specific nature, purpose, and need for equipment or assistance should be described in sufficient detail in the proposal to permit evaluation by knowledgeable reviewers.

There is no separate program for funding of special equipment or assistance. Requests are made in conjunction with regular competitive proposals, or as a supplemental funding request to an existing NSF award. Specific instructions for each type of request are provided below.

a. Requests as part of a competitive proposal submission

Funds may be requested to purchase special equipment, modify equipment or provide services required specifically for the work to be undertaken. Requests for funds for equipment or assistance that compensate in a general way for the disabling condition are not permitted. For example, funds may be requested to provide: prosthetic devices to manipulate a particular apparatus; equipment to convert sound to visual signals, or vice versa, for a particular experiment; access to a special site or to a mode of transportation (except as defined below); a reader or interpreter with special technical competence related to the project; or other special-purpose equipment or assistance needed to conduct a particular project. Items, however, such as standard wheel chairs, prosthetics, hearing aids, TDD/text-phones, or general readers for the blind would not be supported because the need for them is not specific to the proposed project. Similarly, ramps, elevators, or other structural modifications of research facilities are not eligible for direct support under this program.

No maximum funding amount has been established for such requests. It is expected, however, that the cost (including equipment adaptation and installation) will not be a major component of the total proposed budget for the project. Requests for funds for special equipment or assistance to facilitate the participation of individuals with disabilities should be included in the proposed budget for the project and documented in the budget justification. The specific nature, purpose and need for such equipment or assistance should be described in sufficient detail in the Project Description to permit evaluation of the request by knowledgeable reviewers.

b. Supplemental funding requests to existing NSF grants

Supplemental funds for special equipment or assistance to facilitate participation in NSF-supported projects by persons with disabilities may be requested under existing NSF grants. Normally, title is vested in the grantee organization for equipment purchased in conjunction with NSF-supported activities. In accordance with the applicable grant terms and conditions, the grantee organization guarantees use of the equipment for the specific project during the period of work funded by the Foundation, and assures its use in an appropriate manner after project completion. In instances involving special equipment for persons with disabilities, the need for such may be unique to the individual. In such cases, the grantee organization may elect to transfer title to the individual to assure appropriate use after project completion.

Supplemental funding requests should be submitted by using the "Supplemental Funding Request" function in FastLane and should include a brief description of the request, a budget and a budget justification. Requests must be submitted at least two months before funds are needed. Funding decisions will be made on the basis of the justification and availability of program funds with any resultant funding provided through a formal amendment of the existing NSF grant.

7. Conference Proposal[36]

NSF supports conferences in special areas of science and engineering that bring experts together to discuss recent research or education findings or to expose other researchers or students to new research and education techniques. NSF encourages the convening in the US of major international conferences.

A conference proposal will be evaluated through use of the two National Science Board (NSB)-approved merit review criteria of Intellectual Merit and Broader Impacts, and, will be supported only if equivalent results cannot be obtained by attendance at regular meetings of professional societies. Although requests for support of a conference proposal ordinarily originates with educational institutions or scientific and engineering societies, they also may come from other groups. Shared support by several Federal agencies, States or private organizations is encouraged. A conference proposal should generally be submitted at least a year in advance of the scheduled date. Conferences, including the facilities in which they are held, funded in whole or in part with NSF funds, must be accessible to participants with disabilities.

A conference proposal must contain the elements identified below:

- Cover Sheet
- Project Summary (Chapter II.C.2.b) should be consulted to prepare this portion of the proposal)
- Project Description (not to exceed 15 pages) that includes:

a. A statement of the need for such a gathering and a list of topics;

b. Separate statements on the intellectual merit and broader impacts of the proposed activity;

c. A listing of recent meetings on the same subject, including dates and locations;

d. The names of the chairperson and members of organizing committees and their organizational affiliations;

e. Information on the location and probable date(s) of the meeting and the method of announcement or invitation;

[36] This coverage also applies to symposia and workshop proposals.

f. A statement of how the meeting will be organized and conducted, how the results of the meeting will be disseminated and how the meeting will contribute to the enhancement and improvement of scientific, engineering and/or educational activities; and

g. Results from Prior NSF Support (up to five pages): If **any PI or co-PI** identified on the proposal has received prior NSF support including:

- an award with an end date in the past five years; or
- any current funding, including any no cost extensions,

information on the award is required **for each PI and co-PI**, regardless of whether the support was directly related to the proposal or not. See Chapter II.C.2.d.(iii) for additional instructions on preparation of this section.

- Proposal Budget and Budget Justification: A budget, and budget justification for the conference prepared in accordance with Chapter II.C.2.g. The following provides a listing of the types of costs that may be included on a conference budget. Such costs may be included only if they are specifically and clearly identified in the proposed scope of work and budget. Note that registration fees under NSF-supported conferences are considered program income. For additional information on program income generated from conferences, see Chapter VIII.D.4.

a. Conference Facilities. Rental of facilities and necessary equipment.

b. Supplies. Expendable materials and supplies necessary for the meeting.

c. Conference Services. Costs of translation services, audio visual, webcast, and computer services for recording, transmitting and transcribing the proceedings.

d. Publication Costs. Costs of publishing the proceedings.

e. Salaries. Salaries of professional personnel, editorial and clerical assistants and other staff members in proportion to the time or effort devoted to the preparation and conduct of the conference and summarizing its results.

f. Consultant Services and Speaker Fees. Reasonable fees and travel allowances and per diem (or meals provided in lieu of per diem). Consultants' travel costs, including subsistence, may be included. If requested, the proposer must be able to justify that the proposed rate of pay is reasonable. Additional information on the allowability of consultant or professional service costs is available in 2 CFR § 200.459. In addition to other provisions required by the proposing organization, all contracts made under the NSF award must contain the applicable provisions identified in 2 CFR § 200, Appendix II.

g. Meals and Coffee Breaks. Meals that are an integral and necessary part of a conference (e.g., working meals where business is transacted). Funds may be included for furnishing a reasonable amount of hot beverages or soft drinks to conference participants and attendees during periodic coffee breaks. Proposed costs for meals must be reasonable and otherwise allowable to the extent such costs do not exceed charges normally allowed by the grantee organization in its regular operations as the result of the grantee organizations written policies. In the absence of an acceptable, written grantee organizational policy regarding meal costs, 2 CFR §200.474(b)(3) will apply.

h. Participant Support Costs. See Chapter II.C.2.g.(v).

i. Dependent Care Costs. As needed, the costs of identifying, but not providing, locally available dependent care resources may be included.

The following provides a listing of the types of costs that are not allowable for inclusion on a conference budget.

(i) Meals and Coffee Breaks for Intramural Meetings. NSF funds may not be included or spent for meals or coffee breaks for intramural meetings of an organization or any of its components, including, but not limited to, laboratories, departments and centers, as a direct cost.

(ii) Entertainment. Costs of entertainment, amusement, diversion and social activities (such as tickets to shows or sporting events, meals, lodging, rentals, transportation and gratuities) are unallowable and may not be requested. Travel, meal, and hotel expenses of awardee employees who are not on travel status also are not permitted. See also 2 CFR § 200.438.

(iii) Alcoholic Beverages. No NSF funds may be requested or spent for alcoholic beverages.

- Current and Pending Support: The support requested or available from other Federal agencies and other sources. Chapter II.C.2.h should be consulted to prepare this portion of the proposal.

- Facilities, Equipment and Other Resources: If there will be support from other sources for the conference, such information should be included in the Facilities, Equipment and Other Resources section of the proposal. The description should be narrative in nature and must not include any quantifiable financial information Chapter II.C.2.i should be consulted to prepare this portion of the proposal. If included, these resources will not be auditable and must not be included in the proposal budget or budget justification. A description of such support should be included in the Facilities, Equipment and Other Resources section of the proposal.

- Data Management Plan: Plans for management and sharing of any data products resulting from the activity. Chapter II.C.2.j should be consulted to prepare this portion of the proposal.

8. Equipment Proposal

A proposal for specialized equipment may be submitted by an organization for: individual investigators; groups of investigators within the same department; several departments; organization(s) participating in a collaborative or joint arrangement; any components of an organization; or a region. One individual must be designated as PI. Investigators may be working in closely related areas or their research may be multidisciplinary.

An equipment proposal must contain all of the following proposal sections:

- Cover Sheet

- Project Summary: Chapter II.C.2.b should be consulted to prepare this portion of the proposal.

- Project Description (not to exceed 15 pages) that includes:

a. an overall acquisition plan which discusses arrangements for acquisition, maintenance and operation. Equipment to be purchased, modified or constructed must be described in sufficient detail to allow comparison of its capabilities with the needs of the proposed activities;

b. a description, from each potential major user, of the project(s) for which the equipment will be used. This description must be succinct, not necessarily as detailed as in a full research proposal, and must emphasize the intrinsic merit of the activity and the importance of the equipment to it. A brief summary will suffice for auxiliary users; and

c. a description of comparable equipment already at the proposing organization(s), if applicable, and an explanation of why it cannot be used. This includes comparable government-owned equipment that is on-site.

d. Results from Prior NSF Support (up to five pages): If **any PI or co-PI** identified on the proposal has received prior NSF support including:

- an award with an end date in the past five years; or
- any current funding, including any no cost extensions,

information on the award is required **for each PI and co-PI**, regardless of whether the support was directly related to the proposal or not. See Chapter II.C.2.d.(iii) for additional instructions on preparation of this section.

- Biographical Sketch(es) of the person(s) who will have overall responsibility for maintenance and operation of the equipment and a brief statement of qualifications. (Chapter II.C.2.f should be consulted to prepare this portion of the proposal; also see Chapter II.C.2.f.(iii).

- Proposal Budget and Budget Justification: An annual budget and budget justification for the operation, maintenance and administration of the proposed equipment (Chapter II.C.2.g should be consulted to prepare this portion of the proposal).

- Current and Pending Support: Chapter II.C.2.h should be consulted to prepare this portion of the proposal.

- Facilities, Equipment and Other Resources that includes a description of the physical facility, including floor plans or other appropriate information, where the equipment will be located; a narrative description of the source of funds available for operation and maintenance of the proposed equipment; a brief description of other support services available, and a statement of why the equipment is severable or non-severable from the physical facility (Chapter II.C.2.i should be consulted to prepare this portion of the proposal).

- Data Management Plan: Chapter II.C.2.j should be consulted to prepare this portion of the proposal.

- Postdoctoral Mentoring Plan (if applicable): Chapter II.C.2.j should be consulted to prepare this portion of the proposal.

This type of proposal normally competes with proposals for research or education projects.[37]

9. Travel Proposal

A proposal for travel support, either domestic and/or international, for participation in scientific and engineering meetings are handled by the NSF organizational unit with program responsibility for the area of interest.

A group travel proposal is encouraged as the primary means of support for travel. A university, professional society or other non-profit organization may apply for funds to enable it to coordinate and support participation in one or more scientific meeting(s). A proposal submitted for this purpose must contain the elements identified below, with particular attention to plans for composition and recruitment of the travel group. Information on planned speakers should be provided, where available, from the conference organizer.

[37]See Chapter IX.D for additional information on the administration of equipment awards.

- Cover Sheet

- Project Summary (Chapter II.C.2.b should be consulted to prepare this portion of the proposal).

- Project Description (not to exceed 15 pages) that includes:

a. A statement of the need for attending such a gathering and a list of topics;

b. A listing of recent meetings on the same subject, including dates and locations;

c. Information on the location and probable date(s) of the meeting;

d. A statement of how the meeting will be organized and conducted, how the results of the meeting will be disseminated and how the meeting will contribute to the enhancement and improvement of scientific, engineering and/or educational activities; and

e. Results from Prior NSF Support (up to five pages): If **any PI or co-PI** identified on the proposal has received prior NSF support including:

- an award with an end date in the past five years; or
- any current funding, including any no cost extensions,

information on the award is required **for each PI and co-PI**, regardless of whether the support was directly related to the proposal or not. See Chapter II.C.2.d.(iii) for additional instructions on preparation of this section.

- Proposal Budget and Budget Justification: A budget, and budget justification for the travel prepared in accordance with Chapter II.C.2.g. For proposals to support travel to international destinations, in accordance with the Fly America Act (49 USC 40118), any air transportation to, from, between, or within a country other than the US of persons or property, the expense of which will be assisted by NSF funding, must be performed by or under a code-sharing arrangement with a US-flag air carrier if service provided by such a carrier is available (see Comptroller General Decision B-240956, dated September 25, 1991). Tickets (or documentation for electronic tickets) must identify the US flag air carrier's designator code and flight number. See Chapter XI.F for additional information.

- Current and Pending Support: The support requested or available from other Federal agencies and other sources. Chapter II.C.2.h should be consulted to prepare this portion of the proposal.

- Facilities, Equipment and Other Resources: If there will be support from other sources for the travel, such information should be included in the Facilities, Equipment and Other Resources section of the proposal. The description should be narrative in nature and must not include any quantifiable financial information. Chapter II.C.2.i should be consulted to prepare this portion of the proposal. If included, these resources will not be auditable and must not be included in the proposal budget or budget justification. A description of such support should be included in the Facilities, Equipment and Other Resources section of the proposal.

- Data Management Plan: Plans for management and sharing of any data products resulting from the activity. Chapter II.C.2.j should be consulted to prepare this portion of the proposal.

A group travel proposal may request support only for the travel costs of the proposed activity. Group travel grantees are required to retain supporting documentation that funds were spent in accordance with the original intent of the proposal. Such documentation may be required in final reports and is subject to audit.

10. Center Proposal

NSF provides support for a variety of individual Centers and Centers programs that contribute to the Foundation's vision as outlined in the NSF Strategic Plan. Centers exploit opportunities in science, engineering and technology in which the complexity of the research problem(s) or the resources needed to solve the(se) problem(s) require the advantages of scope, scale, change, duration, equipment, facilities, and students that can only be provided by an academic research center. They focus on investigations at the frontiers of knowledge not normally attainable through individual investigations, at the interfaces of disciplines and/or by incorporating fresh approaches to the core of disciplines. Centers focus on integrative learning and discovery and demonstrate leadership in broadening participation through focused investments in a diverse set of partner organizations and individuals. In doing so, they draw upon, and contribute to, the development of the Nation's full intellectual talent. Most Center awards are limited to a maximum duration of ten years and are often subject to mid-course external merit review. Proposers interested in learning more about current or future NSF Centers are encouraged to contact the appropriate disciplinary NSF Program Officer. Centers are not considered research infrastructure, but will often use research infrastructure to meet their objectives.

11. Research Infrastructure Proposal

As an integral part of its responsibility for strengthening the science and engineering capacity of the country, NSF provides support for the design, construction, operation and upgrade of research infrastructure including instrumentation, mid-scale projects and major facilities. NSF depends on the research communities to provide the justification as well as the capabilities to manage the development and implementation of research infrastructure projects. Justification normally occurs through National Academies studies, workshop reports, professional society activities, and other community-based mechanisms, including engineering studies and research projects related to the development of new technologies. Many of these mechanisms are funded by interested NSF Programs on the basis of merit-reviewed proposals. The NSF process and funding mechanisms for development and implementation of research infrastructure projects depends, in part, on the scale of the project. The largest projects, major facilities, are typically supported through the Major Research Equipment and Facilities Construction (MREFC) account. Proposers are strongly encouraged to contact the appropriate NSF Program to discuss the availability of funding and the appropriate funding mechanisms in advance of proposal submission. The Large Facilities Manual (LFM), a public document managed by the Large Facilities Office, contains policies and procedures related to NSF oversight and Recipient management of research infrastructure projects funded by NSF. The purpose of the LFM is to: (1) provide guidance to NSF staff on conducting appropriate oversight and to recipients in carrying out effective project planning and management; and (2) clearly state the required policies and procedures as well as pertinent good practices for each life-cycle stage.

Exhibit II-1: Proposal Preparation Checklist

It is imperative that all proposals conform to the proposal preparation and submission instructions specified in Part I of the *Proposal and Award Policies and Procedures Guide*. Conformance with all preparation and submission instructions is required and will be strictly enforced unless a deviation has been approved in advance of proposal submission. Note that some NSF program solicitations modify standard NSF proposal preparation guidelines, and, in such cases, the guidelines provided in the solicitation must be followed. FastLane uses the rules specified for each type of proposal, (e.g., Research, RAPID, EAGER, RAISE, GOALI, Ideas Lab, FASED, Conference, Equipment, or Travel) to check for compliance prior to submission to NSF. Proposers are strongly advised to review the applicable sections of Chapter II.E. relevant to the type of proposal being developed PRIOR to submission. NSF will not accept[38] or will return without review proposals that are not consistent with these instructions. See Chapter IV.B and visit: http://www.nsf.gov/bfa/dias/policy/autocompliance.jsp for additional information.

Prior to submission, it is strongly recommended that an administrative review be conducted to ensure that proposals comply with the instructions, and the format specified. This checklist is not intended to be an all-inclusive repetition of the required proposal contents and associated proposal preparation guidelines. It is, however, meant to highlight certain critical items so they will not be overlooked when the proposal is prepared.

[ ] General:

[ ] The proposal is compliant with the provisions in the PAPPG and/or the relevant program solicitation.

[ ] The proposal is responsive to the relevant program description or announcement (if applicable).

[ ] If the proposal has been previously declined and is being resubmitted, the proposal has been substantively revised to take into account the major comments from the prior NSF review.

[ ] The proposed work is appropriate for funding by NSF, and is not a duplicate of, or substantially similar to, a proposal already under consideration by NSF from the same submitter.

[ ] The proposal must be submitted by 5 p.m. submitter's local time on the established deadline date.

[ ] Single Copy Documents:

[ ] Authorization to Deviate from NSF Proposal Preparation Requirements is included (if applicable).

[ ] List of Suggested Reviewers, or Reviewers Not To Include has been provided (if applicable).

[ ] SF LLL, *Disclosure of Lobbying Activities* has been provided (if applicable).

[ ] Collaborators and Other Affiliations (COA) Information has been separately provided for each individual identified as senior project personnel through use of the COA template available at: https://nsf.gov/bfa/dias/policy/coa.jsp.

[38] Proposal Not Accepted is defined as FastLane will not permit submission of the proposal.

[ ] Cover Sheet:

[ ] For interdisciplinary proposals, ensure all relevant programs are identified.

[ ] Title includes any necessary prefix, e.g., "Collaborative Proposal:."

[ ] For renewal proposals, previous award numbers have been entered.

[ ] Related preliminary proposal number has been entered (if applicable).

[ ] The "Special Exception to the Deadline Date Policy" box has been checked on the NSF Cover Sheet and the requisite Single Copy Document has been provided (if applicable).

[ ] Appropriate box(es) have been checked, and requisite information has been provided.

[ ] Project Summary:

[ ] The Project Summary may ONLY be uploaded as a Supplementary Document if use of special characters is necessary. Such Project Summaries must be formatted with separate headings for Overview, Intellectual Merit and Broader Impacts.

[ ] The Project Summary must not exceed one page.

[ ] Project Description:

[ ] The Project Description must not exceed the 15-page limitation, the limit specified in a specific program solicitation, or the limit provided in the instructions for types of proposals (e.g., RAPID, EAGER and Ideas Lab).

[ ] Project Description contains, as a separate section within the narrative, a section labeled "Intellectual Merit."

[ ] Project Description contains, as a separate section within the narrative, a section labeled "Broader Impacts".

[ ] Project Description is self-contained, and Uniform Resource Locators (URLs) have not been included.

[ ] Results from Prior NSF Support have been provided for **any PI or co-PI** identified on the proposal that has received prior NSF support including:

- an award with an end date in the past five years; or
- any current funding, including any no cost extensions.

[ ] Results related to Intellectual Merit and Broader Impacts are described under two separate, distinct headings, and are limited to five pages of the Project Description.

[ ] References Cited:

[ ] This section includes bibliographic citations only and does not provide parenthetical information outside of the 15-page Project Description.

[ ] Each reference is in the required format, which may vary according to the norms of the scientific discipline.

[ ] Biographical Sketch(es):

[ ] The content described is in accordance with the instructions, and does not contain additional information beyond that specified.

[ ] Each biographical sketch must not exceed two pages.

[ ] Each individual's biographical sketch must be uploaded as a single PDF file associated with that individual.

[ ] Proposal Budget:

[ ] Each budget line item is documented and justified in the budget justification.

[ ] Any compensation for senior personnel in excess of two months is disclosed in the proposal budget and justified in the budget justification.

[ ] A budget justification must be provided for the proposer AND any proposed subawardee.

[ ] The amount for indirect costs was be calculated by applying the current negotiated indirect cost rate(s) to the approved base(s) and amount is specified in the budget justification.

[ ] Each budget justification must not exceed five pages or the page limitation specified in a specific program solicitation. For proposals that contain a subaward(s), **each subaward** must include a separate budget justification that must not exceed five pages.

[ ] Cost Sharing:

[ ] Unless required by an NSF program solicitation, voluntary committed cost sharing has not been included. Note that voluntary committed cost sharing is prohibited and Line M on the proposal budget will not be available for use by the proposer. While not required by NSF, proposing organizations may, at their own discretion, continue to contribute voluntary uncommitted cost sharing to NSF-sponsored projects. These resources are not auditable by NSF and should not be included in the proposal budget or budget justification.

[ ] Current and Pending Support:

[ ] All current and pending support from whatever source (e.g., Federal, state, local or foreign government agencies, public or private foundations, industrial or other commercial organizations, or internal funds allocated toward specific projects) must be listed. The proposed project and all other projects or activities requiring a portion of time of the PI and other senior personnel must be included, even if they receive no salary support from the project(s).

[ ] Information on this proposal is included.

[ ] Each individual's current and pending support must be uploaded as a single PDF file or inserted as text associated with that individual.

[ ] Facilities, Equipment and Other Resources:

[ ] An aggregated description of the internal and external resources (both physical and personnel) that the organization and its collaborators will provide to the project, should it be funded, has been included.

[ ] No quantifiable financial information has been provided.

[ ] If there are no facilities, equipment or other resources identified, a statement to that effect has been included in this section of the proposal and uploaded into FastLane.

[ ] Special Information and Supplementary Documentation:

[ ] A postdoctoral mentoring plan, limited to one page, has been included, if required.

[ ] A data management plan, limited to two pages, has been included.

[ ] Letters of collaboration documenting collaborative arrangements of significance to the proposal have been included (if applicable).

[ ] Other types of information identified in Chapter II.C.2.j have been included, as appropriate.

[ ] Any additional items specified in a relevant program solicitation have been included.

[ ] Appendices:

[ ] Appendices may not be included unless a deviation has been authorized.

Exhibit II-2: Potentially Disqualifying Conflicts of Interest

Unless a waiver has been granted by NSF, a reviewer cannot review a proposal if:

- the reviewer, the reviewer's spouse, minor child, or business partner;
- an organization where the reviewer is employed, serves as a consultant, has an arrangement for future employment or is negotiating for employment; or
- the organization where the reviewer is an officer, director, trustee, or partner;

has a financial interest in the outcome of the proposal.

Unless a waiver has been granted by NSF, a potential reviewer also may be barred from reviewing a proposal, if it involves individuals with whom he/she has a personal relationship, such as a close relative, current or former collaborator, or former Ph.D. student/advisor.

Unless a waiver has been granted by NSF, a disqualifying conflict may exist, if a proposal involves an organization or other entity with which the potential reviewer has a connection. Such potentially disqualifying connections include:

- a reviewer's recent former employer;
- an organization in which the reviewer is an active participant;
- an institution at which the reviewer is currently enrolled as a student, or at which he/she serves as a visiting committee member; or
- an entity with which the reviewer has or seeks some other business or financial relationship (including receipt of an honorarium).

Exhibit II-3: Drug-Free Workplace Certification

Instructions for Certification

1. By signing the NSF Cover Sheet and submitting this proposal, the Authorized Organizational Representative is providing the certifications set out below.

2. The certification set out below is a material representation of fact upon which reliance was placed when the agency determined to award the grant. If it is later determined that the grantee knowingly rendered a false certification, or otherwise violates the requirements of the Drug-Free Workplace Act, the agency, in addition to any other remedies available to the Federal Government, may take action authorized under the Drug-Free Workplace Act.

3. For grantees other than individuals, Alternate I applies.

4. For grantees who are individuals, Alternate II applies.

Certification Regarding Drug-Free Workplace Requirements

Alternate I (Grantees Other Than Individuals)

The grantee certifies that it will or will continue to provide a drug-free workplace by:

(a) Publishing a statement notifying employees that the unlawful manufacture, distribution, dispensing, possession or use of a controlled substance is prohibited in the grantee's workplace and specifying the actions that will be taken against employees for violation of such prohibition;

(b) Establishing an ongoing drug-free awareness program to inform employees about --

(1) The dangers of drug abuse in the workplace;

(2) The grantee's policy of maintaining a drug-free workplace;

(3) Any available drug counseling, rehabilitation and employee assistance programs; and

(4) The penalties that may be imposed upon employees for drug abuse violations occurring in the workplace;

(c) Making it a requirement that each employee to be engaged in the performance of the grant be given a copy of the statement required by paragraph (a);

(d) Notifying the employee in the statement required by paragraph (a) that, as a condition of employment under the grant, the employee will --

(1) Abide by the terms of the statement; and

(2) Notify the employer in writing of his or her conviction for a violation of a criminal drug statute occurring in the workplace, no later than five calendar days after such conviction;

(e) Notifying the agency in writing, within 10 calendar days after receiving notice under subparagraph (d)(2) from an employee or otherwise receiving actual notice of such conviction.

Employers of convicted employees must provide notice, including position title, to every grant officer or other designee on whose grant activity the convicted employee was working, unless the

Federal agency has designated a central point for the receipt of such notices. Notice shall include the identification number(s) of each affected grant;

(f) Taking one of the following actions, within 30 calendar days of receiving notice under subparagraph (d)(2), with respect to any employee who is so convicted--

(1) Taking appropriate personnel action against such an employee, up to and including termination, consistent with the requirements of the Rehabilitation Act of 1973, as amended; or

(2) Requiring such employee to participate satisfactorily in a drug abuse assistance or rehabilitation program approved for such purposes by a Federal, State, or local health, law enforcement, or other appropriate agency;

(g) Making a good faith effort to continue to maintain a drug-free workplace through implementation of paragraphs (a), (b), (c), (d), (e) and (f).

Alternate II (Grantees Who Are Individuals)

(a) The grantee certifies that, as a condition of the grant, he or she will not engage in the unlawful manufacture, distribution, dispensing, possession or use of a controlled substance in conducting any activity with the grant.

(b) If convicted of a criminal drug offense resulting from a violation occurring during the conduct of any grant activity, he or she will report the conviction, in writing, within 10 calendar days of the conviction, to every grant officer or other designee, unless the Federal agency designates a central point for the receipt of such notices. When notice is made to such a central point, it shall include the identification number(s) of each affected grant.

For NSF, grantee notification should be made to the Cost Analysis & Audit Resolution Branch, Division of Institution & Award Support, NSF, Arlington, VA 22230.

Exhibit II-4: Debarment and Suspension Certification

Instruction on Certification Regarding Debarment and Suspension

1. By signing and submitting this proposal, the prospective primary participant is providing the certification set out below.

2. The inability of a person to provide the certification required below will not necessarily result in denial of participation in this covered transaction. The prospective participant shall submit an explanation of why it cannot provide the certification set out below. The certification or explanation will be considered in connection with the department or agency's determination whether to enter into this transaction. However, failure of the prospective primary participant to furnish a certification or an explanation shall disqualify such person from participation in this transaction.

3. The certification in this clause is any material representation of fact upon which reliance was placed when the department or agency determined to enter into this transaction. If it is later determined that the prospective primary participant knowingly rendered an erroneous certification, in addition to other remedies available to the Federal Government, the department or agency may terminate this transaction for cause or default.

4. The prospective primary participant shall provide immediate written notice to the department or agency to whom this proposal is submitted if at any time the prospective primary participant learns that its certification was erroneous when submitted or has become erroneous by reason of changed circumstances.

5. The terms covered transaction, debarred, suspended, ineligible, lower tier covered transaction, participant, person, primary covered transaction, principal, proposal, and voluntarily excluded, as used in this clause, have the meanings set out in the Definitions and Coverage sections of the rules implementing Executive Order 12549. You may contact the department or agency to which this proposal is being submitted for assistance in obtaining a copy of those regulations.

6. The prospective primary participant agrees by submitting this proposal that, should the proposed covered transaction be entered into, it shall not knowingly enter into any lower tier covered transaction with a person who is debarred, suspended, declared ineligible, or voluntarily excluded from participation in this covered transaction, unless authorized by the department or agency entering into this transaction.

7. The prospective primary participant further agrees by submitting this proposal that it will include the clause titled "Certification Regarding Debarment, Suspension, Ineligibility and Voluntary Exclusion - Lower Tier Covered Transaction", provided by the department or agency entering into this covered transaction, without modification, in all lower tier covered transactions.

8. A participant in a covered transaction may rely upon a certification of a prospective participant in a lower tier covered transaction that it is not debarred, suspended, ineligible, or voluntarily excluded from the covered transaction, unless it knows that the certification is erroneous. A participant may decide the method and frequency by which it determines the eligibility of its principals. Each participant may, but is not required to, check the Nonprocurement List.

9. Nothing contained in the foregoing shall be construed to require establishment of a system of records in order to render in good faith the certification required by this clause. The knowledge and information of a participant is not required to exceed that which is normally possessed by a prudent person in the ordinary course of business dealings.

10. Except for transactions authorized under paragraph 6 of these instructions, if a participant in a covered transaction knowingly enters into a lower tier covered transaction with a person who is suspended, debarred, ineligible, or voluntarily excluded from participation in this transaction, in addition to other remedies available to the Federal Government, the department or agency may terminate this transaction for cause or default.

Certification

(1) The prospective primary participant certifies to the best of its knowledge and belief, that it and its principals: (a) Are not presently debarred, suspended, proposed for debarment, declared ineligible, or voluntarily excluded from a covered transaction by any Federal department or agency; (b) Have not within a three-year period preceding this proposal been convicted of or had a civil judgment rendered against them for commission of fraud or a criminal offense in connection with obtaining, attempting to obtain, or performing a public (Federal, State or local) transaction or contract under a public transaction; violation of Federal or State antitrust statutes or commission of embezzlement, theft, forgery, bribery, falsification or destruction of records, making false statements, or receiving stolen property; (c) Are not presently indicted for or otherwise criminally or civilly charged by a governmental entity (Federal, State or local) with commission of any of the offenses enumerated in paragraph (1)(b) of this certification; and (d) Have not within a three-year period preceding this application/proposal had one or more public transactions (Federal, State or local) terminated for cause or default.

(2) Where the prospective primary participant is unable to certify to any of the statements in this certification, such prospective participant shall include an explanation with this proposal.

Exhibit II-5: Lobbying Certification

Instructions on Certification Regarding Lobbying

This certification is required for an award of a Federal contract, grant or cooperative agreement exceeding $100,000 and for an award of a Federal loan or a commitment providing for the United States to insure or guarantee a loan exceeding $150,000. The *Certification for Contracts, Grants, Loans and Cooperative Agreements* also is included in full text on the FastLane submission screen.

Certification for Contracts, Grants, Loans and Cooperative Agreements

The undersigned certifies, to the best of his or her knowledge and belief, that:

(1) No Federal appropriated funds have been paid or will be paid, by or on behalf of the undersigned, to any person for influencing or attempting to influence an officer or employee of any agency, a Member of Congress, an officer or employee of Congress, or an employee of a Member of Congress in connection with the awarding of any federal contract, the making of any Federal grant, the making of any Federal loan, the entering into of any cooperative agreement, and the extension, continuation, renewal, amendment, or modification of any Federal contract, grant, loan, or cooperative agreement.

(2) If any funds other than Federal appropriated funds have been paid or will be paid to any person for influencing or attempting to influence an officer or employee of any agency, a Member of Congress, an officer or employee of Congress, or an employee of a Member of Congress in connection with this Federal contract, grant, loan, or cooperative agreement, the undersigned shall complete and submit Standard Form LLL, “Disclosure of Lobbying Activities,” in accordance with its instructions.

(3) The undersigned shall require that the language of this certification be included in the award documents for all subawards at all tiers including subcontracts, subgrants, and contracts under grants, loans, and cooperative agreements and that all subrecipients shall certify and disclose accordingly.

This certification is a material representation of fact upon which reliance was placed when this transaction was made or entered into. Submission of this certification is a prerequisite for making or entering into this transaction imposed by Section 1352, Title 31, US Code. Any person who fails to file the required certification shall be subject to a civil penalty of not less than $10,000 and not more than $100,000 for each such failure.

Exhibit II-6: Nondiscrimination Certification

Instructions for Nondiscrimination Certification

1. In accordance with NSF policy, by signing the proposal, the Authorized Organizational Representative is providing the requisite Certification of Compliance with National Science Foundation Nondiscrimination Regulations and Policies. This Certification sets forth the nondiscrimination obligations with which all awardees must comply. These obligations also apply to subrecipients, and contractors under the award. The proposer therefore, shall obtain the NSF Nondiscrimination Certification from each organization that applies to be, or serves as a subrecipient, or contractor under the award (for other than the provision of commercially available supplies, materials, equipment or general support services) prior to entering into the subaward or contract arrangement.

2. The proposer shall provide immediate notice to the Foundation if at any time the proposer learns that its certification was erroneous when submitted, or has become erroneous by reason of changed circumstances.

Certification of Compliance with National Science Foundation Nondiscrimination Regulations and Policies

By signing the proposal, the Authorized Organizational Representative hereby certifies that the organization will comply with Title VI of the Civil Rights Act of 1964 (42 USC § 2000d), Title IX of the Education Amendments of 1972 (20 USC §§ 1681 *et seq.*), the Rehabilitation Act of 1973 (29 USC § 794), the Age Discrimination Act of 1975 (42 USC §§ 6101 *et seq.*) and all regulations and policies issued by NSF pursuant to these statutes.

To that end, in accordance with the above-referenced nondiscrimination statutes, and NSF's implementing regulations and policies, no person in the United States shall, on the ground of race, color, national origin, sex, disability, or age, be excluded from participation in, be denied the benefits of, or be otherwise subjected to discrimination under any program or activity for which the Proposer receives Federal financial assistance from the Foundation; and HEREBY CERTIFIES THAT it will immediately take any measures necessary to effectuate this agreement.

If any real property or structure thereon is provided or improved with the aid of Federal financial assistance extended to the Proposer by the Foundation, this Certification shall obligate the Proposer, or in the case of any transfer of such property, the transferee, for the period during which the real property or structure is used for a purpose for which the Federal financial assistance is extended or for another purpose involving the provision of similar services or benefits. If any personal property is so provided, this Certification shall obligate the Proposer for the period during which it retains ownership or possession of the property. In all other cases, this Certification shall obligate the Proposer for the period during which the Federal financial assistance is extended to it by the Foundation.

THIS CERTIFICATION is given in consideration of and for the purpose of obtaining any and all Federal grants, cooperative agreements, loans, contracts, property, discounts or other Federal financial assistance extended after the date hereof to the Proposer by the Foundation, including installment payments after such date on account of applications for Federal financial assistance which were approved before such date. The Proposer recognizes and agrees that such Federal financial assistance will be extended in reliance on the representations and agreements made in this Certification, and that the United States shall have the right to seek judicial enforcement of this Certification. This Certification is binding on the Proposer, its successors, transferees, and assignees.

Exhibit II-7: Definitions of Categories of Personnel

The personnel categories listed on parts A and B of the Proposal Budget are defined as follows:

A. Senior Personnel

1. (co) Principal Investigator/Project Director (PI/PD) -- the individual(s) designated by the proposer, and approved by NSF, who will be responsible for the scientific or technical direction of the project. NSF does not infer any distinction in scientific stature among multiple PIs, whether referred to as PI or co-PI. If more than one, the first one listed will serve as the contact PI, with whom all communications between NSF program officials and the project relating to the scientific, technical, and budgetary aspects of the project should take place. The PI and any identified co-PIs, however, will be jointly responsible for submission of the requisite project reports. The term "Principal Investigator" generally is used in research projects, while the term "Project Director" generally is used in centers, major facilities, and other projects. For purposes of this Guide, PI/co-PI is interchangeable with PD/co-PD.

2. Faculty Associate (faculty member) (or equivalent) -- an individual other than the Principal Investigator(s) considered by the performing institution to be a member of its faculty (or equivalent) or who holds an appointment as a faculty member at another institution, and who will participate in the project being supported.

B. Other Personnel

1. Postdoctoral (Scholar, Fellow, or Other Postdoctoral Position) -- an individual who has received a doctoral degree (or equivalent) and is engaged in a temporary and defined period of mentored advanced training to enhance the professional skills and research independence needed to pursue his or her chosen career path. Postdoctoral scholars not identified under Senior Personnel above should be listed as Other Personnel.

2. Other Professional -- a person who may or may not hold a doctoral degree or its equivalent, who is considered a professional and is not reported as a Principal Investigator, faculty associate, postdoctoral scholar or student. Examples of persons included in this category are doctoral associates not reported under B1, professional technicians, physicians, veterinarians, system experts, computer programmers and design engineers.

3. Graduate Student (research assistant) -- a part-time or full-time student working on the project in a research capacity who holds at least a bachelor’s degree and is enrolled in a degree program leading to an advanced degree.

4. Undergraduate Student -- a student who is enrolled in a degree program (part-time or full-time) leading to a bachelor’s or associate’s degree.

5. & 6. These categories include persons working on the project in a non-research capacity, such as secretaries, clerk-typists, draftsmen, animal caretakers, electricians and custodial personnel regardless of whether they hold a degree or are involved in degree work.

Any personnel category for which NSF funds are requested must indicate, in the parentheses provided on the Proposal Budget, the number of persons expected to receive some support from those funds.

Chapter III: NSF Proposal Processing and Review

Proposals received by NSF are assigned to the appropriate NSF program and are assessed to ensure that they meet NSF compliance requirements. All compliant proposals are then carefully reviewed by a scientist, engineer, or educator serving as an NSF Program Officer, and usually by three to ten other persons outside NSF either as *ad hoc* reviewers, panelists, or both, who are experts in the particular fields represented by the proposal. Proposers are invited to suggest names of persons they believe are especially well qualified to review the proposal and/or persons they would prefer not review the proposal. These suggestions may serve as one source in the reviewer selection process at the Program Officer's discretion. In addition, Program Officers may obtain comments from site visits before recommending final action on proposals. Senior NSF staff further review recommendations for awards. A flowchart that depicts the entire NSF proposal and award process (and associated timeline) is included as Exhibit III-1.

A comprehensive description of the Foundation's merit review process is available on the NSF website at: http://www.nsf.gov/bfa/dias/policy/merit_review/.

Proposal review is one step in the NSF program planning and implementation process. Embedded in this process are core strategies that are fundamental to the fulfillment of NSF's mission. More information about NSF's mission and strategies can be found in *Investing in Science, Engineering, and Education for the Nation's Future: NSF Strategic Plan for 2014-2018*. NSF's mission is particularly well-implemented through the integration of research and education and broadening participation in NSF programs, projects, and activities.

A. Merit Review Principles and Criteria

The National Science Foundation strives to invest in a robust and diverse portfolio of projects that creates new knowledge and enables breakthroughs in understanding across all areas of science and engineering research and education. To identify which projects to support, NSF relies on a merit review process that incorporates consideration of both the technical aspects of a proposed project and its potential to contribute more broadly to advancing NSF's mission "to promote the progress of science; to advance the national health, prosperity, and welfare; to secure the national defense; and for other purposes." NSF makes every effort to conduct a fair, competitive, transparent merit review process for the selection of projects.

1. Merit Review Principles

These principles are to be given due diligence by PIs and organizations when preparing proposals and managing projects, by reviewers when reading and evaluating proposals, and by NSF program staff when determining whether or not to recommend proposals for funding and while overseeing awards. Given that NSF is the primary Federal agency charged with nurturing and supporting excellence in basic research and education, the following three principles apply:

- All NSF projects should be of the highest quality and have the potential to advance, if not transform, the frontiers of knowledge.

- NSF projects, in the aggregate, should contribute more broadly to achieving societal goals. These broader impacts may be accomplished through the research itself, through activities that are directly related to specific research projects, or through activities that are supported by, but are complementary to, the project. The project activities may be based on previously established and/or innovative methods and approaches, but in either case must be well justified.

- Meaningful assessment and evaluation of NSF funded projects should be based on appropriate metrics, keeping in mind the likely correlation between the effect of broader impacts and the resources provided to implement projects. If the size of the activity is limited, evaluation of that

activity in isolation is not likely to be meaningful. Thus, assessing the effectiveness of these activities may best be done at a higher, more aggregated, level than the individual project.

With respect to the third principle, even if assessment of Broader Impacts outcomes for particular projects is done at an aggregated level, PIs are expected to be accountable for carrying out the activities described in the funded project. Thus, individual projects should include clearly stated goals, specific descriptions of the activities that the PI intends to do, and a plan in place to document the outputs of those activities.

These three merit review principles provide the basis for the merit review criteria, as well as a context within which the users of the criteria can better understand their intent.

2. Merit Review Criteria

All NSF proposals are evaluated through use of two National Science Board approved merit review criteria. In some instances, however, NSF will employ additional criteria as required to highlight the specific objectives of certain programs and activities.

The two merit review criteria are listed below. Both criteria are to be given full consideration during the review and decision-making processes; each criterion is necessary but neither, by itself, is sufficient. Therefore, proposers must fully address both criteria. (Chapter II.C.2.d(i) contains additional information for use by proposers in development of the Project Description section of the proposal.) Reviewers are strongly encouraged to review the criteria, including Chapter II.C.2.d(i), prior to the review of a proposal.

When evaluating NSF proposals, reviewers will be asked to consider what the proposers want to do, why they want to do it, how they plan to do it, how they will know if they succeed, and what benefits could accrue if the project is successful. These issues apply both to the technical aspects of the proposal and the way in which the project may make broader contributions. To that end, reviewers will be asked to evaluate all proposals against two criteria:

- **Intellectual Merit:** The Intellectual Merit criterion encompasses the potential to advance knowledge; and

- **Broader Impacts:** The Broader Impacts criterion encompasses the potential to benefit society and contribute to the achievement of specific, desired societal outcomes.

The following elements should be considered in the review for both criteria:

1. What is the potential for the proposed activity to:

a. Advance knowledge and understanding within its own field or across different fields (Intellectual Merit); and

b. Benefit society or advance desired societal outcomes (Broader Impacts)?

2. To what extent do the proposed activities suggest and explore creative, original, or potentially transformative concepts?

3. Is the plan for carrying out the proposed activities well-reasoned, well-organized, and based on a sound rationale? Does the plan incorporate a mechanism to assess success?

4. How well qualified is the individual, team, or organization to conduct the proposed activities?

5. Are there adequate resources available to the PI (either at the home organization or through collaborations) to carry out the proposed activities?

B. Selection of Reviewers

The NSF guidelines for the selection of reviewers are designed to ensure selection of experts who can give Program Officers the proper information needed to make a recommendation in accordance with the NSB-approved criteria for selection of projects. Optimally, reviewers should have:

1. Special knowledge of the science and engineering subfields involved in the proposals to be reviewed to evaluate competence, intellectual merit, and utility of the proposed activity. Within reasonable limits, reviewers' fields of specialty should be complementary within a reviewer group.

2. Broader or more generalized knowledge of the science and engineering subfields involved in the proposals to be reviewed to evaluate the broader impacts of the proposed activity. Reviewers with broad expertise are required for proposals involving substantial size or complexity, broad disciplinary or multidisciplinary content, or significant national or international implications.

3. Broad knowledge of the infrastructure of the science and engineering enterprise, and its educational activities, to evaluate contributions to societal goals, scientific and engineering personnel, and distribution of resources to organizations and geographical areas.

4. To the extent possible, diverse representation within the review group. The goal is to achieve a balance among various characteristics. Important factors to consider include: type of organization represented, reviewer diversity, age distribution and geographic balance.

C. Proposal File Updates

It is the responsibility of the proposing organization to thoroughly review each proposal prior to submission. On occasion, however, a problem is identified with a portion of the proposal after the proposal has been submitted electronically to NSF.

The FastLane Proposal File Update Module allows the organization to request the replacement of files or revision of other Proposal Attributes, associated with a previously submitted proposal. (Note: The FastLane Proposal File Update module must not be used for submission of revised budgets. All budgetary revisions must be submitted through use of the FastLane Revised Proposal Budget Module. See Section D. below for further information.) A request for a proposal file update must be signed and submitted by the AOR. An update request must contain a justification that addresses:

1. why the changes or file replacements are being requested; and

2. any differences between the original and proposed replacement files.

A request for a proposal file update automatically will be accepted if submitted **prior to:**

- the deadline date;
- initiation of external peer review in cases when a target date is utilized; [39] and
- initiation of external peer review in the case of an unsolicited proposal.

A request for a proposal file update **after** the timeframes specified above will require acceptance by the cognizant NSF Program Officer. Such requests shall be submitted only to correct a technical problem with the proposal (i.e., formatting or print problems). Changes in the content of the proposal should not be requested after the timeframes specified above. When a request is accepted, the proposed files or

[39] The status of a proposal may be found in Research.gov.

revisions to proposal attributes will immediately replace the existing files and become part of the official proposal.

PIs can access the Proposal File Update Module via the "Proposal Functions" section of FastLane. Authorized individuals in the organization's SPO can initiate or review requests for proposal file updates using the "Submit Proposals/Supplements/File Updates/Withdrawals" Module via the FastLane "Research Administration Functions."[40]

NSF will consider only one request for a proposal file update per proposal at a time. It is anticipated that it will be a rare occurrence for more than one file update request to be submitted for a proposal.

D. Revisions to Proposals Made During the Review Process

In the event of a significant development (e.g., research findings, changed circumstances, unavailability of PI or other senior personnel, etc.) that might materially affect the outcome of the review of a pending proposal, the proposer must contact the cognizant NSF Program Officer to discuss the issue. Submitting additional information must not be used as a means of circumventing page limitations or stated deadlines.

Before recommending whether or not NSF should support a particular project, the cognizant NSF Program Officer may, subject to certain constraints outlined below, engage in discussions with the proposing PI(s).

Negotiating budgets generally involves discussing a lower or higher amount of total support for the proposed project. The cognizant NSF Program Officer may suggest reducing or eliminating costs for specific budget items that are clearly unnecessary or unreasonable for the activities to be undertaken, especially when the review process supports such changes; however, this would generally not include faculty salaries, salary rates, fringe benefits, or tuition. Note: indirect cost (F&A) rates are not subject to negotiation. The NSF Program Officer may discuss with PIs the "bottom line" award amount, i.e., the total NSF funding that will be recommended for a project. NSF Program Officers may not renegotiate cost sharing or other organizational commitments.

When such discussions result in a budget reduction of 10% or more from the amount originally proposed, a corresponding reduction should be made in the scope of the project. Proposers must use the FastLane Revised Proposal Budget Module to submit this information.[41] A revised proposal budget also must include a Budget Impact Statement that describes the impact of the budget reduction on the scope of the project.

Revised proposal budgets must be signed and submitted by the AOR.

E. Funding Recommendation

After scientific, technical and programmatic review and consideration of appropriate factors, the NSF Program Officer recommends to the cognizant NSF Division Director whether the proposal should be declined or recommended for award. Normally, final programmatic approval is at the Division/Office level. Because of the large volume of proposals, this review and consideration process may take up to six months. Large or particularly complex proposals may require additional review and processing time.

Should a proposal be recommended for award, the PI may be contacted by the NSF Program Officer for assistance in preparation of the public award abstract and its title. An NSF award abstract, with its title, is an NSF document that describes the project and justifies the expenditure of Federal funds by articulating how the project serves the national interest, as stated by NSF's mission: "to promote the progress of science; to advance the national health, prosperity and welfare; or to secure the national defense."

[40] Detailed instructions on submitting proposer-initiated proposal file updates are available on the FastLane website at: https://www.fastlane.nsf.gov/documents/pfu/pfu.jsp.

[41] Note: All preaward budgetary revisions must be submitted through use of the Revised Budget Module in Fastlane.

Note that a recommendation for an award by an NSF Program Officer does not constitute approval or obligation of Federal funds. Proposers are cautioned that only an appointed NSF Grants Officer may make commitments, obligations or awards on behalf of NSF or authorize the expenditure of funds. No commitment on the part of NSF or the Government should be inferred from technical or budgetary discussions with an NSF Program Officer. A PI or organization that makes financial or personnel commitments in the absence of a grant or cooperative agreement signed by a NSF Grants Officer does so at its own risk.

F. NSF's Risk Management Framework and the Decision to Award or Decline Proposals

If the program recommendation is for an award and final Division/Office or other programmatic approval is obtained, then the recommendation goes to the Division of Grants and Agreements (DGA) or the Division of Acquisition and Cooperative Support (DACS) for review of business, financial and policy implications. After the completion of this review, a final decision will be made to fund or decline the proposal.

1. NSF Risk Management Framework. Consistent with 2 CFR §200.205, NSF's risk-based framework evaluates the risks posed by proposers prior to issuance of an NSF award. This framework includes, but is not limited to, the following:

- conducting pre-award financial and administrative reviews for all proposers recommended for award that have not managed NSF funding in the last five years and whose NSF funding would exceed $225,000;

- conducting pre-award financial and administrative reviews for organizations who have received prior NSF awards whose cumulative NSF funding amount would exceed $225,000 should the proposal recommended for award be funded;

- conducting pre-award financial and administrative reviews for all proposers recommended for Phase II funding under the Small Business Innovation Research (SBIR) or Small Business Technology Transfer (STTR) program;

- considering the proposer's record of how it has managed past and current Federal awards, and leveraging NSF systems and to identify any ongoing issues that need to be considered before proceeding with future awards;

- considering the status of corrective actions necessary to address findings or concerns noted in audits, desk reviews, site visits, or other monitoring activities of the proposer's past and current Federal awards; and

- ensuring that NSF conducts review of information available through any OMB-designated repositories and that no awards are made to proposers that are currently suspended or debarred or otherwise ineligible for participation in Federal programs or activities.

DGA generally makes the decision to award or decline proposals within 30 days after the program Division/Office makes its recommendation. Proposals from organizations that have not received an NSF award within the preceding five years, involve special situations (such as coordination with another Federal agency or a private funding source), cooperative agreements, or unusual arrangements, may require additional review and processing time. NSF will report proposals that are declined for reasons that meet the guidelines set forth by OMB to the OMB-designated integrity and performance system in accordance with Federal regulation, but only after the proposer has had an opportunity to exhaust the review procedures contained in Chapter III.F.2 below.

2. Process to Appeal NSF's Decision to Decline a Proposal for Financial or Administrative Reasons

a. Background

A proposer who has been declined for an NSF award for reasons related to NSF's pre-award financial and administrative reviews will be afforded the opportunity to discuss the decline decision with the cognizant NSF Grants Officer or Branch Chief in DGA or DACS. If, after obtaining further clarification from the cognizant NSF Grants Officer or Branch Chief in DGA or DACS, the proposer believes that NSF made a substantive or procedural error in arriving at its decision to decline an award, the proposer may submit a request for review to the cognizant Division Director of DGA or DACS. The decision made by the cognizant Division Director of DGA or DACS is final.

Award of NSF assistance is discretionary. A formal hearing, therefore, is not provided.

b. Applicability

Chapter III.F.2.c contains the process by which proposers may appeal a DGA or DACS Branch Chief's decision to decline an NSF award arising from NSF's pre-award financial and administrative reviews. This process does not apply to decisions to return or decline a proposal for any other reason.

Proposals declined for administrative or financial reasons are not eligible for reconsideration under Chapter IV.D.

c. Procedures

1. Proposers who are declined for an NSF award for financial or administrative reasons will be identified as such in the declination notice. A proposer who disagrees with NSF's decision should first contact the cognizant NSF Grants Officer or Branch Chief in DGA or DACS, who will afford the proposer an informal opportunity to obtain further clarification.

2. If dissatisfied with the explanation provided by the cognizant NSF Grants Officer or Branch Chief in DGA or DACS, the proposing organization's AOR may submit a request for review to the cognizant Division Director of DGA or DACS. The letter must be addressed to the Division Director, Division of Grants and Agreements (DGA), or the Division Director, Division of Acquisition and Cooperative Support (DACS) as appropriate, and must be received by the Foundation within 30 days after the date of the declination notice. The time for filing a request for review is strictly enforced and no extensions for the purpose of preparing it will be granted.

3. The request for review need not follow any prescribed format. However, it must contain a full statement of the proposer's position with respect to the disputed matter, as well as the facts and reasons supporting the proposer's position that the declination was unwarranted. The request may address any errors made in the financial and administrative review process, and it may contain supporting documentation that was not originally presented as part of the financial and administrative review process. However, NSF will not consider any new information that would not have been available at the time the decision to decline was made. Therefore, new information presented in relation to the proposer's financial stability or the quality of its management systems will not be considered.

4. The Division Director, DGA or DACS, will review or designate one or more individuals to review the matter. In no case will the review be undertaken by any individual involved with the decline decision. The reviewing official(s) may request additional information from the proposer, but only information that would have been available at the time the decision to decline was made will be considered.

5. The designated reviewing official(s) will, within 30 days of NSF's receipt of the request for review, forward a report to the Division Director, DGA or DACS or his/her designee for a final written decision for the agency. The Division Director, DGA or DACS or his/her designee will communicate the decision in

writing to the proposer, normally within 15 days of receipt of the report, unless otherwise specified by NSF. The decision made by the Division Director, DGA or DACS, is final.

G. Review Information Provided to PI

When a decision has been made (whether an award or a declination), the following information is released electronically to the PI:

- description of the context in which the proposal was reviewed;
- copies of all reviews used in the decision (with any reviewer-identifying information redacted);
- copy of panel summary, if the proposal was reviewed by a panel at any point in the process; and/or
- site-visit reports, if applicable.

In addition, if not otherwise provided in the panel summary, the PI is provided an explanation (written or telephoned) of the basis for the declination. A PI also may request and obtain any other releasable material in NSF's file on his/her proposal. Everything in the file, except information that identifies either reviewers or other pending or declined proposals is usually releasable to the PI.

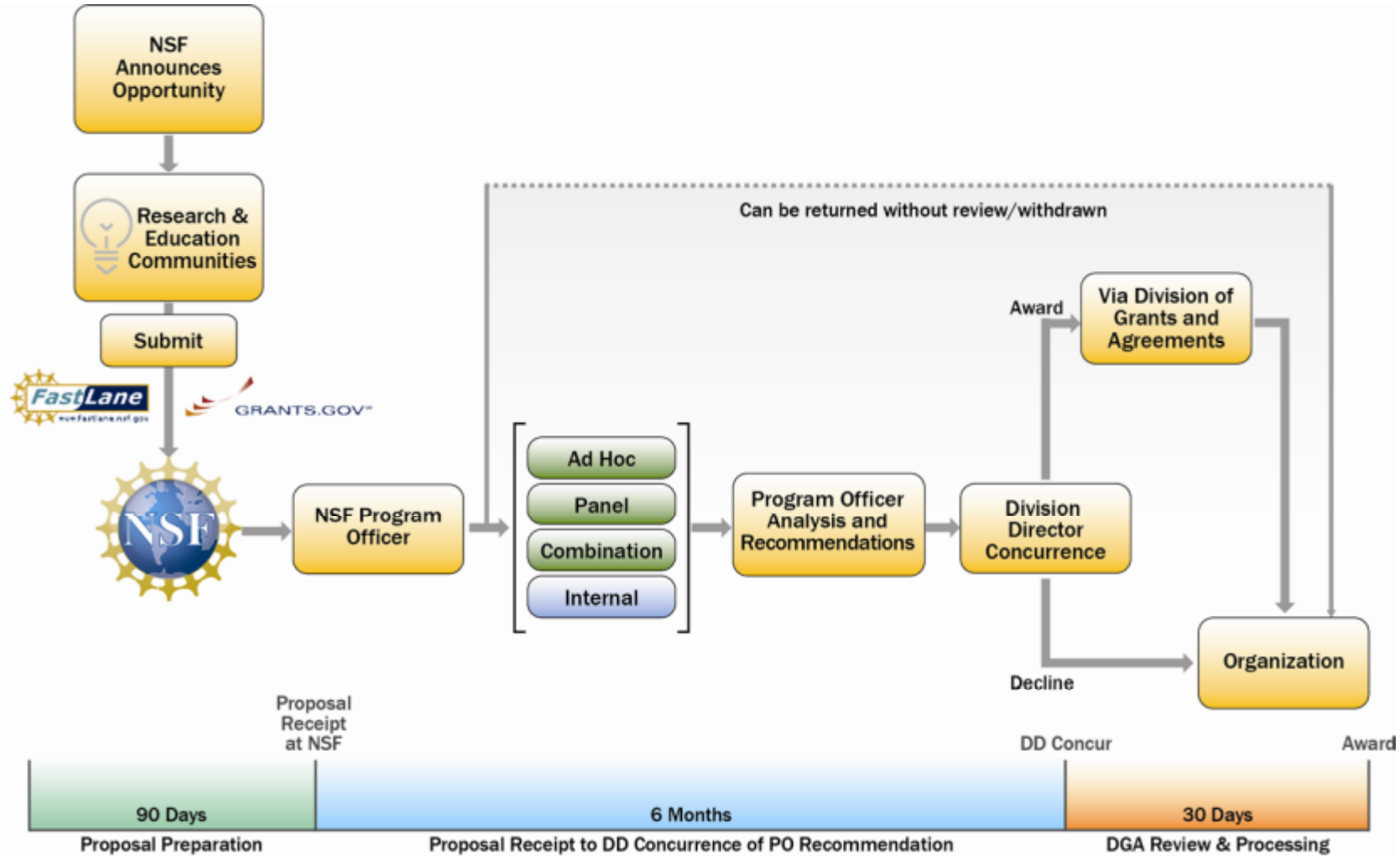
Reviews are made available directly to the PI, to provide feedback for the purpose of improving proposed research and research methods, and to assist in preparation of future proposals. They are not intended for any other purpose.

H. Release of Grantee Proposal Information

A proposal that results in an NSF award will be available to the public on request, except for privileged information or material that is personal, proprietary or otherwise exempt from disclosure under law. Appropriate labeling in the proposal aids identification of what may be specifically exempt. (See Chapter II.D.1) Such information will be withheld from public disclosure to the extent permitted by law, including the Freedom of Information Act. Without assuming any liability for inadvertent disclosure, NSF will seek to limit disclosure of such information to its employees and to outside reviewers when necessary for merit review of the proposal, or as otherwise authorized by law.

Portions of proposals resulting in grants that contain descriptions of inventions in which either the Government or the grantee owns a right, title, or interest (including a non-exclusive license) will not normally be made available to the public until a reasonable time has been allowed for filing patent applications. NSF will notify the grantee of receipt of requests for copies of funded proposals so the grantee may advise NSF of such inventions described, or other confidential, commercial or proprietary information contained in the proposal.

Exhibit III-1: NSF Proposal & Award Process & Timeline



Chapter IV: Non-Award Decisions and Transactions

A. Proposal Withdrawal

A proposal may be withdrawn at any time before a funding recommendation is made by the cognizant NSF Program Officer. FastLane automates the proposal withdrawal process and provides a mechanism that will help organizations to more effectively manage their proposal portfolio, as well as to help eliminate the submission of duplicate proposals to NSF. The Withdrawals Module includes three processes:

- *Principal Investigator's Proposal Withdrawal* allows a PI to initiate a proposal withdrawal and forward it to the organization's AOR for submission to NSF.

- *Sponsored Projects Office (SPO) Proposal Withdrawal* allows an authorized individual in the organization's SPO to initiate a proposal withdrawal and forward it to the AOR for submission to NSF.

- *Proposal Submission Duplicate Withdrawal* prevents the AOR from submitting a new proposal if a duplicate (a proposal from the same organization with the same title and same PI and co-PIs) already has been submitted to NSF within the last two weeks prior to the current submission. If these conditions are met, the system will allow the AOR to either withdraw the previous duplicate proposal, and then proceed with the submission of the new proposal, or to modify the new proposal so it is different from the previous proposal.

Authorized individuals[42] can initiate or review a proposal withdrawal using the " Proposals/File Updates/Supplements/Withdrawals" Module via the FastLane "Research Administration Functions".[43]

In cases where NSF already has made a funding decision, proposals will not be permitted to be withdrawn via the electronic proposal withdrawal system. When a PI or other authorized official attempts to prepare a proposal withdrawal for such a proposal, a message will be displayed to contact the cognizant NSF Program Officer for further assistance.

NSF must be notified if any funding for the proposed project is received from another source or sponsor. If it is brought to NSF's attention that funding for a proposal to NSF has been accepted from another sponsor, NSF will send a withdrawal confirmation to the PI and the SPO without waiting for the official withdrawal notification.

If a proposal withdrawal is submitted for a proposal that is part of a collaborative effort, regardless of whether the organization is the lead or non-lead, the electronic proposal withdrawal system will withdraw that proposal along with the other remaining proposals that are identified as part of the collaborative effort. If the remaining organizations in the collaborative determine that the project can still proceed, a new collaborative proposal must be submitted.

Copies of reviews received by NSF before a proposal is withdrawn will be provided to the PI. NSF provides notice of a withdrawal, return, declination, or reconsideration to both the PI and the SPO.

[42] AORs also can initiate a proposal withdrawal.
[43] Detailed instructions are available in FastLane at: https://www.fastlane.nsf.gov/documents/epw/epw.jsp.

B. Proposal Not Accepted or Returned Without Review

A proposal will not be accepted[44] or will be returned without review by NSF for the following reasons.

The proposal:

1. is inappropriate for funding by the National Science Foundation (see Chapter I.B);
2. is submitted with insufficient lead-time before the activity is scheduled to begin;
3. is a full proposal that was submitted by a proposer that has received a "not invited" response to the submission of a preliminary proposal (see Chapter I.D.2. and Chapter I.D.2.a);
4. is a duplicate of, or substantially similar to, a proposal already under consideration by NSF from the same submitter (see Chapter I.G.2);
5. does not meet NSF proposal preparation requirements, such as page limitations, formatting instructions, and electronic submission, as specified in Part I of the *Proposal and Award Policies and Procedures Guide* (see Chapters II.A, II.B, and II.C), the NSF *Grants.gov Application Guide,* or program solicitation;
6. is not responsive to the NSF funding opportunity;
7. does not meet an announced proposal deadline date;
8. was previously reviewed and declined and has not been substantially revised (see Chapter IV.E);
9. duplicates another proposal that was already awarded; and/or
10. does not contain each of the required sections of the proposal, as described in Chapter II.C.2.

C. Declinations

A PI whose proposal for NSF support has been declined will receive information and an explanation of the reason(s) for declination along with copies of the reviews considered in making the decision. If that explanation does not satisfy the PI, he/she may request additional information from the cognizant NSF Program Officer or Division Director. See Chapter III.G for additional information.

PIs and co-PIs may access review information from NSF after the decision has received the concurrence of the cognizant NSF Division Director, when all the review information has been released for their proposal.

D. Reconsideration

1. Overview

a. A proposer whose proposal has been declined may ask the cognizant NSF Program Officer or the cognizant NSF Division Director for information over and above the explanatory materials received with the declination notice. If the PI/PD is not satisfied that the proposal was fairly handled and reasonably reviewed, he/she may request reconsideration by the cognizant Assistant Director (AD) or Office Head. An organization (or an unaffiliated PI/PD) still not satisfied after reconsideration by the cognizant AD/Office Head may request further reconsideration by the Deputy Director of the Foundation. The decision made by the Deputy Director is final.

b. If a proposal has been declined after review by the NSB, only an explanation will be available.

c. The aim of any reconsideration is to ensure that NSF's review has been fair and reasonable, both substantively and procedurally. The scientific and technical merits may be examined within the context of budget availability and program priorities. Reconsideration also may address any procedural errors in peer review or other aspects of proposal review, including unaccounted-for conflict of interests or inappropriate consideration of records, information or rumor.

[44] Proposal Not Accepted is defined as FastLane will not permit submission of the proposal.

d. Award of NSF assistance is discretionary and reconsideration is not an adversarial process. A formal hearing, therefore, is not provided. Because factors such as program budget and priorities factor into the decision on a proposal, NSF cannot ensure proposers that reconsideration will result in an award even if error is established in connection with the initial review.

e. No revisions made to the proposal after declination will be considered in connection with the original proposal. A substantially revised proposal, however, may be submitted for review as a new proposal under standard procedures. NSF reserves the right to return without review a proposal that is substantially the same as one that was previously reviewed and declined whether or not a request for reconsideration was made.

2. Applicability

NSF's reconsideration process is available to individuals and organizations concerning proposals for grant funding. It does not apply to:

a. "discourage" (i.e., non-binding) decisions resulting from submission of a preliminary proposal;

b. proposals for:

(1) fellowships;
(2) travel grants;
(3) Rapid Response Grants (RAPID);
(4) EArly-concept Grants for Exploratory Research (EAGER);
(5) Research Advanced by Interdisciplinary Science and Engineering (RAISE); or
(6) Phase I proposals submitted under the Small Business Innovation Research (SBIR) program.

c. proposals returned without review by NSF for failure to:

(1) be submitted with sufficient lead time before the activity is to begin;
(2) meet an announced proposal deadline date; or
(3) meet NSF proposal preparation requirements, such as page limitations, formatting instructions, and electronic submission, as specified in Part I of the PAPPG*,* the NSF *Grants.gov Application Guide,* or program solicitation.

3. Reconsideration Process

The following paragraphs highlight the various stages of the NSF Reconsideration Process, including the necessary procedural aspects of each stage of the process.

a. Explanations by the NSF Program Officer or Division Director

When a proposal is declined, the PI/PD receives verbatim but unattributed copies of any *ad hoc* reviews and the panel summary (if applicable), a description of how the proposal was reviewed, and, if not otherwise provided in the panel summary, an explanation (written or telephoned) of the basis for the declination. A returned proposal also will be accompanied by an explanation. A PI/PD who is considering asking for reconsideration should first contact the cognizant NSF Program Officer or Division Director, who will afford the PI/PD an informal opportunity to seek further clarification.

b. Reconsideration by the Cognizant NSF Assistant Director

(1) If dissatisfied with the explanation provided by the NSF Program Officer or Division Director, the PI/PD may request in writing that NSF reconsider its action. Such a request will be considered only if the PI/PD has first sought and obtained further clarification from the cognizant NSF Program Officer or Division Director, and only if the request is received by the Foundation within 90 days after the declination or the

return. The request should be addressed to the AD/Office Head for the Directorate or Office that handled the proposal and should explain why the PI/PD believes that the declination or return was unwarranted.

(2) The AD/Office Head will reconsider the record to determine whether NSF's review of the declined proposal was fair and reasonable, substantively and procedurally, taking into account availability of funds and the policies and priorities of the program and NSF. In the case of a returned proposal, the record will be reviewed to determine whether the proposed project was inappropriate for NSF consideration. The AD/Office Head may request additional information from the PI/PD and may obtain additional reviews. If additional reviews are sought, they are subject to standard review procedures (e.g., instructions must be provided to reviewers and conflicts-of-interest policies must be followed). The AD/Office Head may conduct the reconsideration personally or may designate another NSF official who had no part in the initial review to do so. As used here, "AD/Office Head" includes such a designated official.

(3) Within 45 days after the date of the request, the AD/Office Head will furnish the results of the reconsideration, in writing, to the PI/PD. If results cannot be furnished within 45 days, the AD/Office Head will send the PI/PD a written explanation of the need for more time, indicating the date when the results can be expected. If the AD/Office Head reaffirms the declination or return, he/she will inform the PI/PD that the PI/PD's organization may obtain further reconsideration by the Deputy Director of NSF as provided below.

c. Further Reconsideration by the NSF Deputy Director

(1) Within 60 days after the AD/Office Head has notified the PI/PD of the results of the reconsideration, the proposing organization or an unaffiliated PI/PD may request further reconsideration by the Deputy Director of NSF.

(2) A request for further reconsideration need not be in any particular format, but it must be in writing, and must be signed by the organization's president or other chief executive officer and by the PI/PD. For declinations, it should explain why the organization believes that an error may have occurred in the initial evaluation and why it is not entirely satisfied with the reconsideration by the cognizant AD/Office Head. For returned proposals, it should explain why the organization believes that an error may have occurred in the initial determination that the proposal was inappropriate for NSF consideration.

(3) The Deputy Director will review the request for further reconsideration and the record of earlier NSF actions, including the original review and the reconsideration by the AD/Office Head, to determine whether NSF's review of the declined proposal was fair and reasonable, or, in the case of a returned proposal, whether the proposed project was inappropriate for NSF consideration. The Deputy Director may request additional information from the PI/PD or the proposing organization and may obtain additional reviews. If additional reviews are sought, they are subject to standard review procedures (e.g., instructions must be provided to reviewers and conflicts-of-interest policies must be followed).

(4) The Deputy Director may conduct the further reconsideration personally or may designate another NSF official who had no part in the initial evaluation of the proposal or the earlier reconsideration to do so. As used here, "Deputy Director" includes such a designated official.

(5) Within 30 days after a request for further reconsideration is received at NSF, the Deputy Director will furnish the results of the further reconsideration, in writing, to the organization. If results cannot be furnished within 30 days, the Deputy Director will send the organization a written explanation of the need for more time, indicating the date when the results can be expected.

(6) The decision made by the Deputy Director is final.

E. Resubmission

A declined proposal may be resubmitted, but only after it has undergone substantial revision. A resubmitted proposal that has not clearly taken into account the major comments or concerns resulting from the prior NSF review may be returned without review. The Foundation will treat the revised proposal as a new proposal, subject to the standard review procedures.

Chapter V: Renewal Proposals

A renewal proposal is a request for additional funding for a support period subsequent to that provided by a standard or continuing grant. A renewal proposal competes with all other proposals and must be developed as fully as though the proposer is applying for the first time. Renewal proposals must be submitted at least six months before additional funding is required or consistent with an established deadline, target date or submission window. In preparing a renewal proposal, proposers should assume that reviewers will not have access to previously submitted versions of the proposal. Please note the National Science Board strongly endorses the principle that all expiring awards are to be recompeted.[45]

All proposals for renewed support of research projects, from academic institutions *only*, must include information on human resources development at the postdoctoral, graduate and undergraduate levels as part of Results from Prior NSF Support.[46] This may involve, but is not limited to, the role of research in student training, course preparation and seminars (particularly for undergraduates). Special accomplishments in the development of professional scientists and engineers from underrepresented groups should be described. Graduate students who participated in the research should be identified by name. This requirement does not apply to non-academic organizations.

PIs are encouraged to discuss renewal proposals with the program prior to submission of a proposal. Unless precluded by individual program requirements, PIs may choose either of the following two formats for preparation of a renewal proposal.

A. Traditional Renewal. The “traditional” renewal proposal is developed as fully as though the proposer were applying for the first time. It covers all the information required in a proposal for a new project, including Results from Prior NSF Support. The 15-page limitation on the Project Description applies.

B. Accomplishment-Based Renewal. In an "Accomplishment-Based Renewal" (ABR) proposal, the Project Description (including the Results from Prior NSF Support) is replaced with the following items:

1. copies of no more than six reprints[47] of publications resulting from the research supported by NSF (including research supported by other sources that is closely related to the NSF-supported research) during the preceding period of NSF support. Of the six publications, two preprints (accepted for publication) may be included;

2. information on human resources development at the postdoctoral, graduate and undergraduate levels; and

3. a brief summary (not to exceed four pages) of plans for the proposed support period.

All other information required for NSF proposal submission remains the same.

---

[45]Reference National Science Board Resolution (NSB-2015-45) entitled, *National Science Board Statement on Recompetition of Major Facilities.* For the operation of a major facility, the National Science Board has endorsed the principle that NSF should perform a rigorous review prior to the end date of the award to determine whether it is in the best interest of US science and engineering to recompete that award (Reference NSB-2015-46).

[46]This requirement applies to both types of renewal proposals: Traditional Renewal and Accomplishment-Based Renewal.

[47]Reprints should be provided as supplementary documentation and should be submitted via the Proposal Preparation Module in FastLane.

It must be clearly indicated in the proposal that it is an ABR submission and the box for "Accomplishment-Based Renewal" must be checked on the Cover Sheet. ABR proposals may not be submitted for consecutive renewals.

PIs are advised that the ABR is a special type of renewal proposal appropriate only for an investigator who has made significant contributions, over a number of years, in the area of research addressed by the proposal. Investigators are strongly urged to contact the cognizant NSF Program Officer prior to developing a proposal using this format.

Descriptions of other forms of additional funding support, including continuing grants and supplemental funding requests, are contained in Chapter VI.

PART II: AWARD, ADMINISTRATION AND MONITORING OF GRANTS AND COOPERATIVE AGREEMENTS

Part II of the NSF *Proposal & Award Policies & Procedures Guide* sets forth NSF policies and procedures regarding the award, administration, and monitoring of grants and cooperative agreements. Coverage includes the NSF award process, from issuance and administration of an NSF award through closeout. Guidance is provided regarding other grant requirements or considerations that either are not universally applicable or do not follow the award cycle. Part II also implements other Public Laws, Executive Orders (E.O.) and other directives insofar as they apply to grants, and is issued pursuant to the authority of Section 11(a) of the NSF Act (42 USC §1870). When NSF Grant General Conditions or an award notice reference a particular section of the PAPPG, then that section becomes part of the award requirements through incorporation by reference.

Chapter VI: NSF Awards

A. Acceptance of Assistance Agreements

Grantees[48] are free to accept or reject the grant as awarded. Normally, a request to drawdown NSF funds constitutes acceptance, however, in limited circumstances, NSF may require formal acceptance of a grant. If the grantee chooses not to accept the award, then a written request should be sent to the cognizant NSF Program Officer to withdraw (or terminate) the grant.

B. Award Instrument

1. Composition of an NSF Award

Composition of an NSF award includes:

a. the award notice, including any special conditions applicable to the award and any numbered amendments thereto;

b. general Federal award information as required by 2 CFR § 200.210;

c. the budget, which indicates the amounts, by categories of expense, on which NSF has based its support;

d. the applicable NSF general conditions referenced in the award notice;

e. the proposal referenced in the award notice; and

f. any NSF program announcement, program solicitation or other documents or special requirements incorporated by reference in the award notice.

2. Award Transmission

NSF transmits award notices to organizations via e-mail. In addition to the e-mail notification, grantees and PIs also may access NSF award notices via use of NSF's electronic systems. SPOs are able to view, print and/or download NSF award notices for their organizations. PIs also may access their award notices via FastLane.

[48] For purposes of this Guide, except where explicitly noted, the term "grant" is interchangeable with the term "cooperative agreement", and the term "grantee" is interchangeable with the "awardee" of a cooperative agreement.

C. NSF Award Conditions

Each NSF award notice specifically identifies certain conditions that are applicable to, and become part of, that award. The award conditions are available electronically on NSF's website at: http://www.nsf.gov/awards/managing/. When these conditions reference a particular PAPPG section, that section becomes part of the award requirements through incorporation by reference.

D. NSF Grant Periods

1. Definitions

a. START DATE is the date specified in the award notice on or after which, except for fixed amount awards, expenditures may be charged to the grant. With the exception of PI transfers, the start date used by NSF is either the 1st or the 15th day of the month. If no start date is specified, then the date of the award notice is the start date. (For preaward expenditures, Chapter X.A.2.b.)

b. END DATE is the date specified in the award notice after which expenditures may not be charged against the grant except to satisfy obligations to pay allowable project costs committed on or before that date. The end date is the last day of a month.

c. AWARD DATE is the date when the NSF award is signed by the cognizant NSF Grants Officer.

d. GRANT PERIOD is the period of time between the start date and the end date of an NSF award shown as the duration.

2. Significance of Grant Period

a. Except in fixed amount awards, an NSF grant gives authority to the grantee to commit and expend funds for allowable costs (see Chapter X) in support of the project up to the grant amount specified in the award notice at any time during the grant period.

b. Except as provided in Chapter X.A.2.b or Chapter X.A.2.c, expenditures may not be charged prior to the start date or subsequent to the end date under an NSF cost reimbursement grant.

3. Changes in a Grant Period

a. Start Date

Once an award is made, the start date cannot be changed; however, grantees have the authority to incur pre-award costs as outlined in Chapter X.A.2.b.

b. End Date

The end date may be changed as a result of approval of a request for continued support of a continuing grant, for a no-cost grant extension, or by approval of a request for supplemental support. When appropriate, the NSF Grants Officer will issue an amendment to the grant.

c. No-Cost Extension

(i) Grantee-Approved Extension. Grantees may authorize a one-time extension of the end date of the grant of up to 12 months if additional time beyond the established end date is required to assure adequate completion of the original scope of work within the funds already made available. This one-time extension may not be exercised merely for the purpose of using the unliquidated balances. Grantees are not authorized to extend an award that contains a zero balance. The grantee shall notify NSF, providing supporting reasons for the extension and the revised period of performance, at least ten calendar days prior

to the end date specified in the grant to ensure accuracy of NSF's grant data. All grantee-approved extension notifications must be signed and submitted by the AOR via use of NSF's electronic systems.[49] For grantee-approved extensions, no amendment will be issued. The revised end date can be viewed via NSF's electronic systems.

(ii) NSF-Approved Extension

(a) If additional time beyond the extension provided by the grantee is required and exceptional circumstances warrant, a formal request must be signed and submitted by the AOR via use of NSF's electronic systems. The request should be submitted to NSF at least 45 days prior to the end date of the grant. All late requests must include a strong justification as to why it was not submitted earlier. The request must explain the need for the extension and include an estimate of the unobligated funds remaining and a plan for their use. As indicated above, that unobligated funds may remain at the end of the grant is not in itself sufficient justification for an extension. The plan must adhere to the previously approved objectives of the project. Such requests must be signed and submitted by the AOR via use of NSF's electronic systems.

(b) The first no-cost extension request will be considered for approval by the cognizant NSF Program Officer. The grantee and the PI will be electronically notified of the disposition of this request by the cognizant NSF Program Officer (an amendment to the grant will not be issued). The second (or any subsequent) no-cost extension request will be subject to the approval of an NSF Grants Officer, and, if approved, will be in the form of an amendment to the grant specifying a new end date. Grantees are cautioned not to make new commitments or incur new expenditures after the end date of the award in anticipation of a no-cost extension. In addition, grantees must be aware that most NSF appropriated funds have a limited period of availability for expenditure before the appropriation cancels. No-cost extensions do not extend the period of availability for canceling funds[50].

d. Two-Year Extensions for Special Creativity

A Program Officer may recommend the extension of funding for certain research grants beyond the initial period for which the grant was awarded for a period of up to two years. The objective of such extensions is to offer the most creative investigators an extended opportunity to attack adventurous, "high-risk" opportunities in the same general research area, but not necessarily covered by the original/current award. Awards eligible for such an extension are generally continuing grants. Special Creativity Extensions are normally initiated by the NSF Program Officer based on progress during the first two years of the grant; PIs will be informed of such action a year in advance of the end date of the grant. In response to the Program Officer's recommendation, the required information must be signed and submitted by the AOR via use of NSF's electronic systems.

E. Additional Funding Support

1. Types of Additional Funding Support

Additional funding of a project beyond the original grant period will be in the form of renewed support, continued support or supplemental support. A description of each of these additional funding mechanisms is provided below.

---

[49] NSF has developed a matrix that lists NSF's electronic capabilities, and whether they can be found in FastLane, Research.gov or both. This matrix will be updated as appropriate, independent of the *Proposal & Award Policies & Procedures Guide* revision cycle. The current version is located at: http://www.nsf.gov/bfa/dias/policy/estatus_matrix.jsp.

[50] In accordance with 31 USC 1552(a), funds will no longer be available for expenditure for any purpose beyond September 30th of the fifth fiscal year after the expiration of a fixed appropriation's period of availability for incurring new obligations – see also Chapter VIII.E for Financial Requirements and Payments.)

2. Renewed Support

Renewed support is defined as additional funding for a support period subsequent to that provided by the original grant. Renewals to grants, if any, will be in the form of a new grant with a new grant number. Costs incurred under the old grant cannot be transferred to the new grant. Residual funds remaining in the old grant cannot be transferred to the new grant.

Instructions for preparation of renewal proposals are contained in Chapters II and V.

3. Support under Continuing Grants

a. Funding increments for projects being supported under continuing grants receive high priority within NSF and normally are not considered in competition with proposals for new grants or for grants for renewed support.

b. Unless otherwise provided for in the original award notice, each increment of a continuing grant will be funded at the level indicated in the original award notice without a formal request, subject to NSF's judgment of satisfactory progress, availability of funds, and receipt and approval of the required annual report. NSF makes every attempt to honor continuing grant commitments. In order to adjust to changes in the general level of funds for a particular field of science or engineering or to major new opportunities in that field, however, NSF may reduce continuing grant increments below the levels indicated in original award notice. This requires full written justification by program staff and management review and approval. In the absence of major unanticipated fiscal year constraints, reductions are rare. Continuing grant increments will be released by the cognizant NSF Program Officer upon approval of the annual report. The SPO and PI will be notified by the cognizant NSF Program Officer of NSF's approval via email.

c. In order to obtain a committed funding increment and ensure continuity of funding, an annual project report must be submitted by the PI electronically via use of Research.gov no later than 90 days prior to the end of the current budget period. (See Chapter VII.D for additional information on submission of project reports.)

4. Supplemental Support

a. In unusual circumstances, small amounts of supplemental funding and up to six months of additional support may be requested to assure adequate completion of the original scope of work. Such requests for supplemental funding support must be signed and submitted by the AOR via use of NSF's electronic systems at least two months prior to the need for the additional funds and must be adequately justified. Program Officers may make decisions regarding whether or not to recommend a small supplement without merit review of the supplemental request. Requests for larger supplements may require external merit review.

b. A request for supplemental support must be submitted electronically via FastLane and must include:

(i) A summary of the proposed work;

(ii) A justification of the need for the supplemental funds; and

(iii) A budget and budget justification, highlighting the use by budget category of the additional funding as distinguished from the original funding provided in those categories of cost. AORs are required to electronically sign the supplemental funding request via the Authorized Organizational Representative Functions in FastLane.

c. NSF will not approve requests for supplemental support for such purposes as defraying the costs of increases in salaries, wages or staff benefits or for additional indirect cost (F&A) reimbursement, whether

caused by a change in the indirect cost rate or by changes in direct cost expenditures which affect the indirect cost base. (See Chapter X.A.2.a.)

d. If approved, the NSF Grants Officer will amend the grant to provide additional funding for the current support period. The amendment notice will specify both the amount of supplemental funding and the cumulative amount awarded through the end date, which normally will remain unchanged.

e. Special NSF programs such as *Research Experiences for Undergraduates* may provide their funding through supplements to other NSF grants. In such instances, the guidance in this section may not be applicable. Please see the applicable NSF funding opportunity for the relevant guidance.

Chapter VII: Grant Administration

A. Monitoring Project Performance

1. Grantee Responsibilities

a. The grantee has full responsibility for the conduct of the project or activity supported under an NSF grant and for the results achieved. The grantee should monitor the performance of the project to assure adherence to performance goals, time schedules or other requirements as appropriate to the project or the terms and conditions of the grant. In order to carry out these responsibilities, each grantee organization shall agree to comply with the applicable Federal requirements for grants and to the prudent management of all expenditures and actions affecting the grant. Documentation for each expenditure or action affecting the grant shall reflect appropriate organizational reviews or approvals, which should be made in advance of the action. Organizational reviews are intended to help assure that expenditures are allowable, necessary and reasonable for the conduct of the project, and that the proposed action:

(i) is consistent with grant terms and conditions;

(ii) is consistent with NSF and grantee policies;

(iii) represents effective utilization of resources; and

(iv) does not constitute changes in objectives or scope.

b. Notwithstanding these responsibilities, NSF continues to encourage communication between NSF Program Officers and PI/PDs on the progress of projects supported by NSF as well as on project changes.

c. NSF, through authorized representatives, has the right, at all reasonable times, to make site visits to review project accomplishments, grantee management control systems and administration and management of the grant and to provide technical assistance as may be required. If any site visit is made by the Foundation on the premises of the grantee or a subrecipient under a grant, the grantee shall provide and shall require its subrecipients to provide all reasonable facilities and assistance for the safety and convenience of the NSF representatives.

2. Grantee Notifications to NSF

The following is a listing of grantee notifications to NSF, including a reference to where additional guidance is provided. All notifications must be submitted via use of NSF's electronic systems. With the exception of significant changes in methods or procedures and significant changes, delays or events of unusual interest, all notifications must be signed and submitted by the AOR via use of NSF's electronic systems.

• Grantee-Approved No-Cost Extension	VI.D.3.c(i)
• Significant Changes in Methods or Procedures (Other than Changes in Objectives or Scope)	VII.B.1.b
• Significant Changes, Delays or Events of Unusual Interest (Other than Changes in Objectives or Scope)	VII.B.1.c
• Annual and Final Cost Share Notification by Recipient	VII.C.3
• Conflicts of Interest that cannot be satisfactorily managed, reduced or eliminated and research that proceeds without the imposition of conditions or restrictions when a conflict of interest exists	IX.A

Certain actions require prior approval from NSF. Guidance regarding prior approval requirements is covered in Chapter X.A.3, and the NSF column of the Research Terms and Conditions, Appendix A.

B. Changes in Project Direction or Management

1. Changes in Objectives, Scope or Methods/Procedures

a. Changes in Objectives or Scope

The objectives or scope of the project may not be changed without prior NSF approval. Such change requests must be signed and submitted by the AOR via use of NSF's electronic systems. If approved by NSF, the Grants Officer will amend the grant. Prior written NSF approval also is required for changes to the Facilities, Equipment and Other Resources section of the approved proposal that would constitute changes in objectives or scope (see Chapter II.C.2.i for further information).

b. Significant Changes in Methods or Procedures

NSF believes that the PI/PD and co-PI/co-PD, operating within the established policies of the grantee organization, should feel free to pursue interesting and important leads that may arise during the conduct of a research (or other grant-supported) project or to adopt an alternative approach which appears to be a more promising means of achieving the objectives of the project. Significant changes in methods or procedures should be reported to appropriate grantee official(s). The PI/PD also must notify NSF via use of NSF's electronic systems.

c. Significant Changes, Delays or Events of Unusual Interest

In the event there are problems, delays or adverse conditions that will materially impact the ability to attain the objectives of the project or to meet such time schedules as may have been proposed, the PI/PD should notify the appropriate grantee official(s). The PI/PD also must notify NSF via use of NSF's electronic systems.

NSF should be informed of any events of unusual interest that occur during the course of the project. Reports, communications or photographs should be submitted via the interim report capability in Research.gov.

2. Changes in PI/PD, co-PI/co-PD or Person-Months Devoted to the Project

The NSF decision to support a proposed project is based to a considerable extent upon its evaluation of the proposed PI/PD and any identified co-PI/co-PD's knowledge of the field of study and his/her capabilities to conduct the project in an efficient and productive manner. This is reflected in the NSF merit review criteria (see Chapter III). The named PI/PD (and co-PI/co-PD) should be continuously responsible for the conduct of the project and be closely involved with the effort.

If the grantee determines that there is a need for the addition of a new co-PI/co-PD, or the current PI/PD or co-PI/co-PD plans to, or becomes aware that he/she will: (i) devote substantially less effort to the project than anticipated in the approved proposal; (ii) sever his/her connection with the grantee organization; or (iii) otherwise relinquish active direction of the project, he/she shall advise the appropriate official at the grantee organization, who shall initiate action appropriate to the situation under the guidelines that follow.

a. Long-Term Disengagement of PI/PD or co-PI/co-PD

(i) In the event the PI/PD or co-PI/co-PD will be disengaged from the project for a period greater than three months (e.g. sabbatical leave) but intends to return, arrangements for oversight of the project must be signed and submitted by the AOR via use of NSF's electronic systems. This information must be provided at least 30 days before departure or as soon as practicable after the prospective disengagement is known. The cognizant NSF Program Officer will provide written approval to the grantee if the arrangements are satisfactory, but no formal amendment to the grant will be made. If the arrangements are not satisfactory to NSF, the grant may be terminated as prescribed in Chapter XII.A.

(ii) In the event the PI/PD or co-PI/co-PD will temporarily be working for NSF as an IPA (Intergovernmental Personnel Act employee) or VSEE (Visiting Scientist, Engineer or Educator), the appropriate officials at the

grantee organization **must** contact the cognizant NSF Program Officer for procedural guidance regarding any existing grants or pending proposals of the PI/PD or co-PI/PD.

b. Changes in Person-Months Devoted to the Project

If the PI/PD or co-PI/co-PD will devote substantially less time to the project than anticipated in the approved proposal, (defined in the applicable grant terms and conditions as a reduction of 25% or more in time) he/she should consult with the appropriate officials of the grantee organization. Requests for changes to the person-months devoted to the project must be signed and submitted by the AOR via use of NSF's electronic systems. If the grantee organization or NSF determines that the reduction of effort will substantially impair the successful execution of the project, the NSF Program Officer will consult the NSF Grants Officer. The NSF Grants Officer may:

(i) request the grantee to nominate a replacement PI/PD or co-PI/co-PD acceptable to the cognizant NSF Program Officer;

(ii) initiate the termination procedures described in Chapter XII.A.; or

(iii) negotiate an appropriate modification to the grant.

c. Addition of co-PI/co-PD

In the event the grantee desires to add a new co-PI/co-PD to a project[51], the AOR must sign and submit the request via use of NSF's electronic systems. The new co-PI/co-PD's name, biographical sketch, current and pending support from all ongoing projects and proposals, as well as a justification for the addition must also be included in the request. The contact information for the new co-PI/co-PD also should be included. If approved by NSF, the Grants Officer will amend the grant.

d. Withdrawal of PI/PD or co-PI/co-PD

In the event the PI/PD or co-PI/co-PD severs his/her connection with the grantee organization or otherwise relinquishes active direction of the project, the AOR must sign and submit a notification of the withdrawal of the PI/PD or co-PI/co-PD via use of NSF's electronic systems. The grantee also must:

(i) initiate transfer of the grant as described in Chapter VII.B.2.f;

(ii) nominate a substitute as described in Chapter VII.B.2.e; or

(iii) initiate grant closeout procedures through submission of final reports as described in Chapter VII.D.2.

e. Substitute (Change) PI/PD or co-PI/co-PD

In the event the grantee desires to continue the project with a substitute PI/PD or co-PI/co-PD, the AOR must sign and submit the request via use of NSF's electronic systems. The substitute PI/PD's name or co-PI/PD's name, biographical sketch, current and pending support from all ongoing projects and proposals, as well as a justification for the substitution must also be included in the request. The contact information for the substitute PI/PD or co-PI/co-PD also should be included. If approved by NSF, the Grants Officer will amend the grant. If not approved, NSF may take steps, pursuant to Chapter XII.A to suspend or terminate the grant.

In cases where a former NSF employee or IPA is being reappointed as PI or co-PI to an award they were previously involved with, the grantee must submit a "Change of PI" request using the FastLane Notifications and Requests module. The change in PI request should include documentation from the AOR designating a substitute negotiator for that award under the Associated Documents section. A co-PI request should designate the PI as the substitute negotiator.

[51] NSF's policy limits the number of co-PIs/co-PDs to four per award.

f. Disposition of a Grant When a PI/PD Transfers from One Organization to Another Organization

(i) Policy. When a PI/PD plans to leave an organization during the course of a grant, the organization has the prerogative to nominate a substitute PI/PD or request that the grant be terminated and closed out. In those cases where the PI/PD's original and new organizations agree, NSF will facilitate a transfer of the grant and the assignment of remaining unobligated funds to the PI/PD's new organization. This should normally be done with a tripartite agreement (involving NSF, the PI/PD's original organization and new organization), or by a subaward arrangement between the PI/PD's original and new organizations, subject to NSF's consent. (See Chapter VII.B.3)

(ii) Procedures. When a PI/PD plans to leave an organization during the course of a grant, the PI/PD or the SPO, shall notify the cognizant NSF Program Officer. If the project is to continue with the original organization, the cognizant NSF Program Officer should advise the grantee to nominate a substitute PI/PD (see Chapter VII.B.2.e). If the project is to be continued at the PI/PD's new organization, and if NSF and both organizations agree, formal notification of the impending transfer can be electronically initiated by either the PI/PD or the PI/PD's organization. The amount transferred has to be equal to or less than the unobligated balance. The request shall include a:

(a) brief summary of progress to date;

(b) description of work yet to be accomplished;

(c) completed on-line transfer request, including total disbursements and unpaid obligations to date (transfer amount will be automatically calculated, based on the amount entered in total disbursements). The original organization is responsible for including in the total estimated disbursements, any anticipated costs yet to be incurred against the original grant;

(d) detailed line item budget for the transfer amount and any outstanding continuing grant increments; and

(e) If funding is requested to support a postdoctoral researcher, then the request must include the requisite mentoring plan as described in Chapter II.C.2.j. The plan must be uploaded under "Mentoring Plan" in the Supplementary Documentation section of Fastlane. The PI must report on the mentoring activities provided to the individual(s) in annual and final project reports.

The original organization concurs with the transfer of the award by electronically forwarding the request to the new organization.

The new organization completes the request by providing a detailed budget for the transfer amount agreed to by both organizations. The AOR of the new organization must sign and submit the request via use of NSF's electronic systems. Submission of the request constitutes agreement by the new organization to assume responsibility for completion of the project effort and to administer the grant (as originally awarded) from the transfer date to completion in accordance with any special terms and conditions and the applicable general terms and conditions that normally govern NSF grants made to the new organization. Special terms and conditions, as appropriate, cited in the original award will convey to the new grantee organization.

NSF will assign a proposal number at the time of submission. This proposal number will become the new grant number when the transfer is approved by an NSF Grants Officer.

(iii) Fund Transfer. Upon receipt of the above material, NSF will review the request and, if approved, deduct the specified transfer amount from the original grant and re-establish it under a new grant number at the new organization. Award notification by the NSF Grants Officer will constitute NSF approval of the grant transfer. The award notification also will specify the applicable general terms and conditions that govern the grant.

(iv) Monetary Discrepancies. Upon transfer of the grant to the new organization, any monetary discrepancies must be resolved between the original and the new grantee. NSF will not intervene in any disputes between the two organizations regarding the transferred amount.

(v) Equipment Transfers. Equipment purchased with NSF funds for use in a specific project should remain available for use for the duration of the project. PI/PDs who are in the midst of projects that included funding for equipment and who will continue the project at a new organization with NSF support, should arrange with their original organization to have the equipment transferred with them. Shipping costs for such equipment may be charged to the original or transferred grant as an allowable cost. Budgets should not include funds to "buy" equipment that had been previously obtained with Federal funds.

(vi) Possible Alternatives to the Transfer Process. When the amount of time and funds remaining in a project are modest, and if both the original and new organizations are in agreement, the original organization may issue a subaward to the new organization for completion of the project. This and other possible alternatives should be discussed with the NSF Grants Officer.

3. Subawarding or Transferring Part of an NSF Award (Subaward)

Excluding the purchase of items such as commercially available materials and supplies, equipment or general support services allowable under the grant, no part of an NSF award may be subawarded or transferred to another organization without prior NSF authorization. The intent to enter into such arrangements should be disclosed in the proposal.

If it becomes necessary to subaward or transfer part of an NSF award after a grant has been made, the grantee shall submit, at a minimum:

a. a clear description of the work to be performed by each subrecipient;

b. a separate budget for each subaward; and

c. If funding is requested to support a postdoctoral researcher, and the original proposal did not include a mentoring plan, then the request must include the requisite mentoring plan as described in Chapter II.C.2.j. The plan must be uploaded under "Mentoring Plan" in the Supplementary Documentation section of Fastlane.

The request must be signed and submitted by the AOR via use of NSF's electronic systems, and NSF authorization will be indicated by an amendment to the grant signed by the Grants Officer. The NSF grant terms and conditions will identify which articles flow-down to subrecipients.

Procurements under NSF grants are subject to Chapter X.C as well as 2 CFR § 200.317-326.

4. Postaward Additions of Postdoctoral Scholars

If a grantee rebudgets funds to support a postdoctoral researcher and the original proposal included a mentoring plan, no further documentation is necessary. If the original proposal did not include a mentoring plan, then the grantee must send the cognizant NSF Program Officer the requisite mentoring plan, as described in Chapter II.C.2.j.

If supplemental funding is requested to support a postdoctoral researcher and the original proposal did not include a mentoring plan, then the supplemental funding request must include the requisite mentoring plan, as described in Chapter II.C.2.j. The plan should be uploaded to the "Other Supplementary Documents" section of the FastLane Supplemental Funding Request module.

In all cases, the PI must report on the mentoring activities provided to the individual in the annual and final project reports.

C. Cost Sharing

1. General

The National Science Board issued a report entitled "*Investing in the Future: NSF Cost Sharing Policies for a Robust Federal Research Enterprise"* (NSB 09-20), which contained eight recommendations for NSF regarding cost sharing. In implementation of the Board's recommendations, NSF's revised guidance[52] (see Chapter II.C.2.g(xii)) is as follows:

- inclusion of voluntary committed cost sharing is prohibited. It should be noted that foregoing full indirect cost rate (F&A) recovery is considered voluntary committed cost sharing; and

- when mandatory cost sharing is included on Line M and accepted by NSF, the commitment of funds becomes legally binding and is subject to audit. Failure to provide the level of cost sharing required by the NSF solicitation and reflected in the NSF award budget may result in termination of the NSF award, disallowance of award costs and/or refund of award funds to NSF by the grantee.

2. Mandatory Cost Sharing Commitments

2 CFR § 200.306 prescribes criteria and procedures for the allowability of cash and in-kind contributions. Grantees should be aware that mandatory cost sharing commitments are subject to audit. Audit findings involving cost sharing have pertained to: a) failure to keep adequate source documentation for claimed cost sharing; b) unclear valuation of in-kind donated contributions; c) lack of support for cost sharing contributions by subrecipients; and d) failure to complete annual certifications for awards with mandatory cost sharing requirements. Additional guidance on cost sharing may be obtained at: http://www.nsf.gov/bfa/dias/caar/costrev.jsp.

3. Mandatory Cost Sharing Records and Reports

a. Grantee Records

Grantees shall maintain records of all project costs that are claimed by the grantee to meet mandatory cost sharing requirements specified in an NSF program solicitation. Records for cost share contributed to NSF awards must be compliant with the requirements of 2 CFR § 200.306, and are subject to audit.

b. Grantee Reports

The amount of mandatory cost sharing must be documented (on an annual and final basis), certified by the AOR, and reported to the cognizant NSF Program Officer via use of NSF's electronic systems. Such notifications must be submitted no later than 90 days prior to the end of the current budget period to meet the annual notification requirement, and no later than 120 days following the end date of the of the grant to meet the final notification requirement. The cost share notification is considered due during the 90 or 120 day period respectively. The notification becomes overdue the day after the respective 90 or 120 day period ends.

c. Changes to the Cost Sharing Amount Specified on Line M of the NSF Award Budget

Should the grantee become aware that it may be unable to provide the cost sharing of at least the amount identified on Line M of the NSF award budget, it must immediately provide written notification to the Grants Officer of the situation; and either: indicate steps it plans to take to secure replacement cost sharing; or indicate the plans it has to either continue or phase out the project in the absence of the approved level of cost sharing.

Should NSF agree to the organization's proposed plans, the NSF Grants Officer will modify the award accordingly, including, if appropriate, reducing the amount of NSF support. Should the organization's plans be unacceptable

[52] See NSF's Revised Cost Sharing Policy Statement for the Foundation's overarching policies on cost sharing.

to NSF, the award may be subject to termination. NSF modifications to proposed cost sharing revisions are made on a case-by-case basis.

Failure by the organization to notify NSF, in accordance with the paragraph above, may result in the disallowance of some or all of the costs charged to the grant; the subsequent recovery by NSF of some or all of the NSF funds provided under the grant; possible termination of the grant; and may constitute a violation of the terms of the award so serious as to provide grounds for subsequent suspension or debarment.

D. Technical Reporting Requirements

NSF requires project reports for all assistance awards. Information from these reports is used in annual reports to Congress to demonstrate the Foundation's performance as mandated by the Government Performance and Results Act (GPRA) of 1993. These reports also provide NSF program officers and administrative offices with information on the progress of supported projects and the way these funds are used. Information in these reports may be made available to the general public through the Freedom of Information Act (FOIA). These reports are fully consistent with and implement the Research Performance Progress Report (RPPR), which is the government-wide standard for use with research and research-related activities. Except where another format is approved by OMB for use by an NSF program, this means that the ''where practicable'' requirement specified in 2 CFR § 200.301 is not required as the RPPR does not relate financial information to performance data.

1. Annual Project Reports

Annual Project Reports should address progress in all activities of the project, including any activities intended to address the Broader Impacts criterion that are not intrinsic to the research. These reports are not cumulative, and should be written specifically for the most recently completed budget period. Unless otherwise specified in the grant, annual project reports should be submitted electronically no later than 90 days prior to the end of the current budget period to allow adequate time for the cognizant Program Officer to review and approve the report.

As reflected in the Project Report System, the report is considered due during the 90 day period. The report becomes overdue the day after the 90 day period ends. Failure to submit timely reports will delay processing of additional funding and administrative actions, including, but not limited to, no cost extensions. In the case of continuing grants, failure to submit timely reports may delay processing of funding increments. See also Chapter VI.E.3.

Annual project reports may not be required for fellowship awards. Specific reporting requirements for fellowships are established in the applicable program solicitation and award conditions.

For multi-year standard grants, PIs are required to submit annual reports as outlined above. In the case of cooperative agreements, the annual report is required before NSF approves any future funding increments.

Continuing grants also are subject to the same policies regarding report submission as outlined above. For continuing grants that have a duration of 18 months or more per increment, two annual reports are required. A report must be submitted for the first 12 months of the project, and then another report for the remaining months of the increment. Continuing grants which include an increment of 24 months will require an annual report for each 12 month period.

2. Final Project Report

The Final Project Report should address progress in all activities of the project, including any activities intended to address the Broader Impacts criterion that are not intrinsic to the research. This report is not cumulative; it is the last annual report of the project and should be written specifically for the most recently completed budget period. By submitting the report, the PI is indicating that the scope of work for the project has been completed and that he/she does not anticipate any further action on the grant, including no cost extension requests, supplemental funding, or transfer of grant. Unless otherwise specified in the award, the final project report should be submitted electronically no later than 120 days following the end date of the grant. As reflected in the Project Report System, the report is considered due during the 120 day period. The report becomes overdue the day

after the 120 day period ends. In addition, the grantee also shall provide to the cognizant NSF Program Officer, within 120 days following the end date of the grant:

- any unique reports or other end items specified in the grant, including any reporting requirements set forth in any NSF brochure, guide, solicitation, etc., referenced in the grant as being directly related to either the award or the administration of the grant.

- a final cost share notification documented and certified by the AOR for grants where there is mandatory cost sharing established for the program.

Final project reports may not be required for institutional graduate research fellowships. However, final reporting requirements for individual fellowships are established in the applicable program solicitation.

3. Project Outcomes Report for the General Public

No later than 120 days following expiration of the grant, a project outcomes report for the general public must be submitted electronically. This report serves as a brief summary, prepared specifically for the public, of the nature and outcomes of the project. This report will be posted electronically by NSF **exactly as it is submitted** and will be accompanied by the following disclaimer:

> "This Project Outcomes Report for the General Public is displayed verbatim as submitted by the Principal Investigator (PI) for this award. Any opinions, findings, and conclusions or recommendations expressed in this Report are those of the PI and do not necessarily reflect the views of the National Science Foundation; NSF has not approved or endorsed its content."

Grantees are to ensure that the report does not contain any confidential, proprietary business information; unpublished conclusions or data that might compromise the ability to publish results in the research literature; or invention disclosures that might adversely affect the patent rights or those of the organization, in a subject invention under the award. Project Outcomes Reports are not to contain any personally identifiable information such as home contact information, individual demographic data or individually identifiable information collected from human research participants.

Section Contents:

a. Describe the project outcomes or findings that address the intellectual merit and broader impacts of the work as defined in the NSF merit review criteria. This description should be a brief (generally, two to three paragraphs) summary of the project's results that is written for the lay reader. PIs are strongly encouraged to avoid use of jargon, terms of art, or acronyms.

b. NSF will automatically include all publications associated with the grant that are reported in annual and final project reports. Other products that have resulted from the grant may also be listed. Examples of other products include collections, data sets, software, as well as educational materials.

c. Information regarding anticipated publication of project results, as well as any other information that would be of interest to the public also may be included in this section.

4. Compliance with Technical Reporting Requirements

PIs must submit final technical reports within the time period specified. **Failure to provide these reports on a timely basis will delay NSF review and processing of pending proposals for all identified PIs and co-PIs on a given grant.**

5. Grant Closeout

Grant closeout is the process by which NSF determines that all applicable administrative actions and all required work of the grant have been completed. Grants will be financially closed out on the first day of each month for all

awards with end dates of 120 or more days prior to the financial closeout day. See also Chapter VI.D.3.c for additional information on no cost extensions. The close out amount will be based on the costs recorded at that time. See also Chapter VIII.E.5 for additional information on post closeout adjustments and final disbursements. Grants are administratively closed after receipt of the Final Project Report and after determination that any other administrative requirements in the grant have been met. In the event a final audit has not been performed prior to the closeout of the grant, NSF reserves the right to recover funds after fully considering the recommendations on disallowed costs resulting from the final audit.

E. Record Retention and Audit

1. Financial records, supporting documents, statistical records and all other records pertinent to the NSF grant must be retained by the grantee for a period of three years from award financial closeout described in Chapter VIII.E.3, except as noted in 2 CFR § 200.333.

2. It is the responsibility of grantees that are States, local governments or non-profit organizations to arrange for the conduct of audits as required by 2 CFR § 200, Subpart F. They shall provide copies of the reports of these audits to the Federal Audit Clearinghouse (see 2 CFR § 200.512(b)). Any Federal audit deemed necessary by NSF shall build upon the results of such audit(s).

3. All awards issued by NSF meet the definition of “Research and Development” (R&D) at 2 CFR § 200.87. As such, auditees should identify NSF awards as part of the R&D cluster on the Schedule of Expenditures of Federal Awards (SEFA). The auditor should test NSF awards for compliance as instructed in Part V, Clusters of Programs. NSF recognizes that some awards may have another classification for purposes of indirect costs (F&A). The auditor is not required to report the disconnect (i.e., the award is classified as R&D for audit purposes but non-research for indirect cost rate (F&A) purposes), unless the auditee is charging indirect costs at a rate other than the rate(s) specified in the award document(s).

Chapter VIII - Financial Requirements and Payments

The acceptance of an award from NSF creates a legal obligation on the part of the grantee organization to use the funds or property made available in accordance with the terms and conditions of the award. Payments may be made in advance of work performed or as a reimbursement for work performed and/or costs incurred by the grantee. Payments, however, may not be made prior to an award being signed by an NSF Grants Officer.

NSF has a revisionary interest in any:

(a) unused funds from advance payments;

(b) improperly applied funds (whether or not received as an advance payment); and

(c) property acquired through the award, to which NSF specifically either retains title or reserves the right to require title transfer.

While the provisions of this Chapter cover all NSF assistance awards (i.e., grants and cooperative agreements), the term "grantee" is used throughout. Graduate fellowship awards to domestic colleges and universities are included, but contracts are excluded. All categories of grantees (academic, non-academic, profit and non-profit) are covered by this chapter. The procedures in this chapter apply primarily to the comptroller's office or business office.

A. Standards for Financial Management

NSF grantees must meet the financial management systems requirements of 2 CFR § 200.302.

B. Definitions

The following definitions are either not included elsewhere in Part II of the PAPPG or are repeated in this Chapter because of their special applicability.

1. ADVANCE PAYMENT - means a payment that NSF makes by any appropriate payment mechanism, including a predetermined payment schedule, before the grantee disburses the funds for program purposes.

2. BUSINESS OFFICER – means the financial official of the grantee organization who has primary responsibility for the accountability for, and reporting on, NSF award funds.

3. CASH ON HAND – means a grantee organization's cash position relative to the funds received from NSF minus the costs incurred for the award.

4. DISBURSEMENTS/OUTLAYS/EXPENDITURES – means charges made by the grantee to a project or program for which an NSF award was received.

5. FINANCIAL FUNCTIONS – means the NSF systems and services used by grantees to transmit financial information to NSF. Grantees must access Financial Functions through Research.gov. The primary Financial Functions used by grantees are:

(a) Award Cash Management Service (ACM$) – means NSF's award payment process under which grantees provide award level detail at the time of the payment request. ACM$ replaced both the reporting of expenditures on the Federal Financial Report (FFR) and the Cash Request Function.

(b) Federal Financial Report History – means the electronic version of the standard "Federal Financial Report". This was used by grantees prior to ACM$ implementation to report the financial activity of NSF awards on a quarterly basis.

6. GRANTEE - means the organization or other entity that receives a grant and assumes legal and financial responsibility and accountability both for the awarded funds and for the performance of the grant-supported activity. NSF grants are normally made to organizations rather than to individual PI/PD(s). Categories of eligible proposers may be found in Chapter I.E.

7. NSF OBLIGATIONS – means funds authorized by an NSF Grants Officer for a specific NSF award creating a balance payable to a grantee.

8. GRANTEE OBLIGATIONS – means orders placed for property and services, contracts and subawards made, and similar transactions during a given period that require payment by the grantee during the same or a future period.

9. PAYMENTS – means the funds transferred from NSF to the grantee by electronic funds transfer (Automated Clearing House (ACH)) and on some rare occasions by check or by wire for same day electronic funds transfer or international payments.

10. UNLIQUIDATED OBLIGATIONS –

(a) for grantees operating on a cash basis, means obligations incurred by the grantee that have not been paid (liquidated).

(b) for grantees operating on an accrual expenditure basis, means obligations incurred by the grantee for which an expenditure has not been recorded.

11. UNOBLIGATED BALANCE – means the amount of funds under an NSF award that the grantee has not obligated. The amount is computed by subtracting the cumulative amount of the grantee's unliquidated obligations and expenditures of funds under the NSF award from the cumulative amount of the funds that NSF has authorized the grantee to obligate.

C. Payment Requirements

1. Requesting Payments

NSF grantees, except for some Special Payment grantees (see Chapter VIII.C.4), and some foreign grantees, are required to request payments electronically through ACM$. Under ACM$, grantees must provide award level detail at the time of the payment request.

Certain Special Payment grantees and foreign grantees without access to a US bank are required to request funds by submitting a "Request for Advance or Reimbursement Form", (SF 270), to NSF either through mail, email or by fax.

2. Payment Policies

The purpose of this section is to prescribe the timing of advances and the procedures to be observed to ensure that cash payments occur only when essential to meet the needs of a grantee for its actual disbursements.

a. Timing of Payments. Advance payments to grantees must be limited to the minimum amounts needed. The timing of advanced payments must be in accordance with the actual, immediate cash requirements of the grantee in carrying out the purpose of the approved program or project. The timing and amount of advance payments must be as close as is administratively practicable to the actual disbursements by the grantee for direct program or project costs and the proportionate share of any allowable indirect costs (F&A).

b. Payments to Subrecipients and Contractors. Payments made by NSF grantees to subrecipients and contractors shall conform to the same standards of timing and amount as apply to payments by NSF to its grantees.

c. Withholding Payments. NSF reserves the right, upon written notice, to withhold future payments after a specified date if the grantee either:

(i) fails to comply with the terms and conditions of an NSF award, including the reporting requirements; or

(ii) is indebted to the US Government.

Payments will be released to the grantee upon subsequent compliance.

d. Safeguarding Funds. In no case will NSF funds be commingled with the personal funds of, or be used for personal purposes by, any officer, employee, or agent of the grantee; nor will any of these funds be deposited in personal bank accounts for disbursement by personal check.

3. Request for Payment

a. Grantees may submit requests for payments as often as they like and may submit payment requests for reimbursement or in advance of costs incurred. The following conditions must exist for grantees submitting payment requests in advance of costs incurred:

(i) Funds for the project period have been obligated by a Grants Officer in the form of an electronically signed grant;

(ii) The grantee has established written procedures that will minimize the time elapsing between the transfer of funds from the US Treasury and their disbursement by the grantee; and

(iii) The grantee's financial management system meets the standards for fund control and accountability prescribed in 2 CFR § 200.302.

b. Grantees shall maintain advances of NSF funds in interest bearing accounts as specified in Chapter VIII.D.3.

4. Special Payment Grantees

When grantees do not meet the conditions specified in Chapter VIII.C.3 above, or when otherwise considered appropriate, NSF may restrict their capability to request funds through ACM$ or they may be required to request funds by using a Request for Advance or Reimbursement (SF 270) form. The cognizant NSF Grants Officer is responsible for establishing the documentation requirements for special payment grantees. Documentation may be submitted by email, by fax to 703-292-9142, or through the mail to:

National Science Foundation
Division of Grants & Agreements
2415 Eisenhower Avenue
Alexandria, VA 22314

5. Working Capital Advance

In those cases where the reimbursement method described in Chapter VIII.C.3 is not feasible, arrangements may be made whereby NSF projects are financed on a working capital advance basis. On this basis, funds may be advanced to the grantee to cover estimated disbursement needs for a given initial period. Thereafter, the grantee would be reimbursed for the amount of its actual cash disbursements. The amount of the initial advance shall be geared to the reimbursement cycle so that after the initial period, the advance approximately equals the average amount of the grantees' unreimbursed program disbursements.

6. Grantee Banking Information for Payments

The System for Award Management (SAM) is the NSF system of record for organizational financial information. Once a grant is awarded, failure to maintain current and complete financial information within SAM could prevent the grantee from receiving funds.

D. Cash Refunds and Credits to NSF

1. Final Unobligated Balance

NSF has a reversionary interest in the unobligated balance of an award upon the end date or completion of the award. Based on final payment amounts submitted through ACM$, the final unobligated balance will be computed by NSF and de-obligated from the award amount.

2. Erroneous Payments

Advances or reimbursements made in error must be refunded to the National Science Foundation. Excess funds should be promptly refunded electronically or by check. Electronic remittances should be submitted through Pay.gov at: https://pay.gov/paygov/forms/formInstance.html?agencyFormId=10506005. Checks shall be mailed to NSF, Attn. Cashier, 2415 Eisenhower Avenue, Alexandria, VA 22314. Contact the NSF Division of Financial Management at (703) 292-8280 for additional information.

The only exception to the requirement for prompt refunding is when the funds involved will be disbursed immediately. This exception for prompt refunding should not be construed as approval by NSF for a grantee to maintain excessive funds on hand.

3. Interest Earned on Advance Payments

The following provisions implement the applicable portions of 2 CFR § 200.305 on interest income.

Grantees shall maintain advances of NSF funds in interest bearing accounts, unless any of the following apply:

a. The grantee receives less than $120,000 in Federal awards per year;

b. The best reasonably available interest bearing account would not be expected to earn interest in excess of $500 per year on Federal cash balances;

c. The depository would require an average or minimum balance so high that it would not be feasible within the expected Federal and non-Federal cash resources; or

d. A foreign government or banking system prohibits or precludes interest bearing accounts.

Grantees may retain interest earned amounts up to $500 per year for administrative expenses. Any additional interest earned on Federal advance payments deposited in interest-bearing accounts must be remitted annually to the Department of Health and Human Services Payment Management System (PMS) through an electronic medium using either Automated Clearing House (ACH) network or a Fedwire Funds Service payment. Remittances must include pertinent information of the payee and nature of payment in the memo area (often referred to as "addenda records" by financial institutions) as that will assist in the timely posting of interest earned on Federal funds. The remittance must be submitted as follows:

(i) For ACH Returns:

Routing Number: 051036706

Account number: 303000

Bank Name and Location: Credit Gateway—ACH Receiver St. Paul, MN

(ii) For Fedwire Returns*:

Routing Number: 021030004

Account Number: 75010501

Bank Name and Location: Federal Reserve Bank Treas NYC/Funds Transfer Division New York, NY

(* Please note organization initiating payment is likely to incur a charge from your Financial Institution for this type of payment)

(iii) For International ACH Returns:

Beneficiary Account: Federal Reserve Bank of New York/ITS (FRBNY/ITS)

Bank: Citibank N.A. (New York)

Swift Code: CITIUS33

Account Number: 36838868

Bank Address: 388 Greenwich Street, New York, NY 10013 USA

Payment Details (Line 70): Agency

Name (abbreviated when possible) and ALC Agency POC: Michelle Haney, (301) 492-5065

(iv) For grantees that do not have electronic remittance capability, please make check** payable to: "The Department of Health and Human Services."

Mail Check to Treasury approved lockbox:

HHS Program Support Center, P.O. Box 530231, Atlanta, GA 30353-0231

(** Please allow 4-6 weeks for processing of a payment by check to be applied to the appropriate PMS account)

(v) Any additional information/instructions may be found on the PMS website at: http://www.dpm.psc.gov/.

4. Program Income

a. Background

The following provisions implement the applicable portions of 2 CFR § 200.307 on program income.

b. Definition

PROGRAM INCOME means gross income earned by the grantee that is directly generated by a supported activity or earned as a result of the grant during the period of performance. Program income includes, but is not limited to, income from fees for services performed, the use of rental or real or personal property acquired under the grant, the sale of commodities or items fabricated under the grant, license fees and royalties on patents and copyrights, and principal and interest on loans made with grant funds. Interest earned on advances of Federal funds is not program income. Except as otherwise provided in Federal statutes, regulations, or the terms and conditions of the grant, program income does not include rebates, credits, discounts, and interest earned on any of them.

Note: Registration fees collected under NSF-supported conferences are considered program income.

c. NSF Policy

(i) Standard Treatment

Unless otherwise specified in the grant, program income received or accruing to the grantee during the period of the grant is to be retained by the grantee, added to the funds committed to the project by NSF, and thus used to further project objectives. The grantee has no obligation to NSF with respect to program income received beyond the period of the grant. The grantee also shall have no obligation to NSF with respect to program income earned from license fees and royalties for copyrighted material, patents, patent applications, trademarks, and inventions produced under an award. However, Patent and Trademark Amendments (35 USC 18) shall apply to inventions made under an award.

Efforts should be made to avoid having unexpended program income remaining at the end date of the grant. Program income earned during the project period should be expended prior to requesting reimbursement against the grant. In the event a grantee has unexpended program income remaining at the end of the grant, it must be remitted to NSF by crediting costs otherwise chargeable against the grant. If it is not possible to record the credit via ACM$, the excess program income must be remitted to NSF electronically or by check payable to the National Science Foundation. (See section D.2 above for further information.)

(ii) Special Treatment

In exceptional circumstances, the NSF Grants Officer, in collaboration with Program Officers and other appropriate NSF offices, may approve use of a special grant provision to restrict or eliminate a grantee's control of income earned through NSF-supported activities if it determines that this would best serve the purposes of a particular program or grant. The special provisions may require treatment of the program income via use of the deductive method, the Federal share of program income be kept in a separate account, or reported on and/or remitted for such periods as may be reasonable under the circumstances.

If, in accordance with the grant terms and conditions program income is designated for deductive treatment, it must be remitted to NSF by crediting costs otherwise chargeable against the grant. Program Income in excess of the award will be remitted to NSF electronically or by check payable to the National Science Foundation.

(iii) Program Income Reporting Requirements

On an annual basis, grantees are required to submit a Program Income Reporting Worksheet to NSF in order to report program income earned and expended for any of their grants or to validate that they did not earn and expend program income for any of their grants during the applicable period.

(a) The Program Income Reporting Worksheet utilizes the standard, OMB-approved government-wide data elements from the Program Income section of the Federal Financial Report (SF 425).

(b) The Program Income Reporting Worksheet in Microsoft Excel is available to grantees through the Program Income page of Research.gov. Grantees are required to report the award number, amount of program income earned, amount of program income expended, and the amount of unexpended program income remaining as of the applicable period. Grantees that have no program income to report will be able to validate that status by an email response. The Program Income Reporting Worksheet is due 45 days after the end of the Federal fiscal year.

Failure to report program income or to validate that no program income was earned/expended could result in suspension of future grant payments.

5. Other Cost Credits

Purchase discounts, rebates, allowances, credits resulting from overhead rate adjustments and other credits relating to any allowable cost received by or accruing to the grantee shall be credited against NSF award costs if the grant has not been financially closed out. See also Chapter X.A.2.c.

E. Award Financial Reporting Requirements and Final Disbursements

NSF does not require grantees to submit FFRs for each award for purposes of final award accountability. NSF procedures have been designed to extract the final financial data from the entries in ACM$. This is accomplished as follows:

For any award listed on the ACM$ Payments screen, the grantee will enter the final payment amount in the Payment Amount Requested column to complete final financial reporting to NSF. Considerations for financial closeout are as follows:

1. After closeout, if final disbursements change by $1.00 or more from the final award amount, then the grantee should submit an "Adjustment to a Financially Closed Award" through the ACM$ payment process.

2. Grantees must liquidate all obligations incurred under their awards not later than 120 calendar days after the award end date.

3. NSF will financially close awards 120 days after the award end date and the award will be removed from the ACM$ payment screen for active awards. Any remaining funds that exceed the final payment amount will be deobligated from the award so the net award balance will equal total payments.

4. Grantees also have the option of using ACM$ to designate awards for financial closeout prior to NSF's regularly scheduled close out date. Grantees can complete that action by selecting the Final Flag on the ACM$ payment screen. (Note that the flag cannot be unchecked at a later date and should typically not be used for original awards that transferred to another awardee organization). Selecting the Final Flag will financially close the award upon posting of the ACM$ transaction to the NSF financial accounting system, typically overnight.

5. Grantees are authorized to make upward or downward adjustments to the Federal share of costs for a financially closed award within the following time limits:

- Upward adjustments may be submitted through ACM$ for up to 14 months after the financial closeout date of the award or until the appropriated funds cancel[53], whichever is earlier.

- Downward adjustments may be submitted through ACM$ until the appropriated funding for the award cancels. The time limitation for ACM$ downward adjustments has no effect on the requirement that the grantee return any funds due to NSF as a result of later refunds, corrections, or other transactions including final indirect cost rate adjustments. In cases where the award appropriation has been canceled, the grantee should return the funds associated with the downward adjustment in accordance with section D. Cash Refunds and Credits to NSF.

6. NSF will notify grantees of any canceling appropriations on open awards in order for grantees to properly expend and draw down funds before the end of the Federal fiscal year. Notifications regarding canceling appropriations must not be interpreted as authorization to draw down remaining grant funds simply to avoid losing them. Grantees are reminded that "the timing and amount of advance payments must be as close and as administratively practicable to the actual disbursements…" in accordance with Chapter VIII.C.2, and that all excess funds drawn must be returned to NSF promptly. See section C. above for additional information on grant payments. If only a portion of the awarded funds are canceling, then NSF will reduce the available balance of the award. If all funds under the award are canceling appropriations, then NSF must financially close the award no later than September 30th regardless of the award end date. No extensions, requests for payment, or upward adjustments will be allowed beyond the end of the Federal fiscal year in which the funds cancel. Although in this situation awards may be financially closed early, grantees will still have the full 120-day closeout period to submit final project reports in accordance with the grant terms and conditions.

[53] In accordance with 31 USC 1552(a), funds will no longer be available for expenditure for any purpose beyond September 30th of the fifth fiscal year after the expiration of a fixed appropriation's period of availability for incurring new obligations.

Chapter IX: Grantee Standards

NSF encourages the increased involvement of academic researchers and educators with industry and private entrepreneurial ventures, but recognizes that such interactions carry with them an increased risk of conflicts of interest. Chapter IX.A contains NSF's policy on conflicts of interest.

2 CFR § 200 prescribes three separate sets of standards related to the financial and program management of Federal grants, each governing a different area: financial management, property management, and procurement management. Chapter IX.C, IX.D and IX.E implement these standards, and extend their applicability to all types of grantees of NSF grants, including for-profit organizations.

A. Conflict of Interest Policies

1. NSF requires each grantee organization employing more than fifty persons to maintain an appropriate written and enforced policy on conflict of interest and that all conflicts of interest for each award be managed, reduced or eliminated prior to the expenditure of the award funds. If the organization carries out agency-funded research through subrecipients or collaborators, the organization must take reasonable steps to ensure that:

a. the entity has its own policies in place that meet the requirements of this policy; or

b. investigators working for such entities follow the policies of the primary organization.

Guidance for development of such policies has been issued by university associations and scientific societies.

2. An organizational conflict of interest policy should require that each investigator disclose to a responsible representative of the organization all significant financial interests of the investigator (including those of the investigator's spouse and dependent children): (i) that would reasonably appear to be affected by the research or educational activities funded or proposed for funding by NSF; or (ii) in entities whose financial interests would reasonably appear to be affected by such activities.

The term "investigator" means the PI/PD, co-PI/co-PDs, and any other person identified on the proposed project who is responsible for the design, conduct, or reporting of research or educational activities funded or proposed for funding by NSF.

The term "significant financial interest" means anything of monetary value, including, but not limited to, salary or other payments for services (e.g., consulting fees or honoraria); equity interest (e.g., stocks, stock options or other ownership interests); and intellectual property rights (e.g., patents, copyrights and royalties from such rights).

The term does not include:

a. salary, royalties or other remuneration from the proposing organization;

b. any ownership interests in the organization, if the organization is an applicant under the Small Business Innovation Research Program or Small Business Technology Transfer Program;

c. income from seminars, lectures, or teaching engagements sponsored by public or non-profit entities;

d. income from service on advisory committees or review panels for public or nonprofit entities;

e. an equity interest that, when aggregated for the investigator and the investigator's spouse and dependent children, meets both of the following tests: (i) does not exceed $10,000 in value as determined

through reference to public prices or other reasonable measures of fair market value; and (ii) does not represent more than a 5% ownership interest in any single entity; or

f. salary, royalties or other payments that, when aggregated for the investigator and the investigator's spouse and dependent children, are not expected to exceed $10,000 during the prior twelve-month period.

3. An organizational policy must ensure that investigators have provided all required financial disclosures at the time the proposal is submitted to NSF. It must also require that those financial disclosures are updated during the period of the award, either on an annual basis, or as new reportable significant financial interests are obtained.

4. An organizational policy must designate one or more persons to review financial disclosures, determine whether a conflict of interest exists, and determine what conditions or restrictions, if any, should be imposed by the organization to manage, reduce or eliminate such conflict of interest. A conflict of interest exists when the reviewer(s) reasonably determines that a significant financial interest could directly and significantly affect the design, conduct, or reporting of NSF-funded research or educational activities.

Examples of conditions or restrictions that might be imposed to manage, reduce or eliminate conflicts of interest include, but are not limited to:

a. public disclosure of significant financial interests;

b. monitoring of research by independent reviewers;

c. modification of the research plan;

d. disqualification from participation in the portion of the NSF-funded research that would be affected by significant financial interests;

e. divestiture of significant financial interests; or

f. severance of relationships that create conflicts.

If the reviewer(s) determines that imposing conditions or restrictions would be either ineffective or inequitable, and that the potential negative impacts that may arise from a significant financial interest are outweighed by interests of scientific progress, technology transfer, or the public health and welfare, then the reviewer(s) may allow the research to go forward without imposing such conditions or restrictions.

5. The organizational policy must include adequate enforcement mechanisms, and provide for sanctions where appropriate.

6. The organizational policy must include arrangements for keeping NSF's Office of the General Counsel (OGC) appropriately informed if the organization finds that it is unable to satisfactorily manage a conflict of interest and if the organization finds that research will proceed without the imposition of conditions or restrictions when a conflict of interest exists.[54]

When OGC is notified of an unmanageable conflict of interest by a grantee, OGC will conduct the following review:

[54] Grantee notifications of conflict of interest that cannot be managed, reduced, or eliminated and awardee notifications of situations where research will proceed without the imposition of conditions or restrictions when a conflict of interest exists, must be signed and submitted by the AOR via use of NSF's electronic systems.

a. Examine a copy of the organization's conflict of interest policy to ascertain if the policy includes procedures for addressing unmanageable conflicts.

b. Contact the grantee organization's representative to determine what actions the organization plans/has taken with respect to the reported unmanageable conflict of interest, ensuring consistency with their conflict of interest policy.

c. Request confirmation from the grantee when proposed actions have been accomplished.

7. Organizations must maintain records of all financial disclosures and of all actions taken to resolve conflicts of interest for at least three years beyond the termination or completion of the grant to which they relate, or until the resolution of any NSF action involving those records, whichever is longer.

B. Responsible Conduct of Research (RCR)

1. Background

The responsible and ethical conduct of research (RCR) is critical for excellence, as well as public trust, in science and engineering. Consequently, education in RCR is considered essential in the preparation of future scientists and engineers. Section 7009 of the America Creating Opportunities to Meaningfully Promote Excellence in Technology, Education, and Science (COMPETES) Act (42 USC 1862o–1) requires that ''each institution that applies for financial assistance from the Foundation for science and engineering research or education describe in its grant proposal a plan to provide appropriate training and oversight in the responsible and ethical conduct of research to undergraduate students, graduate students, and postdoctoral researchers participating in the proposed research project."

The language specified below provides NSF's implementation of Section 7009.

2. Institutional Responsibilities

a. An institution must have a plan in place to provide appropriate training and oversight in the responsible and ethical conduct of research to undergraduates, graduate students, and postdoctoral researchers who will be supported by NSF to conduct research. As noted in Chapter II.C.1.d, institutional certification to this effect is required for each proposal.

b. While training plans are not required to be included in proposals submitted to NSF, institutions are advised that they are subject to review, upon request.

c. An institution must designate one or more persons to oversee compliance with the RCR training requirement.

d. Institutions are responsible for verifying that undergraduate students, graduate students, and postdoctoral researchers supported by NSF to conduct research have received training in the responsible and ethical conduct of research.

C. Financial Management Systems Standards

NSF grantees are required to have financial management systems which meet the requirements of 2 CFR § 200.302.

D. Property Management Standards

2 CFR §§ 200.310-316 prescribe standards for managing and disposing of property furnished by the Federal government or whose cost was charged to a project supported by a Federal grant. In the rare

instances where NSF grants involve the acquisition of real property, the real property standards of 2 CFR § 200.311 are applicable to such NSF grants. NSF implementation of the OMB standards on intangible property is contained in Chapter XI.D.

Title to materials developed and supplies purchased under an NSF grant will vest in the grantee, subject to the conditions identified in paragraph 2. below.

2 CFR § 200.312 contains a special category of "exempt property." Under that provision, any Federal agency which has statutory authority (such as NSF) may vest title in an institution of higher education, hospital or other non-profit organization without further obligation to the Federal government and under conditions the agency considers appropriate. NSF's authority is provided by the Federal Technology Transfer Act (15 USC 3710.)

1. Title to Equipment

a. Title to Equipment - Non-Profit Organizations

(i) Normal Situations. Unless otherwise specified in the grant, title to equipment purchased or fabricated with NSF grant funds by a college or university or other non-profit organization will vest in the grantee organization upon acquisition. Such equipment is considered "exempt property" (see Chapter IX.D) and subject to the conditions of Chapter IX.D.2.

(ii) Special Situations. In special situations the grant may require that title to equipment purchased, acquired or fabricated by the grantee with NSF funds pass directly to the government upon acquisition.

b. Title to Equipment – For-Profit Organizations

Unless otherwise specified in the grant, title to equipment purchased or fabricated with NSF grant funds by a small business or other for-profit organization will vest in the government. Such equipment will be acquired and used in accordance with Chapter IX.D.2, and managed in accordance with Chapter IX.D.4.

2. Conditions for Acquisition and Use of Equipment

a. Grantee Assurance. The grantee will assure each purchase of equipment is:

(i) necessary for the research or activity supported by the grant;

(ii) not otherwise reasonably available and accessible;

(iii) of the type normally charged as a direct cost to sponsored agreements; and

(iv) acquired in accordance with organizational practice.

b. General Purpose Equipment. Expenditures for general purpose equipment are typically not available for support.

c. Equipment Usage. The equipment must remain in use for the specific project for which it was obtained in accordance with 2 CFR § 200.313 unless the provision in 2 CFR § 200.313(c)(4) applies.

d. Equipment Sharing. The equipment must be shared on other projects or programs in accordance with 2 CFR § 200.313(c)(1).

e. Property Management Standards. The grantee shall maintain a property management system which, at a minimum, meets the requirements of 2 CFR § 200.313(d).

f. Competition. In accordance with 2 CFR § 200.313(c)(3), grantees shall not use equipment acquired with Federal funds to provide services for a fee that is less than private companies charge for equivalent services, unless specifically authorized by statute, for as long as the Federal government retains an interest in the equipment.

g. Right to Transfer Title.

(i) In accordance with 2 CFR § 200.313(e), NSF may identify items of equipment having a unit acquisition cost of $5,000 or more where NSF reserves the right to transfer the title to the Federal government or to a third party named by the Federal government.

(ii) In such cases where NSF elects to transfer the title, disposition instructions will be issued no later than 120 days after the end date of the NSF-supported project for which it was acquired.

3. Principles Relating to the Use of NSF-Supported Research Instrumentation and Facilities

The following principles on use of NSF-supported instrumentation and facilities were adopted by the NSB:

> The National Science Foundation seeks the maximum productive use of the Nation's scientific instrumentation and research expertise. Ensuring that the highest quality instrumentation, facilities, and services are available to scientific users, both academic and industrial, is a key requirement, as are harmonious relations and cooperation between industry and universities. Private research and testing laboratories, as well as university, government, and industrial laboratories, have a contribution to make.
>
> The National Science Board recognizes that there may be circumstances where NSF grantees use NSF-supported research instrumentation to provide services in commerce for a fee, to an extent that such practice, (1) detracts from the performance of their obligation under the grant, and/or (2) may have a material and deleterious effect on the success of private companies engaged in the provision of equivalent services. It is contrary to the NSF's intent for grantees to use NSF-supported research instrumentation or facilities to provide services for a fee in competition with private companies in a manner that is prohibited by 2 CFR § 200.313(c)(3).

Grantees should implement the above principles and related grant conditions in a reasonable manner. Grantees are expected to provide fair and adequate consideration of any complaints about use of instrumentation and facilities.

4. Property Management Standards When Title Retained by NSF

In the event that title to equipment or property is vested in the Federal government, such property shall be marked, tagged or segregated in such a manner as to indicate clearly its ownership by the government. Unless otherwise provided in the grant, such government property shall be used only for the performance of the project. The grantee shall submit an annual inventory report by NSF grant number of such property having an original acquisition cost of $5,000 or more, to the NSF Property Administrator, Division of Administrative Services (DAS). A physical inventory of Government-Owned Equipment (GOE) shall be conducted every two years pursuant to 2 CFR § 200.313(d)(2). Upon expiration of the grant, the grantee shall report the property to the Property Section for further agency utilization. (See Chapter IX.D.5.)

5. Excess Government Personal Property

a. Policy

(i) As a means of expanding the ability of grantees to accomplish NSF objectives while conserving supply and equipment funds, NSF will continue to sponsor the transfer of excess government personal property to NSF grantees.

(ii) Excess government personal property includes all types of personal equipment and materials (except consumable items such as drugs, paint, etc.), new or used, owned by the Federal government and no longer needed by the holding agency, but having additional useful life. Under regulations established by the General Services Administration (GSA), the agency charged with operating this program, excess government personal property may be reported to, or requested from, GSA by other Federal agencies, including NSF.

(iii) NSF will sponsor the transfer of excess government personal property to eligible organizations under one of the following conditions:

(a) the use of the property significantly furthers an NSF grant-supported activity;

(b) the property is such that it would have been procured under the grant for which property is being requested if additional grant funds had been available;

(c) the property is used as part of the grant activity and subsequently for research or science education purposes; or

(d) the property is classified by GSA as scientific or engineering research equipment and has a unit acquisition cost of $1,000 or more. (See Chapter IX.D.5.f.)

b. Eligibility

Eligible organizations are NSF grantees that are public or private colleges or universities or non-profit organizations whose primary purpose is the conduct of research or science education activities. State and municipal governments, public health units, hospitals, for-profit firms and individual PI/PDs are not eligible for excess government personal property under NSF sponsorship.

c. Procedures

(i) To access a worldwide inventory of available excess personal property, an eligible grantee should contact the Property Administrator (fsrpts@nsf.gov), DAS to become a registered user of GSAXcess® (GSA's online personal property system located at: http://gsaxcess.gov/).

(ii) Grantees can visit GSAXcess® to screen for items that are necessary to accomplish the NSF supported project by searching GSA's inventory and adding items to the Selection Cart. Excess personal property is reported by Category (Federal Supply Classification (FSC)) and includes the item control number, name, location, quantity available, and unit of issue, original acquisition cost, surplus release date and condition of items posted. In some instances a photograph of the property item is available.

(iii) Materials and equipment so selected should be inspected whenever possible (see Chapter IX.D.5.d), or the holding Federal agency should be contacted by the grantee to verify the condition of the items, because interpretation of condition codes can vary among agencies.

(iv) If the condition of the item is acceptable, the grantee should freeze items by Checkout of the Selection Cart to generate a Transfer Control Number/SF 122, *Transfer Order Excess Personal Property*.

(v) The grantee should next submit the SF 122, *Transfer Order Excess Personal Property* and a separate written justification statement, if necessary, to the Property Administrator, DAS. The justification will explain why the property is needed to reduce the cost or enhance the performance under the specific grant for which the property is requested.

(vi) The SF 122 should be signed by either the PI/PD or the AOR. The following information should also be provided on each SF 122:

(a) name of grantee;

(b) grant number;

(c) grant end date;

(d) the statement "The above equipment is requested for use by the grantee in support of research or education as outlined in the grant"; and

(e) the statement "Transfer is in accordance with the provisions of 41 CFR § 101.43." The grantee should also verify the automatic release date with GSA to preclude loss of property before the transaction is processed.

(vii) The written justification should detail the scientific need for the equipment as it relates to the particular grant under which the equipment is to be used, and should cite the conditions of this section as being binding upon the grantee, should the property be acquired.

(viii) Upon receipt by NSF, the SF 122 will be reviewed and forwarded to the appropriate GSA Office. Items are usually allocated on a first come, first served basis. Since GSA may have several freezes on a piece of equipment, first come, first served is interpreted as the first approved SF 122 received by the GSA office and designated Area Property Officer (APO). However, preference will be given by GSA to agencies which do not grant title to equipment. As confirmation of approval, the grantee will receive copies of the SF 122 from the GSA APO. If the request is disapproved, it will be returned to the grantee with an appropriate explanation.

(ix) Upon receipt of the requested property, the grantee should immediately return a copy of the SF 122 to NSF as evidence of delivery. Cancelations by GSA, or nonreceipt within a reasonable period of time should also be reported to NSF after follow-up inquiries have been made to GSA.

d. Visiting Holding Agency Facilities

(i) Under current GSA regulations all non-Federal grantee representatives wishing to visit holding agency facilities to screen or freeze government excess personal property must receive prior certification from GSA. Because of current security protocols, the holding agency may require written authorization for access.

(ii) The number of screeners on a grant should be limited to no more than two persons. The primary screener should be the PI/PD. However, if the PI/PD finds it impossible to screen, he/she may designate, in writing, a substitute.

(iii) Normally, certification will be on a regional basis; however, NSF will, under certain circumstances, sponsor grantee representatives who need to make interregional visits in order to secure equipment not readily available within their region.

e. Dollar Limitation

To ensure equitable distribution of excess government personal property, grantees may be authorized to acquire property under each NSF grant up to a total acquisition cost equal to the dollar value of that particular grant. Any request for excess property which causes the total to exceed the value of the grant will require additional justification beyond that requested in Chapter IX.D.5.c. A higher percentage of excess property requested under a particular grant by a grantee must be approved by an administrative level in NSF which is higher than the cognizant Program Officer. NSF will give full consideration to all factors in determining whether to approve transfers of excess property above the dollar value of a given grant. Grantees are therefore urged to be selective in their requests for excess government personal property to limit quantities of each item where possible and to avoid stockpiling items for future use.

f. Restrictions

(i) NSF will sponsor the transfer of excess government personal equipment to eligible organizations only under project grants. As defined in the GSA regulations, "project grants" refers to grants made for specific purposes with established termination dates, e.g., grants made to specific organizations to perform specific tasks within set time frames and costs. No excess property may be acquired on behalf of conference, publication-support grants or travel grants. In addition, on "summer-type training grants," no property may be acquired after the training period has terminated. Further, grantees should exercise careful judgment on the appropriateness of requesting excess personal property when only a short period of time exists between the date of the property request and the completion or successful accomplishment of the NSF-supported activity. GSA will consider items of personal property as research equipment for transfer without reimbursement to NSF for use by a grantee when the property requested has a unit acquisition cost of $1,000 or more and is within Federal Supply Classification Groups:

(a) 12 Fire Control Equipment;

(b) 14 Guided Missiles;

(c) 43 Pumps and Compressors;

(d) 48 Valves;

(e) 58 Communication, Detection and Coherent Radiation Equipment;

(f) 59 Electrical and Electronic Equipment Components;

(g) 65 Medical, Dental and Veterinary Equipment and Supplies;

(h) 66 Instruments and Laboratory Equipment;

(i) 67 Photographic Equipment;

(j) 68 Chemical and Chemical Products; or

(k) 70 General Purpose Automatic Data Processing Equipment, Software, Supplies and Support Equipment.

Automatic data processing equipment must be acquired under the provisions in 41 CFR § 201.23.

(ii) GSA will give consideration to the transfer without reimbursement of items of excess property in other Federal supply classification groups and items with a unit acquisition cost of less than $1,000, when NSF certifies that the item requested is a component part of or related to a piece of research equipment or is an otherwise difficult to acquire item needed for scientific or engineering research. Items of property determined by GSA to be common-use or general purpose property, regardless of classification or unit acquisition cost, shall not be transferred to a grantee for the purpose of cannibalization, unless the granting agency sends with the transfer request a supporting statement which clearly indicates that disassembly of the requested item for secondary use of its component parts, or for repair and maintenance of a similar item, has greater potential benefit than utilization of the item in its existing form and that a clear cost savings to the government will result, subject to final determination by GSA.

g. Costs

Excess government personal property is usually secured without cost. However, the grantee should specify the method of shipment and must pay all costs of packing, transportation and subsequent installation, rehabilitation and maintenance if required. Grant funds may be used to pay such costs.

h. Title

Title to excess government personal property obtained by colleges, universities, and other non-profit grantees through NSF sponsorship remains with the government until the property is delivered to the grantee organization. Upon delivery, the grantee should forward to NSF a receipted SF 122 which clearly lists the items of property actually obtained by the grantee. Unless NSF informs the grantee to the contrary, when this SF 122 is received at NSF, title to all property acquired will automatically pass to and be vested in the grantee organization, subject to the understanding that the property will be used for research or for science education purposes as long as it has a useful life. At such time as the property is no longer useful for such purposes, it may be disposed of in accordance with organizational practices, but any proceeds therefrom shall be used by the grantee solely for research or science education purposes. Under certain conditions, such as when highly specialized equipment is involved, NSF may retain title to excess property. When such a condition exists, NSF will inform the grantee. Excess government personal property may not be transferred to a foreign country without the express written approval of the Grants Officer.

i. Accountability and Recordkeeping

(i) While no particular type of classification of accounts or inventory system is required, NSF expects that the responsible officials of the grantee organization will exercise careful stewardship of excess government personal property acquired in support of projects undertaken with NSF's financial assistance. In accordance with Chapter IX.D.5.e, relating to the dollar limitation on the amount of excess government personal property provided by NSF, grantees should maintain appropriate inventory procedures that will enable them to identify those requests which require more extensive administrative and scientific justification. In those cases where title to excess government personal property remains with the government, the grantee must maintain suitable records to identify its location, description, utilization and value. The use of excess government personal property under an NSF grant is subject to inspection and audit by representatives of NSF at all reasonable times during the life of the grant under which the property was acquired.

(ii) Further details may be obtained from the Property Administrator, Division of Administrative Services, National Science Foundation, 2415 Eisenhower Avenue, Alexandria, VA 22314.

E. Procurement Standards

NSF grantees shall adhere to the requirements of 2 CFR §§ 200.317-326 which prescribes standards for use by grantees in establishing procedures for the procurement of supplies and other expendable property, equipment, real property and other services with Federal funds.

Chapter X: Allowability of Costs

A. Basic Considerations

Expenditures under NSF cost reimbursement grants are governed by the Federal cost principles and must conform with NSF policies where articulated in the grant terms and conditions, grant special provisions and grantee internal policies. While grantees are encouraged to seek advice regarding the treatment of costs from the NSF Grants Officer identified in the award notice, it is the grantee that is ultimately responsible for ensuring that all costs charged to NSF awards meet the requirements of the cost principles contained in 2 CFR § 200, Subpart E.[55], grant terms and conditions, and any other specific requirements of both the award notice and the applicable program solicitation. In addition, grantees should ensure that their own internal policies and procedures and other requirements are met for all charges to NSF grants. Otherwise such costs may be disallowed during audit resolution or by specific determination of an NSF Grants Officer.

In the event a grantee anticipates charging an item of direct cost that might subsequently be disputed, an authorized official of the grantee organization should discuss the matter with the cognizant NSF Grants Officer and document the conditions or factors surrounding the item in order to avoid possible subsequent disallowance. If the NSF Grants Officer determines that such costs are appropriate considering the special requirements of a particular NSF sponsored activity, this should be documented through an advance agreement or understanding. Advance agreements regarding the treatment of such costs may be incorporated by specific language in the award notice, or by other written correspondence.

1. Conflicting Guidelines

It is NSF's intent that the following guidance on cost allowability be consistent with the Federal cost principles. However, in the event of any discrepancy between the summary information contained in this Chapter and any specific provision of the cost principles contained in 2 CFR § 200, Subpart E, the cost principles in effect as of the start date of the NSF grant will govern. In the case of a discrepancy between the special provisions of an NSF grant and the standards of the cost principles, the special provisions of the grant will govern.

2. Other Considerations

a. Maximum Obligation

The maximum obligation of NSF for support of the project will not exceed the amount specified in the grant, as amended. NSF does not amend grants to provide additional funds for such purposes as reimbursement for unrecovered indirect costs resulting from the establishment of final negotiated rates, or for increases in salaries, fringe benefits and other costs.

b. Pre-Award Costs

(1) Grantees may incur allowable pre-award costs within the 90-day period immediately preceding the start date of the grant providing:

(a) the approval of pre-award spending is made and documented in accordance with the grantee's procedures; and

(b) the advanced funding is necessary for the effective and economical conduct of the project.[56]

[55] Applicable cost principles for for-profit organizations are contained in Federal Acquisition Regulation Part 31.
[56] In the case of a renewal award, grantees are reminded that costs incurred under the old grant cannot be transferred to the new grant.

(2) Pre-award expenditures are made at the grantee's risk. Grantee authority to approve pre-award costs does not impose an obligation on NSF: (a) in the absence of appropriations; (b) if an award is not subsequently made; or (c) if an award is made for a lesser amount than the grantee anticipated.

(3) Requests for pre-award costs for periods exceeding 90 days must be submitted to NSF via use of NSF's electronic systems. Pre-award expenditures prior to funding of an increment within a continuing grant are not subject to this limitation or approval requirement, but are subject to paragraph (2) above.

Additional information on pre-award costs is available at 2 CFR §§ 200.308(d)(1) & (d)(4).

c. Post-End Date Costs

NSF funds may not be expended subsequent to the end date of the grant, except to liquidate valid commitments that were made on or before the end date, e.g., commitment of project funds for subrecipient or contractor for services rendered during that award period but not billed to the grantee until after the grant ended. (See Chapter VIII.E.2.) Generally, the costs of equipment or materials and supplies ordered after the end date may not be charged to the project.

In addition, the grantee typically should not purchase items of equipment, computing devices, or restock materials and supplies in anticipation of the end date of the grant where there is little or no time left for such items to be utilized in the actual conduct of the research.
However, in accordance with 2 CFR § 200.461, Publication and Printing costs, grantees may charge the NSF award before closeout for the costs of publication or sharing of research results, if the costs are not incurred during the period of performance of the award.

3. Prior Written Approvals

The funding of items identified in the NSF grant budget constitutes NSF's authorization for the grantee to incur these costs, provided there is not a specific limitation in the grant language and the costs are otherwise allowable, allocable, and reasonable in accordance with the cost principles contained in 2 CFR § 200, Subpart E.

If required in furtherance of the project, the grantee is authorized to transfer funds from one budget category to another for allowable expenditures. Grantees should refer to the applicable grant terms and conditions referenced in the grant. The Research Terms and Conditions (RTC), Appendix A, (NSF column) provides a consolidated listing of prior approvals that are required to be obtained from NSF. Unless otherwise specified in the grant notice, no additional prior approvals beyond those specified in RTC Appendix A are required. Chapter VII.A.2 also provides a listing of required notifications to NSF.

When a change requires NSF approval, the request must be submitted via NSF's electronic systems. The request should clearly state which budget items, if any, are to be changed and by what amounts, and should explain the reasons for any changes.

B. Direct Costs

1. Compensation - Personal Services

a. Salaries and Wages

Compensation paid or accrued by the organization for employees working on the NSF-supported project during the grant period is allowable, in accordance with 2 CFR § 200.430.

b. Fringe Benefits

If the proposer's usual accounting practices provide that its contributions to employee benefits (leave, employee insurance, social security, retirement, other payroll-related taxes, etc.) be treated as direct costs, NSF grant funds may be requested to fund fringe benefits as a direct cost. These are typically determined by application of a calculated fringe benefit rate for a particular class of employee (full time or part-time) applied to the salaries and wages requested. They also may be paid based on actual costs for individual employees, if that institutional policy has been approved by the cognizant Federal agency for Indirect Costs.

Fringe benefits that are not accrued but are charged as direct costs and incurred under "pay as you go plans" may be subject to reasonableness determination where the benefits are earned under other work and charged to the last activity on which the employee was working. This is of particular concern for large lump sum payments for leave, disability, pregnancy or other employee fringe benefits. See 2 CFR § 200.431 for the definition and allowability of fringe benefits.

2. Intra-University (IHE) Consulting

Since intra-university consulting is assumed to be undertaken as a university obligation requiring no compensation in addition to full-time salary, the principles summarized in Chapter X.B.1, also apply to those who function as consultants or otherwise contribute to a project conducted by another faculty member of the same institution. However, in unusual cases where consultation is across departmental lines or involves a separate or remote operation, and the work performed by the consultant is in addition to his/her regular appointment, any charges for such work representing extra compensation above the institutional base salary are allowable if consistent with established university policy and the applicable cost principles.

3. Federal Employees

Employees of the Federal government (other than NSF) may be utilized as lecturers or staff members on a project and may receive compensation and/or expenses if they obtain prior approval from their agencies to participate, and if services to the project are performed outside their regular working hours or while they are on leave status from official duties. Under no circumstances may NSF employees receive compensation from an NSF-supported project.

C. Other Direct Costs

1. Rearrangement and Reconversion Costs

a. Except under certain programs, NSF does not normally make grants for construction or facility improvements. However, rearrangement and reconversion costs that do not constitute construction (i.e., rearrangement and reconversion costs aggregating $25,000 or less) may be allowable under NSF grants to adapt space or utilities within a completed structure to accomplish the objective of the NSF-supported activity, provided that the:

(i) grantee is not an individual or a foreign organization;

(ii) building has a usable life consistent with project purposes and is architecturally suitable for conversion;

(iii) rearrangement and reconversion costs are essential to the project supported by the grant; and

(iv) space involved will actually be occupied by the project. In situations where the space is rented, in order for the costs of the rearrangement and reconversion to be allowable, the grantee must secure a lease for the length of the project.

b. Rearrangement and reconversion expenditures which do not exceed $25,000 may be approved by grantees. For rearrangement and reconversion expenditures exceeding $25,000, the grantee must request prior approval from NSF via use of NSF's electronic systems. Otherwise, any plans for such rearrangement or reconversion should be clearly set forth in the proposal. If approved by NSF, such approval must be indicated in the award notice. Note that 2 CFR § 200, Appendix II contains provisions that must be included in contracts made by the grantee.

See 2 CFR § 200.462 for additional information on rearrangement and reconversion costs.

2. News Release Costs

Costs of communicating with the public and press to announce the results and accomplishments of an NSF-supported project are allowable. Grantees should be aware of the restrictions on advertising, organizational promotion, and lobbying costs as outlined in the applicable cost principles. See 2 CFR § 200.421 for additional information on advertising and public relations.

In the event the performing organization wishes to collaborate with NSF in a simultaneous news release, arrangements may be made through the NSF's Office of Legislative and Public Affairs, Public Affairs Group, telephone (703) 292-8070.

3. Travel and Temporary Dependent Care Costs

Travel, meal and hotel expenses of grantee employees who are not on travel status are unallowable. Costs of employees on travel status are limited to those specifically authorized by 2 CFR § 200.474.

Temporary dependent care costs (a dependent is defined in 26 USC 152) above and beyond regular dependent care that directly results from travel to conferences are allowable costs provided that the costs are:

a. a direct result of the individual's travel for the NSF conference award;

b. consistent with the grantee's documented travel policy for all employee travel; and

c. only temporary during the travel period.

See 2 CFR § 200.474 for additional information on travel costs.

D. Indirect Costs

1. NSF Policy

a. Except as noted in an NSF program solicitation, it is NSF policy that grantees are entitled to reimbursement from grant funds for indirect costs (F&A) allocable to the NSF share of allowable direct costs of a project. NSF program staff are not authorized to negotiate indirect costs as a discrete item of a proposal

budget. NSF program staff also are not authorized to suggest or request that PI/PDs seek reductions or waivers of indirect costs.

b. The awarded indirect cost rate is generally based upon a grantee's current Federally negotiated indirect cost rate agreement. When establishing an indirect rate for an award where the domestic grantee does not have a current negotiated rate agreement, NSF will consider the rate proposed in the budget, the grantee's indirect cost proposal submission, the amount of total funding requested and other pertinent financial factors. Supporting documentation is not required for organizations that request a *de minimis* rate of 10% (or less) of modified total direct costs. Since some types of rates limit indirect cost recoveries and require adjustments, grantees receiving awards should ensure that they understand the type of indirect cost rate, the applicable base and the type of rate used in the grant.

Foreign grantees that do not have a US Federally negotiated indirect cost rate are limited to a *de minimis* indirect cost rate recovery of 10% of modified total direct costs. Foreign grantees that have a negotiated rate agreement with a US Federal agency may recover indirect costs at the current negotiated rate.

Types of indirect cost rates that are most frequently used on NSF awards are as follows:

(i) Predetermined Rate – As authorized by 41 USC § 4708, and recommended by the Federal cost principles in 2 CFR § 200, Appendices III, IV, and VI, NSF has elected to use predetermined rates, where appropriate. A predetermined rate is an indirect cost rate, applicable to a specified current or future period, usually the organization's fiscal year. The rate is based on an estimate of the costs to be incurred during the period. A predetermined rate is not subject to adjustment.

Special Limitation concerning Predetermined Rates at Colleges and Universities subject to 2 CFR § 200 Appendix III, paragraph C.7 contains an additional restriction on recovery of indirect costs for colleges and universities and other organizations of higher education. These awardees are subject to a further limitation in that the negotiated rate at the time the award is made shall be used throughout the life of the award. The applicable text from 2 CFR § 200 Appendix III, paragraph C.7 is repeated below:

> "7. Fixed rates for the life of the sponsored agreement.
>
> "Federal agencies must use the negotiated rates except as provided in paragraph (e) of §200.414 Indirect (F&A) costs, in effect at the time of the initial award throughout the life of the Federal award. Award levels for Federal awards may not be adjusted in future years as a result of changes in negotiated rates. "Negotiated rates" per the rate agreement include final, fixed, and predetermined rates and exclude provisional rates. "Life" for the purpose of this subsection means each competitive segment of a project. A competitive segment is a period of years approved by the Federal awarding agency at the time of the award. If negotiated rate agreements do not extend through the life of the Federal award at the time of the initial award, then the negotiated rate for the last year of the award must be extended through the end of the life of the Federal award."

Where grantees receive limited NSF funding, the Foundation may elect to set award specific rates as opposed to a formal annual negotiated indirect cost rate agreement. In these cases, the award notice will specify the rate type and application base. If the award notice is silent on the rate type, the rate approved in the award budget will be considered a predetermined rate.

(ii) Provisional (or billing) rate – A provisional rate is a temporary indirect cost rate applicable to a specified period which is used for funding, interim reimbursement, and reporting indirect costs pending the establishment of a final rate for the period.

Grantees with provisional rates are required to submit indirect cost proposals to their cognizant Federal Agency for rate negotiation within six months after the close of each fiscal year. Adjustments to awards for amounts previously billed at provisional rates are required after final indirect cost rates are established.

(iii) Final rate – A final rate is an indirect cost rate applicable to a specified past period which is based on the actual costs of the period. A final rate is not subject to adjustment.

c. NSF will not amend a grant solely to provide additional funds for changes in indirect cost rates.

d. NSF will generally fund continuing grant increments and supplemental support using the negotiated indirect cost rate(s) approved at the time of the initial award. (See Chapter VI.E.)

e. Any negotiations with respect to business and financial matters on specific grants, including the amount of indirect cost reimbursement, are conducted by the cognizant Grants Officer in DGA with an authorized official of the grantee's organization. When other Federal agencies are responsible for negotiating indirect cost rates and an awardee does not have a current negotiated indirect cost rate in effect at the time of award but had a previous indirect cost rate agreement, DGA may elect to use the most recently negotiated rate for award funding purposes. The NSF Cost Analysis and Pre-Award Review Branch in the Division of Institution and Award Support provides advisory assistance to the Grants Officer.

2. NSF Cognizant Organizations

Organizations for which NSF is the cognizant agency for negotiation of indirect cost rates and who do not have approved award specific rates are required to submit indirect cost proposals, reconciled to financial statements, within six months after the close of each accounting year during which NSF has active awards. Indirect cost proposals should be sent to the Cost Analysis and Pre-Award Review Branch. Grantees are responsible for timely submission of indirect cost rate proposals. If the organization is unable to submit their indirect cost rate proposal within the time frame allotted, then an extension should be requested. Failure to submit the indirect cost proposal and to have a negotiated indirect cost rate in place could lead to NSF withholding reimbursement of indirect costs.

See http://www.nsf.gov/bfa/dias/caar/docs/idcsubmissions.pdf for indirect cost rate proposal submission requirements.

Such proposals and requests for extensions in time to submit proposals should be sent electronically to BFACAARMPR@nsf.gov or by paper copy to Lead Analyst Indirect Costs, National Science Foundation, NSF Cost Analysis and Pre-Award Review Branch, 2415 Eisenhower Avenue, Alexandria, Virginia 22314.

E. Fee Payments under NSF Grants

Payment of fees (profit) is allowable only if expressly authorized by the terms and conditions of the NSF award.

Chapter XI: Other Post Award Requirements and Considerations

A. Non-Discrimination Statutes and Regulations

1. General

a. A number of statutes prohibit NSF grantees from discriminating against individuals who participate in any of their programs, services, and activities. These statues include: Title VI of the Civil Rights Act of 1964 (prohibits discrimination on the basis of race, color, or national origin); Section 504 of the Rehabilitation Act of 1973 (prohibits discrimination on the basis of disability); Title IX of the Educational Amendments of 1972 (prohibits discrimination on the basis of sex in Federally assisted education programs or activities); and the Age Discrimination Act of 1975 (prohibits discrimination on the basis of age). In addition to statutory prohibitions, Executive Order (E.O.) 11246 bars various types of discriminatory employment practices under grants for construction, and E.O. 13166 "Improving Access to Services for Persons with Limited English Proficiency (LEP)" requires government agencies to take steps to provide meaningful access to its programs and activities to members of the public who are limited in their English proficiency, including recipients' programs and activities. The following sections discuss the application of each of these Statutes and E.O. 11246 and E.O. 13166 to NSF grantees.

b. When an NSF grantee receives a complaint alleging discrimination under any of these statutes and the complainant requests that NSF conduct the investigation, the original complaint is to be sent to the Director, Office of Diversity and Inclusion (ODI), National Science Foundation, 2415 Eisenhower Avenue, Alexandria, VA 22314

Information on the complaint process can be found at https://www.nsf.gov/od/odi/titlevi_ix.jsp. The complainant may also use the grantee's internal discrimination complaint process. Such processes are required for NSF grantees to implement under Title IX for sex discrimination complaints, including sexual harassment and under Section 504 for disability discrimination complaints. Grantees are prohibited by these regulations from engaging in acts of intimidation, coercion or retaliation against: a) any person who attempts to assert a right protected by these statutes and their implementing regulations; or b) cooperates in any part of NSF's review, investigation and compliance resolution or enforcement process.

c. NSF proposers and grantees are required under these regulations to keep records deemed pertinent to ascertain a grantee's compliance with these regulations. It also requires the grantee to submit to NSF timely, complete and accurate reports, and in such form and containing such information, as NSF may determine to be necessary to ascertain whether the grantee has complied or is complying with these regulations.

d. NSF proposers and grantees are advised that NSF may conduct pre-award and post-award compliance reviews to ensure that the grantee is complying with NSF's civil rights regulations. Grantees are required to provide NSF with access to information, records, grantee officials, grantee program participants, and facilities deemed pertinent by NSF to ascertain a grantee's compliance with these regulations.

e. NSF proposers and grantees are required under these regulations to make available to its participants, beneficiaries, and other interested persons, information regarding these regulations and their applicability to the program(s) under which the grantee receives Federal financial assistance from NSF. Proposers and grantees are also required to make such information available to these parties in such a manner as NSF finds necessary to apprise such persons of the protections against discrimination assured them by these regulations.

f. NSF proposers and grantees may contact ODI at the address in (b) above or at 1-703-292-8020 with any questions regarding these non-discrimination statutes and their implementing NSF regulations and their applicability to their institutions' programs, services and activities.

2. Certification of Compliance/Civil Rights Certifications

Each of these regulations require that as a condition of approval of an assistance proposal, the proposer must execute a Certification of Compliance, whether or not a similar assurance form has been filed with another Federal agency. (See Exhibit II-6 for additional information.). Before any organization serves as a subrecipient on an NSF grant (for other than the purchase of commercially available materials and supplies, equipment or general support services), it must first file an Assurance of Compliance with the grantee. Each NSF grant contains as part of the grant terms and conditions, an article implementing the applicable nondiscrimination statutes and regulations.

3. Title VI of the Civil Rights Act of 1964

a. Background

Section 602 of the Civil Rights Act of 1964 (42 USC § 2000d, et seq.) provides that no person in the US shall, on the grounds of race, color or national origin, be excluded from participation in, be denied the benefits of, or be otherwise subjected to discrimination under any program or activity receiving Federal financial assistance. Section 602 requires that each Government agency which is empowered to extend such financial assistance issue rules or regulations implementing Title VI with respect to such programs or activities administered by the agency.

b. NSF Regulations

NSF regulations implementing Title VI are found at 45 CFR § 611. These regulations apply to grantees, subrecipients and successors in interest. These regulations do not apply to contracts for commercially available materials and supplies, equipment or general support services.

c. Key Procedural and Programmatic Requirements

The procedures for ensuring NSF grantees' compliance with Title VI are found at 45 CFR §§ 611.6-11. These regulations provide for the compliance and investigative actions that NSF may initiate in order to ascertain grantees' compliance with Title VI. These regulations also provide for the enforcement procedures to compel grantee compliance with Title VI if a grantee refuses to take corrective action for violations found during an NSF complaint investigation or compliance review. NSF's Section 504, Title IX and Age Discrimination Act regulations incorporate by reference the Title VI compliance, investigative and enforcement procedures.

4. Section 504 of the Rehabilitation Act of 1973

a. Background

Section 504 of the Rehabilitation Act of 1973, as amended (29 USC § 794) provides that "no otherwise qualified individual with a disability in the United States...shall, solely by reason of her or his disability, be excluded from the participation in, be denied the benefits of, or be subjected to discrimination under any program or activity receiving Federal financial assistance...."

b. NSF Regulations

NSF regulations implementing Section 504 are found at 45 CFR § 605. With a few changes, these regulations mirror the regulations issued by the Department of Health and Human Services (DHHS) at 45 CFR § 84. These regulations also apply to subrecipients and successors in interest. These regulations do not apply to contracts for commercially available materials and supplies, equipment or general support services.

c. Key Procedural and Programmatic Requirements

NSF's Section 504 regulations, in addition to prohibiting discrimination on the basis of disability, requires NSF grantees to designate a Section 504 coordinator, publish and adopt procedures to investigate and adjudicate complaints of internally-filed disability discrimination complaints, and notify the public of its obligation to comply with Section 504. NSF's Section 504 regulation also requires NSF grantees to conduct a self-evaluation to determine the accessibility of its programs, services, activities and facilities, and implement modifications, implement corrective actions identified from the self-evaluation and conduct a transition plan to identify those physical elements of the NSF grantee's facilities that are inaccessible to individuals with disabilities and require retrofit. NSF's Section 504 regulations also require that all new construction and alterations to existing facilities are accessible to individuals with disabilities and meet current accessibility standards. See 45 CFR § 605 for all of the regulatory requirements for NSF grantees.

5. Title IX of the Education Amendments of 1972

a. Background

Subject to exceptions regarding admission policies at certain religious and military organizations, Title IX of the Education Amendments of 1972 (20 USC §§ 1681-1686) prohibits discrimination on the basis of sex in any education program or activity receiving Federal financial assistance.

b. NSF Regulations

NSF regulations implementing Title IX are found at 45 CFR § 618. All NSF grantees who provide or operate educational programs as defined in 45 CFR 618.105 must comply with Title IX. These regulations also apply to subrecipients and successors in interest who operate such educational programs. These regulations do not apply to contracts for commercially available materials and supplies, equipment or general support services.

c. Key Procedural and Programmatic Requirements

NSF's Title IX regulations, in addition to prohibiting discrimination on the basis of sex in education programs, requires NSF grantees to designate a Title IX coordinator, publish and adopt procedures to investigate and adjudicate complaints of internally-filed sex discrimination complaints, notify the public of its obligation to comply with Title IX, conduct a self-evaluation to determine whether its programs, services, activities and facilities comply with Title IX and implement corrective actions identified from the self-evaluation. See 45 CFR § 618 for all of the regulatory requirements for NSF grantees under Title IX.

6. Age Discrimination Act of 1975

a. Background

The Age Discrimination Act of 1975, as amended (42 USC § 6101 et seq.), provides that pursuant to regulations issued by DHHS "no person in the United States shall, on the basis of age, be excluded from participation in, be denied the benefits of, or be subjected to discrimination under, any program or activity receiving Federal financial assistance."

b. NSF Regulations

NSF regulations implementing the Age Discrimination Act are found at 45 CFR § 617. These regulations also apply to subrecipients and successors in interest. These regulations do not apply to contracts for commercially available materials and supplies, equipment or general support services.

c. Key Procedural and Programmatic Requirements

NSF's Age Discrimination Act regulations, in addition to prohibiting discrimination on the basis of age in education programs, requires NSF grantees conduct a self-evaluation to determine each recipient shall identify all age distinctions it uses and justify each age distinction it imposes on the program or activity receiving Federal financial assistance from NSF. See 45 CFR § 617 for all of the regulatory requirements under the Age Discrimination Act for NSF grantees.

7. Equal Employment Opportunity under E.O. 11246

a. Background

E.O. 11246, as amended, requires contractors and subcontractors performing Federally assisted construction projects to provide equal opportunity, without regard to race, color, religion, sex or national origin, to persons employed or seeking employment with them. This E.O. may apply to some NSF grants for construction. E.O. 11246 is enforced by the US Department of Labor's Office of Federal Contract Compliance Programs.

b. Grant Condition

In instances when E.O. 11246 is applicable, the grant will include an equal opportunity clause in conformance with regulations issued by the Secretary of Labor at 41 CFR § 60.

8. Limited English Proficiency under E.O. 13166

a. Background

E.O. 13166 requires Federal agencies and NSF grantees to take steps to provide meaningful access to its programs and activities to program participants who are limited English proficient (LEP). The failure to ensure that LEP persons can effectively participate in or benefit from Federally-assisted programs and activities may violate the prohibition under Title VI of the Civil Rights Act of 1964 and NSF's Title VI implementing regulations against national origin discrimination. The US Department of Justice has published guidance to assist NSF grantees in their efforts to comply with Title VI when providing LEP individuals with access to recipient programs, services and activities.

b. Grant Condition

In instances when E.O. 13166 is applicable, NSF grantees should contact NSF's LEP Coordinator within the Office of Diversity and Inclusion for guidance at2415 Eisenhower Avenue, Alexandria, VA 22314.

B. Protection of Living Organisms

1. Human Subjects

a. Background

The grantee is responsible for the protection of the rights and welfare of human subjects involved in activities supported by NSF. All research involving human subjects supported or otherwise subject to regulation by any Federal agency is covered by a policy set forth in the Common Rule, "Protection of Human Subjects" For projects lacking definite plans for the use of human subjects pursuant to 45 CFR § 690.118, NSF can accept a preliminary approval from an IRB that establishes a limited approval period, requires the PI to submit an amendment or new IRB application prior to the expiration date, and stipulates that no work with human subjects, including recruitment, may be conducted until full IRB approval is obtained. Complete

instructions on preparation and submission of proposals that involve use of human subjects are available in Chapter II.D.5.

b. NSF Regulation

(i) NSF's Common Rule on Protection of Human Subjects is available on the NSF website at: https://www.nsf.gov/bfa/dias/policy/docs/45cfr690.pdf. Guidance about the regulation is available at: https://www.nsf.gov/bfa/dias/policy/human.jsp.

(ii) During the life of the award, additional IRB approval must be obtained if the protocols for the use of human subject has been changed substantively from those originally proposed and approved. When an additional IRB approval is required, the organization must provide to the cognizant NSF Program Officer a signed copy of the new IRB approval letter indicating approval of the covered activities and explicitly referencing the title of the award. In the event the grantee's FWA is canceled or lapses, the grantee must immediately notify the NSF Grants Officer identified in the award.

(iii) Supplements to existing awards do not require a separate IRB approval letter. If the scope of the project has been substantively changed from the award, however, a new signed IRB approval letter is required, indicating approval of the covered activities and explicitly referencing the title of the award.

c. Grant Conditions

Each NSF grant contains, as part of the grant terms and conditions, an article implementing NSF regulations on projects involving research with human subjects. In addition, each award notice contains the following language relating to a grantee's responsibilities regarding the use of human subjects during the life of the grant:

> "It is the grantee's responsibility to ensure that any human subjects work conducted under this award has an Institutional Review Board (IRB) approval, where required, and that such approval remains valid at all times that human subjects work is conducted under the award. Failure to comply with this condition will result in suspension and/or termination of the award."

2. Research Involving Recombinant or Synthetic Nucleic Acid Molecules

a. Applicability

This section applies to all research, for which NSF grant funds are used, that falls within the scope of the Guidelines for Research Involving Recombinant DNA or Synthetic Nucleic Acid Molecules (NIH Guidelines) (http://oba.od.nih.gov/rdna/nih_guidelines_oba.html), as amended in March 2013, hereafter referred to as the "Guidelines".

b. Policy

NSF grantees performing research within the US that falls within the scope of the Guidelines shall comply with the Guidelines, including the procedural requirements and any subsequent revisions as they are published in the Federal Register. Grantee responsibilities include:

(i) Each organization involved in the conduct of NSF-supported recombinant or synthetic nucleic acid research subject to the Guidelines must have a standing Institutional Biosafety Committee (IBC) as specified in Section IV of the Guidelines.

(ii) Recombinant or synthetic nucleic acid research subject to the Guidelines must be registered with the IBC indicating compliance with the containment requirements specified in Part III of the Guidelines.

IBCs are required to keep records of recombinant or synthetic nucleic acid research conducted at their organization in a form that is available to NSF upon request.

c. Research Requiring Prior Approval

In certain instances research should not be initiated or registered with IBCs prior to approval and determination of containment level by the Director, NIH. Normally such experiments are reviewed by the Recombinant DNA Advisory Committee before a decision is made by the Director, NIH. Such instances are:

(i) research for which containment levels are not explicitly specified by the Guidelines;

(ii) research involving experiments prohibited by the Guidelines, i.e., requests for exceptions to the Guidelines; and

(iii) requests to perform experiments without regard to the containment specified in the Guidelines, i.e., exemptions from the Guidelines.

3. Vertebrate Animals

a. Background

The grantee is responsible for the humane care and treatment of any vertebrate animal used or intended for use in such activities as field or laboratory research, development, training, experiments, biological testing or for related purposes supported by NSF grants.

b. Federal Regulations

(i) Any grantee performing research on vertebrate animals[57] shall comply with the Animal Welfare Act [7 USC 2131 et seq.] and the regulations promulgated thereunder by the Secretary of Agriculture [9 CFR 1.1-4.11] pertaining to the humane care, handling, and treatment of vertebrate animals held or used for research, teaching, or other activities supported by Federal awards. The grantee is expected to ensure that the guidelines described in the National Academies of Science, Engineering and Medicine (NASEM) Publication, "*Guide for the Care and Use of Laboratory Animals*" are followed and to comply with the *Public Health Service Policy and Government Principles Regarding the Care and Use of Animals* (included as Appendix D to the NAS Guide). Taxon-specific guidelines may be used as supplemental references.[58] Departures from the Guide must be approved by the Institutional Animal Care and Use Committee (IACUC) and based on scientific, veterinary, medical, or animal welfare issues (for more information, see OLAW-Departures from the Guide).

(ii) During the life of the award, additional IACUC approval must be obtained if the protocols for the care of use of vertebrate animals have been changed substantively from those originally proposed and approved. When an additional IACUC approval is required, the organization must provide to the cognizant NSF Program Officer a signed copy of a new IACUC approval letter indicating approval of the covered activities and explicitly referencing the title of the award. In the event the grantee's PHS Assurance is canceled or lapses, the grantee must immediately notify the NSF Grants Officer identified in the award.

[57] In addition to vertebrate animals covered by the Animal Welfare Act, the requirements specified in this coverage also are extended to rats, birds, and mice.

[58] Guidelines to the Use of Wild Birds in Research; Guidelines of the American Society of Mammalogists for the Use of Wild Mammals in Research; Guidelines for the Use of Fishes in Research; Guidelines for the Use of Live Amphibians and Reptiles in Field and Laboratory Research.

(iii) Supplements to existing awards do not require a separate IACUC approval letter. If the scope of the project has been substantively changed from the parent award, however, a new signed IACUC approval letter is required.

(iv) Research facilities subject to the Animal Welfare Act using or intending to use live animals in research and who receive Federal funding are required to register the facility with the Animal and Plant Health Inspection Service (APHIS), US Department of Agriculture. A current listing of licensed animal dealers may also be obtained from APHIS. The location of the nearest APHIS Regional Office, as well as information concerning this and other APHIS activities, may be obtained at: http://www.aphis.usda.gov/.

(v) Awards to US grantees for projects involving the care or use of vertebrate animals at a foreign institution or foreign field site also require approval of research protocols by the US grantee's IACUC. If the project is to be funded through a grant to a foreign institution or through an individual fellowship award that will support activities at a foreign institution, NSF will require a statement from the international organization explicitly listing the proposer's name and referencing the title of the award to confirm that activities will be conducted in accordance with all applicable laws in the foreign country and that the *International Guiding Principles for Biomedical Research Involving Animals* (see http://www.cioms.ch/) will be followed.

c. Grant Conditions

Each NSF grant contains, as part of the grant terms and conditions, an article implementing NSF regulations on projects involving research with vertebrate animals. In addition, each award notice contains the following language relating to a grantee's responsibilities regarding the care or use of vertebrate animals during the life of the grant:

> "It is the grantee's responsibility to ensure that any vertebrate animal work conducted under this award has an Institutional Animal Care and Use Committee (IACUC) approval, where required, and that such approval remains valid at all times that vertebrate animal work is conducted under the award. Failure to comply with this condition will result in suspension and/or termination of the award."

4. Government Permits and Activities Abroad

a. For awards that include activities requiring permits from appropriate Federal, State, or local government authorities, the grantee should obtain any required permits prior to undertaking the proposed activities.

b. The grantee should ensure that activities carried on outside the US are coordinated as necessary with appropriate US and foreign government authorities and that necessary licenses, permits or approvals are obtained prior to undertaking proposed activities. NSF does not assume responsibility for grantee compliance with the laws and regulations of the country in which the work is to be conducted.

5. Life Sciences Dual Use Research of Concern (DURC)

a. Applicability

This section applies to all research, for which NSF grant funds may be used, that potentially falls within the scope of the *US Government Policy for Institutional Oversight of Life Sciences Dual Use Research of Concern* (http://www.phe.gov/s3/dualuse/Pages/default.aspx) as published in September 2014, hereafter referred to as the "Policy".

b. NSF Implementation of the Policy

NSF is committed to preserving the benefits of life sciences research while minimizing the risk of misuse of the knowledge, information, products, or technologies provided by such research. The purpose of NSF's implementation of this Policy is to clarify grantee expectations about NSF-funded research with certain high-consequence pathogens and toxins with potential to be considered dual use research of concern.

Grantees are responsible for monitoring the research progress and for implementation of all appropriate biosafety and biosecurity risk mitigation measures including compliance with all applicable laws and regulations related to that implementation, including the Policy specified above. While the White House Office of Science and Technology Policy has lifted the research funding pause on gain of function research as of January 2017 (https://www.phe.gov/s3/dualuse/Pages/GainOfFunction.aspx), NSF will not consider funding research that would lead to a gain of function for agents of concern.

C. Construction, Rearrangements and Reconversions

1. Davis-Bacon Act

The Davis-Bacon Act (40 USC §§ 276a et seq.) establishes minimum wages to be paid to laborers and mechanics on construction contracts to which the US is a party involving public buildings or public works within the US. A number of other statutes have extended this provision to specific Federal grant programs involving construction. However, unless specifically stated in the grant, the Davis-Bacon Act does not normally apply to NSF grants since grantees normally retain title to property acquired under the grant and the construction, if any, is normally on non-Government land.

2. Bonding and Insurance

The NSF Act (42 USC § 1870c) provides NSF with authority to enter into grants, contracts or other agreements without performance or other bonds. Therefore, unless the grant specifically provides otherwise, NSF does not require performance or other bonds or insurance.

3. Seismic Safety of Buildings

E.O. 12699 *Seismic Safety of Federal and Federally-assisted or Regulated New Building Construction* dated January 5, 1990, requires that consideration be given to seismic hazards in the design of buildings. Very seldom are NSF grantees involved with Federally-assisted construction and rarely are new buildings involved. NSF ensures compliance with the Order through its grantees by inclusion of a specific grant condition in any appropriate grant.

D. Intellectual Property

1. Patents and Inventions

a. Background

(i) The disposition of rights to inventions made by small business firms and non-profit organizations, including universities and other institutions of higher education, during NSF-assisted research is governed by Chapter 18 of Title 35 of the USC, commonly called the Bayh-Dole Act. In accordance with a Presidential Memorandum entitled *Government Patent Policy* issued on February 18, 1983 and under the authority of Section 12 of the National Science Foundation Act of 1950, as amended (42 USC § 1871), NSF applies the policies of the Bayh-Dole Act to all its grantees. The Department of Commerce (DOC) is the lead agency for implementing the Bayh-Dole Act and has published guidance to Federal agencies in 37 CFR §401. NSF's implementing rules are published in 45 CFR § 650.

(ii) NSF's standard Patent Rights clause, published at 45 CFR § 650.4(a) and below at Chapter XI.D.1.c, "Standard Patent Rights Clause" is identical to that prescribed in the DOC guidance (37 CFR § 401.14(a)) except that:

(a) NSF has tailored the clause to apply to grants and to identify NSF;

(b) pursuant to Section 401.5(d) of the DOC guidance (37 CFR § 401.5(d)), NSF has added to paragraph b. of the clause a stipulation that NSF reserves the right to direct a grantee to transfer to a foreign government or research performer such rights to any subject invention as are required to comply with any international treaty or agreement identified when the grant is made as being applicable to the assisted research;

(c) as permitted by Section 401.5(f) of the DOC guidance (37 CFR § 401.5(f)), NSF has added a subparagraph to the end of paragraph f. of the clause to require grantees or their representatives to send NSF confirmations of the Government licenses for subject inventions and of the page of any United States patent application that contains the Federal support clause;

(d) since NSF normally uses the same clause for all subrecipients, the first two subparagraphs of paragraph g. of the clause specified in the DOC guidance have been reduced to one;

(e) paragraph c.1 of the clause has been changed to require that invention disclosures be submitted electronically via the iEdison system; and

(f) paragraph L. of the clause has been changed to require that all communications required by the Patents Rights clause be submitted electronically via the iEdison system unless prior permission is obtained from the NSF Patent Assistant.

b. National Science Foundation Patent Policy

As authorized by the NSB, the Director of the NSF has adopted the following NSF patent policy.

(i) In accordance with the Bayh-Dole Act and the Presidential Memorandum entitled *Government Patent Policy* issued February 18, 1983, NSF will use the Patent Rights clause prescribed by DOC in all its funding agreements for the performance of experimental, developmental or research work, including grants made to foreign entities, unless NSF determines that some other provision would better serve the purposes of that Act or the interests of the US and the general public.

(ii) In funding agreements covered by a treaty or agreement that provide that an international organization or foreign government, research institute or inventor will own or share patent rights, NSF will acquire such patent rights as are necessary to comply with the applicable treaty or agreement.

(iii) If a grantee elects not to retain rights to an invention, NSF will allow the inventor to retain the principal patent rights unless the grantee, or the inventor's employer, if other than the grantee, shows that it would be harmed by that action.

(iv) NSF will normally allow any patent rights not wanted by the grantee or inventor to be dedicated to the public through publication in scientific or engineering journals or as a statutory invention registration. If another Federal agency is known to be interested in the relevant technology, however, NSF may give it an opportunity to review and patent the invention so long as that does not inhibit the dissemination of the research results to the research community.

c. Standard Patent Rights Clause

Each NSF grant contains as part of the grant terms and conditions, an article implementing the standard patent rights clause.

d. Electronic Invention Handling

Grantees are required to use the iEdison System to disclose NSF subject inventions. Detailed instructions for use of that system are provided at: http://s-edison.info.nih.gov/iEdison/ and should be followed for NSF subject inventions except that:

a. All communications required must be provided electronically as a PDF or TIFF file through iEdison unless prior permission for another form of submission is obtained from the NSF Patent Assistant.

b. NSF may request a Utilization or other report or statement regarding a subject invention.

Questions on use of iEdison and requests for permission to submit material in other forms may be sent to the NSF Patent Assistant at patents@nsf.gov, or at the Office of the General Counsel, National Science Foundation, 2415 Eisenhower Avenue, Alexandria, VA 22314.

2. Copyright

a. Rights to Copyrighted Material

The following principles governing the treatment of copyrighted material produced under NSF grants were adopted by the NSB.

(i) NSF normally will acquire only such rights to copyrighted material as are needed to achieve its purposes or to comply with the requirements of any applicable government-wide policy or international agreement.

(ii) To preserve incentives for private dissemination and development, NSF normally will not restrict, or take any part of income earned from, copyrighted material except as necessary to comply with the requirements of any applicable government-wide policy or international agreement.

(iii) In exceptional circumstances, NSF may restrict or eliminate a grantee's control of NSF-supported copyrighted material and of income earned from it, if NSF determines that this would best serve the purposes of a particular program or grant.

b. Standard Copyrighted Material Clause

Unless a special clause has been negotiated, each NSF grant that relates to scientific or engineering research contains as part of the grant terms and conditions, an article implementing the standard copyrighted material requirements.

c. Public Access to Copyrighted Material

NSF's policy on public access to copyrighted material (Public Access Policy) reflects the Foundation's commitment to making certain that, to the extent possible, the American public, industry and the scientific community have access to the results of Federally funded scientific research. Pursuant to this policy, grantees must ensure that articles in peer-reviewed scholarly journals and papers in juried conference proceedings:

- are deposited in a public access compliant repository (as identified in the Public Access Policy);
- are available for download, reading, and analysis within 12 months of publication;
- possess a minimum set of machine-readable metadata elements as described in the Public Access Policy; and

- are reported in annual and final reports with a persistent identifier.

Either the final printed version or the final peer-reviewed manuscript is acceptable for deposit. NSF's Public Access Policy applies to awards, funded in whole or in part, as a result of proposals submitted, or due, on or after January 25, 2016. NSF's Public Access Policy may be viewed at: http://www.nsf.gov/news/special_reports/public_access/index.jsp.
Each NSF grant contains as part of the grant terms and conditions, an article implementing the public access requirements.

3. Special Patent and Copyright Situations

a. Special Grant Provisions

At the request of the prospective grantee or on recommendation from NSF staff, the NSF Grants Officer, with the concurrence of the cognizant NSF Program Officer, may negotiate special patent or copyright provisions when he/she determines that exceptional circumstances require restriction or elimination of the right of a prospective grantee to control principal rights to subject inventions or writings in order to better achieve the objectives of the program, the National Science Foundation Act, or (in the case of inventions) Chapter 18 of Title 35 of the USC. Every special copyright or patent provision will allow the grantee, after an invention has been made or copyrighted material created, to request that it be allowed to retain principal rights to that invention or material, unless doing so would be inconsistent with an obligation imposed on NSF by statute, international agreement or pact with other participants in, or supporters of, the research.

b. Grants Not Primarily for Research

(i) Grants not primarily intended to support scientific or engineering research generally do not contain patent or copyrighted material provisions. Examples of such grants are travel, conference and equipment grants.

(ii) NSF Fellowships and Traineeships. In accordance with Section 212 of Title 35 of the USC, NSF claims no rights to inventions made by fellows or trainees. The following provision will be included in each fellowship or traineeship program solicitation and made part of the grant:

> **INTELLECTUAL PROPERTY RIGHTS**
>
> The National Science Foundation claims no rights to any inventions or writings that might result from its fellowship or traineeship grants. However, fellows and trainees should be aware that the NSF, another Federal agency, or some private party may acquire such rights through other support for particular research. Also, fellows and trainees should note their obligation to include an Acknowledgment and Disclaimer in any publication.

[END OF PROVISION]

c. Grants Affected by International Agreements

(i) Many of the bilateral and multilateral treaties and agreements underlying NSF's international cooperative research programs contain provisions on allocation of rights to inventions or writings. These sometimes require an allocation of rights different from that provided by the standard Copyrighted Material or Patent Rights clauses. In those cases, the standard clauses will be modified through the addition of the following to the grant:

> "This project is supported under the cooperative program listed below. Your rights in inventions, writings, and data may be affected."

The applicable agreement or treaty will be identified immediately beneath that sentence.

(ii) After an invention is disclosed to the Patent Assistant, the recipient of a grant subject to an international agreement will be informed as to what rights, if any, it must transfer to foreign participants. Grantees also may ask the NSF Program Officer for copies of the identified international agreement before or after accepting a grant.

4. Dissemination and Sharing of Research Results

a. Investigators are expected to promptly prepare and submit for publication, with authorship that accurately reflects the contributions of those involved, all significant findings from work conducted under NSF grants. Grantees are expected to permit and encourage such publication by those actually performing that work, unless a grantee intends to publish or disseminate such findings itself.

b. Investigators are expected to share with other researchers, at no more than incremental cost and within a reasonable time, the primary data, samples, physical collections and other supporting materials created or gathered in the course of work under NSF grants. Grantees are expected to encourage and facilitate such sharing. Privileged or confidential information should be released only in a form that protects the privacy of individuals and subjects involved. General adjustments and, where essential, exceptions to this sharing expectation may be specified by the funding NSF Program or Division/Office for a particular field or discipline to safeguard the rights of individuals and subjects, the validity of results, or the integrity of collections or to accommodate the legitimate interest of investigators. A grantee or investigator also may request a particular adjustment or exception from the cognizant NSF Program Officer.

c. Investigators and grantees are encouraged to share software and inventions created under the grant or otherwise make them or their products widely available and usable.

d. NSF normally allows grantees to retain principal legal rights to intellectual property developed under NSF grants to provide incentives for development and dissemination of inventions, software and publications that can enhance their usefulness, accessibility and upkeep. Such incentives do not, however, reduce the responsibility that investigators and organizations have as members of the scientific and engineering community, to make results, data and collections available to other researchers.

e. NSF program management will implement these policies for dissemination and sharing of research results, in a way appropriate to field and circumstances, through the proposal review process; through award negotiations and grant conditions; and through appropriate support and incentives for data cleanup, documentation, dissemination, storage and the like.

(f) Each NSF grant contains, as part of the grant terms and conditions, an article implementing dissemination and sharing of research results.

5. Tangible Property

a. Background

Some NSF grants support collection or creation of tangible property, such as insects, marine life, drilling core samples and genetically-altered microorganisms. As used in this section “tangible property” means any personal property other than equipment (see Chapter II.C.2.g.(iii)), and intellectual property.

b. Legal Rights to Tangible Property

Unless otherwise provided in the grant, all legal rights to tangible property collected or created during NSF-assisted research remain with the grantee or investigators as determined by the policies of the organization. As members of the scientific and engineering community, both grantees and investigators are responsible for making such tangible property appropriately available to other researchers.

E. Publication/Distribution of Grant Materials

1. NSF Policy

NSF advocates and encourages open scientific and engineering communication. NSF expects significant findings from research it supports to be promptly submitted for publication, with authorship that accurately reflects the contributions of those involved. Copyrighted material published in peer-reviewed scholarly journals and papers included in juried conference proceedings must comply with NSF's Public Access Policy as implemented in the grant terms and conditions.

2. Costs

Cost of documenting, preparing, publishing, disseminating and sharing research findings and supporting material are allowable charges against the grant. (See Chapter X.A.2.c.)

3. Responsibilities

Unless otherwise provided in the grant, preparation, content, editing, identification of authorship and submission for publication of significant research findings are the responsibility of the investigators, consistent with such policies and procedures as the grantee may prescribe.

4. Grantee Obligations

a. Acknowledgement of Support. Unless otherwise provided in the grant, the grantee is responsible for assuring that an acknowledgment of NSF support is made:

(i) in any publication (including web pages) of any material based on or developed under this project, in the following terms:

> "This material is based upon work supported by the National Science Foundation under Grant No. (NSF grant number)."

(ii) NSF support also must be orally acknowledged during all news media interviews, including popular media such as radio, television and news magazines.

b. Disclaimer. The awardee is responsible for assuring that every publication of material (including web pages) based on or developed under this award, except scientific articles or papers appearing in scientific, technical or professional journals, contains the following disclaimer:

> "Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation."

c. Copies for NSF. The grantee is responsible for assuring that the cognizant NSF Program Officer is provided access to, either electronically or in paper form, a copy of every publication of material based on or developed under this award, clearly labeled with the award number and other appropriate identifying information, promptly after publication.

d. Compliance with NSF Public Access Policy. The grantee is responsible for ensuring that copyrighted material published in peer-reviewed scholarly journals and papers in juried conference proceedings are accessible to the public in accordance with the grant terms and conditions.

e. Grantees also should note their obligations in regard to copyrights (see Chapter XI.D.2) and their responsibilities as members of the scientific and engineering community to disseminate and share research results (see Chapter XI.D.4).

f. Each NSF grant contains, as part of the grant terms and conditions, an article implementing the grantee obligations regarding the acknowledgement of support and disclaimer.

F. International Considerations

1. Travel to Foreign Countries

a. Policy

(i) Expenses for transportation, lodging, subsistence and related items incurred by project personnel and by outside consultants employed on the project, (see Chapter II.C.2.g.(iv)) who are on travel status on business related to an NSF-supported project are allowable as prescribed in the governing OMB cost principles.

(ii) Except as provided in the governing OMB cost principles, the difference between economy airfare and a higher-class airfare is unallowable. A train, bus or other surface carrier may be used in lieu of, or as a supplement to, air travel at the lowest first-class rate by the transportation facility used. If such travel, however, could have been performed by air, the allowance will not normally exceed that for jet economy airfare.

(iii) Support for the foreign travel of an investigator's dependents is allowable only under the conditions identified in Chapter X.C.5.

b. Use of US-Flag Air Carriers

(i) In accordance with the Fly America Act (49 USC 40118), any air transportation to, from, between, or within a country other than the US of persons or property, the expense of which will be assisted by NSF funding, must be performed by or under a code-sharing arrangement with a US-flag air carrier if service provided by such a carrier is available (see Comptroller General Decision B-240956, dated September 25, 1991). Tickets (or documentation for electronic tickets) must identify the US flag air carrier's designator code and flight number.

(ii) For the purposes of this requirement, US-flag air carrier service is considered available even though:

(a) comparable or a different kind of service can be provided at less cost by a foreign-flag air carrier;

(b) foreign-flag air carrier service is preferred by, or is more convenient for, NSF or traveler; or

(c) service by a foreign-flag air carrier can be paid for in excess foreign currency.

(iii) The following rules apply unless their application would result in the first or last leg of travel from or to the US being performed by a foreign-flag air carrier:

(a) a US-flag air carrier shall be used to destination or, in the absence of direct or through service, to the farthest interchange point on a usually traveled route.

(b) if a US-flag air carrier does not serve an origin or interchange point, a foreign-flag air carrier shall be used only to the nearest interchange point on a usually traveled route to connect with a US flag air carrier.

c. Use of Foreign-Flag Air Carriers

There are certain circumstances under which use of a foreign-flag air carrier is permissible. These circumstances are outlined below:

(i) Airline "Open Skies" Agreement*:*

A foreign-flag air carrier may be used if the transportation is provided under an air transportation agreement between the United States and a foreign government, which the Department of Transportation has determined meets the requirements of the Fly America Act. For information on "Open Skies" agreements in which the United States has entered, please refer to the GSA website at: http://www.gsa.gov/portal/content/103191.

Note on US/European Union Open Skies Agreement

In 2007, the US entered into an “Open Skies” Agreement with the European Union (EU). This agreement was modified in June 2010. The current Agreement gives European Community airlines (airlines of Member States) the right to transport passengers and cargo on flights funded by the US government, when the transportation is between: (1) any two points outside the United States; or (2) a point in the United States and any point outside the United States that the EU airline is authorized to serve under the “Open Skies” Agreement.

In 2011, two significant changes were made to the US/EU Open Skies Agreement. First, EU airlines are now granted the right to transport civilian agency-funded passengers who are NOT eligible to travel on GSA Airline City Pair Contract fares (e.g., grantees) between a point in the United States and a point outside the United States even if there is a GSA Airline City Pair Contract fare in effect between the origin and destination points. An individual, however, who is traveling on a route for which there is a City Pair Contract fare in effect, *and who is eligible for such a fare* (e.g., Federal employee), are required to fly on a US carrier, absent another applicable exception.

Second, under the amended Agreement, EU airlines are now authorized to transport passengers between points in the United States and points outside the EU if the EU airline is authorized to serve the route under the Agreement. This includes flights that originate, arrive, or stop in the EU. Prior to this change, EU airlines were limited to flying passengers between points in the US and points in the EU.

(ii) Involuntary Rerouting

Travel on a foreign-flag carrier is permitted if a US-flag air carrier involuntarily reroutes the traveler via a foreign-flag air carrier, notwithstanding the availability of alternative US-flag air carrier service.

(iii) Travel To and From the US on non-European Community Airlines

Use of a non-European Community foreign-flag air carrier is permissible if the airport abroad is:

(a) the traveler's origin or destination airport, and use of US-flag air carrier service would extend the time in a travel status by at least 24 hours more than travel by a foreign-flag air carrier; or

(b) an interchange point, and use of US-flag air carrier service would increase the number of aircraft changes the traveler must make outside of the US by two or more, would require the traveler to wait four hours or more to make connections at that point, or would extend the time in a travel status by at least six hours more than travel by a foreign-flag air carrier.

(iv) Travel Between Points Outside the US on non-European Community Airlines

Use of a non-European Community foreign-flag air carrier is permissible if:

(a) travel by a foreign-flag air carrier would eliminate two or more aircraft changes en route;

(b) travel by a US-flag air carrier would require a connecting time of four hours or more at an overseas interchange point; or
(c) the travel is not part of the trip to or from the US, and use of a US-flag air carrier would extend the time in a travel status by at least six hours more than travel by a foreign-flag air carrier.

(v) Short Distance Travel

For all short distance travel, regardless of origin and destination, use of a foreign-flag air carrier is permissible if the elapsed travel time on a scheduled flight from origin to destination airport by a foreign-flag air carrier is three hours or less and service by a US-flag air carrier would double the travel time.

2. Charter Flights

Because of the risk of catastrophic loss, NSF does not encourage the use of charter flights as a means of mass transportation for groups of scientists and engineers nor does it make arrangements for purchase of charter flight airline tickets.

3. Projects in a Foreign Country

a. For awards that include activities requiring permits from appropriate Federal, State, or local government authorities, the grantee should obtain any required permits prior to undertaking the proposed activities.

b. The grantee must comply with the laws and regulations of any foreign country in which research is to be conducted. Areas of potential concern include: (1) requirements for advance approval to conduct research or surveys; (2) special arrangements for the participation of foreign scientists and engineers; and (3) special visas for persons engaged in research or studies. NSF does not assume responsibility for grantee compliance with the laws and regulations of the country in which the work is to be conducted.

c. The grantee also should assure that activities carried on outside the US are coordinated as necessary with appropriate US and foreign government authorities and that necessary licenses, permits or approvals are obtained prior to undertaking the proposed activities.

4. Passports and Visas

NSF assumes no responsibility for securing passports or visas required by any person because of participation in an NSF-supported project. For restrictions concerning directly charging visa costs to an NSF award, see 2 CFR § 200.463.

It should be noted that some countries that normally do not require visas for tourists do require special visas for scientists and engineers engaged in research or studies.

G. Intergovernmental Review of Federal Programs

The Foundation's regulation on Intergovernmental Review of National Science Foundation Programs and Activities is published in 45 CFR Part 660.

H. Handling of Information

1. Questionnaires: Data Collection Under NSF Grants

a. Paperwork Control

Under the OMB regulation, *Controlling Paperwork Burdens on the Public* (5 CFR § 1320), if a grantee obtains information from ten or more persons by means of identical questions, it is considered to be "sponsored" by NSF only if:

(i) the grantee is collecting the information at the specific request of NSF; or

(ii) the terms of the grant require specific approval by NSF of the collection or its procedures.

If either of these conditions is met, OMB approval of the data collection is generally required and grantees should obtain the necessary control number from the cognizant NSF Program Officer.

b. NSF Policy

Data collection activities of NSF grantees are the responsibility of grantees, and NSF support of a project does not constitute NSF approval of the survey design, questionnaire content or data collection procedures. No representation may be made to respondents that such data are being collected for, or in association with, NSF or the government. However, this requirement is not intended to preclude mention of NSF support of the project in response to an inquiry or acknowledgment of such support in any publication of this data (see Chapter XI.E.4).

2. Release of Information by NSF

a. Press Releases

Grants for projects which appear to be of special interest to the general public may be made the subject of an NSF or joint NSF/grantee press release to the news media. (See Chapter X.C.2.)

b. Open Government Legislation

(i) Pursuant to the Freedom of Information Act (FOIA) (5 USC § 552), NSF will provide agency records, with certain exceptions, if the request is in writing, includes the requester's name and mailing address, is clearly identified as a FOIA request, and describes the records sought with sufficient specificity to permit identification. The requester also must agree to pay fees that are chargeable under the NSF regulations. Detailed procedures are contained in 45 CFR § 612. Further information is available at: http://www.nsf.gov/policies/foia.jsp.

(ii) The Government in the Sunshine Act (5 USC § 552b) requires that all meetings of the NSB be open to public observation unless the subject falls within one of ten exemptions. NSF's Sunshine Act regulations are contained in 45 CFR § 614. The Sunshine Act and the FOIA cited above, may require NSF to release to the public information, correspondence and documents received by NSF from grantees, unless they fall within the Acts' limited exceptions.

c. Release of Project Reports

NSF expects significant findings from research it supports to be promptly submitted for publication. To the extent permitted by law, NSF will honor requests from grantees that release of Annual and Final Project Reports be delayed to permit orderly dissemination of significant findings through refereed channels. Unless such a request is received or material is marked as proprietary, such reports may be made available

to others without notice to the grantee. Information, the disclosure of which might invade personal privacy, will be redacted before release.

I. Tax Status

Determination of the tax status of an organization or person receiving compensation in any form as a result of an NSF grant is the responsibility of the IRS, State and local tax authorities and the courts.

J. Protection of Properties in the National Register of Historic Places

NSF is required by 16 USC § 470f to take into account the effect of grant activities on properties included in the National Register of Historic Places. Occasionally, an NSF grant may involve activities that require mitigation or other actions under the National Historic Preservation Act and implementing regulations of the Advisory Council on Historic Preservation (36 CFR § 800). In such cases, as deemed appropriate by NSF, conditions executing the goals of the National Historic Preservation Act may be included in the grant and grantees or prospective grantees may be required to coordinate with State or local historical preservation officers.

K. Environmental Compliance

Prior to making an award, NSF considers the anticipated environmental impacts associated with the activities described in the proposal. The assessment of environmental impacts is based, in part, on information provided by the organization. If, while performing the work required by the grant, the grantee learns of any potential or actual unanticipated environmental impacts, the grantee shall immediately notify NSF of such impacts. The grantee also shall cease all work anticipated to cause or causing such unanticipated environmental impacts until NSF has had a reasonable opportunity to assess the situation, comply with all relevant environmental laws, and provide further direction to the grantee.

L. National Security

NSF grants are intended for unclassified, publicly releasable research. The grantee will not be granted access to classified information. NSF does not expect that the results of the research project will involve classified information.

If, however, in conducting the activities supported under a grant, the PI/PD is concerned that any of the research results involve potentially classifiable information that may warrant Government restrictions on the dissemination of the results, the PI/PD should promptly notify the cognizant NSF Program Officer.

M. Miscellaneous

1. Liabilities and Losses

NSF cannot assume any liability for accidents, bodily injury, illness, breach of contract, any other damages or loss, or any claims arising out of any activities undertaken pursuant to the grant, whether with respect to persons or property of the grantee or third parties. The grantee is advised to insure or otherwise protect itself or others, as it may deem desirable.

2. Pre-College Students and Experimental Curriculum Development Projects

As required by 42 USC § 1869 a and b, grantees of projects which involve pre-college students in research or development, or pilot-testing, evaluation or revision of, experimental or innovative curriculum development projects will:

a. provide to the school board, or comparable authority responsible for the schools considering participation in the project, information concerning the need for and purposes of, the particular education project, the proposed content of the material to be used, the expected benefits to be derived and other information to assist the jurisdiction in arriving at a decision on participation;

b. obtain written approval for participation in the project activities after the responsible authority has carried out its procedures;

c. provide information and materials to the responsible school authority to assist it in carrying out its own established procedures regarding the participation of students in project activities;

d. provide information to NSF describing compliance with the above provisions; and

e. provide in every publication, testing or distribution agreement involving instructional material developed under a grant (including but not limited to teacher's manuals, textbooks, films, tapes or other supplementary material) that such material will be made available within the school district using such material, for inspection by parents or guardians of children engaged in educational programs or projects of that school district.

3. Use of Metric Measurements

The Metric Conversion Act of 1975 (15 USC §§ 205a-k) and E.O. 12770 (3 CFR § 1991 comp.) encourage Federal agencies to use the Metric System in procurement, grants and other business-related activities. The NSF grant will contain a provision encouraging PI/PDs to submit project reports, final reports, other reports and publications produced under grants that employ the metric system of measurements.

4. Executive Order 13788, Buy American and Hire American

Pursuant to Executive Order 13788, dated April 18, 2017, it is the policy of the Executive Branch of the Federal Government to maximize, consistent with law and regulation, the use of goods, products, and materials produced in the US. Further, it is the policy of the Executive Branch that relevant agencies will rigorously enforce and administer the laws governing entry into the US of workers from abroad.

Chapter XII: Grant Administration Disputes and Misconduct

A. Suspension and Termination Procedures

1. Definitions

a. SUSPENSION means an action by NSF that temporarily withholds Federal support of a project pending corrective action by the grantee or a decision by NSF to terminate the grant.

b. TERMINATION means the ending of the NSF award, in whole or in part at any time prior to the planned end of the period of performance.

2. Suspension and Termination

a. NSF Policy

(i) A grant may be suspended or terminated in whole or in part in any of the following situations by:

(a) NSF when the grantee fails to comply with the terms and conditions of the grant;

(b) NSF when the Foundation has cause;

(c) NSF when ordered by the Deputy Director under NSF's Regulation on Research Misconduct (45 CFR § 689);

(d) mutual agreement of NSF and the grantee; or

(e) the grantee on written notice to NSF setting forth the reasons for such action, the effective date, and, in the case of partial termination, the portion to be terminated or suspended (with the understanding that if NSF determines that the unterminated portion will not accomplish the purposes of the grant it may suspend or terminate the entire grant).

(ii) Normally, action by NSF to suspend or terminate a grant will be taken only after the grantee has been informed by NSF of the proposed action, or informed of any deficiency on its part and given an opportunity to correct it. NSF, however, may immediately suspend or terminate a grant without notice when it believes such action is reasonable to protect the interests of the government.

(iii) No costs incurred during a suspension period or after the effective date of a termination will be allowable, except those costs which, in the opinion of NSF, the grantee could not reasonably avoid or eliminate, or which were otherwise authorized by the suspension or termination notice, provided such costs would otherwise be allowable under the terms of the grant and the governing cost principles.

(iv) Within 30 days of the termination date the grantee will furnish a summary of progress under the grant and an itemized accounting of costs incurred prior to the termination date or pursuant to (iii) above. Final allowable costs under a termination settlement shall be in accordance with the terms of the grant, including this section, and the governing cost principles, giving due consideration to the progress under the grant. In no event will the total of NSF payments under a terminated grant exceed the grant amount or the NSF pro rata share when cost sharing was anticipated, whichever is less.

(v) A notice of termination other than by mutual agreement and/or the final settlement amount may be subject to review pursuant to Chapter XII.B.

(vi) NSF will report award terminations to the OMB-designated integrity and performance system in accordance with Federal regulation, but only after the grantee has had an opportunity to exhaust the review procedures contained in Chapter XII.B.

b. Procedures for Suspension or Termination by NSF

(i) When it is believed that a grantee has failed to comply with one or more of the terms and conditions of a grant, the NSF Grants Officer will normally advise the grantee in writing of the nature of the problem and that failure to correct the deficiency may result in suspension or termination of the grant. The grantee will be requested to respond in writing within 30 calendar days of the date of such letter, describing the action taken or the plan designed to correct the deficiency. Copies of such correspondence will be furnished to the PI/PD and to the cognizant NSF Program Officer. NSF, however, may immediately suspend or terminate a grant without notice when it believes such action is reasonable to protect the interests of the government.

(ii) If a satisfactory response is not received within the above period, the NSF Grants Officer may issue a notice immediately suspending authority to further obligate grant funds, in whole or in part. Notice of suspension is sent to the AOR, with a copy to the PI/PD. Within NSF, copies are furnished to the NSF Division of Financial Management and to the cognizant NSF Program Officer. The notice will set forth the terms of the suspension and its effective date.

(iii) Normally, the suspension will remain in effect for a maximum of 60 days to allow the grantee to take corrective action. In the event that the deficiency is not corrected to the satisfaction of NSF, the NSF Grants Officer may issue a notice of termination. The notice will set forth the reasons for the action and its effective date.

(iv) The remedies described in Chapter XII.B do not preclude a grantee from being subject to debarment and suspension under the OMB Guidelines to Agencies on Government-wide Debarment and Suspension (Nonprocurement) published at 2 CFR § 180 and NSF's Implementation of the OMB Guidelines, located at 2 CFR Chapter XXV.

(v) Suspension or termination due to research misconduct will be imposed as provided in that regulation.

3. Termination by Mutual Agreement

a. NSF Policy

Circumstances may arise in which either NSF or the grantee wishes to terminate a project. If both parties agree that continuation of the project would not produce results commensurate with the further expenditure of funds, or if there arises any other reason, the grant may be terminated by mutual agreement.

b. Procedures

(i) If the grantee wishes to terminate the project, the AOR should advise the NSF Grants Officer in writing and send a copy to the cognizant NSF Program Officer.

(ii) If NSF wishes to terminate the project, the NSF Grants Officer will advise the grantee's AOR in writing and send copies to the PI/PD and the cognizant NSF Program Officer.

(iii) Within 30 days after receipt of request from either party for termination by mutual agreement, the other party will provide an appropriate written response. In the event of disagreement between the parties, the NSF Grants Officer will make a final decision, subject to the review procedures prescribed by Chapter XII.B.

(iv) Following termination, grant closeout procedures will be initiated.

4. NSF Suspension or Termination Review Procedure

Grantees should refer to Chapter XII.B.3 for procedures to request review of a suspension or termination notice. Pending resolution of the request for review, a notice of termination shall remain in effect.

B. Informal Resolution of Grant Administration Disputes

1. Background

Consistent with the *Recommendation on Grant Disputes* by the Administrative Conference of the US, and with the intent of the provisions of Alternative Dispute Resolution, the Foundation provides the informal resolution processes described below concerning disputes or disagreements that may arise over Grants Officer post-award decisions under an NSF grant.

2. Scope of Post-Award Disputes Covered

The disputes below are covered under the process described in Chapter XII.B.3:

a. cost disallowances pursuant to a Grants Officer's decision (e.g., specific disallowances under an individual grant or as a result of an audit report);

b. termination orders; and

c. the final settlement amount under a termination.

3. Procedures

a. The grantee should submit a letter addressed to both the Division Director, Division of Grants and Agreements (DGA) and the Division Director, Division of Acquisition and Cooperative Support (DACS), National Science Foundation, 2415 Eisenhower Avenue, Alexandria, VA 22314. This letter must specify the grantee's disagreement or dispute and identify the NSF Grants Officer's decision in question, giving reasons for the request for review and providing any other material pertinent to the request. NSF will determine the appropriate Division to respond to the dispute.

b. The letter to the Division Directors, DGA and DACS, must be either postmarked or stamped received by NSF no later than 30 days after the date of the NSF letter notifying the grantee of the decision in question. The time for filing a request for review is strictly enforced and no extensions will be granted.

c. The request for review need not follow a prescribed format; however, it must contain a full statement of the grantee's position with respect to the disputed matter and the facts and rationale that support the grantee's position. Dispute requests will be reviewed if the grantee:

- submits new information (which was unavailable at the time of the original decision);
- identifies an error in fact or application of NSF policy in the original decision; or
- improper procedures were followed in the original decision.

d. The Division Director, DGA or DACS, will review or designate one or more individuals to review the matter. One reviewing official will be at least at a management level equivalent to the official who made the decision that is being reviewed. In no case, will the review be conducted by any individual involved with

the original decision or involved in recommending and/or monitoring the programmatic aspects of the project, or responsible for negotiating and/or administering its business or financial aspects.

e. The designated individual(s) will review and consider all relevant information available. A report which identifies the conclusion and recommendation will be completed, and in disputes covered under:

(i) Chapter XII.B.2.a and Chapter XII.B.2.b, the report will be completed within 30 days and forwarded to the Director, DGA or DACS or their designee for a final and unappealable written decision for the agency. The Director, DGA or DACS or their designee will communicate the decision in writing to the grantee, normally within 15 days of receipt of the report, unless otherwise specified by NSF.

(ii) Chapter XII.B.2.c, the report will be completed within 90 days and forwarded to the NSF Deputy Director or his/her designee. The NSF Deputy Director or his/her designee will make the final and unappealable decision for the agency and will communicate the decision in writing to the grantee within 15 days of receipt of the report unless otherwise specified by NSF.

C. Research Misconduct

RESEARCH MISCONDUCT means fabrication, falsification, or plagiarism in proposing or performing research funded by NSF, reviewing research proposals submitted to NSF, or in reporting research results funded by NSF.

1. NSF Policies and Responsibilities

a. NSF will take appropriate action against individuals or organizations upon a determination that misconduct has occurred. It may also take interim action during an investigation. Possible actions include sending a letter of reprimand to the individual or organization, requiring prior NSF approval of particular activities by an individual or organization, requiring special assurances of compliance with particular policies, restricting designated activities or expenditures under particular grants, suspending or terminating grants, debarring or suspending an individual or organization and prohibiting participation by an individual as an NSF reviewer, advisor or consultant.

b. NSF will find misconduct only after careful inquiry and investigation by a grantee, by another Federal agency or by NSF. An “inquiry” consists of preliminary information-gathering and preliminary fact-finding to determine whether an allegation or apparent instance of misconduct has substance. An investigation must be undertaken if the inquiry determines the allegation or apparent instance of misconduct has substance. An “investigation” is a formal development, examination, and evaluation of a factual record to determine whether misconduct has taken place or, if misconduct has already been confirmed, to assess its extent and consequences or determine appropriate action.

c. Before NSF makes any final finding of misconduct or takes any final action on such a finding, NSF will normally afford the accused individual or organization notice, a chance to provide comments and rebuttal and a chance to appeal. In structuring procedures in individual cases, NSF may take into account procedures already followed by other entities investigating or adjudicating the same allegation of misconduct.

d. Debarment or suspension for misconduct will be imposed only after further procedures described in applicable debarment and suspension regulations (2 CFR § 180 and 2 CFR Chapter XXV).

e. The OIG oversees investigations of research misconduct and conducts any NSF inquiries and investigations into suspected or alleged research misconduct.

f. The Deputy Director adjudicates research misconduct proceedings and the Director decides appeals.

g. After receiving an investigation report, the subject's rebuttal and recommendations of the OIG, the NSF Deputy Director may initiate further investigation or hearings or order interim or final actions. A written disposition specifying actions to be taken will be sent to affected individuals or organizations and will include instructions on how to pursue an appeal to the Director of the Foundation.

2. Role of Grantees

a. Grantees bear primary responsibility for prevention and detection of misconduct. In most instances, NSF will rely on grantees to promptly:

(i) initiate an inquiry into any suspected or alleged misconduct;

(ii) conduct a subsequent investigation, if the inquiry finds substance;

(iii) take action necessary to ensure the integrity of research, the rights and interests of research subjects and the public and the observance of legal requirements or responsibilities; and

(iv) provide appropriate safeguards for subjects of allegations as well as informants.

b. If a grantee wishes NSF to defer independent inquiry or investigation, it should:

(i) inform NSF immediately if an initial inquiry finds substance;

(ii) keep NSF informed during such an investigation;

(iii) notify NSF even before deciding to initiate an investigation or as required during an investigation:

(a) if there is reasonable indication of possible violations of civil or criminal law;

(b) if public health or safety is at risk;

(c) if NSF's resources, reputation, or other interests need protecting;

(d) if Federal action may be needed to protect the interests of a subject of the investigation or of others potentially affected;

(e) if the research community or the public should be informed; or

(f) if research activities should be suspended.

(iv) provide NSF with the final report from any investigation.

c. If a grantee wishes NSF to defer independent inquiry or investigation, it should complete any inquiry and decide whether an investigation is warranted within 90 days. It should similarly complete any investigation and reach a disposition within 180 days. If completion of an inquiry or investigation is delayed, but the grantee wishes NSF deferral to continue, NSF may require submission of periodic status reports.

d. Grantees should maintain and effectively communicate to their staff appropriate policies and procedures relating to misconduct, which should indicate when NSF must or should be notified.

e. Online research ethics training is available through the Department of Health and Human Services, Office of Research Integrity.

3. Reporting Possible Misconduct

Possible misconduct in activities funded by NSF should be reported to the Office of the Inspector General, National Science Foundation, 2415 Eisenhower Avenue, Alexandria, VA 22314, (703) 292-7100 or (800) 428-2189 or via e-mail at: oig@nsf.gov.

Subject Index

PAPPG SECTION

A

Absence (Disengagement) of Principal Investigator/Project Director or co-PI/co-PD VII.B.2
Acceptance of Assistance Agreements ..... VI.A
Accomplishment-Based Renewal ..... II.C.2.d(vi), V.B
Acknowledgment and Disclaimer XI.E.4
Acquisition Cost of Equipment ..... II.C.2.g(iii)
Additional Funding Support ..... VI.E, X.A.2, X.D.1
Addition of co-PI/ co-PD VII.B.2.c
Administrative and Clerical Salaries and Wages ..... II.C.2.g(i)(b), X.B.2
Advance Payments ..... VIII.C, VIII.D
Advanced Visualization Resources II.D.7
Age Discrimination ..... Exhibit II-6; XI.A.5
Alcoholic Beverages ..... II.C.2.g(xiii)(c)
Allowability of Costs ..... II.C.2.g, Chapter X
Animal Welfare II.C.2.j, II.D.4, XI.B.3
Annual Project Report VI.E.3, VII.D.1, XI.H.2.c
Antarctic Proposals I.E.7, II.C.2.j
Anthropogenic Disasters I.F.2, II.E.1
Anti-discrimination Statutes ..... II.C.1.d, Exhibit II-6, XI.A
Appeal Process for Proposals Declined for Financial or Administrative Reasons… III.F.2
Appeals ..... III.F.2, Chapter XII
Appendices II.C.2.k
Applicable Credits VIII.D.4, VIII.D.5
Appointments ..... II.C.2.f, II.C.2.g(i)(a)
Approvals, Requests for NSF X.A.3
Audits ..... VII.E
Authorized Organizational Representative (AOR) ..... I.D.2, I.G.2, II.C.1.d, III.C, III.D, IV.A, ... VII.A.2, Exhibit II-1
Award Abstract… ..... III.E
Award Cash Management Service ..... Chapter VIII
Award Conditions ..... VI.C
Award Instrument ..... VI.B
Award Recommendations ..... III.E, Exhibit III-1
Awardee Organization II.C.2.a

B

Bayh-Dole Act XI.D.1
Beginning Investigator ..... II.C.2.a(iv)(a), II.D.2
Biographical Sketch(es) ..... II.C.2.f, II.D.3.b, Exhibit II-1
Biological Sciences Directorate Duplicate Proposal Policy II.D.2
Bonding and Insurance XI.C.2
Broader Impacts ..... II.C.2.b, II.C.2.d, II.C.2.i, II.C.2.j, III.A
Budget II.C.2.g
Budget Changes ..... VII.B.3, VII.C
Business Officer VIII.B.2
Buy American and Hire American XI.M.4

C

Cash
- Advances ..... VIII.C, VIII.D
- Contributions ..... VIII.D
- Refunds ..... VIII.D
- Reports ..... VIII.E

Center Proposal II.E.10
Certifications I.G.2, II.C.1, II.C.1.d
- Authorized Organizational Representative or Individual Proposer II.C.1.d
- Conflict of Interest ..... II.C.1.d, Exhibit II-2
- Contracts, Grants, Loans and Cooperative Agreements ..... Exhibit II-5
- Criminal Conviction ..... II.C.1.d, Exhibit II-4
- Debarment or Suspension ..... II.C.1.d, Exhibit II-4
- Drug-Free Workplace ..... II.C.1.d, Exhibit II-3
- Federal Tax Obligations II.C.1.d
- Flood Hazard Insurance II.C.1.d
- Lobbying ..... II.C.1.d, Exhibit II-1, Exhibit II-5
- Nondiscrimination ..... II.C.1.d, Exhibit II-6
- Organizational Support II.C.1.d
- Responsible Conduct of Research II.C.1.d
- Unpaid Federal Tax Liability II.C.1.d

Changes
- Grant Budget ..... VI.B.1.c, Exhibit II-1, VII.B.3, VII.C
- Grant Periods ..... VI.D
- Grantee Notifications to and Requests for Approval from the National Science Foundation ..... Chapter VII
- Project Direction or Management ..... VII.B
- Objectives, Scope, or Methods/Procedures VII.B.1
- Principal Investigator/Project Director VII.B.2

Charter Flights XI.F.2
Civil Rights Act of 1964 ..... Exhibit II-6, XI.A.2
Classified Research ..... XI.L
Clerical (Administrative & Clerical Salaries & Wages) ..... II.C.2.g(i)(b), X.B.2
Closeout ..... VII.D.5, VIII.E
Closure of NSF I.F.2
Co-Editors II.C.1.e
Coffee Breaks and Meals ..... II.C.2.g(xiii)(b), X.C.5
Collaborative Arrangements ..... II.C.2.g(vi)(e), II.C.2.j, II.D.3
Collaborative Proposals ..... II.D.3, IV.A
- Submission of a collaborative proposal from one organization II.D.3.a
- Submission of a collaborative proposal from multiple organizations II.D.3.b

Collaborators II.C.1.e, II.D.3.b
Collaborators and Other Affiliations Information II.C.1.e
Colleges ..... I.E.1, II.C.2.g(i)(a), II.C.2.g(viii)
Common Rule for the Protection of Human Subjects II.D.5
Compensation for Personal Services X.B.1
Compliance, Environmental ..... II.C.2.j, XI.K
Computer Services ..... II.C.2.g(vi), II.C.2.g(vi)(d)
Computing Devices ..... II.C.2.g(vi)(a)
Conferences ..... I.B, II.C.2.g(iv), II.C.2.g(v), II.C.2.j, II.E.7, II.E.9
Confidential Budgetary Information ..... II.C.1.c, II.C.2.g(i)(d), II.D.1
Conflict of Interest Policies ..... IX.A

Conflicting Guidelines X.A.1
Conflicts of Interest (COIs) I.D.1, II.C.1.d, II.C.1.e
Potentially Disqualifying COIs ..... Exhibit II-2
Construction, Rearrangements and Reconversions ..... Exhibit II-1, X.C.1, XI.C
Consultant Services ..... II.C.2.g(vi), X.B.3
Continuing Grant VI.E.3
Contracts under Grants (Subawards) ..... II.C.2.g(vi)(e), Exhibit II-5, VII.B.3
Contributions ..... VIII.D
Cooperative Agreements ..... I.C.4, I.H, Exhibit II-5, III.F, Chapter VI
Copies of Reviews ..... III.G, IV.A, IV.C, IV.D.3
Copyright XI.D.2
Copyrightable Material Clause XI.D.2.b
Cost Principles ..... II.C.2.g, Chapter X
Cost Sharing ..... II.C.2.g(xii), II.C.2.i, III.D, VII.C
Costs, Visa ..... II.C.2.g(vi)(f), X.C.4, XI.F.4
Cover Sheet ..... I.D.2, I.F.2, I.G.2, II.A, II.C.1.d, II.C.2.a, II.D, Exhibit II-1
Credits ..... VIII.D
Current and Pending Support ..... II.C.2.h, II.D.3.b, Exhibit II-1
Curriculum Development Projects XI.M.2

D

Data Collection XI.H.1
Data Management Plans ..... II.C.2.d(i), II.C.2.j, II.D.3.b, II.E.7, II.E.8, II.E.9
Data Rights ..... XI.D
Data Storage II.D.7
Data Universal Numbering System (DUNS) I.G.3, II.C.2.a
Davis-Bacon Act XI.C.1
Deadline Dates I.F.2
Dear Colleague Letters I.C.1
Debarment and Suspension ..... II.C.1.d, Exhibit II-4, XII.A, XII.C.1.d
Declination of Proposal ..... III.E., III.F, IV.C, IV.D
Decline for Financial or Administrative Reasons ..... III.F
Definitions of Categories of Personnel ..... Exhibit II-7
Delays VII.B.1.c
Delinquent Reports VII.D.4
Dependent Care Travel Costs ..... II.C.2.g(iv)(a), X.C.5
Deviation Authorization ..... I.F, II.A, II.C.2.d, II.C.2.k, Exhibit II-1
Direct Costs ..... II.C.2.g X.B, X.C
Disability Regulations ..... Exhibit II-6, XI.A
Disasters (Natural or anthropogenic) I.F.2, II.E.1
Disbursements ..... VIII.B.4, VIII.E
Disclaimer XI.E.4.b
Disclosure of Lobbying Activities (SF LLL) ..... II.C.1.d, Exhibit II-1, Exhibit II-5
Discrimination ..... II.C.1.d, Exhibit II-6, XI.A
Disputes Resolution ..... XII.B
Dissemination Costs ..... II.C.2.g(vi)(b)
Dissemination of Research Results ..... II.C.2.d, II.C.2.g(vi)(b), XI.D.4, XI.E, XI.H.2.c
Distribution of Grant Materials ..... XI.E
DNA Guidelines XI.B.2
Documentation Costs ..... II.C.2.g(vi)(b), X.A.2.c, XI.E.2
Domestic Travel ..... II.C.2.g(iv)(b), X.C.5

Dual Use Research of Concern II.C.1.d, II.D.6, XI.B.5
Duration Information II.C.2.a

E

EArly-concept Grants for Exploratory Research (EAGER) II.E.2
Education Amendments of 1972 (Title IX) ..... Exhibit II-6, XI.A.4
Effective Date ..... see "Start Date"
Electronic Submission - Financial ..... VIII.B.5, VIII.C.6, VIII.E
Electronic Submission Requirements ..... I.A, I.B, I.D, I.G.1, II.B, II.C, VII.A.2
Encourage/Discourage Decisions I.D.2.b, IV.D.2.a
End Date VI.D.1.b, VI.D.3.b
Endangered Species II.C.2.j
Entertainment Costs ..... II.C.2.g(xiii)(a), II.E.7
Environmental Compliance ..... II.C.2.j, XI.K
Environmental Impact ..... II.C.2.j, XI.K
Equal Employment Opportunity XI.A.1.b, XI.A.6
Equipment
- Acquisition ..... IX.D
- Allowability of Costs ..... II.C.2.g(iii), VII.B.2.e, IX.D.2, X.A.2.c, X.C.3
- Conditions for Acquisition and Use IX.D.2
- Definition ..... II.C.2.g(iii)
- For-profit Organizations IX.D.1.b
- General Purpose ..... II.C.2.g(iii), II.C.2.g(vi)(d), IX.D.2.b
- Government-Owned IX.D.2.g, IX.D.4, IX.D.5
- Non-profit Organizations IX.D.1.a
- Proposals II.E.6, II.E.8, II.E.11
- Rental or Lease X.C.3
- Shared Use IX.D.2.d
- Special Purpose ..... II.C.2.g(iii), II.E.6, II.E.8, II.E.11, X.C.3
- Title IX.D.1, IX.D.2.g, IX.D.4
- Transfer VII.B.2.e, IX.D.2.g

Erroneous Payments VIII.D.2
Excess Government Personal Property IX.D.5
Executive Order
- 12372, Intergovernmental Review of Federal Programs ..... II.C.2.j, XI.G
- 12770, Metric Usage in Federal Government Programs I.D.3, XI.M.3
- 13788, Buy American and Hire American XI.M.4

Expendable Personal Property ..... see "Materials and Supplies"
Expenditures
- Allowability of Costs ..... II.C.2.g, Chapter X
- Grantee Responsibility VII.A.1
- Reports ..... VIII.E
- Outlays VIII.B.4

Experimental Curriculum XI.M.2
Expiration Date ..... see "End Date"
Extension of Grant Period VI.D.3.c

F

Facilitation Award for Scientists and Engineers with Disabilities (FASED) II.C.2, II.C.2.j, II.E.6
Facilities and Administrative Costs (F&A) ..... II.C.2.g(v), II.C.2.g(vi), II.C.2.g(viii), II.C.2.g(ix), III.D, X.D
Facilities Capital Cost of Money ..... X.D.1.b(iii)
Facilities, Equipment and Other Resources ..... II.C.2.i, II.D.3.b, Exhibit II-1
Faculty Associate, Definition ..... Exhibit II-7
FastLane System ..... I.A, I.G.1, II.B, II.C, IV.A, Chapter V
    Proposal Preparation Module ..... I.A, I.G.3, II.C
Federal Acquisition Regulation ..... II.C.2.g, X.A
Federal Agencies ..... I.E.7, I.H, II.D.2
Federal Awardee Performance and Integrity Information System (FAPIIS) ..... III.F, XII.A.2.a(vi)
Federal Cost Principles (now consolidated in 2 CFR § 200, Subpart E) ..... II.C.2.g, Chapter X
Federal Emergency Management Agency II.C.1.d
Federal Financial Reports ..... VII.D, VIII.E
Federal Funding Accountability and Transparency Act (FFATA) I.G.3
Federally Funded Research and Development Centers (FFRDCs) I.E.7
Federal Wide Assurance (FWA) II.D.5
Fee Payments ..... II.C.2.g(x), X.E
Fellowships ..... II.D.2, II.D.4, IV.D.2, VII.D.1, VII.D.2, XI.B.3.b(iv), XI.D.3.b(ii)
Final Disbursement Reporting ..... VIII.E
Final Project Report VII.D.2, XI.H.2.c
Final Technical Information Items ..... VII.D
Financial Disclosure ..... II.C.1.d, Exhibit II-2, IX.A
Financial Management System Standards ..... VIII.A, IX.C
Fiscal Reports ..... VIII.E
Fixed Amount Award ..... VI.D
Flood Hazard Insurance II.C.1.d
Fonts ..... II.B, Exhibit II-1
Foreign Organizations ..... I.E.6, II.C.2.g(viii), II.D.4, XI.B.3.b(iv)
Foreign Projects ..... II.C.2.a(4)(i), II.C.2.j, II.D.4, XI.B.3.b(iv), XI.B.4, XI.D.1, XI.D.3.c, XI.F.3
Foreign Travel ..... II.C.2.g(iv)(c), XI.F
Foreign-Flag Air Carriers ..... XI.F
For-profit Organizations I.E.3, IX.D.1.b
Freedom of Information Act XI.H.2.b
Fringe Benefits ..... II.C.2.g(ii), III.D, X.B.1.b
Full Proposal ..... I.D, II.C.2, IV.B
Funding Opportunities ..... I.A, I.B, I.C, II.C.2.a(2)
Funding Recommendations ..... Chapter III, Exhibit III-1
Funds
    Allowability of Costs/Expenditures ..... II.C.2.g, Chapter X
    Payment of ..... VIII.C
    Residual VI.E.2
    Safeguarding VIII.C.2
    Transfer of VII.B.3
    Withholding VIII.C.2

G

General Purpose Equipment....II.C.2g(iii), IX.D.2.b
Government-Owned Equipment....IX.D
Graduate Advisors....II.C.1.e, Exhibit II-2
Graduate Students....I.E, II.C.2.g(i)(c), Exhibit II-7, Chapter V
Grant
- Budget....II.C.2.g, VI.B.1.c
- Changes....VII.B
- Closeout....VII.D.5
- Conditions, General and Standard....VI.C
- Disputes Resolution....XII.B
- Financial Disclosure....II.C.1.d, Exhibit II-2, IX.A
- Financial Reporting....VIII.E
- International Travel....II.C.2.g(iv)(c), XI.F
- Instrument, Defined....VI.B
- Opportunities for Academic Liaison with Industry (GOALI)....II.C.2, II.E.4
- Periods....VI.D
- Renewal....II.C.2.a, II.C.2.d(vi), Exhibit II-1, Exhibit II-5, Chapter V, VI.E.2
- Suspension....XII.A
- Termination....XII.A
- Transfer....VII.B.3

Grantee
- Definition....VIII.B.6
- Organization....II.C.2.a, II.E.6.b, VIII.B.6
- Prior Approvals....Chapter VII
- Share....see “Cost Sharing”
- Standards....Chapter IX

Grants and Agreements, Division of....I.H, III.F
Grants.gov....I.A, I.G.1, II.A, II.B, II.C.2, IV.B, IV.D.2
Group Proposals....II.C.2.d(v)

H

Hazardous Materials....II.C.2.j
High Performance Computing Resources....II.D.7
Higher Education, Institutions of....I.E.1
Hire American, Buy American and....XI.M.4
Historic Places....II.C.2.a., II.C.2.j, XI.J
Human Subjects....II.C.2.a, II.C.2.g(vi), II.C.2.j, II.D.5, XI.B.1

I

Ideas Lab....II.E.5
Income....VIII.D.4, XI.D.2.a
Indirect Costs....II.C.2.g(v), II.C.2.g(viii), II.C.2.g(ix), III.D, X.D
Informal Resolution of Grant Administration Disputes....XII.B
Information About Principal Investigators/Project Directors and co-Principal Investigators/ co-Project Directors....II.C.2.a, Exhibit II-1
Information Release....XI.H.2
In-Kind Contributions....II.C.2.g(xii), VII.C.2
Inspector General, Office of....XII.C

Institutional Animal Care and Use Committee II.D.4, XI.B.3
Institutional Review Board II.D.5, XI.B.1
Institutions of Higher Education I.E.1
Instrumentation and Facilities Guidelines IX.D.3
Insurance II.C.1.d, XI.C.2
Intangible Property ..... XI.D
Intellectual Merit ..... II.C.1.d, II.C.2.b, II.C.2.d, II.C.2.i, II.C.2.j, Exhibit II-1, III.A, III.B
Intellectual Property ..... XI.D
Interest VIII.D.3
Intergovernmental Review of Federal Programs ..... II.C.2.j, XI.G
International
- Activities ..... I.E.6, II.C.2.a, II.C.2.j, XI.F
- Agreements XI.F.3
- Charter Flights XI.F.2
- Copyright XI.D.2.a, XI.D.3
- Patents XI.D.1.b, XI.D.3
- Projects ..... see "Foreign Projects"
- Travel ..... II.C.2.g(iv)(c), XI.F
- Travel Grants IV.D.2.b
- Travel Proposals II.E.9

Intra-University Consulting X.B.3
Inventions and Patents XI.D.1
Investigator Financial Disclosure ..... II.C.1.d, Exhibit II-2, IX.A
Invite/Not Invite Decisions I.D.2.a

L

Large Facilities Projects II.E.11
Leased Facilities and Equipment X.C.3
Leave, Sabbatical VII.B.2.a
Letters of Collaboration ..... II.C.2.d(iv), II.C.2.j
Letters of Intent I.C.4, I.D.1
Letters of Support II.C.2.j
Liabilities and Losses XI.M.1
License Rights XI.D.1
Limited English Proficiency (LEP) XI.A.7
List of Suggested Reviewers or Reviewers Not to Include ..... II.C.1.b, Exhibit II-1
Living Organisms ..... XI.B
Lobbying .....
- Certification ..... II.C.1.d, Exhibit II-5
- Disclosure of Lobbying Activities ..... II.C.1.d, Exhibit II-1, Exhibit II-5

Local Governments I.E.4
Long-Term Absence of PI/PD VII.B.2.a

M

Major Research Facilities II.D.7, II.E.11
"March-in" Rights XI.D.1.c
Margin and Spacing Requirements ..... II.B, Exhibit II-1
Materials and Supplies ..... II.C.2.g(vi)(a), IX.D
Maximum Obligation of NSF X.A.2.a
Meals and Coffee Breaks ..... II.C.2.g(xiii)(b), X.C.5

Meetings and Conferences .....I.B, II.C.2.g(iv), II.C.2.g(v), II.C.2.j, II.E.7, II.E.9, X.C.5
Mentoring .....II.C.2.j, II.D.3, Exhibit II-1, VI.E.4.f
Merit Review Criteria.....I.C, I.D.3, II.C.2.b, II.C.2.d, Chapter III, VI.E.4
Merit Review Principles.....Chapter III.A
Methods/Procedures, Changes in.....VII.B.1.b
Metric Conversion Act of 1975.....I.D.3, XI.M.3
Minority-Owned Business II.C.2.a
Misconduct in Science .....I.D.3, XII.C

N

National Center for Atmospheric Research……II.D.7
National Environmental Policy Act of 1969 .....II.C.2.j, XI.K
National Flood Insurance Act.....II.C.1.d
National Register of Historic Places.....II.C.2.a, II.C.2.j, XI.J
National Science Board.....I.C.2, I.C.3, II.C.2.g(xii), III.A, III.B, IV.D.1, Chapter V, ...XI.D.1, XI.H.2
National Science Foundation
Act XI.C.2, XI.D.1
Approvals .....Chapter VII
National Security .....XI.L
Natural Disasters.....I.F, II.E.1
New Awardee.....Chapter II
News Release X.C.2, XI.H.2
No-Cost Extension VI.D.3.c
Non-academic Organization.....I.E.2, II.C.2.g(i)(a), Chapter V, Chapter VIII
Non-award Decisions and Transactions .....Chapter IV
Non-Discrimination Statutes and Regulations ..... II.C.1.d, Exhibit II-6, XI.A
Non-profit Organization .....I.E.2, II.E.9, VII.E, Chapter VIII, IX.D.1.a, XI.D.1.a
Notifications to NSF .....Chapter VII
NSF ID I.G.4, II.C.2.a
NSF Prospective New Awardee Guide .....Chapter II
NSF Unit of Consideration .....II.C.2.a.
NSF Update .....I.B
NSF-Use Only II.C.1

O

Objectives, Changes in VII.B.1.a
Obligations VIII.B.7
Off-Campus (Off Site) Activities .....II.C.2.a.1, II.D.4.d, XI.B.3(iv)
Office of Inspector General (NSF) .....XII.C
Open Government Legislation XI.H.2.b
Other Direct Costs.....II.C.2.g(vi), X.B
Other Personnel .....II.C.2.f(ii), Exhibit II-7
Other Professional, Definition .....Exhibit II-7
Outlays VIII.B.4
Overhead.....see “Indirect Costs”

P

Page Formatting Instructions ..... II.B
Page Limitations ..... II.C, II.E, Exhibit II-1, III.D, IV.B, IV.D.2, V.A, V.B
Pagination Instructions ..... II.B
Participant Support ..... II.C.2.g(v), II.E.7, X.A.3.b
Passports and Visas X.C.4, XI.F.4
Patent Rights Clause XI.D.1.c
Patents and Inventions XI.D.1
Payments ..... VIII.B.9, VIII.C
Permits XI.B.4
Person Months ..... II.C.2.g(i)(c), II.C.2.h, VII.B.2
Personnel, Definitions ..... Exhibit II-7
PI/PD (co-PI/co-PD)
- Addition of co-PI/co-PD VII.B.2.c
- Change in Person-Months VII.B.2
- Compensation ..... II.C.2.g(i), X.B.1.a
- Definition ..... Exhibit II-7
- Information about PI/PDs and co-PIs/co-PDs ..... II.C.2.a.4(d)
- Long-Term Disengagement VII.B.2.a
- Substitute (Change) VII.B.2.e
- Transfer to Another Institution VII.B.2.f
- Withdrawal VII.B.2.d

Postdoctoral Associate ..... II.C.2.f(ii)
Postdoctoral Scholar ..... Exhibit II-7
Postdoctoral Sponsor II.C.1.e
Post-Expiration Costs X.A.2.c
Postgraduate-Scholar Sponsor II.C.1.e
Potentially Disqualifying Conflicts of Interest ..... Exhibit II-2
Pre-Award Costs X.A.2.b
Pre-Award Financial and Administrative Reviews III.F.1
Pre-College Students XI.M.2
Preliminary Proposals I.C.4, I.D.2
Preservation of Historic Places ..... II.C.2.a, II.C.2.j, XI.J
Press Releases X.C.2, XI.H.2.a
Previous NSF Award ..... II.C.2.a.4(e)
Principal Investigator (PI) ..... II.C.2.a.4(d), Exhibit II-7
Principles, Federal Cost (now consolidated in 2 CFR § 200, Subpart E) ..... II.C.2.g, Chapter X
Prior NSF Approval ..... Chapter VII, X.A.3.b
Privileged Information ..... II.C.1.c, II.C.2.g(i)(d), II.D.1, III.H, XI.H.2.c
Procurement Standards ..... IX.E
Products ..... II.C.2.f(i)(c)
Professional Preparation ..... II.C.2.f(i)(a)
Professional Service Costs ..... II.C.2.g(vi)(c)
Program Announcements I.C.3
Program Descriptions I.C.2
Program Income VIII.D.4
Program Solicitations I.C.4
Project Descriptions ..... II.C.2.d, II.C.2.e, II.C.2.j, II.D.3.b, II.E, Exhibit II-1
Project Income VIII.D.4
Project Performance ..... VII.A
Project/Performance Site Primary Location II.C.2.a.1

Project Reports ..... VII.D
Project Summary ..... II.C.2.b, Exhibit II-1
Property
- Acquisition IX.D.2
- Copyright and Publication ..... XI.D.2, XI.E
- Data and Software XI.D.4
- Equipment ..... II.C.2.g(iii), IX.D.1, X.C.3
- Excess Government Personal Property IX.D.5
- Intellectual ..... XI.D
- Inventions and Patents XI.D.1
- Management Standards ..... IX.D
- Materials and Supplies ..... II.C.2.g(vi)(a), IX.D
- Protection of Historic Places ..... II.C.2.a.4(i), II.C.2.j, XI.J
- Title ..... IX.D, IX.D.1, IX.D.4, XI.D

Proposal
- Certifications ..... I.G.2, II.C.1, II.C.1.d, Exhibit II-1, Exhibit II-3, Exhibit II-4, Exhibit II-5, Exhibit II-6
- Conference ..... II.C.2.g(iv), II.C.2.g(v), II.C.2.j, II.E.7, II.E.9
- Contents ..... II.C
- Cover Sheet ..... II.C.2.a, Exhibit II-1, Exhibit II-3
- Declination of Proposals for Financial or Administrative Reasons ..... III.F
- Declinations ..... III.E., III.F, Chapter IV
- Deviations ..... I.F, II.A, II.C.2.d, II.C.2.j, Exhibit II-1
- File Updates ..... III.C, IV.A
- Format ..... II.B
- International Travel ..... II.C.2.g(iv)(c), II.C.2.j, II.E.9
- Margin and Spacing Requirements ..... II.B, Exhibit II-1
- Not Accepted ..... IV.B
- Page Formatting ..... II.B
- Pagination Instructions ..... II.B
- Preparation Checklist ..... Exhibit II-1
- Processing ..... I.H
- Proposal & Award Process & Timeline ..... Exhibit III-1
- Receipt I.G.5
- Reconsideration ..... IV.D
- Renewed Support ..... II.C.2.a.4(e), II.C.2.d(vi), II.E.1, II.E.2, Chapter V
- Return without Review ..... II.A, II.B, II.C, Exhibit II-1, Exhibit III-1, IV.B, IV.D.2, IV.E
- Revisions Made During the Review Process ..... III.D
- Sections II.C.2
- Status I.G.5
- Submission ..... Chapter I, Chapter II, Exhibit II-1, IV.A, IV.E, Chapter V
- Symposia ..... see “Conference”
- Time Sensitive Proposals ..... I.H, II.E.1
- Title ..... I.D.1, II.C.2.a.4(a)
- Types ..... II.E
- Withdrawal ..... Chapter IV.A

Proprietary Information ..... II.C.1.d, II.C.2.g(i)(d), II.D.1, Exhibit II-1, III.H, XI.H.2.c
Prospective New Awardee Guide ..... Chapter II
Provisional Rate X.D.1
Public Access Policy XI.D.2.c, XI.E.1, XI.E.4.d
Publication(s) ..... II.C.2.d(iii), II.C.2.f(i)(c), II.C.2.g(vi)(b), XI.D.4, XI.E, XI.H.2

Q

Questionnaires XI.H.1

R

Rapid Response Research Grants (RAPID) II.E.1
Rearrangements and Reconversion Costs ..... X.C.1, XI.C
Rebudgeting X.A.3, X.B.2
Recombinant DNA XI.B.2
Recompetition ..... Chapter V
Reconsideration of Proposals ..... IV.D
Records Retention ..... VII.E
References Cited ..... II.C.2.e, II.D.3.b, Exhibit II-1
Refunds to NSF ..... VIII.D
Rehabilitation Act of 1973 ..... Exhibit II-6, XI.A.3
Reimbursements VIII.C.4, VIII.C.5
Release of Grantee Proposal Information ..... III.H
Release of Information XI.H.2
Relocation Costs X.C.4
Renewed Support ..... II.C.2.a.4(e), II.C.2.d(vi), VI.E.2
    Accomplishment-Based Renewal ..... V.B
    Traditional Renewal ..... V.A
Rental or Lease of Facilities/Equipment X.C.3
Reporting Requirements ..... VII.D, VIII.E, XII.C.3
Reports
    Annual Project VII.D.1, XI.H.2.c
    Cost Sharing VII.C.3
    Final Disbursement Reporting ..... VIII.E
    Final Project VII.D.2, XI.H.2.c
    Project Outcomes Report for the General Public VII.D.3
    Technical Information Items ..... VII.D
Request for Advance or Reimbursement ..... VIII.C
Research Advanced by Interdisciplinary Science and Engineering (RAISE) II.E.3
Research Assistant, Definition ..... Exhibit II-7
Research Equipment ..... II.C.2.g(iii), II.C.2.i, II.E.8, II.E.11, IX.D
Research Experiences for Undergraduates II.C.2.j
Research Infrastructure Proposal II.E.11
Research in Undergraduate Institutions II.C.2.j
Research Involving Wildlife II.D.4, X.B.3
Research Misconduct ..... I.D.3, XII.C
Research Opportunity Awards II.C.2.j
Residual Funds VI.E.2
Resolution of Grant Administration Disputes ..... XII.B
Responsible Conduct of Research ..... II.C.1.d, IX.B
Resubmission of Proposal ..... IV.E
Results from Prior NSF Support ..... II.C.2.d, Exhibit II-1, Chapter V
Return without Review ..... II.A, II.B, II.C, Exhibit II-1, Exhibit III-1, IV.B, IV.D.2, IV.E
Review Criteria ..... I.D.3, Exhibit II-1, Chapter III
Reviews, Copies of ..... III.G, IV.A, IV.C, IV.D.3
Revisions to Proposals (Made During the Review Process) ..... III.D
Revocation ..... XII.A

Rights in Data and Software ..... XI.D
Risk Management Framework ..... III.F
Royalties under Grants ..... XI.D

S

Sabbatical Leave VII.B.2.a
Salaries and Wages ..... II.C.2.g(i), X.B
Administrative and Clerical Salaries and Wages Policy ..... II.C.2.g(i)(b), X.B.2
Confidential Budgetary Information ..... II.C.1.c, II.C.2.g(i)(d), II.D.1
Policies ..... II.C.2.g(i)(a), II.C.2.g(i)(b), X.B
Procedures ..... II.C.2.g(i)(c), X.B
Scope, Change in VII.B.1.a,
Security, National ..... XI.L
Seismic Safety XI.C.3
Selection of Reviewers ..... II.C.1.b, III.B
Senior Personnel ..... II.C.2.f(i), II.C.2.g(i)(a), II.C.2.h, Exhibit II-2
Sex Discrimination ..... I.E, Exhibit II-6, XI.A.4
SF LLL, Disclosure of Lobbying Activities ..... II.C.1.d, Exhibit II-1, Exhibit II-5
Shared Use of Equipment IX.D.2.d, IX.D.3
Sharing Research Results ..... XI.D.4, XI.E
Short-Term Absence of PI/PD VII.B.2
Significant Change, Delay or Event of Unusual Interest VII.B.1.c
Single Copy Documents ..... II.C.1, Exhibit II-1
Site Visits VII.A.1.c
Small Business ..... I.E.3, II.C.2.a(4)(g), IV.D.2.b, IX.D.1.b
Software Rights ..... XI.D
Special Exceptions (to Deadline Date Policy) I.F.2
Special Processing Instructions ..... II.E, Exhibit II-1
Special Information ..... II.C.2.j, Exhibit II-1
Sponsored Projects Office ..... I.D.2, II.C.2.j, IV.A
Staff Benefits ..... see “Fringe Benefits”
Standards
Financial Management Systems ..... VIII.A, IX.C
Grantee ..... Chapter IX
Procurement ..... IX.E
Property Management ..... IX.D
Start Date VI.D.1.a
State Governments I.E.4, II.C.2.h,
Students ..... I.E, II.C.2.f(ii), II.C.2.g(i)(c), II.E.6, II.E.7, Exhibit II-1, Exhibit II-7, Chapter V
Subawards ..... II.C.2.g(vi)(e), II.D.3, Exhibit II-5, Exhibit II-6, VII.B.3, VIII.C.2.b
Submission
Instructions ..... I.G.2, Chapter II, Exhibit II-1
Windows I.F.3
Substitute Principal Investigator/Project Director VII.B.2.e
Sunshine Act ..... XI.H.2.b(ii)
Supplemental Support II.E.6, VI.E.4
Supplementary Documentation ..... II.B.2, II.C.2, II.C.2.d, II.C.2.j, Exhibit II-1
Supplies ..... see “Materials and Supplies”
Suspension of Award ..... XII.A
Synergistic Activities ..... II.C.2.f(i)(d)
System for Award Management (SAM) I.G.3

T

Table of Contents II.C.2, II.C.2.c
Tangible Property ..... XI.D
Target Dates I.F.1
Tax Status ..... XI.I
Technical Reporting Requirements ..... VII.D
Temporary Dependent Care Travel Costs ..... II.C.2.g(iv)(a), II.E.7, X.C.5
Termination of Award ..... XII.A
Thesis Advisor ..... II.C.1.e, Exhibit II-2
Title
- Equipment IX.D.1
- Patents and Inventions XI.D.1.c
- Supplies and Materials ..... IX.D
- Tangible Property XI.D.5.b

Title of Proposed Project ..... I.D.1, II.C.2.a.(4)(a)
Total Direct Costs ..... II.C.2.g(vii)
Traditional Renewal ..... V.A
Transfer of
- Equipment VII.B.2.f, IX.D.2.g
- Grant VII.B.2.f
- Funds VII.B.2.f, VII.B.31
- Principal Investigator/Project Director VII.B.2
- Project Effort VII.B.2

Travel Allowances
- Dependent of Key Project Personnel ..... II.C.2.g(iv)(a), II.E.7, X.C.5
- Domestic ..... II.C.2.g(iv)(b), II.E.9
- Foreign ..... II.C.2.g(iv)(c), II.E.9

Types of Proposals ..... II.E
- Conference II.E.7
- EArly-concept Grants for Exploratory Research (EAGER) II.E.2
- Equipment II.E.8
- Facilitation Awards for Scientists and Engineers with Disabilities (FASED) II.E.6
- Grant Opportunities for Academic Liaison with Industry (GOALI) II.E.4
- Ideas Lab II.E.5
- Center II.E.10
- Rapid Response Research (RAPID) II.E.1
- Research Advanced by Interdisciplinary Science and Engineering (RAISE) II.E.3
- Research Infrastructure II.E.11
- Travel II.E.9

U

Unaffiliated Individuals I.E.5, IV.D.1
Unallowable Costs ..... II.C.2.g(xiii)
- Alcoholic Beverages ..... II.C.2.g(xiii)(c)
- Entertainment Costs ..... II.C.2.g(xiii)(a)
- Meals and Coffee Breaks ..... II.C.2.g(xiii)(b), X.C.5

Undergraduate Student, Definition ..... Exhibit II-7
Underrecovery of Indirect Costs ..... II.C.2.g(viii), X.D.1.c
Unfunded Collaborations ..... II.C.2.d(iv)

Uniform Resource Locators (URLs) ..... II.C.2.d(ii), II.C.2.f(c)
Unit of Consideration ..... II.C.2.a(3)
Universities, Definition I.E.1
Unliquidated Obligations VIII.B.10
Unobligated Balance VIII.B.11, VIII.D.1
Unused Award Funds VIII.D.1
US-Flag Air Carriers ..... II.C.2.g(iv)(a), XI.F.1.b

V

Vertebrate Animals ..... II.C.2.a(4)(i), II.C.2.j, II.D.4, XI.B.3
Veterans Administration I.E.7
Visas X.C.4, XI.F.4
Visual Materials ..... II.C.2.d(ii)

W

Wages ..... see “Salaries and Wages”
Wildlife Research II.D.4, XI.B.3
Withdrawal of Principal Investigator/Project Director VII.B.2.d
Withdrawal of Proposal ..... IV.A
Withholding Payments VIII.C.2.c
Woman-Owned Business ..... II.C.2.a(4)(g)
Working Capital Advance VIII.C.5

X

XSEDE… II.D.7

Privacy Act and Public Burden Statements

The information requested on proposal forms and project reports is solicited under the authority of the National Science Foundation Act of 1950, as amended. The information on proposal forms will be used in connection with the selection of qualified proposals; and project reports submitted by awardees will be used for program evaluation and reporting within the Executive Branch and to Congress. The information requested may be disclosed to qualified reviewers and staff assistants as part of the proposal review process; to proposer institutions/grantees to provide or obtain data regarding the proposal review process, award decisions, or the administration of awards; to government contractors, experts, volunteers and researchers and educators as necessary to complete assigned work; to other government agencies or other entities needing information regarding Proposers or nominees as part of a joint application review process, or in order to coordinate programs or policy; and to another Federal agency, court, or party in a court or Federal administrative proceeding if the government is a party. Information about Principal Investigators may be added to the Reviewer file and used to select potential candidates to serve as peer reviewers or advisory committee members. See Systems of Records, NSF-50, "Principal Investigator/Proposal File and Associated Records," 79 Federal Register 76398 (December 22, 2014), and NSF-51, "Reviewer/Proposal File and Associated Records," 79 Federal Register 76398 (December 22, 2014). Submission of the information is voluntary. Failure to provide full and complete information, however, may reduce the possibility of receiving an award.

An agency may not conduct or sponsor, and a person is not required to respond to, an information collection unless it displays a valid OMB control number. The OMB control number for this collection is 3145-0058. Public reporting burden for this collection of information is estimated to average 120 hours per response, including the time for reviewing instructions. Send comments regarding the burden estimate and any other aspect of this collection of information, including suggestions for reducing this burden, to:

Suzanne H. Plimpton
Reports Clearance Officer
Office of the General Counsel
National Science Foundation
Alexandria, VA 22314

Please wait...

If this message is not eventually replaced by the proper contents of the document, your PDF viewer may not be able to display this type of document.

You can upgrade to the latest version of Adobe Reader for Windows®, Mac, or Linux® by visiting http://www.adobe.com/go/reader_download.

For more assistance with Adobe Reader visit http://www.adobe.com/go/acrreader.

Windows is either a registered trademark or a trademark of Microsoft Corporation in the United States and/or other countries. Mac is a trademark of Apple Inc., registered in the United States and other countries. Linux is the registered trademark of Linus Torvalds in the U.S. and other countries.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME:

eRA COMMONS USER NAME (credential, e.g., agency login):

POSITION TITLE:

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY

A. Personal Statement

B. Positions and Honors

C. Contributions to Science

D. Additional Information: Research Support and/or Scholastic Performance

ScInterinstitutional Assurance

The Interinstitutional Assurance is used by U.S. institutions that receive Public Health Service (PHS) funds through a grant or contract award when the institution has neither its own animal care and use program, facilities to house animals, nor an Institutional Animal Care and Use Committee (IACUC) and will conduct the animal activity at an Assured institution (named as a performance site).

I. Awardee Institution

Name of Awardee Institution:
Address: *(street address, city, state, zip code)*
Project Title: *(from grant application/contract proposal)*
Grant/Contract Number:
Principal Investigator:

A. Applicability

This Interinstitutional Assurance between the awardee institution and the Assured institution is applicable to research, research training, and biological testing involving live vertebrate animals supported by the PHS and conducted at the Assured institution.

B. Awardee and Assured Institutional Responsibilities

i. The institutions agree to comply with all applicable provisions of the Animal Welfare Act and other Federal statutes and regulations relating to animals.

ii. The institutions agree to be guided by the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training and comply with the PHS Policy on Humane Care and Use of Laboratory Animals (Policy).

iii. The institutions acknowledge and accept responsibility for the care and use of animals involved in activities covered by this Assurance. As partial fulfillment of this responsibility, the institutions will make a reasonable effort to ensure that all individuals involved in the care and use of laboratory animals understand their individual and collective responsibilities for compliance with this Assurance, as well as all other applicable laws and regulations pertaining to animal care and use.

iv. The awardee institution acknowledges and accepts the authority of the IACUC of the Assured institution where the animal activity will be performed and agrees to abide by all conditions and determinations as set forth by that IACUC.

Name of Assured Institution:
Address: *(street address, city, state, zip code)*

II. Institutional Endorsement

By signing this document, the authorized official at the awardee institution and the Institutional Official and IACUC Chairperson at the Assured institution (performance site) provide their assurances that the project identified in Part I will be conducted in compliance with the PHS Policy and the Assurance of the Assured institution.

A. Endorsement of Awardee Institution	
Name of Awardee Institution:	
Authorized Official:	
Signature:	Date:
Title:	
Address: *(street address, city, state, zip code)*	
Phone:	Fax:
E-mail:	
B. Endorsement of Assured Institution	
Name of Assured Institution:	
Institutional Official:	
Signature:	Date:
Title:	
Address: *(street address, city, state, zip code)*	
Phone:	Fax:
E-mail:	
IACUC Chairperson:	
Signature:	Date:
Title:	
Address: *(street address, city, state, zip code)*	
Phone:	Fax:
E-mail:	
Date of IACUC Approval: *(within 3 years, pending not acceptable)*	

III. PHS Approval *(to be completed by OLAW)*

Signature of OLAW Official:	Date:
Office of Laboratory Animal Welfare (OLAW) National Institutes of Health 6705 Rockledge Drive RKL1, Suite 360, MSC 7982 Bethesda, MD 20892-7982 (express mail zip code 20817) Phone: (301) 496-7163 Fax: (301) 915-9465	
Grant/Contract #:	Animal Welfare Assurance #:
Effective Date:	Expiration Date: *(duration of project, up to 5 years)*

Please wait...

If this message is not eventually replaced by the proper contents of the document, your PDF viewer may not be able to display this type of document.

You can upgrade to the latest version of Adobe Reader for Windows®, Mac, or Linux® by visiting http://www.adobe.com/go/reader_download.

For more assistance with Adobe Reader visit http://www.adobe.com/go/acrreader.

Windows is either a registered trademark or a trademark of Microsoft Corporation in the United States and/or other countries. Mac is a trademark of Apple Inc., registered in the United States and other countries. Linux is the registered trademark of Linus Torvalds in the U.S. and other countries.

Department of Health and Human Services

Part 1. Overview Information

Participating Organization(s)

National Institutes of Health (NIH (http://www.nih.gov))

Components of Participating Organizations

National Institute of Allergy and Infectious Diseases (NIAID (http://www.niaid.nih.gov))

Funding Opportunity Title

Emerging Infectious Diseases Research Centers (U01 Clinical Trial Not Allowed)

Activity Code

U01 (//grants.nih.gov/grants/funding/ac_search_results.htm?text_curr=u01&Search.x=0&Search.y=0&Search_Type=Activity) Research Project – Cooperative Agreements

Announcement Type

New

Related Notices

- **April 04, 2019** - Notice of Budget Change in RFA-AI-19-028. See Notice NOT-AI-19-056 (/grants/guide/notice-files/NOT-AI-19-056.html).

Funding Opportunity Announcement (FOA) Number

RFA-AI-19-028

Companion Funding Opportunity

RFA-AI-19-029 (https://grants.nih.gov/grants/guide/rfa-files/rfa-ai-19-029.html), U01 (//grants.nih.gov/grants/funding/ac_search_results.htm?text_curr=u01&Search.x=0&Search.y=0&Search_Type=Activity) Research Project – Cooperative Agreements

Number of Applications

See Section III. 3. Additional Information on Eligibility.

Catalog of Federal Domestic Assistance (CFDA) Number(s)

93.855

Funding Opportunity Purpose

The purpose of this Funding Opportunity Announcement (FOA) is to establish a coordinated network of Emerging Infectious Disease Research Centers (EIDRCs) in regions around the globe where emerging and re-emerging infectious disease outbreaks are likely to occur. Multidisciplinary teams of investigators will conduct pathogen/host surveillance, study pathogen transmission, pathogenesis and immunologic responses in the host, and will develop reagents and diagnostic assays for improved detection for important emerging pathogens and their vectors.

Key Dates

Posted Date

March 5, 2019

Open Date (Earliest Submission Date)

May 28, 2019

Letter of Intent Due Date(s)

30 days prior to the application due date

Application Due Date(s)

June 28, 2019, by 5:00 PM local time of applicant organization. All types of non-AIDS applications allowed for this funding opportunity announcement are due on this date.

Applicants are encouraged to apply early to allow adequate time to make any corrections to errors found in the application during the submission process by the due date.

AIDS Application Due Date(s)

Not Applicable

Scientific Merit Review

November 2019

Advisory Council Review

(http://grants1.nih.gov/grants/funding/submissionschedule.htm#reviewandaward) January 2020

Earliest Start Date

March 2020

Expiration Date

June 29, 2019

Due Dates for E.O. 12372

Not Applicable

Required Application Instructions

It is critical that applicants follow the Research (R) Instructions in the SF424 (R&R) Application Guide (//grants.nih.gov/grants/guide/url_redirect.htm?id=12000), except where instructed to do otherwise (in this FOA or in a Notice from the *NIH Guide for Grants and Contracts (//grants.nih.gov/grants/guide/)*). Conformance to all requirements (both in the Application Guide and the FOA) is required and strictly enforced. Applicants must read and follow all application instructions in the Application Guide as well as any program-specific instructions noted in Section IV. When the program-specific instructions deviate from those in the Application Guide, follow the program-specific instructions. **Applications that do not comply with these instructions may be delayed or not accepted for review.**

There are several options available to submit your application through Grants.gov to NIH and Department of Health and Human Services partners. You **must** use one of these submission options to access the application forms for this opportunity.

1. Use the NIH ASSIST system to prepare, submit and track your application online.

   Apply Online Using ASSIST

2. Use an institutional system-to-system (S2S) solution to prepare and submit your application to Grants.gov and eRA Commons (http://public.era.nih.gov/commons/) to track your application. Check with your institutional officials regarding availability.

3. Use Grants.gov (http://www.grants.gov/web/grants/applicants/download-application-package.html#search=true&oppNum=RFA-AI-19-028) Workspace to prepare and submit your application and eRA Commons (http://public.era.nih.gov/commons/) to track your application.

Table of Contents

Part 1. Overview Information
Part 2. Full Text of the Announcement

Section I. Funding Opportunity Description
Section II. Award Information
Section III. Eligibility Information
Section IV. Application and Submission Information
Section V. Application Review Information

Section VI. Award Administration Information
Section VII. Agency Contacts
Section VIII. Other Information

Part 2. Full Text of Announcement

Section I. Funding Opportunity Description

Purpose

The purpose of this Funding Opportunity Announcement (FOA) is to establish a coordinated network of Emerging Infectious Disease Research Centers (EIDRCs) in regions around the globe where emerging and re-emerging infectious disease outbreaks are likely to occur. Multidisciplinary teams of investigators will conduct pathogen/host surveillance, study pathogen transmission, including evaluating zoonotic hosts and reservoirs, study pathogenesis and immunologic responses in the host, and will develop reagents and diagnostic assays for improved detection for important emerging pathogens and their vectors. These EIDRC will conduct research project(s) and will collaborate under the direction of a separate EIDRC Coordinating Center (EIDRC CC, RFA-AI-19-029 (https://grants.nih.gov/grants/guide/rfa-files/rfa-ai-19-029.html)), to develop and formulate strategies for detecting, controlling and preventing emergence or re-emergence of infectious diseases. Rapid translation of these findings to development of reagents, diagnostic tools, etc. that will be provided to downstream partners to advance therapeutic and preventive interventions is key.

Background

A core component of the NIAID mission in the 2017 Strategic Plan is to "develop flexible domestic and international capacity to efficiently undertake research required in response to newly emerging threats wherever they occur." Clearly, recent infectious pathogens with significant potential to threaten the United States are emerging /re-emerging with increasing frequency. Most of these naturally occurring outbreaks have been caused by RNA viral pathogens characterized by zoonotic reservoirs and/or arthropod vectors propagating transmission to humans. These outbreaks have revealed gaps in the research infrastructure that impact the ability to respond rapidly and effectively to these emerging pathogens; obtaining contemporary knowledge of the pathophysiology and natural history, accurate detection/diagnosis of the infections, and development of critical research and product development reagents, were challenges during previous outbreaks.

This FOA and its companion (https://grants.nih.gov/grants/guide/rfa-files/rfa-ai-19-029.html), will serve as important components of the broader NIAID strategy for pandemic preparedness by establishing infrastructure and scientific expertise in geographical regions of the world that are prone to emerging and re-emerging infectious diseases. These Centers will draw on skills from teams of multidisciplinary scientists, including infectious disease clinicians, epidemiologists, virologists, clinical microbiologists, veterinarians, and entomologists, to conduct field studies of endemic diseases that have the greatest potential of becoming pandemic threats, such as zoonotic and vector-borne viruses, develop reagents for the broader research community and have the flexibility and ability to re-direct work among the EIDRC in the event of an outbreak to mount a rapid and effective research response.

Research Objectives and Scope

The Emerging Infectious Diseases Program comprised of the EIDRC CC and the EIDRCs, is designed to improve our knowledge of emerging and re-emerging infectious diseases, complement

and leverage existing NIAID international research efforts when possible, and allow NIAID to develop the flexibility and capacity to respond rapidly and effectively to outbreaks where they occur. The program as a whole is designed to improve our knowledge of the natural history, incidence, and prevalence of emerging/re-emerging infectious diseases. In many instances, re-emerging pathogens circulate at low levels for several years before breaking out and causing major public health problems, in other cases new pathogens emerge rapidly.

Research activities at the centers may include pathogen discovery; surveillance in humans, animal reservoirs, and vectors as it relates to assessment of prevalence and molecular epidemiology of specific pathogens in their geographic regions; evaluation of factors related to transmission, emergence and adaptation to new hosts; natural history of infection and contemporary clinical disease in humans as well as immunologic responses to the infection; development of reagents, new diagnostic methods for improved detection of infectious agents, and development of appropriate animal models, if/when necessary. Clinical cohorts with symptoms of acute infections may be used to define the pathophysiology and clinical outcomes of the infection and disease, describe the spectrum of disease presentation and severity, elucidate factors associated with disease transmission and progression, evaluate important immune responses to the emerging pathogen, determine clinical and molecular diagnostic criteria, and provide the basis for sample collection.

Importantly, the centers will provide reagents, tools and assays to NIAID and partners to support the development of translational products and will readily share information, data, samples, diagnostics, reagents and tools across the network in the event of an outbreak. In that regard, the centers will provide a clear plan for accomplishing effective coordination and collaboration with the entire network and the EIDRC CC.

The EIDRCs will complement and leverage existing NIAID international research efforts- such as the Centers of Excellence for Influenza Research and Surveillance (CEIRS), International Centers of Excellence for Malaria Research (ICEMR), Tropical Medicine Research Centers (TMRC), and Centers for AIDS Research (CFAR)- and/or other US Government or internationally funded research infrastructure as needed to conduct proposed research and exhibit the flexibility to expand capacity in response to outbreaks in their geographic area, as directed by NIAID and the EIDRC CC. Each EIDRC will have the flexibility to study any emerging pathogen (viral, bacterial or eukaryotic), however, the initial focus will be on infectious pathogens that are most likely to emerge/remerge including, but not limited to: Flaviviruses, Filoviruses, Alphaviruses, Coronaviruses, Bunyaviruses, and Enteroviruses that are not already studied by other NIAID funded networks (e.g. HIV, influenza, malaria) and for which countermeasures are not developed or are sub-optimal.

Each EIDRC will have relevant scientific expertise and experience working in one or more geographic areas located within the tropical or subtropical regions of the world (Tropics: latitude between 23.5o -Tropic of Cancer and Capricorn-Subtropics: between tropic and temperate zones (35-66.5o N and S of the equator) with the goal to establish multiple sites in targeted areas of the globe such as South and Central America, West/Central Africa and Southeast Asia. In an effort to train the next generation of investigators, develop expertise in emerging infectious disease research and expand regional capacity in these countries, the centers will be required to conduct a pilot project research program, that will be coordinated, including the awarding of projects, by the EIDRC CC.

EIDRC Structure

Administration and Leadership Team

The Administration and Leadership Team, led by the Program Director(s)/Principal Investigator(s), will be responsible for organizing, coordinating, and providing oversight for the implementation of activities that facilitate progress and completion of the research project(s) and conduct of pilot research projects, if awarded. This team will provide administrative oversight, coordination and facilitated communications with other EIDRCs and interact directly with the EIDRC CC and NIAID.

Data Management and Analysis Team

The Data Management and Analysis Team will be responsible for implementing standard procedures provided by the EIDRC CC for the collection, oversight and inventory of data and biological samples, including; harmonization, quality control, and uniformity of data collection processes, troubleshooting data system problems and developing solutions. Team members will also work with the Administration and Leadership Team, to develop efficient study designs and statistical calculations and provide support for the preliminary and final data analyses for the study. This team will serve as the hub for expertise with sharing data portal(s) as approved by NIAID. This team is responsible for directly depositing all relevant data, samples, reagents, tools, etc. as outlined to the EIDRC CC

Clinical Research Support Team

The Clinical Research Support Team will be responsible for coordinating the functions at all enrollment sites to recruit, enroll, and collect data and biological samples from participants. This Team will ensure adherence to NIH and EIDRC CC guidelines on research and data quality and all local and other regulatory oversight pertaining to all human subjects' research. This team will serve as the process and procedure hub for the interaction among the clinical sites and other functional work areas.

External Advisory Committee (EAC)

One External Advisory Committee, comprised of experts in the field outside of the EIDRCs and EIDRC CC, will be established after award to review progress for the Emerging Infectious Diseases Program (EIDRCs and EIDRC CC) and to share recommendations for the program with NIAID as part of the annual programmatic meeting. **Note that applicants should not name or contact potential EAC members in their application.**

Annual Programmatic Meetings

Each EIDRC will attend a combined kick-off meeting and annual program meetings under the leadership of the EIDRC CC to articulate and establish the major roles and functions of the program and to facilitate collaborations, provide progress reporting, seek new research directions and ideas, and update NIAID on issues of need. These meetings will be attended by the PD(s)/PI(s), Key personnel, NIAID staff, and the EAC membership and will be held in the Washington DC area.

Applications proposing any of the following topic areas will be considered nonresponsive and will not be reviewed:

- Applications that focus on pathogens that are not on the NIAID list of Emerging diseases (https://www.niaid.nih.gov/research/emerging-infectious-diseases-pathogens (https://www.niaid.nih.gov/research/emerging-infectious-diseases-pathogens)). Research focus should be emerging/remerging viruses, but if studying coinfected population with a

pathogen not on the Emerging Diseases list, application should include a strong justification and rationale for inclusion.
- Pathogens already studied by other NIAID funded networks, such as influenza, tuberculosis, malaria and antibiotic resistant bacteria. Please contact the Scientific/Research Contact if further clarification is needed.
- HIV, SIV or AIDS studies.
- Genome-wide association studies (GWAS).
- Behavioral research.

See Section VIII. Other Information for award authorities and regulations.

Section II. Award Information

Funding Instrument

Cooperative Agreement: A support mechanism used when there will be substantial Federal scientific or programmatic involvement. Substantial involvement means that, after award, NIH scientific or program staff will assist, guide, coordinate, or participate in project activities. See Section VI.2 for additional information about the substantial involvement for this FOA.

Application Types Allowed

New

The OER Glossary (//grants.nih.gov/grants/guide/url_redirect.htm?id=11116) and the SF424 (R&R) Application Guide provide details on these application types.

Clinical Trial?

Not Allowed: Only accepting applications that do not propose clinical trials

Need help determining whether you are doing a clinical trial? (https://grants.nih.gov/grants/guide/url_redirect.htm?id=82370)

Funds Available and Anticipated Number of Awards

NIAID intends to commit $6M in FY 2020 to fund 2-4 awards.

Award Budget

Applications budget are limited to up to $850,000 per year in direct costs and need to reflect the actual needs of the proposed project.

Award Project Period

The scope of the proposed project should determine the project period. The maximum period is 5 years.

NIH grants policies as described in the *NIH Grants Policy Statement (//grants.nih.gov/grants/guide/url_redirect.htm?id=11120)* will apply to the applications submitted and awards made from this FOA.

Section III. Eligibility Information

1. Eligible Applicants

Eligible Organizations

Higher Education Institutions

- Public/State Controlled Institutions of Higher Education
- Private Institutions of Higher Education

The following types of Higher Education Institutions are always encouraged to apply for NIH support as Public or Private Institutions of Higher Education:

- Hispanic-serving Institutions
- Historically Black Colleges and Universities (HBCUs)
- Tribally Controlled Colleges and Universities (TCCUs)
- Alaska Native and Native Hawaiian Serving Institutions
- Asian American Native American Pacific Islander Serving Institutions (AANAPISIs)

Nonprofits Other Than Institutions of Higher Education

- Nonprofits with 501(c)(3) IRS Status (Other than Institutions of Higher Education)
- Nonprofits without 501(c)(3) IRS Status (Other than Institutions of Higher Education)

For-Profit Organizations

- Small Businesses
- For-Profit Organizations (Other than Small Businesses)

Governments

- State Governments
- County Governments
- City or Township Governments
- Special District Governments
- Indian/Native American Tribal Governments (Federally Recognized)
- Indian/Native American Tribal Governments (Other than Federally Recognized)
- Eligible Agencies of the Federal Government
- U.S. Territory or Possession

Other

- Independent School Districts
- Public Housing Authorities/Indian Housing Authorities
- Native American Tribal Organizations (other than Federally recognized tribal governments)
- Faith-based or Community-based Organizations
- Regional Organizations
- Non-domestic (non-U.S.) Entities (Foreign Institutions)

Foreign Institutions

Non-domestic (non-U.S.) Entities (Foreign Institutions) **are** eligible to apply.

Non-domestic (non-U.S.) components of U.S. Organizations **are** eligible to apply.

Foreign components, as defined in the *NIH Grants Policy Statement* (//grants.nih.gov/grants/guide/url_redirect.htm?id=11118), **are** allowed.

Required Registrations

Applicant Organizations

Applicant organizations must complete and maintain the following registrations as described in the SF 424 (R&R) Application Guide to be eligible to apply for or receive an award. All registrations must be completed prior to the application being submitted. Registration can take 6 weeks or more, so applicants should begin the registration process as soon as possible. The NIH Policy on Late Submission of Grant Applications (//grants.nih.gov/grants/guide/notice-files/NOT-OD-15-039.html) states that failure to complete registrations in advance of a due date is not a valid reason for a late submission.

- Dun and Bradstreet Universal Numbering System (DUNS) (http://fedgov.dnb.com/webform) - All registrations require that applicants be issued a DUNS number. After obtaining a DUNS number, applicants can begin both SAM and eRA Commons registrations. The same DUNS number must be used for all registrations, as well as on the grant application.
- System for Award Management (SAM) (https://www.sam.gov/portal/public/SAM/)– Applicants must complete and maintain an active registration, **which requires renewal at least annually**. The renewal process may require as much time as the initial registration. SAM registration includes the assignment of a Commercial and Government Entity (CAGE) Code for domestic organizations which have not already been assigned a CAGE Code.
    - NATO Commercial and Government Entity (NCAGE) Code (//grants.nih.gov/grants/guide/url_redirect.htm?id=11176) – Foreign organizations must obtain an NCAGE code (in lieu of a CAGE code) in order to register in SAM.
- eRA Commons (//grants.nih.gov/grants/guide/url_redirect.htm?id=11123) - Applicants must have an active DUNS number to register in eRA Commons. Organizations can register with the eRA Commons as they are working through their SAM or Grants.gov registration, but all registrations must be in place by time of submission. eRA Commons requires organizations to identify at least one Signing Official (SO) and at least one Program Director/Principal Investigator (PD/PI) account in order to submit an application.
- Grants.gov (//grants.nih.gov/grants/guide/url_redirect.htm?id=82300) – Applicants must have an active DUNS number and SAM registration in order to complete the Grants.gov registration.

Program Directors/Principal Investigators (PD(s)/PI(s))

All PD(s)/PI(s) must have an eRA Commons account. PD(s)/PI(s) should work with their organizational officials to either create a new account or to affiliate their existing account with the applicant organization in eRA Commons. If the PD/PI is also the organizational Signing Official, they must have two distinct eRA Commons accounts, one for each role. Obtaining an eRA Commons account can take up to 2 weeks.

Eligible Individuals (Program Director/Principal Investigator)

Any individual(s) with the skills, knowledge, and resources necessary to carry out the proposed research as the Program Director(s)/Principal Investigator(s) (PD(s)/PI(s)) is invited to work with his/her organization to develop an application for support. Individuals from underrepresented racial

and ethnic groups as well as individuals with disabilities are always encouraged to apply for NIH support.

For institutions/organizations proposing multiple PDs/PIs, visit the Multiple Program Director/Principal Investigator Policy and submission details in the Senior/Key Person Profile (Expanded) Component of the SF424 (R&R) Application Guide.

Applicants for the EIDRC CC (RFA-AI-19-029 (https://grants.nih.gov/grants/guide/rfa-files/rfa-ai-19-029.html)) may also apply to this FOA.

2. Cost Sharing

This FOA does not require cost sharing as defined in the *NIH Grants Policy Statement. (//grants.nih.gov/grants/guide/url_redirect.htm?id=11126)*

3. Additional Information on Eligibility

Number of Applications

Applicant organizations may submit more than one application, provided that each application is scientifically distinct.

The NIH will not accept duplicate or highly overlapping applications under review at the same time. This means that the NIH will not accept:

- A new (A0) application that is submitted before issuance of the summary statement from the review of an overlapping new (A0) or resubmission (A1) application.
- A resubmission (A1) application that is submitted before issuance of the summary statement from the review of the previous new (A0) application.
- An application that has substantial overlap with another application pending appeal of initial peer review (see NOT-OD-11-101 (//grants.nih.gov/grants/guide/notice-files/NOT-OD-11-101.html)).

Section IV. Application and Submission Information

1. Requesting an Application Package

The application forms package specific to this opportunity must be accessed through ASSIST, Grants.gov Workspace or an institutional system-to-system solution. Links to apply using ASSIST or Grants.gov Workspace are available in Part 1 of this FOA. See your administrative office for instructions if you plan to use an institutional system-to-system solution.

2. Content and Form of Application Submission

It is critical that applicants follow the Research (R) Instructions in the SF424 (R&R) Application Guide (//grants.nih.gov/grants/guide/url_redirect.htm?id=12000), except where instructed in this funding opportunity announcement to do otherwise. Conformance to the requirements in the Application Guide is required and strictly enforced. Applications that are out of compliance with these instructions may be delayed or not accepted for review.

Letter of Intent

Although a letter of intent is not required, is not binding, and does not enter into the review of a subsequent application, the information that it contains allows IC staff to estimate the potential review workload and plan the review.

By the date listed in Part 1. Overview Information, prospective applicants are asked to submit a letter of intent that includes the following information:

- Descriptive title of proposed activity
- Name(s), address(es), and telephone number(s) of the PD(s)/PI(s)
- Names of other key personnel
- Participating institution(s)
- Number and title of this funding opportunity

The letter of intent should be sent to:

Eleazar Cohen, Ph.D.
Telephone: 240-669-5081
Email:ecohen@niaid.nih.gov (mailto:ecohen@niaid.nih.gov)

Page Limitations

All page limitations described in the SF424 Application Guide and the Table of Page Limits (//grants.nih.gov/grants/guide/url_redirect.htm?id=11133) must be followed, with the following additional instructions.

For this specific FOA, the Research Strategy is limited to 30 pages.

Instructions for Application Submission

The following section supplements the instructions found in the SF424 (R&R) Application Guide and should be used for preparing an application to this FOA.

SF424(R&R) Cover

All instructions in the SF424 (R&R) Application Guide must be followed.

SF424(R&R) Project/Performance Site Locations

All instructions in the SF424 (R&R) Application Guide must be followed.

SF424(R&R) Other Project Information

All instructions in the SF424 (R&R) Application Guide must be followed. With the following additional instructions:

Facilities and Other Resources:

In a separate section entitled "Information Technology Access" include a brief description of the features of the institutional environment that are relevant to the effective implementation of the proposed unified collaborations across Teams and functions. Describe in general available IT resources associated with the enrollment and field sites with respect to access to computers, source and status of reliable, secure internet connections, and other communications.

In a separate section entitled "Biological Sample Storage and Access", describe the facilities available to store, manage and retrieve biological specimens for use by the PD(s)/PI(s) and key personnel.

SF424(R&R) Senior/Key Person Profile

All instructions in the SF424 (R&R) Application Guide must be followed. With the following additional instructions:

- Within the Biosketch under Personal Statement, describe the leadership approach and experience of the PD(s)/PI(s) with respect to developing and executing multi-disciplinary research program(s) of a similar size and nature.
- Within the Biosketch under Personal Statement, all Key Personnel should demonstrate strong administrative, technical, and management expertise in areas critical to the success of the application, including experience working productively in team collaborative environments. Demonstrate how specific expertise supports the multi-disciplinary approach to guarantee a successful, integrated effort towards the goals of the research project.

R&R or Modular Budget

All instructions in the SF424 (R&R) Application Guide must be followed. With the following additional instructions:

In Year 01, include funds in the budget for the PD(s)/PI(s), collaborators, key personnel to travel and attend a kickoff meeting to be held shortly after the award over 1 full day in the Bethesda, MD area. Beginning in Year 02 include funds in the budget for an annual program meeting to be held over two full days in the Bethesda, MD area. Do not include costs associated with organizing and holding the kickoff or annual program meetings.

Include costs associated with submission of data into publicly accessible portal(s) approved by NIAID. Also include costs associated with biological sample collection, processing, shipping, and storage as well as costs for depositing data, reagents and tools to the EIDRC CC.

R&R Subaward Budget

All instructions in the SF424 (R&R) Application Guide must be followed.

PHS 398 Cover Page Supplement

All instructions in the SF424 (R&R) Application Guide must be followed.

PHS 398 Research Plan

All instructions in the SF424 (R&R) Application Guide must be followed, with the following additional instructions:

Specific Aims: List in priority order, the broad, long-range objectives and goals of the proposed research including surveillance, transmission, pathogenesis and immunologic responses in the host, and reagent and diagnostic assay development for improved detection for important emerging pathogens and their vectors and indicate how these goals will be accomplished. Concisely describe the hypothesis or hypotheses to be tested with an initial emphasis on RNA viruses and plans for incorporation of other pathogens and emerging infectious diseases. Indicate how the work proposed dovetails to address the overall goals and objectives of the research.

Research Strategy:

In a clearly labeled section entitled "Administrative Plan":

- Describe the administrative and organizational structure of the EIDCRC Administrative and Leadership Team. Include the unique features of the organizational structure that serve to facilitate accomplishment of the long-range goals and objectives.

- Describe how communications will be planned, implemented, and provided to collaborators, teams, or sites. In addition, describe plans to achieve synergy and interaction within the EIDRC to ensure efficient cooperation, communication and coordination.
- Specifically address how the EIDRC Team will communicate with other EIDRCs, the EIDRC CC and NIAID and how the EIDRC will implement plans and guidance from the EIDRC CC to ensure coordination and collaboration across the Emerging Infectious Disease Program as a whole. Describe how the study site will provide information, if any, back to the local health authorities and the study subjects as needed. Note any adjustments or changes needed for rapid and flexible responses to outbreaks in emerging infectious diseases.
- Describe the overall management approach, including how resources will be managed, organized and prioritized, including pilot research projects.
- Highlight how the proposed staffing plan incorporates scientific and administrative expertise to fulfill the goal to develop the flexibility and capacity to respond rapidly and effectively to outbreaks in emerging infectious diseases in an international setting.

In a clearly labeled section entitled "Data Management Plan":

- Describe internal and external data acquisition strategies to achieve harmonization of systems and procedures for data management, data quality, data analyses, and dissemination for all data and data-related materials generated by the research study with the EIDRC CC.
- Within the plan, indicate the extent to which dedicated systems or procedures will be utilized to harmonize the acquisition, curation, management, inventory and storage of data and samples. Describe how training for the data and sample collection, in terms of the use of electronic data capture systems, will be provided to all staff including those at enrollment sites.
- Describe the quality control procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens.

In a clearly labeled section entitled "Clinical Management Plan":

- Describe plans, processes, and procedures for the following activities: clinical site selection including feasibility, capacity, and capabilities, and study development, conduct, and oversight.
- Describe strategies for oversight and implementation of standardized approaches in the recruitment and clinical characterization of adequate numbers of relevant patient populations to ensure the prompt screening, enrollment, retention and completion of studies. Describe novel approaches and solutions to study enrollment. including the clinical meta-data that will be captured and describe how the staff at each enrollment/collection site will be trained and comply with the quality standards for data and sample collection and compliance with the standardized procedures. Provide a general description of the methods for establishing clinical cohorts and how cohorts may be leveraged for new emerging pathogens.
- Provide general approaches to identification of the types of clinical cohorts needed to fulfill the goals of the EIDRC. Discuss types of clinical cohorts and clinical assessments that will be utilized to study natural history and pathophysiology of disease, including characteristics of the cohort, site for recruitment, types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.

- Describe methods that might be applied to either elucidate a zoonotic source or a vector of transmission.
- Describe capacity for and approaches to clinical, immunologic and biologic data and specimen collection, and how these data and specimens will address and accomplish the overall goals and objectives of the research study.
- Provide plans for the potential addition of new sites and expanding scientific areas of research when needed for an accelerated response to an outbreak or newly emerging infectious disease.

In a clearly labeled section within the Research Strategy entitled "Statistical Analysis Plan":

- Describe the overall plan for statistical analysis of preliminary research data, including observational cohort data.
- Describe the overall staffing plan for biostatistical support and systems available for following functions: 1) preliminary data analyses, 2) estimates of power and sample size, 3) research study design and protocol development.
- Describe the quality control and security procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens.
- Describe any unique or innovative approaches to statistical analysis that may be utilized.

In a clearly labeled section entitled "Project Milestones and Timelines":

- Describe specific quantifiable milestones by annum and include annual timelines for the overall research study and for tracking progress from individual sites, collaborators, and Teams. Milestones must specify the outcome(s) for each activity. Milestones should be quantifiable and scientifically justified, and include the completion of major research study activities, including, for example, protocol development, case report forms, scheduled clinical visits, obtaining clearances study completion, and analysis of final data. Milestone criteria should not simply be a restatement of the specific aims. Using a Gantt chart or equivalent tool, describe the associated timelines and identified outcomes for the research study.

Note: if the proposed research includes a clinical study, specific information for that study will be entered using the PHS Human Subjects and Clinical Trials Information and should not be duplicated in the Research Strategy.

Letters of Support: Include any letter necessary to demonstrate the support of consortium/site participants.

Resource Sharing Plan: Individuals are required to comply with the instructions for the Resource Sharing Plans as provided in the SF424 (R&R) Application Guide, with the following modification:

- All applications, regardless of the amount of direct costs requested for any one year, should address a Data Sharing Plan that details the rapid sharing, release and access of datasets, analysis tools, computational models, reagents, and other resources, which are generated by the Center to the broader scientific community in adherence to the requirements and timelines described in the NIAID Data and Reagents Sharing and Release Guidelines (https://www.niaid.nih.gov/research/data-sharing-and-release-guidelines) and the NIH Genomic Data Sharing (GDS) Policy (https://grants.nih.gov/grants/guide/notice-files/NOT-OD-14-124.html).
- NIAID encourages the use of NIH supported open access Data Sharing Repositories as a first choice and a list of them can be found at

https://www.nlm.nih.gov/NIHbmic/nih_data_sharing_repositories.html. (https://www.nlm.nih.gov/NIHbmic/nih_data_sharing_repositories.html) Provide a timeline for the planned acceptance, quality check, transfer and upload of data of all types.

- All investigators funded under this FOA will be expected to share their data through a portal (s) approved by NIAID as well as any collected biological samples, including clinical and animal research specimens. Therefore, the Resource Sharing plan should include a summary of how the applicant will manage data submission and interactions with the chosen portal(s), as well as sharing of biological samples with the scientific community.
- All Investigators are expected to be aware of and abide by all applicable NIH guidance for sharing of research resources and data, consistent with existing laws, regulations, and policies.
- All investigators should provide a plan for how to accomplish sharing of critical diagnostic reagents and tools when necessary, to NIAID, NIAID supported repositories, the other EIDRCs, the CC, and the broader research community.

Appendix:

Only limited Appendix materials are allowed. Follow all instructions for the Appendix as described in the SF424 (R&R) Application Guide.

PHS Human Subjects and Clinical Trials Information

When involving NIH-defined human subjects research, clinical research, and/or clinical trials (and when applicable, clinical trials research experience) follow all instructions for the PHS Human Subjects and Clinical Trials Information form in the SF424 (R&R) Application Guide, with the following additional instructions:

If you answered "Yes" to the question "Are Human Subjects Involved?" on the R&R Other Project Information form, you must include at least one human subjects study record using the **Study Record: PHS Human Subjects and Clinical Trials Information** form or **Delayed Onset Study** record.

Study Record: PHS Human Subjects and Clinical Trials Information

All instructions in the SF424 (R&R) Application Guide must be followed with the following additional instructions:

Section 3 - Protection and Monitoring Plans

3.1 Protection of Human Subjects

3.1.1 Risks to Human Subjects

3.1.1.b Study Procedures, Materials and Potential Risks

Additional Instructions

For applications proposing to use samples from ongoing or completed clinical research, provide a timeline for the request, transportation and receipt of the biological samples, and the timeline associated with the preparation and use of the biological samples.

For all research projects provide information on:

- Describe the type(s) of biosamples to be used

- Describe the handling and processing of biosamples at the collection site (collection, processing, storage, transportation and quality control measures to ensure sample integrity)
- List the research laboratory(ies) to be used and describe the selection rationale and qualifications
- Describe the laboratory methodology(ies) to be used for each proposed assay/test and provide evidence of assay feasibility

Delayed Onset Study

Note: Delayed onset (https://grants.nih.gov/grants/glossary.htm#DelayedOnsetHumanSubjectStudy) does NOT apply to a study that can be described but will not start immediately (i.e., delayed start).

All instructions in the SF424 (R&R) Application Guide must be followed.

PHS Assignment Request Form

All instructions in the SF424 (R&R) Application Guide must be followed.

Foreign Institutions

Foreign (non-U.S.) institutions must follow policies described in the *NIH Grants Policy Statement (//grants.nih.gov/grants/guide/url_redirect.htm?id=11137)*, and procedures for foreign institutions.

3. Unique Entity Identifier and System for Award Management (SAM)

See Part 1. Section III.1 for information regarding the requirement for obtaining a unique entity identifier and for completing and maintaining active registrations in System for Award Management (SAM), NATO Commercial and Government Entity (NCAGE) Code (if applicable), eRA Commons, and Grants.gov

4. Submission Dates and Times

Part I. Overview Information contains information about Key Dates and times. Applicants are encouraged to submit applications before the due date to ensure they have time to make any application corrections that might be necessary for successful submission. When a submission date falls on a weekend or Federal holiday (https://grants.nih.gov/grants/guide/url_redirect.htm?id=82380), the application deadline is automatically extended to the next business day.

Organizations must submit applications to Grants.gov (//grants.nih.gov/grants/guide/url_redirect.htm?id=11128) (the online portal to find and apply for grants across all Federal agencies). Applicants must then complete the submission process by tracking the status of the application in the eRA Commons (//grants.nih.gov/grants/guide/url_redirect.htm?id=11123), NIH's electronic system for grants administration. NIH and Grants.gov systems check the application against many of the application instructions upon submission. Errors must be corrected and a changed/corrected application must be submitted to Grants.gov on or before the application due date and time. If a Changed/Corrected application is submitted after the deadline, the application will be considered late. Applications that miss the due date and time are subjected to the NIH Policy on Late Application Submission.

Applicants are responsible for viewing their application before the due date in the eRA Commons to ensure accurate and successful submission.

Information on the submission process and a definition of on-time submission are provided in the SF424 (R&R) Application Guide.

5. Intergovernmental Review (E.O. 12372)

This initiative is not subject to intergovernmental review. (//grants.nih.gov/grants/guide/url_redirect.htm?id=11142)

6. Funding Restrictions

All NIH awards are subject to the terms and conditions, cost principles, and other considerations described in the *NIH Grants Policy Statement (//grants.nih.gov/grants/guide/url_redirect.htm?id=11120)*.

Pre-award costs are allowable only as described in the *NIH Grants Policy Statement (//grants.nih.gov/grants/guide/url_redirect.htm?id=11143)*.

7. Other Submission Requirements and Information

Applications must be submitted electronically following the instructions described in the SF424 (R&R) Application Guide. Paper applications will not be accepted.

Applicants must complete all required registrations before the application due date. Section III. Eligibility Information contains information about registration.

For assistance with your electronic application or for more information on the electronic submission process, visit How to Apply – Application Guide (https://grants.nih.gov/grants/how-to-apply-application-guide.html). If you encounter a system issue beyond your control that threatens your ability to complete the submission process on-time, you must follow the Dealing with System Issues (https://grants.nih.gov/grants/how-to-apply-application-guide/due-dates-and-submission-policies/dealing-with-system-issues.htm) guidance. For assistance with application submission, contact the Application Submission Contacts in Section VII.

Important reminders:

All PD(s)/PI(s) must include their eRA Commons ID in the Credential field of the Senior/Key Person Profile Component of the SF424(R&R) Application Package. Failure to register in the Commons and to include a valid PD/PI Commons ID in the credential field will prevent the successful submission of an electronic application to NIH. See Section III of this FOA for information on registration requirements.

The applicant organization must ensure that the DUNS number it provides on the application is the same number used in the organization's profile in the eRA Commons and for the System for Award Management. Additional information may be found in the SF424 (R&R) Application Guide.

See more tips (//grants.nih.gov/grants/guide/url_redirect.htm?id=11146) for avoiding common errors.

Upon receipt, applications will be evaluated for completeness and compliance with application instructions by the Center for Scientific Review and responsiveness by components of participating

organizations, NIH. Applications that are incomplete, non-compliant and/or nonresponsive will not be reviewed.

Post Submission Materials

Applicants are required to follow the instructions for post-submission materials, as described in the policy (//grants.nih.gov/grants/guide/url_redirect.htm?id=82299). Any instructions provided here are in addition to the instructions in the policy.

Section V. Application Review Information

1. Criteria

Only the review criteria described below will be considered in the review process.

Applications submitted to the NIH in support of the NIH mission (//grants.nih.gov/grants/guide/url_redirect.htm?id=11149) are evaluated for scientific and technical merit through the NIH peer review system.

Overall Impact

Reviewers will provide an overall impact score to reflect their assessment of the likelihood for the project to exert a sustained, powerful influence on the research field(s) involved, in consideration of the following review criteria and additional review criteria (as applicable for the project proposed).

Scored Review Criteria

Reviewers will consider each of the review criteria below in the determination of scientific merit, and give a separate score for each. An application does not need to be strong in all categories to be judged likely to have major scientific impact. For example, a project that by its nature is not innovative may be essential to advance a field.

Significance

Does the project address an important problem or a critical barrier to progress in the field? Is the prior research that serves as the key support for the proposed project rigorous? If the aims of the project are achieved, how will scientific knowledge, technical capability, and/or clinical practice be improved? How will successful completion of the aims change the concepts, methods, technologies, treatments, services, or preventative interventions that drive this field?

Investigator(s)

Are the PD(s)/PI(s), collaborators, and other researchers well suited to the project? If Early Stage Investigators or those in the early stages of independent careers, do they have appropriate experience and training? If established, have they demonstrated an ongoing record of accomplishments that have advanced their field(s)? If the project is collaborative or multi-PD/PI, do the investigators have complementary and integrated expertise; are their leadership approach, governance and organizational structure appropriate for the project?

Specific for this FOA: Is the level of commitment of the PD(s)/PI(s) and key personnel adequate to manage the overall project? Do the investigators/collaborators have a previous history of translational research efforts?

Do the investigators have relevant scientific expertise and experience working in one or more geographic areas located within the tropical or subtropical regions of the world (Tropics: latitude

between 23.5o -Tropic of Cancer and Capricorn- Subtropics: between tropic and temperate zones (35-66.5o N and S of the equator)? Does the team have a multidisciplinary expertise that could successfully be applied to characterizing the clinical features and transmission cycle of a newly emerging infectious disease?

Innovation

Does the application challenge and seek to shift current research or clinical practice paradigms by utilizing novel theoretical concepts, approaches or methodologies, instrumentation, or interventions? Are the concepts, approaches or methodologies, instrumentation, or interventions novel to one field of research or novel in a broad sense? Is a refinement, improvement, or new application of theoretical concepts, approaches or methodologies, instrumentation, or interventions proposed?

Approach

Are the overall strategy, methodology, and analyses well-reasoned and appropriate to accomplish the specific aims of the project? Have the investigators included plans to address weaknesses in the rigor of prior research that serves as the key support for the proposed project? Have the investigators presented strategies to ensure a robust and unbiased approach, as appropriate for the work proposed? Are potential problems, alternative strategies, and benchmarks for success presented? If the project is in the early stages of development, will the strategy establish feasibility and will particularly risky aspects be managed? Have the investigators presented adequate plans to address relevant biological variables, such as sex, for studies in vertebrate animals or human subjects?

If the project involves human subjects and/or NIH-defined clinical research, are the plans to address

1) the protection of human subjects from research risks, and

2) inclusion (or exclusion) of individuals on the basis of sex/gender, race, and ethnicity, as well as the inclusion or exclusion of individuals of all ages (including children and older adults), justified in terms of the scientific goals and research strategy proposed?

Specific to this FOA: Do the investigators provide a clear plan for how to add new sites and/or expand scientific areas of research when needed for an accelerated response to an outbreak? Has the investigator described the establishment of a clinical cohort (or cohorts) that is likely to improve our understanding of the natural history of an emerging pathogen? Is the rationale for eligibility for inclusion in the cohort sound? Are the recruitment and retention plans and study timelines reasonable?

Do the targeted pathogen(s)/disease(s) address the FOA's intent to focus on the most relevant emerging pathogens? Are the contributions of each individual activity proposed within the research design well integrated with the aims of the project? Are the coordination and synergy within the project adequate to ensure the achievement of its central objectives?

Are the proposed host systems adequate, well-documented and characterized? If using animal models, are the models relevant and appropriate to investigate emerging ID infections?

Are the proposed technologies well justified, critical and relevant to the project aims?

Do the investigators provide a clear plan for achieving defined project milestones and timelines?

Are the described data management activities sufficient?

Environment

Will the scientific environment in which the work will be done contribute to the probability of success? Are the institutional support, equipment and other physical resources available to the investigators adequate for the project proposed? Will the project benefit from unique features of the scientific environment, subject populations, or collaborative arrangements?

Specific to this FOA: Do the investigators plan to complement and leverage existing NIAID international research efforts- such as the Centers of Excellence for Influenza Research and Surveillance (CEIRS), International Centers of Excellence for Malaria Research (ICEMR), Tropical Medicine Research Centers (TMRC), and Centers for AIDS Research (CFAR) and/or other US and internationally funded existing infrastructure as needed to conduct proposed research?

Additional Review Criteria

As applicable for the project proposed, reviewers will evaluate the following additional items while determining scientific and technical merit, and in providing an overall impact score, but will not give separate scores for these items.

Protections for Human Subjects

For research that involves human subjects but does not involve one of the categories of research that are exempt under 45 CFR Part 46, the committee will evaluate the justification for involvement of human subjects and the proposed protections from research risk relating to their participation according to the following five review criteria: 1) risk to subjects, 2) adequacy of protection against risks, 3) potential benefits to the subjects and others, 4) importance of the knowledge to be gained, and 5) data and safety monitoring for clinical trials.

For research that involves human subjects and meets the criteria for one or more of the categories of research that are exempt under 45 CFR Part 46, the committee will evaluate: 1) the justification for the exemption, 2) human subjects involvement and characteristics, and 3) sources of materials. For additional information on review of the Human Subjects section, please refer to the Guidelines for the Review of Human Subjects (//grants.nih.gov/grants/guide/url_redirect.htm?id=11175).

Inclusion of Women, Minorities, and Individuals Across the Lifespan

When the proposed project involves human subjects and/or NIH-defined clinical research, the committee will evaluate the proposed plans for the inclusion (or exclusion) of individuals on the basis of sex/gender, race, and ethnicity, as well as the inclusion (or exclusion) of individuals of all ages (including children and older adults) to determine if it is justified in terms of the scientific goals and research strategy proposed. For additional information on review of the Inclusion section, please refer to the Guidelines for the Review of Inclusion in Clinical Research (//grants.nih.gov/grants/guide/url_redirect.htm?id=11174).

Vertebrate Animals

The committee will evaluate the involvement of live vertebrate animals as part of the scientific assessment according to the following criteria: (1) description of proposed procedures involving animals, including species, strains, ages, sex, and total number to be used; (2) justifications for the use of animals versus alternative models and for the appropriateness of the species

proposed; (3) interventions to minimize discomfort, distress, pain and injury; and (4) justification for euthanasia method if NOT consistent with the AVMA Guidelines for the Euthanasia of Animals. Reviewers will assess the use of chimpanzees as they would any other application proposing the use of vertebrate animals. For additional information on review of the Vertebrate Animals section, please refer to the Worksheet for Review of the Vertebrate Animal Section (//grants.nih.gov/grants/guide/url_redirect.htm?id=11150).

Biohazards

Reviewers will assess whether materials or procedures proposed are potentially hazardous to research personnel and/or the environment, and if needed, determine whether adequate protection is proposed.

Resubmissions

Not Applicable

Renewals

Not Applicable

Revisions

Not Applicable

Additional Review Considerations

As applicable for the project proposed, reviewers will consider each of the following items, but will not give scores for these items, and should not consider them in providing an overall impact score.

Applications from Foreign Organizations

Reviewers will assess whether the project presents special opportunities for furthering research programs through the use of unusual talent, resources, populations, or environmental conditions that exist in other countries and either are not readily available in the United States or augment existing U.S. resources.

Select Agent Research

Reviewers will assess the information provided in this section of the application, including 1) the Select Agent(s) to be used in the proposed research, 2) the registration status of all entities where Select Agent(s) will be used, 3) the procedures that will be used to monitor possession use and transfer of Select Agent(s), and 4) plans for appropriate biosafety, biocontainment, and security of the Select Agent(s).

Resource Sharing Plans

Reviewers will comment on whether the following Resource Sharing Plans, or the rationale for not sharing the following types of resources, are reasonable: (1) Data Sharing Plan (//grants.nih.gov/grants/guide/url_redirect.htm?id=11151); (2) Sharing Model Organisms (//grants.nih.gov/grants/guide/url_redirect.htm?id=11152); and (3) Genomic Data Sharing Plan (GDS) (//grants.nih.gov/grants/guide/url_redirect.htm?id=11153). Do the investigators provide a clear and concise plan for sharing of information, data, and biological samples, and the development and sharing of critical research reagents and tools when necessary, according to specified timelines to NIAID supported repositories, the other EIDRCs, the CC, and the broader research community?

Authentication of Key Biological and/or Chemical Resources:

For projects involving key biological and/or chemical resources, reviewers will comment on the brief plans proposed for identifying and ensuring the validity of those resources.

Budget and Period of Support

Reviewers will consider whether the budget and the requested period of support are fully justified and reasonable in relation to the proposed research.

2. Review and Selection Process

Applications will be evaluated for scientific and technical merit by (an) appropriate Scientific Review Group(s) convened by the National Institute of Allergy and Infectious Diseases, in accordance with NIH peer review policy and procedures (//grants.nih.gov/grants/guide/url_redirect.htm?id=11154), using the stated review criteria. Assignment to a Scientific Review Group will be shown in the eRA Commons.

As part of the scientific peer review, all applications:

- May undergo a selection process in which only those applications deemed to have the highest scientific and technical merit (generally the top half of applications under review) will be discussed and assigned an overall impact score.
- Will receive a written critique.

Appeals (//grants.nih.gov/grants/guide/notice-files/NOT-OD-11-064.html) of initial peer review will not be accepted for applications submitted in response to this FOA.

Applications will be assigned to the appropriate NIH Institute or Center. Applications will compete for available funds with all other recommended applications submitted in response to this FOA. Following initial peer review, recommended applications will receive a second level of review by the National Advisory Allergy and Infectious Diseases Council. The following will be considered in making funding decisions:

- Scientific and technical merit of the proposed project as determined by scientific peer review.
- Availability of funds.
- Relevance of the proposed project to program priorities.

3. Anticipated Announcement and Award Dates

After the peer review of the application is completed, the PD/PI will be able to access his or her Summary Statement (written critique) via the eRA Commons (//grants.nih.gov/grants/guide/url_redirect.htm?id=11123). Refer to Part 1 for dates for peer review, advisory council review, and earliest start date.

Information regarding the disposition of applications is available in the *NIH Grants Policy Statement* (//grants.nih.gov/grants/guide/url_redirect.htm?id=11156).

Section VI. Award Administration Information

1. Award Notices

If the application is under consideration for funding, NIH will request "just-in-time" information from the applicant as described in the *NIH Grants Policy Statement* (//grants.nih.gov/grants/guide/url_redirect.htm?id=11157).

A formal notification in the form of a Notice of Award (NoA) will be provided to the applicant organization for successful applications. The NoA signed by the grants management officer is the authorizing document and will be sent via email to the grantee's business official.

Awardees must comply with any funding restrictions described in Section IV.5. Funding Restrictions. Selection of an application for award is not an authorization to begin performance. Any costs incurred before receipt of the NoA are at the recipient's risk. These costs may be reimbursed only to the extent considered allowable pre-award costs.

Any application awarded in response to this FOA will be subject to terms and conditions found on the Award Conditions and Information for NIH Grants (//grants.nih.gov/grants/guide/url_redirect.htm?id=11158) website. This includes any recent legislation and policy applicable to awards that is highlighted on this website.

2. Administrative and National Policy Requirements

All NIH grant and cooperative agreement awards include the *NIH Grants Policy Statement (//grants.nih.gov/grants/guide/url_redirect.htm?id=11120)* as part of the NoA. For these terms of award, see the *NIH Grants Policy Statement Part II: Terms and Conditions of NIH Grant Awards, Subpart A: General (//grants.nih.gov/grants/guide/url_redirect.htm?id=11157)* and Part II: Terms and Conditions of NIH Grant Awards, Subpart B: Terms and Conditions for Specific Types of Grants, Grantees, and Activities (//grants.nih.gov/grants/guide/url_redirect.htm?id=11159). More information is provided at Award Conditions and Information for NIH Grants (//grants.nih.gov/grants/guide/url_redirect.htm?id=11158).

Recipients of federal financial assistance (FFA) from HHS must administer their programs in compliance with federal civil rights law. This means that recipients of HHS funds must ensure equal access to their programs without regard to a person's race, color, national origin, disability, age and, in some circumstances, sex and religion. This includes ensuring your programs are accessible to persons with limited English proficiency. HHS recognizes that research projects are often limited in scope for many reasons that are nondiscriminatory, such as the principal investigator's scientific interest, funding limitations, recruitment requirements, and other considerations. Thus, criteria in research protocols that target or exclude certain populations are warranted where nondiscriminatory justifications establish that such criteria are appropriate with respect to the health or safety of the subjects, the scientific study design, or the purpose of the research.

For additional guidance regarding how the provisions apply to NIH grant programs, please contact the Scientific/Research Contact that is identified in Section VII under Agency Contacts of this FOA. HHS provides general guidance to recipients of FFA on meeting their legal obligation to take reasonable steps to provide meaningful access to their programs by persons with limited English proficiency. Please see https://www.hhs.gov/civil-rights/for-individuals/special-topics/limited-english-proficiency/index.html (https://www.hhs.gov/civil-rights/for-individuals/special-topics/limited-english-proficiency/index.html). The HHS Office for Civil Rights also provides guidance on complying with civil rights laws enforced by HHS. Please see https://www.hhs.gov/civil-rights/for-individuals/section-1557/index.html (https://www.hhs.gov/civil-rights/for-individuals/section-1557/index.html); and https://www.hhs.gov/civil-rights/for-providers/laws-regulations-guidance/index.html (https://www.hhs.gov/civil-rights/for-providers/laws-regulations-guidance/index.html). Recipients of FFA also have specific legal obligations for serving qualified individuals with disabilities. Please see https://www.hhs.gov/civil-rights/for-individuals/disability/index.html (https://www.hhs.gov/civil-

rights/for-individuals/disability/index.html). Please contact the HHS Office for Civil Rights for more information about obligations and prohibitions under federal civil rights laws at https://www.hhs.gov/ocr/about-us/contact-us/index.html (https://www.hhs.gov/ocr/about-us/contact-us/index.html) or call 1-800-368-1019 or TDD 1-800-537-7697. Also note it is an HHS Departmental goal to ensure access to quality, culturally competent care, including long-term services and supports, for vulnerable populations. For further guidance on providing culturally and linguistically appropriate services, recipients should review the National Standards for Culturally and Linguistically Appropriate Services in Health and Health Care at http://minorityhealth.hhs.gov/omh/browse.aspx?lvl=2&lvlid=53 (http://minorityhealth.hhs.gov/omh/browse.aspx?lvl=2&lvlid=53).

In accordance with the statutory provisions contained in Section 872 of the Duncan Hunter National Defense Authorization Act of Fiscal Year 2009 (Public Law 110-417), NIH awards will be subject to the Federal Awardee Performance and Integrity Information System (FAPIIS) requirements. FAPIIS requires Federal award making officials to review and consider information about an applicant in the designated integrity and performance system (currently FAPIIS) prior to making an award. An applicant, at its option, may review information in the designated integrity and performance systems accessible through FAPIIS and comment on any information about itself that a Federal agency previously entered and is currently in FAPIIS. The Federal awarding agency will consider any comments by the applicant, in addition to other information in FAPIIS, in making a judgement about the applicant's integrity, business ethics, and record of performance under Federal awards when completing the review of risk posed by applicants as described in 45 CFR Part 75.205 "Federal awarding agency review of risk posed by applicants." This provision will apply to all NIH grants and cooperative agreements except fellowships.

Cooperative Agreement Terms and Conditions of Award

The following special terms of award are in addition to, and not in lieu of, otherwise applicable U.S. Office of Management and Budget (OMB) administrative guidelines, U.S. Department of Health and Human Services (DHHS) grant administration regulations at 45 CFR Part 75, and other HHS, PHS, and NIH grant administration policies.

The administrative and funding instrument used for this program will be the cooperative agreement, an "assistance" mechanism (rather than an "acquisition" mechanism), in which substantial NIH programmatic involvement with the awardees is anticipated during the performance of the activities. Under the cooperative agreement, the NIH purpose is to support and stimulate the recipients' activities by involvement in and otherwise working jointly with the award recipients in a partnership role; it is not to assume direction, prime responsibility, or a dominant role in the activities. Consistent with this concept, the dominant role and prime responsibility resides with the awardees for the project as a whole, although specific tasks and activities may be shared among the awardees and the NIH as defined below.

The PD(s)/PI(s) will have the primary responsibility for:

- Serving as the administration oversight for the proposed research, including overseeing, managing, and coordinating the overall Centers/proposed research.
- Sharing data collected under this award through a NIAID approved portal(s), as well as sharing any collected biological samples with the scientific community, as appropriate and consistent with achieving the goals of the program.

- Awardees will retain custody of and have primary rights to the data and software developed under these awards, subject to Government rights of access consistent with current DHHS, PHS, and NIH policies.
- Rapidly responding to outbreaks as directed by NIAID and the EIDRC CC. Examples include but are not limited to; providing information, samples, data, reagents, etc. to another Center, the Coordination Center and/or other collaborators, in addition to planning and/or performing critical experiments to advance the development of new diagnostic tools and reagents.
- Publishing research results, presenting at professional meetings, developing quality diagnostic capability and managing pilot projects.
- Demonstrating collaborative effort within the larger EIDRC network, including the other EIDRCs, the CC and NIAID

NIH staff have substantial programmatic involvement that is above and beyond the normal stewardship role in awards, as described below:

- Coordinate NIAID staff assistance, including participation in periodic on-site monitoring with respect to compliance with Federal regulations, quality control, accuracy of data recording, sample accrual, enrollment, etc.
- Facilitate collaborations with and access to other NIAID-supported research resources and services.
- Serve as liaison/facilitator between the awardee and the EIDRC CC, related to database and biological sample storage (approved by NIAID).
- Assist in establishing an External Advisory Committee (EAC).
- The NIH Project Scientist will review and assist in developing the operating guidelines and consistent policies for dealing with situations that require coordinated action.
- The NIH Project Scientist will periodically review the data generated under this award.
- Additionally, an agency program official or IC program director will be responsible for the normal scientific and programmatic stewardship of the award and will be named in the award notice.

Areas of Joint Responsibility include:

- The PD(s)/PI(s) and NIH Project Scientist will review the entirety of program milestones annually and update them based on recommendations from the EAC.
- The NIH Project Scientist and the PD(s)/PI(s) will coordinate the overall Center objectives and progress at the annual workshop to facilitate the achievement of program goals.

Dispute Resolution:

Any disagreements that may arise in scientific or programmatic matters (within the scope of the award) between award recipients and the NIH may be brought to Dispute Resolution. A Dispute Resolution Panel composed of three members will be convened. It will have three members: a designee of the Steering Committee chosen without NIH staff voting, one NIH designee, and a third designee with expertise in the relevant area who is chosen by the other two; in the case of individual disagreement, the first member may be chosen by the individual awardee. This special dispute resolution procedure does not alter the awardee's right to appeal an adverse action that is otherwise appealable in accordance with PHS regulation 42 CFR Part 50, Subpart D and DHHS regulation 45 CFR Part 16.

3. Reporting

When multiple years are involved, awardees will be required to submit the Research Performance Progress Report (RPPR) (//grants.nih.gov/grants/rppr/index.htm) annually and financial statements as required in the NIH Grants Policy Statement. (//grants.nih.gov/grants/guide/url_redirect.htm?id=11161)

A final RPPR, invention statement, and the expenditure data portion of the Federal Financial Report are required for closeout of an award, as described in the *NIH Grants Policy Statement (//grants.nih.gov/grants/guide/url_redirect.htm?id=11161)*.

The Federal Funding Accountability and Transparency Act of 2006 (Transparency Act), includes a requirement for awardees of Federal grants to report information about first-tier subawards and executive compensation under Federal assistance awards issued in FY2011 or later. All awardees of applicable NIH grants and cooperative agreements are required to report to the Federal Subaward Reporting System (FSRS) available at www.fsrs.gov (//grants.nih.gov/grants/guide/url_redirect.htm?id=11170) on all subawards over $25,000. See the *NIH Grants Policy Statement (//grants.nih.gov/grants/guide/url_redirect.htm?id=11171)* for additional information on this reporting requirement.

In accordance with the regulatory requirements provided at 45 CFR 75.113 and Appendix XII to 45 CFR Part 75, recipients that have currently active Federal grants, cooperative agreements, and procurement contracts from all Federal awarding agencies with a cumulative total value greater than $10,000,000 for any period of time during the period of performance of a Federal award, must report and maintain the currency of information reported in the System for Award Management (SAM) about civil, criminal, and administrative proceedings in connection with the award or performance of a Federal award that reached final disposition within the most recent five-year period. The recipient must also make semiannual disclosures regarding such proceedings. Proceedings information will be made publicly available in the designated integrity and performance system (currently FAPIIS). This is a statutory requirement under section 872 of Public Law 110-417, as amended (41 U.S.C. 2313). As required by section 3010 of Public Law 111-212, all information posted in the designated integrity and performance system on or after April 15, 2011, except past performance reviews required for Federal procurement contracts, will be publicly available. Full reporting requirements and procedures are found in Appendix XII to 45 CFR Part 75 – Award Term and Conditions for Recipient Integrity and Performance Matters.

Section VII. Agency Contacts

We encourage inquiries concerning this funding opportunity and welcome the opportunity to answer questions from potential applicants.

Application Submission Contacts

eRA Service Desk (Questions regarding ASSIST, eRA Commons, application errors and warnings, documenting system problems that threaten submission by the due date, and post-submission issues)

Finding Help Online: http://grants.nih.gov/support/ (preferred method of contact)
Telephone: 301-402-7469 or 866-504-9552 (Toll Free)

General Grants Information (Questions regarding application instructions, application processes, and NIH grant resources)

Email: GrantsInfo@nih.gov (mailto:GrantsInfo@nih.gov) (preferred method of contact)
Telephone: 301-945-7573

Grants.gov Customer Support (Questions regarding Grants.gov registration and Workspace)
Contact Center Telephone: 800-518-4726
Email: support@grants.gov (mailto:support@grants.gov)

Scientific/Research Contact(s)

Jean Patterson, Ph.D.
National Institute of Allergy and Infectious Diseases (NIAID)
Telephone: 240-292-4830
Email: jean.patterson@nih.gov (mailto:jean.patterson@nih.gov)

Peer Review Contact(s)

Eleazar Cohen, Ph.D.
National Institute of Allergy and Infectious Diseases (NIAID)
Telephone: 240-669-5081
Email: ecohen@niaid.nih.gov (mailto:ecohen@niaid.nih.gov)

Financial/Grants Management Contact(s)

Bora Mpinja
National Institute of Allergy and Infectious Diseases (NIAID)
Telephone: 301-761-5434
Email address: bora.mpinja@nih.gov (mailto:bora.mpinja@nih.gov)

Section VIII. Other Information

Recently issued trans-NIH policy notices (//grants.nih.gov/grants/guide/url_redirect.htm?id=11163) may affect your application submission. A full list of policy notices published by NIH is provided in the *NIH Guide for Grants and Contracts (//grants.nih.gov/grants/guide/url_redirect.htm?id=11164)*. All awards are subject to the terms and conditions, cost principles, and other considerations described in the NIH Grants Policy Statement (//grants.nih.gov/grants/guide/url_redirect.htm?id=11120).

Authority and Regulations

Awards are made under the authorization of Sections 301 and 405 of the Public Health Service Act as amended (42 USC 241 and 284) and under Federal Regulations 42 CFR Part 52 and 45 CFR Part 75.

Weekly TOC for this Announcement (/grants/guide/WeeklyIndex.cfm?03-08-19)
NIH Funding Opportunities and Notices (/grants/guide/index.html)

National Institutes of Health (/grants/oer.htm)
Office of Extramural Research

(http://www.hhs.gov/) Department of Health and Human Services (HHS)

(http://www.usa.gov/)

NIH... Turning Discovery Into Health®

Note: For help accessing PDF, RTF, MS Word, Excel, PowerPoint, Audio or Video files, see Help Downloading Files (/grants/edocs.htm).

[Insert Letterhead here]

06 June 2019

Dr. Peter Daszak
President
EcoHealth Alliance
460 W 34th St. 17th Floor
New York, NY 10001 USA

Dear Dr. Daszak,

This letter expresses the high interest that staff at [insert name of institution] has in collaborative research with EcoHealth Alliance and other partners on the proposed NIAID Emerging Infectious Diseases Research Center (EIDRC) submission (FOA: RFA-AI-19-028), titled “Understanding risk of zoonotic virus emergence in EID hotspots of Southeast Asia”.

The [insert name of institution] recognizes the mutual benefits to be gained through research cooperation and a successful partnership in this center, including sharing of technology, samples, reagents, data and research results. The research proposed will advance our understanding of the ability of wildlife-origin coronaviruses, henipaviruses and filoviruses to spillover into high-risk human populations and cause illnesses that may have been previously unreported, or undiagnosed. We believe the results will be scientifically important, and have great public health value in the region, and globally, by identifying key pandemic threats in an EID hotspot region.

We at [insert name of institution] have collaborated successfully with all partners on this proposal, and look forward to continuing our work together. Our work will include managing [Insert sentence on key roles in project, e.g. “human surveillance, sample collection, testing, and development of laboratory diagnostics”]. We will also actively take part in technology transfer and research cross-training with other partners, and are ready, willing and able to work in surge capacity to conduct research during outbreak situations, in collaboration with all other EIDRCs and the EIDCC.

Sincerely,

[Insert signature]

[Insert full name, title, institution, address, and email]

Equipment

EcoHealth Alliance, New York, USA (Drs. Daszak and Olival) EA1

EcoHealth Alliance is equipped with fiber optic Internet access and video conferencing facilities to facilitate easy communication between collaborators. EcoHealth Alliance employees have around-the-clock access to servers, VPNs, encryption software, IT support, and all necessary software including Git and Github (Hosted software revision/audit service), Sublime and Vim text editors, Vagrant and Oracle Virtualbox virtual machines, Google Apps (Hosted email and collaboration web based software), Ansible (Server provisioning software framework), Python, NodeJS, and R programming languages, Meteor (Javascript framework), Bash shell scripts, Jenkins (Continuous Integration server), Microsoft Office and Adobe CS6 running on both Apple Mac OS X, Ubuntu linux, and Windows Operating Systems. EcoHealth Alliance has a dedicated quad-core Linux server and another dedicated dual quad-core Mac Pro Server - each with 4TB hard drives. Either server individually or in combination may be used for intensive computational modeling and/or database processing by all the grantees. Access to the cloud and supercomputing services (Amazon) is provided by core funding to EcoHealth Alliance. EA2

Page 1 Comments

EA1 Here, list (1) name of institution, (2) location of institution, and (3) name(s) of Key Personnel from your institution contributing to this proposal

EcoHealth Alliance, 6/4/2019 09:40 AM

EA2 List major items of equipment already available for this project and, if appropriate, identify the equipment's location and pertinent capabilities

EcoHealth Alliance, 6/4/2019 09:40 AM

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Daszak, Peter

eRA COMMONS USER NAME (credential, e.g., agency login): daszak

POSITION TITLE: President & Chief Scientist

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Bangor University, UK	B.S. (hons)	07/1986	Zoology
University of East London, UK	Ph.D.	03/1993	Infectious Diseases

A. Personal Statement

I have 20+ years NIH-funded research experience on emerging viral zoonoses, and the scientific management experience to support this proposed work that involves international interdisciplinary teams working on field surveillance in wild mammals, human behavioral risk surveys and clinical sampling, development of novel diagnostic approaches, and viral characterization *in vitro* and *in vivo*. I am President and Chief Scientist of EcoHealth Alliance, a US-based 501(c)(3) institution that conducts research on emerging zoonoses. My research background is focused on understanding the process of zoonotic disease emergence, particularly viral zoonoses. This includes identifying the bat origin of SARS-CoV and SADS-CoV, analyzing the causes of West Nile, Nipah and Hendra virus emergence and spread, publishing the first unbiased analysis of global emerging disease hotspots, and analyzing patterns of viral spillover into human populations in EID hotspots. I have been the PI on 5 multidisciplinary R01s that use modeling, epidemiology, laboratory and field science to test hypotheses on the emergence of wildlife-origin viral zoonoses, including SARS-CoV, Nipah and Hendra virus, Avian influenza and novel viruses from bats. I have also led large contracts from USAID (institutional lead on $75 million PREDICT-1 and $138 million PREDICT-2) involving successful management of teams of virologists, field biologists, mathematical modelers, veterinarians, epidemiologists, laboratorians and anthropologists. Much of the groundwork for the current proposed work has developed from my previous collaborative research with each member of our proposed EIDRC consortium.

1. Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, Wang H, Crameri G, Hu Z, Zhang H, Zhang J, McEachern J, Field H, **Daszak P**, Eaton BT, Zhang S & Wang L-F (2005). Bats are natural reservoirs of SARS-like coronaviruses. **Science** 310: 676-679.
2. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, and **Daszak P*** (2008). Global trends in emerging infectious diseases. **Nature** 451:990-993
3. Olival KJ*, Hosseini PR, Zambrana-Torrelio C, Ross N, Bogich TL, **Daszak P*** (2017). Host and viral traits predict zoonotic spillover from mammals. **Nature** 546, 646–650.
4. Carroll D, **Daszak P***, Wolfe ND, Gao GF, Morel C, Morzaria S, Pablos-Méndez A, Tomori O, Mazet JAK (2018). The global virome project. **Science** 359: 872-874.

B. Positions and Honors

Positions and Employment

1993 -98 Senior Faculty Research Scientist, Kingston University UK
1998 Guest Researcher, Centers for Disease Control and Prevention (CDC)
1999 -01 Faculty Research Scientist, University of Georgia
2001 - Sr. Adjunct Faculty, Columbia University
2001 -09 Executive Director, Consortium for Conservation Medicine, EcoHealth Alliance, New York
2009 - President & Chief Scientist, EcoHealth Alliance New York

Other Experience and Professional Membership

2003 -07 NIH: ad hoc member, ZRG1 IDM-G 90 (2003-5) ZRG1 IRAP-Q (2005-7)
2004 - Editorial Board, *Conserv. Biol.*
2005 NIAID: Steering Committee, workshop on virus-host shifts & emergence of new pathogens
2010 - Editor-in-Chief, *EcoHealth*; Member of IOM Forum on Microbial Threats; External Advisory Board, DHS and Kansas State Univ. Ctr. of Excellence for Emerg. & Zoonotic Animal Diseases (CEEZAD)
2011 Steering Committeee, NIAID Workshop on Arboviruses
2014 - Member NRC Advisory Committee to advise the US Global Change Research Program (USGCRP)
2015 - Member of Supervisory Board, One Health Platform; Editorial Board *One Health*
2016 - Member, WHO Expert group on Public Health Emergency Disease Prioritization
2016 - Member, Core Steering Committee & Co-Chair, Science & Technol WG, Global Virome Project
2017 External Review Committee, CSIRO Health & Biosecurity Business Unit
2017 - Chair, Forum on Microbial Threats, National Academies of Science, Engineering & Medicine

Honors

1999 Meritorious service award, CDC
2000 CSIRO silver medal for collaborative research
2002 Honored by the naming of a new species of centipede, *Cryptops daszaki* (*J Nat Hist* 36: 76-106)
2003 6th Annual Lecturer, Medicine & Humanities, Texas A&M
2007 Finalist, Director's Pioneer Award
2008 Presidential Lecturer, University of Montana
2012 Elected member of the Cosmos Club, Washington DC
2013 Honored by the naming of a new parasite species, *Isospora daszaki* (*Parasit. Res.* 111:1463-1466)
2013 Hsu-Li Distinguished Lectureship in International Epidemiology, Univ. Iowa
2015 Robert Leader Endowed Lecture in Food Safety, Michigan State Univ.
2018 - Member, National Academy of Medicine (NAM), USA.

C. Contribution to Science

1. Research on the bat origins of emerging viruses. Numerous high impact emerging viruses appear to have bat reservoirs (e.g. SARS-CoV, EBOV, NiV, HeV, MERS-CoV, SADS-CoV). As PI on four prior R01s, my work has helped demonstrate their bat origin (SARS-CoV, SADS-CoV), analyze the drivers of their emergence and risk factors for spillover. Collaborating with virologists in China, we have isolated and characterized SARS-like CoVs from bats that use the same human host cell receptor (ACE-2) as SARS-CoV. This work provides critical reagents and resources that have helped advance understanding of virus-host binding and may contribute to vaccine development. My other work identified factors leading to the emergence of NiV from *Pteropus* bats in Malaysia and Bangladesh; a likely bat origin for MERS-CoV; and proof that bats harbor a significantly higher proportion of zoonoses than all other mammalian groups.

a. Ge X-Y, Li J-L, Yang X-L, Chmura AA, Zhu G, Epstein JH, Mazet JK, Hu B, Zhang W, Peng C, Zhang Y-J, Luo C-M, Tan B, Wang N, Zhu Y, Crameri G, Zhang S-Y, Wang L-F, **Daszak P***, Shi Z-L* (2013).

Isolation and characterization of a bat SARS-like Coronavirus that uses the ACE2 receptor. **Nature** 503: 535-538.

b. Memish ZA, Mishra N, Olival KJ, Fagbo SF, Kapoor V, Epstein JH, Al Hakeem R, Durosinloun A, Al Asmari M, Islam A, Kapoor A, Briese T, **Daszak P**, Al Rabeeah A, Lipkin WI. (2013). Middle East respiratory syndrome coronavirus in bats, Saudi Arabia. **EID** 19(11): 1819-1823.

c. Zhou P, Fan H, Lan T, Yang X-L, Shi W-F, Zhang W, Zhu Y, Zhang Y-W, Xie Q-M, Mani S, Zheng X-S, Li B, Li J-M, Guo H, Pei G-Q, An X-P, Chen J-W, Zhou L, Mai K-J, Wu Z-X, Li D, Anderson DE, Zhang L-B, Li S-Y, Mi Z-Q, He T-T, Cong F, Fuo P-J, Huang R, Luo Y, Liu X-L, Chen J, Huang Y, Sun Q, Zhang X-L-L, Wang Y-Y, Xing S-Z, Chen Y-S, Sun Y, Li J, **Daszak P***, Wang L-F*, Shi Z-L*, Tong Y-G*, Ma J-Y* (2018). Fatal Swine Acute Diarrhea Syndrome caused by an HKU2-related Coronavirus of Bat Origin. **Nature** 556: 255-258.

d. Nikolay B, Salje H, Hossain MJ, Khan AKMD, Sazzad HMS, Rahman M, **Daszak P**, Ströher U, Pulliam JRC, Kilpatrick AM, Nichol ST, Klena JD, Sultana S, Afroj S, Luby SP, Cauchemez S & Gurley ES. (2019). Transmission of Nipah Virus - 14 Years of Investigations in Bangladesh. **NEJM** 380:1804-1814

2. **Analyzing the process of disease emergence.** Emerging infectious diseases are a significant threat to global health. However, their emergence is sporadic, complex, and seemingly unpredictable. In the early 2000s I started to use analytical approaches to identify predictable patterns in the process of disease emergence. By collating a database of all known prior EID events, identifying their point origins, and correcting for reporting biases, I published the first ever predictive 'hotspots' maps of where future disease emergence is most likely. I have continued this line of research, publishing spatial analyses of the drivers of disease spread, and outlining strategies to predict pandemic emergence.

   a. Kilpatrick AM, Chmura AA, Gibbons DW, Fleischer RC, Marra PP & **Daszak P** (2006). Predicting the global spread of H5N1 avian influenza. **PNAS** 103: 19368-19373.

   b. Morse SS, Mazet JAK, Woolhouse M, Parrish CR, Carroll D, Karesh WB, Zambrana-Torrelio C, Lipkin WI, **Daszak P*** (2012). Prediction and prevention of the next pandemic zoonosis. **Lancet** 380:1956-1965.

   c. **Daszak P***, Zambrana-Torellio C, Bogich TL, Fernandez M, Epstein JH, Murray KA, Hamilton H (2013). Interdisciplinary approaches to understanding disease emergence: The past, present and future drivers of Nipah virus emergence. **PNAS** 110: 3681-3688

   d. Allen T, Murray KA, Zambrana-Torrelio C, Morse SS, Rondinini C, Di Marco M, Breit N, Olival KJ, **Daszak P*** (2017). Global hotspots and correlates of emerging zoonotic diseases. **Nature Comm** 8: 1124

3. **Studies of wildlife disease ecology to understand emerging zoonoses.** The majority of EIDs are zoonotic, with the majority of these originating in wildlife. I reviewed this field in a paper in *Science* in 2000 and in a more recent paper in *Nature* on the links among biodiversity and health. During the last two decades, I have led collaborative research programs on how the ecology of specific wildlife-origin zoonoses can help explain patterns of risk to people. This includes my role as PI on 4 R01s, as institutional lead for USAID-EPT-PREDICT, and Chief of Party for USAID-IDEEAL. This work includes estimations of the diversity of yet-to-be discovered viruses which forms the rationale for the Global Virome Project.

   a. **Daszak P***, Cunningham AA, Hyatt AD (2000). Emerging infectious diseases of wildlife - threats to biodiversity and human health. **Science** 287: 443-449

   b. Keesing F, Belden LK, **Daszak P**, Dobson A, Harvell CD, Holt RD, Hudson P, Jolles A, Jones KE, Mitchell CE, Myers SS, Bogich T & Ostfeld RS. (2010). Impacts of biodiversity on the emergence and transmission of infectious diseases. **Nature** 468:647-652.

c. Anthony SJ, Epstein JH, Murray KA, Navarrete-Macias I, Zambrana-Torrelio CM, Solovyov A, Ojeda-Flores R, Arrigo NC, Islam A, Ali Khan S, Hosseini P, Bogich TL, Olival KJ, Sanchez-Leon MD, Karesh W, Goldstein T, Luby SP, Morse SS, Mazet JAK, **Daszak P***, Lipkin WI. (2013). A strategy to estimate unknown viral diversity in mammals. **MBio** 4(5): e00598-13.

d. Mandl JN, Ahmed R, Barreiro LB, **Daszak P**, Epstein JH, Virgin HW, Feinberg MB. (2015). Reservoir host immune responses to emerging zoonotic viruses. **Cell** 160: 20-35

D. Additional Information: Research Support and/or Scholastic Performance

Ongoing Research Support

USAID Emerging Pandemic Threats — Mazet (PI) — 10/01/14 – 09/30/19
PREDICT-2
The goal is to conduct surveillance for novel pathogens in wildlife, livestock and people; characterize human risk behavior; analyze EID risk; and design interventions in >20 countries
Role: PI on Subcontract

2R01 AI110964 — Daszak (PI) — 06/01/19 – 05/31/24
Understanding the Risk of Bat Coronavirus Emergence (Renewal)
The goal of this work is to characterize the virological, behavioral and demographic factors that present a high risk of future emergence of SARSr-CoVs in people in southern China, and identify clinical outcomes.
Role: PI

Completed Research Support (last 3 years only) out of 14 prior awards

1R01 AI110964 — Daszak (PI) — 06/01/14 – 05/31/19
Understanding the Risk of Bat Coronavirus Emergence
The goal was to conduct ecological and virological studies on bat-origin SARSr-CoVs in China, behavioral risk surveys and testing in people, to identify risk of future spillover of these viruses.
Role: PI

USAID 1414374 (RDMA, Thailand) — Daszak (CoP) — 10/01/13 - 03/30/19
Infectious Disease Emergence and Economics of Altered Landscapes (IDEEAL)
Cooperative agreement to analyze how land use change affects economics of disease risk in SE Asia.
Role: Chief of Party

NSF DEB 1414374 — Perrings (PI) — 10/15/14 - 04/14/18
US-UK Collab: Risks of Animal and Plant Infectious Diseases through Trade (RAPID Trade)
The goal is to analyze and model how policy changes to trade affect emerging disease risk globally.
Role: Co-Investigator

HDTRA1 — Allen (PI) — 04/15/15 - 04/14/17
Global Rapid Identification of undiagnosed EID Events
The goal was to design software for the DoD biosurveillance ecosystem to diagnose novel EID events.
Role: Co-Investigator

1R01GM100471 (NIGMS) — Perrings (PI) — 09/15/11-06/30/15
MASpread: Modeling Anthropogenic Effects in the Spread of Infectious Disease
The goal was to analyze the social decisions involved in disease spread through trade.
Role: Co-Investigator

NSF — Daszak (PI) — 07/01/10-06/30/15
EcoHealthNet - a Research Coordination Network
Funding for student exchange and workshops to fuse veterinary science, ecology and human medical sciences
Role: PI

Commons ID	Senior/Key Personnel (Y/N)	Name	Degree(s)	Role	Person Months		
					Cal	Aca.	Sum.

Received: 25 March 2019 | Revised: 26 May 2019 | Accepted: 11 June 2019

DOI: 10.1111/tbed.13270

Check for updates

SHORT COMMUNICATION

Transboundary and Emerging Diseases WILEY

The re-emerging of SADS-CoV infection in pig herds in Southern China

Ling Zhou[1,2] | Qian N. Li[1,2] | Jiang N. Su[1] | Gui H. Chen[1] | Zi X. Wu[1] | Ying Luo[1] | Rui T. Wu[1,2] | Yuan Sun[1,2] | Tian Lan[1] | Jing Y. Ma[1,2]

[1]College of Animal Science, South China Agricultural University, Guangzhou, China

[2]Key Laboratory of Animal Health Aquaculture and Environmental Control, Guangzhou, China

Correspondence
Jing Y. Ma, College of Animal Science, South China Agricultural University, Tianhe District, Wushan Road 483, Guangzhou 510642, China.
Email: majy2400@scau.edu.cn

Funding information
National Key Research and Development Program of China, Grant/Award Number: 2016YFD0501304; Science and Technology Program of Guangzhou City of China, Grant/Award Number: 201904010433

Abstract

A new highly virulent swine acute diarrhoea syndrome coronavirus (SADS-CoV) emerged in Guangdong province in 2017 followed by fatal diarrhoea that involved the death of 24,693 piglets. And yet from May 2017 to January 2019, there were no new SADS cases arising in pig herds in Guangdong. In this study, we reported the recent diarrhoea outbreak of SADS-CoV in Southern China on February 2019. Intestinal samples collected from diarrhoeal piglets were detected for common swine virus and confirmed that SADS-CoV was responsible for the diarrhoea case. Meanwhile, serological investigation of sows' sera implied that SADS-CoV has existed in the farm and PEDV antibody may not directly contribute to the amplification of SADS-CoV. Homology and phylogenetic analysis of the whole genome showed that the re-emerging SADS-CoV strain shared high sequence identities with existing SADS-CoV strains and all strains clustered together in Alpha coronavirus. All in all, the report herein emphasized the re-emerging of SADS-CoV and highlights continuous monitoring for this virus.

KEYWORDS
phylogenetic analysis, re-emerging, SADS-CoV, Southern China

1 | INTRODUCTION

Swine acute diarrhoea syndrome (SADS) was first identified in South China in January 2017 as a disease causing high mortality in neonatal piglets (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018). It is quickly demonstrated that the causative agent of SADS is a novel HKU2-related coronavirus, swine acute diarrhoea syndrome coronavirus (SADS-CoV) which originated from the same genus of horseshoe bats (*Rhinolophus*) as severe acute respiratory syndrome coronavirus (SARS-CoV), but the mechanism of transmission of bat-originated coronavirus in pigs needs further illumination (Cui, Li, & Shi, 2019; Zhou et al., 2018). Based on the analysis of genomes, SADS-CoV is classified into the genus *Alphacoronavirus* within the subfamily *Orthocoronavirinae* of the family *Coronaviridae* (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018). So far, besides porcine epidemic diarrhoea virus (PEDV), transmissible gastroenteritis virus (TGEV) and porcine deltacoronavirus (PDCoV), SADS-CoV is the fourth porcine enteric coronavirus that circulated in pigs in China (Dong et al., 2015; Xuan et al., 1984). A retrospective investigation showed that SADS-CoV emerged in China at least since August 2016 (Zhou et al., 2019). Li et al. (2018) reported one SADS-CoV strain that was isolated from fecal and small intestinal samples collected from Fujian province and suggested that SADS-CoV might have spread from Guangdong to Fujian province.

In the first outbreak of SADS-CoV occurring between January and May 2017, in order to reduce mortality in suckling pigs, the minced intestines from acutely infected piglets were filtered, centrifuged and inactivated before feeding back to pregnant sows to allow maternal antibodies to be obtained in neonatal piglets. Hence, after May 2017, the epidemic was controlled. And with a period of quiescence, SADS-CoV seemed to have disappeared from pig herds in South China. While on February 2019, there was a large-scale

 © 2019 Blackwell Verlag GmbH

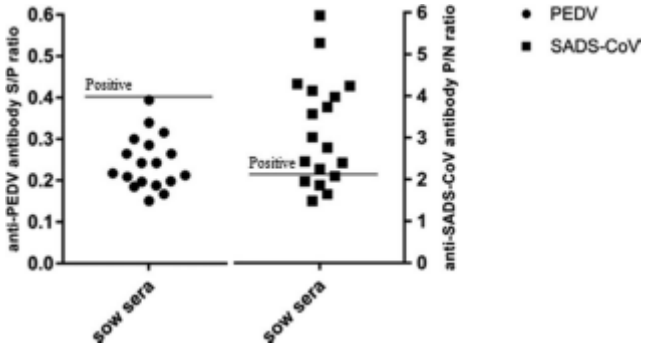


FIGURE 1 The results of ELISA test, *n* = 18 sows sera from outbreak farm. Detection of PEDV antibodies, sample S/P ratios ≥ 0.4 were considered positive. Detection of SADS-CoV antibodies, P/N ratios ≥ 2.1 were considered positive

outbreak of approximately 2000 piglets dying of diarrhoea in one farm, which was near the origin of the SADS pandemic in 2017. The extensive aetiological investigation for common swine viruses suggested that the causative agent of this recent diarrhoea outbreak was SADS-CoV. In this study, we compared the infections of piglets with the original and re-emerging SADS-CoV strains by clinical signs, epidemiological investigation, serological investigation and phylogenetic analysis. The results highlight the significance of SADS-CoV and the disease it causes.

At the beginning of the latest outbreak on February 2019, infected suckling piglets developed acute diarrhoea, vomiting and died after 2–6 days of the diarrhoea, which were consistent with the clinical signs reported in SAD-CoV outbreaking farms (Zhou et al., 2018). Two weeks after the onset, mortality did not increase due to the effective inactivated SADS-CoV vaccine which was propagated in vitro (data unpublished). Clinical small intestine samples were collected rapidly from piglets, and intestinal samples were homogenized, centrifuged and filtered for virus isolate. Viral nucleic acid was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instruction. The potential swine viral pathogens were examined, including SADS-CoV, PEDV, PDCoV, porcine rotavirus (RV), TGEV, reovirus (REO), senecavirus A (SVA), foot and mouth disease virus (FMDV), porcine pseudorabies virus (PRV), porcine circovirus 2 (PCV2), porcine circovirus 3 (PCV3), porcine reproductive

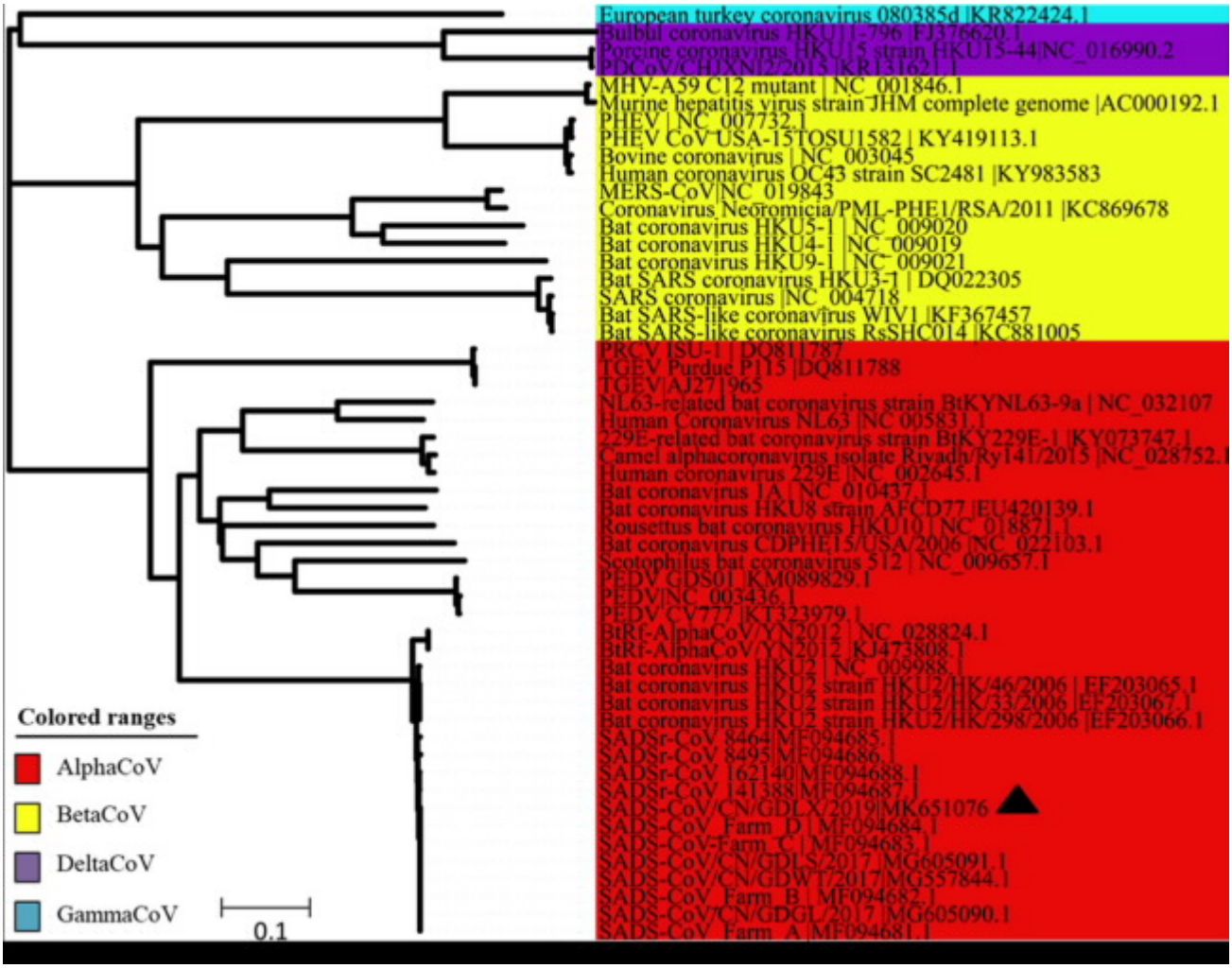


FIGURE 2 Phylogenetic analysis of the complete genome of SADS-CoV and reference coronavirus species. The tree was constructed using MEGA 7.0 software with neighbour-joining methods and 1,000 replicate sets on bootstrap analysis [Colour figure can be viewed at wileyonlinelibrary.com]

and respiratory syndrome virus (PRRSV), porcine parainfluenza virus 1 (PPIV1), swine influenza virus (SIV), atypical porcine pestivirus (APPV), haemophilus parasuis (HPS), hog cholera (HC), porcine parvovirus (PPV), porcine bocavirus (PBoV), porcine sapelovirus (PSV) and porcine kobuvirus (PKV) by PCR or RT-PCR following the established protocol of Key Laboratory of Animal Health Aquaculture and Environmental Control. The extensive aetiological investigations for common swine viruses showed that only SADS-CoV was detected positively, which suggested that the causative agent of the most recent diarrhoea outbreak on February was SADS-CoV.

Furthermore, a total of 18 blood samples were simultaneously collected from healthy sows for serological investigation. Sera samples were obtained after coagulation and centrifugation (2,000 *g*, 10 min) and stored at −20°C until ELISA implemented. The PEDV antibody production was detected by means of commercial ELISA test kit (ZhenRui, China), of which, sample S/P ratios with ≥0.4 were considered positive and <0.4 were considered negative. And for SADS-CoV, ELISA was conducted following the modified procedures established in Key Laboratory of Animal Health Aquaculture and Environmental Control (P/N ≥ 2.1 considered positive). The absorbance at 450 nm wavelength was measured (Multiskan™ FC, Thermo Fisher). The results of ELISA assay revealed that all sera (100%) were negative for the PEDV and 13 of 18 (72.2%) were positive for SADS-CoV (Figure 1). In our previously clinical investigations, PEDV had caused prior outbreaks at most farms with SADS-CoV outbreaks (Zhou et al., 2018). It is possible that the presence of PEDV antibody may have somehow facilitated the amplification of the SADS-CoV. However, our results showed that PEDV antibodies were undetectable in all SADS-CoV antibody-positive sera samples, suggesting that it is SADS-CoV that caused the lethal infection in pigs on February 2019. Collectively, the re-emergence of SADS-CoV in pig herds in South China was confirmed by ELISA test and the association between PEDV and SADS-CoV infection requires further validation.

Subsequently, one SADS-CoV genome was amplified as previously described (Zhou et al., 2019). The novel SADS-CoV strain was named as SADS-CoV/CN/GDLX/2019 (accession number: MK651076). Sequence alignment and phylogenetic analysis were performed with Lasergene coresuite 10 (DNASTAR Inc.). Phylogenetic tree was constructed using the neighbour-joining method with MEGA 7.0 software (Kumar, Stecher, & Tamura, 2016). Bootstrap analysis was carried out on 1,000 replicate data sets

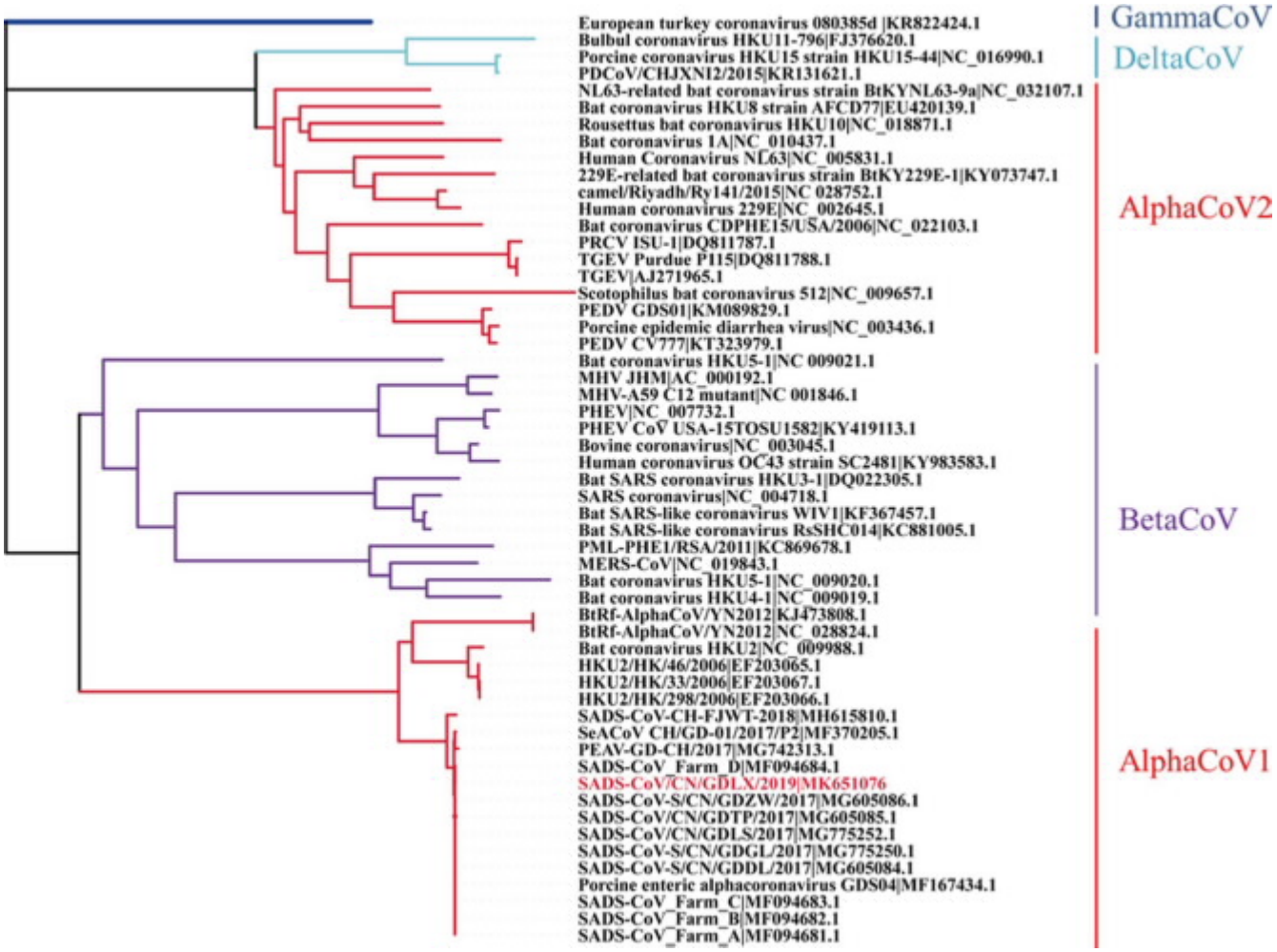


FIGURE 3 Phylogenetic analysis of the S gene of SADS-CoV and reference coronavirus species. The tree was constructed using MEGA 7.0 software with neighbour-joining methods and 1,000 replicate sets on bootstrap analysis [Colour figure can be viewed at wileyonlinelibrary.com]

(Felsenstein, 1985). For the whole genomes, sequence alignment results revealed that the nucleotide identities between SADS-CoV/CN/GDLX/2019 and previously reported SADS-CoV strains ranged from 99.3% to 100%. Moreover, the phylogenetic tree indicated that the novel SADS-CoV complete genome clustered together with other reported SADS-CoV strains and located in the Alpha coronavirus branch (Figure 2). Phylogenetic analysis based on S genes was also conducted, and the result showed that all SADS-CoV, bat HKU2 and BtRf-AlphaCoV strains were separated from other AlphaCoVs and clustered within BetaCoVs (Figure 3), which is consistent with previous studies (Pan et al., 2017; Xu et al., 2019; Zhou et al., 2019). Notably, the S gene of SADS-CoV/CN/GDLX/2019 had high identities of 99.2%–99.9% with other SADS-CoV strains detected in Guangdong and lowest identities of 97.5% with SADS-CoV-CH-FJWT-2018 reported from Fujian. Additionally, the comparative analysis of amino acids encoded by S gene revealed that the S protein of the novel strain had a two-amino-acid mutation (F306 and I882) compared with that of other SADS-CoV strains. It is well known that the S protein of coronavirus play an important role in cellular entry and determining the immunogenicity (Li, Frank, He, Peter, & Berend-Jan, 2016). In view of the lowest identities of Spike gene between the strains from Guangdong and Fujian, continuous monitoring of SADS-CoV genetic evolution is important for better understanding its epidemiology and establishing efficient strategies against its further circulating in pigs in China.

Here, we reported a new case of acute diarrhoea outbreak from a swine farm in Guangdong province on February 2019. Pathogenic and serologic analysis determined that SADS-CoV re-emerged in Guangdong and was responsible for the recent acute diarrhoea outbreak. Phylogenetic analysis based on whole genomes showed that SADS-CoV/CN/GDLX/2019 shared high nucleotide identities with previously reported SADS-CoV strains. Taken together, our study highlighted the significant threat of SADS-CoV and the potential outbreak of SADS epidemic in following months in Guangdong. Therefore, the persistent surveillance and vaccination countermeasures will be necessary to control the spread of SADS-CoV.

ACKNOWLEDGEMENTS

This work was supported by the National Key Research and Development Program of China (No. 2016YFD0501304). The Research and Extension of Major Animal Epidemic Prevention and Control Technologies in the Strategic Project of Rural Revitalization of Guangdong Agricultural Department of China (Building Modern Agricultural System) (2018–2020) and the Science and Technology Program of Guangzhou City of China (No. 201904010433).

CONFLICT OF INTEREST

The authors declare no conflict of interests with any organization.

REFERENCES

Cui, J., Li, F., & Shi, Z. (2019). Origin and evolution of pathogenic coronavirus. *Nat Rew Microbiol*, *17*, 181–192.

Dong, N., Fang, L., Zeng, S., Sun, Q., Chen, H., & Xiao, S. (2015). Porcine Deltacoronavirus in mainland China. *Emerging Infectious Diseases*, *21*, 2254–2255. https://doi.org/10.3201/eid2112.150283

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, *39*, 783–791.

Gong, L., Li, J., Zhou, Q., Xu, Z., Chen, L., Zhang, Y., … Cao, Y. (2017). A new bat-HKU–like coronavirus in swine, China, 2017. *Emerging Infectious Diseases*, *23*, 1607–1609.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* , *33*, 1870–1874.

Li, K., Li, H., Bi, Z., Gu, J., Gong, W., Luo, S., … Tang, Y. (2018). Complete genome sequence of a novel swine acute diarrhea syndrome coronavirus, CH/FJWT/2018, isolated in fujian, china, in 2018. *Microbiol Resour Announc*, *7*(22), e01259–18.

Li, W., Frank, J., He, Q., Peter, J., & Berend-Jan, B. (2016). Cellular entry of the porcine epidemic diarrhea virus. *Virus Research*, *226*, 117–127. https://doi.org/10.1016/j.virusres.2016.05.031

Pan, Y., Tian, X., Qin, P., Wang, B., Zhao, P., Yang, Y.-L., … Huang, Y.-W. (2017). Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. *Veterinary Microbiology*, *211*, 15–21. https://doi.org/10.1016/j.vetmic.2017.09.020

Xu, Z., Zhang, Y., Gong, L., Huang, L., Lin, Y., Qin, J., … Cao, Y. (2019). Isolation and characterization of a highly pathogenic strain of Porcine enteric alphacoronavirus causing watery diarrhoea and high mortality in newborn piglets. *Transboundary and Emerging Diseases*, *66*, 119–130.

Xuan, H., Xing, D., Wang, D., Zhu, W., Zhao, F., Gong, H., & Fei, S. (1984). Study on the culture of porcine epidemic diarrhea virus adapted to fetal porcine intestine primary cell monolayer. *Chin. J. Vet. Sci.*, *4*, 202–208.

Zhou, L., Sun, Y., Lan, T., Wu, R., Chen, J., Wu, Z., … Ma, J. (2019). Retrospective detection and phylogenetic analysis of swine acute diarrhoea syndrome coronavirus in pigs in southern China. *Transboundary and Emerging Diseases*, *66*, 687–695. https://doi.org/10.1111/tbed.13008

Zhou, P., Fan, H., Lan, T., Yang, X.-L., Shi, W.-F., Zhang, W., … Ma, J.-Y. (2018). Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. *Nature*, *556*, 255–258. https://doi.org/10.1038/s41586-018-0010-9

How to cite this article: Zhou L, Li QN, Su JN, et al. The re-emerging of SADS-CoV infection in pig herds in Southern China. *Transbound Emerg Dis*. 2019;66:2180–2183. https://doi.org/10.1111/tbed.13270

JVI Accepted Manuscript Posted Online 25 September 2019
J. Virol. doi:10.1128/JVI.01448-19
Copyright © 2019 American Society for Microbiology. All Rights Reserved.

Accepted Manuscript Posted Online
Journal of Virology
JVI
Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Broad cross-species infection of cultured cells by the bat HKU2-related swine acute diarrhea syndrome coronavirus (SADS-CoV) and identification of its replication in murine dendritic cells *in vivo* highlight its potential for diverse interspecies transmission

Yong-Le Yang,[a]* Pan Qin,[a]* Bin Wang,[a] Yan Liu,[a] Guo-Han Xu,[a] Lei Peng,[a] Jiyong Zhou,[a] Shu Jeffrey Zhu,[a]# Yao-Wei Huang[a]#

[a]Key Laboratory of Animal Virology of Ministry of Agriculture and Institute of Preventive Veterinary Medicine, Department of Veterinary Medicine, Zhejiang University, Hangzhou 310058, Zhejiang, China.

*These authors contributed equally to this work.

#Address correspondence to

Dr. Yao-Wei Huang, Zhejiang University, yhuang@zju.edu.cn

Dr. Shu Jeffrey Zhu, Zhejiang Univeristy, shuzhu@zju.edu.cn

Running title: Broad species tropism and a mouse model of SADS-CoV

Word counts: manuscript text (5,908 words); abstract (250 words); importance (139 words).

Figure number: 6; **Table number**: 1

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

ABSTRACT

Outbreaks of severe diarrhea in neonatal piglets in Guangdong, China in 2017 resulted in isolation and discovery of a novel swine enteric alphacoronavirus (SeACoV) derived from the species *Rhinolophus bat coronavirus HKU2* (Vet Microbiol, 2017, 211:15-21). SeACoV was later referred to as swine acute diarrhea syndrome (SADS) CoV by another group (Nature, 2018, 556:255-258). The present study was set up to investigate potential species barriers of SADS-CoV *in vitro* and *in vivo*. We first demonstrated that SADS-CoV possesses a broad species tropism and is able to infect cell lines from diverse species including bats, mice, rats, gerbils, hamsters, pigs, chickens, nonhuman primates and humans. Trypsin contributes to, but is not essential for SADS-CoV propagation *in vitro*. Furthermore, C57BL/6J mice were inoculated with the virus via oral or intraperitoneal routes. Although the mice exhibited only subclinical infection, they supported viral replication and prolonged infection in the spleen. SADS-CoV nonstructural proteins and double-stranded RNA were detected in splenocytes of the marginal zone on the edge of lymphatic follicles, indicating active replication of SADS-CoV in the mouse model. We identified that splenic dendritic cells (DCs) are the major targets of virus infection by immunofluorescence and flow cytometry approaches. Finally, we demonstrated that SADS-CoV does not utilize known CoV receptors for cellular entry. The ability of SADS-CoV to replicate in various cells lines from a broad range of species and the unexpected tropism for murine DCs provide important insights into the biology of this bat-origin CoV, highlighting its possible ability to cross interspecies barriers.

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

43 **IMPORTANCE**

44 Infections with bat-origin CoVs (SARS-CoV and MERS-CoV) have caused severe illness
45 in humans after "host jump" events. Recently, a novel bat-HKU2-like CoV named swine acute
46 diarrhea syndrome CoV (SADS-CoV) has emerged in southern China, causing a lethal diarrhea
47 in newborn piglets. It is important to assess the species barriers of SADS-CoV infection since the
48 animal hosts (other than pigs and bats) and zoonotic potential are still unknown. An *in vitro*
49 susceptibility study revealed a broad species tropism of SADS-CoV, including various rodent
50 and human cell lines. We established a mouse model of SADS-CoV infection, identifying its
51 active replication in splenic dendritic cells, which suggests that SADS-CoV has the potential to
52 infect rodents. These findings highlight the potential cross-species transmissibility of SADS-CoV,
53 though further surveillance in other animal populations is needed to fully understand the ecology
54 of this bat-HKU2-origin CoV.

55

56 **Keywords:** Interspecies transmission; Coronavirus; SADS-CoV; Mouse infection model

57

58

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

INTRODUCTION

The spread of zoonotic pathogens remains among the leading threats to global public health . Coronaviruses (CoVs) can infect a wide variety of animals and humans, resulting in several diseases with respiratory, enteric, and neurological pathologies of varying severity (1-4). Because of the various routes of infection and extensive phagocytosis in tissues, close contact between humans and animals provides potential scenarios for adaptive mutation and interspecies transmission (5).

The source of the severe acute respiratory syndrome (SARS)-CoV was traced to civets in animal markets and ultimately to bats, leading to more than 8,000 human infections and 774 deaths after its emergence in 2002 (5-7). The emergence of Middle East respiratory syndrome (MERS)-CoV in 2012 (2) resulted in more than 1,000 clinical cases with a mortality rate of 35%, making it the second marked threatening CoV of the 21st century (8, 9). Although camels can be infected with MERS-CoV, bats are also thought to be the original host of MERS-CoV (5). Both SARS-CoV and MERS-CoV originated in bats, illustrating the damage caused by CoVs during interspecies transmission events, and highlighting the need for increased global vigilance of CoV-associated disease (1, 5, 10).

In February 2017, outbreaks of severe diarrhea of suckling piglets occurred in swine herds in Guangdong Province, China (11). Clinical signs consisted of acute vomiting and watery diarrhea, but porcine viruses commonly associated with diarrhea including porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV) and porcine deltacoronavirus (PDCoV) were not detected in any of the clinical samples (11). The new enteric pathogen of

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

commercial pigs that was isolated was finally identified as a new porcine CoV belonging to the species *Rhinolophus bat coronavirus HKU2* (11-13). Our research group tentatively designated this newly emerged virus as swine enteric alphacoronavirus (SeACoV) (11), and it was later named swine acute diarrhea syndrome (SADS)-CoV by Zhou *et al.* (14). It is also known by other names, such as porcine enteric alphacoronavirus (PEAV) (13). For purposes of unity, SADS-CoV is the name used to refer to this new virus in the current study. The expanded host range of bat-origin HKU2 to pigs indicates that bats play an important role in the ecology and evolution of SADS-CoV, though the mechanism of bat-to-swine transmission remains unclear. In view of the damage caused by SARS and MERS for both animal and public health, careful attention must be paid to the prevalence of CoV-associated disease among humans and domestic animals (15).

Therefore, there is an urgent need for more information on the details of SADS-CoV infection. It is critically important to assess potential species barriers of SADS-CoV transmission since the animal hosts (other than pigs and bats) and zoonotic potential are still unknown. In the present study, we demonstrated that SADS-CoV possesses a very broad species tropism *in vitro* and is able to infect cell lines from diverse species including rodents and humans. Furthermore, *in vivo* evidence from experimental infection of mice with SADS-CoV identified splenic dendritic cells (DCs) as the major site of SADS-CoV replication in mice. Finally, we demonstrated that SADS-CoV does not utilize known CoV protein receptors for cellular entry. These results present the possibility that rodents are among the susceptible hosts of SADS-CoV, highlighting the potential cross-species transmissibility of SADS-CoV.

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

101

102 **MATERIALS AND METHODS**

103 **Virus stocks and viral antibodies.** The SADS-CoV isolate CH/GD-01/2017 at passage 10
104 was used in all experiments and cultured in Vero cells (16). The virus was passaged serially using
105 the culture supernatant to infect fresh Vero cells at a multiplicity of infection (MOI) of 0.1, and
106 viral titers were determined in Vero cells by endpoint dilution as the 50% tissue culture infective
107 dose 50% ($TCID_{50}$). Rabbit polyclonal antibodies (pAb) against the membrane (M),
108 nucleocapsid (N) and the nonstructural protein 3 (Nsp3) acidic domain (AC) of SADS-CoV were
109 generated in-house, and validated in SADS-CoV-infected Vero cells (16). A mouse
110 anti-SADS-CoV-N pAb was also produced to allow double staining when mixed with the rabbit
111 pAb. A monoclonal antibody (MAb) against dsRNA (anti-dsRNA mAb J2, Cat# J2-1702,
112 SCICONS, Hungary) was used to specifically detect viral replication of SADS -CoV.

113 **Cell lines and cell culture.** Twenty-four cell lines derived from tissues of different species
114 were used (Table 1), including human (Huh-7, HepG2/C3A, 293T, A549 and HeLa), monkey
115 (Marc-145, Cos-7, BSC-40, Vero), swine [ST, PK15, LLC-PK1, IPEC-J2 (17)], bat [BFK (18),
116 Tb-1], canine (MDCK), mouse (NIH/3T3, RAW 264.7), hamster (BHK -21, CHO), rat (BRL-3A,
117 NRK-52E), chicken (DF-1) cell lines and a primary kidney cell line from Mongolian gerbils
118 (prepared in-house). The BFK cell line was a generous gift from Dr. Changchun Tu at the
119 Institute of Military Veterinary Medicine, Changchun, China. Each cell line was cultured in
120 Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented wit h 10% (v/v) fetal
121 bovine serum (FBS, Biological Industries), 100 U/m1 penicillin and 100 U/ml streptomycin at

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

122 37 °C, 5% CO_2, and water-saturated humidity conditions.

123 To determine viral susceptibility, each cell line was cultured at 70% confluence in 12-well
124 plates with maintenance medium (MM) containing DMEM, 0.3% tryptose phosphate broth
125 (TPB), and 1% penicillin/streptomycin, or MM with addition of 5 μg/ml trypsin (MMT) (Sigma,
126 Cat#T7186-50TAB, St Louis, MO, USA). After washing with phosphate buffered saline (PBS),
127 cells were inoculated with SADS-CoV diluted in MM or MMT at an MOI of 0.01 for 2 h.
128 Non-attached viruses were removed by washing the cells three times with DMEM, and cell
129 monolayers were subsequently incubated in MM or MMT at 37 °C for 5 days. To determine the
130 effect of trypsin on viral entry, cell monolayers were infected by SADS-CoV in three conditions:
131 1) no trypsin treatment, infected with SADS-CoV diluted in MM, subsequently incubated in MM;
132 2) pre-trypsin treatment, inoculated with SADS-CoV diluted in MMT, subsequently incubated in
133 MM; and 3) double-trypsin treatment, inoculated with SADS-CoV in MMT, subsequently
134 incubated in MMT. Supernatants from cells were collected at 12, 24, 36, 48, 72, and 120 hours
135 post-infection (hpi) for one-step quantitative RT-PCR analysis. Cell cultures were examined for
136 cytopathic effects (CPE) and immunofluorescence assay at 48-72 hpi.

137 **Immunofluorescence assay (IFA) for cell line susceptibility.** Different cells infected with
138 SADS-CoV in 12-well plates were washed twice with PBS and fixed in 4% paraformaldehyde in
139 PBS and then permeabilized with 0.1% Triton X-100 in PBS. Cells were then incubated with the
140 rabbit anti-SADS-CoV-M pAb at 1:5000 dilution for 1 h at 37 °C, washed with PBS and stained
141 with the Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Thermo Fisher
142 Scientific, USA) at 1:1000 dilution. After incubation for 1 h at 37 °C, the cells were washed with

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

143 PBS, stained with 4′,6-diamidino-2-phenylindole (DAPI) at 1:1000 dilution and visualized on a
144 fluorescence microscope.

145 **One-step quantitative RT-PCR analysis targeting the N gene.** The full-length SADS-CoV
146 N gene was inserted into an appropriately digested pET-28a vector using two unique restriction
147 sites, *Nde*I and *Xho*I, and then linearized with *Xho*I. The N gene was *in vitro* transcribed using
148 the T7 High Efficiency Transcription Kit (TransGen Biotech Co., LTD, Beijing China). Standard
149 curves were generated using dilutions of known quantity of N gene RNA to allow absolute
150 quantitation of SADS-CoV RNA copy numbers in samples.

151 Total RNA was extracted from culture supernatants or tissue homogenates using Trizol
152 (ThermoFisher Scientific, USA) following the manufacturer's instructions. SADS-CoV RNA
153 titer was determined by one-step qRT-PCR (TOYOBO Co., LTD) targeting the N gene with the
154 primers: 5'-CTAAAACTAGCCCCACAGGTC-3' and 5'-TGATTGCGAGAACGAGACTG-3',
155 and the probe FAM-GAAACCCAAACTGAGGTGTAGCAGG-TAMRA. Samples with a cycle
156 threshold value <35 were considered positive based upon validation data using the RNA
157 standards.

158 **Mouse infections, tissue harvest and viral load determination.** Wild-type C57BL/6J mice
159 (Jackson no. 000664) were purchased from the Model Animal Research Center of Nanjing
160 University and housed in animal facilities at the Zhejiang University under
161 specific-pathogen-free condition. For SADS-CoV infections, 6- to 8-week-old female and male
162 mice were inoculated with 5×10^5 $TCID_{50}$ (equal to 6×10^8 genome copies) of SADS-CoV, either
163 per oral infection (p.o.) with 25 μl inoculum (2×10^7 $TCID_{50}$/ml) or intraperitoneal infection (i.p.)

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

with 200 µl inoculum (2.5×10^6 $TCID_{50}$/ml). For viral load determination in specific tissues, mice were euthanized at 1, 3, 5, 7, 14, 21, and 28 days post-infection (dpi), and tissues were harvested including stomach, duodenum, jejunum, ileum, cecum, colon, mesenteric lymph nodes, spleen, kidney, liver, heart, lung, blood and feces. Tissues were weighed and homogenized in medium (DMEM contained 2% FBS) by bead beating using sterile zirconium oxide beads (Cat# ZrOB20 MidSci). Total RNA was extracted from tissue homogenates and tested by quantitative RT-PCR analysis targeting the SADS-CoV N gene, as described above. Blood samples were collected from the heart and serum was separated for virus-specific antibody detection.

Enzyme-linked immunosorbent assay (ELISA). SADS-CoV virus particles were purified from infected cell culture supernatants by sucrose density gradient centrifugation, and protein concentration was determined by the BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China). The optimal dilution of antigen was determined by square titration. The IgG antibodies contained in serum at a 1:100 dilution were detected in wells coated with purified SADS-CoV virus particles (6.25 ng/well) as antigen.

Histopathology, immunohistochemistry and immunofluorescence assay for murine spleen. Mice were infected i.p. with SADS-CoV and at 3 dpi, spleens were harvested and fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Tissue sections were then deparaffinized and rehydrated in three changes of xylene, 15 min each, dehydrated in two changes of pure ethanol for 5 min, followed by rehydration in an ethanol gradient of 85% and 75% ethanol. After washing in distilled water, tissues were subjected to hematoxylin and eosin staining for histopathological examinations.

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

185 For antigen retrieval, deparaffinized and rehydrated sections were immersed in sodium
186 citrate antigen retrieval solution (pH 6.0) and maintained at a sub-boiling temperature for 8 min,
187 let stand at 98 °C for 8 min and then incubated again at sub-boiling temperature for 7 min. After
188 allowing to cool to room temperature (RT) and washing three times with PBS (pH 7.4),
189 endogenous peroxidase was blocked by immersion in 3% hydrogen peroxide at RT for 30 min
190 and again washed with PBS. Tissue sections were blocked in 3% BSA at RT for 30 min, then
191 incubated with 1:500 dilution of each primary antibody (anti-dsRNA MAb, anti-SADS-CoV-M
192 pAb or anti-SADS-CoV-AC pAb) overnight at 4 °C. After washing slides three times with PBS
193 (pH 7.4), they were stained with appropriate secondary antibodies labeled with horseradish
194 peroxidase at RT for 50 min. Freshly prepared diaminobenzidine chromogenic reagent was added
195 and counterstained with hematoxylin, then dehydrated and visualized on a light microscope.
196 Spontaneous fluorescence quenching reagent (Wuhan servicebio technology Co., Ltd,
197 Wuhan, China) was added to the tissue sections and incubated for 5 min after antigen retrieval.
198 The sections were then washed in running water, followed with blocking and antibody staining
199 as described above. In addition, the primary antibody was supplement with a CD11c antibody
200 (Wuhan servicebio technology Co., Ltd, Wuhan, China) at a 1:200 dilution, then stained with
201 appropriate secondary antibodies. Finally, DAPI was added and sections were visualized on a
202 fluorescence microscope; nuclei labeled with DAPI appear blue, positive cells are green by
203 labeling with CD11/c or red by labeling with virus-specific antibody.
204 **Preparation of murine splenocytes and flow cytometry.** Mice infected with SADS-CoV
205 were euthanized at 3 dpi, and spleens were removed and placed in 5 ml complete DMEM. After

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

grinding the excised spleen through a 100-μm cell strainer using the plunger end of a 5-ml syringe, cells were washed with an excess of DMEM and centrifuged at 200 ×g for 5 min. After resuspending cells in 3 ml of red blood cell lysis buffer (Solarbio Life Sciences, Beijing, China) and incubation at RT for 10 min, 5 ml of DMEM was added and cells were passed through another strainer to remove clumps. After centrifugation at 200 ×g for 5 min, the supernatant was discarded and cells were resuspended in 10 ml fresh DMEM for cell counting and viability checks using trypan blue and a hemocytometer.

For flow cytometry, cultured cells were resuspended in Fc Block buffer (containing anti-mouse CD16 Fc Block Antibody at 1:500 dilution) and incubated on ice. Cells in Fc Block buffer were added to 96-well plates at 1×10^6 cells/well. After 30 min incubation, cells were centrifuged at 200 ×g for 10 min, the supernatant was discarded and pellets resuspended in 100 μl Cytofix/Cytoperm solution (Cytofix/Cytoperm Soln Kit; BD Biosciences, San Jose, CA, USA) to fix cells. After incubation on ice for 20 min protected from light , cells were centrifuged at 800 ×g for 5 min at 4 °C, supernatant was removed without disturbing cell pellets and cells were washed twice in 150 μl of 1x Perm/Wash buffer. After addition of 50 μl virus-specific primary antibody (anti-dsRNA MAb, anti-SADS-CoV-N pAb or anti-SADS-CoV-AC pAb) diluted in 1× Perm/Wash buffer with 3% BSA and incubation at 4 °C for 30 min, cells were centrifuged at 200 ×g for 10 min. Cells were washed twice in 150 μl of 1× Perm/Wash buffer followed by staining with appropriate secondary antibodies conjugated to Alexa Fluor 488 (Thermo Fisher Scientific, USA) at 4 °C for 30 min. After centrifuging at 800 ×g for 5 min at 4 °C and washing with 1× Perm/Wash buffer, pellets were resuspended in 0.2 ml FACS buffer and analyzed by flow

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

227 cytometry.

228 **Infection of splenocytes *in vitro*.** To detect replication of SADS-CoV in mouse splenic cells
229 *in vitro*, splenocytes were extracted from naïve mice, plated in 100- or 35-mm dishes and
230 infected with SADS-CoV at an MOI of 0.1. At 48 hpi, splenocytes were harvested and placed in
231 a 15-ml tube, centrifuged at 200 ×g for 10 min at 4 °C, and analyzed by flow cytometry as
232 described above. Infected mouse splenic cells in 35-mm dishes were detected by
233 immunofluorescence assay with anti-SADS-CoV-N antibodies, and infection supernatants were
234 collected at 0, 12, 24, 36, 48 and 72 hpi for one-step quantitative RT-PCR analysis.

235 **FACS analysis of splenocytes with cell marker staining.** Mice infected with SADS-CoV
236 were euthanized at 3 dpi, and splenocytes were prepared for flow cytometry by staining with
237 appropriate antibodies: anti-SADS-CoV-AC following secondary antibodies conjugated to Alexa
238 Fluor 647 (Thermo Fisher Scientific, USA); anti-CD19-FITC (eBioscience, Catalog no.4318813)
239 for B cells; anti-CD4-PE (eBioscience, Catalog no.4329629) for T cells; anti-CD11/c-PE-Cy7
240 (BD Bioscience, Catalog no.561022) for DCs; and anti-F4/80-PE/Cy5 (Biolegend, Catalog
241 no.123111) for macrophages. Stained cells were resuspended in 0.2 ml FACS buffer and
242 analyzed by flow cytometry.

243 **Production and transduction of S protein-pseudotyped lentiviruses.** Pseudovirions with
244 various CoV spike proteins were produced as described previously (19). Briefly, each of the
245 plasmids encoding TGEV, SARS-CoV, MERS-CoV, and mouse hepatitis virus (MHV) S proteins
246 were cotransfected into 293T cells with pLenti-Luc-green fluorescent protein (GFP) and psPAX2
247 plasmids (kindly provided by Dr. Zhaohui Qian, Chinese Academy of Medical Sciences &

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

248 Peking Union Medical College) at a molar ratio of 1:1:1 by using polyethylenimine (PEI). The
249 cells were fed with fresh medium in the next 24 h and the supernatant media containing
250 pseudovirions were then collected and centrifuged at 800 ×g for 5 min to remove debris. To
251 quantify S protein-mediated entry of pseudovirions, MDCK cells were seeded at about 25-30%
252 confluency in 24-well plates and transfected with either pAPN-Flag, hDPP4-Flag,
253 mCEACAM1a-Flag, hACE2-GFP (kindly provided by Dr. Zhaohui Qian) (19) or the control
254 backbone vector by using Lipofectamine 3000 (Thermo Fisher). The MDCK cells
255 overexpressing each receptor were inoculated with 500 µl of 1:1 diluted corresponding
256 pseudovirions at 24 h post-transfection. At 40 hpi, cells were lysed at room temperature with 110
257 µl of medium with an equal volume of Steady-Glo (Promega, Madison, WI). The cell lysates
258 were also used to confirm the expression of each receptor by using western blotting.
259 Transduction efficiency was monitored by quantitation of luciferase activity using a Modulus II
260 microplate reader (Turner Biosystems, Sunnyvale, CA). On the other hand, the MDCK cells
261 overexpressing each receptor were inoculated with SADS-CoV (MOI=1) at 24 h
262 post-transfection. IFA was performed to test for SADS-CoV susceptibility using anti-N pAb. The
263 replication competency of SADS-CoV in MDCK cells was further determined by a reverse
264 genetics system. Development of a DNA-launched SADS-CoV (SeACoV) infectious cDNA
265 clone (named pSEA) and rescue of SADS-CoV by transfection of cultured cells with pSEA
266 followed by passaging on Vero cells have been described recently by our lab (16).

267 **Ethics statement.** All animal experiments were performed in strict accordance with the
268 Experimental Animal Ethics Committee of Zhejiang University (approval no. ZJU20170026).

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

RESULTS

SADS-CoV can infect cell lines originating from various species. Previously, we reported that SADS-CoV was isolated in Vero cells supplemented with trypsin (11). Since exogenous trypsin is essential for propagation of PEDV isolates *in vitro* (20), likely by mediating activation of membrane fusion by S glycoprotein proteolysis (21), we were interesting to know whether it is also required for SADS-CoV growth in cell culture. A total of 24 cell lines originating in various tissues of humans and different animal species were tested for susceptibility to SADS-CoV treated with or without trypsin (Table 1). In brief summary of the results, 21 of the 24 cell lines showed significant susceptibility to SADS-CoV infection, defined by efficient viral replication, antigen expression and the appearance of CPE. The three cell lines that were not infected by SADS-CoV were MDCK, BFK and RAW 264.7.

First, CPE was examined by inverted light microscopy at 48 hpi, and scores are shown in Table 1. As the 293T, NIH/3T3, CHO, BRL-3A and NRK-52E cell lines were sensitive to trypsin, they couldn't be tested for SADS-CoV infection in MMT. Apart from that, CPE was visible in Vero, ST and BRL-3A cell lines without trypsin, and prominent CPE appeared or was enhanced with trypsin in Marc-145, Cos-7, BSC-40, Vero, ST, PK15, LLC-PK1 and BHK-21 cell lines (Table 1).

As some cells did not display CPE after SADS-CoV infection, all cell lines were subsequently tested for viral M protein expression by IFA (Fig. 1), revealing the same range as seen by CPE in the different cell lines (data not shown). Syncytia formation was prominent in

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

290 Huh-7, Vero and BHK-21 cells, whereas in MDCK, BFK and RAW 264.7 cells the antigen
291 expression was much less prominent than in the other cell lines (Figs. 1A, 1C and 1I). Most cell
292 lines tested showed evidence of productive infection as indicated by expression of the M protein,
293 while the inefficient antigen expression in Marc-145, LLC-PK1 and IPEC-J2 cells suggested
294 only a limited infection.

295 Next, viral load in the culture supernatants was detected over 5 dpi by quantitative RT-PCR
296 (Fig. 2A-2C). A higher mean viral load was detected by qRT-PCR after trypsin treatment in
297 HepG2, HeLa, Marc-145, Cos-7, BSC-40, Vero, LLC-PK1, IPEC-J2, BHK-21 and DF-1 cells.
298 Therefore, trypsin contributes to, but is not essential for SADS-CoV propagation in these cell
299 lines. There was no difference after trypsin treatment in the other cell lines, though Huh-7 and
300 Tb-1 cells had high levels of SADS-CoV RNA regardless of trypsin treatment.

301 The progressive release of infectious SADS-CoV into the culture medium of six
302 representative cell lines infected with SADS-CoV was determined by titration of supernatants in
303 Vero cells (Fig. 2D). Unlike in MDCK cells, SADS-CoV infection of HeLa, Vero, Tb-1,
304 BHK-21 and PK-15 cells was productive, with HeLa cells showing the greatest susceptibility
305 (Fig. 2D).

306 **Wild-type C57BL/6J mice can be infected by SADS-CoV via oral and intraperitoneal**
307 **routes.** With the observation that SADS-CoV could infect diverse rodent cell lines (from mice,
308 rats and hamsters as well as gerbil primary kidney cells), we hypothesized that mice may be
309 susceptible to SADS-CoV. To test this, we inoculated 6- to 8-week-old wild-type B6 mice with
310 5×10^5 $TCID_{50}$ of SADS-CoV by the p.o. or i.p. route and monitored them for 28 days for clinical

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

311 symptoms. The mice did not succumb to the infection nor did they develop diarrhea or
312 experience weight loss during the incubation period (data not shown).

313 To determine whether SADS-CoV infected the animals asymptomatically, tissue and fecal
314 samples from inoculated mice were collected at 1, 3, 5, 7, 14, 21 and 28 dpi to determine viral
315 growth kinetics and shedding. Analysis of tissue samples by qRT-PCR suggested that
316 SADS-CoV replicated modestly in the stomach early after i.p. or p.o. infection, declining and
317 reaching undetectable levels at 7 or 14 dpi and thereafter (Fig. 3A). A very limited viral
318 replication was observed in each region of the small intestine, with the ileum via i.p. infection
319 showing continuous and decent detectable viral RNA (Fig. 3B). In the large intestine, i.p.
320 infection also resulted in viral RNA loads slightly above the limit of detection at each time point
321 in the ceca, whereas it led to higher viral RNA levels at 1-3 dpi, and much lower viral RNA at
322 21-28 dpi in the colon compared to the p.o. route (Fig. 3C). However, this replication in the large
323 intestine did not translate into higher shedding, as hardly any viral genomes were detected even
324 at 1 dpi in the fecal samples collected from i.p.-infected mice (Fig. 3F). On the contrary,
325 significantly more virus was detected in the feces of p.o.-infected mice at 1 and 3 dpi, indicating
326 that i.p. inoculation does not lead to higher virus shedding.

327 Finally, SADS-CoV replicated more efficiently in the spleen following the i.p. route, with
328 significantly higher viral RNA loads at 21 dpi (Fig. 3D). More importantly, the virus was not
329 cleared from this tissue by 28 dpi in the i.p.-infected group and by 14 dpi in the p.o.-infected
330 group, suggestive of a SADS-CoV prolonged infection in the spleen independent of inoculation
331 route. In contrast to the spleen, only very low levels of viral RNA were detected in the local

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

lymphoid tissue of mesenteric lymph nodes (MLNs) at 1-3 dpi, and no virus was detectable at later time points (Fig. 3D). We also looked for virus in other extraintestinal sites including the heart, lungs, liver, kidneys and blood, but they were all negative or had extremely low levels (Fig. 3E). IgG antibody levels after 7 days detected by SADS-CoV virion-based ELISA showed that the i.p. route could effectively elicit host immune responses (Fig. 3G).

Splenocytes support SADS-CoV replication. With the mouse infection model described above, our next step was to determine the cell tropism of SADS-CoV *in vivo*. Thus, we performed immunohistochemistry (IHC) on sections of small and large intestine and spleen from mice infected i.p. with 5×10^5 $TCID_{50}$ of SADS-CoV at 3 dpi. A monoclonal antibody against dsRNA was used to identify cells that supported active virus replication, as dsRNA is an intermediate that only exists during intracellular viral replication. SADS-CoV dsRNA signals were observed in the splenic white pulp in the marginal zone on the edge of lymphatic follicles, and in the margins of the periarteriolar lymphocyte sheath (Fig. 4A). Staining of tissue sections from mock-infected mice were used as a control (Fig. 4B). In addition to dsRNA, we also used rabbit pAbs to detect expression of viral structural protein (M) or nonstructural protein (Nsp3-AC). At 3 dpi, anti-M or anti-AC staining was observed in the white pulp around the lymphatic nodules (Fig. 4C), similar to the localization of dsRNA staining (Fig. 4A). Tissue sections from SADS-CoV or mock-infected mice probed with preimmune sera were negative, indicating the specificity of the SADS-CoV antibody. Unfortunately, neither viral proteins (structural or nonstructural) nor dsRNA were detected in the intestine of infected mice, consistent with the detection of only very low levels of viral RNA in these tissues by qRT-PCR

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

353 (Fig. 3).

354 Next, SADS-CoV infection was quantified in the spleen using flow cytometry. We
355 inoculated B6 wild-type mice with 5×10^5 $TCID_{50}$ of virus either i.p. or p.o., and extracted the
356 bulk immune cells from the spleen of infected animals at 3 dpi. The flow cytometry method was
357 first validated in Vero cells infected with SADS-CoV at an MOI of 0.01 followed by staining
358 with pAb against the N or AC protein at 24 hpi (Fig. 4D). As the anti-AC pAb exhibited optimal
359 intracellular staining for viral signals (Fig. 4D), it was used to determine the percentage of
360 infected splenocytes. Approximately 1.5- and 2.5-fold increase of total splenocytes were positive
361 for virus replication after p.o. and i.p. inoculation, respectively (Fig. 4E, the left panel; Fig. 4F),
362 with a significant increase in the total number of AC-positive splenocytes in i.p.-infected mice
363 compared to p.o. (Fig. 4E, the right panel). This data is consistent with the significantly lower
364 viral loads in the spleen at 1 and 3 dpi in p.o.-inoculated mice (Fig. 3D), suggesting better virus
365 dissemination and replication and escape from mucosal immune clearance.

366 We then evaluated the growth characteristics of SADS-CoV in splenocytes by assessing
367 antigen production and replication kinetics *ex vitro*. Splenocytes were first extracted from naïve
368 mice, plated in 100 mm dishes and infected with 1×10^5 $TCID_{50}$ of SADS-CoV. We observed
369 clusters of infected cells that appeared to have been engulfed by phagocytes (Fig. 4G, the middle
370 panel), and the structural N protein was shown in the cytoplasm of infected cells by confocal
371 microscopy (Fig. 4G, the middle and right panels). The percentage of infected cells was
372 quantified by flow cytometry using anti-AC pAb, revealing that nearly 2-fold increase of the
373 splenocytes were positive for viral signals (Fig. 4H), very similar to the percentage of infection

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

374 observed *in vivo*. To further characterize the growth kinetic of SADS-CoV in primary
375 splenocytes, cells were infected with 1×10^5 $TCID_{50}$ of SADS-CoV, and culture supernatants
376 were harvested at 0, 12, 24, 48 and 72 hpi. Active viral replication was confirmed, with a 1.5-log
377 time-dependent increase in genomic RNA equivalents, plateauing from 24 to 72 hpi (Fig. 4I).
378 This data suggests that although only ~2% of splenocytes were infected, these cells supported a
379 decent level of viral replication. Together, these results indicate that SADS-CoV productively
380 infects mouse splenocytes.

381 **Splenic DCs support SADS-CoV replication.** Splenocytes were harvested from
382 i.p.-infected mice at 3 dpi, and the extracted cells were co-stained with antibodies against
383 SADS-CoV-AC and each of four cell surface markers (anti-CD19 for B cells; anti-CD4 for T
384 cells; anti-$CD11/c^+$ for DCs and anti-$F4/80^+$ for macrophages) using flow cytometry (Fig. 5A).
385 The percentage of infected $CD11/c^+$ cells was significantly higher than the other cell subgroups,
386 indicating that DCs are the major targets of SADS-CoV infection in the spleen.

387 The phenotype was further confirmed by double-staining IFA with anti-dsRNA, anti-M or
388 anti-AC antibody plus anti-$CD11/c^+$ in splenic sections. As expected, dsRNA staining
389 overlapped with the CD11/c surface marker on the edges of lymphatic follicles (Fig. 5B),
390 whereas no viral signals were seen in the mock-infected control (Fig. 5C). Similar patterns of
391 co-staining were detected by M and AC antibodies (Fig. 5D). To gain insight into the relative
392 quantity of DCs compared to other undefined target cells, cells positive for dsRNA and CD11/c
393 were counted in 10-15 different microscope fields of spleens from 3 infected mice (Fig. 5E),
394 showing that 61.76% of SADS-CoV-infected cells were DCs (Fig. 5F).

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

395 **SADS-CoV does not utilize known CoV protein receptors for cellular entry.** To our
396 knowledge, these results reveal the most extensive cell tropism among known CoVs, suggesting
397 the functional receptor(s) for SADS-CoV is likely to be a very common molecule. In order to test
398 this hypothesis, it was first necessary to find a cell line that was refractory to infection only at the
399 internalization step. MDCK cells, which showed undetectable virus production in early infection
400 tests (Fig. 1 and Fig. 2), were chosen as a potential candidate. There are four known types of
401 functional CoV protein receptors, including angiotensin converting enzyme 2 (ACE2) for
402 SARS-CoV (22), dipeptidyl peptidase 4 (DPP4) for MERS-CoV (23), aminopeptidase N (APN)
403 for TGEV (24) and PDCoV (25, 26), and mouse carcinoembryonic antigen-related cell adhesion
404 molecule 1a (mCEACAM1a) for MHV (27). To test whether one of these molecules serves as the
405 SADS-CoV receptor, we attempted to inoculate non-susceptible MDCK cells overexpressing
406 porcine APN, human DPP4, mouse CEACAM1a, or human ACE2 with SADS-CoV, but none of
407 them allowed infection as staining with anti-SADS-CoV-N pAb was negative (Fig. 6A).
408 Meanwhile, the expression of each receptor in MDCK cells was confirmed by IFA (Fig. 6A) and
409 western blot analysis (Fig. 6B) using antibodies against the tags fused to the receptors. As
410 positive controls, we confirmed that lentiviruses pseudotyped with TGEV, SARS-CoV,
411 MERS-CoV or MHV spike (i.e., pseudoviruses) efficiently entered MDCK cells exogenously
412 expressing the respective receptors (Fig. 6C).

413 Next, we demonstrated that MDCK cells can confer SADS-CoV replication competency by
414 transfection of a SADS-CoV/SeACoV infectious cDNA clone established recently (16), as
415 simultaneous expression of Nsp3-AC and N proteins were clearly detected by IFA (Fig. 6D).

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Moreover, passaging of supernatants from pSEA-transfected MDCK cells onto fresh Vero cells resulted in progeny SADS-CoV infection, as evidenced by expression of the N protein (Fig. 6E), indicating that MDCK cells can also support infectious SADS-CoV production without cell-to-cell spread. Therefore, SADS-CoV apparently does not utilize any of the known CoV receptors for cellular entry. The same conclusion was reached using HeLa cells overexpressing each of the four classical CoV receptors followed by SADS-CoV inoculation by Zhou et al (14); however, the HeLa cell line itself was most susceptible to SADS-CoV infection in the present study (Fig. 2D).

DISCUSSION

In order to assess the potential species barriers of SADS-CoV infection, a cell line susceptibility study was first conducted using 24 different cell lines. As SADS-CoV probably originated from a bat SADSr-CoV (14) derived from HKU2-CoV identified in *Rhinolophus sinicus* (Chinese horseshoe bats) (12), we commenced testing viral susceptibility in two available bat cell lines, BFK from *Myotis daubentonii* (18) and Tb-1 from *Tadarida brasiliensis*. Although BFK cells did not support SADS-CoV replication, it replicated efficiently in Tb-1 cells (Fig. 1A and Fig. 2), suggesting that other bat species in addition to horseshoe bats are likely susceptible to SADS-CoV infection.

Interestingly, SADS-CoV protein expression was detected in almost all of the rodent cells (hamster, gerbil, mouse and rat) including BHK-21, which is not susceptible to other known human CoVs such as SARS-CoV and MERS-CoV (28, 29) as well as three swine enteric CoVs,

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

437 PEDV, PDCoV and TGEV (25). Given the fact that SADS-CoV infects both primary and
438 passaged or primary cell lines originating from rodents, we hypothesized that rodents may be
439 susceptible to SADS-CoV infection. To explore this possibility, we challenged wild-type B6
440 mice with SADS-CoV by two different inoculation routes.

441 The challenged animals neither succumbed to infection nor manifested any signs of
442 gastroenteritis. In fact, experimental infection of neonatal piglets with a higher dose of purified
443 SADS-CoV in our laboratory only resulted in mild diarrheal signs or subclinical infection (11).
444 Also, there was a lack of robust viral replication in the intestines during infection, and no tissue
445 damage was detected throughout the intestines (Fig. 3B and 3C), reflecting the suboptimal
446 infection by SADS-CoV in immunocompetent wild-type mice. On the contrary, the virus had
447 more efficient replication within the spleen, reflected by a continuous detection of viral genomic
448 RNA in the immune cells at all time points over a 28-day period (Fig. 3D). The phenotype was
449 also consistent with the replication kinetics in extracted splenocytes *in vitro*, in which viral
450 genomic RNA peaked and plateaued at 72 hpi (Fig. 4G and 4I). This data collectively led to
451 speculation that SADS-CoV favors splenic cells over other tissues. The most logical explanation
452 for these tissue-specific discrepancies in virus replication is: i) target cells are more concentrated
453 in the spleen and more sporadic in the intestine; or ii) splenic immune cells have enhanced
454 expression of the unknown receptor(s) over intestinal cells. The animals were more susceptible
455 to i.p. infection, resulting in higher virus replication in the distal section of the small intestine,
456 large intestine and spleen, and perhaps a delayed clearance of viral infection in the cecum (Fig.
457 3B to 3E), suggesting the important role of mucosal immunity for controlling early infection in

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

458 SADS-CoV in mice. It should be note that mice (C57BL/6J mice in this study) may not be the
459 optimal rodent species for SADS-CoV infection, as wild rats are more commonly seen in
460 Chinese pig farms. In addition, other transmission routes may be considered. Recently, PDCoV
461 has been shown to possibly spread via the respiratory route in addition to fecal-oral transmission
462 (30). Therefore, it will be interesting to try intranasal route for inoculation in rats or the other
463 rodent species to mimic SADS-CoV natural transmission in future studies.

464 More interestingly, we identified DCs to be the precise cell population that supported
465 SADS-CoV replication (Fig. 5). There have been a few reports of immune cell tropism for CoVs.
466 Macrophages are susceptible to MHV infection, representing the largest group of innate immune
467 cells that infiltrate the central nervous system after infection with neurotropic MHV strains (31).
468 In addition, based on the fact that SARS-CoV spike-pseudotyped HIV-based vectors can
469 efficiently transduce human DCs, Kobinger *et al*. hypothesized that SARS-CoV infection in
470 immature DCs contributes to viral pathogenesis (32). Yang *et al*. demonstrated that SARS-CoV
471 can infect myeloid DCs via S glycoprotein-associated cell entry, and DC infection mediated viral
472 transmission to other cells *in vivo* (33). These previous evidences support our present results,
473 showing that SADS-CoV can efficiently replicate in DCs.

474 Furthermore, this study gives us a novel inspiration that rodents may potentially serve as
475 susceptible hosts for SADS-CoV in addition to bats and pigs. Of note, the species *Rhinolophus*
476 *bat α-CoV HKU2*, including SADS-CoV, possesses unique S genes closely related to the
477 betacoronavirus (β-CoV), in a manner similar to some globally distributed rodent α-CoVs (11, 34,
478 35), implying an unknown evolutionary connection between the bat α-CoV HKU2 and rodents

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

479 α-CoVs. In the field conditions of China, direct contact between pigs and flying bats is a low
480 probability; however, rodents (especially rats) are frequently visible in the swine industry,
481 causing great nuisance due to feed loss. It is possible that as bats prey on insects near pig
482 facilities, they leave feces containing HKU2-like CoVs that contaminate pig feed, which is then
483 eaten by pig and rodents that subsequently become carriers of SADS-CoV. Rats and mice are
484 increasingly implicated as external vectors for a wide range of different pig pathogens, such as *L.*
485 *intracellularis* (36). Rodents not only spread pathogens, but also harm the practitioners of the
486 swine industry, as they are thought to be the major source of leptospirosis in pigs and piggery
487 workers (37). Future study on identifying SADS-CoV-positive samples in rodents near pig farms
488 are warranted to test this hypothesis.

489 In addition to rodents, we also measured the SADS-CoV susceptibility of cell lines from
490 humans, monkeys, chickens and dogs, revealing a remarkably broad spectrum of tropism (Table
491 1 and Fig. 1). As for the ability of SADS-CoV to grow efficiently in human cell lines, we should
492 not underestimate the risk that this bat-origin CoV may 'jump' from pigs to humans. It is
493 noteworthy that camel workers with high rates of exposure to camel nasal and oral secretions had
494 evidence of MERS-CoV infection (38). Considering that SARS-CoV and MERS-CoV originated
495 from bats and spread from one species to another through intermediate hosts (civets and camels,
496 respectively), SADS-CoV may pose a similar risk to human health through transmission from
497 pigs or other susceptible hosts.

498 The cell susceptibility study and testing of overexpression of four known CoV receptors in
499 non-susceptible MDCK cells (Fig. 6) demonstrated that SADS-CoV might use a new receptor

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

molecule that is conserved in bats, pigs, rodents, chickens, monkeys and humans, indicating a low barrier to cross-species transmission. This is in line with the unusual feature of SADS-CoV's apparently broad species tropism.

In summary, these results provide important insights into the ecology of this bat-origin CoV, highlighting the possibility of its jumping interspecies barriers and the potential role of rodents as susceptible hosts in the field. Identification of the unknown SADS-CoV cellular receptor and further surveillance of other animal populations are needed to fully understand the biology of SADS-CoV.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program of China (2016YFD0500102), the National Natural Science Foundation of China (31872488) and the Fundamental Research Funds for the Central Universities of China (2019FZA6014). The professional editing service NB Revisions was used for technical preparation of the text prior to submission.

References

1. **Graham RL, Baric RS.** 2010. Recombination, reservoirs, and the modular spike: mechanisms of coronavirus cross-species transmission. J Virol **84:**3134-3146.

2. **van Boheemen S, de Graaf M, Lauber C, Bestebroer TM, Raj VS, Zaki AM, Osterhaus AD, Haagmans BL, Gorbalenya AE, Snijder EJ, Fouchier RA.** 2012. Genomic characterization of a newly

Accepted Manuscript Posted Online

discovered coronavirus associated with acute respiratory distress syndrome in humans. MBio **3:**e00473-00412.

3. **Soma T, Saito N, Kawaguchi M, Sasai K.** 2018. Feline coronavirus antibody titer in cerebrospinal fluid from cats with neurological signs. J Vet Med Sci **80:**59-62.

4. **Huang YW, Dickerman AW, Pineyro P, Li L, Fang L, Kiehne R, Opriessnig T, Meng XJ.** 2013. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. MBio **4:**e00737-00713.

5. **Lu G, Wang Q, Gao GF.** 2015. Bat-to-human: spike features determining 'host jump' of coronaviruses SARS-CoV, MERS-CoV, and beyond. Trends Microbiol **23:**468-478.

6. **Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, Tong S, Urbani C, Comer JA, Lim W, Rollin PE, Dowell SF, Ling AE, Humphrey CD, Shieh WJ, Guarner J, Paddock CD, Rota P, Fields B, DeRisi J, Yang JY, Cox N, Hughes JM, LeDuc JW, Bellini WJ, Anderson LJ, Group SW.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med **348:**1953-1966.

7. **Drexler JF, Corman VM, Drosten C.** 2014. Ecology, evolution and classification of bat coronaviruses in the aftermath of SARS. Antiviral Res **101:**45-56.

8. **Chan JF, Lau SK, To KK, Cheng VC, Woo PC, Yuen KY.** 2015. Middle East respiratory syndrome coronavirus: another zoonotic betacoronavirus causing SARS-like disease. Clin Microbiol Rev **28:**465-522.

9. **Yin Y, Wunderink RG.** 2018. MERS, SARS and other coronaviruses as causes of pneumonia. Respirology **23:**130-137.

10. **Hulswit RJ, de Haan CA, Bosch BJ.** 2016. Coronavirus Spike Protein and Tropism Changes. Adv Virus

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Accepted Manuscript Posted Online

Res **96:**29-57.

11. **Pan Y, Tian X, Qin P, Wang B, Zhao P, Yang YL, Wang L, Wang D, Song Y, Zhang X, Huang YW.** 2017. Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. Vet Microbiol **211:**15-21.

12. **Lau SK, Woo PC, Li KS, Huang Y, Wang M, Lam CS, Xu H, Guo R, Chan KH, Zheng BJ, Yuen KY.** 2007. Complete genome sequence of bat coronavirus HKU2 from Chinese horseshoe bats revealed a much smaller spike gene with a different evolutionary lineage from the rest of the genome. Virology **367:**428-439.

13. **Gong L, Li J, Zhou Q, Xu Z, Chen L, Zhang Y, Xue C, Wen Z, Cao Y.** 2017. A New Bat-HKU2-like Coronavirus in Swine, China, 2017. Emerg Infect Dis **23:**1607-1609.

14. **Zhou P, Fan H, Lan T, Yang XL, Shi WF, Zhang W, Zhu Y, Zhang YW, Xie QM, Mani S, Zheng XS, Li B, Li JM, Guo H, Pei GQ, An XP, Chen JW, Zhou L, Mai KJ, Wu ZX, Li D, Anderson DE, Zhang LB, Li SY, Mi ZQ, He TT, Cong F, Guo PJ, Huang R, Luo Y, Liu XL, Chen J, Huang Y, Sun Q, Zhang XL, Wang YY, Xing SZ, Chen YS, Sun Y, Li J, Daszak P, Wang LF, Shi ZL, Tong YG, Ma JY.** 2018. Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. Nature **556:**255-258.

15. **Wang L, Su S, Bi Y, Wong G, Gao GF.** 2018. Bat-Origin Coronaviruses Expand Their Host Range to Pigs. Trends Microbiol **26:**466-470.

16. **Yang YL, Liang QZ, Xu SY, Mazing E, Xu GH, Peng L, Qin P, Wang B, Huang YW.** 2019. Characterization of a novel bat-HKU2-like swine enteric alphacoronavirus (SeACoV) infection in cultured cells and development of a SeACoV infectious clone. Virology **536:**110-118.

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Accepted Manuscript Posted Online

563 17. **Ji CM, Wang B, Zhou J, Huang YW.** 2018. Aminopeptidase-N-independent entry of porcine epidemic
564 diarrhea virus into Vero or porcine small intestine epithelial cells. Virology **517:**16-23.
565 18. **He B, Yang F, Yang W, Zhang Y, Feng Y, Zhou J, Xie J, Feng Y, Bao X, Guo H, Li Y, Xia L, Li N,**
566 **Matthijnssens J, Zhang H, Tu C.** 2013. Characterization of a novel G3P[3] rotavirus isolated from a
567 lesser horseshoe bat: a distant relative of feline/canine rotaviruses. J Virol **87:**12357-12366.
568 19. **Ou XY, Zheng WL, Shan YW, Mu ZX, Dominguez SR, Holmes KV, Qian ZH.** 2016. Identification of
569 the Fusion Peptide-Containing Region in Betacoronavirus Spike Glycoproteins. Journal of Virology
570 **90:**5586-5600.
571 20. **Hofmann M, Wyler R.** 1988. Propagation of the virus of porcine epidemic diarrhea in cell culture. J Clin
572 Microbiol **26:**2235-2239.
573 21. **Wicht O, Li W, Willems L, Meuleman TJ, Wubbolts RW, van Kuppeveld FJ, Rottier PJ, Bosch BJ.**
574 2014. Proteolytic activation of the porcine epidemic diarrhea coronavirus spike fusion protein by trypsin in
575 cell culture. J Virol **88:**7952-7961.
576 22. **Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga**
577 **K, Greenough TC, Choe H, Farzan M.** 2003. Angiotensin-converting enzyme 2 is a functional receptor
578 for the SARS coronavirus. Nature **426:**450-454.
579 23. **Raj VS, Mou H, Smits SL, Dekkers DH, Muller MA, Dijkman R, Muth D, Demmers JA, Zaki A,**
580 **Fouchier RA, Thiel V, Drosten C, Rottier PJ, Osterhaus AD, Bosch BJ, Haagmans BL.** 2013.
581 Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. Nature
582 **495:**251-254.
583 24. **Delmas B, Gelfi J, L'Haridon R, Vogel LK, Sjostrom H, Noren O, Laude H.** 1992. Aminopeptidase N

Journal of Virology
JVI
Journal of Virology
Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Accepted Manuscript Posted Online

is a major receptor for the entero-pathogenic coronavirus TGEV. Nature **357:**417-420.

25. **Wang B, Liu Y, Ji CM, Yang YL, Liang QZ, Zhao P, Xu LD, Lei XM, Luo WT, Qin P, Zhou J, Huang YW.** 2018. Porcine deltacoronavirus engages the transmissible gastroenteritis virus functional receptor porcine aminopeptidase N for infectious cellular entry. J Virol **92:**e00318-00318.

26. **Li W, Hulswit RJG, Kenney SP, Widjaja I, Jung K, Alhamo MA, van Dieren B, van Kuppeveld FJM, Saif LJ, Bosch BJ.** 2018. Broad receptor engagement of an emerging global coronavirus may potentiate its diverse cross-species transmissibility. Proc Natl Acad Sci U S A **115:**E5135-E5143.

27. **Williams RK, Jiang GS, Holmes KV.** 1991. Receptor for mouse hepatitis virus is a member of the carcinoembryonic antigen family of glycoproteins. Proc Natl Acad Sci U S A **88:**5533-5536.

28. **Chan JF, Chan KH, Choi GK, To KK, Tse H, Cai JP, Yeung ML, Cheng VC, Chen H, Che XY, Lau SK, Woo PC, Yuen KY.** 2013. Differential cell line susceptibility to the emerging novel human betacoronavirus 2c EMC/2012: implications for disease pathogenesis and clinical manifestation. J Infect Dis **207:**1743-1752.

29. **Muller MA, Raj VS, Muth D, Meyer B, Kallies S, Smits SL, Wollny R, Bestebroer TM, Specht S, Suliman T, Zimmermann K, Binger T, Eckerle I, Tschapka M, Zaki AM, Osterhaus AD, Fouchier RA, Haagmans BL, Drosten C.** 2012. Human coronavirus EMC does not require the SARS-coronavirus receptor and maintains broad replicative capability in mammalian cell lines. MBio **3:**e00515-00512.

30. **Woo PC, Lau SK, Tsang CC, Lau CC, Wong PC, Chow FW, Fong JY, Yuen KY.** 2017. Coronavirus HKU15 in respiratory tract of pigs and first discovery of coronavirus quasispecies in 5'-untranslated region. Emerg Microbes Infect **6:**e53.

31. **Mazaleuskaya L, Veltrop R, Ikpeze N, Martin-Garcia J, Navas-Martin S.** 2012. Protective role of

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Journal of Virology JVI Journal of Virology

Accepted Manuscript Posted Online

605 Toll-like Receptor 3-induced type I interferon in murine coronavirus infection of macrophages. Viruses
606 **4:**901-923.

607 32. **Kobinger GP, Limberis MP, Somanathan S, Schumer G, Bell P, Wilson JM.** 2007. Human
608 immunodeficiency viral vector pseudotyped with the spike envelope of severe acute respiratory syndrome
609 coronavirus transduces human airway epithelial cells and dendritic cells. Hum Gene Ther **18:**413-422.

610 33. **Yang ZY, Huang Y, Ganesh L, Leung K, Kong WP, Schwartz O, Subbarao K, Nabel GJ.** 2004.
611 pH-dependent entry of severe acute respiratory syndrome coronavirus is mediated by the spike glycoprotein
612 and enhanced by dendritic cell transfer through DC-SIGN. J Virol **78:**5642-5650.

613 34. **Tsoleridis T, Chappell JG, Onianwa O, Marston DA, Fooks AR, Monchatre-Leroy E, Umhang G,**
614 **Muller MA, Drexler JF, Drosten C, Tarlinton RE, McClure CP, Holmes EC, Ball JK.** 2019. Shared
615 Common Ancestry of Rodent Alphacoronaviruses Sampled Globally. Viruses **11**.

616 35. **Wang W, Lin XD, Guo WP, Zhou RH, Wang MR, Wang CQ, Ge S, Mei SH, Li MH, Shi M, Holmes**
617 **EC, Zhang YZ.** 2015. Discovery, diversity and evolution of novel coronaviruses sampled from rodents in
618 China. Virology **474:**19-27.

619 36. **Collins AM, Fell S, Pearson H, Toribio JA.** 2011. Colonisation and shedding of Lawsonia intracellularis
620 in experimentally inoculated rodents and in wild rodents on pig farms. Vet Microbiol **150:**384-388.

621 37. **Everard CO, Ferdinand GA, Butcher LV, Everard JD.** 1989. Leptospirosis in piggery workers on
622 Trinidad. J Trop Med Hyg **92:**253-258.

623 38. **Alshukairi AN, Zheng J, Zhao J, Nehdi A, Baharoon SA, Layqah L, Bokhari A, Al Johani SM,**
624 **Samman N, Boudjelal M, Ten Eyck P, Al-Mozaini MA, Zhao J, Perlman S, Alagaili AN.** 2018. High
625 Prevalence of MERS-CoV Infection in Camel Workers in Saudi Arabia. MBio **9:**e01985-01918.

Journal of Virology
JVI
Journal of Virology
Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

626

627 # FIGURE LEGENDS

628

629 **Figure 1. Immunofluorescence assay showing susceptibility of different cell lines to**
630 **SADS-CoV infection.** Immunofluorescence assay of cells infected with SADS-CoV at an
631 MOI=0.01 was performed using rabbit anti-SADS-CoV-M polyclonal Ab (200× magnification)
632 and Alexa Fluor 488-conjugated anti-rabbit IgG as secondary antibody, with DAPI for
633 visualization of cell nuclei. Mock-infected cells were treated with the same procedures as
634 appropriate. Cells were tested from different species of origin, including: **(A)** Bats (BFK and
635 Tb-1); **(B)** Hamsters (CHO and BHK-21); **(C)** Mice (NIH/3T3 and RAW264.7); **(D)** Rats
636 (BRL-3A and NRK-52E); **(E)** Humans (Huh-7, HepG2, 293T, A549, and HeLa); **(F)** Monkeys
637 (Marc-145, Cos-7, BSC-40, and Vero); **(G)** Pigs (ST, PK15, LLC-PK1, and IPEC-J2); **(H)**
638 Chickens (DF-1); **(I)** Dogs (MDCK); and **(J)** Gerbil primary kidney cells.

639

640 **Figure 2. Growth of SADS-CoV in different cell lines through five days post-infection.** To
641 determine the effect of trypsin on SADS-CoV infection, each cell line was infected in three
642 conditions: **(A)** "No trypsin" treatment: inoculated with SADS-CoV diluted in maintenance
643 medium (MM) for 2 h, and subsequently replaced with MM; **(B)** "Pre-trypsin" treatment:
644 inoculated with SADS-CoV diluted in MM containing 5 μg/ml trypsin (MMT) for 2 h, and
645 subsequently replaced with MM; and **(C)** "Double-trypsin" treatment: inoculated with
646 SADS-CoV in MMT, and subsequently replaced with MMT. Infection supernatants were

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

collected at 12, 24, 36, 48, 72 and 120 hpi for viral load detection by a qRT-PCR assay targeting the viral N gene. Data is expressed as the mean viral load (log_{10} copies/µl) ± standard deviation (SD), and all experiments were performed in triplicate. The 293T, CHO, BRL-3A and NRK-52E cell lines did not survive in the presence of trypsin. **(D)** Infectious titers ($TCID_{50}$/ml) of SADS-CoV secreted from HeLa, Vero, Tb-1, BHK-21 PK-15 and MDCK cells were determined on Vero cells.

Figure 3. SADS-CoV infection of mice. C57BL/6J WT mouse were infected per orally (p.o; black) or intraperitoneally (i.p; red) with 5×10^5 $TCID_{50}$ of purified SADS-CoV. Viral loads in different tissue samples including **(A)** stomach, **(B)** small intestinal segments, **(C)** large intestinal segments, **(D)** lymphoid tissues, **(E)** the other organs (liver, kidney, heart and lung) and **(F)** feces collected at 1, 3, 5, 7, 14, 21, and 28 days post-infection (dpi) were determined by qRT-PCR; MLN: Mesenteric lymph nodes. Data are from three independent experiments, and each symbol represents titers from an individual sample (*: $p<0.05$). The limit of detection was 1×10^2 genome copies/mg. **(G)** SADS-CoV IgG antibodies were detected in serum samples collected at euthanasia, using an ELISA based on purified SADS-CoV virus particles.

Figure 4. SADS-CoV replication in mouse splenocytes. (A) Hematoxylin & eosin staining (HE) and immunohistochemistry (IHC) were performed on sections of spleen from intraperitoneally infected mice and **(B)** Mock-infected mice at 3 dpi, using anti-dsRNA antibodies to identify splenic cells that support active virus replication. **(C)** SADS-CoV infection could also be

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

668 detected by IHC using SADS-CoV nonstructural protein antibody (anti-AC) and structural
669 protein antibody (anti-M). Scale bars=50 μm, except for magnified fields shown on the right side,
670 with scale bars=10 μm. **(D)** SADS-CoV antibody validation in Vero cells for developing the flow
671 cytometry assay. Flow cytometry plots of Vero cells infected with SADS-CoV (MOI=0.1) at 24
672 hpi, staining with anti-N or anti-AC. **(E)** Flow cytometry detection of Nsp3-AC antigens of
673 SADS-CoV in splenocytes from infected mice using anti-AC antibody at 3 dpi. The data are
674 presented as the fold-increase in staining splenocytes from infected mice relative to the
675 mock-infected group for statistical purposes (left panel); *: $p<0.05$. **(F)** Representative
676 FACS plots of panel (E). The solid-line frame gated anti-AC positive splenocytes from p.o. or i.p.
677 inoculated mice. The plot of mock-infected cells stained with secondary antibody only was also
678 shown. **(G)** Isolated mouse splenocytes were infected with SADS-CoV at an MOI=1, and
679 SADS-CoV N protein expression was detected by IFA with anti-N antibody. **(H)** Flow cytometry
680 detection of nonstructural protein antigens of SADS-CoV in infected splenocytes at 48 hpi using
681 anti-AC antibody. The data are presented as the fold-increase in staining cells relative to the
682 mock-infected cells for statistical purposes (left panel); *: $p<0.05$. **(I)** Growth of SADS-CoV in
683 *ex vivo* splenocytes was monitored over 72 hpi by qRT-PCR targeting the SADS-CoV N gene.
684
685 **Figure 5. SADS-CoV infection of dendritic cells in the spleen of mice inoculated via i.p.**
686 **route. (A)** Splenocytes were extracted from infected mice at 3 dpi, and flow cytometry was used
687 to detect nonstructural antigen AC of SADS-CoV with immune cell markers on splenocytes
688 including B cells ($CD19^+$), T cells ($CD4^+$), macrophages ($F4/80^+$), and dendritic cells (DCs,

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

689 $CD11c^{+}$). The data are presented as the fold-increase in positive staining cells from infected mice
690 relative to the mock-infected cells for statistical purposes; ***: $p<0.001$. **(B)**
691 Immunofluorescence assay of SADS-CoV dsRNA and DC marker CD11c in sections of spleen
692 from intraperitoneally (i.p.) infected mice and **(C)** mock-infected mice at 3 dpi. **(D)** SADS-CoV
693 infection could also be detected by IFA using anti-AC and anti-M antibodies. **(E)** The numbers of
694 SADS-CoV-positive DCs in i.p.-infected mice were counted and averaged from 10-15 different
695 visual fields, and **(F)** the proportion of DCs in infected cells was presented with a Venn diagram.
696 Scale bars = 50 μm, except for magnified fields shown on the right side, with scale bars =10 μm.
697
698 **Figure 6. SADS-CoV utilizes an unknown receptor for cellular entry. (A)** MDCK cells
699 overexpressing each of the four known CoV receptors fused with detectable tags (pAPN-Flag,
700 hDPP4-Flag, mCEACAM1a-Flag, or hACE2-GFP) did not confer SADS-CoV infection at 24 h
701 post-transfection of the expression plasmids. At 48 h, SADS-CoV-inoculated cells transfected
702 with pAPN-Flag, hDPP4-Flag, or mCEACAM1a-Flag were co-stained with a mouse anti-FLAG
703 MAb and a rabbit anti-SADS-CoV-N pAb. Alexa Fluor 488-conjugated anti-mouse IgG and
704 Alexa Fluor 594-conjugated anti-rabbit IgG were co-stained for secondary antibody detection,
705 followed by DAPI incubation. For challenged cells transfected with hACE2-GFP,
706 anti-SADS-CoV-N pAb and Alexa Fluor 594-conjugated anti-rabbit IgG were used;
707 magnification=200×. **(B)** Western blot analysis also confirmed the expression of CoV receptors
708 in transfected MDCK cells. **(C)** TGEV-, SARS-CoV-, MERS-CoV- or MHV-spike-mediated
709 pseudovirus entry into MDCK cells overexpressing the corresponding receptor. The pseudovirus

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

entry efficiency was characterized as luciferase activity accompanying the entry. Cells transfected with the empty backbone vector were used as controls. **(D)** Rescue of SADS-CoV in MDCK cells transfected with a SeACoV infectious cDNA clone. Detection of expression of Nsp3-AC and N proteins of SADS-CoV was conducted at 72 h post-transfection by co-staining with a rabbit anti-AC pAb and a mouse anti-N pAb (magnification = 200×). **(E)** Infection of fresh Vero cells with progeny SADS-CoV rescued in MDCK cells. The expression of SADS-CoV N protein was detected by staining with anti-N pAb at 36 hpi.

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Table 1. Summary of human and animal cell lines and their susceptibility to SADS-CoV infection as determined by cytopathic effect (CPE) and IFA.

Cell lines			Without Trypsin		With Trypsin	
Species and/or tissue origin	Name	ATCC® Number	IFA	CPE	IFA	CPE
Human						
Hepatocellular carcinoma	Huh-7	N/A	++	-	++	-
Hepatocellular carcinoma	HepG2/C3A	HB-8065	+	-	++	-
Embryonic kidney	293T	CRL-11268	+	-	N/D	N/D
Lung carcinoma	A549	CCL-185EMT	+	-	++	-
Cervix adenocarcinoma	HeLa	CCL-2	+	-	+	-
Monkey						
African green monkey kidney	Marc-145	N/A	+	-	++	+
African green monkey kidney	Cos-7	CRL-1651	++	-	+++	++
African green monkey kidney	BSC-40	CRL-2761	++	-	+++	++
African green monkey kidney	Vero	CRL-1586	++	+	+++	+++
Swine						
Testis	ST	CRL-1746	+	+	++	++
Kidney	PK15	CCL-33	+	-	++	+
Kidney	LLC-PK1	CL-101	+	-	++	+
Small intestinal epithelium	IPEC-J2	N/A	+	-	+	-
Bat						
Myotis petax, fetal kidney	BFK	N/A	-	-	-	-
Tadarida brasiliensis, lung	Tb-1	CCL-88	+	-	++	-
Canine						
Kidney	MDCK	CCL-34	-	-	-	-
Mouse						
Embryo fibroblasts	NIH/3T3	CRL-1658	+	-	N/D	N/D
Monocyte/macrophage	RAW 264.7	TIB-71	-	-	-	-
Hamster						
Syrian golden hamster, kidney	BHK-21	CCL-10	+	-	+++	+
Chinese hamster, ovary	CHO	CCL-61	+	-	N/D	N/D
Rat						
Liver	BRL 3A	CRL-1442	++	+	N/D	N/D
Kidney	NRK-52E	CRL-1571	+	-	N/D	N/D
Gerbil						
Primary kidney cells		N/A	++	-	N/D	N/D
Chicken						
Embryo fibroblasts	DF-1	CRL-12203	+	-	N/D	N/D

Degree of infection as determined by IFA or CPE (-: No infection or obvious lesion≤1%; +: ≤25%; ++: ≤50%; +++: ≤75%; ++++: ≤100%);

N/A: Not available; N/D: Not detected due to cell sensitivity to trypsin.

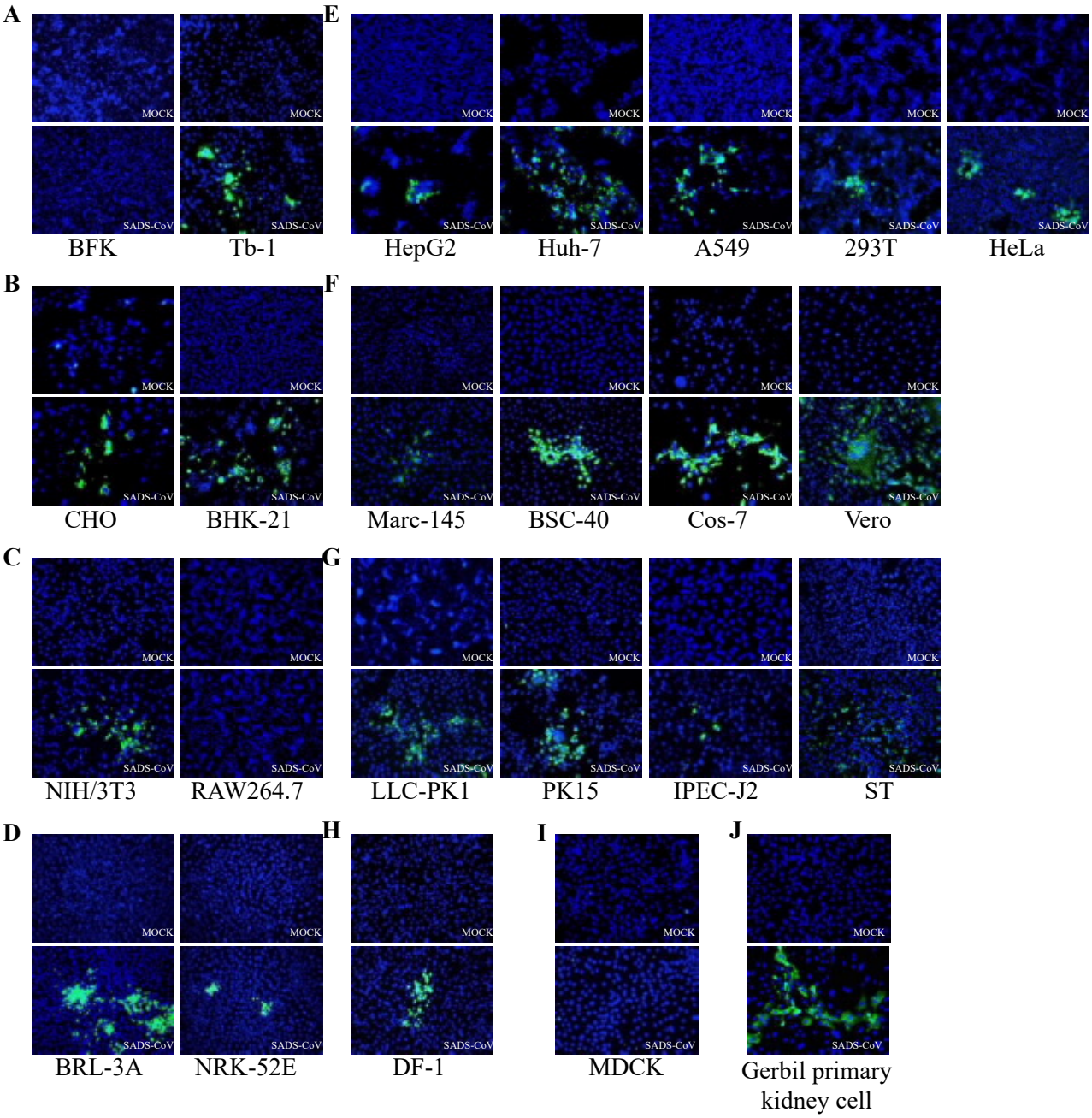
Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Figure 1



Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

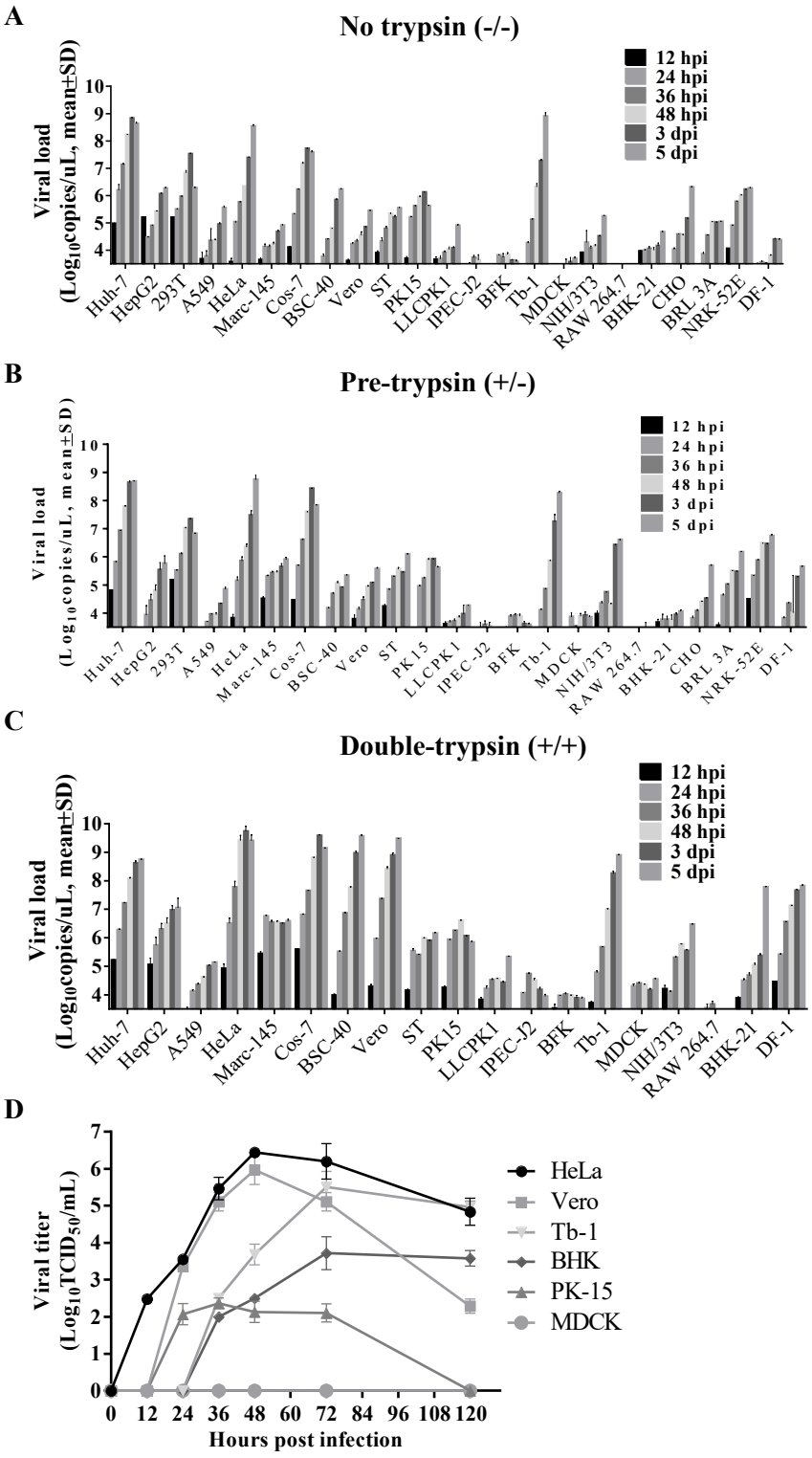
Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

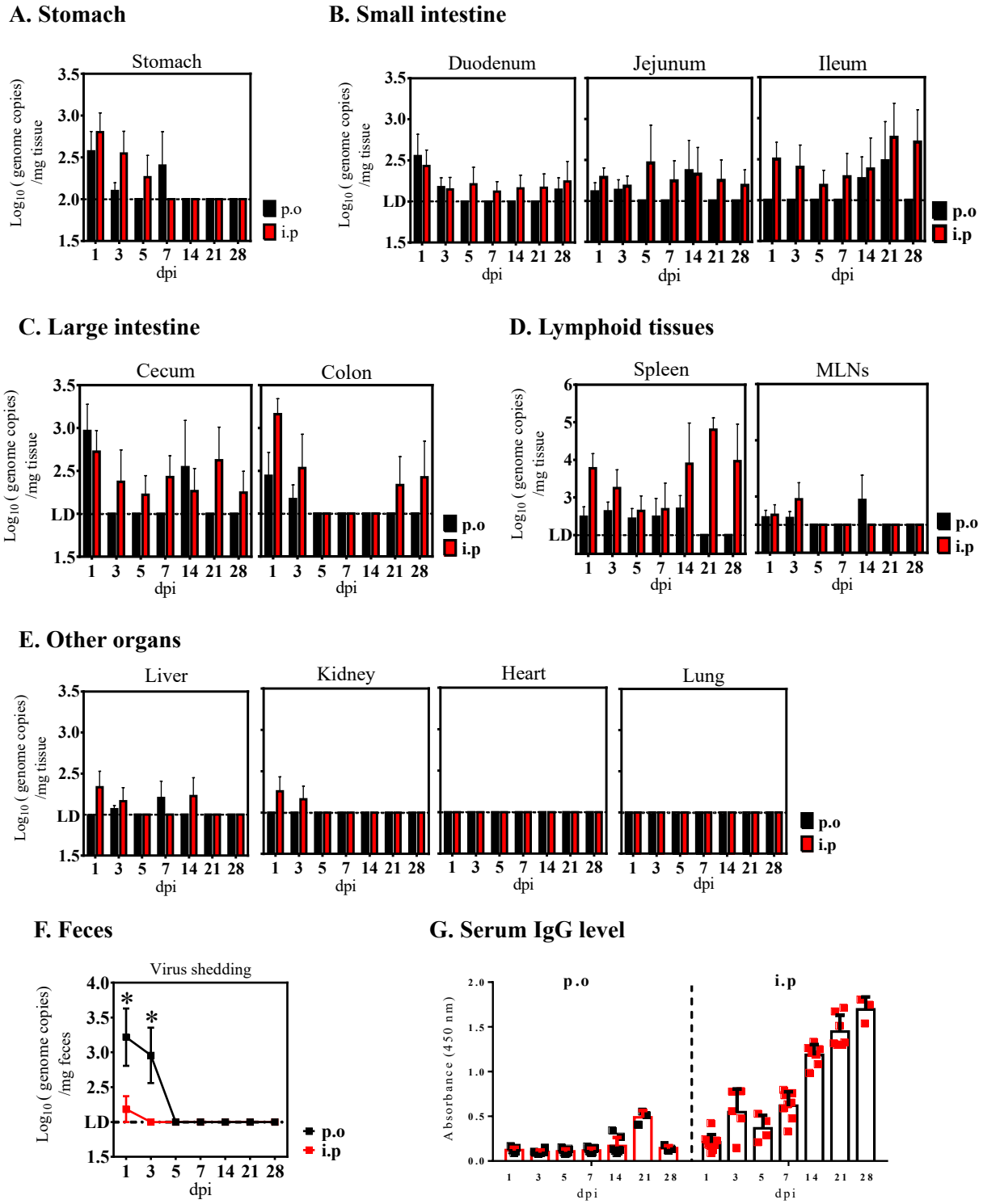
Figure 2



Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Accepted Manuscript Posted Online
Journal of Virology
JVI
Journal of Virology

Figure 3



Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

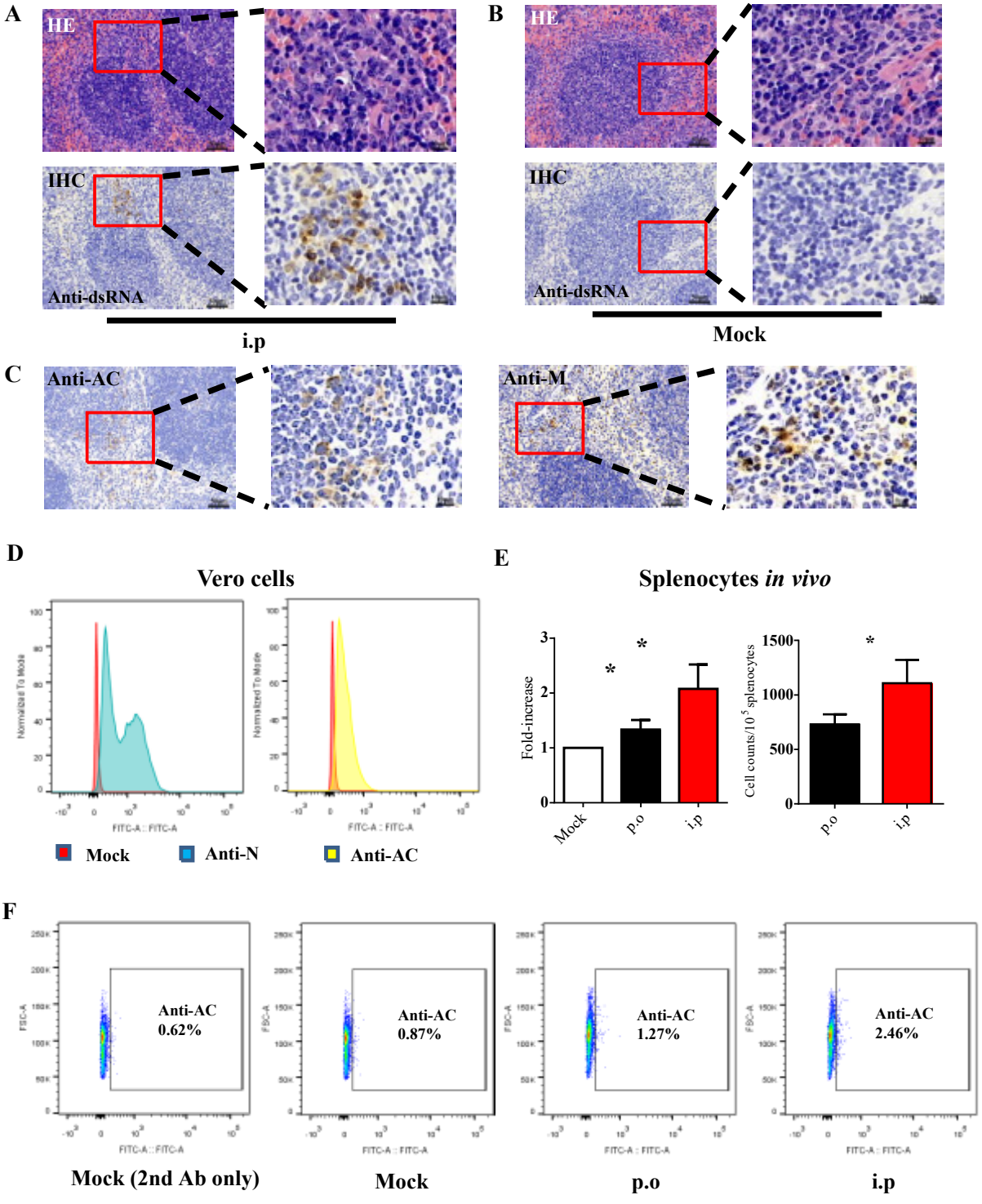
Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

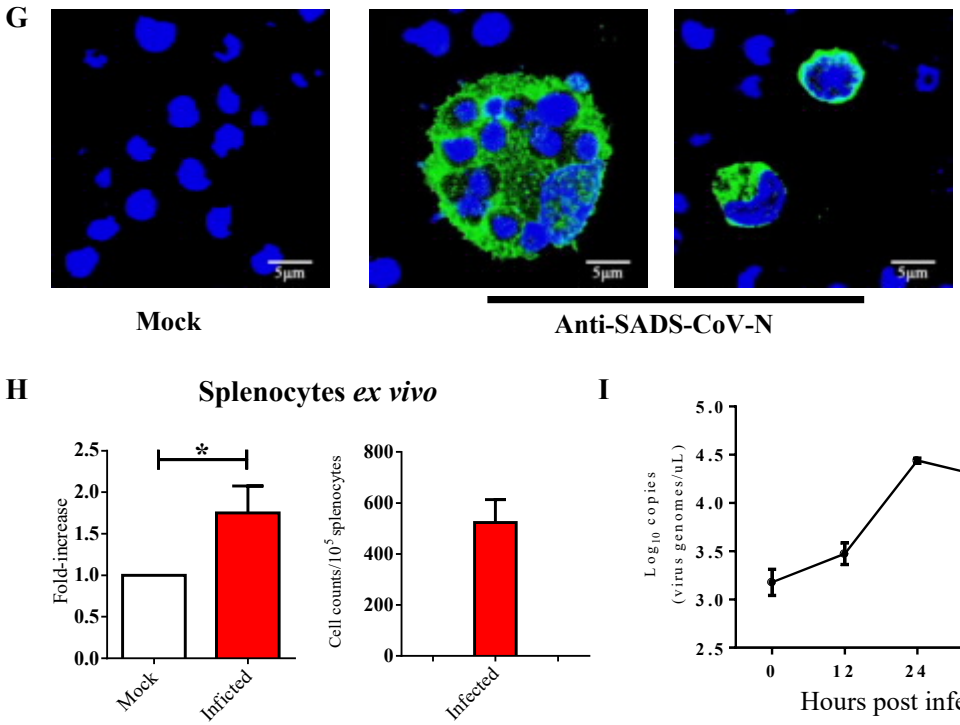
Figure 4



Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Accepted Manuscript Posted Online

Figure 4 (continued)



Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Journal of Virology

JVI

Journal of Virology

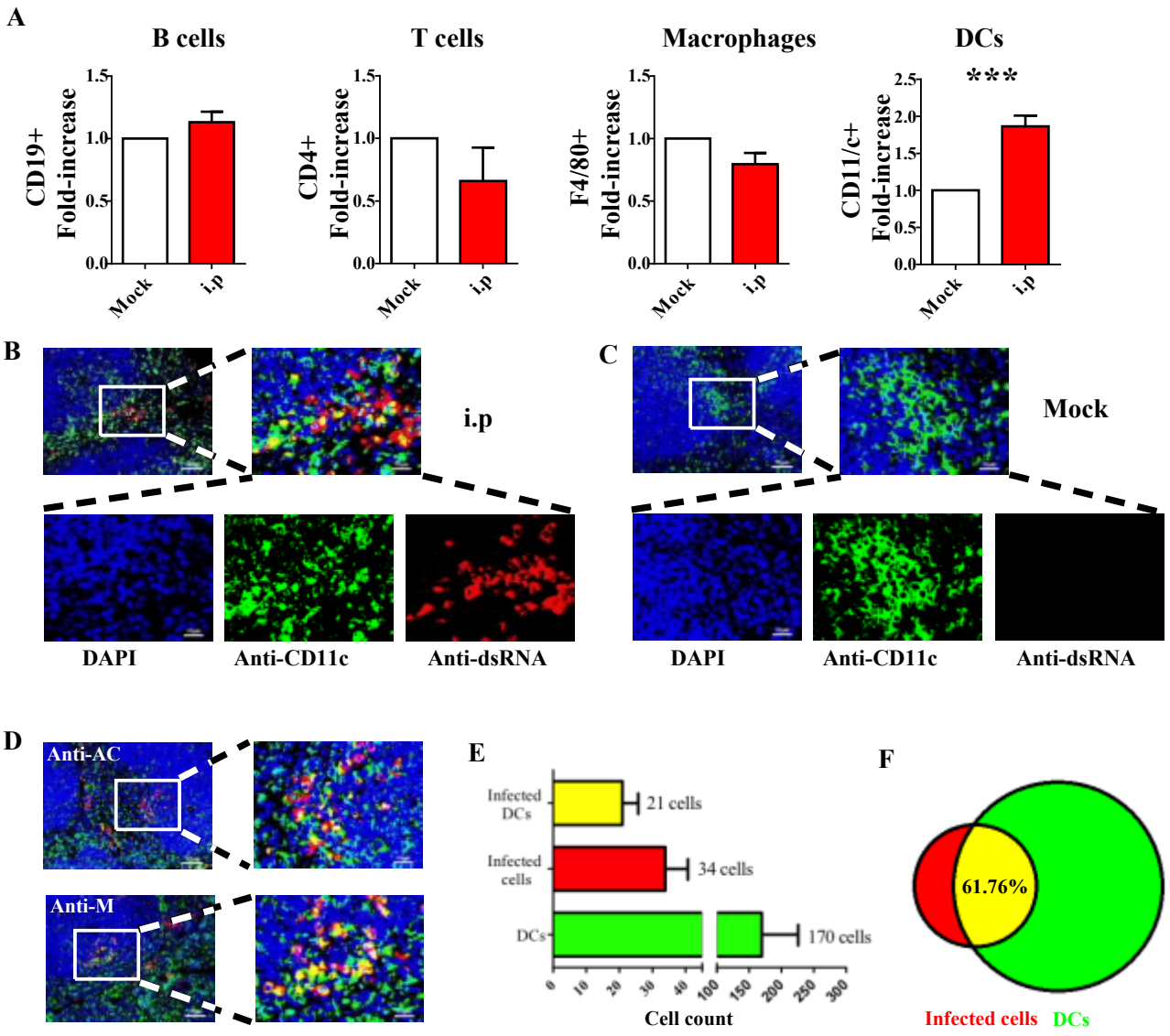
Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Figure 5



Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

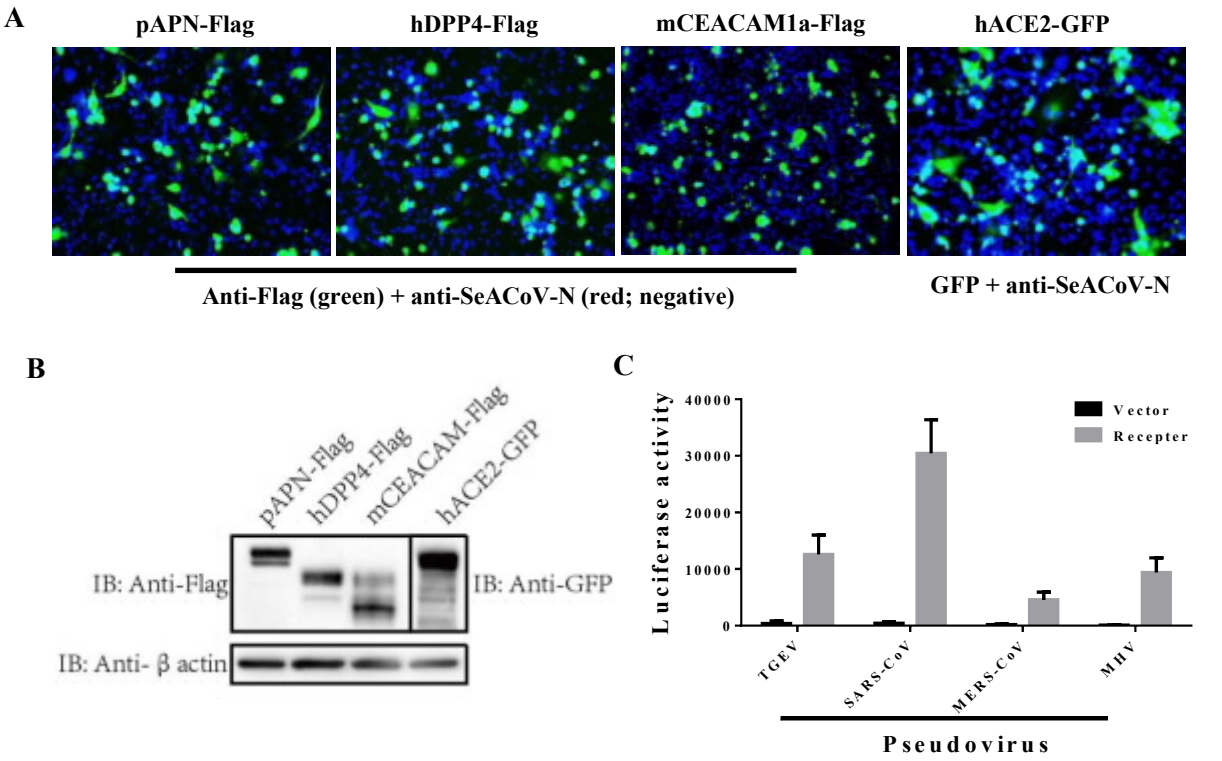
Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Figure 6



Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Received: 25 March 2019 | Revised: 26 May 2019 | Accepted: 11 June 2019

DOI: 10.1111/tbed.13270

Check for updates

SHORT COMMUNICATION

Transboundary and Emerging Diseases WILEY

The re-emerging of SADS-CoV infection in pig herds in Southern China

Ling Zhou[1,2] | Qian N. Li[1,2] | Jiang N. Su[1] | Gui H. Chen[1] | Zi X. Wu[1] | Ying Luo[1] | Rui T. Wu[1,2] | Yuan Sun[1,2] | Tian Lan[1] | Jing Y. Ma[1,2]

[1]College of Animal Science, South China Agricultural University, Guangzhou, China

[2]Key Laboratory of Animal Health Aquaculture and Environmental Control, Guangzhou, China

Correspondence
Jing Y. Ma, College of Animal Science, South China Agricultural University, Tianhe District, Wushan Road 483, Guangzhou 510642, China.
Email: majy2400@scau.edu.cn

Funding information
National Key Research and Development Program of China, Grant/Award Number: 2016YFD0501304; Science and Technology Program of Guangzhou City of China, Grant/Award Number: 201904010433

Abstract

A new highly virulent swine acute diarrhoea syndrome coronavirus (SADS-CoV) emerged in Guangdong province in 2017 followed by fatal diarrhoea that involved the death of 24,693 piglets. And yet from May 2017 to January 2019, there were no new SADS cases arising in pig herds in Guangdong. In this study, we reported the recent diarrhoea outbreak of SADS-CoV in Southern China on February 2019. Intestinal samples collected from diarrhoeal piglets were detected for common swine virus and confirmed that SADS-CoV was responsible for the diarrhoea case. Meanwhile, serological investigation of sows' sera implied that SADS-CoV has existed in the farm and PEDV antibody may not directly contribute to the amplification of SADS-CoV. Homology and phylogenetic analysis of the whole genome showed that the re-emerging SADS-CoV strain shared high sequence identities with existing SADS-CoV strains and all strains clustered together in Alpha coronavirus. All in all, the report herein emphasized the re-emerging of SADS-CoV and highlights continuous monitoring for this virus.

KEYWORDS
phylogenetic analysis, re-emerging, SADS-CoV, Southern China

1 | INTRODUCTION

Swine acute diarrhoea syndrome (SADS) was first identified in South China in January 2017 as a disease causing high mortality in neonatal piglets (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018). It is quickly demonstrated that the causative agent of SADS is a novel HKU2-related coronavirus, swine acute diarrhoea syndrome coronavirus (SADS-CoV) which originated from the same genus of horseshoe bats (*Rhinolophus*) as severe acute respiratory syndrome coronavirus (SARS-CoV), but the mechanism of transmission of bat-originated coronavirus in pigs needs further illumination (Cui, Li, & Shi, 2019; Zhou et al., 2018). Based on the analysis of genomes, SADS-CoV is classified into the genus *Alphacoronavirus* within the subfamily *Orthocoronavirinae* of the family *Coronaviridae* (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018). So far, besides porcine epidemic diarrhoea virus (PEDV), transmissible gastroenteritis virus (TGEV) and porcine deltacoronavirus (PDCoV), SADS-CoV is the fourth porcine enteric coronavirus that circulated in pigs in China (Dong et al., 2015; Xuan et al., 1984). A retrospective investigation showed that SADS-CoV emerged in China at least since August 2016 (Zhou et al., 2019). Li et al. (2018) reported one SADS-CoV strain that was isolated from fecal and small intestinal samples collected from Fujian province and suggested that SADS-CoV might have spread from Guangdong to Fujian province.

In the first outbreak of SADS-CoV occurring between January and May 2017, in order to reduce mortality in suckling pigs, the minced intestines from acutely infected piglets were filtered, centrifuged and inactivated before feeding back to pregnant sows to allow maternal antibodies to be obtained in neonatal piglets. Hence, after May 2017, the epidemic was controlled. And with a period of quiescence, SADS-CoV seemed to have disappeared from pig herds in South China. While on February 2019, there was a large-scale

 © 2019 Blackwell Verlag GmbH

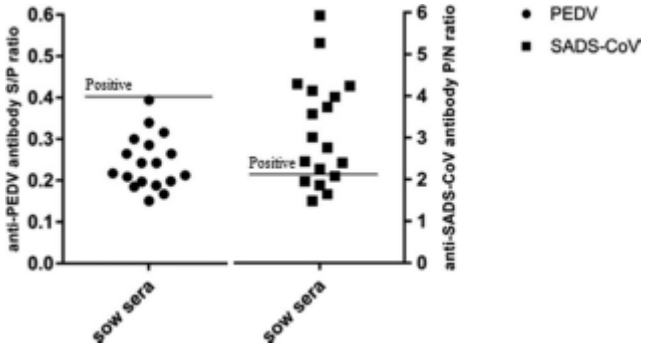


FIGURE 1 The results of ELISA test, *n* = 18 sows sera from outbreak farm. Detection of PEDV antibodies, sample S/P ratios ≥ 0.4 were considered positive. Detection of SADS-CoV antibodies, P/N ratios ≥ 2.1 were considered positive

outbreak of approximately 2000 piglets dying of diarrhoea in one farm, which was near the origin of the SADS pandemic in 2017. The extensive aetiological investigation for common swine viruses suggested that the causative agent of this recent diarrhoea outbreak was SADS-CoV. In this study, we compared the infections of piglets with the original and re-emerging SADS-CoV strains by clinical signs, epidemiological investigation, serological investigation and phylogenetic analysis. The results highlight the significance of SADS-CoV and the disease it causes.

At the beginning of the latest outbreak on February 2019, infected suckling piglets developed acute diarrhoea, vomiting and died after 2–6 days of the diarrhoea, which were consistent with the clinical signs reported in SAD-CoV outbreaking farms (Zhou et al., 2018). Two weeks after the onset, mortality did not increase due to the effective inactivated SADS-CoV vaccine which was propagated in vitro (data unpublished). Clinical small intestine samples were collected rapidly from piglets, and intestinal samples were homogenized, centrifuged and filtered for virus isolate. Viral nucleic acid was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instruction. The potential swine viral pathogens were examined, including SADS-CoV, PEDV, PDCoV, porcine rotavirus (RV), TGEV, reovirus (REO), senecavirus A (SVA), foot and mouth disease virus (FMDV), porcine pseudorabies virus (PRV), porcine circovirus 2 (PCV2), porcine circovirus 3 (PCV3), porcine reproductive

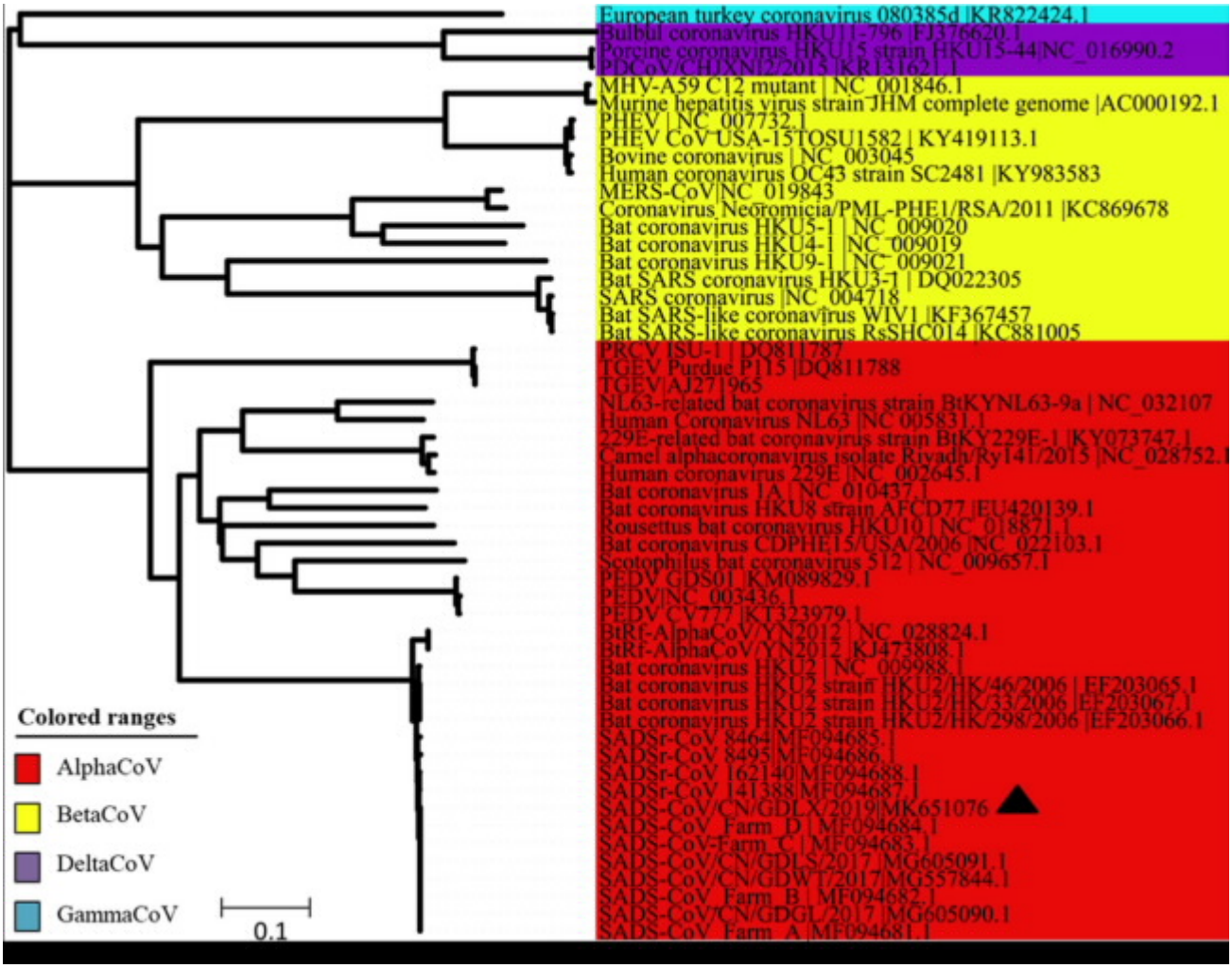


FIGURE 2 Phylogenetic analysis of the complete genome of SADS-CoV and reference coronavirus species. The tree was constructed using MEGA 7.0 software with neighbour-joining methods and 1,000 replicate sets on bootstrap analysis [Colour figure can be viewed at wileyonlinelibrary.com]

and respiratory syndrome virus (PRRSV), porcine parainfluenza virus 1 (PPIV1), swine influenza virus (SIV), atypical porcine pestivirus (APPV), haemophilus parasuis (HPS), hog cholera (HC), porcine parvovirus (PPV), porcine bocavirus (PBoV), porcine sapelovirus (PSV) and porcine kobuvirus (PKV) by PCR or RT-PCR following the established protocol of Key Laboratory of Animal Health Aquaculture and Environmental Control. The extensive aetiological investigations for common swine viruses showed that only SADS-CoV was detected positively, which suggested that the causative agent of the most recent diarrhoea outbreak on February was SADS-CoV.

Furthermore, a total of 18 blood samples were simultaneously collected from healthy sows for serological investigation. Sera samples were obtained after coagulation and centrifugation (2,000 *g*, 10 min) and stored at −20°C until ELISA implemented. The PEDV antibody production was detected by means of commercial ELISA test kit (ZhenRui, China), of which, sample S/P ratios with ≥0.4 were considered positive and <0.4 were considered negative. And for SADS-CoV, ELISA was conducted following the modified procedures established in Key Laboratory of Animal Health Aquaculture and Environmental Control (P/N ≥ 2.1 considered positive). The absorbance at 450 nm wavelength was measured (Multiskan™ FC, Thermo Fisher). The results of ELISA assay revealed that all sera (100%) were negative for the PEDV and 13 of 18 (72.2%) were positive for SADS-CoV (Figure 1). In our previously clinical investigations, PEDV had caused prior outbreaks at most farms with SADS-CoV outbreaks (Zhou et al., 2018). It is possible that the presence of PEDV antibody may have somehow facilitated the amplification of the SADS-CoV. However, our results showed that PEDV antibodies were undetectable in all SADS-CoV antibody-positive sera samples, suggesting that it is SADS-CoV that caused the lethal infection in pigs on February 2019. Collectively, the re-emergence of SADS-CoV in pig herds in South China was confirmed by ELISA test and the association between PEDV and SADS-CoV infection requires further validation.

Subsequently, one SADS-CoV genome was amplified as previously described (Zhou et al., 2019). The novel SADS-CoV strain was named as SADS-CoV/CN/GDLX/2019 (accession number: MK651076). Sequence alignment and phylogenetic analysis were performed with Lasergene coresuite 10 (DNASTAR Inc.). Phylogenetic tree was constructed using the neighbour-joining method with MEGA 7.0 software (Kumar, Stecher, & Tamura, 2016). Bootstrap analysis was carried out on 1,000 replicate data sets

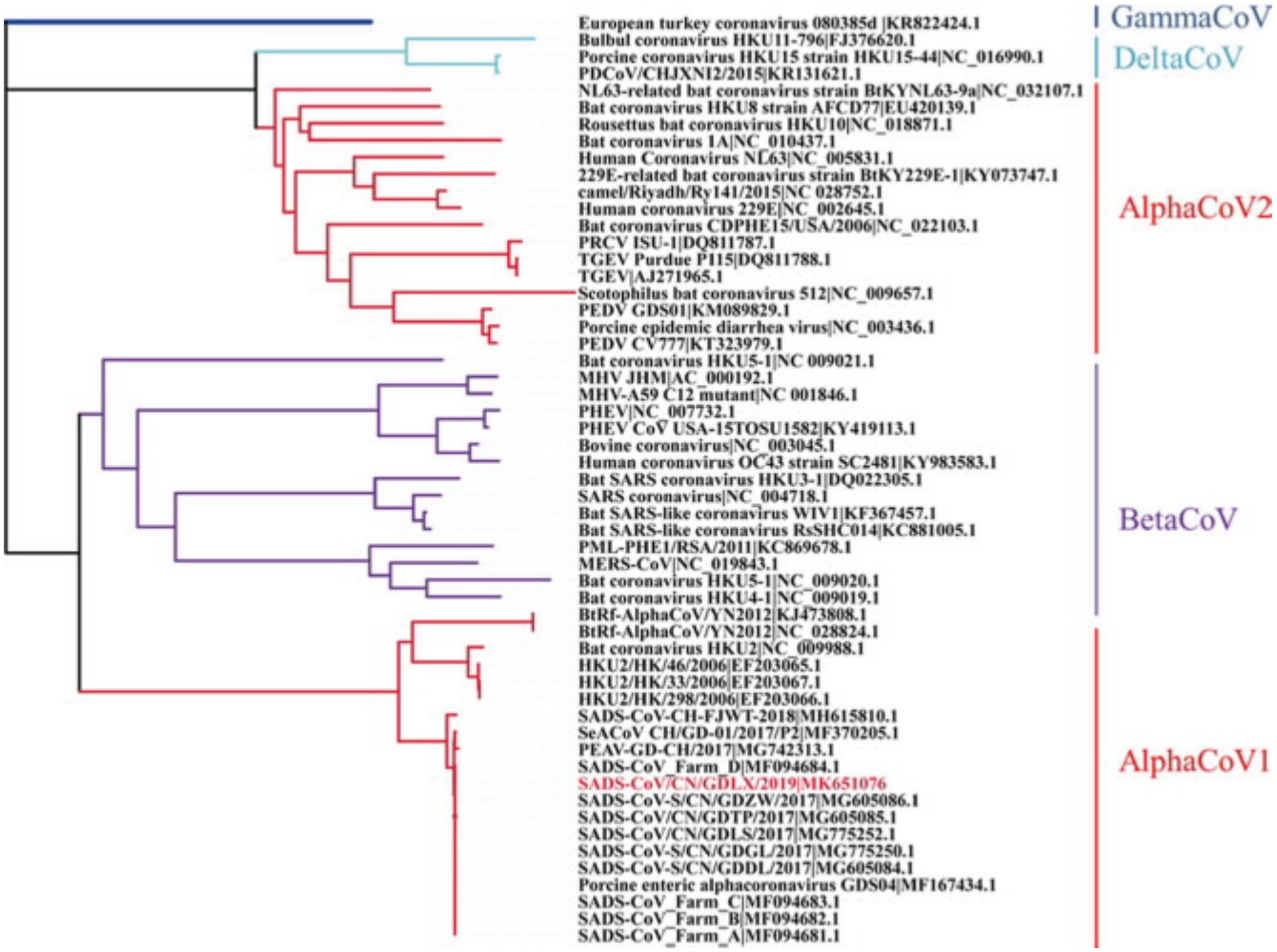


FIGURE 3 Phylogenetic analysis of the S gene of SADS-CoV and reference coronavirus species. The tree was constructed using MEGA 7.0 software with neighbour-joining methods and 1,000 replicate sets on bootstrap analysis [Colour figure can be viewed at wileyonlinelibrary.com]

(Felsenstein, 1985). For the whole genomes, sequence alignment results revealed that the nucleotide identities between SADS-CoV/CN/GDLX/2019 and previously reported SADS-CoV strains ranged from 99.3% to 100%. Moreover, the phylogenetic tree indicated that the novel SADS-CoV complete genome clustered together with other reported SADS-CoV strains and located in the Alpha coronavirus branch (Figure 2). Phylogenetic analysis based on S genes was also conducted, and the result showed that all SADS-CoV, bat HKU2 and BtRf-AlphaCoV strains were separated from other AlphaCoVs and clustered within BetaCoVs (Figure 3), which is consistent with previous studies (Pan et al., 2017; Xu et al., 2019; Zhou et al., 2019). Notably, the S gene of SADS-CoV/CN/GDLX/2019 had high identities of 99.2%–99.9% with other SADS-CoV strains detected in Guangdong and lowest identities of 97.5% with SADS-CoV-CH-FJWT-2018 reported from Fujian. Additionally, the comparative analysis of amino acids encoded by S gene revealed that the S protein of the novel strain had a two-amino-acid mutation (F306 and I882) compared with that of other SADS-CoV strains. It is well known that the S protein of coronavirus play an important role in cellular entry and determining the immunogenicity (Li, Frank, He, Peter, & Berend-Jan, 2016). In view of the lowest identities of Spike gene between the strains from Guangdong and Fujian, continuous monitoring of SADS-CoV genetic evolution is important for better understanding its epidemiology and establishing efficient strategies against its further circulating in pigs in China.

Here, we reported a new case of acute diarrhoea outbreak from a swine farm in Guangdong province on February 2019. Pathogenic and serologic analysis determined that SADS-CoV re-emerged in Guangdong and was responsible for the recent acute diarrhoea outbreak. Phylogenetic analysis based on whole genomes showed that SADS-CoV/CN/GDLX/2019 shared high nucleotide identities with previously reported SADS-CoV strains. Taken together, our study highlighted the significant threat of SADS-CoV and the potential outbreak of SADS epidemic in following months in Guangdong. Therefore, the persistent surveillance and vaccination countermeasures will be necessary to control the spread of SADS-CoV.

ACKNOWLEDGEMENTS

This work was supported by the National Key Research and Development Program of China (No. 2016YFD0501304). The Research and Extension of Major Animal Epidemic Prevention and Control Technologies in the Strategic Project of Rural Revitalization of Guangdong Agricultural Department of China (Building Modern Agricultural System) (2018–2020) and the Science and Technology Program of Guangzhou City of China (No. 201904010433).

CONFLICT OF INTEREST

The authors declare no conflict of interests with any organization.

REFERENCES

Cui, J., Li, F., & Shi, Z. (2019). Origin and evolution of pathogenic coronavirus. *Nat Rew Microbiol*, *17*, 181–192.

Dong, N., Fang, L., Zeng, S., Sun, Q., Chen, H., & Xiao, S. (2015). Porcine Deltacoronavirus in mainland China. *Emerging Infectious Diseases*, *21*, 2254–2255. https://doi.org/10.3201/eid2112.150283

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, *39*, 783–791.

Gong, L., Li, J., Zhou, Q., Xu, Z., Chen, L., Zhang, Y., … Cao, Y. (2017). A new bat-HKU–like coronavirus in swine, China, 2017. *Emerging Infectious Diseases*, *23*, 1607–1609.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* , *33*, 1870–1874.

Li, K., Li, H., Bi, Z., Gu, J., Gong, W., Luo, S., … Tang, Y. (2018). Complete genome sequence of a novel swine acute diarrhea syndrome coronavirus, CH/FJWT/2018, isolated in fujian, china, in 2018. *Microbiol Resour Announc*, *7*(22), e01259–18.

Li, W., Frank, J., He, Q., Peter, J., & Berend-Jan, B. (2016). Cellular entry of the porcine epidemic diarrhea virus. *Virus Research*, *226*, 117–127. https://doi.org/10.1016/j.virusres.2016.05.031

Pan, Y., Tian, X., Qin, P., Wang, B., Zhao, P., Yang, Y.-L., … Huang, Y.-W. (2017). Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. *Veterinary Microbiology*, *211*, 15–21. https://doi.org/10.1016/j.vetmic.2017.09.020

Xu, Z., Zhang, Y., Gong, L., Huang, L., Lin, Y., Qin, J., … Cao, Y. (2019). Isolation and characterization of a highly pathogenic strain of Porcine enteric alphacoronavirus causing watery diarrhoea and high mortality in newborn piglets. *Transboundary and Emerging Diseases*, *66*, 119–130.

Xuan, H., Xing, D., Wang, D., Zhu, W., Zhao, F., Gong, H., & Fei, S. (1984). Study on the culture of porcine epidemic diarrhea virus adapted to fetal porcine intestine primary cell monolayer. *Chin. J. Vet. Sci.*, *4*, 202–208.

Zhou, L., Sun, Y., Lan, T., Wu, R., Chen, J., Wu, Z., … Ma, J. (2019). Retrospective detection and phylogenetic analysis of swine acute diarrhoea syndrome coronavirus in pigs in southern China. *Transboundary and Emerging Diseases*, *66*, 687–695. https://doi.org/10.1111/tbed.13008

Zhou, P., Fan, H., Lan, T., Yang, X.-L., Shi, W.-F., Zhang, W., … Ma, J.-Y. (2018). Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. *Nature*, *556*, 255–258. https://doi.org/10.1038/s41586-018-0010-9

How to cite this article: Zhou L, Li QN, Su JN, et al. The re-emerging of SADS-CoV infection in pig herds in Southern China. *Transbound Emerg Dis*. 2019;66:2180–2183. https://doi.org/10.1111/tbed.13270

Received: 19 March 2018 | Revised: 3 August 2018 | Accepted: 29 August 2018

DOI: 10.1111/tbed.13008

ORIGINAL ARTICLE

WILEY Transboundary and Emerging Diseases

Retrospective detection and phylogenetic analysis of swine acute diarrhoea syndrome coronavirus in pigs in southern China

Ling Zhou[1,2,*] | Yuan Sun[1,2,*] | Tian Lan[1] | Ruiting Wu[1,2] | Junwei Chen[3] | Zixian Wu[3] | Qingmei Xie[1,2] | Xiangbin Zhang[1,2,3] | Jingyun Ma[1,2]

[1]College of Animal Science, South China Agricultural University, Guangzhou, China

[2]Key Laboratory of Animal Health Aquaculture and Environmental Control, Guangzhou, Guangdong, China

[3]Guangdong Wen's Foodstuffs Group Co., Ltd., Yunfu, Guangdong, China

Correspondence
Xiangbin Zhang and Jingyun Ma, College of Animal Science, South China Agricultural University, Guangzhou, China.
Emails: zhangxb@scau.edu.cn; majy2400@scau.edu.cn

Funding information
National Key Researchand Development Program of China, Grant/Award Number: 2016YFD0501304

Summary

Swine acute diarrhoea syndrome coronavirus (SADS-CoV), a novel coronavirus, was first discovered in southern China in January 2017 and caused a large scale outbreak of fatal diarrheal disease in piglets. Here, we conducted a retrospective investigation of 236 samples from 45 swine farms with a clinical history of diarrheal disease to evaluate the emergence and the distribution of SADS-CoV in pigs in China. Our results suggest that SADS-CoV has emerged in China at least since August 2016. Meanwhile, we detected a prevalence of SADS-CoV (43.53%), porcine deltacoronavirus (8.83%), porcine epidemic diarrhoea virus (PEDV) (78.25%), rotavirus (21.77%), and transmissible gastroenteritis virus (0%), and we also found the co-infection of SADS-CoV and PEDV occurred most frequently with the rate of 17.65%. We screened and obtained two new complete genomes, five N and five S genes of SADS-CoV. Phylogenetic analysis based on these sequences revealed that all SADS-CoV sequences in this study clustered with previously reported SADS-CoV strains to form a well defined branch that grouped with the bat coronavirus HKU2 strains. This study is the first retrospective investigation for SADS-CoV and provides the epidemiological information of this new virus in China, which highlights the urgency to develop effective measures to control SADS-CoV.

KEYWORDS
phylogenetic analysis, prevalence, retrospective detection, Swine Acute Diarrhoea Syndrome Coronavirus

1 | INTRODUCTION

Swine acute diarrhoea syndrome coronavirus (SADS-CoV) is a newly discovered coronavirus which is an enveloped, positive and single-stranded sense RNA virus with a genome size of approximately 27 kb (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018). SADS-CoV belongs to the family Coronaviridae which contains four genera, Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus (Woo, Huang, Lau, & Yuen, 2010; Woo et al., 2012). So far, six coronaviruses have been identified from pigs, which include porcine epidemic diarrhoea virus (PEDV), porcine respiratory coronavirus (PRCV), SADS-CoV and transmissible gastroenteritis virus (TGEV) that all belong to the Alphacoronavirus genus, as well as one betacoronavirus, porcine hemagglutinating encephalomyelitis virus (PHEV) and one deltacoronavirus, porcine deltacoronavirus (PDCoV) (Lin, Saif, Marthaler, & Wang, 2016; Wesley, Woods, & Cheung, 1991; Woo et al., 2010). Among these viruses, SADS-CoV is the most newly discovered coronavirus, which has been first reported in 2017 in China and is considered to be an HKU2-related coronavirus with a bat-origin (Gong et al., 2017; Zhou et al., 2018). In January 2017, SADS-CoV was detected in a swine farm and subsequently spread rapidly to three other farms in

*These authors contributed equally to this work.

 © 2018 Blackwell Verlag GmbH

Guangdong Province and caused the fatal swine acute diarrhoea syndrome (SADS) characterized by the clinical signs with severe, acute diarrhoea and rapid weight loss of piglets. The symptoms of SADS-CoV are similar to those that caused by other swine enteric coronaviruses such as PDCoV and PEDV, but SADS-CoV is more harmful than these viruses because it has led to the death of almost 25,000 piglets in a short time and resulted in more significant economic losses (Dong et al., 2015; Sun, Wang, Wei, Chen, & Feng, 2016; Zhou et al., 2018). So, it is urgent to investigate the molecular epidemiology and transmission patterns of SADS-CoV for establishing effective controls for this new coronavirus.

In the present study, we performed the retrospective PCR testing on diarrheal samples from 45 swine farms in Guangdong Province to evaluate the emergence and the distribution of SADS-CoV in pigs in China. The prevalence and co-infection information of SADS-CoV from eleven SADS-CoV-positive farms was provided. The sequences of SADS-CoV, including two complete genomes, five nucleocapsid protein (N) genes and five spike protein (S) genes, were also identified and characterized to investigate the phylogenetic relationships of SADS-CoV.

2 | MATERIALS AND METHODS

2.1 | Sample collection

A total of 236 clinical diarrhoea samples including faeces and intestinal contents were collected from piglets and sows from 45 swine farms in the cities Qingyuan and Shaoguan of North Guangdong Province between August 2016 and May 2017 in accordance with the recommendations of National Standards for Laboratory Animals of the People's Republic of China (GB149258-2010). Samples were preserved at - 80 °C from the time of original receipt until use.

2.2 | Nucleic acid extraction and molecular diagnosis

Samples were homogenized in phosphate-buffered saline (PBS) (20% w/v), frozen and thawed three times, then centrifuged for 10 min at 10,000 *g*. Viral nucleic acid was extracted following the manufacturer's recommendations of AxyPrep™ Body Fluid Viral DNA/RNA Miniprep Kit (Axygen Scientific, Inc). The virus nucleic acid was stored at - 80°C until PCR was performed. A pair of primers (forward primer 5′-GGTCCCTGTGACCGAAGTTTTA-3′, reverse primer 5′- GCGTTCTGCGATAAAGCTTAAAACTATTA-3′) was designed to detected SADS-CoV based on the conserved N gene of this virus. One step RT-PCR using PrimeScript™ One Step RT-PCR Kit Ver.2 with Dye Plus (Takara, Biotechnology, Dalian, China) was carried out to amplify the target fragments by the following thermal profile of 50°C for 30 min, 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, an extension at 72°C for 30 s, and a final step of 72°C for 5 min. Four other diarrheal pathogens including PEDV, PDCoV, rotavirus (RV), and TGEV from SADS-CoV-positive farms were also tested by RT-PCR according to the previously described methods (Liu, Zhu, Liao, Xu, & Zhou, 2015; Mai et al., 2017; Stevenson et al., 2013).

2.3 | Amplification of the N gene, the S gene, and the complete genome of SADS-CoV

Specific primer pairs based on reported SADS-CoV strains (GenBank accession numbers: MF094681-MF094684) were designed for S genes, N genes and complete genome amplifications, respectively (Table S1). PCR assays were performed with the following thermal profile: 95°C for 5 min, 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min 15 s, followed by a final 10 min extension at 72°C. The products were purified following the manufacturer's instructions of Gel Band Purification Kit (Omega Bio-tek, USA) and then cloned into the PMD19-T vector (Takara, Biotechnology, Dalian, China) and transformed *E. coli* DH5α competent cells. The positive clones were screened out and sent to Beijing Genomics Institute (Shenzhen, Guangdong, China) for further sequencing.

2.4 | Sequence alignment and phylogenetic analysis

The nucleotide sequences were assembled and aligned using the DNASTAR program (DNAStar V7.1, Madison, WI, USA). Phylogenetic trees were constructed using the neighbour-joining method in MEGA 7.0 software with bootstrap analysis of 1,000 replicates. Percentages of replicate trees in which the associated taxa clustered are shown as nearby branches (Chenna et al., 2003; Kumar, Stecher, & Tamura, 2016; Tamura, Nei, & Kumar, 2004).

3 | RESULTS

3.1 | SADS-CoV detection

Of 236 clinical diarrhoea samples from 45 swine farms, 53 samples collected from eleven swine farms between August 2016 and May 2017 were tested positive for SADS-CoV and the locations of these eleven farms were indicated in Figure 1. The farms SM, GL, DL, DE, TP, WT, DCD, and SC were in Qingyuan, and the farms LS, ZW, and YX were in Shaoguan. From these eleven SADS-CoV-positive farms, a total of 170 archived diarrheal samples including 55 faeces and 115 intestinal contents were collected to further investigate diarrhoeal pathogens including SADS-CoV, PEDV, RV, PDCoV, and TGEV. The infection rates of these five pathogens in order were 43.5% (74/170), 78.2% (133/170), 21.8% (37/170), 8.8% (15/170), and 0% (0/170). SADS-CoV was tested positively both in the intestinal and faecal samples and the detection rates were 49.6% (57/115) and 30.9% (17/55), respectively, which were both the second highest among the five tested pathogens. PEDV had the highest positive rate at 84.3% (97/115) for intestinal and 65.5% (36/55) for faecal samples. TGEV was tested negatively in all 170 diarrheal samples (Table S2).

The cases of individual infection or co-infection for SADS-CoV, PDCoV, PEDV, and RV were also detected. More than half the

FIGURE 1 Locations of positive farms for study of SADS-CoV in Guangdong Province, China [Colour figure can be viewed at wileyonlinelibrary.com]

samples were only infected by one of the four viruses at a rate of 58.82% (100/170), and the individual infection of PEDV had the highest rate of 37.06% (63/170). In the 74 SADS-CoV-positive samples, 28 samples were infected alone by SADS-CoV, and the rest 46 samples were infected by SADS-CoV combined with one to three other pathogens. The co-infection of SADS-CoV and PEDV occurred most frequently with the rate of 17.65% (30/170) (Table 1).

The earliest cases with SADS-CoV-positive infection were found in the farms LS, TP and ZW on August 2016 according to Table S2. The intestinal contents from these eleven farms were tested positive for SADS-CoV and the positive rates ranged from 25% to 77.78%. While the faecal samples from two farms ZW and YX were tested negative for SADS-CoV, but positive for PDCoV, PEDV, and RV infections. The positive rates of SADS-CoV in the faecal samples of other nine farms varied from 13.33% to 100%. The farm LS had the highest positive rate of 77.8% (7/9) for intestinal samples and the farms DCD and SC had 100% positive rates for faecal samples.

3.2 | Sequence identities and phylogenetic analyses

The two complete genomes (accession number MG605090 and MG605091), five N genes (accession number MG605087, MG605088, MG605089, MG775251, and MG775253) and five S genes (accession number MG605084, MG605085, MG605086, MG775250 and MG775252) were sequenced and available in GenBank. The lengths of the complete genome, N gene and S gene were 27,163 bp, 1,128 bp, and 3,390 bp, respectively. The two new complete sequences, five N genes and five S genes sequences each shared high sequence identities of 99.8%–100%, 99.8%–100%, and 99.6%–100% with previous reported complete genomes, N genes and S genes of SADS-CoV. The two full-length sequences of SADS-CoV showed 95%–95.1% nucleotide identities with four bat coronavirus HKU2 strains, and the nucleotide identities between the N genes of SADS- CoV studied here and those of bat coronavirus HKU2 strains ranged from 94.2% to 94.3%. The S genes of SADS-CoV in this paper shared low nucleotide identities of 79.9%–80% with the S genes of bat coronavirus HKU2 strains. Phylogenetic analyses based on the complete genomes, N genes and S genes indicated that all SADS-CoV sequences clustered together to form a well-defined branch and group with four bat coronavirus HKU2 strains In the phylogenetic trees of the full-length genomes and N genes, all SADS-CoV strains were phylogenetically located within the AlphaCoVs, while the phylogenetic tree of S genes exhibited that all SADS-CoV sequences, four bat HKU2 and two BtRf-AlphaCoV strains separated from other AlpaCoVs sequences and clustered with the BeltaCoV sequences (Figures 2, 3 and 4).

4 | DISCUSSION

In this study, we performed the retrospective investigation of SADS-CoV in 45 pig farms from Guangdong Province based on 236

TABLE 1 Screening information of SADS-CoV, and co-infections of different pathogens detected in north Guangdong Province of China between August 2016 and May 2017

Months of year	One pathogen positive samples no.(%, *n* = 170)					Two pathogens positive samples no.(%, *n* = 170)			Three pathogens positive samples no.(%, *n* = 170)			Four pathogens positive samples no.(%, *n* = 170)	SADS-CoV positive farms (Positive/Total)
	SADS-CoV	PEDV	RV	PDCoV	TGEV	SADS-CoV+ PEDV	PEDV+ RV	PEDV+ PDCoV	SADS-CoV+ PEDV+ RV	SADS-CoV+ PEDV+ PDCoV	PEDV+ RV+ PDCoV	SADS-CoV+ PEDV+ RV+ PDCoV	
Aug, 2016	3	4	0	0	0	2	3	1	0	0	1	0	11/45
Sep, 2016	0	6	0	2	0	0	1	1	0	0	0	0	
Oct, 2016	0	2	1	0	0	0	3	0	0	0	0	0	
Nov, 2016	1	8	1	1	0	1	4	1	1	1	0	2	
Dec, 2016	2	8	0	1	0	3	0	1	3	0	0	0	
Jan,2017	2	3	2	0	0	10	3	0	2	0	0	0	
Feb, 2017	6	7	0	0	0	4	3	1	2	0	0	0	
Mar, 2017	3	9	0	1	0	4	0	0	4	0	0	0	
Apr, 2017	10	10	0	0	0	6	0	0	0	1	0	0	
May,2017	1	6	0	0	0	0	1	0	0	0	0	0	
Total	28 (16.47%)	63 (37.06%)	4 (2.35%)	5 (2.94%)	0 (0%)	30 (17.65%)	18 (10.59%)	5 (2.94%)	12 (7.06%)	2 (1.18%)	1 (0.59%)	2 (1.18%)	
	SADS-CoV positive samples: 74(43.53%)												

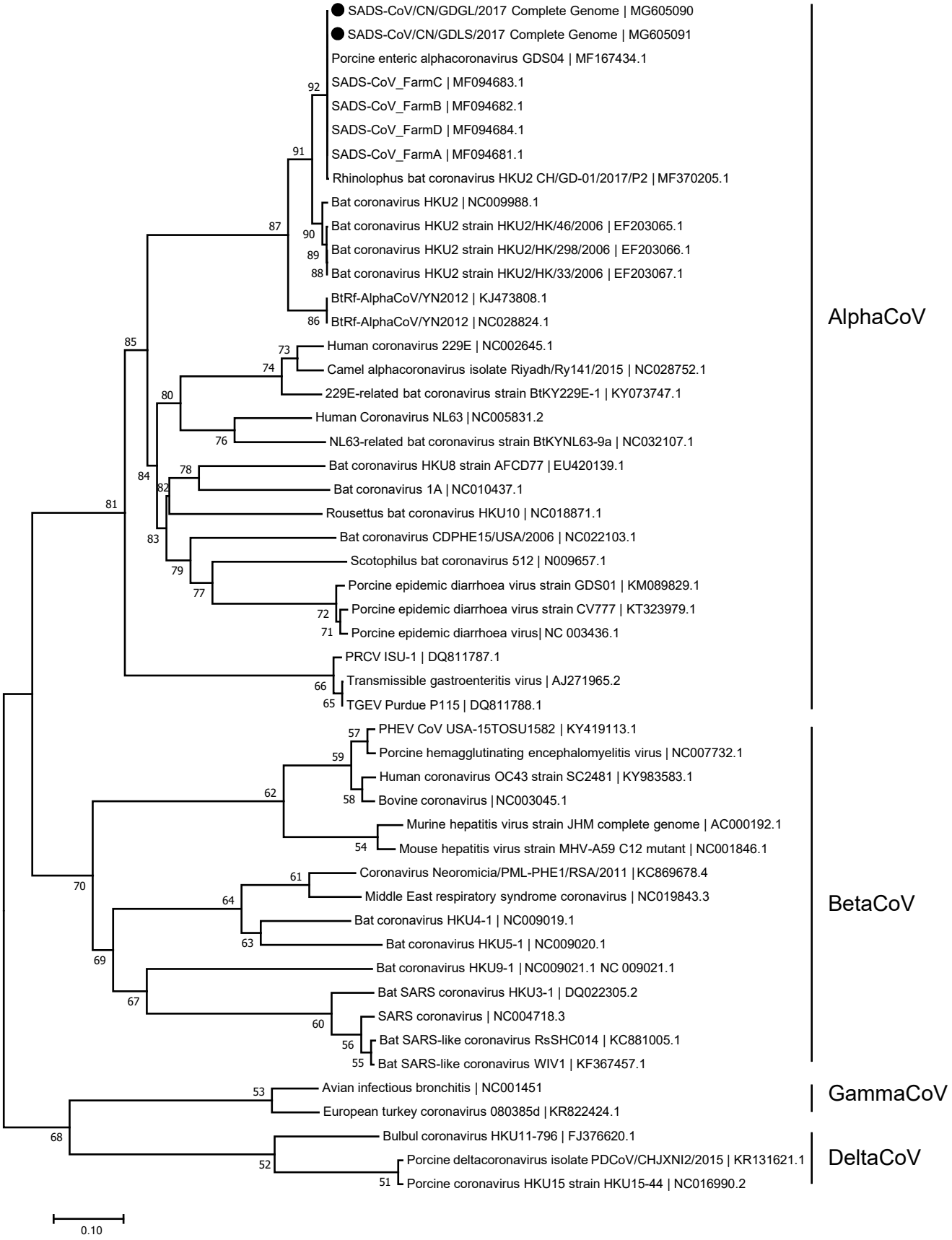


FIGURE 2 Phylogenetic analysis of the complete genomes of SADS-CoV and reference coronavirus species. The tree was constructed using MEGA 7.0 software with neighbour-joining methods and 1,000 replicate sets on bootstrap analysis. Two new complete genomes studied in this work were indicated with "black solid circles"

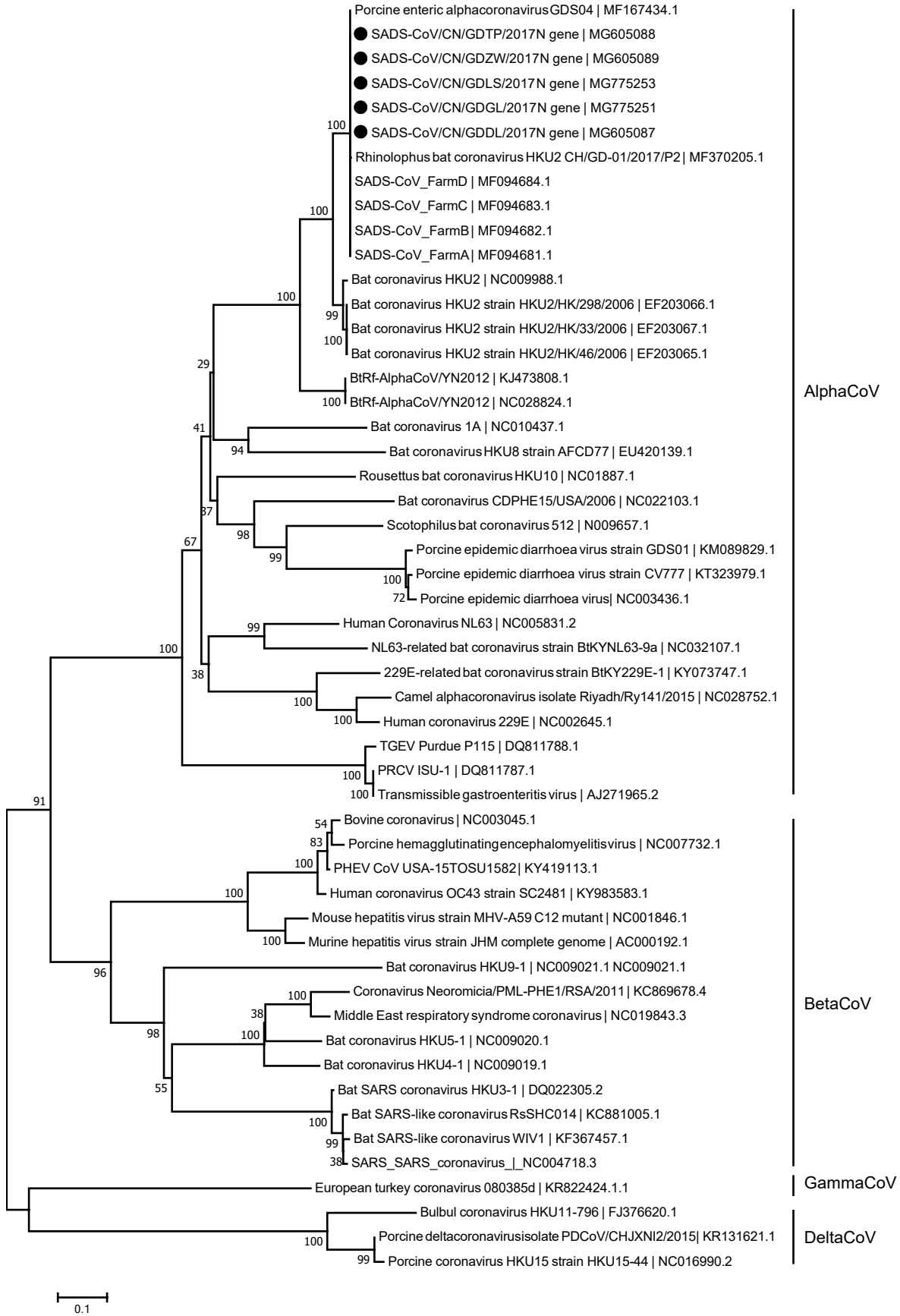


FIGURE 3 Phylogenetic analysis of the N genes of SADS-CoV and reference coronavirus species. The tree was constructed as per Figure 2 above. Five new sequences of N genes studied in this work were indicated with "black solid circles"

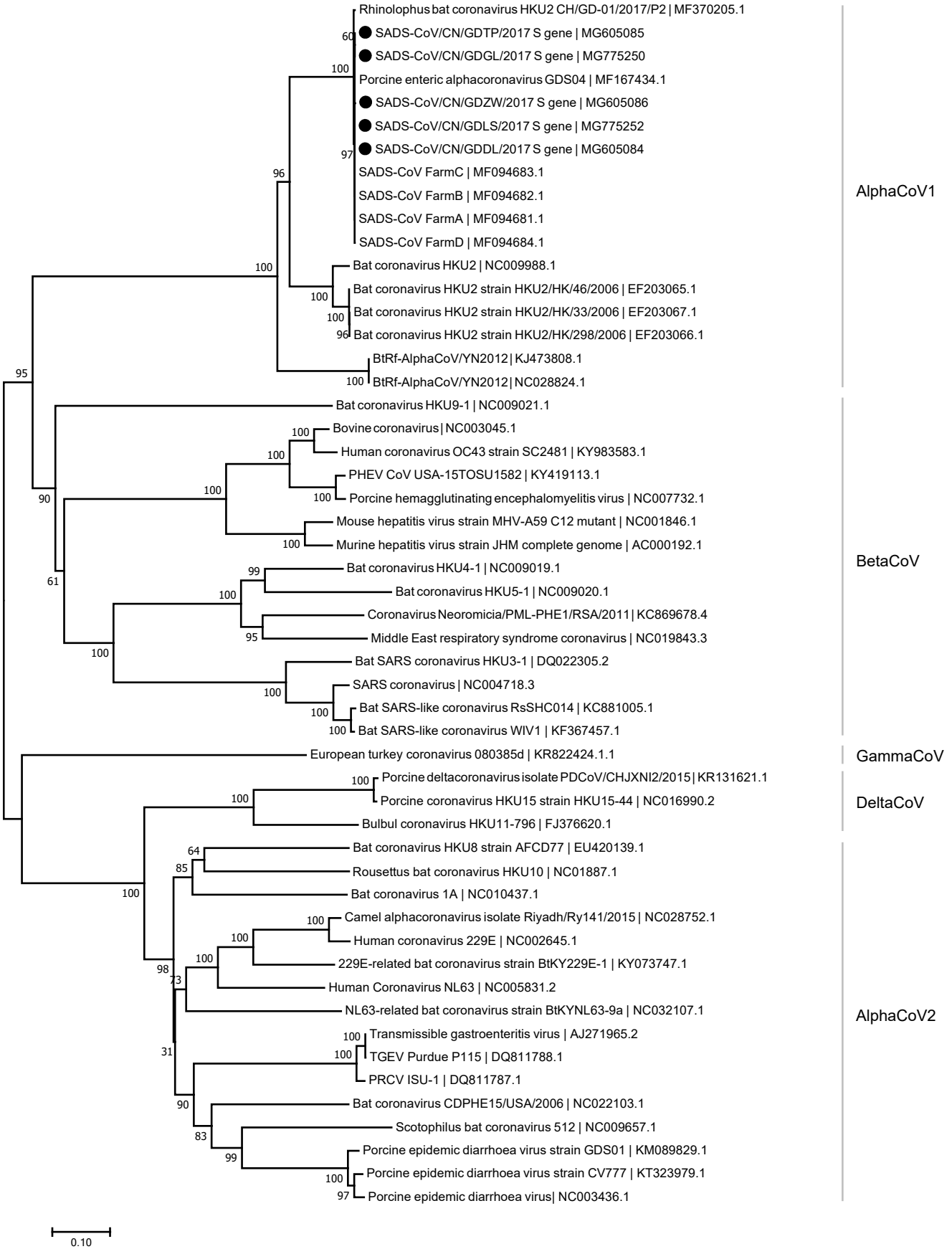


FIGURE 4 Phylogenetic analysis of the S genes of SADS-CoV and reference coronavirus species. The tree was constructed as per Figure 2 above. Five new sequences of S genes studied in this work were indicated with "black solid circles"

diarrhoea samples.Our results showed that the first SADS-CoV positive sample was collected in August 2016 from the farm LS with a history of diarrhoea, as well as from other two farms TP and ZW, which indicates that SADS-CoV has emerged in pigs in China at least since August 2016. And this time point is 5 months earlier than the first discovered time reported by our previous study (Zhou et al., 2018). As the same time, clinical signs of SADS-CoV during the retrospective investigation included sever and acute vomiting and diarrhoea, leading to death in piglets that were less than 5 days of age with a mortality rate of around 50%. These clinical presentations were similar to those signs in the large scale outbreak of SADS-CoV reported by Zhou et al. (2018), except the mortality rate in piglets later increased to 90%.

Based on the rates of infection documented in our work, it revealed that PEDV (78.25%) was still the primary cause of the porcine diarrhoea, which is consistent with previous studies that PEDV has been considered to be the major pathogen responsible for the porcine diarrhoea epidemic in China since 2010 (Ge et al., 2013; Sun et al., 2012; Zhao et al., 2016). Except PEDV, SADS-CoV had the second high infection rate (43.53%),which exhibits an evidently prevailing tendency in pigs. Meanwhile, of the SADS-CoV positive samples, 62.2% were co-infections with one to four other viruses, revealing a high prevalence of co-infection in the sampled farms. Notably, our results also showed that SADS-CoV and PEDV were simultaneously present in all co-infection samples of SADA-CoV. Considering the fact that PEDV has caused prior outbreaks of the porcine diarrhoea at the pig farm where Zhou et al. (2018) later reported the occurrence of SADS-CoV, PEDV seems to be able to contribute to the infection of SADS-CoV in pigs. Thus, SADS-CoV may be pathogenic as secondary infection following the infection of PEDV, which needs further studies to better understand the pathogenesis of this novel coronavirus.

The phylogenetic relationships of SADS-CoV sequences were also identified in this study. The results showed that all SADS-CoV sequences clustered together to form an independent branch and separated from other viral sequences in the genus Alphacoronavirus. Our results also indicated that both the complete genomes, N genes and S genes of all SADS-CoV strains shared the highest nucleotides identifies with those corresponding sequences of four bat coronavirus HKU2 strains. In this work, The phylogenetic trees of full length genomes and S genes of SADS-CoV sequences showed that the SADS-CoV branch clustered with these four HKU2 strains, which is same to previous results (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018). Besides the genomes and S genes, the tree of N genes in our study revealed the identical result too. So far, a total of eight full-length genomes of SADS-CoV have been reported in Guangdong Province of China (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018; this study). The two new genomes of SADS-CoV sequences in this work shared 100% nucleotides identities with the sequence MF167434 published by Gong et al. (2017) and our four previously reported sequences (Zhou et al., 2018), and shared 99.8% nucleotides identities with the sequence MF370205 studied by Pan et al. (2017). The results suggest that these eight SADS-CoV sequences may come from the same origin. Only the phylogenetic tree of S genes in our work showed that sequences of the AlphaCoV were divided into two sublineages,AlphaCoV1 which contained all SADS-CoV sequences and AlphaCoV2, clustering together with sequences of the BeltaCoV and the DelatCov, respectively. And this result was consistent with the study of Pan et al. (2017). As a newly discovered coronavirus, the availability of SADS-CoV sequences data is limited which prevents better understandings of the molecular epidemiology of this virus. Meanwhile, being a RNA virus, SADS-CoV may mutate rapidly and exhibit high genetic differences (Drummond, Pybus, Rambaut, Forsberg, & Rodrigo, 2003; Kühnert, Wu, & Drummond, 2011). So, new more available sequences data are warranted to give a deep insight of viral genetic origin, evolution, and transmission patterns of SADS-CoV.

In summary, our retrospective study suggests that SADS-CoV has emerged in pigs in China at least since August 2016. The severe clinical symptoms and the strong transmission in short term highlights the urgency to develop effective measures to control this new discovered virus. Further studies, such as the epidemiology, virology and pathobiology should be performed for better understanding of SADS-CoV in China.

ACKNOWLEDGEMENTS

This work was supported by the National Key Research and Development Program of China (No. 2016YFD0501304). We would acknowledge Guangdong Wen's Foodstuffs Group Co., Ltd. China, for providing us with pigs' tissue samples.

CONFLICT OF INTEREST

The authors declare no conflict of interests with any organization.

ORCID

JingyunMa https://orcid.org/0000-0001-6285-312X

REFERENCES

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., & Thompson, J. D. (2003). Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research*, *31*, 3497–3500. https://doi.org/10.1093/nar/gkg500

Dong, N., Fang, L., Zeng, S., Sun, Q., Chen, H., & Xiao, S. (2015). Porcine Deltacoronavirus in Mainland China. *Emerging Infectious Diseases*, *21*, 2254–2255. https://doi.org/10.3201/eid2112.150283

Drummond, A., Pybus, O., Rambaut, A., Forsberg, R., & Rodrigo, A. (2003). Measurably evolving populations. *Trends in Ecology & Evolution*, *18*, 481–488. https://doi.org/10.1016/S0169-5347(03)00216-7

Ge, F. F., Yang, D. Q., Ju, H. B., Wang, J., Liu, J., Liu, P. H., & Zhou, J. P. (2013). Epidemiological survey of porcine epidemic diarrhea virus in swine farms in Shanghai, China. *Archives of Virology*, *158*, 2227–2231. https://doi.org/10.1007/s00705-013-1722-7

Gong, L., Li, J., Zhou, Q., Xu, Z., Chen, L., Zhang, Y., … Cao, Y. (2017). A new bat-HKU2–like coronavirus in swine, China, 2017. *Emerging*

Infectious Diseases, *23*, 1607–1609. https://doi.org/10.3201/eid2309.170915

Kühnert, D., Wu, C., & Drummond, A. J. (2011). Phylogenetic and epidemic modeling of rapidly evolving infectious diseases. *Infection, Genetics and Evolution*, *11*, 1825–1841. https://doi.org/10.1016/j.meegid.2011.08.005

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

Lin, C., Saif, L. J., Marthaler, D., & Wang, Q. (2016). Evolution, antigenicity and pathogenicity of global porcine epidemic diarrhea virus strains. *Virus Research*, *226*, 20–39. https://doi.org/10.1016/j.virusres.2016.05.023

Liu, X., Zhu, L., Liao, S., Xu, Z., & Zhou, Y. (2015). The porcine microRNA transcriptome response to transmissible gastroenteritis virus infection. *PLoS One*, *10*(3), e0120377. https://doi.org/10.1371/journal.pone.0120377

Mai, K., Feng, J., Chen, G., Li, D., Zhou, L., Bai, Y., … Ma, J. (2017). The detection and phylogenetic analysis of porcine deltacoronavirus from Guangdong Province in Southern China. *Transboundary and Emerging Diseases*, *65*(1), 166–173.

Pan, Y., Tian, X., Qin, P., Wang, B., Zhao, P., Yang, Y., … Huang, Y. (2017). Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. *Veterinary Microbiology*, *211*, 15–21. https://doi.org/10.1016/j.vetmic.2017.09.020

Stevenson, G. W., Hoang, H., Schwartz, K. J., Burrough, E. R., Sun, D., Madson, D., … Yoon, K. J. (2013). Emergence of Porcine epidemic diarrhea virus in the United States: Clinical signs, lesions, and viral genomic sequences. *Journal of veterinary Diagnostic Investigation*, *25*, 649–654. https://doi.org/10.1177/1040638713501675

Sun, R. Q., Cai, R. J., Chen, Y. Q., Liang, P. S., Chen, D. K., & Song, C. X. (2012). Outbreak of porcine epidemic diarrhea in suckling piglets, China. *Emerging Infectious Diseases*, *18*, 161–163. https://doi.org/10.3201/eid1801.111259

Sun, D., Wang, X., Wei, S., Chen, J., & Feng, L. (2016). Epidemiology and vaccine of porcine epidemic diarrhea virus in China: A mini-review. *Journal of Veterinary Medical Science*, *78*, 355–363. https://doi.org/10.1292/jvms.15-0446

Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences*, *101*, 11030–11035. https://doi.org/10.1073/pnas.0404206101

Wesley, R. D., Woods, R. D., & Cheung, A. K. (1991). Genetic analysis of porcine respiratory coronavirus, an attenuated variant of transmissible gastroenteritis virus. *Journal of Virology*, *65*, 3369–3373.

Woo, P. C., Huang, Y., Lau, S. K., & Yuen, K. Y. (2010). Coronavirus genomics and bioinformatics analysis. *Viruses*, *2*, 1804–1820.

Woo, P. C., Lau, S. K., Lam, C. S., Lau, C. C., Tsang, A. K., Lau, J. H., … Yuen, K. Y. (2012). Discovery of seven novel Mammalian and Avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. *Journal of Virology*, *86*, 3995–4008. https://doi.org/10.1128/JVI.06540-11

Zhao, Z. P., Yang, Z., Lin, W. D., Wang, W. Y., Yang, J., Jin, W. J., & Qin, A. J. (2016). The rate of co-infection for piglet diarrhea viruses in China and the genetic characterization of porcine epidemic diarrhea virus and porcine kobuvirus. *Acta Virologica*, *60*, 55–61. https://doi.org/10.4149/av_2016_01_55

Zhou, P., Fan, H., Lan, T., Yang, X., Zhang, W., Zhu, Y., … Ma, J. (2018). Fatal Swine Acute Diarrhea Syndrome caused by an HKU2-related Coronavirus of Bat Origin. *Nature*, *556*, 255–258. https://doi.org/10.1038/s41586-018-0010-9

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Zhou L, Sun Y, Lan T, et al. Retrospective detection and phylogenetic analysis of swine acute diarrhoea syndrome coronavirus in pigs in southern China. *Transbound Emerg Dis*. 2019;66:687–695. https://doi.org/10.1111/tbed.13008

Origin and evolution of pathogenic coronaviruses

Jie Cui[1], Fang Li[2] and Zheng-Li Shi[1]

Abstract | Severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) are two highly transmissible and pathogenic viruses that emerged in humans at the beginning of the 21st century. Both viruses likely originated in bats, and genetically diverse coronaviruses that are related to SARS-CoV and MERS-CoV were discovered in bats worldwide. In this Review, we summarize the current knowledge on the origin and evolution of these two pathogenic coronaviruses and discuss their receptor usage; we also highlight the diversity and potential of spillover of bat-borne coronaviruses, as evidenced by the recent spillover of swine acute diarrhoea syndrome coronavirus (SADS-CoV) to pigs.

Severe acute respiratory syndrome
A serious form of pneumonia that is characterized by diffuse alveolar damage and that has the potential to progress to acute respiratory distress.

Type II pneumocytes
Epithelial cells that line the lung alveoli; type II cells are round and produce surfactants to lower the surface tension of water and allow the membrane to separate, thereby increasing the capability to exchange gases.

[1]*CAS Key Laboratory of Special Pathogens and Biosafety, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, Hubei, China.*

[2]*Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, Saint Paul, MN, USA.*

**e-mail: zlshi@wh.iov.cn*

https://doi.org/10.1038/s41579-018-0118-9

Coronaviruses cause respiratory and intestinal infections in animals and humans[1]. They were not considered to be highly pathogenic to humans until the outbreak of severe acute respiratory syndrome (SARS) in 2002 and 2003 in Guangdong province, China[2–5], as the coronaviruses that circulated before that time in humans mostly caused mild infections in immunocompetent people. Ten years after SARS, another highly pathogenic coronavirus, Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in Middle Eastern countries[6]. SARS coronavirus (SARS-CoV) uses angiotensin-converting enzyme 2 (ACE2) as a receptor and primarily infects ciliated bronchial epithelial cells and type II pneumocytes[7,8], whereas MERS-CoV uses dipeptidyl peptidase 4 (DPP4; also known as CD26) as a receptor and infects unciliated bronchial epithelial cells and type II pneumocytes[9–11]. SARS-CoV and MERS-CoV were transmitted directly to humans from market civets and dromedary camels, respectively[12–14], and both viruses are thought to have originated in bats[15–21]. Extensive studies of these two important coronaviruses have not only led to a better understanding of coronavirus biology but have also been driving coronavirus discovery in bats globally[21–31]. In this Review, we focus on the origin and evolution of SARS-CoV and MERS-CoV. Specifically we emphasize the ecological distribution, genetic diversity, interspecies transmission and potential for pathogenesis of SARS-related coronaviruses (SARSr-CoVs) and MERS-related coronaviruses (MERSr-CoVs) found in bats, as this information can help prepare countermeasures against future spillover and pathogenic infections in humans with novel coronaviruses.

Coronavirus diversity

Coronaviruses are members of the subfamily *Coronavirinae* in the family *Coronaviridae* and the order *Nidovirales* (International Committee on Taxonomy of Viruses). This subfamily consists of four genera—*Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus*—on the basis of their phylogenetic relationships and genomic structures (Fig. 1). The alphacoronaviruses and betacoronaviruses infect only mammals. The gammacoronaviruses and deltacoronaviruses infect birds, but some of them can also infect mammals[24]. Alphacoronaviruses and betacoronaviruses usually cause respiratory illness in humans and gastroenteritis in animals. The two highly pathogenic viruses, SARS-CoV and MERS-CoV, cause severe respiratory syndrome in humans, and the other four human coronaviruses (HCoV-NL63, HCoV-229E, HCoV-OC43 and HKU1) induce only mild upper respiratory disease in immunocompetent hosts, although some of them can cause severe infections in infants, young children and elderly individuals[1,28,29]. Alphacoronaviruses and betacoronaviruses can pose a heavy disease burden on livestock; these viruses include porcine transmissible gastroenteritis virus[32], porcine enteric diarrhoea virus (PEDV)[33] and the recently emerged swine acute diarrhoea syndrome coronavirus (SADS-CoV)[34]. On the basis of current sequence databases, all human coronaviruses have animal origins: SARS-CoV, MERS-CoV, HCoV-NL63 and HCoV-229E are considered to have originated in bats; HCoV-OC43 and HKU1 likely originated from rodents[28,29]. Domestic animals may have important roles as intermediate hosts that enable virus transmission from natural hosts to humans. In addition, domestic animals themselves can suffer disease caused by bat-borne or closely related coronaviruses; genomic sequences highly similar to PEDV were detected in bats[35–38], and SADS-CoV is a recent spillover from bats to pigs[34] (Fig. 2). Currently, 7 of 11 ICTV-assigned *Alphacoronavirus* species and 4 of 9 *Betacoronavirus* species were identified only in bats (Fig. 3). Thus, bats are

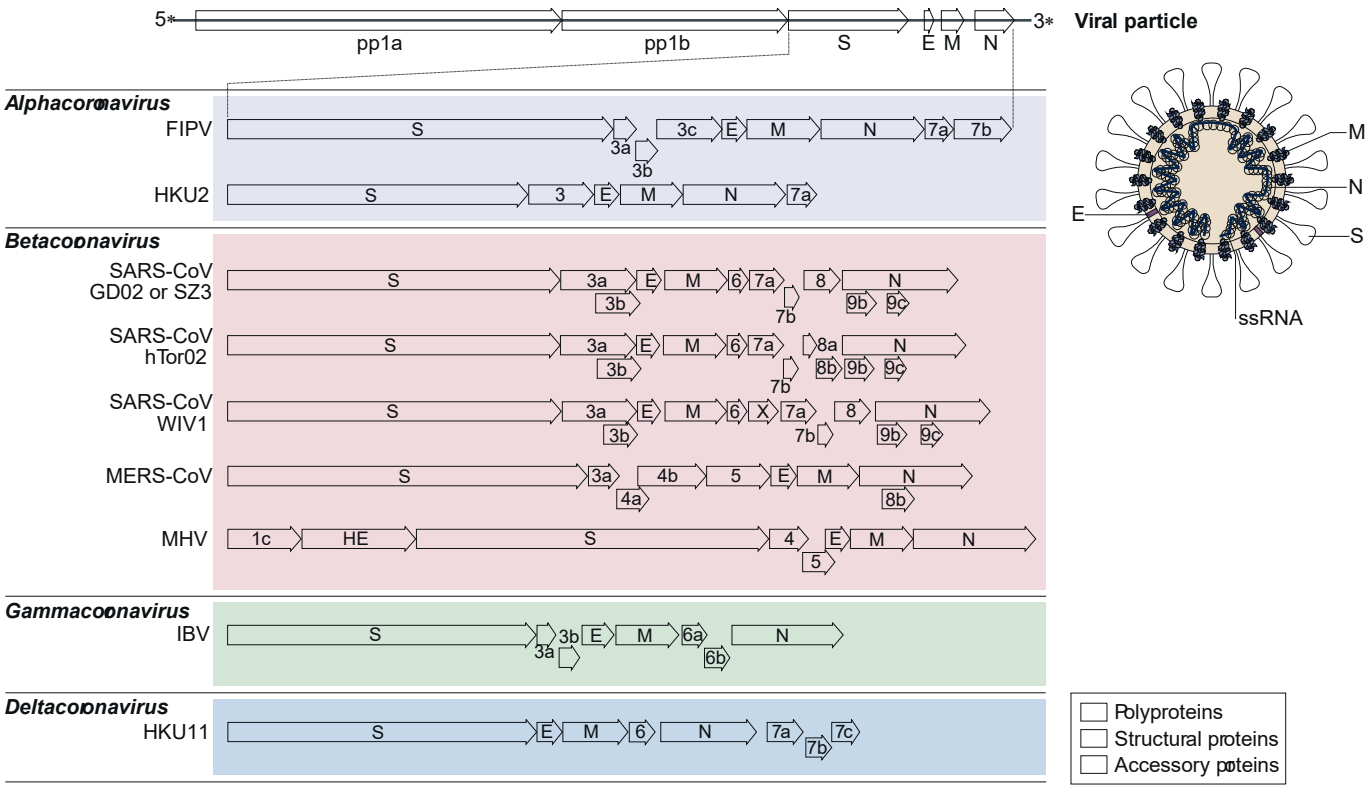


Fig. 1 | **The genomes, genes and proteins of different coronaviruses.** Coronaviruses form enveloped and spherical particles of 100–160 nm in diameter. They contain a positive-sense, single-stranded RNA (ssRNA) genome of 27–32 kb in size. The 5′-terminal two-thirds of the genome encodes a polyprotein, pp1ab, which is further cleaved into 16 non-structural proteins that are involved in genome transcription and replication. The 3′ terminus encodes structural proteins, including envelope glycoproteins spike (S), envelope (E), membrane (M) and nucleocapsid (N). In addition to the genes encoding structural proteins, there are accessory genes that are species specific and dispensable for virus replication. Here, we compare prototypical and representative strains of four coronavirus genera: feline infectious peritonitis virus (FIPV), *Rhinolophus* bat coronavirus HKU2, severe acute respiratory syndrome coronavirus (SARS-CoV) strains GD02 and SZ3 from humans infected during the early phase of the SARS epidemic and from civets, respectively, SARS-CoV strain hTor02 from humans infected during the middle and late phases of the SARS epidemic, bat SARS-related coronavirus (SARSr-CoV) strain WIV1, Middle East respiratory syndrome coronavirus (MERS-CoV), mouse hepatitis virus (MHV), infectious bronchitis virus (IBV) and bulbul coronavirus HKU11.

likely the major natural reservoirs of alphacoronaviruses and betacoronaviruses[24].

Animal origin and evolution of SARS-CoV

At the beginning of the SARS epidemic, almost all early index patients had animal exposure before developing disease. After the causative agent of SARS was identified, SARS-CoV and/or anti-SARS-CoV antibodies were found in masked palm civets (*Paguma larvata*) and animal handlers in a marketplace[12,16,39–42]. However, later, wide-reaching investigations of farmed and wild-caught civets revealed that the SARS-CoV strains found in market civets were transmitted to them by other animals[16,39]. In 2005, two teams independently reported the discovery of novel coronaviruses related to human SARS-CoV, which were named SARS-CoV-related viruses or SARS-like coronaviruses in horseshoe bats (genus *Rhinolophus*)[15,43]. These discoveries suggested that bats may be the natural hosts for SARS-CoV and that civets were only intermediate hosts. Subsequently, many coronaviruses phylogenetically related to SARS-CoV (SARSr-CoVs) were discovered in bats from different provinces in China and also from European, African and Southeast Asian countries[15,20,38,43–54] (Fig. 4; Supplementary Fig. S1a). According to the ICTV criteria, only the strains found in *Rhinolophus* bats in European countries, Southeast Asian countries and China are SARSr-CoV variants. Those from *Hipposideros* bats in Africa are less closely related to SARS-CoV and should be classified as a new coronavirus species[54]. These data indicate that SARSr-CoVs have wide geographical spread and might have been prevalent in bats for a very long time. A 5-year longitudinal study revealed the coexistence of highly diverse SARSr-CoVs in bat populations in one cave of Yunnan province, China[18,20,55]. This location is a diversity hot spot, and the SARSr-CoVs in this location contain all the genetic diversity found in other locations of China. Furthermore, the viral strains that exist in this one location contain all genetic elements that are needed to form SARS-CoV (Fig. 5). As no direct progenitor of SARS-CoV was found in bat populations despite 15 years of searching and as RNA recombination is frequent within coronaviruses[56], it is highly likely that SARS-CoV newly emerged through recombination of

bat SARSr-CoVs in this or other yet-to-be-identified bat caves. This hypothesis is consistent with previous data showing that a direct progenitor of SARS-CoV emerged before 2002 (refs[42,57,58]). Recombination analysis also strongly supported the hypothesis that the civet SARS-CoV strain SZ3 arose through recombination of two existing bat strains, WIV16 and Rf4092 (ref.[20]).

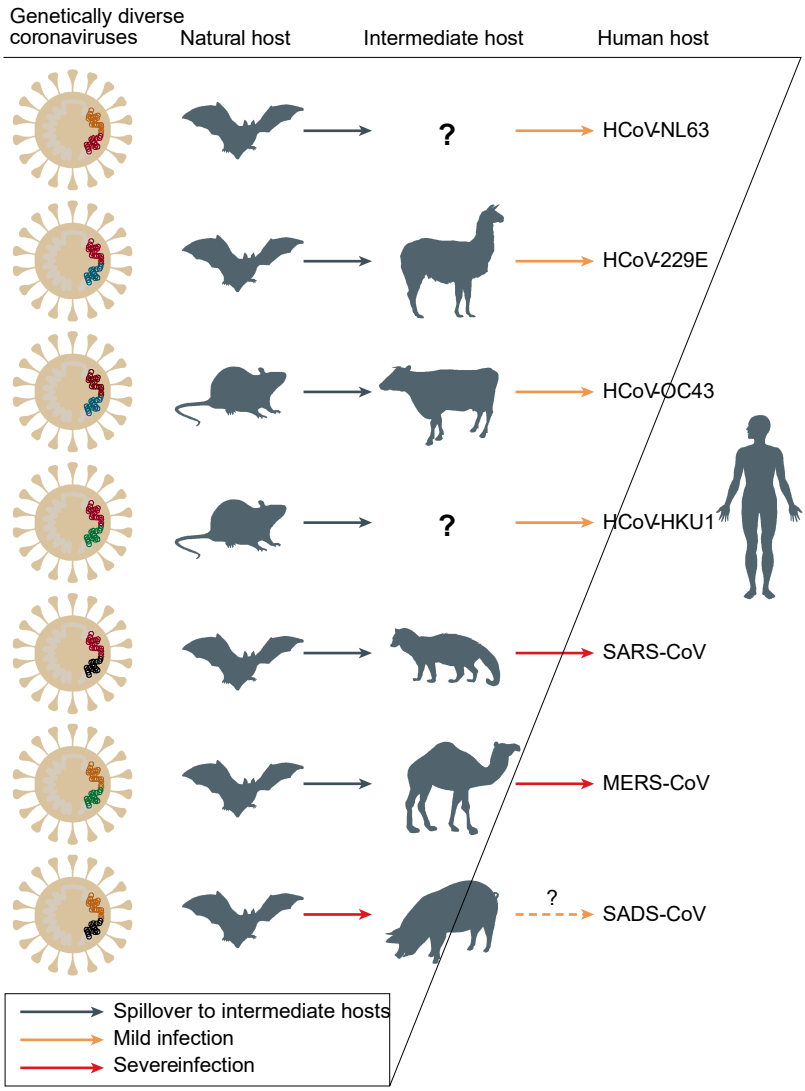


Fig. 2 | **Animal origins of human coronaviruses.** Severe acute respiratory syndrome coronavirus (SARS-CoV) is a new coronavirus that emerged through recombination of bat SARS-related coronaviruses (SARSr-CoVs)[20]. The recombined virus infected civets and humans and adapted to these hosts before causing the SARS epidemic[42,62]. Middle East respiratory syndrome coronavirus (MERS-CoV) likely spilled over from bats to dromedary camels at least 30 years ago[100] and since then has been prevalent in dromedary camels. HCoV-229E and HCoV-NL63 usually cause mild infections in immunocompetent humans. Progenitors of these viruses have recently been found in African bats[133,134], and the camelids are likely intermediate hosts of HCoV-229E[134,135]. HCoV-OC43 and HKU1, both of which are also mostly harmless in humans, likely originated in rodents. Recently, swine acute diarrhoea syndrome (SADS) emerged in piglets. This disease is caused by a novel strain of *Rhinolophus* bat coronavirus HKU2, named SADS coronavirus (SADS-CoV)[84]; there is no evidence of infection in humans. Solid arrows indicate confirmed data. Broken arrows indicate potential interspecies transmission. Black arrows indicate infection in the intermediate animals, yellow arrows indicate a mild infection in humans, and red arrows indicate a severe infection in humans or animals.

Furthermore, WIV16, the closest relative to SARS-CoV found in bats, likely arose through recombination of two other prevalent bat SARSr-CoV strains[20]. The most frequent recombination breakpoints are within the *S* gene, which encodes the spike (S) protein that contains the receptor-binding domain (RBD), and upstream of *orf8*, which encodes an accessory protein[20,58,59]. Given the prevalence and great genetic diversity of bat SARSr-CoVs, their close coexistence and the frequent recombination of the coronaviruses it is expected that novel variants will emerge in the future[60,61]. Because there were no SARS cases in Yunnan province during the SARS outbreak, we hypothesize that the direct progenitor of SARS-CoV was produced by recombination within bats and then transmitted to farmed civets or another mammal, which then transmitted the virus to civets by faecal–oral transmission. When the virus-infected civets were transported to Guangdong market, the virus spread in market civets and acquired further mutations before spillover to humans.

Variability of SARS-CoV in humans and civets

The genome sequences of SARS-CoVs from market civets are almost identical to the genomes of human SARS-CoVs[42,62]. However, two genes show major variation. The first variable region is located in the *S* gene. The SARS-CoV S protein is functionally divided into two subunits, denoted S1 and S2, which are responsible for receptor binding and fusion with the cellular membrane, respectively[1]. S1 is further divided into the amino-terminal domain (S1-NTD) and the carboxy-terminal domain (S1-CTD). The S1-CTD functions as the RBD and is responsible for binding ACE2 and entering cells[7,63,64]. Two amino acid residues in the RBD, 479 and 487, were identified to be essential for ACE2-mediated SARS-CoV infection and critical for virus transmission from civets to humans[36,78].

The second major location of variation is the accessory gene *orf8* (Fig. 5). On the basis of SARS spread, the SARS 2002–2003 outbreak could be divided into three phases, with the early phase characterized by a limited number of localized cases, followed by a middle phase during which a superspreader event occurred in a hospital and finally the late phase of international spread[92]. The viral genomes from early-phase patients contain two genotypes of *orf8*, one with a complete *orf8* (369 nucleotides) and the other containing an 82-nucleotide deletion. By contrast, viral genomes from late-phase patients and most of the genomes from middle-phase patients contain a split *orf8* (*orf8a* and *orf8b*) owing to a 29-nucleotide deletion; two exceptions were found in middle-phase genomes, one containing an 82-nucleotide deletion in *orf8* and the other with the whole *orf8* deleted. The human isolates from 2004 and all civet SARS-CoV genomes have a complete *orf8* except one civet strain with an 82-nucleotide deletion[62]. These data indicate that *orf8* genes underwent adaptations during transmission from animals to humans during the SARS epidemic. A limited functional analysis suggested that the ORF8a protein is dispensable for SARS-CoV replication in Vero E6 cells but may have a role in modulating endoplasmic reticulum

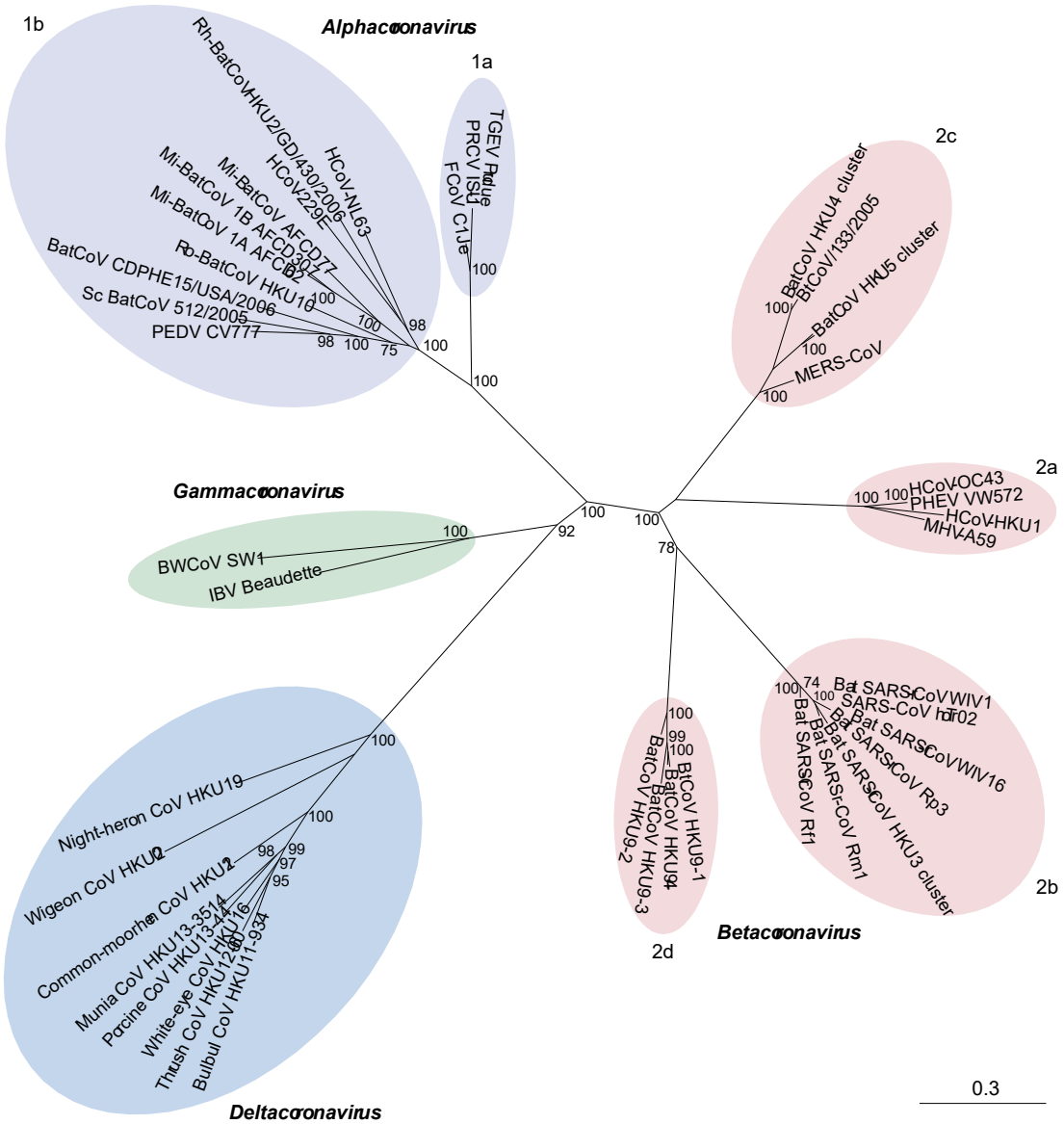


Fig. 3 | **Phylogenetic relationships in the *Coronavirinae* subfamily.** The highly human-pathogenic coronaviruses belong to the subfamily *Coronavirinae* from the family *Coronaviridae*. The viruses in this subfamily group into four genera (prototype or representative strains shown): *Alphacoronavirus* (purple), *Betacoronavirus* (pink), *Gammacoronavirus* (green) and *Deltacoronavirus* (blue). Classic subgroup clusters are labelled 1a and 1b for the alphacoronaviruses and 2a–2d for the betacoronaviruses. The tree is based on published trees of *Coronavirinae*[25,136] and reconstructed with sequences of the complete RNA-dependent RNA polymerase coding region of the representative coronaviruses (maximum-likelihood method under the GTR + I + Γ model of nucleotide substitution as implemented in PhyML, version 3.1 (rEF.[137])). Only nodes with bootstrap support above 70% are shown. IBV, infectious bronchitis virus; MERS-CoV, Middle East respiratory syndrome coronavirus; MHV, mouse hepatitis virus; PEDV, porcine enteric diarrhoea virus; SARS-CoV, severe acute respiratory syndrome coronavirus; SARSr-CoV, SARS-related coronavirus.

stress, inducing apoptosis and inhibiting interferon responses in host cells[20,65–69]. Whether and how these adaptations were involved in SARS-CoV virulence are not fully clarified.

Variability of bat SARSr-CoVs

SARS-CoVs and bat SARSr-CoVs mainly vary in three regions: S, ORF8 and ORF3 (Fig. 5). Bat SARSr-CoVs share high sequence identity with SARS-CoV in the S2 region but are highly variable in the S1 region. Compared with human and civet SARS-CoV, bat SARSr-CoV S1 can be divided into two clades: clade 1, which is found only in Yunnan province, has the same size S protein as human and civet isolates[8–20,51], whereas clade 2, which is found in many locations, has a shorter size S protein owing to deletions of 5, 12 or 13 amino acids in length[15,43–45,48,50]. Among the sequenced bat SARSr-CoVs, those with deletions in their RBDs show 78.2–80.2% amino acid sequence identity with SARS-CoV in the S protein, whereas those without deletions are much more closely related to SARS-CoV, with 90.0–97.2% amino acid sequence identity.

The second variable region is located in ORF8. Most of bat SARSr-CoVs retain an intact *orf8* (366 or 369 nucleotides) and share 47.7–100% sequence identity among themselves and 50.6–98.4% with SARS-CoV in civets and early-phase patients. A split *orf8* (364 nucleotides) owing to a 5-nucleotide deletion was found in one bat SARSr-CoV strain, similar to that of SARS-CoVs from middle-phase and late-phase patients[20]. The European bat SARSr-CoV has completely lost *orf8* (rEF.[45]). These data show that the *orf8* gene in bat SARSr-CoVs are constantly evolving in their natural reservoirs. Considering the variability of *orf8* in bats, civets and humans, investigating the function of *orf8* is a priority, particularly the contribution of these different variants to viral pathogenesis.

The third variable region is in ORF3. The SARS-CoV genome encodes a 154-amino acid ORF3b, which is an interferon antagonist. Bat SARSr-CoVs and SARS-CoV are highly similar in ORF3a (96.4–98.9% amino acid identity), but bat SARSr-CoVs have different sizes of ORF3b (54–154 amino acids) (a large part of the region encoding ORF3b overlaps with the ORF3a coding region)[20,70]. ORF3b retains the anti-interferon function in some bat SARSr-CoVs but has lost the function in other bat SARSr-CoVs[70].

A novel accessory gene, named *orfx* and located between *orf6* and *orf7*, was identified in the genomes of several bat SARSr-CoVs from Yunnan province[18–20] (FIg. 5). A preliminary study indicated that ORFX is involved in an anti-interferon response[71].

Receptor usage of SARS-CoV and SARSr-CoV

ACE2 binding is a critical determinant for the host range of SARS-CoV[72,73]. Electron microscopic studies have shown that the SARS-CoV S protein forms a clover-shaped trimer, with three S1 heads and a trimeric S2 stalk[74,75]. The RBD is located on the tip of each S1 head. The RBD binds to the outer surface of ACE2, away from its zinc-chelating enzymatic site[77,141] (FIg. 6a). Different SARS-CoV strains isolated from several hosts vary in their binding affinities for human ACE2 and consequently in their infectivity of human cells[76,78] (FIg. 6b). The epidemic strain hTor02 was isolated from humans during the late phase of the outbreak in 2002–2003. It has a high affinity for human ACE2 and high infectivity in human cells, and consequently, it was transmitted efficiently between humans[82]. Strains cSz02 and cHb05 were isolated from palm civets in 2002–2003 and 2005, respectively. Both have low affinity for human ACE2 and low infectivity in human cells but have high affinity for civet ACE2 and high infectivity in civet cells[12,79]. Strain hcGd03 was isolated from both humans and palm civets in 2003–2004 and has moderate affinity for human ACE2 and moderate infectivity in human cells; it infected humans but did not transmit between humans[80]. Strain hHae08 was isolated from human cell culture and has high affinity for human ACE2 and high infectivity in human cells[81]. Understanding the molecular basis for human receptor usage by different SARS-CoV strains is crucial for understanding the cross-species transmission of SARS-CoV and for epidemiological monitoring of potential future outbreaks.

SARS-CoV mutations that affect human and civet receptor binding. Crystal structures of the SARS-CoV RBD complexed with human ACE2 revealed that the SARS-CoV RBD contains a core structure and a receptor-binding

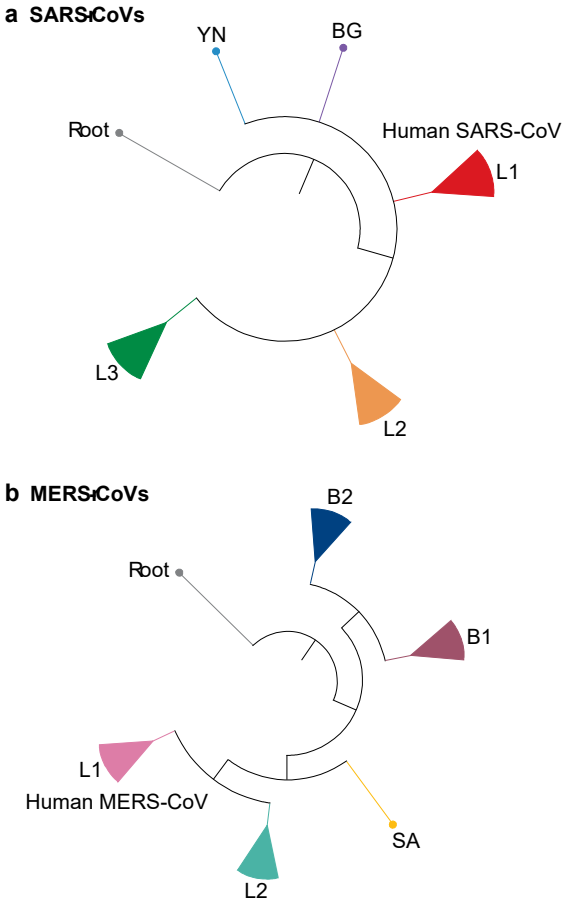


Fig. 4 | **Phylogenetic analysis of SARSr-CoVs and MERSr-CoVs. a** | The figure shows a simplified phylogenetic tree of severe acute respiratory syndrome-related coronaviruses (SARSr-CoVs) from bats. SARSr-CoVs cluster into three lineages, L1–L3, and human severe acute respiratory syndrome coronaviruses (SARS-CoVs) embed in L1. Two individual SARSr-CoVs do not cluster into these lineages: YN, a virus isolated from Yunnan province, China, and BG, a virus from Bulgaria, Europe. The tree is based on published trees[20,138] and reconstructed using sequences of the complete RNA-dependent RNA polymerase-coding region (maximum-likelihood method under the GTR + I + Γ model of nucleotide substitution as implemented in PhyML, version 3.1 (rEF.[137])). The strain Zhejiang2013 (GenBank No. KF636752) was used as a root. **b** | By contrast, Middle East respiratory syndrome-related coronaviruses (MERSr-CoVs) form two major viral lineages, L1 and L2. L1 is found in humans and camels, and L2 is found only in camels. Two small clusters, B1 (bat 1) and B2, and one single virus, SA, from South Africa, were found in bats. The phylogenetic tree of MERSr-CoVs is based on a published trees[94,139] and reconstructed using full-genome alignment of all coding regions using the same method as above. HKU4-1 (EF065505) and HKU5-1 (EF065509), two 2c betacoronaviruses, served as the root of the tree. Detailed phylogenetic trees and grouping information can be found in Supplementary Fig. S1. MERS-CoV, Middle East respiratory syndrome coronavirus.

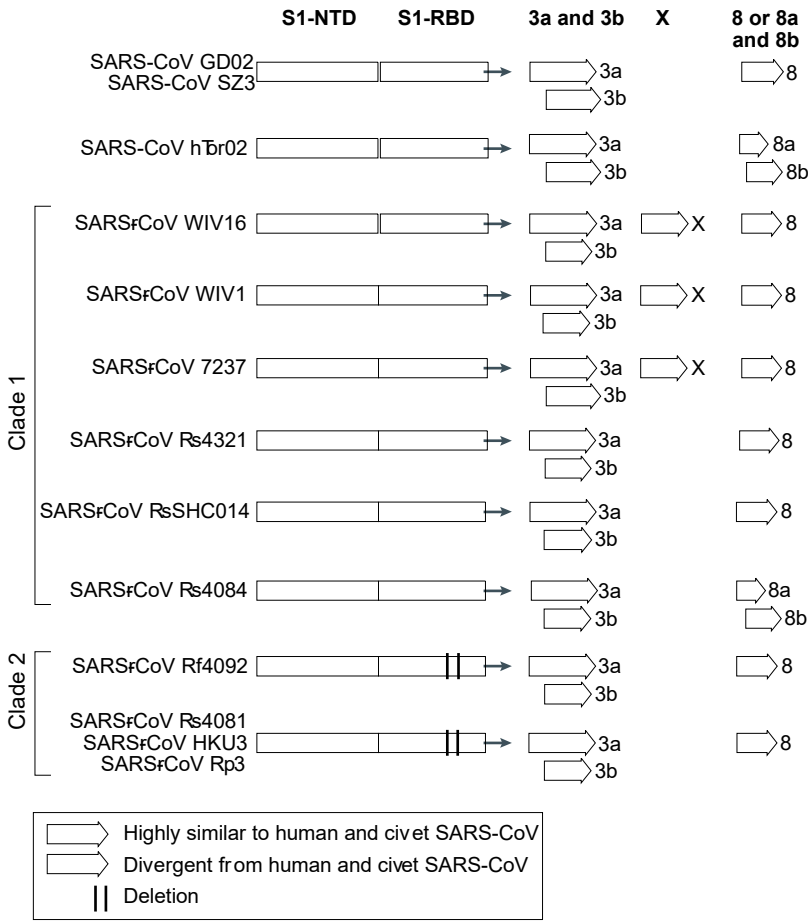


Fig. 5 | **Variable regions in different SARS-CoV and bat SARSr-CoV isolates.** Variability and thus species adaptation majorly affect three severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-related coronavirus (SARSr-CoV) proteins: the spike protein (S) (both the S1 amino-terminal domain (S1-NTD) and the S1 receptor-binding domain (S1-RBD) show variability), ORF3 (3a and 3b) and ORF8 (8a and b). SARS-CoV GD02 and hTor02 represent strains that were isolated from patients during the early, and middle or late phase of the SARS epidemic in 2002–2003, respectively; SARS-CoV CZ3 is a representative of strains isolated from civets in 2003 and 2004 (rEFs [42,62]). All bat SARSr-CoVs, except HKU3 and Rp3, were discovered in Yunnan province during 2011–2015. On the basis of deletions in the RBD, bat SARSr-CoVs can be divided into two clades. Those without a deletion and thus an identical size in S1 to SARS-CoV can be further divided into four genotypes: genotype 1, represented by WIV16, is highly similar to SARS-CoV in both the NTD and the RBD; genotype 2, represented by WIV1, differs in NTD from SARS-CoV; genotype 3, represented by Rs4231, differs in RBD from SARS-CoV; and genotype 4, represented by SHC014 and Rs4084, differs in both NTD and RBD from SARS-CoV [20]. The differences in S influence species-specific receptor binding, whereas differences in the accessory proteins, including potentially the newly discovered ORFX (X), mainly affect immune responses and viral immune evasion. Adapted from rEF. [20], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

motif (RBM) [82,141] (Fig. 6a). Two virus-binding hot spots have been identified at the interface of the RBD and human ACE2, centring on ACE2 residues Lys31 (hot spot 31) and Lys353 (hot spot 353) [83,84] (Fig. 6b). They both consist of a salt bridge (between Lys31 and Glu35 for hot spot 31 and between Lys353 and Asp38 for hot spot 353); both salt bridges are buried in hydrophobic pockets and contribute a substantial amount of energy to RBD–ACE2 binding as well as filling voids at the RBD–ACE2 interface. Naturally selected RBM mutations all interact with the hot spots (Fig. 6b; TAbIE 1) and affect RBD–ACE2 binding.

Salt bridge
A structure in proteins that forms a bond between oppositely charged residues that are sufficiently close to each other to experience electrostatic attraction.

Mutations in RBM residue 479 had an important role in the civet-to-human transmission of SARS-CoV [42,76,78,85]. Residue 479 is an asparagine in strains hTor02, hGd03 and hHae08 but is a lysine in strain cSz02 and an arginine in strain cHb05 (TAbIE 1). Asn479 is located near hot spot 31, without interfering with the structure of hot spot 31 (rEF. [85]) (Fig. 6b, c). However, a change to Lys479 leads to steric and electrostatic interference with hot spot 31, reducing the binding affinity between the SARS-CoV RBD and human ACE2. By contrast, Arg479 reaches the vicinity of hot spot 353 and forms a salt bridge with ACE2 residue Asp38 (rEF. [83]) (Fig. 6d). Hence, strains hTor02, hGd03 and hHae08 (all of which contain Asn479) and strain cHb05 (which contains Arg479) recognize human ACE2 and infect human cells efficiently, whereas strain cSz02 (which contains Lys479) recognizes human ACE2 inefficiently and infects human cells inefficiently. The above structural analyses are supported by biochemical, functional and epidemiological data [42,76,78,83–85]. Because of residue differences between human ACE2 and civet ACE2, both Asn479 and Lys479 fit well into the interface between the RBD and civet ACE2, although Arg479 fits even better [83,85]; consequently, strains hTor02, cSz02, hGd03 and cHb05 (which contain either Asn479, Lys479 or Arg479) recognize civet ACE2 and infect civet cells efficiently [79]. In sum, Asn479 and Arg479 are viral adaptations to human ACE2, whereas Lys479 is incompatible with human ACE2; Arg479 is a viral adaptation to civet ACE2, whereas Asn479 and Lys479 are also compatible with civet ACE2.

Mutations in RBM residue 487 had an important role in the human-to-human transmission of SARS-CoV. Residue 487 is a threonine in strain hTor02 but is a serine in the other strains isolated from humans and civets. The methyl group of Thr487 interacts with hot spot 353 in human ACE2 by providing stacking support for the formation of the salt bridge between Lys353 and Asp38; consequently, strain hTor02 recognizes human ACE2 efficiently and was transmitted between humans during the 2002–2003 SARS epidemic. By contrast, Ser487 cannot provide support to hot spot 353, and hence the other strains isolated from humans and civets recognize human ACE2 inefficiently. Consequently, neither cSz02 nor hGd03 was transmitted between humans. The above structural analyses are supported by biochemical, functional and epidemiological data [42,76,78,83–85]. Because of residue differences between human ACE2 and civet ACE2, Ser487 fits well into the RBD–civet ACE2 interface although still not as well as Thr487 (rEFs [83,85]); consequently, strains SZ02, hGd03 and cHb05 (which contain Ser487) recognize civet ACE2 and infect civet cells efficiently [79]. In sum, Thr487 is a viral adaptation to both human and civet ACE2, and Ser487 is much more compatible with civet ACE2 than with human ACE2 (Fig. 6b).

RBM residues 442, 472 and 480 also contribute to receptor recognition and host range of SARS-CoV although not as much as residues 479 and 487. Detailed structural, biochemical and functional analyses showed that Phe442, Phe472 and Asp480 are viral adaptations to human ACE2, whereas Tyr442, Leu472 or Pro472, and Gly480 are viral adaptations to civet ACE2 (rEFs [72,83]).

To corroborate the importance of these residues for SARS-CoV binding to either human or civet ACE2, two SARS-CoV S proteins, hOptimize and cOptimize, were rationally designed: the former contains all of the human ACE2-adapted residues (Phe442, Phe472, Asn479, Asp480 and Thr487), whereas the latter contains the civet ACE2-adapted residues (Tyr442, Pro472, Arg479, Gly480 and Thr487). These two S proteins demonstrate exceptionally high affinity for human ACE2 and civet ACE2, confirming that the human ACE2-adapted and civet ACE2-adapted RBM residues help determine SARS-CoV host range[72,83]. In addition to

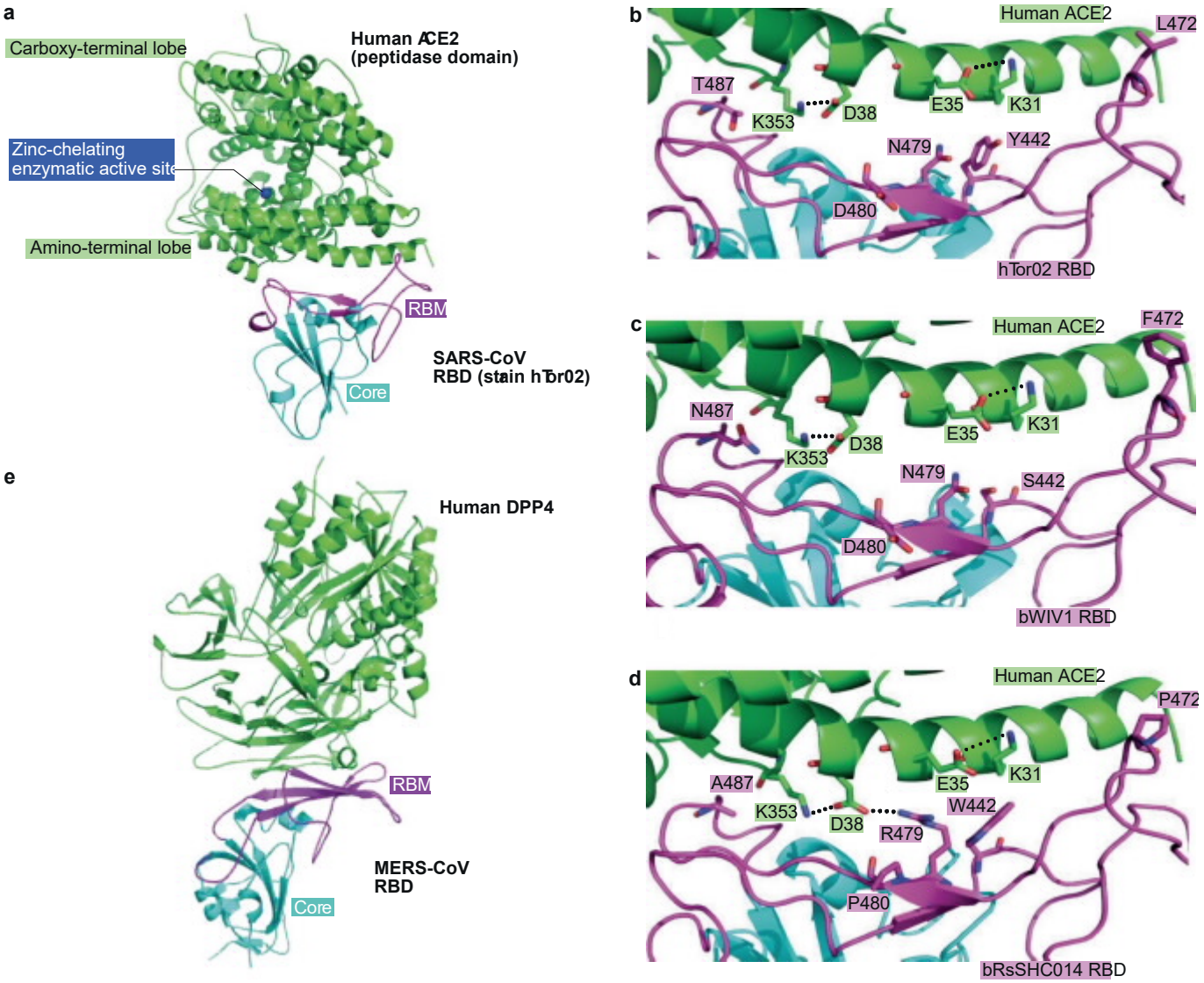


Fig. 6 | **Receptor recognition by SARS-CoV and MERS-CoV. a** | Severe acute respiratory syndrome coronavirus (SARS-CoV) uses its receptor-binding domain (RBD) (as shown in the structure of strain hTor02, containing core structure (cyan) and receptor-binding motif (RBM; magenta)) to bind human angiotensin-converting enzyme 2 (ACE2 green; Protein Data Bank ID: 2AJF). ACE2 is a peptidase with zinc (blue) in its active centre. **b** | Several residues in the host and viral receptor, as well as two salt bridges that stabilize the structure (dotted lines) and form two binding hot spots, are crucial for binding of the severe acute respiratory syndrome (SARS) epidemic strain hTor02. Hydrophobic residues surrounding the two salt bridges are present in the structure but are not shown in the figure. **c** | By contrast, the SARS-related coronavirus (SARS-CoV) strain bWIV1, which was isolated from bats and can infect both civet and human cells, differs in residues 442, 472 and 487. The mutation from threonine to asparagine in residue 487 introduces a polar side chain and is predicted to interfere with binding at hot spot 353. The model shown here was built on the basis of the structure of hTor02 RBD complexed with human ACE2 (Protein Data Bank ID: 2AJF) in which residues 442, 472 and 487 were mutated from those in strain hTor02 to those in strain bWIV1. **d** | The bat SARS-CoV strain bRsSHC014 can also infect human and civet cells; it carries an alanine in position 487, and the short side chain of this residue does not support the structure of hot spot 353. The model was built on the basis of the structure of cOptimize RBD complexed with human ACE2 (Protein Data Bank ID: 3SCJ) in which residues 442, 480 and 487 were mutated from those in strain cOptimize to those in strain bWIV1. **e** | The Middle East respiratory syndrome coronavirus (MERS-CoV) RBD (core structure in cyan and RBM in magenta) binds human dipeptidyl peptidase 4 (DPP4 green; Protein Data Bank ID: 4KR0). Structure figures were made using PyMOL[141]. Modelled mutations in panels **c** and **d** were performed using Coot[140]. Panels **a**–**d** are adapted from rEF.[83]: this research was originally published in *The Journal of Biological Chemistry* Wu, K. L., Peng, G. Q., Wilken, M., Geraghty, R. J. & Li, F. Mechanisms of host receptor adaptation by severe acute respiratory syndrome coronavirus. *J. Biol. Chem.* 2012; **287**: 8904–8911 © American Society for Biochemistry and Molecular Biology.

Table 1 | **Mutations in the receptor-binding motif of SARS-CoV**

Strain	Host	Year	Truncation in receptor-binding motif	Residue 442	Residue 472	Residue 479	Residue 480	Residue 487
hTor02	Human	2002–2003	No	Tyr	Leu	Asn	Asp	Thr
cSz02	Civet	2002–2003	No	Tyr	Leu	Lys	Asp	Ser
hcGd03	Human and civet	2003–2004	No	Tyr	Pro	Asn	Gly	Ser
cHb05	Civet	2005	No	Tyr	Pro	Arg	Gly	Ser
hHae08	Human	2008	No	Phe	Phe	Asn	Asp	Ser
hOptimize	Human	In vitro design	No	Phe	Phe	Asn	Asp	Ser
cOptimize	Civet	In vitro design	No	Tyr	Pro	Arg	Gly	Thr
bHKU3	Bat	2005	Yes	Ser	Gly	Ser	Thr	Val
bWIV1	Bat	2013	No	Ser	Phe	Asn	Asp	Asn
bRsSHC014	Bat	2013	No	Trp	Pro	Arg	Pro	Ala

Data from Hu et al. 2012 (rEF.[83]). SARS-CoV, severe acute respiratory syndrome coronavirus.

receptor binding, proteolytic cleavage of S and potentially other mutations that affect virion and trimer stability may also be important for virus transmissibility in different hosts, and these factors need to be studied further.

SARSr-CoV mutations that affect receptor binding. To date, numerous SARSr-CoV strains have been identified from bats[5,16,18–20]. These bat SARSr-CoVs are the likely progenitors of SARS-CoV that infected humans and civets, and hence understanding their interactions with human or civet ACE2 is critical for tracing the origins of SARS-CoV and for preventing and controlling future SARS-CoV outbreaks in humans. The RBD sequences of these bat SARSr-CoVs fall into three major groups; the representative strains from each group are bHKU3 (isolated in 2005), bWIV1 (isolated in 2013) and bRsSHC014 (isolated in 2013) (TAbIE 1). Strains bWIV1 and bRsSHC014, but not strain bHKU3, use both human and civet ACE2 and hence can infect both human and civet cells[16,18–20,86,87]. Strain bHKU3 has a truncated RBM (TAbIE 1), which distorts the structure of the RBM and abolishes its binding to human and civet ACE2. Neither strain bWIV1 nor strain bRsSHC014 contains truncations in its RBM, and hence, their RBMs likely retain the same structure as SARS-CoV RBMs. Here, we analysed the potential interactions between these two strains (bWIV1 and bRsSHC014) and human ACE2 by building homology structural models of their RBDs complexed with human ACE2, focusing on residues 479 and 487 (FIg. 6c, d). Strain bWIV1 contains Asn479 and Asn487 in its RBM. Whereas Asn479 is a viral adaptation to human ACE2, the polar side chain of Asn487 may have unfavourable interactions with the aliphatic portion of residue Lys353 in human ACE2, which is part of hot spot 353 (FIg. 6c). Strain bRsSHC014 contains Arg479 and Ala487 in its RBM. Whereas Arg479 is a viral adaptation to human ACE2, the small side chain of Ala487 does not provide support to the structure of hot spot 353 (FIg. 6d). Therefore, although both bWIV1 and bRsSHC014 can infect human airway cells, they bind human ACE2 less well than hTor02 and produce less severe symptoms than the epidemic strain of SARS-CoV in vivo[88,89]. Similarly, both bWIV1 and bRsSHC014 can infect civet cells, but they bind civet ACE2 less well than cSz02. Thus, it is predicted that both strains will be attenuated compared with early-phase or late-phase human SARS epidemic viruses. Future evolution of bat SARSr-CoV strains bWIV1 and bRsSHC014 in crucial RBM residues may allow them to cross the species barriers between bats, civets and humans, posing potential health threats.

Origin and evolution of MERS-CoV

Whereas the emergence of SARS involved palm civets, most of the early MERS index cases had contact with dromedary camels. Indeed, MERS-CoV strains isolated from camels were almost identical to those isolated from humans[90–95]. Moreover, MERS-CoV-specific antibodies were highly prevalent in camels from the Middle East, Africa and Asia[13,14,96–103]. MERS-CoV infections were detected in camel serum samples collected in 1983 (rEF.[100]), suggesting that MERS-CoV was present in camels at least 30 years ago. Genomic sequence analysis indicated that MERS-CoV, *Tylonycteris* bat coronavirus HKU4 and *Pipistrellus* bat coronavirus HKU5 are phylogenetically related (denoted as beta coronavirus lineage C)[21]. The viruses in this lineage have identical genomic structures and are highly conserved in their polyproteins and most structural proteins, but their S proteins and accessory proteins are highly variable. MERSr-CoVs were found in at least 14 bat species from two bat families, Vespertilionidae and Nycteridae. However, none of these MERSr-CoVs is a direct progenitor of MERS-CoV, as their S proteins differ substantially from that of MERS-CoV[98,104–106].

To understand the evolutionary relationships between MERS-CoV and MERSr-CoVs, we constructed a phylogenetic tree on the basis of the alignment of all the coding regions (FIg. 4b; Supplementary Fig. S1b). The phylogenetic tree contains two main clusters and several small clades or strains. Overall, the genetic diversity within the L1 and L2 viral lineages is low, indicating that humans and camels have been infected by viruses from the same source within a short time period. The L1 viruses include human and camel MERS-CoVs mainly

from the Middle East (the United Arab Emirates, the Kingdom of Saudi Arabia, Oman and Jordan) and two Asian countries (South Korea and Thailand) that had caused outbreaks in human populations. It is worth noting that the cases reported in South Korea and Thailand were related to those in the Middle East. The L2 viruses include camel MERS-CoVs from Africa (Nigeria, Burkina Faso and Ethiopia) and one Middle East country (Morocco); these viruses have not caused any human infection. Clearly, these two viral lineages share a common ancestor but have diverged in their potential to cause human infections. The MERSr-CoV strain Neoromicia/5038 (GenBank No. MF593268) isolated in South Africa was the closest relative to MERS-CoVs in the phylogenetic tree. Overall, all the MERSr-CoV isolated from bats support the hypothesis that MERS-CoV originated from bats. However, given the phylogenetic gap between the bat MERSr-CoVs and human and camel MERS-CoVs, there should be other yet-to-be-identified viruses that are circulating in nature and directly contributed to the emergence of MERS-CoV in humans and camels. Hopefully, such viruses will be found in bats in the future.

Not surprisingly, recombination events have taken place in the evolution and emergence of MERS-CoV[94,105,107–109]. Phylogenetic trees constructed using genes encoding *orf1ab* and *S* were incongruent with the tree topology of the complete genomes, suggesting potential recombination in these genes[108]. Numerous recombinations imply that MERS-CoV originated from the exchange of genetic elements between different viral ancestors, including those isolated from camels and the assumed natural host bats[94,105,107,110,111].

Variability of human and camel MERS-CoV

The full-length genomic sequences of MERS-CoVs isolated from humans and camels are almost identical (>99% identity). The major variations are located in S, ORF4b and ORF3, particularly in African camel MERS-CoVs[94]. Substitutions of a few amino acid residues were found in the S protein of some camel MERS-CoVs, but none of them was located in the RBD[94,112]. Neutralization assays indicated that camel sera that are positive for MERS-CoV can completely neutralize the human MERS-CoV strains, suggesting that MERS-CoVs isolated from humans and camels are antigenically similar to each other[94]. MERS-CoVs from both humans and camels contain variable ORF3 and ORF4 proteins with different lengths owing to either terminal truncations or internal deletions[94]. ORF4b is known to be an interferon antagonist[113,114]. MERS-CoV isolates from West African camels with a truncated ORF4b gene replicate less efficiently in human cell culture and are less pathogenic in human DPP4 transgenic mice[94]. Curiously, deletion of the *orf4* gene in the human MERS-CoV strain EMC did not substantially reduce virus replication, although it induced a stronger interferon response[94]. Another study demonstrated that the deletion of *orf3–orf5* dramatically attenuated MERS-CoV virulence, primarily through increased host responses, including disrupted cellular processes, increased activation of the interferon pathway and robust inflammation[115].

Variability of bat MERSr-CoVs

To date, bat MERSr-CoVs and human and camel MERS-CoVs share the same genomic structures but differ substantially in their genomic sequences[105,106,110,111,116]. The highest overall genomic sequence identity between bat MERSr-CoV and human and camel MERS-CoV is ~85%. On the basis of their genomic sequences, several bat MERSr-CoV strains discovered in China, such as li-MERSr-CoV, Ve-MERSr-CoV and Hy-MERSr-CoV, have just reached the taxonomic threshold to be considered the same species as MERS-CoV[106,110,111].

Compared with human and camel MERS-CoV, bat MERSr-CoVs vary most in *S* and accessory genes. The sequence identity of the S protein between bat MERSr-CoVs and human and camel MERS-CoVs is approximately 45–65%, with even lower sequence identity in the RBD region[110,111]. The size of these S proteins differs in these strains, mainly because of deletions in their RBD region and/or the S1 and S2 boundary. These deletions are considered to be related to the differences in receptor binding and cell entry[111,116]. The accessory genes, including those encoding ORF3, ORF4a, ORF4b and ORF5, are also highly variable in length and sequence between bat MERSr-CoVs and human and camel MERS-CoVs, suggesting substantial evolution of these genes in their natural hosts[105,106,110,111,116].

Receptor usage of MERS-CoV and MERSr-CoV

In contrast to SARS-CoV, which uses ACE2 as its receptor, MERS-CoV uses DPP4. Similar to SARS-CoV S1-CTD, MERS-CoV S1-CTD functions as the viral RBD[10,117]. Like the SARS-CoV S1-CTD, the MERS-CoV S1-CTD also contains two subdomains, a core structure and an RBM[9,118–120] (Fig. 6e). The core structures of these two S1-CTDs are similar to each other, with both containing a five-stranded β-sheet as the main scaffold. However, their RBMs differ substantially whereas the SARS-CoV RBM mainly contains loops, the MERS-CoV RBM mainly contains a four-stranded β-sheet. The structural differences between MERS-CoV and SARS-CoV RBMs account for the different receptor specificities of the two viruses[121].

Like the interactions between SARS-CoV and ACE2, the interactions between MERS-CoV and DPP4 have been extensively examined. DPP4 from humans, camels, horses and bats can function as a receptor for MERS-CoV, whereas DPP4 from mice, hamsters and ferrets cannot[112,122–125]. Key residue differences between human DPP4 and the DPP4 from other species affect the species specificities of MERS-CoV. For example, two residues (288 and 330) in mouse DPP4 and five residues (291, 295, 336, 341 and 346) in hamster DPP4 are largely responsible for the incompatibility of mouse and hamster DPP4s with MERS-CoV[112,123]. Mutating these residues to the corresponding residues in human DPP4 makes mouse and hamster DPP4 functional receptors for MERS-CoV. On the other hand, MERS-CoV and MERSr-CoVs have been isolated from camels and bats, respectively. MERS-CoV strains isolated from humans and camels are highly similar to each other, and they both use human DPP4 efficiently[112]. MERSr-CoVs from bats in general share only ~60–70% sequence identity with MERS-CoV in the RBD, and only some of these bat viruses, including HKU4,

recognize DPP4 as the receptor[110,111,126]. However, they bind DPP4 less efficiently than MERS-CoV. Mutating three residues in the HKU4 RBD (540, 547 and 558) substantially increased its affinity for human DPP4 (rEF.[127]). Overall, as in the case of SARS-CoV, receptor recognition is a crucial determinant of the host range of MERS-CoV.

SADS-CoV

From 28 October 2016 to 2 May 2017, swine acute diarrhoea syndrome (SADS) was observed in four pig breeding farms in Guangdong province, with a mortality up to 90% for piglets 5 days or younger. A novel HKU2-related bat coronavirus, named SADS-CoV, was identified as the causative agent[34,128,129]. The SADS-CoV isolates from piglets of the four farms were almost identical and shared 95% identity with *Rhinolophus* bat coronavirus HKU2 (rEF.[130]), indicating the bat origin of this pig virus. Immediately after the SADS outbreak, SADS-related CoVs (SADSr-CoVs) with 96–98% sequence identity to SADS-CoV were detected in 9.8% of anal swabs collected from different *Rhinolophus* species in Guangdong province during 2013–2016. Although genetically highly similar, bat SADSr-CoVs show high genetic diversity in the *S* gene, with 72–92% nucleotide and 80–98% amino acid identity to SADS-CoV. Receptor analysis indicated that none of the known coronavirus receptors, ACE2, DPP4 and aminopeptidase N, are essential for SADS-CoV entry[34]. The mechanism of transmission of SADS-CoV from bats to pigs and the pathogenesis of bat-originated SADSr-CoVs in pigs need further exploration. This is the first documented spillover of a bat coronavirus that caused severe disease in domestic animals, although molecular evolution data suggested PEDV probably originated in bats[37,38].

Conclusions and future perspectives

The collected data on genetic evolution, receptor binding and pathogenesis demonstrated that SARS-CoV most likely originated in bats through sequential recombination of bat SARSr-CoVs. Recombination likely occurred in bats before SARS-CoV was introduced into Guangdong province through infected civets or other infected mammals from Yunnan. The introduced SARS-CoV underwent rapid mutations in *S* and *orf8* and successfully spread in market civets. After several independent spillovers to humans, some of the strains underwent further mutations in *S* and became epidemic during the SARS outbreak in 2002–2003. However, a recent serological investigation revealed the presence of antibodies against the SARSr-CoV nucleocapsid in humans living around a bat cave but who had not shown clinical signs of disease, suggesting that the virus can infect humans through frequent contact[31].

A similar scenario might have happened for MERS-CoV. Since its outbreak in 2012, MERSr-CoVs and related viruses (HKU4 and HKU5) have been found in different bat species in five continents[17,21,106,110,111,116,126,127,132]. The ORF1ab of these viruses is highly similar to MERS-CoV ORF1ab, but they are highly diverse in their S proteins. Surprisingly, some bat MERSr-CoVs and HKU can use the same receptor, DPP4, as MERS-CoV[110,111,126,127]. Given the massive number of coronaviruses carried by different bat species, the high plasticity in receptor usage and other features such as adaptive mutation and recombination, frequent inter-species transmission from bats to animals and humans is expected.

Currently, no clinical treatments or prevention strategies are available for any human coronavirus. Given the conserved RBDs of SARS-CoV and bat SARSr-CoVs, some anti-SARS-CoV strategies in development, such as anti-RBD antibodies or RBD-based vaccines, should be tested against bat SARSr-CoVs. Recent studies demonstrated that anti-SARS-CoV strategies worked against only WIV1 and not SHC014 (r EFs[71,88,89]). In addition, little information is available on HKU3-related strains that have much wider geographical distribution and bear truncations in their RBD. Similarly, anti-S antibodies against MERS-CoV could not protect from infection with a pseudovirus bearing the bat MERSr-CoV S[111]. Furthermore, little is known about the replication and pathogenesis of these bat viruses. Thus, future work should be focused on the biological properties of these viruses using virus isolation, reverse genetics and in vitro and in vivo infection assays. The resulting data would help the prevention and control of emerging SARS-like or MERS-like diseases in the future.

It is widely accepted that many viruses have existed in their natural reservoirs for a very long time. The constant spillover of viruses from natural hosts to humans and other animals is largely due to human activities, including modern agricultural practices and urbanization. Therefore, the most effective way to prevent viral zoonosis is to maintain the barriers between natural reservoirs and human society, in mind of the 'one health' concept.

Published online 10 December 2018

1. Masters, P. S. & Perlman, S. in *Fields Virology* Vol. 2 (eds Knipe, D. M. & Howley, P. M.) 825–858 (Lippincott Williams & Wilkins, 2013).
2. Zhong, N. S. et al. Epidemiology and cause of severe acute respiratory syndrome (SARS) in Guangdong, People's Republic of China, in February, 2003. *Lancet* **362**, 1353–1358 (2003).
3. Drosten, C. et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N. Engl. J. Med.* **348**, 1967–1976 (2003).
4. Fouchier, R. A. et al. Aetiology: Koch's postulates fulfilled for SARS virus. *Nature* **423**, 240 (2003).
5. Ksiazek, T. G. et al. A novel coronavirus associated with severe acute respiratory syndrome. *N. Engl. J. Med.* **348**, 1953–1966 (2003).
6. Zaki, A. M., van Boheemen, S., Bestebroer, T. M., Osterhaus, A. D. & Fouchier, R. A. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N. Engl. J. Med.* **367**, 1814–1820 (2012).
7. Li, W. H. et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* **426**, 450–454 (2003).
8. Qian, Z. et al. Innate immune response of human alveolar type II cells infected with severe acute respiratory syndrome-coronavirus. *Am. J. Respir. Cell. Mol. Biol.* **48**, 742–748 (2013).
9. Lu, G. et al. Molecular basis of binding between novel human coronavirus MERS-CoV and its receptor CD26. *Nature* **500**, 227–231 (2013).
10. Raj, V. S. et al. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature* **495**, 251–254 (2013).
11. Scobey, T. et al. Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus. *Proc. Natl Acad. Sci. USA* **110**, 16157–16162 (2013).
12. Guan, Y. et al. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science* **302**, 276–278 (2003). **This paper is the first demonstration of SARS-CoV transmission from animals.**
13. Alagaili, A. N. et al. Middle East respiratory syndrome coronavirus infection in dromedary camels in Saudi Arabia. *MBio* **5**, e00884–14 (2014).
14. Hemida, M. G. et al. Middle East Respiratory Syndrome (MERS) coronavirus seroprevalence in domestic livestock in Saudi Arabia, 2010 to 2013. *Euro. Surveill.* **18**, 21–27 (2013).
15. Lau, S. K. et al. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proc. Natl Acad. Sci. USA* **102**, 14040–14045 (2005).

16. Kan, B. et al. Molecular evolution analysis and geographic investigation of severe acute respiratory syndrome coronavirus-like virus in palm civets at an animal market and on farms. *J. Virol.* **79**, 11892–11900 (2005).
17. Ithete, N. L. et al. Close relative of human Middle East respiratory syndrome coronavirus in bat, South Africa. *Emerg. Infect. Dis.* **19**, 1697–1699 (2013).
18. Ge, X. Y. et al. Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. *Nature* **503**, 535–538 (2013).
This paper provides the first identification of human ACE2 as the receptor for bat SARS-like coronavirus.
19. Yang, X. L. et al. Isolation and characterization of a novel bat coronavirus closely related to the direct progenitor of severe acute respiratory syndrome coronavirus. *J. Virol.* **90**, 3253–3256 (2016).
20. Hu, B. et al. Discovery of a rich gene pool of bat SARS-related coronaviruses provides new insights into the origin of SARS coronavirus. *PLOS Pathog.* **13**, e1006698 (2017).
This paper identifies a gene pool of SARS-CoVs in bats.
21. Lau, S. K. et al. Genetic characterization of Betacoronavirus lineage C viruses in bats reveals marked sequence divergence in the spike protein of pipistrellus bat coronavirus HKU5 in Japanese pipistrelle: implications for the origin of the novel Middle East respiratory syndrome coronavirus. *J. Virol.* **87**, 8638–8650 (2013).
22. Woo, P. C., Lau, S. K., Huang, Y. & Yuen, K. Y. Coronavirus diversity, phylogeny and interspecies jumping. *Exp. Biol. Med. (Maywood)* **234**, 1117–1127 (2009).
23. Perlman, S. & Netland, J. Coronaviruses post-SARS: update on replication and pathogenesis. *Nat. Rev. Microbiol.* **7**, 439–450 (2009).
24. Woo, P. C. et al. Discovery of seven novel mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. *J. Virol.* **86**, 3995–4008 (2012).
This paper describes coronavirus origins by phylogenetic analysis.
25. Graham, R. L., Donaldson, E. F. & Baric, R. S. A decade after SARS: strategies for controlling emerging coronaviruses. *Nat. Rev. Microbiol.* **11**, 836–848 (2013).
26. Hu, B., Ge, X., Wang, L. F. & Shi, Z. Bat origin of human coronaviruses. *Virol. J.* **12**, 221 (2015).
27. de Wit, E., van Doremalen, N., Falzarano, D. & Munster, V. J. SARS and MERS: recent insights into emerging coronaviruses. *Nat. Rev. Microbiol.* **14**, 523–534 (2016).
28. Su, S. et al. Epidemiology, genetic recombination, and pathogenesis of coronaviruses. *Trends Microbiol.* **24**, 490–502 (2016).
29. Forni, D., Cagliani, R., Clerici, M. & Sironi, M. Molecular evolution of human coronavirus genomes. *Trends Microbiol.* **25**, 35–48 (2017).
30. Anthony, S. J. et al. Global patterns in coronavirus diversity. *Virus Evol.* **3**, vex012 (2017).
31. Wang, L., Su, S., Bi, Y., Wong, G. & Gao, G. F. Bat-origin coronaviruses expand their host range to pigs. *Trends Microbiol.* **26**, 466–470 (2018).
32. Brian, D. A. & Baric, R. S. Coronavirus genome structure and replication. *Curr. Top. Microbiol. Immunol.* **287**, 1–30 (2005).
33. Lin, C. M., Saif, L. J., Marthaler, D. & Wang, Q. Evolution, antigenicity and pathogenicity of global porcine epidemic diarrhea virus strains. *Virus Res.* **226**, 20–39 (2016).
34. Zhou, P. et al. Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. *Nature* **556**, 255–258 (2018).
This paper describes bat origin of a swine coronavirus.
35. Huang, Y. W. et al. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. *MBio* **4**, e00737–00713 (2013).
36. Liu, C. et al. Receptor usage and cell entry of porcine epidemic diarrhea coronavirus. *J. Virol.* **89**, 6121–6125 (2015).
37. Simas, P. V. et al. Bat coronavirus in Brazil related to appalachian ridge and porcine epidemic diarrhea viruses. *Emerg. Infect. Dis.* **21**, 729–731 (2015).
38. Lacroix, A. et al. Genetic diversity of coronaviruses in bats in Lao PDR and Cambodia. *Infect. Genet. Evol.* **48**, 10–18 (2017).
39. Tu, C. et al. Antibodies to SARS coronavirus in civets. *Emerg. Infect. Dis.* **10**, 2244–2248 (2004).
40. Wang, M. et al. [Analysis on the risk factors of severe acute respiratory syndromes coronavirus infection in workers from animal markets]. *Zhonghua Liu Xing Bing Xue Za Zhi* **25**, 503–505 (2004).
41. Xu, H. F. et al. [An epidemiologic investigation on infection with severe acute respiratory syndrome coronavirus in wild animals traders in Guangzhou]. *Zhonghua Yu Fang Yi Xue Za Zhi* **38**, 81–83 (2004).
42. Song, H. D. et al. Cross-host evolution of severe acute respiratory syndrome coronavirus in palm civet and human. *Proc. Natl Acad. Sci. USA* **102**, 2430–2435 (2005).
This paper describes genetic evolution of SARS-CoV during transmission from animals and humans.
43. Li, W. et al. Bats are natural reservoirs of SARS-like coronaviruses. *Science* **310**, 676–679 (2005).
44. Ren, W. et al. Full-length genome sequences of two SARS-like coronaviruses in horseshoe bats and genetic variation analysis. *J. Gen. Virol.* **87**, 3355–3359 (2006).
45. Drexler, J. F. et al. Genomic characterization of severe acute respiratory syndrome-related coronavirus in European bats and classification of coronaviruses based on partial RNA-dependent RNA polymerase gene sequences. *J. Virol.* **84**, 11336–11349 (2010).
46. Lau, S. K. et al. Ecoepidemiology and complete genome comparison of different strains of severe acute respiratory syndrome-related Rhinolophus bat coronavirus in China reveal bats as a reservoir for acute, self-limiting infection that allows recombination events. *J. Virol.* **84**, 2808–2819 (2010).
47. Rihtaric, D., Hostnik, P., Steyer, A., Grom, J. & Toplak, I. Identification of SARS-like coronaviruses in horseshoe bats (Rhinolophus hipposideros) in Slovenia. *Arch. Virol.* **155**, 507–514 (2010).
48. Yuan, J. et al. Intraspecies diversity of SARS-like coronaviruses in Rhinolophus sinicus and its implications for the origin of SARS coronaviruses in humans. *J. Gen. Virol.* **91**, 1058–1062 (2010).
49. Balboni, A., Gallina, L., Palladini, A., Prosperi, S. & Battilani, M. A real-time PCR assay for bat SARS-like coronavirus detection and its application to Italian greater horseshoe bat faecal sample surveys. *Sci. World J.* **2012**, 989514 (2012).
50. Yang, L. et al. Novel SARS-like betacoronaviruses in bats, China, 2011. *Emerg. Infect. Dis.* **19**, 989–991 (2013).
51. He, B. et al. Identification of diverse alphacoronaviruses and genomic characterization of a novel severe acute respiratory syndrome-like coronavirus from bats in China. *J. Virol.* **88**, 7070–7082 (2014).
52. Gouilh, M. A. et al. SARS-coronavirus ancestor's foot-prints in South-East Asian bat colonies and the refuge theory. *Infect. Genet. Evol.* **11**, 1690–1702 (2011).
53. Wacharapluesadee, S. et al. Diversity of coronavirus in bats from Eastern Thailand. *Virol. J.* **12**, 57 (2015).
54. Tong, S. et al. Detection of novel SARS-like and other coronaviruses in bats from Kenya. *Emerg. Infect. Dis.* **15**, 482–485 (2009).
55. Wang, M. N. et al. Longitudinal surveillance of SARS-like coronaviruses in bats by quantitative real-time PCR. *Virol. Sin.* **31**, 78–80 (2016).
56. Lai, M. M. & Cavanagh, D. The molecular biology of coronaviruses. *Adv. Virus Res.* **48**, 1–100 (1997).
57. Zhao, Z. et al. Moderate mutation rate in the SARS coronavirus genome and its implications. *BMC Evol. Biol.* **4**, 21 (2004).
58. Hon, C. C. et al. Evidence of the recombinant origin of a bat severe acute respiratory syndrome (SARS)-like coronavirus and its implications on the direct ancestor of SARS coronavirus. *J. Virol.* **82**, 1819–1826 (2008).
59. Wu, Z. et al. ORF8-related genetic evidence for chinese horseshoe bats as the source of human severe acute respiratory syndrome coronavirus. *J. Infect. Dis.* **213**, 579–583 (2016).
60. Nagy, P. D. & Simon, A. E. New insights into the mechanisms of RNA recombination. *Virology* **235**, 1–9 (1997).
61. Rowe, C. L. et al. Generation of coronavirus spike deletion variants by high-frequency recombination at regions of predicted RNA secondary structure. *J. Virol.* **71**, 6183–6190 (1997).
62. Chinese, S. M. E. C. Molecular evolution of the SARS coronavirus during the course of the SARS epidemic in China. *Science* **303**, 1666–1669 (2004).
This paper describes the genetic evolution of human SARS-CoV during SARS outbreaks.
63. Babcock, G. J., Esshaki, D. J., Thomas, W. D. & Ambrosino, D. M. Amino acids 270 to 510 of the severe acute respiratory syndrome coronavirus spike protein are required for interaction with receptor. *J. Virol.* **78**, 4552–4560 (2004).
64. Wong, S. K., Li, W. H., Moore, M. J., Choe, H. & Farzan, M. A. 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. *J. Biol. Chem.* **279**, 3197–3201 (2004).
This paper identifies the receptor-binding domain of the SARS-CoV spike protein.
65. Le, T. M. et al. Expression, post-translational modification and biochemical characterization of proteins encoded by subgenomic mRNA8 of the severe acute respiratory syndrome coronavirus. *FEBSJ.* **274**, 4211–4222 (2007).
66. Oostra, M., de Haan, C. A. & Rottier, P. J. The 29-nucleotide deletion present in human but not in animal severe acute respiratory syndrome coronaviruses disrupts the functional expression of open reading frame 8. *J. Virol.* **81**, 13876–13888 (2007).
67. Wong, H. H. et al. Accessory proteins 8b and 8ab of severe acute respiratory syndrome coronavirus suppress the interferon signaling pathway by mediating ubiquitin- dependent rapid degradation of interferon regulatory factor 3. *Virology* **515**, 165–175 (2018).
68. Sung, S. C., Chao, C. Y., Jeng, K. S., Yang, J. Y. & Lai, M. M. The 8ab protein of SARS-CoV is a luminal ER membrane-associated protein and induces the activation of ATF6. *Virology* **387**, 402–413 (2009).
69. Chen, C. Y. et al. Open reading frame 8a of the human severe acute respiratory syndrome coronavirus not only promotes viral replication but also induces apoptosis. *J. Infect. Dis.* **196**, 405–415 (2007).
70. Zhou, P., Li, H., Wang, H., Wang, L. F. & Shi, Z. Bat severe acute respiratory syndrome-like coronavirus ORF3b homologues display different interferon antagonist activities. *J. Gen. Virol.* **93**, 275–281 (2012).
71. Zeng, L. P. et al. Cross-neutralization of SARS coronavirus-specific antibodies against bat SARS-like coronaviruses. *Sci. China Life Sci.* **60**, 1399–1402 (2017).
72. Li, F. Receptor recognition and cross-species infections of SARS coronavirus. *Antiviral Res.* **100**, 246–254 (2013).
73. Li, W. H. et al. Animal origins of the severe acute respiratory syndrome coronavirus: insight from ACE2-S-protein interactions. *J. Virol.* **80**, 4211–4219 (2006).
74. Li, F. et al. Conformational states of the severe acute respiratory syndrome coronavirus spike protein ectodomain. *J. Virol.* **80**, 6794–6800 (2006).
75. Yuan, Y. et al. Cryo-EM structures of MERS-CoV and SARS-CoV spike glycoproteins reveal the dynamic receptor binding domains. *Nat. Commun.* **8**, 15092 (2017).
76. Li, W. H. et al. Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2. *EMBO J.* **24**, 1634–1643 (2005).
This paper identifies key residues involved in SARS-CoV adaptation to humans.
77. Towler, P. et al. ACE2 X-ray structures reveal a large hinge-bending motion important for inhibitor binding and catalysis. *J. Biol. Chem.* **279**, 17996–18007 (2004).
78. Qu, X. X. et al. Identification of two critical amino acid residues of the severe acute respiratory syndrome coronavirus spike protein for its variation in zoonotic tropism transition via a double substitution strategy. *J. Biol. Chem.* **280**, 29588–29595 (2005).
79. Liu, L. et al. Natural mutations in the receptor binding domain of spike glycoprotein determine the reactivity of cross-neutralization between palm civet coronavirus and severe acute respiratory syndrome coronavirus. *J. Virol.* **81**, 4694–4700 (2007).
80. Liang, G. D. et al. Laboratory diagnosis of four recent sporadic cases of community- acquired SARS, guangdong province, china. *Emerg. Infect. Dis.* **10**, 1774–1781 (2004).
81. Sheahan, T. et al. Mechanisms of zoonotic severe acute respiratory syndrome coronavirus host range expansion in human airway epithelium. *J. Virol.* **82**, 2274–2285 (2008).
82. Li, F., Li, W. H., Farzan, M. & Harrison, S. C. in *The Nidoviruses: Toward Control of SARS and Other Nidovirus Diseases* (Advances in Experimental Medicine and Biology Vol. 581), 229–234 (Springer, 2006).
83. Wu, K. L., Peng, G. Q., Wilken, M., Geraghty, R. J. & Li, F. Mechanisms of host receptor adaptation by severe acute respiratory syndrome coronavirus. *J. Bio. Chem.* **287**, 8904–8911 (2012).

84. Wu, K. et al. A virus-binding hot spot on human angiotensin-converting enzyme 2 Is critical for binding of two different coronaviruses. *J. Virol.* **85**, 5331–5337 (2011).
85. Li, F. Structural analysis of major species barriers between humans and palm civets for severe acute respiratory syndrome coronavirus infections. *J. Virol.* **82**, 6984–6991 (2008).
86. Becker, M. M. et al. Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice. *Proc. Natl Acad. Sci. USA* **105** , 19944–19949 (2008).
87. Ren, W. et al. Difference in receptor usage between severe acute respiratory syndrome (SARS)coronavirus and SARS-like coronavirus of bat origin. *J. Virol.* **82**, 1899–1907 (2008).
88. Menachery, V. D. et al. A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence. *Nat. Med.* **21** , 1508–1513 (2015).
89. Menachery, V. D. et al. SARS-like WIV1-CoV poised for human emergence. *Proc. Natl Acad. Sci. USA* **113** , 3048–3053 (2016).
90. Haagmans, B. L. et al. Middle East respiratory syndrome coronavirus in dromedary camels: an outbreak investigation. *Lancet Infect. Dis.* **14**, 140–145 (2014).
This paper provides the first identification of MERS-CoV in camels.
91. Azhar, E. I. et al. Evidence for camel-to-human transmission of MERS coronavirus. *N. Engl. J. Med.* **370** , 2499–2505 (2014).
92. Raj, V. S. et al. Isolation of MERS coronavirus from a dromedary camel, Qatar, 2014. *Emerg. Infect. Dis.* **20**, 1339–1342 (2014).
93. Sabir, J. S. et al. Co-circulation of three camel coronavirus species and recombination of MERS-CoVs in Saudi Arabia. *Science* **351** , 81–84 (2016).
94. Chu, D. K. W. et al. MERS coronaviruses from camels in Africa exhibit region-dependent genetic diversity. *Proc. Natl Acad. Sci. USA* **115** , 3144–3149 (2018).
This paper describes the genetic diversity of MERS-CoV in African camels.
95. Paden, C. R. et al. Zoonotic origin and transmission of Middle East respiratory syndrome coronavirus in the UAE. *Zoo. Pub. Health* **65**, 322–333 (2018).
96. Perera, R. A. et al. Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013. *Euro. Surveill.* **18**, 20574 (2013).
97. Reusken, C. B. et al. Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study. *Lancet Infect. Dis.* **13**, 859–866 (2013).
98. Hemida, M. G. et al. MERS coronavirus in dromedary camel herd, Saudi Arabia. *Emerg. Infect. Dis.* **20**, 1231–1234 (2014).
99. Corman, V. M. et al. Antibodies against MERS coronavirus in dromedary camels, Kenya, 1992–2013. *Emerg. Infect. Dis.* **20**, 1319–1322 (2014).
100. Muller, M. A. et al. MERS coronavirus neutralizing antibodies in camels, Eastern Africa, 1983–1997. *Emerg. Infect. Dis.* **20**, 2093–2095 (2014).
101. Muller, M. A. et al. Presence of Middle East respiratory syndrome coronavirus antibodies in Saudi Arabia: a nationwide, cross-sectional, serological study. *Lancet Infect. Dis.* **15**, 559–564 (2015).
102. Saqib, M. et al. Serologic evidence for MERS-CoV infection in dromedary camels, Punjab, Pakistan, 2012–2015. *Emerg. Infect. Dis.* **23**, 550–551 (2017).
103. Harcourt, J. L. et al. The prevalence of Middle East respiratory syndrome coronavirus (MERS-CoV) antibodies in dromedary camels in Israel. *Zoonoses Public Health* **65**, 749–754 (2018).
104. de Groot, R. J. et al. Middle East respiratory syndrome coronavirus (MERS-CoV): announcement of the Coronavirus Study Group. *J. Virol.* **87**, 7790–7792 (2013).
105. Corman, V. M. et al. Rooting the phylogenetic tree of middle East respiratory syndrome coronavirus by characterization of a conspecific virus from an African bat. *J. Virol.* **88**, 11297–11303 (2014).
106. Yang, L. et al. MERS-related betacoronavirus in Vespertilio superans bats. *China. Emerg. Infect. Dis.* **20**, 1260–1262 (2014).
107. Dudas, G. & Rambaut, A. MERS-CoV recombination: implications about the reservoir and potential for adaptation. *Virus Evol.* **2** , vev023 (2016).
108. Wang, Y. et al. Origin and possible genetic recombination of the Middle East respiratory syndrome coronavirus from the first imported case in China: phylogenetics and coalescence analysis. *MBio* **6** , e01280–15 (2015).
109. Zhang, Z., Shen, L. & Gu, X. Evolutionary dynamics of MERS-CoV: potential recombination, positive selection and transmission. *Sci. Rep.* **6** , 25049 (2016).
110. Lau, S. K. P. et al. Receptor usage of a novel bat lineage C betacoronavirus reveals evolution of Middle East respiratory syndrome-related coronavirus spike proteins for human dipeptidyl peptidase 4 binding. *J. Infect. Dis.* **218** , 197–207 (2018).
111. Luo, C. M. et al. Discovery of novel bat coronaviruses in South China that use the same receptor as Middle East respiratory syndrome coronavirus. *J. Virol.* **92**, e00116–18 (2018).
112. Barlan, A. et al. Receptor variation and susceptibility to Middle East respiratory syndrome coronavirus infection. *J. Virol.* **88**, 4953–4961 (2014).
113. Matthews, K. L., Coleman, C. M., van der Meer, Y., Snijder, E. J. & Frieman, M. B. The ORF4b-encoded accessory proteins of Middle East respiratory syndrome coronavirus and two related bat coronaviruses localize to the nucleus and inhibit innate immune signalling. *J. Gen. Virol.* **95**, 874–882 (2014).
114. Yang, Y. et al. Middle East respiratory syndrome coronavirus ORF4b protein inhibits type I interferon production through both cytoplasmic and nuclear targets. *Sci. Rep.* **5** , 17554 (2015).
115. Menachery, V. D. et al. MERS-CoV accessory ORFs play key role for infection and pathogenesis. *MBio* **8** , e00665–17 (2017).
116. Anthony, S. J. et al. Further evidence for bats as the evolutionary source of Middle East respiratory syndrome coronavirus. *MBio* **8** , e00373–17 (2017).
117. Du, L. et al. Identification of a receptor-binding domain in the S protein of the novel human coronavirus Middle East respiratory syndrome coronavirus as an essential target for vaccine development. *J. Virol.* **87**, 9939–9942 (2013).
118. Mou, H. et al. The receptor binding domain of the new Middle East respiratory syndrome coronavirus maps to a 231-residue region in the spike protein that efficiently elicits neutralizing antibodies. *J. Virol.* **87**, 9379–9383 (2013).
119. Wang, N. et al. Structure of MERS-CoV spike receptor-binding domain complexed with human receptor DPP4. *Cell Res.* **23**, 986–993 (2013).
120. Chen, Y. et al. Crystal structure of the receptor-binding domain from newly emerged Middle East respiratory syndrome coronavirus. *J. Virol.* **87**, 10777–10783 (2013).
121. Li, F. Receptor recognition mechanisms of coronaviruses: a decade of structural studies. *J. Virol.* **89**, 1954–1964 (2015).
122. Cockrell, A. S. et al. Mouse dipeptidyl peptidase 4 is not a functional receptor for Middle East respiratory syndrome coronavirus infection. *J. Virol.* **88**, 5195–5199 (2014).
123. van Doremalen, N. et al. Host species restriction of Middle East respiratory syndrome coronavirus through its receptor, dipeptidyl peptidase 4. *J. Virol.* **88**, 9220–9232 (2014).
124. Peck, K. M. et al. Glycosylation of mouse DPP4 plays a role in inhibiting Middle East respiratory syndrome coronavirus infection. *J. Virol.* **89**, 4696–4699 (2015).
125. Peck, K. M. et al. Permissivity of dipeptidyl peptidase 4 orthologs to Middle East respiratory syndrome coronavirus is governed by glycosylation and other complex determinants. *J. Virol.* **91** , e00534–17 (2017).
126. Yang, Y. et al. Receptor usage and cell entry of bat coronavirus HKU4 provide insight into bat-to-human transmission of MERS coronavirus. *Proc. Natl Acad. Sci. USA* **111** , 12516–12521 (2014).
127. Wang, Q. et al. Bat origins of MERS-CoV supported by bat coronavirus HKU4 usage of human receptor CD26. *Cell Host Microbe* **16**, 328–337 (2014).
128. Gong, L. et al. A new bat-HKU2-like coronavirus in swine, China, 2017. *Emerg. Infect. Dis.* **23**, 9 (2017).
129. Pan, Y. et al. Discovery of a novel swine enteric alphacoronavirus (SeACoV)in southern China. *Vet. Microbiol.* **211** , 15–21 (2017).
130. Lau, S. K. et al. Complete genome sequence of bat coronavirus HKU2 from Chinese horseshoe bats revealed a much smaller spike gene with a different evolutionary lineage from the rest of the genome. *Virology* **367**, 428–439 (2007).
131. Wang, N. et al. Serological evidence of bat SARS-related coronavirus infection in humans. *China. Virol. Sin.* **33**, 104–107 (2018).
132. Memish, Z. A. et al. Middle East respiratory syndrome coronavirus in bats, Saudi Arabia. *Emerg. Infect. Dis.* **19**, 1819–1823 (2013).
133. Huynh, J. et al. Evidence supporting a zoonotic origin of human coronavirus strain NL63. *J. Virol.* **86**, 12816–12825 (2012).
134. Tao, Y. et al. Surveillance of bat coronaviruses in Kenya identifies relatives of human coronaviruses NL63 and 229E and their recombination history. *J. Virol.* **91** , e01953–16 (2017).
This paper describes the bat origins of two human coronaviruses, NL63 and 229E.
135. Corman, V. M. et al. Link of a ubiquitous human coronavirus to dromedary camels. *Proc. Natl Acad. Sci. USA* **113** , 9864–9869 (2016).
136. Drexler, J. F., Corman, V. M. & Drosten, C. Ecology, evolution and classification of bat coronaviruses in the aftermath of SARS. *Antiviral Res.* **101** , 45–56 (2014).
137. Guindon, S. et al. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* **59**, 307–321 (2010).
138. Lin, X. D. et al. Extensive diversity of coronaviruses in bats from China. *Virology* **507** , 1–10 (2017).
139. Mackay, I. M. & Arden, K. E. MERS coronavirus: diagnostics, epidemiology and transmission. *Virol. J.* **12**, 222 (2015).
140. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2126–2132 (2004).
141. Li, F. et al. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science* **309**, 1864–1868 (2005).

Acknowledgements
This work was jointly funded by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB29010000), the National Natural Science Foundation of China (31621061) and the US National Institutes of Health (NIH) National Institute of Allergy and Infection Diseases (R01AI110964) to Z.-L.S; NIH grants (R01AI089728 and R01AI110700) to F.L.; the CAS Pioneer Hundred Talents Program to J.C.; and the Wuhan Institute of Virology (WIV) "One-Three-Five" Strategic Program (WIV-135-TP1) to J.C. and Z.-L.S.

Author contributions
All authors researched data for the article, contributed substantially to discussion of the content, wrote the article and reviewed and edited the manuscript before submission.

Competing interests
The authors declare no competing interests.

Publisher's note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Reviewer information
Nature Reviews Microbiology thanks R. Baric, B. Haagmans and K.-Y. Yuen for their contribution to the peer review of this work.

Supplementary information
Supplementary information is available for this paper at https://doi.org/10.1038/s41579-018-0118-9.

RELAtED linkS
international Committee on Taxonomyof viruses: http://www.ictvonline.org/

Journal of Virological Methods 265 (2019) 66–70

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development of a SYBRgreen-basedreal-time RT-PCRassayfor rapid detection of the emerging swine acute diarrhea syndrome coronavirus

Lei Ma[a,1], Fanwen Zeng[b,1], Feng Cong[a], Bihong Huang[a], Ren Huang[a], Jingyun Ma[c,*], Pengju Guo[a,*]

[a] GuangdongKey Laboratory of Laboratory Animals, GuangdongLaboratory Animals Monitoring Institute, Guangzhou,China
[b] Collegeof Veterinary Medicine,South China Agricultural University, Guangzhou,China
[c] Collegeof Animal Science,South China Agricultural University, Guangzhou,China

ARTICLEINFO

Keywords:
Swine acute diarrhea syndrome coronavirus (SADS-CoV)
Quantitative PCR
Detection

ABSTRACT

Swine acute diarrhea syndrome coronavirus (SADS-CoV)is a novel coronavirus which was associatedwith severe diarrhea diseasein pigs. SADS-CoVwas first detected and identified as the causative agent of a devastating swine disease outbreak in southern China in 2017. Routine monitoring and early detection of the source of infection is therefore integral to the prevention and control of SADS-CoVinfection. In this study, a SYBRgreen-based real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR)technique was established for rapid detection and monitoring of this emerging virus. Specific primers were designed based on the conservedregion within the M geneof the viral genome.The lowest detection limit of the RT-qPCRassaywas 10 copies/μL. This assaywas specific and had no cross-reactionwith other 11 swine viruses. The positive rate of 84 clinical samples for the SYBRgreen-basedRT-qPCRand the conventional RT-PCRwas 73.81% (62/84) and 53.57% (45/84), respectively. Theseresults demonstrated that the SYBRgreen-basedRT-qPCRtechnique was an effectively diagnostic method with higher sensitivity than probe-basedRT-qPCRand gel-basedRT-PCRfor detection and epidemiological investigations of SADS-CoV.

Swine acute diarrhea syndrome coronavirus (SADS-CoV), a new member of the genus Alphacoronavirus, contributed to a devastating swine disease outbreak in Guangdong province, China, which caused death to more than twenty thousand piglets acrossfour farms and huge economic losses(Zhou et al., 2018b). The piglets infected with SADS-CoV mainly exhibited the clinical manifestations of acute vomiting and diarrhea and death (Gong et al., 2017; Xu et al., 2018; Zhou et al., 2018b). It was notable that the genome sequenceof the causative agent of this large-scale outbreak shares remarkable homology with a bat coronavirus detected in a cave near the pig farm, implying SADS-CoV most likely originated from bats (Gong et al., 2017; Lau et al., 2007; Zhou et al., 2018b). Considering the high lethality of this new emerging virus and the clinical signs of the infected pigs that are similar to that caused by other porcine enteric coronaviruses, there is an urgent need to establish a sensitive and reliable diagnostic method that will be of help to early discovery and identification of SADS-CoVinfection.

Cytopathic effect (CPE) could be observed only after serial passages of SADS-CoVin Vero cells, so isolation and detection of the virus by cell culture are time-consuming (Pan et al., 2017; Zhou et al., 2018b). Immunofluorescence (IF) staining has been used for the detection of SADS-CoV(Pan et al., 2017; Zhou et al., 2018b), however virus-specific antibody and fluorescently-labeled antibody are required to react with the SADS-CoVantigen, this procedure makes the IF method complex and time-consuming. Recently, a reverse transcription loop-mediated isothermal amplification method (RT-LAMP) was established for the detection of SADS-CoV,however it couldn't be used for quantitative analysis of the viral copiesin the samples(Wang et al., 2018). Real-time quantitative PCR(qPCR)is a powerful diagnostic method and has been widely used for detection and quantitation of pathogenic microorganism becauseof high sensitivity and reproducibility (Jensen et al., 2018; Yang et al., 2018; Yuan et al., 2016; Zhou et al., 2018a). Recently, a study reported a probe-based real time RT-qPCRfor the detection of SADS-CoV,with a detection limit of 30 DNA copies/μL (Zhou et al., 2018a). Whereas,the SYBRgreen-basedqPCRassaydoes not require an expensive probe, and is more cost-effective and easier to design than probe-based RT-qPCR.However, up to now, there is no report about SYBRgreen-basedreal-time RT-qPCRfor the detection of SADS-CoV.

In this study, a SYBRgreen-basedreal-time RT-qPCRassaytargeted

* Corresponding authors.
E-mail addresses:majy2400@scau.edu.cn(J. Ma), vetbio2016@hotmail.com (P. Guo).
[1] Contributed equally to this study.

https://doi.org/10.1016/j.jviromet.2018.12.010
Received 17 August 2018; Received in revised form 5 November 2018; Accepted 17 December 2018
Available online 26 December 2018
0166-0934/ © 2018 Elsevier B.V. All rights reserved.

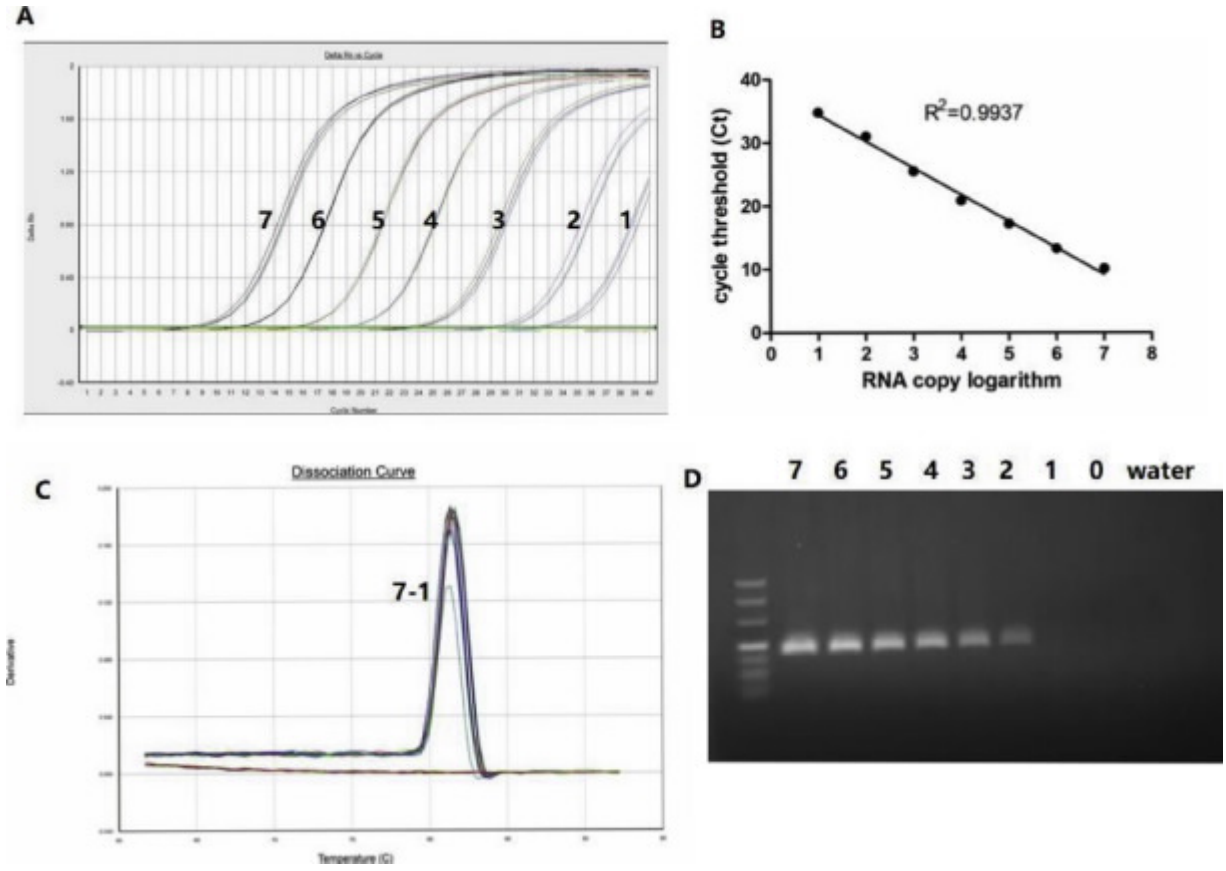


Fig. 1. Sensitivity of SYBRgreen-basedRT-qPCR.A: Amplification of ten-fold dilutions of transcribed RNA by SYBRgreen-basedRT-qPCRin triplicates. B: A standard curve of RT-qPCR,which was generatedby plotting mean Ct values versusRNA copy number in triplicates. C: Dissociation curve analysis of RT-qPCRproducts. D: The detection limit of gel-basedRT-PCR.0–7: a serial of ten-fold RNA dilutions (1×10^0–1×10^7 copies/μL).

Table 1
The specific primers.

Primers	Primer sequence(5′–3′)
SADS-CoV-F	ATGACTGATTCTAACAACAC
SADS-CoV-R	TTAGACTAAATGCAGCAATC
PRRSV-F	ATTTGAATGTTCGCACGGTCTC
PRRSV-R	CCGCTGAAACTCTGGTTAAAGG
PEDV-F	AACACGGCGACTACTCAGC
PEDV-R	GCCTTCTTTAGCAACCCAG
PRV-F	CCACGCGGTCGATGTCGTAG
PRV-R	GGTGCGCGGCGGCGTGTACG
CSFV-F	ATGCCCATAGTAGGACTAGCA
CSFV-R	TCAACTCCATGTGCCATGTAC
PCV2-F	AAGAAGCGGACCCCAAC
PCV2-R	AGGTGGCCCCACAATGA
TGEV-F	GTGGTGTTAGGTGATTATTTTCC
TGEV-R	TATGGTTTAACCTGCACTCACTA
RV-F	TATGCAATACCAGTAGGACCAG
RV-R	GCTCTACGTAGCGAGTATGAAATC
PPV-F	AGCCAAAAATGCAAACCCCAATA
PPV-R	CTTTAGCCTTGGAGCCGTGGAG
FMDV-F	GACAAAGGTTTTGTTCTTGGTCA
FMDV-R	TGCGAGTCCTGCCACGGA
PDCoV-F	TTTCAGGTGCTCAAAGCTCA
PDCoV-R	GCGAAAAGCATTTCCTGAAC
SIV-F	GGACATGTTACCCAGGAGA
SIV-R	ACTGGTAGGTGGATGGTGAA

the mostly conserved gene of SADS-CoVwas established for rapid, sensitive and quantitative detection of the virus infection.

Complete genome sequencesof 34 SADS-CoVisolates in total retrieved from GenBank were aligned using MegAlign7.1.0 (DNAStar, USA), and Matrix (M) gene sequencewas found to be highly conserved among all the strains of SADS-CoVin the database.The primers named as SADS-F (5′-ATGACTGATTCTAACAACAC-3′) (covering nucleotides 24,784–24,803) and SADS-R (5′-TTAGACTAAATGCAGCAATC-3′) (complementary to nucleotides 25,451–25,470) were designed and used to amplify the M gene. Viral RNA was isolated from the cells infected with SADS-CoVisolate SADS-CoV/CN/GDWT/2017 (Accession number: MG557844) by TGuide virus DNA/RNA kit (TIANGEN, China) following the manufacturer's instruction. The viral RNA was transcribed and amplified by using PrimeScript™ One Step RT-PCR Kit (Takara, China) with the primers SADS-Fand SADS-R.The amplicon was subjected to gel electrophoresis and purified by using DNA Gel Extraction Kit (Beyotime, China). The purified amplicon was inserted into pGEM-T vector and transformed to Escherichiacoli DH5α competent cells. The insert of positive clone was confirmed by sequencingand named as SADS-N. SADS-N plasmid was linearized by endonuclease digestion and transcribed in vitro by RiboMAX™ Large Scale RNA Production System (Promega, USA) according to the instruction of the kit manual. The in vitro transcribed RNA was purified, quantified and converted into copy number as the previously reported method (Yuan et al., 2016). Ten-fold serially diluted RNA ranged from 1×10^0–1×10^7 copies/μL were prepared with DEPC-treated water and used as the molecular standards in the following assays.

For the SYBRgreen-basedRT-qPCR,the forward primer (5′-ATGTGGCTCCTATGGCCCTT-3′) (covering nucleotides 24,943–24,962) and the reverse primer (5′-ACGTTCGCGTTCTGCGATAA-3′) (complementary to nucleotides 25,095–25,114) were used to optimize the performance of the assay.The SYBRgreen-basedRT-qPCRwas carried out using the one step SYBRPrimeScript RT-PCRKit (Takara, China) following the kit manual. The RT-qPCRassayin a 20 μL reaction volume contained 10 μL of 2×one step SYBRRT-PCRBuffer, 0.4 μL of

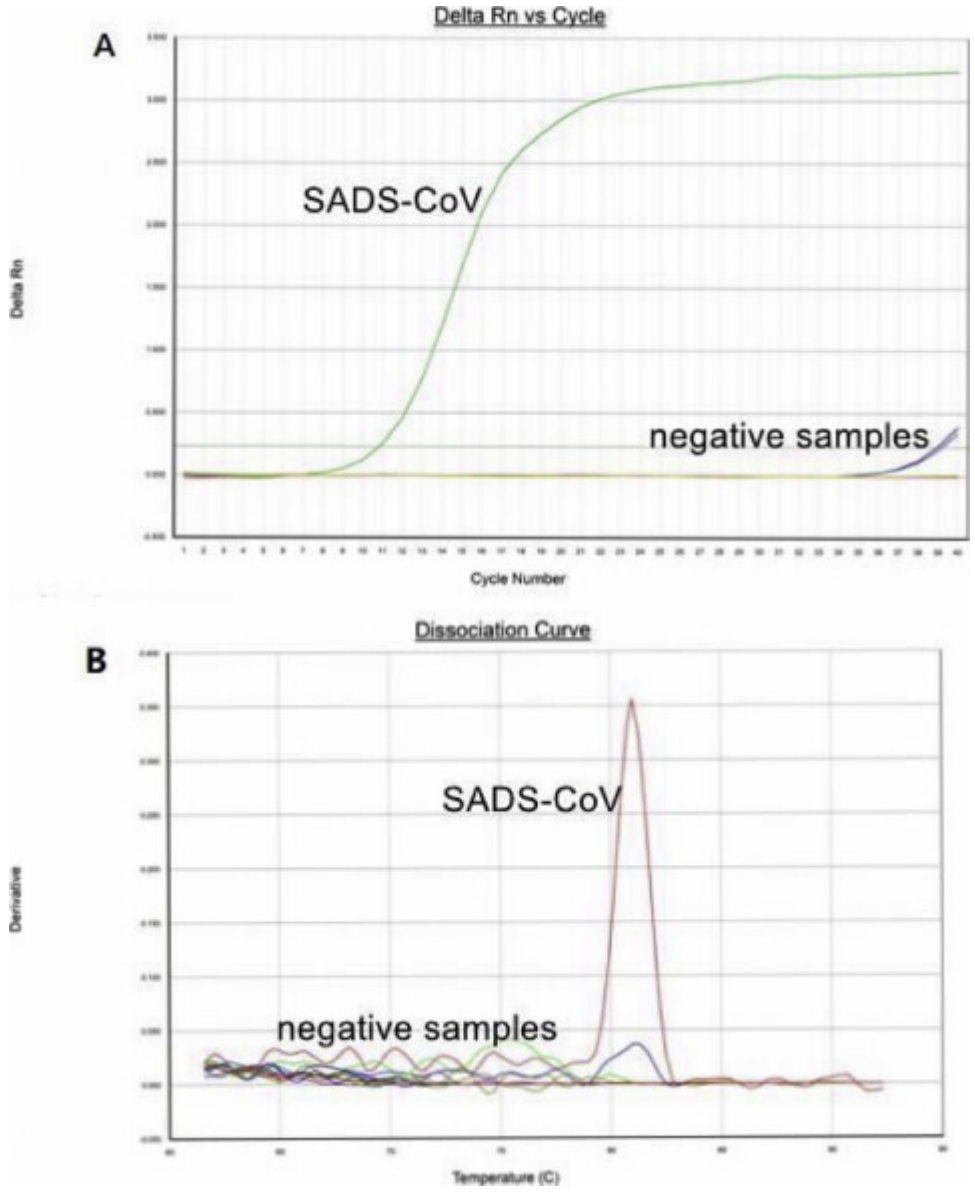


Fig. 2. Specificity of SYBRgreen-basedRT-qPCR.SADS-CoVand negative sampleswere testedby the RT-qPCRassay.Negative samplesincluded PRRSV,PEDV,PRV, CSFV,PCV2,TGEV,RV, PPV,FMDV, PDCoV,SIV and distilled water. A: Only SADS-CoVproduced a strong fluorescencesignal under RT-qPCR.B: Melting peak was observed at 81 °C for only SADS-CoVin RT-qPCR.

TaKaRaEx Taq HS, 0.4 μL of PrimeScript RT Enzyme Mix, 0.4 μL of ROX Reference Dye II, 0.4 μL (10 μM) of each primer, 7.0 μL of RNase free water and 1.0 μL of template. Amplification reaction was performed by Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, USA). Amplification condition was set up as follows: reverse transcription (RT) step at 42 °C for 5 min; initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 34 s. After the cycles completed, a dissociation analysis step was added: 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s. Data were collected and analyzed by the Applied Biosystems7500 SDSSoftware (Version 1.4).

The limit of detection of the RT-qPCRwas determined by testing the serially diluted RNA standards in triplicate. Meanwhile, the detection limit of gel-based RT-PCRwas also determined. As shown in Fig. 1A, RT-qPCRcould detect as few as 10 copies RNA molecule, but standard sample containing one RNA molecule or distilled water only didn't generate positive signals. The dissociation curve analysis indicated the RNA dilutions range from 1×10^1–$1 \times 1\ 0^7$ copies/μL showed almost the same melting temperature (Tm) value (mean Tm values ± standard deviation: 81.57 ± 0.15 °C), meanwhile sample containing one RNA molecule and distilled water as a negative control didn't generate the similar dissociation curve (Fig. 1C). The detection limit of the conventional RT-PCRin this study was 100 copies (Fig. 1D), which was 10 times less sensitive than RT-qPCR.Fig. 1B shows the standard curve generated by plotting the RNA copy number against the cycle (Ct) values of the RNA dilutions tested by RT-qPCRin triplicate using Prism 5.0 software (GraphPad, USA). The Ct values displayed a strong linear correlation (R^2 = 0.9937) with the logarithm of the RNA copy numbers (Fig. 1B), indicating robust amplification efficiency of the assay. RNA dilutions ranged from 1×10^7–$1 \times 1\ 0^2$ copies/μL were used to evaluate the reproducibility of the RT-qPCRassay. Intra- and inter-assay coefficients of variation (CV) were calculated as the method previously described (Zhou et al., 2018a). The intra- and inter-assay CVs of the SYBRgreen-basedRT-qPCRranged from 0.04% to 2.63% and 0.69% to 3.57% respectively, suggesting remarkable reproducibility and high reliability of the assay.

To assessthe specificity of this RT-qPCRmethod, a panel of viruses including porcine reproductive respiratory syndrome virus (PRRSV), porcine epidemic diarrhea virus (PEDV), pseudorabies virus (PRV),

Table 2
Comparative results of clinical samples tested by conventional RT-PCR, SYBR green-based RT-qPCR and probe-based RT-qPCR for SADS-CoV detection.

Samples	Number of samples	Number of SADS-CoV positive samples		
		Conventional RT-PCR	SYBR-based RT-qPCR	probe-based RT-qPCR
Heart	10	3 (13.36–26.16)[a]	5 (13.36–33.67)[b]	5 (18.28–34.41)[c]
Lung	10	4 (14.61–25.23)	6 (14.61–34.45)	6 (20.23–34.98)
Liver	10	4 (15.06–28.63)	7 (15.06–33.23)	7 (20.83–34.04)
Spleen	10	3 (17.23–26.18)	7 (17.23–32.54)	7 (21.35–33.78)
Kidney	10	4 (11.57–27.63)	6 (11.57–31.67)	6 (16.73–32.39)
Jejunum	10	8 (9.36–28.58)	8 (9.36–28.58)	8 (15.28–30.35)
Duodenum	12	9 (8.67–27.78)	11 (8.67–29.67)	11 (14.16–31.41)
Ileum	12	10 (9.17–28.58)	12 (9.17–30.24)	12 (14.79–31.89)
Total	84	45	62	62

[a] Ct values of the positive samples in both SYBR green-based RT-qPCR and conventional RT-PCR.
[b] Ct values of the positive samples in SYBR green-based RT-qPCR.
[c] Ct values of the positive samples in probe-based RT-qPCR.

classical swine fever virus (CSFV), porcine circovirus type 2 (PCV2), transmissible gastroenteritis virus of swine (TGEV), porcine rotavirus (RV), porcine parvovirus (PPV), pig foot-and-mouth disease virus (FMDV), porcine deltacoronavirus (PDCoV) and swine influenza virus (SIV) were tested. All viruses were positive in conventional PCR by their own specific primers and negative by SADS-CoV specific primers. The specific primers were included in Table 1. Distilled water was used as the negative control. The viral genome was extracted by using TGuide virus DNA/RNA kit (TIANGEN, China) following the instruction. When these viruses were tested by the SYBR green-based RT-qPCR assay developed in present study, strong fluorescence signal was only observed for SADS-CoV. No amplification signal was observed throughout the experiment for most of the viruses. Although the fluorescence signal slightly increased after 35 cycles for the PCV2 and PRRSV (Fig. 2A), no specific melting peaks at about 81.57 °C were observed (Fig. 2B), further confirming the specificity of the assay.

To assess the clinical performance of the RT-qPCR for the detection of SADS-CoV, 84 tissue samples from 84 piglets with diarrheal symptoms were collected from a commercial farm in Guangdong, China, and analyzed by the assay. The SADS-CoV-positive status of the samples was determined by the combination of the amplification curve and the melting curve analysis. The samples were also evaluated by conventional RT-PCR and probe-based RT-qPCR which were reported before (Pan et al., 2017; Zhou et al., 2018a). Of 84 samples, 62 samples were SADS-CoV-positive confirmed by SYBR RT-qPCR. When these samples were tested by gel-based RT-PCR, only 45 samples were positive. The positive samples determined by conventional RT-PCR were all positive by SYBR RT-qPCR. Seventeen samples determined negative by conventional RT-PCR were identified as positive samples by SYBR RT-qPCR. When these samples were tested by probe-based RT-qPCR, the detection result was consistent with result by SYBR RT-qPCR. The detection results were summarized in Table 2.

SADS-CoV is a recently discovered virus that has resulted in serious outbreaks and huge financial losses to pig farming industry in south China (Fu et al., 2018; Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018b). Most of these studies applied the conventional RT-PCR to detection of SADS-CoV. However, RT-PCR is time-consuming and cannot determine the amounts of viral copies. A RT-qPCR assay is required for rapid and quantitative detection of SADS-CoV which has been epidemic in the south China.

Recently Zhou et al. reported a probed-based RT-qPCR technique with a detection limit of 30 RNA copies/μL (Zhou et al., 2018a). However the hydrolysis probe increased the experimental cost and the difficulty of assay design. Thus we established a cost-effective SYBR green-based RT-qPCR assay with high sensitivity and reliability for detection of SADS-CoV. This assay doesn't require a probe and is cost-effective. The limit of detection of the SYBR green-based RT-qPCR is 10 RNA copies/μL, 10 times and 3 times more sensitive than that of the conventional RT-PCR and probed-based RT-qPCR, respectively (Zhou et al., 2018a). The detection limit with 10 RNA copies in this study was theoretical analytical sensitivity, since that the sensitivity experiment was done by serial dilution of RNA standard in water, as the method described in a previous study (Zhou et al., 2018a). To determine more realistic sensitivity of the assay, the sensitivity experiment would be done by spiked RNA from negative tissue homogenate in the future. The positive rate of the clinical samples determined by the SYBR green-based RT-qPCR assay was 73.81%, whereas that of the conventional RT-PCR was merely 53.57%, further demonstrating the higher sensitivity of the SYBR green-based RT-qPCR assay. In the future, it is necessary to detect rectal swabs by the SYBR green-based RT-qPCR assay to evaluate the effect of bacteria in the samples on the detection capacity of the method. The sequences of the primers used in the SYBR RT-qPCR assay are 100% identity with all known SADS-CoV strains and a coronavirus isolated from the bat, suggesting the RT-qPCR assay not only can be used to detect the clinical samples from the swine, but also that from the bats.

In summary, a rapid, sensitive, reliable and cost-effective SYBR green-based RT-qPCR assay was established for the detection of SADS-CoV that poses a huge threat to pig industry. The RT-qPCR not only can be applied to the monitoring of SADS-CoV infection, but also the epidemiology study of the virus. In addition, this assay is of great use for the pathogenicity study of SADS-CoV in pigs. It will prompt the understanding of the tissue tropism of the virus and viral distribution in specific organs.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The study was supported by the Science and Technology Research Program (2018B030317001, 2017B030314171, 2017A070702001 and 201707010440) from the Department of the Science and Technology of Guangdong Province and Guangzhou Municipal. We also like to acknowledge Guangdong Wen's Foodstuffs Group Co., Ltd. China, for providing us with piglets' tissue samples.

References

Fu, X., Fang, B., Liu, Y., Cai, M., Jun, J., Ma, J., Bu, D., Wang, L., Zhou, P., Wang, H., Zhang, G., 2018. Newly emerged porcine enteric alphacoronavirus in southern China: identification, origin and evolutionary history analysis. Infect. Genet. Evol. 62, 179–187.

Gong, L., Li, J., Zhou, Q., Xu, Z., Chen, L., Zhang, Y., Xue, C., Wen, Z., Cao, Y., 2017. A new bat-HKU2-like coronavirus in swine, China, 2017. Emerg. Infect. Dis. 23, 1607–1609.

Jensen, K.S., Adams, R., Bennett, R.S., Bernbaum, J., Jahrling, P.B., Holbrook, M.R., 2018. Development of a novel real-time polymerase chain reaction assay for the quantitative detection of Nipah virus replicative viral RNA. PLoSOne 13 e0199534.

Lau, S.K., Woo, P.C., Li, K.S., Huang, Y., Wang, M., Lam, C.S., Xu, H., Guo, R., Chan, K.H., Zheng, B.J., Yuen, K.Y., 2007. Complete genome sequence of bat coronavirus HKU2 from Chinese horseshoe bats revealed a much smaller spike gene with a different evolutionary lineage from the rest of the genome. Virology 367, 428–439.

Pan, Y., Tian, X., Qin, P., Wang, B., Zhao, P., Yang, Y.L., Wang, L., Wang, D., Song, Y., Zhang, X., Huang, Y.W., 2017. Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. Vet. Microbiol. 211, 15–21.

Wang, H., Cong, F., Zeng, F., Lian, Y., Liu, X., Luo, M., Guo, P., Ma, J., 2018. Development of a real time reverse transcription loop-mediated isothermal amplification method (RT-LAMP) for detection of a novel swine acute diarrhea syndrome coronavirus (SADS-CoV). J. Virol. Methods 260, 45–48.

Xu, Z., Zhang, Y., Gong, L., Huang, L., Lin, Y., Qin, J., Du, Y., Zhou, Q., Xue, C., Cao, Y., 2018. Isolation and characterization of a highly pathogenic strain of porcine enteric alphacoronavirus causing watery diarrhea and high mortality in newborn piglets.

Transbound. Emerg. Dis. https://doi.org/10.1111/tbed.12992 .
Yang, F., Chen, B., Liu, F., Peng, X., Sun, T., Yao, H., Wu, H., Wu, N., 2018. Development of a TaqMan MGB RT-PCR assay for the detection of type A and subtype H10 avian influenza viruses. Arch. Virol. 163, 2497–2501.
Yuan, W., Wang, J., Xu, F., Huang, B., Lian, Y., Rao, D., Yin, X., Wu, M., Zhu, Y., Zhang, Y., Huang, R., Guo, P., 2016. Development of a duplex real-time RT-PCR for the simultaneous detection and differentiation of Theiler's murine encephalomyelitis virus and rat theilovirus. J. Virol. Methods 236, 139–146.
Zhou, L., Sun, Y., Wu, J.L., Mai, K.J., Chen, G.H., Wu, Z.X., Bai, Y., Li, D., Zhou, Z.H., Cheng, J., Wu, R.T., Zhang, X.B., Ma, J.Y., 2018a. Development of a TaqMan-based real-time RT-PCR assay for the detection of SADS-CoV associated with severe diarrhea disease in pigs. J. Virol. Methods 255, 66–70.
Zhou, P., Fan, H., Lan, T., Yang, X.L., Shi, W.F., Zhang, W., Zhu, Y., Zhang, Y.W., Xie, Q.M., Mani, S., Zheng, X.S., Li, B., Li, J.M., Guo, H., Pei, G.Q., An, X.P., Chen, J.W., Zhou, L., Mai, K.J., Wu, Z.X., Li, D., Anderson, D.E., Zhang, L.B., Li, S.Y., Mi, Z.Q., He, T.T., Cong, F., Guo, P.J., Huang, R., Luo, Y., Liu, X.L., Chen, J., Huang, Y., Sun, Q., Zhang, X.L., Wang, Y.Y., Xing, S.Z., Chen, Y.S., Sun, Y., Li, J., Daszak, P., Wang, L.F., Shi, Z.L., Tong, Y.G., Ma, J.Y., 2018b. Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. Nature 556, 255–258.

Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Virology

Emerging and re-emerging coronaviruses in pigs

Qiuhong Wang, Anastasia N Vlasova, Scott P Kenney and Linda J Saif

Porcine epidemic diarrhea virus (PEDV), porcine deltacoronavirus (PDCoV), and swine acute diarrhea syndrome-coronavirus (SADS-CoV) are emerging/reemerging coronaviruses (CoVs). They cause acute gastroenteritis in neonatal piglets. Sequence analyses suggest that PEDV and SADS-CoV may have originated from bat CoVs and PDCoV from a sparrow CoV, reaffirming the interspecies transmission of CoVs. The clinical signs and pathogenesis of the three viruses are similar. Necrosis of infected intestinal epithelial cells occurs, causing villous atrophy that results in malabsorptive diarrhea. The severe diarrhea and vomiting may lead to dehydration and death of piglets. Natural infection induces protective immunity, but there is no cross-protection among the three viruses. Besides strict biosecurity measures, individual vaccines are needed for each virus for disease prevention and control.

Address
Food Animal Health Research Program, Ohio Agricultural Research and Development Center, College of Food, Agriculture and Environmental Sciences, Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, Wooster, OH, USA

Corresponding author: Wang, Qiuhong (wang.655@osu.edu)

Current Opinion in Virology 2019, 34:39–49

This review comes from a themed issue on Emerging viruses: inter-species transmission

Edited by Adolfo García-Sastre and Juergen A Richt

For a complete overview see the Issue and the Editorial

Available online 14th January 2019

https://doi.org/10.1016/j.coviro.2018.12.001

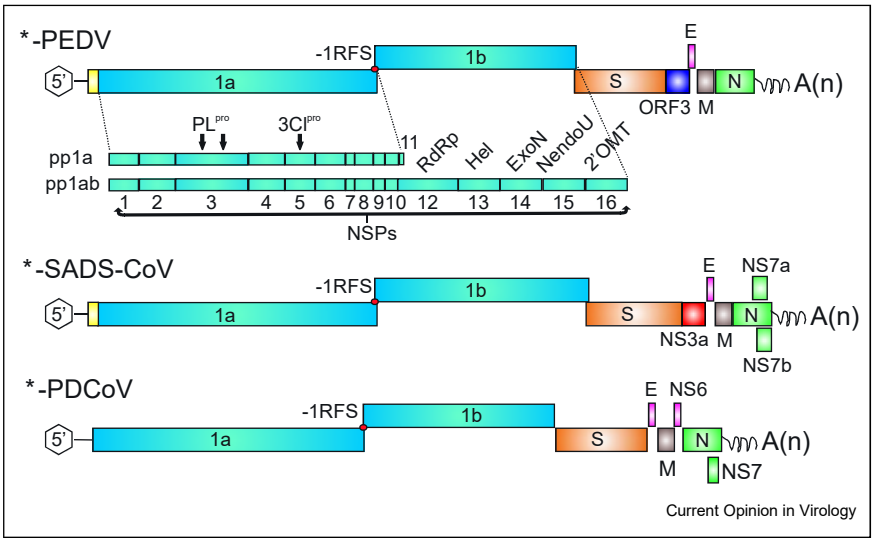
1879-6257/ã 2018 Elsevier B.V. All rights reserved.

Introduction

Coronaviruses (CoVs) are the largest positive-sense, single-stranded RNA (+RNA) viruses. They belong to the Coronaviridae family of the Nidovirales order. Four genera are established: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus. Currently, six CoVs infect pigs, including four alphacoronaviruses [transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), porcine epidemic diarrhea virus (PEDV), and swine acute diarrhea syndrome-coronavirus (SADS-CoV)], one betacoronavirus [porcine hemagglutinating encephalomyelitis virus (PHEV)], and a porcine deltacoronavirus (PDCoV) (Figures 1 and 2). Among them, TGEV, PRCV, and PHEV have been circulating in pigs for decades, whereas PEDV, PDCoV, and SADS-CoV are considered as emerging coronaviruses. Although classical PEDV was first discovered in Europe in the early 1970s [1], and it later spread to Asia in 1970s [2], a re-emerging highly virulent PEDV caused massive outbreaks, with high mortality in suckling piglets, in late 2010 [3]. In 2013–2014, PEDV caused nationwide outbreaks in the US, culminating in major losses to the pork industry. PDCoV was initially detected from pig fecal samples in 2009 in Asia, but its etiological role was not identified until 2014 when it caused diarrhea in pigs in the US [4,5]. Most recently, another highly pathogenic enteric CoV, SADS-CoV, emerged in China in 2016 with high mortality among baby pigs [6,7,8]. All three newly emerged porcine enteric CoVs were first detected in China. To date, the emerging PEDV strains have spread to other Asian and North American countries and to Europe. PEDV has become the second most important pathogen for swine in China (containing > 50% of the world's pig population), after porcine reproductive and respiratory syndrome virus (PRRSV). PDCoV has spread to the US, Canada, and other Asian countries [9]. No SADS-CoV has been reported from other countries besides China. In addition, recombinant viruses between TGEV and PEDV had been reported in Italy, Germany, and Slovakia [10–12]. These recombinant swine enteric coronaviruses (SeCoV), Italy/213306/2009 and GER/L00930/2012, share similar recombination pattern and 99.5% nucleotide identity and have potential parental strains TGEV H16 strain (major, backbone) and classical PEDV CV777 strain (minor, S gene). Because there were no more reports on SeCoV outbreaks, virulence, and tissue tropism, we will not discuss SeCoV further in this review.

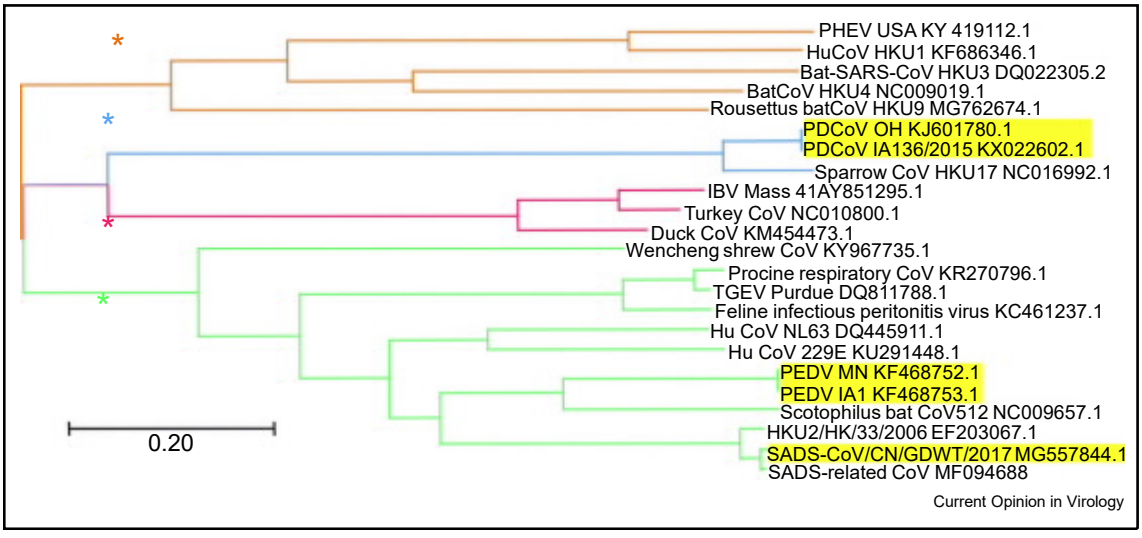
The basic genomic organization is shared among CoVs [13]. The genome size is about 25–30 kb. It contains at least six open reading frames (ORFs) in the order: ORF1a, ORF1b, spike (S), envelope (E), membrane (M), and nucleocapsid (N) (Figure 1). Each virus contains one to several accessory genes, the ORF3 of PEDV, NS3a, NS7a and NS7b of SADS-CoV, and NS6 and NS7 of PDCoV. ORF1a and ORF1b are at the 5^0-end and comprise two-thirds of the genome. They encode two non-structural polyproteins that are processed to generate an estimated 14–16 nonstructural proteins (NSPs) involved in proteolytic processing, genome replication and transcription [13]. Among the structural proteins, the

Figure 1



Genomic organization of alphacoronavirus porcine epidemic diarrhea virus (a-PEDV), swine acute diarrhea syndrome-coronavirus (a-SADS-CoV), and porcine deltacoronavirus (d-PDCoV). Each genome is 5^0capped and 3^0 polyadenylated. Viral genomes are flanked by UTRs and are polycistronic encoding replicase ORFs1a and 1b followed by the genes encoding spike glycoprotein (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. Among the structural protein genes, the genome also contains accessory genes: ORF3 of PEDV; NS3a, NS7a and NS7b of SADS-CoV; and NS6 and NS7 of PDCoV. Expression of ORF 1a and 1b yields 2 polyproteins (pp1a and pp1ab) through a -1 programmed ribosomal frameshift (-1RFS). The polyprotein is co-translationally or post-translationally processed into an estimated 14–16 nonstructural proteins (NSPs): PLpro, papain-like cysteine protease; 3CLpro, main 3C-like cysteine protease; RdRp, RNA-dependent RNA polymerase; Hel, helicase; ExoN, 3^0-5^0 exonuclease; NendoU, nidovirus uridylate-specific endoribonuclease; 2^0OMT, ribose-2^0-O-methyltransferase.

Figure 2



Phylogenetic comparison of the full-length genomes of PEDV, SADS-CoV, and PDCoV with other coronavirus species. Evolutionary history was inferred using the Maximum Likelihood method [112]. The tree with the highest log likelihood (511751.49) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 23 nucleotide sequences. There were a total of 32,538 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [113].

S glycoprotein is most important to elicit virus neutralizing antibodies and is diverse among strains. The N protein, which is the most abundant structural protein, binds to the viral genomic RNA and is packed into the nucleocapsid, while the M and E are membrane proteins.

In this review, we will focus on the origin and potential for intra/interspecies transmission, host receptors for virus replication, antigenic relationships, comparative pathogenesis, and disease control and prevention of the three emerging porcine CoVs. For PEDV, we will focus on the highly virulent PEDV strains.

Origin and potential for intra/interspecies transmission

Coronaviruses, including emerging porcine CoVs of two different genera, alphacoronaviruses and deltacoronaviruses, exhibit an increased propensity for interspecies transmission [14,15]. Scattered genome-wide point mutations normally appear within host evolution of CoVs and are not associated with major changes in their biological functions [16,17]. In contrast, significant gene modifications (deletions or insertions in accessory or spike protein genes) are often involved in or occur following CoV host-shift or tissue tropism change events [18,19,20,21,22–24]. However, in the case of PEDV, a large deletion (194-216-aa Del) in the N terminus of S protein results in partial attenuation of the virus but did not alter tissue tropism [25,26]. This differs from PRCV whereby the large S deletion (207-227-aa) changes the major tissue tropism from enteric to respiratory compared with the parental TGEV. This may reflect the different receptor usage of these two alphacoronaviruses. TGEV uses aminopeptidase N (APN) as the receptor, and APN is distributed ubiquitously in many tissues. PEDV does not use APN as the receptor, which was confirmed by the fact that PEDV infects APN-knockout pigs [27]. Also, the size and localization of the deletions in the spike gene are virus species-specific with variable biological outcomes [28]. Frequent host-shifting (animal-to-animal and occasionally animal-to-human) events are characteristic for CoV evolution, with severe acute respiratory syndrome (SARS)-and Middle East respiratory syndrome (MERS)-CoVs being the most prominent recent examples [29–32]. While palm civets, raccoon dogs, Chinese ferret badgers, or dromedary camels were implicated as incidental hosts that facilitated SARS- or MERS-CoV spread to humans, respectively, bats are considered the natural reservoirs for these viruses [33,34]. Besides SARS- and MERS-CoVs, molecular analyses confirmed that numerous animal and human CoVs emerged as a result of recombination and interspecies transmission events. Those CoVs include alphacoronaviruses, betacoronaviruses, and deltacoronaviruses and are summarized below [14]. Canine CoV (CCoV) II has reportedly emerged via recombination between the original CCoV-I and an unknown CoV, while feline CoV (FCoV) II acquired the CCoV-II spike gene and the backbone from FCoV-I [20]. Another alphacoronavirus, TGEV, is suggested to have originated from CCoV-II because of the high genetic relatedness between the two viruses and by the presence of ORF3 remnants in both genomes [20,35].

Recent studies have further emphasized that bats are important reservoirs contributing to the immense diversity of human and animal CoVs. For example, it was demonstrated that Ghana bat CoV group I and HCoV-229E share a common ancestor [36,37]. Additionally, a recent transmission of bat-CoV-HKU10 from Leschenault's rousettes to Pomona leaf-nosed bats has been reported [38]. Among betacoronaviruses, there are the two human CoVs, respiratory HCoV OC43 associated with common colds and human enteric CoV (HECoV-4408) isolated from a child with diarrhea that are suggested to have been introduced into human population from bovine species [23,39]. Additionally, bovine-like CoVs were identified in captive wild ruminants and camelids such as waterbuck, sambar deer, white-tailed deer, elk, giraffe, sable antelope [18,40–42], alpaca, llama, and dromedary camel [43]. Deltacoronaviruses are genetically highly heterogenous and represent the only genus in the Coronaviridae family that infects avian and mammalian species with frequent host-shift events [44,45]. Recent molecular analysis of avian deltacoronaviruses in the Middle East provided long-sought evidence of interspecies transmission between falcons and their prey, houbara bustards and pigeons, as well as avian-to-swine transmission associated with recombination events in the spike gene [46].

There are no historic large-scale surveillance data that definitively establish that PEDV was introduced into the pig population from bats in the 1970s [47]. However, recently, it was demonstrated that PEDV is genetically more closely related to BtCoV/512/2005 than to TGEV, leading to the hypothesis that PEDV may have originated from bats (Figure 2) [47]. Additionally, PEDV infects cells from pigs, humans, monkeys, duck, and bats, further supporting the theory that PEDV had crossed the bat-swine interspecies barrier sometime in the past (Table 1) [48,49]. PEDV has been detected from feral pigs in Korea and the US [50,51], with the latter study suggesting that PEDV may have spilled over from domestic to feral pigs. However, because there are no control measures to prevent PEDV circulation in feral pigs, they may represent a PEDV reservoir that can lead to PEDV outbreaks in the future.

The SADS-CoV genome was shown to be highly similar (95% nucleotide identity) to another bat CoV (HKU2-CoV) identified in 2007 (Figure 2) [6,7,8,52]. However, the S gene sequence identity is only 86%, suggesting

Table 1

Summary of the three emerging enteric coronaviruses in pigs

Coronavirus (Genus)	Year of emergence (re-emergence	Prototype (GenBank Accession no.)	Receptor	Mortality in neonatal piglets	Infect other animal species besides pigs	Cell lines for virus propagation	Potential ancestor (GenBank Accession no.)
PEDV (Alphacoronavirus)	1971 (2010)	CV777 (NC_003436)	Unknown	Approaching 100% [71]	No	African green monkey kidney (Vero and MARC-145), swine intestine (IECs), bladder, kidney (PK15 and IB-RS-2), testis (ST), and alveolar macrophage (3D4), bat lung (Tb1-Lu), duck intestine (MK-DIEC), and human liver (HuH-7) and lung (MRC-5) [49]	CHN/BtCoV/512/2005 (DQ648858) [47]
PDCoV (Deltacoronavirus)	2009	HKU15-44 (JQ065042)	APN; others?	Up to 40% [9]	Bovine [111]	Swine ST, LLC-PK1, IPEC-J2, PKFA, SK6, PD5, and PK15, galline hepatoma (LMH) and fibroblast (DF-1), human hepatoma (Huh7), A549, HeLa, and HRT-18 [53 ,114,115]	Recombinant between Sparrow CoV HKU17/2007 (NC_016992) and bulbul CoV HKU11 (FJ376619)[4 ,46]
SADS-CoV (Alphacoronavirus)	2016	GDS04 (MF167434); CH/GD-01/2017 (MF370205); CH/GDWT/2017 (MG557844)	Unknown	90-100% in pigs ! 5 days of age and 5% in pigs > 8 days of age [8 ,72]	unknown	Vero [7,8 ,72]	BtCoV/HKU2/HK/33/2006 (EF203067); Bt/SADSr-CoV 162140/2016 (MF094688) [8 ,52]

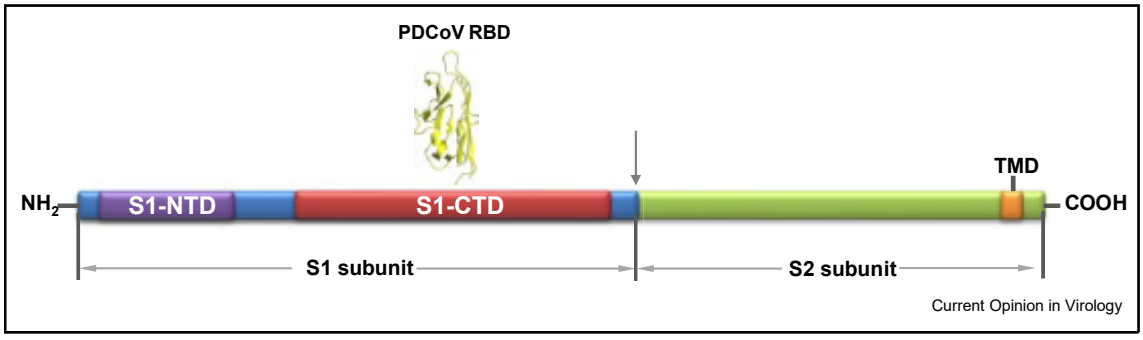
that HKU2-CoV may not be the direct progenitor of SADS-CoV, but that they may share a common ancestor. This prompted the search for new CoVs in bat specimens collected in Guangdong Province between 2013 and 2016. SADS-related CoVs (sharing 96–98% sequence identity with SADS-CoV) were identified in 9.8% of bats, predominantly in horseshoe bats (Rhinolophus spp) that are known reservoirs of SARS-related CoV (Figure 2) [8].

In contrast, molecular clock analyses suggest that PDCoV originated relatively recently from a host-switching event between avian and mammalian species (Figure 2) [4 ,44]. Most recent phylogenetic analyses have confirmed that quail deltacoronavirus UAE-HKU30 belongs to the same deltacoronavirus species as porcine coronavirus HKU15 (Por CoV HKU15) and sparrow coronavirus HKU17 (SpCoV HKU17) [46]. In addition, PDCoV was shown to efficiently infect cells of broad host range, including swine, humans, and chickens (Table 1) [53]. Accordingly, PDCoV S protein was found to target the phylogenetically conserved catalytic domain of APN [53]. Moreover, transient expression of porcine, feline, human, and chicken APN renders cells susceptible to PDCoV infection [53].

Host receptors

The major determinant for attachment and entry is binding of the S glycoprotein to receptors on the cell surface. This step is one of the key divergence points among CoVs, contributing significantly to species tropism, tissue tropism, the resultant pathogenesis in the host, and the ability to cross between species. The S protein is a type I glycoprotein consisting of S1 and S2 subunits present on the virion surface as a trimer (Figure 3) [54,55]. The S1 is involved in receptor binding and contains N-terminal and C-terminal domains (S1-NTD and S1-CTD, respectively), both of which may serve as a receptor-binding domain (RBD) [56]. The S2 subunit forms the stalk of the spike trimer, involved in triggering the fusion of the viral envelope and target cell membrane [53 ,55]. Identification of specific viral receptors can often be difficult due to the S protein potentially binding to multiple cell receptors, the necessity for co-receptors, and the involvement of potential host receptor proteins in viral replication stages other than initial binding. In the case of PEDV, there appears to be promiscuous binding to cell receptors [48]. APN and sialic acid have both been suggested as binding receptors for PEDV [48,57]. Further research suggests that porcine APN (pAPN) is not a functional receptor, but rather it contributes to PEDV infection

Figure 3



Schematic diagram of S protein of emerging porcine coronaviruses. S1-NTD – S1 subunit N terminal domain is shown in purple; S1-CTD – S1 subunit C terminal domain is shown in red; RBD – PDCoV receptor binding domain is shown in yellow; TMD – trans-membrane domain is shown in orange.

through its protease activity [27, 58 ,59,60]. This is confirmed by the recent report that APN-knockout pigs became resistant to TGEV infection, but remained susceptible to PEDV infection [27]. Furthermore, the binding efficiency of PEDV to sialic acid appears to be strain-specific. Sialic acid binding is variably required for other CoV infections in cell culture with sialic acid serving as a primary receptor in some instances (A1 lineage betacoronaviruses) [57], as a potential secondary receptor (TGEV) [61,62,63], or it may be dispensable for entry into cells as noted in cell culture (TGEV) [64]. A similar quandary exists for PDCoV where the S1 domain binds to APN of many different species, allowing functional infection of diverse cell types; however, removal of APN does not render cells completely resistant to infection [53 ,65]. These findings suggest that PDCoV not only utilizes APN, but it can also utilize another unknown receptor(s) in the absence of APN. Less research has been done on the emerging SADS-CoV binding receptor due to its recent identification [6,7,8].

Antigenic relationships

The antigenic relationships among porcine CoVs are complex and include intergenus and intragenus cross-reactivity [66–68]. One-way cross-reactivity was observed between TGEV (Miller strain) and classical and current US PEDV strains. This cross-reactivity was due to at least one epitope on the N-terminal region of the N protein [67] and is similar to the N protein-mediated antigenic cross-reactivity between SARS-CoV and alphacoronaviruses of porcine, canine and feline origin reported previously [66,68]. Because it was confirmed that PEDV does not utilize pAPN as a cellular receptor [27,58 ,59,60], whereas TGEV does, the biological relevance of the observed antigenic cross-reactivity between TGEV and PEDV needs to be evaluated further, but could interfere in diagnostic tests based on whole virus or N protein.

The fact that pAPN acts as a cross-genus CoV functional receptor for both the enteropathogenic PDCoV and alphacoronavirus (TGEV) suggests that some sequence/structural homology may exist to enable this functional interaction between pAPN and the PDCoV/TGEV S protein receptor binding domain region [53]. The existence and extent of antigenic cross-reactivity between TGEV and PDCoV are unknown; however, N-protein mediated two-way cross-reactivity between PEDV and PDCoV was recently confirmed [69]. Thus, the above data suggest that receptor selection in the emergence of porcine CoVs is subject to certain restrictions and may lead to structural re-arrangements in the spike as well as other viral proteins, including the highly conserved (and therefore cross-reactive) N protein. Furthermore, recent experiments demonstrated that angiotensin-converting enzyme 2 (ACE2), pAPN and dipeptidyl peptidase 4 (DPP4) do not function as receptors for SADS-CoV [8]. Thus, additional experimental work is needed to identify cellular receptors for PEDV and SADS-CoV, as well as to gain mechanistic understanding of antigenic interrelationships among emerging porcine CoVs. Specifically, additional information is needed to establish whether there is co-regulation between receptor usage and antigenic cross-reactivity among these CoVs.

Comparative pathogenesis

PEDV, PDCoV, and SADS-CoV are all enteric pathogens, causing indistinguishable acute gastroenteritis in all ages of pigs (Table 1). Clinical signs include mainly anorexia, diarrhea and vomiting that may lead to dehydration, loss of body weight, lethargy, and death. Those clinical signs generally do not last over 10 days. Disease is generally more severe and often lethal in neonatal piglets, especially those born from seronegative sows. In older pigs, such as weaned pigs and sows, morbidity is high but the mortality rate is low. In general, the emerging highly virulent PEDV is more virulent than PDCoV strains tested [9]. During the first year of outbreaks in the US,

the pig morbidity rate due to PEDV and PDCoV was about 100% and 30%, respectively [70]. PEDV and PDCoV caused up to 100% and up to 40% mortality rates in neonatal piglets, respectively [9,71]. The mortality rates of SADS-CoV in piglets less than five days of age and older than eight days of age were 90–100% and 5%, respectively [8 ,72]. Diseases caused by porcine enteric CoVs (TGEV, PEDV, SADS-CoV, and PDCoV) cannot be differentiated without laboratory diagnosis. Transmission is mainly via the fecal-oral route. Vehicles include contaminated feed/ingredients, transportation trucks/trailers, environment, etc. [71]. Airborne transmission was reported for both PEDV and PDCoV, probably via ingestion of aerosolized virus particles [71]. Recently, Li et al. [73] investigated the potential mechanisms of airborne transmission of PEDV. They concluded that the virions can replicate in the nasal epithelial cells, then are carried by dendritic cells (DCs) and passed to CD3+ T cells that transport PEDV to the gut to infect intestinal cells. However, such a conclusion cannot be made based on the data presented in the paper. The major concern is that the authors used extremely high inoculum dose (7 $\log_{10}$ PFU/pig) to intranasally inoculate five-day-old CDCD piglets. Considering the very low infectious dose for neonatal piglets of the emerging highly virulent PEDV, ingestion of even a very small portion (1/10 million, 1 PFU) of this inoculum, which was highly possible, can result in a 100% infection rate in piglets within 24 hpi, as described previously [74,75]. Therefore, although PEDV can replicate in nasal epithelial cells and be taken up by DCs and T cells, whether the virions carried by the DCs and T cells can cause intestinal infection and diarrhea should be investigated further. Also, PEDV is more prevalent than PDCoV in the field [76]. Infectious PEDV can be shed in the feces of infected pigs weeks after clinical signs cease posing difficulties for disease management on farms [71]. Co-infections with more than one pig enteric pathogen are common and often more severe clinically [77,78].

The pathogenesis of PEDV and PDCoV has been studied in different ages of pigs [9,71,79,80]. Disease is more severe in younger pigs than in older pigs, which is called age-dependent resistance probably due to the immature immune responses and lower rate of enterocyte regeneration in younger pigs [81 ,82–84]. In general, pathological changes in young pigs are similar between these two viruses. Clinical signs appear at 1–3 days post-inoculation. Viral replication occurs quickly and mainly in the villous epithelial cells of the small intestine, resulting in villous atrophy. Therefore, diarrhea is probably a consequence of malabsorption due to massive loss of absorptive enterocytes. PEDV and PDCoV antigens also were detected occasionally in crypt cells, but may not contribute significantly to pathogenesis since enterocyte regeneration capacity was preserved [9,79]. No histopathologic changes have been observed in the caecum/colon, although PEDV and PDCoV antigens were detected in the epithelial cells of these intestinal sections. During the acute phase of virus infection, large number of viruses are shed in feces. Viral RNA was also detected transiently in the serum. Low levels of viral RNA were detected in multiple organs by real-time reverse transcription (RT)-PCR possibly due to viremia, since no viral antigen was detected. A recent study showed significantly lower levels of serotonin-secreting enteroendocrine cells in duodenum, mid-jejunum, ileum, or colon in PEDV-infected pigs that had vomiting than in negative control pigs, suggesting that serotonin secretion during PEDV infection might contribute to vomiting [85]. The pathogenesis of the recently emerging SADS-CoV was studied in piglets by three laboratories [7,8 ,72]. Pan et al. [7] noted that SADS-CoV caused only mild-moderate intestinal lesions in three-day-old piglets. In the second study, SADS-CoV proteins were detected in only a few jejunal epithelial cells, which may not correlate with the 50% (3/6) mortality in the inoculated three-day-old piglets [8]. In the most recent study Xu et al. [72] reported that SADS-CoV mainly infects the intestine, causing 100% (12/12) and 50% (2/4) mortality rates in five-day-old and contact control conventional piglets (infected at 7 days of age based on fecal shedding data), respectively. SADS-CoV RNA was also detected from the heart, liver, spleen, kidneys, stomach, and lungs, but not the blood of piglets euthanized at 7 dpi when pigs still had severe diarrhea. Therefore, more detailed pathogenesis studies are warranted to understand SADS-CoV replication in vivo, factors contributing to disease severity, tissue tropism, etc.

Immune responses and disease control and prevention

Immune responses have been studied for PEDV infection, but there are few reports for PDCoV and SADS-CoV [86–89]. Serum PEDV IgG Ab responses were studied in seven-week-old conventional pigs inoculated with non-S INDEL PEDV via the oragastric route at 7 to 42 days post-inoculation (dpi). IgG antibodies were detected as early as 7 dpi for the S1 subunit of the S protein and 10 dpi for the N and M structural proteins and the whole virus particles [90]. The antibody titers peaked at 14–17 dpi and remained detectable through 42 dpi. However, no E protein-specific IgG antibodies were detected throughout the study. Also, the PEDV S1 does not cross-react with IgG antibodies against the other porcine enteric coronaviruses, TGEV (and its variant PRCV) and PDCoV. Therefore, the S protein is the most sensitive and specific marker for PEDV infection. In 11-day-old conventional pigs, a strong positive correlation exists between protection against PEDV challenge and the levels of IgA and IgG antibody secreting cells (ASCs) in the gut associated lymphoid tissues (duodenum and ileum lamina propria and mesenteric lymph nodes) and the blood as well as between protection and serum IgG

and IgA antibody titers [91,92]. In sows exposed to PEDV in the field, IgA and IgG PEDV ASCs were detected in the intestine of sows at one month post-PEDV exposure, then waned, but remained detectable at six months post-PEDV exposure [93]. Additionally, PEDV IgA antibody was detectable in oral fluids [93]. Several experimental challenge studies using naive animals of different ages demonstrated that serum viral neutralizing antibodies became detectable around 10 dpi, peaked around 18 dpi, decreased slightly around 21 dpi, and remained at high level through 42 dpi [88].

T cell immune responses may play an important role in inducing protective immunity because pigs infected with the attenuated and highly virulent PEDV PC22A strain generated similar level of humoral immune responses, but only pigs infected with the virulent PEDV were protected from challenge with homologous virulent virus [94]. However, limited experimental data are available on cellular immune responses focusing mainly on cytokine responses [82]. PEDV-infected suckling pigs had significantly lower natural killer (NK) cell frequencies, undetectable NK cell activity and lower IFN-γ producing CD3$^-$ CD4$^-$ CD8$^+$ NK cells in blood and ileum compared with PEDV-infected weaned pigs [82]. Deficiency in the innate immune function of neonatal NK cells may contribute to the more severe PEDV infection in suckling pigs compared with weaned pigs as also reported for TGEV infections [95]. Inflammatory responses play a significant role in the pathogenesis of enteric coronaviruses. Compared with suckling pigs, weaned pigs had a delayed pro-inflammatory cytokine induction that coincided with the delayed onset of infection, disease and shedding of PEDV RNA in feces [82]. Toll-like receptor 2 (TLR2), TLR3, and TLR9 may contribute to NF-κB activation in response to PEDV infection in small intestinal epithelial cells in vitro [96]. The viral proteins E and N upregulated IL-8 expression by inducing endoplasmic reticulum stress and subsequent activation of the NF-κB pathway [97,98]. PEDV can evade host innate immune responses via at least 11 proteins (nsp1, nsp3, nsp5, nsp7, nsp14, nsp15, nsp16, ORF3, E, M, and N) that function as interferon (IFN) antagonists [99–102]. Identification of the virus-encoded IFN antagonists and understanding their mechanism of action may lead to novel therapeutic targets and more effective vaccines. In summary, infection of PEDV induces protection against homologous virus challenge in neonatal, growing and adult pigs [89,103,104]. Although there is only one serotype of PEDV, only incomplete cross-protection exists among different genetic clusters of PEDV variants [3,103]. Sows previously infected with PEDV can generate lactogenic immunity and passively protect nursing piglets from disease [86,104]. Mucosal IgA antibody levels correlate to protective viral neutralizing antibody levels and the neutralizing epitopes are mainly located in the S glycoprotein [105–107].

Rapid diagnosis is critical for the control of these diseases to prevent them from spreading. Diagnostic methods for PEDV and PDCoV for the detection of viral RNA, viral antigens, and antibodies have been reviewed [76,88]. No specific antiviral drugs are available to treat infected pigs. High level biosecurity and vaccines are still the best choice to prevent these enteric infections [104]. No cross-neutralization exists between antibodies to PEDV and PDCoV [69]. SADS-CoV outbreaks occurred at a swine farm that suffered from recent PEDV infections, suggesting no cross-protection between PEDV and SADS-CoV [8]. Therefore, the prevention of these three emerging porcine CoV diseases will require the development of separate virus-specific vaccines. Although PED occurs in pigs of all ages, piglets up to one week of age experience high mortality and need to be protected by transfer of maternal antibodies, especially neutralizing antibodies, via colostrum and milk from immunized dams. The mechanisms of lactogenic protection described for TGEV and other enteric viral infections apply to PEDV as well [86,108]. Colostrum and milk IgA or neutralizing antibody titers are better markers for lactogenic immunity than antibodies in serum [109]. Live, inactivated, and subunit (S) PEDV vaccines targeting sows have been developed in China, Japan, Korea and the US [104,110]. However, their efficacy has not been sufficient to control PEDV outbreaks that still occur on farms with PEDV-vaccinated pigs. No reports are yet available on vaccine development for PDCoV or SADS-CoV. Some farms use feedback methods with intentional exposure of pregnant sows pre-farrowing to autogenous virus using minced intestines from acutely infected piglets [108]. The goal is to stimulate lactogenic immunity in sows and reduce mortality in nursing pigs. The problem with these methods is that PEDV infection may become persistent on farms and other enteric pathogens could be transmitted to the entire herd.

Conclusions

PDCoV, SADS-CoV, and highly virulent PEDV emerged or reemerged recently (Table 1). PEDV has spread to Asian, North American, and European countries, causing huge economic losses in the swine industry. Among the three enteric CoVs, PEDV has been studied more extensively and some vaccines have been developed, although their efficacy in the field is still questionable. For PDCoV and SADS-CoV, more research is needed to better understand their emergence, evolution, pathogenesis, and immune responses. Currently, disease control and prevention mainly depend on swine farm management, focusing on high biosecurity measures and disease containment within and among farms. Coronaviruses often spill over to other species: PEDV and PDCoV can infect cells from different species, and PDCoV can also infect calves [111]. Currently, these emerging porcine CoVs are only known to infect pigs, but not humans [48,53].

Nevertheless, continued monitoring of these porcine CoVs is necessary for both swine and public health.

Conflict of interest statement

Nothing declared.

Acknowledgements

This work was partially supported by National Institute of Food and Agriculture, U.S. Department of Agriculture (USDA) [award number 2015-67015-23067 2015] and USDA Agricultural Research Service [award number 58-3625-4-087 2014]. Salaries and research support were provided by state and federal funds provided to the Ohio Agricultural Research and Development Center (OARDC), The Ohio State University.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Wood EN: An apparently new syndrome of porcine epidemic diarrhoea . Vet Rec 1977, 100:243-244.

2. Sun D, Wang X, Wei S, Chen J, Feng L: Epidemiology and vaccine of porcine epidemic diarrhea virus in China: a mini-review . J Vet Med Sci 2016, 78:355-363.

3. Sun RQ, Cai RJ, Chen YQ, Liang PS, Chen DK, Song CX: Outbreak of porcine epidemic diarrhea in suckling piglets, China . Emerg Infect Dis 2012, 18:161-163.

4. Woo PC, Lau SK, Lam CS, Lau CC, Tsang AK, Lau JH, Bai R, Teng JL, Tsang CC, Wang M et al.: Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus . J Virol 2012, 86:3995-4008.
It is a nice study on the evolution of coronaviruses.

5. Wang L, Byrum B, Zhang Y: Porcine coronavirus HKU15 detected in 9 US states, 2014. Emerg Infect Dis 2014, 20: 1594-1595.

6. Gong L, Li J, Zhou Q, Xu Z, Chen L, Zhang Y, Xue C, Wen Z, Cao Y: A new Bat-HKU2-like Coronavirus in Swine, China, 2017. Emerg Infect Dis 2017, 23.

7. Pan Y, Tian X, Qin P, Wang B, Zhao P, Yang YL, Wang LX, Wang D, Song Y, Zhang X et al.: Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China . Vet Microbio 2017, 211:15-21.

8. Zhou P, Fan H, Lan T, Yang XL, Shi WF, Zhang W, Zhu Y, Zhang YW, Xie QM, Mani S et al.: Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin . Nature 2018, 556:255-258.
Comprehensively, investigation of a deadly pig diarrhea outbreak caused by a novel alphacoronavirus. They also identified SADS-related CoVs from bats, highlighting the interspecies transmission of bat CoVs to pigs.

9. Jung K, Hu H, Saif LJ: Porcine deltacoronavirus infection: etiology, cell culture for virus isolation and propagation, molecular epidemiology and pathogenesis . Virus Res 2016, 226:50-59.

10. Akimkin V, Beer M, Blome S, Hanke D, Hoper D, Jenckel M, Pohlmann A: New Chimeric Porcine Coronavirus in Swine Feces, Germany, 2012. Emerg Infect Dis 2016, 22:1314-1315.

11. Boniotti MB, Papetti A, Lavazza A, Alborali G, Sozzi E, Chiapponi C, Faccini S, Bonilauri P, Cordioli P, Marthaler D: Porcine epidemic diarrhea virus and discovery of a recombinant swine enteric coronavirus, Italy . Emerg Infect Dis 2016, 22:83-87.

12. Mandelik R, Sarvas M, Jackova A, Salamunova S, Novotny J, Vilcek S: First outbreak with chimeric swine enteric coronavirus (SeCoV) on pig farms in Slovakia - lessons to learn . Acta Veterinaria Hungarica 2018, 66:488-492.

13. Brian DA, Baric RS: Coronavirus genome structure and replication . Curr Topics Microbiol Immunol 2005, 287:1-30.

14. Chan JF, To KK, Tse H, Jin DY, Yuen KY: Interspecies transmission and emergence of novel viruses: lessons from bats and birds . Trends Microbiol 2013, 21:544-555.

15. Vlasova AN, Saif LJ: Biological aspects of the interspecies transmission of selected coronaviruses . In Viral Infections and Global Change. Edited by Singh SK. Wiley; 2013:393-418.

16. Zhang CY, Wei JF, He SH: Adaptive evolution of the spike gene of SARS coronavirus: changes in positively selected sites in different epidemic groups . BMC Microbiol 2006, 6:88.

17. Zhang X, Hasoksuz M, Spiro D, Halpin R, Wang S, Vlasova A, Janies D, Jones LR, Ghedin E, Saif LJ: Quasispecies of bovine enteric and respiratory coronaviruses based on complete genome sequences and genetic changes after tissue culture adaptation . Virology 2007, 363:1-10.

18. Hasoksuz M, Alekseev K, Vlasova A, Zhang X, Spiro D, Halpin R, Wang S, Ghedin E, Saif LJ: Biologic, antigenic, and full-length genomic characterization of a bovine-like coronavirus isolated from a giraffe . J Virol 2007, 81:4981-4990.

19. Laude H, Van Reeth K, Pensaert M: Porcine respiratory coronavirus: molecular features and virus-host interactions . Vet Res 1993, 24:125-150.

20. Lorusso A, Decaro N, Schellen P, Rottier PJ, Buonavoglia C, Haijema BJ, de Groot RJ: Gain, preservation, and loss of a group 1a coronavirus accessory glycoprotein . J Virol 2008, 82:10312-10317.
A nice study on the evolutionary history of alphacoronaviruses based on accessory gene ORF3.

21. Vaughn EM, Halbur PG, Paul PS: Sequence comparison of porcine respiratory coronavirus isolates reveals heterogeneity in the S, 3, and 3-1 genes . J Virol 1995, 69:3176-3184.

22. Vennema H, Poland A, Foley J, Pedersen NC: Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronaviruses . Virology 1998, 243:150-157.

23. Vijgen L, Keyaerts E, Moes E, Thoelen I, Wollants E, Lemey P, Vandamme AM, Van Ranst M: Complete genomic sequence of human coronavirus OC43: molecular clock analysis suggests a relatively recent zoonotic coronavirus transmission event . J Virol 2005, 79:1595-1604.

24. Zhang X, Hasoksuz M, Spiro D, Halpin R, Wang S, Stollar S, Janies D, Hadya N, Tang Y, Ghedin E et al.: Complete genomic sequences, a key residue in the spike protein and deletions in nonstructural protein 3b of US strains of the virulent and attenuated coronaviruses, transmissible gastroenteritis virus and porcine respiratory coronavirus . Virology 2007, 358:424-435.

25. Hou Y, Lin CM, Yokoyama M, Yount BL, Marthaler D, Douglas AL, Ghimire S, Qin Y, Baric RS, Saif LJ et al.: Deletion of a 197-amino-acid region in the N-terminal domain of spike protein attenuates porcine epidemic diarrhea virus in piglets . J Virol 2017, 91.

26. Suzuki T, Shibahara T, Yamaguchi R, Nakade K, Yamamoto T, Miyazaki A, Ohashi S: Pig epidemic diarrhoea virus S gene variant with a large deletion non-lethal to colostrum-deprived newborn piglets . J Gen Virol 2016, 97:1823-1828.

27. Whitworth KM, Rowland RRR, Petrovan V, Sheahan M, Cino-Ozuna AG, Fang Y, Hesse R, Mileham A, Samuel MS, Wells KD et al.: Resistance to coronavirus infection in amino peptidase N-deficient pigs . Transgenic Res 2018.

28. Terada Y, Shiozaki Y, Shimoda H, Mahmoud HYAH, Noguchi K, Nagao Y, Shimojima M, Iwata H, Mizuno T, Okuda M et al.: Feline infectious peritonitis virus with a large deletion in the 5'-terminal region of the spike gene retains its virulence for cats . J Gen Virol 2012, 93:1930-1934.

29. Graham RL, Baric RS: Recombination, reservoirs, and the modular spike: mechanisms of coronavirus cross-species transmission . J Virol 2010, 84:3134-3146.

30. Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, Wong SS, Leung SY, Chan KH, Yuen KY: Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc Natl Acad Sci U S A 2005, 102:14040-14045.

31. Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, Luo SW, Li PH, Zhang LJ, Guan YJ et al.: Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. Science 2003, 302:276-278.

32. Rota PA, Oberste MS, Monroe SS, Nix WA, Campagnoli R, Icenogle JP, Penaranda S, Bankamp B, Maher K, Chen MH et al.: Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science 2003, 300: 1394-1399.

33. de Wit E, van Doremalen N, Falzarano D, Munster VJ: SARS and MERS: recent insights into emerging coronaviruses. Nat Rev Microbiol 2016, 14:523-534.

34. Drexler JF, Corman VM, Drosten C: Ecology, evolution and classification of bat coronaviruses in the aftermath of SARS. Antiviral Res 2014, 101:45-56.

35. Decaro N, Mari V, Elia G, Addie DD, Camero M, Lucente MS, Martella V, Buonavoglia C: Recombinant canine coronaviruses in dogs, Europe. Emerg Infect Dis 2010, 16:41-47.

36. Pfefferle S, Oppong S, Drexler JF, Gloza-Rausch F, Ipsen A, Seebens A, Muller MA, Annan A, Vallo P, Adu-Sarkodie Y et al.: Distant relatives of severe acute respiratory syndrome coronavirus and close relatives of human coronavirus 229E in bats, Ghana. Emerg Infect Dis 2009, 15:1377-1384.

37. Corman VM, Baldwin HJ, Tateno AF, Zerbinati RM, Annan A, Owusu M, Nkrumah EE, Maganga GD, Oppong S, Adu-Sarkodie Y et al.: Evidence for an ancestral association of human coronavirus 229E with bats. J Virol 2015, 89:11858-11870.

38. Lau SK, Li KS, Tsang AK, Shek CT, Wang M, Choi GK, Guo R, Wong BH, Poon RW et al.: Recent transmission of a novel alphacoronavirus, bat coronavirus HKU10, from Leschenault's rousettes to pomona leaf-nosed bats: first evidence of interspecies transmission of coronavirus between bats of different suborders. Edited by Lam CS. 2012, 86:11906-11918.

39. Han MG, Cheon DS, Zhang X, Saif LJ: Cross-protection against a human enteric coronavirus and a virulent bovine enteric coronavirus in gnotobiotic calves. J Virol 2006, 80:12350-12356.

40. Alekseev KP, Vlasova AN, Jung K, Hasoksuz M, Zhang X, Halpin R, Wang S, Ghedin E, Spiro D, Saif LJ: Bovine-like coronaviruses isolated from four species of captive wild ruminants are homologous to bovine coronaviruses, based on complete genomic sequences. J Virol 2008, 82:12422-12431.

41. Tsunemitsu H, el-Kanawati ZR, Smith DR, Reed HH, Saif LJ: Isolation of coronaviruses antigenically indistinguishable from bovine coronavirus from wild ruminants with diarrhea. J Clin Microbiol 1995, 33:3264-3269.

42. Majhdi F, Minocha HC, Kapil S: Isolation and characterization of a coronavirus from elk calves with diarrhea. J Clin Microbiol 1997, 35:2937-2942.

43. Kin N, Miszczak F, Diancourt L, Caro V, Moutou F, Vabret A, Ar Gouilh M: Comparative molecular epidemiology of two closely related coronaviruses, bovine coronavirus (BCoV) and human coronavirus OC43 (HCoV-OC43), reveals a different evolutionary pattern. Infect, Genet Evol 2016, 40:186-191.

44. Ma Y, Zhang Y, Liang X, Lou F, Oglesbee M, Krakowka S, Li J: Origin, evolution, and virulence of porcine deltacoronaviruses in the United States. mBio 2015, 6:e00064.

45. Chu DK, Leung CY, Gilbert M, Joyner PH, Ng EM, Tse TM, Guan Y, Peiris JS, Poon LL: Avian coronavirus in wild aquatic birds. J Virol 2011, 85:12815-12820.

46. Lau SKP, Wong EYM, Tsang CC, Ahmed SS, Au-Yeung RKH, Yuen KY, Wernery U, Woo PCY: Discovery and sequence analysis of four deltacoronaviruses from birds in the middle east reveal interspecies jumping with recombination as a potential mechanism for avian-to-avian and avian-to-mammalian transmission. J Virol 2018, 92.

47. Huang YW, Dickerman AW, Pineyro P, Li L, Fang L, Kiehne R, Opriessnig T, Meng XJ: Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. mBio 2013, 4:e00737-00713.
A nice study on the origin of emerging PEDV in the USA.

48. Liu C, Tang J, Ma Y, Liang X, Yang Y, Peng G, Qi Q, Jiang S, Li J, Du L et al.: Receptor usage and cell entry of porcine epidemic diarrhea coronavirus. J Virol 2015, 89:6121-6125.

49. Teeravechyan S, Frantz PN, Wongthida P, Chailangkarn T, Jaru-Ampornpan P, Koonpaew S, Jongkaewwattana A: Deciphering the biology of porcine epidemic diarrhea virus in the era of reverse genetics. Virus Res 2016, 226:152-171.

50. Bevins SN, Lutman M, Pedersen K, Barrett N, Gidlewski T, Deliberto TJ, Franklin AB: Spillover of swine coronaviruses, United States. Emerg Infect Dis 2018, 24:1390-1392.

51. Lee DU, Kwon T, Je SH, Yoo SJ, Seo SW, Sunwoo SY, Lyoo YS: Wild boars harboring porcine epidemic diarrhea virus (PEDV) may play an important role as a PEDV reservoir. Vet Microbiol 2016, 192:90-94.
This is the first study reporting PEDV infection of wild pigs, which can be an important PEDV reservoir.

52. Lau SK, Woo PC, Li KS, Huang Y, Wang M, Lam CS, Xu H, Guo R, Chan KH, Zheng BJ et al.: Complete genome sequence of bat coronavirus HKU2 from Chinese horseshoe bats revealed a much smaller spike gene with a different evolutionary lineage from the rest of the genome. Virology 2007, 367:428-439.

53. Li W, Hulswit RJG, Kenney SP, Widjaja I, Jung K, Alhamo MA, van Dieren B, van Kuppeveld FJM, Saif LJ, Bosch BJ: Broad receptor engagement of an emerging global coronavirus may potentiate its diverse cross-species transmissibility. Proc Natl Acad Sci U S A 2018, 115:E5135-E5143.
This study reported that PDCoV uses APN as an entry receptor via binding to the phylogenetically conserved catalytic domain of APN. It partially explains the fact that PDCoV efficiently infects cells of a broad species range and the mechanism of interspecies transmission of the virus from birds to pigs.

54. Li F: Structure, function, and evolution of coronavirus spike proteins. Annu Rev Virol 2016, 3:237-261.

55. Hulswit RJ, de Haan CA, Bosch BJ: Coronavirus spike protein and tropism changes. Adv Virus Res 2016, 96:29-57.
Nice and comprehensive review of the coronavirus spike protein and inter- and intraspecies tropism changes.

56. Walls AC, Tortorici MA, Bosch BJ, Frenz B, Rottier PJM, DiMaio F, Rey FA, Veesler D: Cryo-electron microscopy structure of a coronavirus spike glycoprotein trimer. Nature 2016, 531:114-117.

57. Li W, van Kuppeveld FJM, He Q, Rottier PJM, Bosch BJ: Cellular entry of the porcine epidemic diarrhea virus. Virus Res 2016, 226:117-127.

58. Shirato K, Maejima M, Islam MT, Miyazaki A, Kawase M, Matsuyama S, Taguchi F: Porcine aminopeptidase N is not a cellular receptor of porcine epidemic diarrhea virus, but promotes its infectivity via aminopeptidase activity. J Gen Virol 2016, 97:2528-2539.
It is the first report showing experimental evidence that the previously claimed PEDV receptor APN is not the cellular receptor for PEDV.

59. Li W, Luo R, He Q, van Kuppeveld FJM, Rottier PJM, Bosch BJ: Aminopeptidase N is not required for porcine epidemic diarrhea virus cell entry. Virus Res 2017, 235:6-13.

60. Ji CM, Wang B, Zhou J, Huang YW: Aminopeptidase-N-independent entry of porcine epidemic diarrhea virus into Vero or porcine small intestine epithelial cells. Virology 2018, 517:16-23.

61. Reguera J, Santiago C, Mudgal G, Ordono D, Enjuanes L, Casasnovas JM: Structural bases of coronavirus attachment to host aminopeptidase N and its inhibition by neutralizing antibodies. PLoS Pathog 2012, 8:e1002859.

62. Schultze B, Krempl C, Ballesteros ML, Shaw L, Schauer R, Enjuanes L, Herrler G: Transmissible gastroenteritis coronavirus, but not the related porcine respiratory coronavirus, has a sialic acid (N-glycolylneuraminic acid) binding activity . J Virol 1996, 70:5634-5637.

63. Krempl C, Schultze B, Laude H, Herrler G: Point mutations in the S protein connect the sialic acid binding activity with the enteropathogenicity of transmissible gastroenteritis coronavirus . J Virol 1997, 71:3285-3287.
A nice study on the connection between sialic acid binding activity and the enteropathogenicity of TGEV.

64. Schwegmann-Wessels C, Herrler G: Sialic acids as receptor determinants for coronaviruses . Glycoconjugate J 2006, 23:51-58.

65. Zhu X, Liu S, Wang X, Luo Z, Shi Y, Wang D, Peng G, Chen H, Fang L, Xiao S: Contribution of porcine aminopeptidase N to porcine deltacoronavirus infection . Emerg Microbes Infect 2018, 7:65.

66. Vlasova AN, Zhang X, Hasoksuz M, Nagesha HS, Haynes LM, Fang Y, Lu S, Saif LJ: Two-way antigenic cross-reactivity between severe acute respiratory syndrome coronavirus (SARS-CoV) and group 1 animal CoVs is mediated through an antigenic site in the N-terminal region of the SARS-CoV nucleoprotein . J Virol 2007, 81:13365-13377.

67. Lin CM, Gao X, Oka T, Vlasova AN, Esseili MA, Wang Q, Saif LJ: Antigenic relationships among porcine epidemic diarrhea virus and transmissible gastroenteritis virus strains . J Virol 2015, 89:3332-3342.

68. Sun ZF, Meng XJ: Antigenic cross-reactivity between the nucleocapsid protein of severe acute respiratory syndrome (SARS) coronavirus and polyclonal antisera of antigenic group I animal coronaviruses: implication for SARS diagnosis . J Clin Microbiol 2004, 42:2351-2352.

69. Ma Y, Zhang Y, Liang X, Oglesbee M, Krakowka S, Niehaus A, Wang G, Jia A, Song H, Li J: Two-way antigenic cross-reactivity between porcine epidemic diarrhea virus and porcine deltacoronavirus . Vet Microbiol 2016, 186:90-96.

70. Marthaler D, Raymond L, Jiang Y, Collins J, Rossow K, Rovira A: Rapid detection, complete genome sequencing, and phylogenetic analysis of porcine deltacoronavirus . Emerg Infect Dis 2014, 20:1347-1350.

71. Niederwerder MC, Hesse RA: Swine enteric coronavirus disease: a review of 4 years with porcine epidemic diarrhoea virus and porcine deltacoronavirus in the United States and Canada . Transboundary and Emerg Dis 2018, 65:660-675.

72. Xu Z, Zhang Y, Gong L, Huang L, Lin Y, Qin J, Du Y, Zhou Q, Xue C, Cao Y: Isolation and characterization of a highly pathogenic strain of Porcine enteric alphacoronavirus causing watery diarrhoea and high mortality in newborn piglets . Transboundary Emerg Dis 2018.

73. Li Y, Wu Q, Huang L, Yuan C, Wang J, Yang Q: An alternative pathway of enteric PEDV dissemination from nasal cavity to intestinal mucosa in swine . Nat Commun 2018, 9:3811.

74. Liu X, Lin CM, Annamalai T, Gao X, Lu Z, Esseili MA, Jung K, El-Tholoth M, Saif LJ, Wang Q: Determination of the infectious titer and virulence of an original US porcine epidemic diarrhea virus PC22A strain . Vet Res 2015, 46:109.

75. Thomas JT, Chen Q, Gauger PC, Gimenez-Lirola LG, Sinha A, Harmon KM, Madson DM, Burrough ER, Magstadt DR, Salzbrenner HM et al.: Effect of porcine epidemic diarrhea virus infectious doses on infection outcomes in naive conventional neonatal and weaned pigs . PloS One 2015, 10:e0139266.

76. Zhang J: Porcine deltacoronavirus: overview of infection dynamics, diagnostic methods, prevalence and genetic evolution . Virus Res 2016, 226:71-84.

77. Chen Q, Wang L, Zheng Y, Zhang J, Guo B, Yoon KJ, Gauger PC, Harmon KM, Main RG, Li G: Metagenomic analysis of the RNA fraction of the fecal virome indicates high diversity in pigs infected by porcine endemic diarrhea virus in the United States . Virol J 2018, 15:95.

78. Zhang Q, Hu R, Tang X, Wu C, He Q, Zhao Z, Chen H, Wu B: Occurrence and investigation of enteric viral infections in pigs with diarrhea in China . Arch Virol 2013, 158:1631-1636.

79. Lin CM, Saif LJ, Marthaler D, Wang Q: Evolution, antigenicity and pathogenicity of global porcine epidemic diarrhea virus strains . Virus Res 2016, 226:20-39.

80. Jung K, Saif LJ: Porcine epidemic diarrhea virus infection: etiology, epidemiology, pathogenesis and immunoprophylaxis . Vet J 2015, 204:134-143.

81. Moon HW, Kemeny LJ, Lambert G, Stark SL, Booth GD: Age-dependent resistance to transmissible gastroenteritis of swine. III. Effects of epithelial cell kinetics on coronavirus production and on atrophy of intestinal villi . Vet Pathol 1975, 12:434-445.
A nice study on innate age-dependent resistance to transmissible gastroenteritis.

82. Annamalai T, Saif LJ, Lu Z, Jung K: Age-dependent variation in innate immune responses to porcine epidemic diarrhea virus infection in suckling versus weaned pigs . Vet Immunol Immunopathol 2015, 168:193-202.

83. Jung K, Annamalai T, Lu Z, Saif LJ: Comparative pathogenesis of US porcine epidemic diarrhea virus (PEDV) strain PC21A in conventional 9-day-old nursing piglets vs. 26-day-old weaned pigs . Vet Microbiol 2015, 178:31-40.

84. Hammerberg C, Schurig GG, Ochs DL: Immunodeficiency in young pigs . Am J Vet Res 1989, 50:868-874.

85. Jung K, Miyazaki A, Saif LJ: Immunohistochemical detection of the vomiting-inducing monoamine neurotransmitter serotonin and enterochromaffin cells in the intestines of conventional or gnotobiotic (Gn) pigs infected with porcine epidemic diarrhea virus (PEDV) and serum cytokine responses of Gn pigs to acute PEDV infection . Res Vet Sci 2018, 119:99-108.

86. Langel SN, Paim FC, Lager KM, Vlasova AN, Saif LJ: Lactogenic immunity and vaccines for porcine epidemic diarrhea virus (PEDV): Historical and current concepts . Virus Res 2016, 226:93-107.

87. Song Q, Stone S, Drebes D, Greiner LL, Dvorak CM, Murtaugh MP: Characterization of anti-porcine epidemic diarrhea virus neutralizing activity in mammary secretions . Virus Res 2016, 226:85-92.

88. Diel DG, Lawson S, Okda F, Singrey A, Clement T, Fernandes MHV, Christopher-Hennings J, Nelson EA: Porcine epidemic diarrhea virus: an overview of current virological and serological diagnostic methods . Virus Res 2016, 226:60-70.

89. Crawford K, Lager K, Miller L, Opriessnig T, Gerber P, Hesse R: Evaluation of porcine epidemic diarrhea virus transmission and the immune response in growing pigs . Vet Res 2015, 46:49.

90. Gimenez-Lirola LG, Zhang J, Carrillo-Avila JA, Chen Q, Magtoto R, Poonsuk K, Baum DH, Pineyro P, Zimmerman J: Reactivity of porcine epidemic diarrhea virus structural proteins to antibodies against porcine enteric coronaviruses: diagnostic implications . J Clin Microbiol 2017, 55:1426-1436.
A nice study on the immunogenicity and reactivity among porcine enteric CoV structural proteins.

91. de Arriba ML, Carvajal A, Pozo J, Rubio P: Mucosal and systemic isotype-specific antibody responses and protection in conventional pigs exposed to virulent or attenuated porcine epidemic diarrhoea virus . Vet Immunol Immunopathol 2002, 85:85-97.

92. de Arriba ML, Carvajal A, Pozo J, Rubio P: Isotype-specific antibody-secreting cells in systemic and mucosal associated lymphoid tissues and antibody responses in serum of conventional pigs inoculated with PEDV. Vet Immunol Immunopathol 2002, 84:1-16.

93. Ouyang K, Shyu DL, Dhakal S, Hiremath J, Binjawadagi B, Lakshmanappa YS, Guo R, Ransburgh R, Bondra KM, Gauger P et al.: Evaluation of humoral immune status in porcine epidemic diarrhea virus (PEDV) infected sows under field conditions . Vet Res 2015, 46:140.

94. Lin CM, Ghimire S, Hou Y, Langel SN, Vlasova AN, Saif LJ, Wang Q: Pathogenicity and immunogenicity of attenuated porcine epidemic diarrhea virus PC22A strain in conventional weaned pigs. BMC Vet Res 2019. (in press).

95. Derbyshire JB, Jessett DM, Newman G: An experimental epidemiological study of porcine transmissible gastroenteritis. J Comp Pathol 1969, 79:445-452.

96. Cao L, Ge X, Gao Y, Ren Y, Ren X, Li G: Porcine epidemic diarrhea virus infection induces NF-kappaB activation through the TLR2, TLR3 and TLR9 pathways in porcine intestinal epithelial cells. J Gen Virol 2015, 96:1757-1767.

97. Xu X, Zhang H, Zhang Q, Huang Y, Dong J, Liang Y, Liu HJ, Tong D: Porcine epidemic diarrhea virus N protein prolongs S-phase cell cycle, induces endoplasmic reticulum stress, and up-regulates interleukin-8 expression. Vet Microbiol 2013, 164:212-221.

98. Xu X, Zhang H, Zhang Q, Dong J, Liang Y, Huang Y, Liu HJ, Tong D: Porcine epidemic diarrhea virus E protein causes endoplasmic reticulum stress and up-regulates interleukin-8 expression. Virol J 2013, 10:26.

99. Wang D, Fang L, Shi Y, Zhang H, Gao L, Peng G, Chen H, Li K, Xiao S: Porcine epidemic diarrhea virus 3C-like protease regulates its interferon antagonism by cleaving NEMO. J Virol 2015, 90:2090-2101.

100. Ding Z, Fang L, Jing H, Zeng S, Wang D, Liu L, Zhang H, Luo R, Chen H, Xiao S: Porcine epidemic diarrhea virus nucleocapsid protein antagonizes beta interferon production by sequestering the interaction between IRF3 and TBK1. J Virol 2014, 88:8936-8945.

101. Zhang Q, Shi K, Yoo D: Suppression of type I interferon production by porcine epidemic diarrhea virus and degradation of CREB-binding protein by nsp1. Virology 2016, 489:252-268.

102. Zhang Q, Ke H, Blikslager A, Fujita T, Yoo D: Type III interferon restriction by porcine epidemic diarrhea virus and the role of viral protein nsp1 in IRF1 signaling. J Virol 2018, 92.

103. Lin CM, Annamalai T, Liu X, Gao X, Lu Z, El-Tholoth M, Hu H, Saif LJ, Wang Q: Experimental infection of a US spike-insertion deletion porcine epidemic diarrhea virus in conventional nursing piglets and cross-protection to the original US PEDV infection. Vet Res 2015, 46:134.

104. Crawford K, Lager KM, Kulshreshtha V, Miller LC, Faaberg KS: Status of vaccines for porcine epidemic diarrhea virus in the United States and Canada. Virus Res 2016, 226:108-116.

105. Song D, Park B: Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. Virus Genes 2012, 44:167-175.

106. Okda FA, Lawson S, Singrey A, Nelson J, Hain KS, Joshi LR, Christopher-Hennings J, Nelson EA, Diel DG: The S2 glycoprotein subunit of porcine epidemic diarrhea virus contains immunodominant neutralizing epitopes. Virology 2017, 509:185-194.

107. Li C, Li W, Lucio de Esesarte E, Guo H, van den Elzen P, Aarts E, van den Born E, Rottier PJM, Bosch BJ: Cell attachment domains of the porcine epidemic diarrhea virus spike protein are key targets of neutralizing antibodies. J Virol 2017, 91.

108. Chattha KS, Roth JA, Saif LJ: Strategies for design and application of enteric viral vaccines. Annu Rev Anim Biosci 2015, 3:375-395.

109. Bjustrom-Kraft J, Woodard K, Gimenez-Lirola L, Setness B, Ji J, Lasley P, Nelson E, Zhang JQ, Baum D, Gauger P et al.: Serum and mammary secretion antibody responses in porcine epidemic diarrhea-immune gilts following porcine epidemic diarrhea vaccination. JSHAP 2018, 26:34-40.

110. Song D, Moon H, Kang B: Porcine epidemic diarrhea: a review of current epidemiology and available vaccines. Clin Exp Vac Res 2015, 4:166-176.

111. Jung K, Hu H, Saif LJ: Calves are susceptible to the newly emerged porcine deltacoronavirus, but not to the swine enteric alphacoronavirus, porcine epidemic diarrhea virus. Arch Virol 2017. Submitted for publication.

112. Tamura K, Nei M, Kumar S: Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci U S A 2004, 101:11030-11035.

113. Kumar S, Stecher G, Li M, Knyaz C, Tamura K: MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 2018, 35:1547-1549.

114. Hu H, Jung K, Vlasova AN, Chepngeno J, Lu Z, Wang Q, Saif LJ: Isolation and characterization of porcine deltacoronavirus from pigs with diarrhea in the United States. J Clin Microbiol 2015, 53:1537-1548.

115. Jung K, Miyazaki A, Hu H, Saif LJ: Susceptibility of porcine IPEC-J2 intestinal epithelial cells to infection with porcine deltacoronavirus (PDCoV) and serum cytokine responses of gnotobiotic pigs to acute infection with IPEC-J2 cell culture-passaged PDCoV. Vet Microbiol 2018, 221:49-58.

Received: 9 April 2018 | Revised: 3 August 2018 | Accepted: 6 August 2018

DOI: 10.1111/tbed.12992

ORIGINAL ARTICLE

WILEY Transboundary and Emerging Diseases

Isolation and characterization of a highly pathogenic strain of Porcine enteric alphacoronavirus causing watery diarrhoea and high mortality in newborn piglets

Zhichao Xu[1] | Yun Zhang[1] | Lang Gong[1] | Licheng Huang[1] | Ying Lin[1] | Jianru Qin[1] | Yunping Du[2] | Qingfeng Zhou[2] | Chunyi Xue[1] | Yongchang Cao[1]

[1]State Key Laboratory of Biocontrol, School of Life Science, Sun Yat-sen University, Guangzhou, China

[2]Guangdong Wen's Group Academy, Guangdong Wen's Foodstuffs Group Co., Ltd, Xinxing, China

Correspondence
Yongchang Cao, School of Life Science, Higher Education Mega Center, Sun Yat-sen University, Guangzhou 510006, China.
Email: caoych@mail.sysu.edu.cn

Funding information
National Key Researchand Development Program, Grant/Award Number: 2016YFD0500101; National Natural Science Foundation, Grant/Award Number: 31741118; Guangdong Natural Science Foundation, Grant/Award Number: 2018B030314003

Abstract

Porcine enteric alphacoronavirus (PEAV) was first discovered in China in February 2017, and the origin and virulence of this novel porcine coronavirus were not fully characterized. Here, we isolated a strain of PEAV, named GDS04 that is identified by immunofluorescence and typical crown-shaped particles observed with electron microscopy. Genomic analysis reveals that PEAV GDS04 shares a close relationship with SADS-CoV and SeACoV. Furthermore, newborn piglets orally challenged with PEAV GDS04 developed typical clinical symptoms as watery diarrhoea in neonatal piglets. Viral RNA was detected in faeces and various tissues of the infected piglets. Moreover, macroscopic and microscopic lesions in whole intestinal tract were observed, and viral antigen could be detected in the small intestines by immunohistochemical staining and electron microscopy. Importantly, the mortality rate of inoculated-newborn piglets was 100% and half of the cohabiting piglets died. Collectively, we demonstrate that PEAV is highly pathogenic in newborn piglets.

KEYWORDS
newborn piglets, pathogenicity, Porcine enteric alphacoronavirus (PEAV)

1 | INTRODUCTION

Coronaviruses (CoVs), belonging to the subfamily Coronavirinae in the family Coronaviridae within the order Nidovirales (Zhang, 2016), are found in a wide variety of animals (Felippe, da Silva, Santos, Spilki, & Arns, 2010; Pan et al., 2012; Rihtaric, Hostnik, Steyer, Grom, & Toplak, 2010; Stohlman, Brayton, Fleming, Weiner, & Lai, 1982; Tsunemitsu, el-Kanawait, Smith, Reed, & Saif, 1995). Porcine CoVs are significant enteric and respiratory pathogens of swine. Six porcine CoVs have so far been identified: transmissible gastroenteritis virus (TGEV) (Doyle & Hutchings, 1946), porcine respiratory coronavirus (PRCV) (Wesley, Woods, Hill, & Biwer, 1990), porcine epidemic diarrhoea virus (PEDV) (Pensaert & de Bouck, 1978), and porcine enteric alphacoronavirus (PEAV) (Gong et al., 2017) in the Alphacoronavirus genus; porcine haemagglutinating encephalomyelitis virus (PHEV) (Sasseville, Boutin, Gelinas, & Dea, 2002) in the Betacoronavirus genus; porcine deltacoronavirus (PDCoV) (Woo et al., 2012) in the Deltacoronavirus genus. PEAV as the newest member was first detected by our team by genomic analysis of samples collected from a diarrhoea-outbreak swine herds routinely vaccinated with PEDV vaccine in a farm in Guangdong, China in February 2017, and complete genome of the PEAV strain GDS04 was then sequenced (Gong et al., 2017).

Porcine enteric alphacoronavirus is an enveloped, single-stranded, positive-sense RNA virus with a genome of appropriately 27 kb in length (Gong et al., 2017). The genome organization of PEAV is similar to that of bat-like HKU2 strains of coronavirus, with an order of: 5′ untranslated region (UTR), open reading frame 1a/1b (ORF1a/1b), spike (S), nonstructural protein 3 (NS3), envelope (E), membrane (M), nucleocapsid (N), nonstructural protein 7a (NS7a), and 3′ UTR (Lau et al., 2007). The S protein of CoVs is the pivotal surface glycoprotein involved in virus attachment and entry, and induction of neutralizing

 © 2018 Blackwell Verlag GmbH

antibodies in vivo (Cruz, Kim, & Shin, 2008; Woo, Huang, Lau, & Yuen, 2010). GDS04 strain of PEAV has the smallest S protein among all coronaviruses (Gong et al., 2017).

The clinical symptoms in newborn piglets from pig farm with reported PEAV are similar to that by other porcine enteric pathogens such as PEDV and TGEV, which include vomiting, diarrhoea, dehydration, and mortality rate as high as 90% in piglets (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018). Since the new bat-HKU2-like coronavirus (PEAV) was detected in pigs with severe diarrhoea (Gong et al., 2017), another two swine enteric HKU2-related CoV (SADS-CoV and SeACoV) strains were identified in the same region, which reproduced clinical diarrhoeal disease by experimentally infecting piglets with isolated SADS-CoV and SeACoV strains (Pan et al., 2017; Zhou et al., 2018).

Although PEAV GDS04 was detected by genomic analysis in pigs (Gong et al., 2017), detailed information remains unclear. In this study, we isolated a PEAV strain from Guangdong province of China using Vero cells, characterized its genome based on s genes, n genes, and whole-genome, and investigated its pathogenicity in 5-day-old conventional pigs by clinical assessment,virus shedding, virus distribution, histological test, immunohistochemical study, and the mortality rate of inoculated piglets. The results suggest that the isolate of PEAV GDS04 is closely related to SADS-CoV and SeACoV but caused 100% mortality in neonatal piglets, indicating its potential role as pathological agent responsible for severe watery diarrhoea and death in neonatal piglets in the field case.

2 | MATERIALSAND METHODS

2.1 | PEAV-positive specimens

In early February 2017, an outbreak of PEAV was reported in swine herds in Guangdong, China (Gong et al., 2017), with a mortality rate ranging up to 90% (Zhou et al., 2018). To increase the virus titres for isolation, fresh excreta from infected PEDV-vaccinated newborn piglets were inoculated into 5-day-old nonvaccinated piglets as described previously (Gong et al., 2017). Intestinal contents were collected from an inoculated piglet developing symptoms of severe diarrhoea and vomiting, and stored at - 80°C until further use. Prior to virus isolation, intestinal contents were diluted one time using sterile 1× phosphate buffer saline (PBS) (pH 7.4). The supernatants were then collected by centrifugation at 6000× g for 5 min at 4°C, and filtered through 0.22-μm-pore-size filters (Millipore, USA).

2.2 | Virus isolation, plaque purification, and propagation in Vero cells

Vero cells were obtained from ATCC (ATCC number: CCL-81) (USA) and were used to isolate PEAV from the intestinal contents of piglet. Vero cells were cultured in DMEM (Hyclone, USA) supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), and 10% foetal bovine serum (FBS) (BOVOGEN, Australia). The maintenance medium for PEAV propagation was DMEM supplemented with 10 μg/ml trypsin (Gibco, USA).

Virus isolation, plaque purification, and propagation were performed as previously described with some modifications (Lee, Kim, & Lee, 2015; Oka et al., 2014; Pan et al., 2017). Briefly, for the first inoculation, Vero cells were cultured in 6-well plates, and washed three times with sterile 1× PBS (pH 7.4) at a confluency of 90%. Two hundred microlitres of the filtered inoculums, together with 300 μl maintenance medium was added to each well. After adsorption for 1.5 hr at 37°C in 5% CO_2, cells were washed three times with the maintenance medium, and then 2 ml maintenance medium was added. The cells were cultured continuously at 37°C in 5% CO_2 for cytopathic effect (CPE) observation. The plates were frozen at - 80°C and thawed twice around 4 day postinoculation (d.p.i). The cells and supernatant termed as "passage 1 (P1)" were harvested together. Samples collected at 0-hr postinoculation and 4 d.p.i. were tested by PEAV-specific RT-PCR as described previously (Gong et al., 2017). The RT-PCR positive samples were used as seed stocks for the next passageand plaque purification. For virus plaque purification, supernatants from virus-infected cells were serially diluted and used to infect Vero cells in the maintenance medium for 1.5 hr at 37°C in 5% CO_2 and then the maintenance medium was discarded, followed by overlaying 2 ml maintenance medium containing 1.25% Agarose LM GQT (TaKaRa, Dalian) to immobilize the virus. After 24 hr, cells were fixed and visualized with 2 ml maintenance medium containing 1.25% Agarose LM GQT and 0.01% Neutral red solution (Sigma,USA). The plaques were picked by using sterile pipette tips, and the agaroseplaque was placed into a microcentrifuge tube containing 0.5 ml maintenance medium. The selected plaques of PEAV were named GDS04 and used for viral propagation. Vero cells were cultured in T175 flasks, and washed three times with sterile 1× PBS (pH 7.4) at a confluency of 90%. One mL of PEAV together with 50 ml maintenance medium was added into the flask. The cell pellets and supernatant were cultured continuously at 37°C in 5% CO_2 to observe CPE. When CPE was evident in the inoculated cell monolayers (around 1 d.p.i.), the plates were frozen at - 80°C and thawed twice. The cells and supernatant were harvested together to determine viral titres.

2.3 | Infectious-virus titrations by a $TCID_{50}$ assay

Vero cells were seeded on 96-well plates and cultured overnight before washed two times with sterile 1× PBS (pH 7.4). One hundred microlitre of 10-fold dilutions of PEAV was inoculated in eight replicates per dilution, then the cells were cultured continuously at 37°C in 5% CO_2. Viral CPE was observed for 5–7 days, and virus titre was calculated using the Reed-Muench method (Reed, 1938) and expressed as $TCID_{50}$ per millilitre.

2.4 | Immunofluorescence assay (IFA)

Immunofluorescence assay was conducted to observe PEAV-infected Vero cells as described previously with some modifications (Dong et al., 2016). Briefly, Vero cells (1×10^5) were seeded on 24-well plates

and cultured overnight, then infected with PEAV at a multiplicity of infection (MOI) of 1. At 24 hr after inoculation, the cells were fixed with 4% paraformaldehyde for 15 min and then permeabilized with 0.2% Triton X-100 for 15 min at room temperature. The cells were then blocked with 1% bovine serum albumin (BSA), and incubated with PEAV-specific mouse antisera (Guangdong Wen' s Foodstuffs Group Co., Ltd, China) (1:250), followed by fluoresceinisothiocyanate (FITC)-labelled goat anti-mouse secondary antibody (KPL, USA) (1:1,000) for 1 hr. Then, the stained cells were observed with a fluorescence microscope (LEICADMi8, Germany).

2.5 | Electron microscopic observation

Electron microscopy (EM) was conducted to observe virus samples as described previously with some modifications (Alsaad et al., 2018; Hu, Jung et al., 2015; Kong et al., 2010). For visualization of the viral particles in infected-cell culture medium, PEAV-infected Vero cells were frozen at - 80°C and thawed twice, and the cell culture was centrifugated at 7,000× g for 30 min at 4°C. The supernatant was supplemented with 6% PEG6000 for 12 hr at 4°C. The mixture was centrifuged at 12,000× g for 1 hr at 4°C, and the pellet was resuspended in sterile 1× PBS (pH 7.4) buffer, followed by equilibrium in 8 mL nonlinear 20%–60% sucrose-TNE gradients by centrifugation at 110,000× g for 2 hr at 4°C with an ultracentrifuge (Hitachi Koki himac CP 100WX, Japan). After purification by sucrose gradient centrifugation, purified virions were diluted with sterile 1× PBS (pH 7.4) buffer and the sucrose was removed by centrifugation at 7,000× g for 2 hr at 4°C with centrifugal filter units (Millipore, USA). The purified virus pellets were resuspended in sterile 1× PBS (pH 7.4) buffer and negatively stained with 3% phosphotungstic acid. After blotting and drying, the grids were examined with a JEM-100 CX-II electron microscope (JEOLLTD,Japan).

For visualization of the viral particles in jejunum of PEAV-inoculated piglets, portion of jejunum was fixed in 5% glutaraldehyde fixative prepared in sterile 1× PBS (pH 7.4) for 4 weeks and underwent postfixation in 1% osmium tetraoxide for 1 hr. The samples were washed twice by sterile 1× PBS (pH 7.4) and dehydrated using increasing concentrations of acetone. The samples were then embedded in Araldit 520 resin and polymerized in oven at 90°C for 24 hr. Tissue semithin sections (1 μm) were prepared, stained by Toludin blue stain for 5 min and inspected under conventional light microscope. Ultrathin sections (70 nm) were prepared using Leica EM UC7 ultramicrotome, collected on 200 mesh copper grid (PELCO) and stained with uranyl acetate for 15 min and lead citrate for 5 min. The ultrathin sections were screened by 120-kV JOEL1230 transmission electron (TEM) (Akishima, Japan) and images were obtained using side-mounted digital camera (Gatan780AJ03FA, Pleasanton, CA, USA).

2.6 | Genomic cloning and phylogenetic analysis of the s genes, n genes, and whole-genome

Total RNA was prepared from the isolated virus using a RNeasy kit (Magen, China) and was treated with DNase I. The cDNA was synthesized by reverse transcription using RT-PCR kit (TaKaRa,Dalian). A total of 18 primer pairs based upon the PEAV GDS04 (GenBank accession no. MF167434.1; Supporting information Table S1) were designed to amplify the complete genome of PEAV GDS04 "passage 12 (P12)". The PCR products were cloned into the pMD19-T (TaKaRa, Dalian) and sequenced to determine the consensus sequence. The sequences were assembled and analysed using the DNASTAR program. Sequence alignment analysis was performed using the Clustal W program implemented in DNAStar software Lasergene 7.0. A phylogenetic tree was then constructed by the neighbour-joining method using the Molecular Evolutionary Genetics Analysis (MEGA) software version 5 (http://www.megasoftware.net/) based on the s genes, n genes, and whole-genome from PEAV GDS04 strain P12 together with other different CoVs (alpha, beta, gamma, and delta), like PEDV, PDCoV, and TGEV.

2.7 | Experimental infection with the PEAV GDS04 strain in newborn piglets

The animal study was approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University and performed in accordance with regulation and guidelines of this committee. Twenty-four 4-day-old conventional newborn piglets were randomly divided into two groups (8 piglets in group 1 and 16 piglets in group 2). Piglets were fed a mixture of skim milk powder (Inner Mongolia Yi Li Industrial Group Co., Ltd, China) with warm water. Prior to inoculation, piglets were confirmed negative for the major porcine enteric viruses (PDCoV, PEDV, TGEV, PRoV) by testing of rectal swabs using specific RT-PCR according to previously described method (Hu, Li et al., 2015; Jeong et al., 2009; SaengChuto et al., 2017). After 1-day acclimation, piglets in group 1 were orally inoculated with 5 ml of maintenance medium and served as uninfected controls. Twelve piglets in group 2 were orally challenged with 5 ml of maintenance medium containing 5×10^5 $TCID_{50}$ of the PEAV GDS04 P12 and the remaining four piglets served as cohabitation control.

All piglets were observed daily for clinical signs of vomiting, diarrhoea, lethargy, and body condition. Diarrhoea severity was scored with the following criteria (Chen et al., 2015): 0 = normal, 1 = soft (cowpie), 2 = liquid with some solid content, 3 = watery with no solid content.

Rectal swabs were collected daily from each piglet from 1 d.p.i. to 14 d.p.i. and were submerged into 1 ml sterile 1× PBS (pH 7.4) immediately after collection. Two piglets from each group were necropsied at 7 d.p.i. At necropsy, the fresh samples (serum, heart, liver, spleen, lung, kidney, stomach, duodenum, jejunum, ileum, caecum, and colon) were collected and then formalin-fixed. The fresh samples were stored at - 80°C for viral RNA distribution analysis and formalin-fixed samples were used for histopathology and immunohistochemistry analysis. In addition, the mortality of newborn piglets in each group was recorded daily.

2.8 | Real-time RT-PCR analysis

Rectal swabs, serum, and various tissues were tested by a PEAV n gene-based real-time RT-PCR including viral standards with known

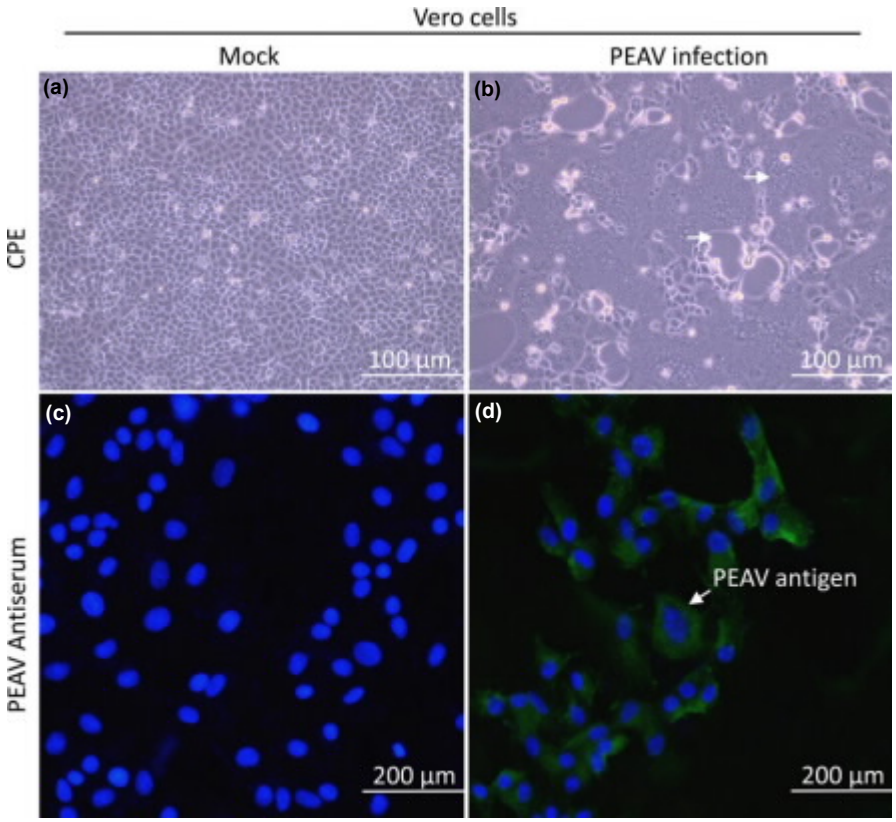


FIGURE 1 Cytopathic effects (CPE)and IFA staining on PEAV-inoculated Vero cells. (a) Mock-inoculated Vero cell culture showing normal cells. (b) PEAV-inoculated Vero cells at 1 d.p.i. showing syncytium and cells detachment (indicated by arrows). Vero cells were mock-inoculated (c) or inoculated with PEAV GDS04 (d). At 24 hr postinoculation, an immunofluorescence assay (IFA) was performed [Colour figure can be viewed at wileyonlinelibrary.com]

plasmid concentration for quantification. Briefly, the homogenates from serum, various tissues, and the supernatants of rectal swab from each piglet were centrifuged at 6,000× g for 5 min, respectively. Total RNA was prepared and used for cDNA synthesis as described above. Specific primers for the nucleocapsid (N) gene of PEAV (sense: 5′-GCACTTTTATTACCTTGGTA8′; antisense: 5′-GTAGCAGGTTCTTTGTTAC-3′), and probe (5′-FAM-TCCTCACGCAGATGCTCCTTTAMRA-3′) were designed according to reference sequence (GenBank, Accession no: MF167434.1) and synthesized by TaKaRa (Dalian, China). The real-time PCR assay was carried out with an Applied Biosystem 7500 Fast instrument (Life Technologies, USA). The PCR was performed in a 20-μl volume containing 1 μl of cDNA, 10 μl of Thunderbird Probe qPCR Mix, 0.04 μl 50× Rox reference dye (TOYOBO, Shanghai),0.2 μM of probe, and a 0.3 μM of each gene-specific primer. The thermal cycling parameters were as follows: 95°C for 20 s; 40 cycles of 95°C for 3 s, 60°C for 30 s. The standard curve was generated by construction of plasmids. Briefly, the n gene was amplified from PEAV GDS04 P12 strain using the specific primers as described above, and the PCR products were cloned into the pMD19-T (TaKaRa, Dalian). The known plasmid concentration was 10-fold serially diluted for generating a standard curve in each plate. The quantity of PEAV viral RNA in tested samples was calculated based on the cycle threshold (Ct) values for the standard curve.

2.9 | Histology and immunohistochemistry

At necropsy, tissue samples of heart, lung, spleen, liver, kidney, stomach, duodenum, jejunum, ileum, caecum, and colon of the piglets from the challenged and control groups were collected separately and routinely fixed in 10% formalin for 36 hr at room temperature (Hu, Jung, Vlasova,& Saif, 2016), and then dehydrated in graded ethanol, embedded in paraffin, cut in 5-μm sectioned, and mounted onto glass slides. Afterwards, the sections were deparaffinized, rehydrated, and stained with haematoxylin and eosin (H&E); the slides were examined and analysed with conventional light microscopy. Sections (5 μm) of formalin-fixed paraffin-embedded tissues were placed onto positively charged glass slides and the slides were air-dried for 120 min at 60°C. The tissue sections were deparaffinized, and then rinsed and incubated with target retrieval solution (Servicebio, China). After being blocked with 1% BSA (Solarbio, China), the sections were incubated with PEAV-specific mouse antisera (1:400) as the primary antibody for 12 hr at 4°C. They were then incubated with peroxidase-labelled goat anti-mouse IgG secondary antibody (Dako, Denmark) for 50 min at room temperature, and the samples were finally visualized with a 3, 3′-diaminobenzidine (DAB) chromogen kit (Dako, Denmark). Haematoxylin was used for counterstaining. Tissues of piglets from negative control groups were used as negative samples.

3 | RESULTS

3.1 | A strain of PEAV was isolated from an inoculated-newborn piglet with fresh excreta from ill newborn piglets

We attempted to isolate PEAV, a new bat-HKU2-like coronavirus from the positive samples since its first detection in China (Gong et

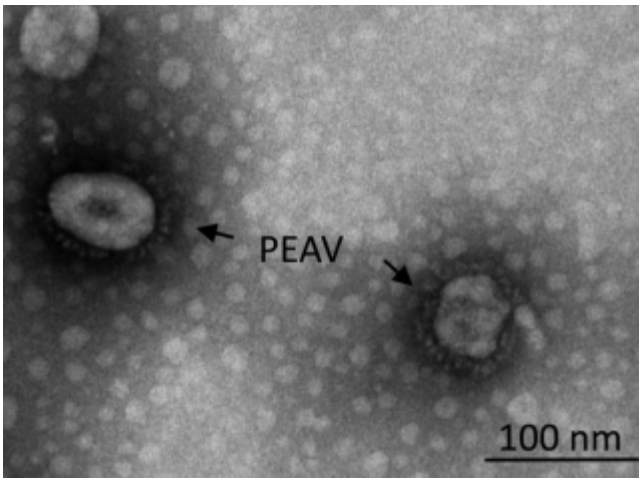


FIGURE 2 Electron micrographs of PEAV-inoculated Vero cells. Crown-shaped spikes of PEAV are visible (arrows). The sample was negatively stained with 3% phosphotungstic acid

al., 2017). As shown in Figure 1b, one inoculated cell monolayer with the supernatant from virus-infected cells of passage 10 (P10) showed visible CPE in the form of syncytium and detachment at 1 d.p.i. as compared with the control in Figure 1a. To confirm PEAV replication in Vero cells, viral RNA was extracted from the inoculated cells at 1 d.p.i. and tested by specific RT-PCR. This cell culture-passaged sample was positive for PEAV but negative for other porcine enteric coronaviruses (data not shown). PEAV from the first passage in Vero cells was named GDS04 (Gong et al., 2017). After blindly passaged in Vero cells for a total of 20 passages, the PEAV GDS04 could still be detected by specific RT-PCR (data not shown), indicating that the proliferation ability of the strain in Vero cells. Plaque-purified PEAV in Vero cells was further confirmed by IFA with PEAV-specific mouse antisera. As shown in Figure 1d, PEAV-specific immunofluorescence was detected in infected cells, as compared to the control (Figure 1c). To characterize the morphology and size of the virus particles, the PEAV GDS04 virus purified from infected Vero cells were examined with EM. Typical crown-shaped particles with spiky surface projections as in other coronaviruses were observed by negative staining on EM. The size of the viral particles was 80–160 nm in diameter (Figure 2). Taken together, these results suggest that a PEAV strain was successfully isolated from the intestinal contents of a newborn diarrheic piglet in China.

3.2 | Phylogenetic analysis of the s genes, n genes, and whole-genome of PEAV GDS04 P12

Since complete genome of the GDS04 was fully described (Gong et al., 2017), to further understand the origin of the virus, phylogenetic trees of complete s genes, n genes, and whole-genome of PEAV GDS04 strains together with other CoVs were constructed. Phylogenetic analysis of the s genes shows that the PEAV GDS04 strains along with four bat coronavirus HKU strains from Hong Kong, a BtRf-AlphaCoV strain from Beijing clustered in one group. Furthermore, the PEAV GDS04 strains, SADS-CoV, and SeACoV clustered into a subclade between bat coronavirus HKU strains and BtRf-AlphaCoV strain (Figure 3a). Consistent with the results of the s genes, phylogenetic analysis of the n genes and whole-genome of the PEAV GDS04 strains and other CoVs reveals that PEAV GDS04 strains, SADS-CoV, and SeACoV belong to the same subclade (Figure 3b&c). Therefore, these data suggest that the GDS04 strains were closely relative to SADS-CoV and SeACoV strains from Guangdong, China.

3.3 | Clinical manifestations of newborn piglets challenged with PEAV GDS04 P12

In order to determine whether PEAV GDS04 was the causative agent for diarrhoea, we experimentally infected newborn piglets with the isolated virus. Twelve newborn piglets inoculated with GDS04 P12 at a dose of 5×10^5 $TCID_{50}$/head via oral feeding showed mild diarrhoea from 1 to 4 d.p.i., and all developed severe watery diarrhoea, together with vomiting, and dehydration from 5 to 12 d.p.i., as compared with controls (Figure 4a–e), indicating a role of PEAV as an important causative agent for severe watery diarrhoea in newborn piglets. Furthermore, four piglets from group 2 as cohabitation controls also developed watery diarrhoea from 5 to 12 d.p.i. (Figure 4e). Since PEAV GDS04 caused severe watery diarrhoea in newborn piglets, we also recorded the mortality of newborn piglets in each group. As shown in Figure 5, in PEAV-inoculated groups, except that two piglets were necropsied at 7 d.p.i., all remaining 10 piglets died from 5 to 12 d.p.i. And the PEAV-inoculated piglets had 1, 1, 3 (+2 euthanized), 1, 1, 2, and 1 death (s) at 5, 6, 7, 8, 9, 10 and 12 d.p.i., respectively. In addition, 2/4 piglets from group 2 as cohabitation controls also died at 7 and 9 d.p.i. No piglets (6/6) died in control group except that two piglets were necropsied at 7 d.p.i. Taken together, these results suggest that PEAV GDS04 is highly pathogenic to the newborn piglets.

3.4 | Faecal shedding and virus distribution in newborn piglets challenged with PEAV GDS04 P12

Since PEAV GDS04 caused watery diarrhoea in newborn piglets, we explored the faecal viral shedding in PEAV-challenged piglets. As shown in Figure 6a, the PEAV RNA was detected by qRT-PCR in faecal swabs collected from orally inoculated piglets from 1 to 11 d.p.i., and peaked on 4 d.p.i. and kept up until 11 d.p.i. We also examined the faecal viral shedding in piglets from group 2 as cohabitation controls. PEAV RNA was detected in rectal samples collected from these piglets from 3 to 13 d.p.i. (Figure 6a), indicating that these piglets may be infected by the PEAV-challenged piglets. No PEAV RNA was detected in the negative control piglets during the study. To examine the distribution of the PEAV virus in different tissues in PEAV-challenged piglets, two piglets from each group were necropsied at 7 d.p.i. As shown in Figure 6b, the PEAV RNA was detected in all collected samples of duodenums, jejunums, ileums, caecum, and colons. The virus was also detected in 2/2 hearts, 2/2

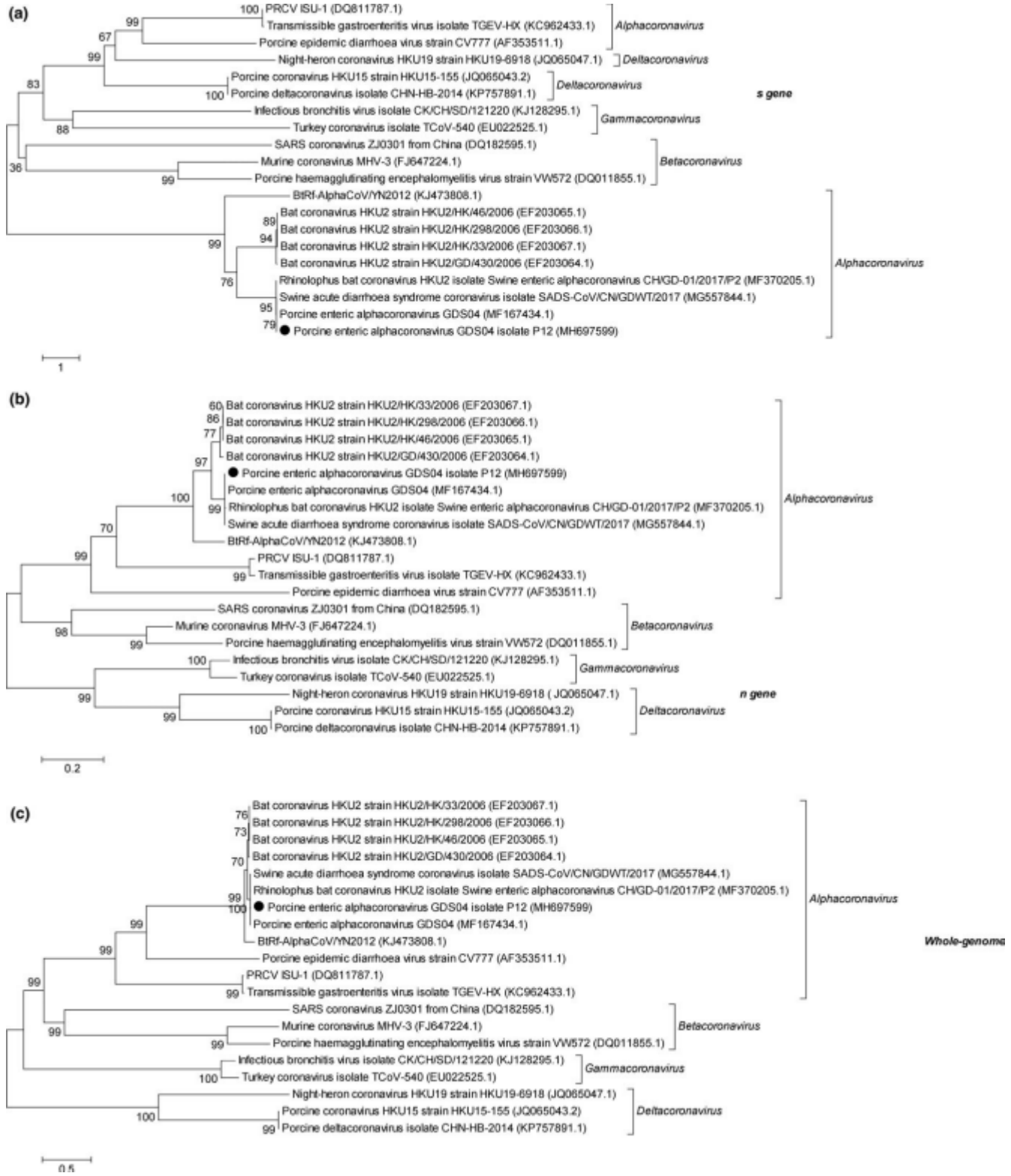


FIGURE 3 Phylogenetic trees constructed on the basis of the s genes, n genes, and whole-genome nucleotide sequences of PEAV GDS04 or other coronaviruses (CoVs). (a) Phylogenetic tree of the s gene. (b) Phylogenetic tree of the n gene. (c) Phylogenetic tree of the whole-genome. The dendrogram was constructed using the neighbour-joining method in the MEGA software package, version 5 (http://www.megasoftware.net). Bootstrap resampling (1,000 replication) was performed, and bootstrap values are indicated for each node. Reference sequence obtained from GenBank is indicated by strain name. The scale bar represents 1, 0.2, or 0.5 nucleotide substitutions per site

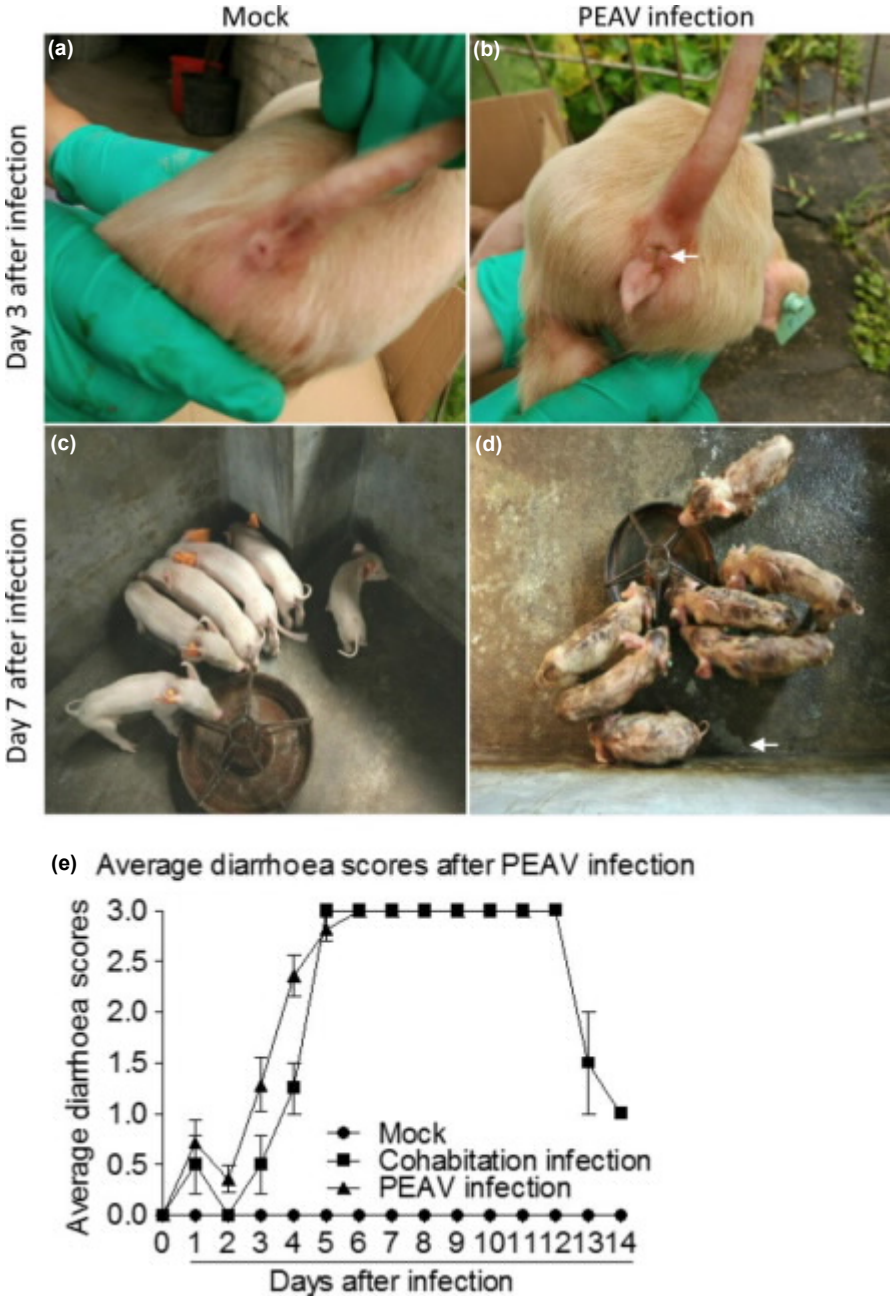


FIGURE 4 Reproduction of watery diarrhoea and faecal viral shedding in newborn piglets inoculated with PEAV GDS04 strain P12 via oral feeding. (a & c) Newborn piglets uninfected as control. (b & d) Watery diarrhoea (indicated by arrows) was observed at 3 and 7 d.p.i. with PEAV infection. (e) Average diarrhoea scores after PEAV infection [Colour figure can be viewed at wileyonlinelibrary.com]

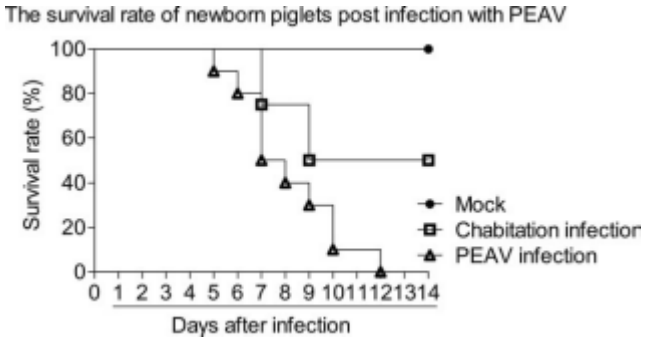


FIGURE 5 The survival rate of newborn piglets postinfection with PEAV GDS04 P12. The mortality of newborn piglets in each group was recorded from 1 to 14 d.p.i

livers, 2/2 spleens, 2/2 kidneys, 2/2 stomachs, and 1/2 lungs, but no viral RNA was detected in blood. No PEAV RNA was detected in the tissue samplesfrom the control piglets. Taken together, these results demonstrate that PEAV GDS04 strain could be widely distributed in different tissues, but mainly concentrated in the intestines of pig.

3.5 | Virus particles in jejunum of inoculated-PEAV piglets

To observe virus particles in vivo, the jejunum of piglets from each group was examined with EM. EM demonstrated that the virion without membrane in the vesicle (Figure 7b) and full virus particles in the intercellular space (Figure 7c) with typical crown-shape of

80–160 nm in diameter and spiky surface projections of CoV, indicating that PEAV GDS04 strain could replicate in the jejunum of pig. In addition, the virus-infected cells showed atrophied, ruptured cell morphology (Figure 7b&c). No virus particles or pathological lesions were detected in the jejunum from the control piglets (Figure 7a). Taken together, these results show that PEAV GDS04 could replicate and cause jejunum lesions in newborn piglets.

3.6 | Gross pathology, histopathology, and immunohistochemistry in newborn piglets infected with PEAV GDS04

To determine the gross pathological and histological changes in piglets infected with the PEAV GDS04 strain, two piglets from each group were necropsied at 7 d.p.i. Gross findings were similar in both piglets orally inoculated with PEAV GDS04. The whole intestinal tract, where yellow watery contents accumulated, were transparent, thin-walled, and gas-distended (Figure 8b). No lesions were observed in any other organs of the PEAV-challenged piglets (data not shown) or the organs in the negative control piglets (Figure 8a), indicating that intestinal tract is the target organ of PEAV infection. Microscopic lesions were also analysed. As shown in Figure 8h–l, abruption of intestinal villus was observed, whereas the intestinal in negative control was normal (Figure 8c–g). Consistent with the histopathological results, PEAV antigen was detected in the cytoplasm of the villous enterocytes of the PEAV-challenged piglets by immunohistochemical analysis (Figure 8o&p). Taken together, these results indicate that PEAV GDS04 could cause intestinal lesions in newborn piglets.

4 | DISCUSSION

The widely distributed CoVs could be isolated from a variety of animal hosts and products with animal origin (Felippe et al., 2010; Pan et al., 2012; Rihtaric et al., 2010; Stohlman et al., 1982; Tsunemitsu et al., 1995), as well as from human (Larson, Reed, & Tyrrell, 1980). Bats are thought to be the natural reservoir of a range of CoVs (Cui et al., 2007). The pathogenicity of five porcine CoVs has so far been confirmed (Doyle & Hutchings, 1946; Pensaert & de Bouck, 1978; Sasseville et al., 2002; Wesley et al., 1990; Woo et al., 2012). In February 2017, a new bat-HKU-like porcine coronavirus (PEAV) was detected by genomic analysis in swine herds (Gong et al., 2017). Although PEAV has been detected in piglets with severe diarrhoea (Gong et al., 2017), little information is known regarding the pathogenicity of PEAV strains in animals. In the present study, we reported that a PEAV strain was successfully isolated from a case of piglet diarrhoea in Guangdong, and showed high pathogenicity to

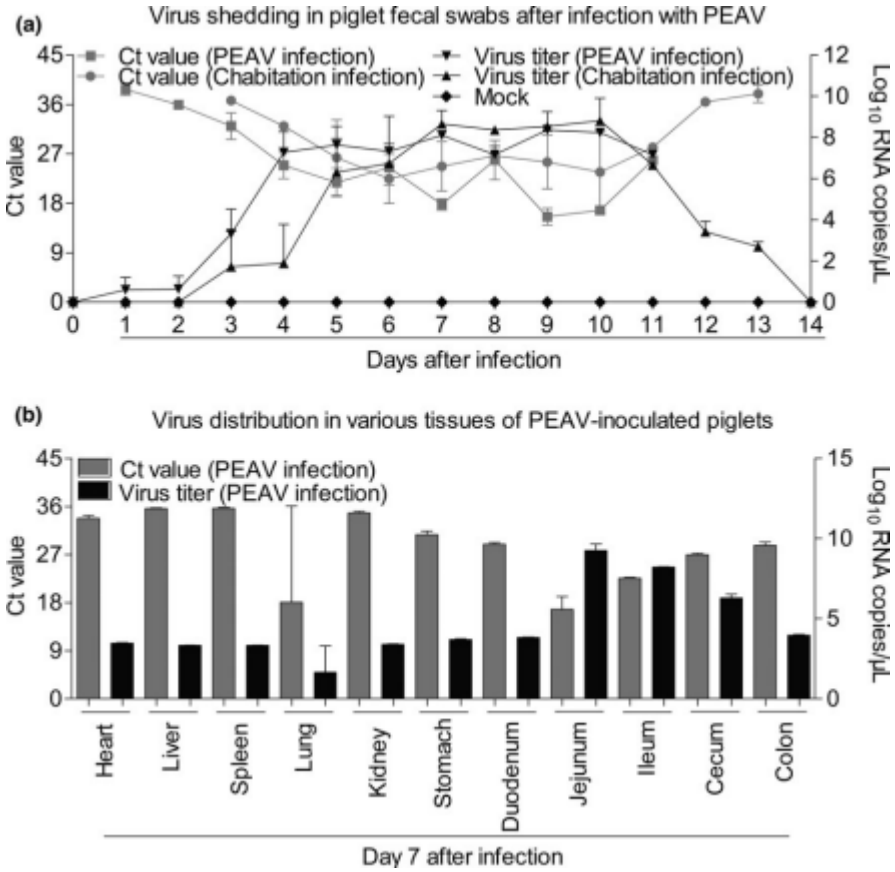


FIGURE 6 Virus shedding in rectal swabs and various tissues of PEAV-inoculated piglets. (a) Ct values of group PEAV inoculation or as cohabitation contrast newborn piglet faecal swabs and viral RNA shedding in faecal swabs after PEAV inoculation or mock inoculation. (b) Virus distribution at 7 d.p.i. in newborn piglets challenged with PEAV

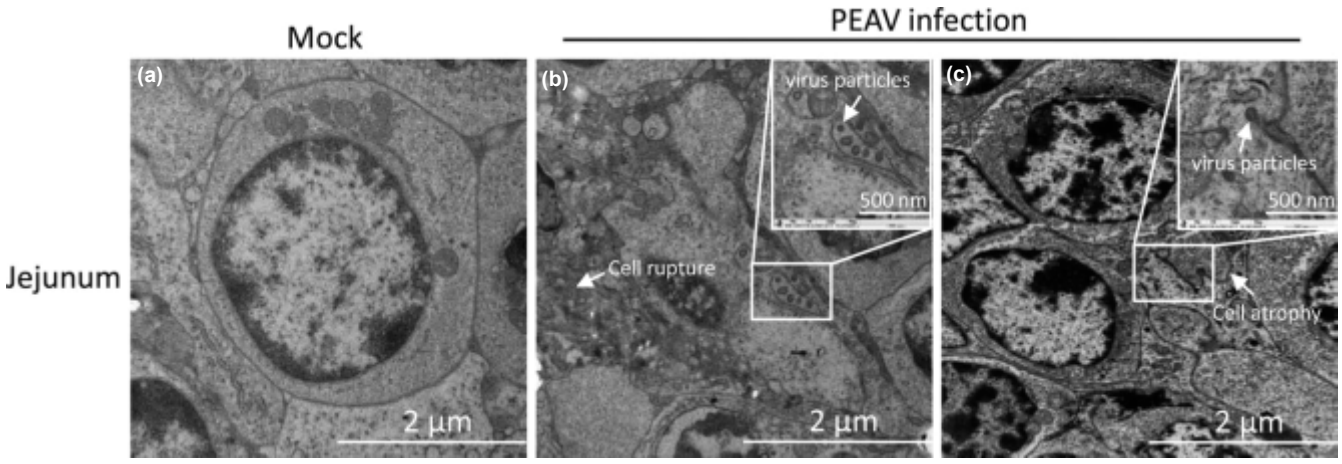


FIGURE 7 Electron micrographs of PEAV on jejunum of PEAV-inoculated newborn piglet. (a) Electron micrographs of jejunum of a control newborn piglet at 7 d.p.i. (b & c) Electron micrographs of PEAV (indicated by arrows) on jejunum of a PEAV-challenged newborn piglets at 7 d.p.i

newborn piglets. This PEAV strain can be further used for virological and serological assay development, as well as vaccine development.

A stable African green monkey kidney cell line (Vero cells) (Rhim, Schell, Creasy, & Case, 1969) is commonly used to isolate CoVs like PEDV or bat coronavirus HKU2 (Lau et al., 2007; Lee et al., 2015). Since PEAV was first detected by genomic analysis in pigs (Gong et al., 2017), we attempted to isolate virus from PEAV-positive samples using Vero cells. The virus could only be isolated in circumstance of inoculation with fresh homogenate in piglets. The difficulty to isolate PEAV from positive samples might be associated with the fact that positive samples characterized by RT-PCR may contain noninfectious or low amount of virus. During virus isolation, the DMEM supplemented with 10 μg/ml trypsin performed better than 7 μg/ml in PEAV propagation with more evident CPE, which indicates that the amount of trypsin in Vero cells might also contribute to the successful PEAV isolation. CPE was firstly observed in inoculated Vero cells until the passage 10. After plaque purification and several passages, the viral titre reached 5.13×10^5 $TCID_{50}$/ml, showing that the PEAV GDS04 strain was highly replicative in Vero cells. The plaque-purified PEAV strain in Vero cells was further verified by IFA with PEAV-specific mouse antisera. The characteristic crown-like particles of the purified PEAV GDS04 strain was observed by EM. Although there were many PEAV-positive samples by genomic analysis, only GDS04 was isolated, indicating that the success rate of isolation PEAV strains was very low. Thus, further attempts are needed to improve PEAV isolation.

To determine the complete genome of P12 of GDS04 strain, we amplified and sequenced the complete genome of PEAV GDS04 P12 by RT-PCR. We found that PEAV GDS04 P12 strain (accession no. MH697599) shares 99.79% nucleotide identity with PEAV GDS04 strain (accession no. MF167434), and compared to the complete genome of the virus in the original small intestinal homogenate, the PEAV GDS04 P12 strain possesses 35 point mutations, a 1-nt deletion (A) in nt 77, a 3-nt deletion (GTA) in nt 24,790–24,792, a 1-nt deletion (A) in nt 27,071, a 1-nt deletion (T) in nt 27,081, a 1-nt deletion (A) in nt 27,089, a 3-nt insertion (TTG) in nt 4,554–4,556, 10-nt insertion (GACTAGAGCC) in nt 12483-12492, indicating that these mutations might be related to cellular adaptation. Based on the phylogenetic tree analysis of s genes, the PEAV strain GDS04 shares 36.23%–99.66% nucleotide identity with other 18 CoVs in GenBank. Notably, the PEAV GDS04 strains can be clustered into one clade with four bat coronavirus HKU strains from Hong Kong, a BtRf-AlphaCoV strain from Beijing, SADS-CoV strain from Guangdong, and SeACoV strain from Guangdong, indicating a close relationship of these strains. In addition, the PEAV GDS04 strains, SADS-CoV, and SeACoV clustered into a subclade, indicating that these three viruses might have similar origins. Consistent with the results of the s genes, phylogenetic analysis of the n genes and whole-genome of the PEAV GDS04 and other CoVs revealed that PEAV GDS04, SADS-CoV, and SeACoV belong to the same subclade. Previous studies have shown that the s gene, the most variable region in the CoV genomes, belongs to type I membrane glycoproteins family (Woo et al., 2010) and is involved in receptor binding and viral entry (Woo et al., 2010). Sequencing of s genes revealed that GDS04 had the smallest S protein among all coronaviruses (Gong et al., 2017). Compared to the s gene of the SeACoV and SADS-CoV strains, the PEAV GDS04 possesses 11 point mutations, 3 point mutations with SeACoV and SADS-CoV, respectively. Whether these unique variations contribute to the efficiency of viral replication and virulence needs to be further investigated.

We further investigated whether the PEAV GDS04 strain was responsible for causing clinical symptoms as severe diarrhoea and death in piglets. As a result, we infected 5-day-old newborn piglets with the PEAV GDS04 strain P12 via oral feeding. The sequential severe diarrhoea and vomiting in piglets by oral infection strongly suggest the pathogenicity of PEAV to the newborn piglets. Furthermore, PEAV RNA was detected from 1 to 11 d.p.i. in faecal of GDS04 challenged piglets, while no RNA was detected in the negative control piglets. Four piglets from group 2 as cohabitation contrast also developed profuse watery diarrhoea and viral shedding, suggesting the possibility of faecal–oral transmission of PEAV. Results from this study may help guide future PEAV experimental designs as the clinical diarrhoea and virus shedding patterns.

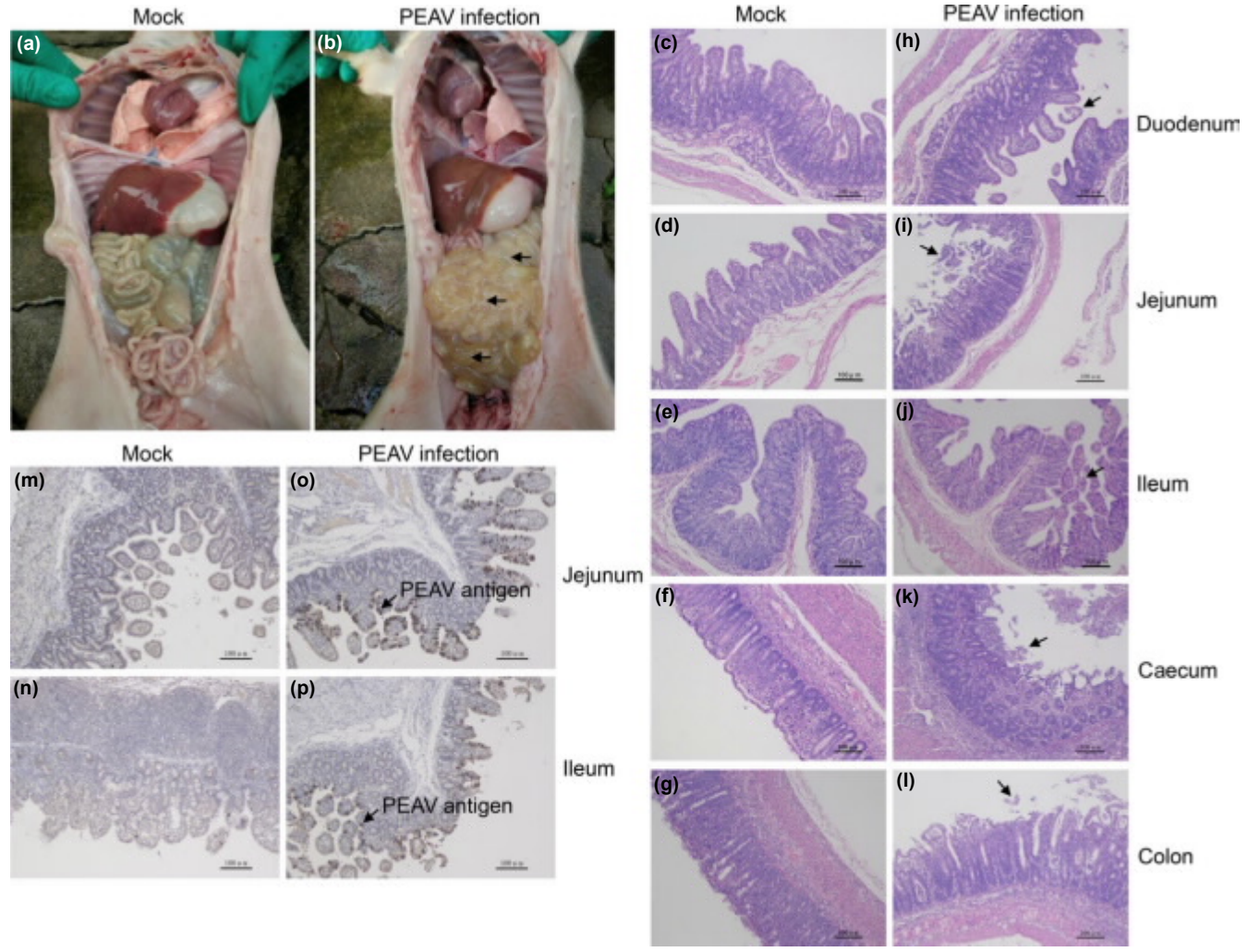


FIGURE 8 Intestinal changes in newborn piglets inoculated with PEAV strain GDS04 P12. (a) Macroscopic picture of a control piglet at 7 d.p.i. (b) Thin-walled intestinal tract (indicated by arrows) of a PEAV-challenged newborn piglets at 7 d.p.i. (c-g) Haematoxylin and eosin (H & E)-stained intestinal tissue section of a control piglet at 7 d.p.i. (h-l) H&E-stained intestinal tissue section of a PEAV-challenged piglet at 7 d.p.i. (Blunt intestinal villus was indicated by arrows). (m&n) Immunohistochemically stained jejunum or ileum tissue section of a control piglet at 7 d.p.i. (o&p) Immunohistochemically stained jejunum or ileum tissue section of a PEAV-challenged piglet at 7 d.p.i [Colour figure can be viewed at wileyonlinelibrary.com]

In addition, the viral RNA distribution in the PEAV-challenged piglets was also tested. The intestines contained higher levels of viral RNA copies compared with the other tissues, and no PEAV viral RNA was detected in the blood, indicating that PEAV might infect multiple organs in pigs, but the intestinal tract is the major target organ of PEAV. Furthermore, we confirmed that PEAV could replicate in pig intestines by EM. These results suggested that PEAV has similar infection process with other porcine CoVs like PEDV or PDCoV. Gross lesions by virus infection were obviously observed in the small intestines, caeca and colons of the 5-day-old piglets at necropsy at 7 d.p.i., similar to observations in PEDV or PDCoV infection (Chen et al., 2015; Lee et al., 2015). While microscopic lesions were observed in the jejunum and ileum infected by PDCoV in previous report (Chen et al., 2015), we found microscopic lesions distributed in the whole intestinal tract in GDS04 infected piglets, suggesting a more deteriorative effect by PEAV than that caused by PDCoV. What is more, no microscopic lesions were observed in any other organs of the PEAV-challenged piglets (data not shown), similar to observations in PDCoV infection (Chen et al., 2015). Consistent with the histopathological results, the PEAV antigen was detected in the cytoplasm of the villous enterocytes of challenged piglets by immunohistochemical analysis. This information is useful for choosing appropriate tissues for PEAV diagnostic investigations.

Since PEAV GDS04 caused severe watery diarrhoea in newborn piglets, we also recorded the mortality of newborn piglets from 1 to 14 d.p.i.. The pathogenicity of a SeACoV strain CH/GD/01/2017/P2 and SADS-CoV in neonatal piglets were described (Pan et al., 2017; Zhou et al., 2018). Results showed that SeACoV and SADS-CoV caused severe diarrhoea and vomiting in pigs of 3 days old. However, SeACoV infection did not cause fatality in piglets in 5 days (Pan et al., 2017), while 50% died after SADS-CoV infection within the same period (Zhou et al., 2018). When infected with PEAV

GDS04, one piglet died in dpi 5, and all PEAV-inoculated piglets and half piglets from cohabitation contrast died in 2 weeks, indicating that PEAV GDS04 strain and SADS-CoV were more pathogenic than SeACoV CH/GD/01/2017/P2. In addition, compared to the complete genome of the SeACoV CH/GD/01/2017/P2, the PEAV GDS04 P12 possesses82 point mutations, a 1-nt deletion (A) in nt 177, 10-nt insertion (GACTAGAGCC)in nt 12483-nt 12492, a 1-nt insertion (A) in nt 27068, a 1-nt insertion (T) in nt 27078, and a 1-nt insertion (A) in nt 27086, presenting a clue of different pathogenicity of the two strains. And compared to the complete genome of the SADS-CoV, the PEAV GDS04 P12 possesses41 point mutations, a 1-nt deletion (A) in nt 176, a 1-nt insertion (A) in nt 27067, a 1-nt insertion (T) in nt 27077, and a 1-nt insertion (A) in nt 27085. In addition, the PEAV strain GDS04 P12 shares 99.58%, 99.66% nucleotide identity with SeACoV and SADS-CoV, respectively, indicating that PEAV has more similarities with SADS-CoV than SeACoV. The details of genome comparison might also help explain the high mortality rate as high as 90% in piglets in PEAV-reported swine herds (Zhou et al., 2018). Together, all these results confirm that the PEAV GDS04 strain isolated in this study could cause enteric diseases and death in newborn piglets. However, there are still several important questions needed to be addressed. For instance, what is the prevalence of PEAV as a new CoV in herds? What are the molecular mechanisms of pathogenesis of PEAV infection? How to prepare effective vaccines against the PEAV? Elucidation of these questions will elevate our understandings of the pathogenicity of PEAV infection and help to develop better strategies to control PEAV.

In summary, we isolated a field strain of PEAV from the intestinal content of an inoculated-newborn piglet with fresh excreta from ill newborn piglets. Genomic analysis shows that the isolate manifests close relationship with SADS-CoV and SeACoV apart from several unique genetic characteristics. Remarkably, inoculation of newborn piglets with PEAV GDS04 P12 by oral feeding reproduced clinical symptoms, including vomiting, dehydration, and severe diarrhoea with a mortality of 100% in neonatal piglets. Collectively, these findings suggest that PEAV GDS04 is highly virulent in piglets.

5 | IMPORTANCE

In this work, a PEAV strain GDS04 was successfully isolated from a case of piglet diarrhoea in Guangdong, China. Newborn piglets orally challenged with PEAV GDS04 developed typical clinical symptoms as watery diarrhoea and high mortality, confirming PEAV is potential role of important pathogen in newborn piglets. The isolation and characterization would lay solid foundation for understanding this virus and the strain GDS04 can be further used for virological and serological assays, as well as vaccine development.

AUTHOR CONTRIBUTIONS

YC and ZX conceived and designed the experiments; ZX, YL, LH, and QZ performed the experiments; ZX analysed the data; YC, YZ, LG, LH, YL, JQ, YD, and QZ contributed reagents/materials/analysis tools; ZX and YZ wrote the paper; YC and CX revised the paper.

ACKNOWLEDGEMENTS

This work was supported by the National Key Research and Development Program (2016YFD0500101), National Natural Science Foundation (31741118), and Guangdong Natural Science Foundation (2018B030314003).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

The animal study was supervised by the Institutional Animal Care and Use Committee of Sun Yat-sen University (IACUC DD-17-1003) and used in accordance with regulation and guidelines of this committee.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

ORCID

ZhichaoXu http://orcid.org/0000-0002-5697-5734
YongchangCao http://orcid.org/0000-0002-0311-5474

REFERENCES

Alsaad, K. O., Hajeer, A. H., Al Balwi, M., Al Moaiqel, M., Al Oudah, N., Al Ajlan, A., … Arabi, Y. M. (2018). Histopathology of Middle East respiratory syndrome coronovirus (MERS-CoV) infection - clinicopathological and ultrastructural study. Histopathology 72, 516–524. https://doi.org/10.1111/his.13379

Chen, Q., Gauger, P., Stafne, M., Thomas, J., Arruda, P., Burrough, E., … Zhang, J. (2015). Pathogenicity and pathogenesis of a United States porcine deltacoronavirus cell culture isolate in 5-day-old neonatal piglets. Virology, 482, 51–59. https://doi.org/10.1016/j.virol.2015.03.024

Cruz, D. J., Kim, C. J., & Shin, H. J. (2008). The GPRLQPY motif located at the carboxy-terminal of the spike protein induces antibodies that neutralize Porcine epidemic diarrhea virus. Virus research, 132, 192–196. https://doi.org/10.1016/j.virusres.2007.10.015

Cui, J., Han, N., Streicker, D., Li, G., Tang, X., Shi, Z., … Zhang, S. (2007). Evolutionary Relationships between bat coronaviruses and their hosts. Emerging Infectious Diseases, 13, 10.

Dong, N., Fang, L., Yang, H., Liu, H., Du, T., Fang, P., … Xiao, S. (2016). Isolation, genomic characterization, and pathogenicity of a Chinese porcine deltacoronavirus strain CHN-HN-2014. Veterinary microbiology, 196, 98–106. https://doi.org/10.1016/j.vetmic.2016.10.022

Doyle, L. P., & Hutchings, L. M. (1946). A transmissible gastroenteritis in pigs. Journal of the American Veterinary Medical Association, 108, 257–259.

Felippe, P. A., da Silva, L. H., Santos, M. M., Spilki, F. R., & Arns, C. W. (2010). Genetic diversity of avian infectious bronchitis virus isolated from domestic chicken flocks and coronaviruses from feral pigeons in Brazil between 2003 and 2009. Avian diseases, 54, 1191–1196. https://doi.org/10.1637/9371-041510-Reg.1

Gong, L., Li, J., Zhou, Q., Xu, Z., Chen, L., Zhang, Y., … Cao, Y. (2017). A new bat-HKU2-like coronavirus in Swine, China, 2017. Emerging InfectiousDiseases, 23, 9.

Hu, H., Jung, K., Vlasova, A. N., Chepngeno, J., Lu, Z., Wang, Q., & Saif, L. J. (2015). Isolation and characterization of porcine deltacoronavirus from pigs with diarrhea in the United States. Journal of clinical microbiology, 53, 1537–1548. https://doi.org/10.1128/JCM.00031-15

Hu, H., Jung, K., Vlasova, A. N., & Saif, L. J. (2016). Experimental infection of gnotobiotic pigs with the cell-culture-adapted porcine deltacoronavirus strain OH-FD22. Archives of virology, 161, 3421–3434. https://doi.org/10.1007/s00705-016-3056-8

Hu, X. Jr, Li, N. Jr, Tian, Z. Jr, Yin, X. Jr, Qu, L., & Qu, J. (2015). Molecular characterization and phylogenetic analysis of transmissible gastroenteritis virus HX strain isolated from China. BMC veterinary research, 11, 72. https://doi.org/10.1186/s12917-015-0387-8

Jeong, Y. J., Park, S. I., Hosmillo, M., Shin, D. J., Chun, Y. H., Kim, H. J., … Cho, K. O. (2009). Detection and molecular characterization of porcine group C rotaviruses in South Korea. Veterinary microbiology 138, 217–224. https://doi.org/10.1016/j.vetmic.2009.03.024

Kong, Q., Xue, C., Ren, X., Zhang, C., Li, L., Shu, D., … Cao, Y. (2010). Proteomic analysis of purified coronavirus infectious bronchitis virus particles. Proteome Science, 8, 29. https://doi.org/10.1186/1477-5956-8-29

Larson, H. E., Reed, S. E., & Tyrrell, D. A. (1980). Isolation of rhinoviruses and coronaviruses from 38 colds in adults. Journal of Medical Virology, 5, 221–229. https://doi.org/10.1002/(ISSN)1096-9071

Lau, S. K., Woo, P. C., Li, K. S., Huang, Y., Wang, M., Lam, C. S., … Yuen, K. Y. (2007). Complete genome sequence of bat coronavirus HKU2 from Chinese horseshoe bats revealed a much smaller spike gene with a different evolutionary lineage from the rest of the genome. Virology, 367, 428–439. https://doi.org/10.1016/j.virol.2007.06.009

Lee, S., Kim, Y., & Lee, C. (2015). Isolation and characterization of a Korean porcine epidemic diarrhea virus strain KNU-141112. Virus research, 208, 215–224. https://doi.org/10.1016/j.virusres.2015.07.010

Oka, T., Saif, L. J., Marthaler, D., Esseili, M. A., Meulia, T., Lin, C. M., … Wang, Q. (2014). Cell culture isolation and sequence analysis of genetically diverse US porcine epidemic diarrhea virus strains including a novel strain with a large deletion in the spike gene. Veterinary microbiology 173, 258–269. https://doi.org/10.1016/j.vetmic.2014.08.012

Pan, Y., Tian, X., Li, W., Zhou, Q., Wang, D., Bi, Y., … Song, Y. (2012). Isolation and characterization of a variant porcine epidemic diarrhea virus in China. Virology Journal, 9, 195. https://doi.org/10.1186/1743-422X-9-195

Pan, Y., Tian, X., Qin, P., Wang, B., Zhao, P., Yang, Y. L., … Huang, Y. W. (2017). Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. Veterinary microbiology 211, 15–21. https://doi.org/10.1016/j.vetmic.2017.09.020

Pensaert, M. B., & de Bouck, P. (1978). A new coronavirus-like particle associated with diarrhea in swine. Archives of virology, 58, 243–247. https://doi.org/10.1007/BF01317606

Reed, L. J. (1938). A simple method of estimating fifty per endpoints. The American Journal of Hygiene, 27, 493–497.

Rhim, J. S., Schell, K., Creasy, B., & Case, W. (1969). Biological characteristics and viral susceptibility of an African green monkey kidney cell line (Vero). Proceedings of the Society for Experimental Biology and Medicine, 132, 670–678. https://doi.org/10.3181/00379727-132-34285

Rihtaric, D., Hostnik, P., Steyer, A., Grom, J., & Toplak, I. (2010). Identification of SARS-like coronaviruses in horseshoe bats (Rhinolophus hipposideros) in Slovenia. Archives of virology, 155, 507–514. https://doi.org/10.1007/s00705-010-0612-5

Saeng-Chuto, K., Lorsirigool, A., Temeeyasen, G., Vui, D. T., Stott, C. J., Madapong, A., … Nilubol, D. (2017). Different lineage of porcine deltacoronavirus in Thailand, Vietnam and Lao PDR in 2015. Transboundary and Emerging Diseases, 64, 3–10. https://doi.org/10.1111/tbed.12585

Sasseville, A. M., Boutin, M., Gelinas, A. M., & Dea, S. (2002). Sequence of the 3'-terminal end (8.1 kb) of the genome of porcine haemagglutinating encephalomyelitis virus: Comparison with other haemagglutinating coronaviruses. Journal of General Virology, 83, 2411–2416. https://doi.org/10.1099/0022-1317-83-10-2411

Stohlman, S. A., Brayton, P. R., Fleming, J. O., Weiner, L. P., & Lai, M. M. (1982). Murine coronaviruses: Isolation and characterization of two plaque morphology variants of the JHM neurotropic strain. Journals of General Virology, 63, 265–275. https://doi.org/10.1099/0022-1317-63-2-265

Tsunemitsu, H., el-Kanawait, Z. R., Smith, D. R., Reed, H. H., & Saif, L. J. (1995). Isolation of coronaviruses antigenically indistinguishable from bovine coronavirus from wild ruminants with diarrhea. Journal of clinical microbiology 33, 3264–3269.

Wesley, R. D., Woods, R. D., Hill, H. T., & Biwer, J. D. (1990). Evidence for a porcine respiratory coronavirus, antigenically similar to transmissible gastroenteritis virus, in the United States. Journal of Veterinary Diagnostic Investigation, 2, 312–317. https://doi.org/10.1177/104063879000200411

Woo, P. C., Huang, Y., Lau, S. K., & Yuen, K. Y. (2010). Coronavirus genomics and bioinformatics analysis. Viruses, 2, 1804–1820. https://doi.org/10.3390/v2081803

Woo, P. C., Lau, S. K., Lam, C. S., Lau, C. C., Tsang, A. K., Lau, J. H., … Yuen, K. Y. (2012). Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. Journal of virology, 86, 3995–4008. https://doi.org/10.1128/JVI.06540-11

Zhang, J. (2016). Porcine deltacoronavirus: Overview of infection dynamics, diagnostic methods, prevalence and genetic evolution. Virus research, 226, 71–84. https://doi.org/10.1016/j.virusres.2016.05.028

Zhou, P., Fan, H., Lan, T., Yang, X. L., Shi, W. F., Zhang, W., … Ma, J. Y. (2018). Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. Nature, 556, 255–258. https://doi.org/10.1038/s41586-018-0010-9

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Xu Z, Zhang Y, Gong L, et al. Isolation and characterization of a highly pathogenic strain of Porcine enteric alphacoronavirus causing watery diarrhoea and high mortality in newborn piglets. Transbound Emerg Dis. 2019;66:119–130. https://doi.org/10.1111/tbed.12992

AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Resource Announcements

GENOMESEQUENCES

Complete Genome Sequence of a Novel Swine Acute Diarrhea Syndrome Coronavirus, CH/FJWT/2018, Isolated in Fujian, China, in 2018

Kai Li,[a,b] Hao Li,[a,b] Zhen Bi,[a,b] Jun Gu,[a,b] Wang Gong,[a,b] Suxian Luo,[a,b] Fanfan Zhang,[a,b] Deping Song,[a,b] Yu Ye,[a,b] Yuxin Tang[a,b]

[a]Key Laboratory for Animal Health of Jiangxi Province, Nanchang, Jiangxi, China

[b]Department of Preventive Veterinary Medicine, College of Animal Science and Technology, Jiangxi Agricultural University, Nanchang, Jiangxi, China

ABSTRACT The full-length genome sequence of a novel swine acute diarrhea syndrome coronavirus (SADS-CoV), CH/FJWT/2018, was determined, which was genetically most closely related to CN/GDWT/2017, recently discovered in Fujian, China. The indel sites of the spike (S) gene of CH/FJWT/2018 were most similar to those of bat-origin SADS-related coronaviruses.

Swine acute diarrhea syndrome coronavirus (SADS-CoV) belongs to the genus *Alphacoronavirus* in the family *Coronaviridae* and is an enveloped, positive-sense, single-stranded RNA virus (1). In January 2017, SADS-CoV was first discovered in suckling piglets with severe enteritis in South China after ruling out the presence of porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine deltacoronavirus (PDCoV), and other known pathogens associated with piglet diarrhea (2–8). SADS-CoV had 95% nucleotide (nt) identity at the full-genome level with the previously reported bat-HKU2 strains (3, 4).

In this study, fecal and small intestinal samples (*n* ffi 168) collected from diarrheal piglets from seven farms in Fujian Province, China, in 2018 were tested for the presence of SADS-CoV based on reverse transcription-PCR (RT-PCR), as described previously (6). A SADS-CoV, designated CH/FJWT/2018, was identified, and this virus was isolated in Vero cell cultures according to the established protocol in the Key Laboratory for Animal Health of Jiangxi Province. To determine the complete genome sequence of CH/FJWT/2018, total RNA was extracted with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions, and 36 sets of primers covering the complete genome of CH/FJWT/2018 were synthesized and then employed for RT-PCR. Each amplicon, including the 5= and 3= ends amplified by a Clontech SMARTer rapid amplification of cDNA ends (RACE) 5=/3= kit (TaKaRa, Japan), was sequenced with the Sanger sequencing method (Sangon Biotech, Ltd., China). The full-length genome sequence was then assembled and analyzed by Lasergene v7.10 (DNAStar, Inc., USA).

The complete genome sequence of CH/FJWT/2018 determined was 27,169 nt in length, excluding the 3= poly(A) tail. The genomic arrangement and corresponding nucleotide positions were as follows: open reading frame 1a (ORF1a), nucleotides 304 to 12456; ORF1b, nucleotides 12456 to 20489; spike gene, nucleotides 20486 to 23881; ORF3, nucleotides 23881 to 24570; envelope gene, nucleotides 24551 to 24778; membrane gene, nucleotides 24787 to 25476; and nucleocapsid gene, nucleotides 25488 to 26615. The results of a phylogenetic analysis using Molecular Evolutionary Genetics Analysis (MEGA) v7.0 (9), based on CH/FJWT/2018 and other reference sequences of SADS-CoVs retrieved from GenBank, showed that CH/FJWT/2018 had 99.6% nt identity with CN/GDWT/2017 (GenBank accession number MG557844), a swine-origin SADS-

Received 19 September 2018 **Accepted** 5 November 2018 **Published** 5 December 2018

Citation Li K, Li H, Bi Z, Gu J, Gong W, Luo S, Zhang F, Song D, Ye Y, Tang Y. 2018. Complete genome sequence of a novel swine acute diarrhea syndrome coronavirus, CH/FJWT/2018, isolated in Fujian, China, in 2018. Microbiol Resour Announc 7:e01259-18. https://doi.org/10.1128/MRA.01259-18.

Editor Jelle Matthijnssens, KU Leuven

Copyright © 2018 Li et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Address correspondence to Yu Ye, yy6157832@163.com or Yuxin Tang, tang53ster@gmail.com.

K.L. and H.L. contributed equally to this work.

Downloaded from http://mra.asm.org/ on October 24, 2019 at Columbia University Libraries

CoV, and 98.9% nt identity with bat CoV HKU2 (EF203064) (10, 11), respectively. The complete genome sequence of CH/FJWT/2018 is 3 nt longer than that of HKU2. The mutations that occurred included a 3-nt (TTG) insertion between nucleotides 4554 and 4555 in the ORF1a gene, a 6-nt (GGCCTC) insertion between nucleotides 20504 and 20505, a 3-nt (GGC) deletion between nucleotides 22463 and 22465 in the spike (S) gene, and a 3-nt (GTA) deletion between nt 24773 and 24775 in the membrane (M) gene. Interestingly, the insertion/deletion patterns of CH/FJWT/2018 were more similar to those of bat-origin SADS-related coronaviruses (SADSr-CoVs) (6) than to those of swine-origin SADS-CoVs, suggesting that CH/FJWT/2018 might originate from bats. The findings determined in this study suggest that CH/FJWT/2018 was a novel SADS-CoV, and further study is required to address its biological properties, etiologies, and pathogenesis.

Data availability. The CH/FJWT/2018 sequence data have been deposited in GenBank under the accession number MH615810.

ACKNOWLEDGMENTS

This study was supported by the National Key Research and Development Program (grant 2017YFD0500600), the Foundation of the Educational Commission of Jiangxi Province (grants GJJ150388 and GJJ160399), the Natural Science Foundation of Jiangxi Province (grant 20161BAB214169), and the Graduate Innovative Foundation of Jiangxi Province (grants YC2017-B036 and YC2018-S200).

REFERENCES

1. Gong L, Li J, Zhou Q, Xu Z, Chen L, Zhang Y, Xue C, Wen Z, Cao Y. 2017. A new bat-HKU2-like coronavirus in swine, China, 2017. Emerg Infect Dis 23. https://doi.org/10.3201/eid2309.170915.
2. Wang L, Su S, Bi Y, Wong G, Gao GF. 2018. Bat-origin coronaviruses expand their host range to pigs. Trends Microbiol 26:466–470. https://doi.org/10.1016/j.tim.2018.03.001.
3. Fu X, Fang B, Liu Y, Cai M, Jun J, Ma J, Bu D, Wang L, Zhou P, Wang H, Zhang G. 2018. Newly emerged porcine enteric alphacoronavirus in southern China: identification, origin and evolutionary history analysis. Infect Genet Evol 62:179–187. https://doi.org/10.1016/j.meegid.2018.04.031.
4. Pan Y, Tian X, Qin P, Wang B, Zhao P, Yang YL, Wang L, Wang D, Song Y, Zhang X, Huang YW. 2017. Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. Vet Microbiol 211:15–21. https://doi.org/10.1016/j.vetmic.2017.09.020.
5. Zhou L, Sun Y, Wu JL, Mai KJ, Chen GH, Wu ZX, Bai Y, Li D, Zhou ZH, Cheng J, Wu RT, Zhang XB, Ma JY. 2018. Development of a TaqMan-based real-time RT-PCR assay for the detection of SADS-CoV associated with severe diarrhea disease in pigs. J Virol Methods 255:66–70. https://doi.org/10.1016/j.jviromet.2018.02.002.
6. Zhou P, Fan H, Lan T, Yang XL, Shi WF, Zhang W, Zhu Y, Zhang YW, Xie QM, Mani S, Zheng XS, Li B, Li JM, Guo H, Pei GQ, An XP, Chen JW, Zhou L, Mai KJ, Wu ZX, Li D, Anderson DE, Zhang LB, Li SY, Mi ZQ, He TT, Cong F, Guo PJ, Huang R, Luo Y, Liu XL, Chen J, Huang Y, Sun Q, Zhang XL, Wang YY, Xing SZ, Chen YS, Sun Y, Li J, Daszak P, Wang LF, Shi ZL, Tong YG, Ma JY. 2018. Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. Nature 556:255–258. https://doi.org/10.1038/s41586-018-0010-9.
7. Qin P, Li H, Wang JW, Wang B, Xie RH, Xu H, Zhao LY, Li L, Pan Y, Song Y, Huang YW. 2017. Genetic and pathogenic characterization of a novel reassortant mammalian orthoreovirus 3 (MRV3) from a diarrheic piglet and seroepidemiological survey of MRV3 in diarrheic pigs from East China. Vet Microbiol 208:126–136. https://doi.org/10.1016/j.vetmic.2017.07.021.
8. Xu Z, Zhang Y, Gong L, Huang L, Lin Y, Qin J, Du Y, Zhou Q, Xue C, Cao Y. 2018. Isolation and characterization of a highly pathogenic strain of porcine enteric alphacoronavirus causing watery diarrhea and high mortality in newborn piglets. Transbound Emerg Dis https://doi.org/10.1111/tbed.12992.
9. Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874. https://doi.org/10.1093/molbev/msw054.
10. Lau SKP, Woo PCY, Li KSM, Huang Y, Wang M, Lam CSF, Xu H, Guo R, Chan K, Zheng B, Yuen K. 2007. Complete genome sequence of bat coronavirus HKU2 from Chinese horseshoe bats revealed a much smaller spike gene with a different evolutionary lineage from the rest of the genome. Virology 367:428–439. https://doi.org/10.1016/j.virol.2007.06.009.
11. Vassilara F, Spyridaki A, Pothitos G, Deliveliotou A, Papadopoulos A. 2018. A rare case of human coronavirus 229E associated with acute respiratory distress syndrome in a healthy adult. Case Rep Infect Dis 2018:6796839. https://doi.org/10.1155/2018/6796839.

Downloaded from http://mra.asm.org/ on October 24, 2019 at Columbia University Libraries

Journal of Virological Methods 260 (2018) 45–48

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development of a real time reverse transcription loop-mediated isothermal amplification method (RT-LAMP) for detection of a novel swine acute diarrhea syndrome coronavirus (SADS-CoV)

Huanan Wang[a,1], Feng Cong[b,c,1], Fanwen Zeng[b,c,1], Yuexiao Lian[b,c], Xiangnan Liu[b,c], Manlin Luo[c,*], Pengju Guo[b,*], Jingyun Ma[d,*]

[a] Department of Veterinary Medicine, College of Animal Sciences, Zhejiang University, Hangzhou, 310058, China
[b] Guangdong Laboratory Animals Monitoring Institute and Guangdong Provincial Key Laboratory of Laboratory Animals, Guangzhou 510633, China
[c] Guangdong Provincial Key Laboratory of Zoonosis Prevention and Control, College of Veterinary Medicine, South China Agricultural University, Guangzhou 510640, China
[d] College of Animal Science, South China Agricultural University, Wushan Road 483, Tianhe District, Guangzhou 510642, China

ARTICLE INFO

Keywords:
SADS-CoV
Real-time LAMP
Diagnosis

ABSTRACT

A novel swine acute diarrhea syndrome Coronavirus (SADS-CoV) that causes severe diarrhea in suckling piglets was identified in southern China in 2017. A simple and rapid detection test was developed for this virus using real-time RT-LAMP based on the conserved N gene of the virus. The method had a detection limit of 1.0×10^1 copies/μL with no cross-reactions with classical swine fever virus, porcine and respiratory syndrome virus NA, porcine and respiratory syndrome virus EU, transmissible gastroenteritis coronavirus, foot and mouth disease virus, porcine epidemic diarrhea virus (S-INDEL and non-S-INDEL), swine influenza virus subtype H1N1, porcine circovirus type 2, senecavalley virus, porcine parvovirus, porcine deltacoronavirus and rotavirus. This method was also reproducible. Twenty of 24 clinical samples were identified as SADS-CoV RNA-positive by the real-time RT-LAMP and the results were consistent with that of the real time RT-PCR method. This new method for detecting SADS-CoV is specific and sensitive for the detection of SADS-CoV.

1. Introduction

Outbreaks of diarrhea in newborn piglets occurred on a pig farm in Guangdong province of southern China in February 2017 (Gong et al., 2017). At that time, several pathogens were tested for these cases of diarrhea, including enteric coronaviruses porcine deltacoronavirus (PDCoV), porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV). However, all samples were negative for the presence of these three pathogens (Pan et al., 2017). In September 2017, a novel swine enteric alphacoronavirus (SeACoV) also named as bat-HKU2–like porcine coronavirus (PEAV) or swine acute diarrhea syndrome coronavirus (SADS-CoV) was identified in southern China (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018a,b). The mortality rate was more than 35% in swine that were less than 10 days old and caused serious economic losses to the swine industry (Pan et al., 2017). All affected pigs showed acute vomiting and severe watery diarrhea, similar to clinical signs caused by PEDV, TGEV and PDCoV. A reliable and simple diagnostic method for the surveillance of SADS-CoV was needed to distinguish between these viral pathogens.

Loop-mediated isothermal amplification (LAMP) is a simple isothermal method for in vitro nucleic acid amplification. The only equipment needed is a constant temperature heating block or water bath. In addition, the results of the reaction can be directly observed with the naked eye when performed in the presence of a fluorescent dye (Notomi et al., 2000; Yuan et al., 2014). In addition, the dye enables the use of a fluorescent detector that can be used to follow the progress of the reaction (real-time LAMP) (Oscorbin et al., 2016).

LAMP assays have been successfully used for other pathogens (de Souza et al., 2012; Kurosaki et al., 2017; Li et al., 2012; Zhao et al., 2011). In this study, a real time RT-LAMP assay was developed for rapid diagnosis of novel SADS-CoV.

* Corresponding authors.
E-mail addresses: 710510116@qq.com (M. Luo), vetbio2016@hotmail.com (P. Guo), majy2400@scau.edu.cn (J. Ma).
[1] Contributed equally to this research.

https://doi.org/10.1016/j.jviromet.2018.06.010
Received 2 March 2018; Received in revised form 21 June 2018; Accepted 21 June 2018
Available online 21 June 2018
0166-0934/ © 2018 Elsevier B.V. All rights reserved.

2. Materials and methods

2.1. Virus strains and clinical samples

Clinical samples were collected from suckling piglets with diarrhea on commercial pig farms in Guangdong, China. All specimens were suspended in phosphate-buffered saline (PBS) and stored at -80 °C until use. The SADS-CoV virus strain was isolated from diarrheal pigs in Guangdong Province of China (Zhou et al., 2018a). Other porcine viruses were used to assess the specificity of the real-time LAMP method. Classical swine fever virus (CSFV) C strain, porcine respiratory and reproductive syndrome virus (PRRSV) JXA1-R vaccine strain (NA type), foot-and-mouth disease virus (FMDV) O/HB/HK99 vaccine strain, porcine circovirus type 2 (PCV2) LG vaccine strain and swine influenza virus H1N1 TJ vaccine strain, were obtained from Guangdong Animal Epidemic Prevention and Material Reserve Center (Guangzhou, China). Porcine epidemic diarrhea virus (PEDV) CV777 vaccine strain (non-S-INDEL), rotavirus (RV) NX vaccine virus and transmissible gastroenteritis virus (TGEV) Huadu vaccine strain were purchased from Harbin Weike Biological Technology (Harbin, China). PRRSV ZD-1 strain (EU type) was acquired from Prof. Zhijun Tian of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. PEDV GD1 strain (S-INDEL), seneca valley virus (SVV) CH-01–2015 strain and porcine deltacoronavirus (PDCoV) CHN-GD16-03 strain were stored in our lab. Porcine parvovirus (PPV) vaccine virus were obtained from Qilu Animal Health Products CO.,LTD. (Jinan, China).

2.2. RNA extraction

Frozen fecal samples were thawed and centrifuged for 10 min at 10,000 × g. Total RNA was extracted from the supernatants using a TGUide Virus DNA/RNA Kit and T-Guide instrument according to the manufacturer's instructions (Tiangen, Beijing, China). RNA was eluted into RNAse-free ddH_2O and stored at - 80 °C until use.

2.3. Design of primers for the real-time RT-LAMP

The genome sequences of SADS-CoV (Accession numbers: MF167434.1, MF370205.1, MF094681, MF094682, MF094683, MF094684, MG557844.1) were retrieved from GenBank. Based on the N gene region of SADS-CoV, six real-time LAMP primers were designed using the software http://primerexplorer.jp/e/ and synthesized by Shanghai Sangon Biotech (Shanghai, China) (GenBank: MG557844.1, Swine acute diarrhea syndrome coronavirus isolate SADS-CoV/CN/GDWT/2017). The primers included two inner primers (SADS-FIP and SADS-BIP) and two outer primers (SADS-F3 and SADS-B3) (Table 1).

2.4. Construction of plasmids containing the N gene of SADS-CoV

Reverse transcription of SADS-CoV RNA templates was performed with random primers following the manufacturer's recommendations using a One Step Prime Script RT-PCR kit (Takara, Shanghai, China). The target fragment was amplified using primers SADS-F3 and SADS-B3 (Table 1). PCR amplification from SADS-CoV N gene (1128bp) were amplified using standard PCR conditions and cloned into pGEM T easy vector (Promega, Madison, USA) between T7 RNA polymerase and SP6 polymerase promoter. The pGEM T easy plasmid was in vitro transcribed (IVT) by RiboMax Large Scale RNA production system T7 (Promega, Madison, USA) according to the manufacturer's instructions. To remove plasmid DNA, 40 units RNase-free DNase (Promega, Madison, USA) enzyme was used. Trizol LS reagent (Invitrogen, Carlsbad, CA) was used for RNA isolation according to manufacturer's instructions. The RNA concentrations were measured by UV spectrophotometry and converted into copy numbers as previously described (Parida et al., 2011). All RNA templates of SADS-CoV N gene were stored at - 80 °C until use.

Table 1
Primers used to amplify the SADS-CoV N gene.

Primers' name	Sequence	Location in genome
SADS-F3	5′- CAGCCTTCTAACTGGCACT-3′	25707bp–25726bp
SADS-B3	5′- ACAGTCAGGTCTGGTGGTAA-3′	25884bp–25903bp
SADS-FIP	5′-CGTCAACAGCGACCCAATGCA-TCCTCACGCAGATGCTC-3′	(25786bp–25806bp) (25745bp–25762bp)
SADS-BIP	5′-AACTAGCCCCACAGGTCTTGGT-AACCCAAACTGAGGTGTAG-3′	(25814bp–25835bp) (25860bp–25879bp)
SADS-LB	5′- TCGCAATCGTAACAAAGAACCT-3′	25838bp–25859bp
SADS-LF	5′- CACCCTGAATCCGTTTCCTC-3′	25766bp–25785bp

Note: outer primers (SADS-F3 and SADS-B3); inner primers (SADS-FIP and SADS-BIP).
Loop primer (LF/LB).

2.5. Development of real-time RT-LAMP assay

The real-time RT-LAMP reaction was carried out in a 25 μL volume containing 2.5 μL 10 × Buffer (B0537S, New England Biolabs, Beverley, MA, USA), 0.2 mM outer primers (OF/OB), 1.6 mM inner primers (FIP/ BIP), 0.8 mM Loop primer (LF/LB), 1 μL Bst DNA polymerase (M5038 L, New England Biolabs), 3.5 μL dNTP Mix (D7373, Beyotime Biotechnology, Beijing, China), 4 μL betaine (BCBS087V, Sigma, St. Louis, MO USA), 0.5 μL SYTO 9 green fluorescent nucleic acid stain (S34854, Invitrogen), 0.5 μL AMV Reverse Transcriptase (M510 A Promega) and 2 μL RNA template. Amplification reactions were carried out at 63 °C for 60 min and a Thermostatic Fluorescence Detector (DEAOU-308C, Diao, Guangzhou, China) was used to monitor reaction progress. Non-template control was used in each reaction.

Specificity of the method was determined using RNA templates of SADS-CoV and other swine viral RNA samples including CSFV, TGEV, FMDV, PEDV (S-INDEL and non-S-INDEL), PRRSV NA and EU, PCV2, SVV, PPV, PDCoV, RV, and SIV H1N1. Ten-fold serial dilutions of N gene RNA templates were used to calculate the analytical sensitivity and assess assay reproducibility. The method was further evaluated using 24 clinical specimens from pigs with diarrhea that had been previously evaluated by a real-time RT-PCR assay (Zhou et al., 2018a). Vero cell nucleic acid was used as a mock control. Nuclease free water was used as a negative control. All reactions were carried out in triplicate.

3. Results

3.1. Specificity, sensitivity and reproducibility

Only samples that contained SADS-CoV were positive while there was no amplification of other tested viruses (Fig. 1). This indicated that the assay was highly specific for the detection of SADS-CoV.

To evaluate RT-LAMP sensitivity, the SADS-CoV N gene was in vitro transcribed from a cloned fragment. The detection limit of this assay is 1.0×10^1 copies / μL (Fig. 2). This indicated that the real-time LAMP assay was a sensitive method for SADS-CoV detection. To investigate the reproducibility of the reaction, different concentrations of the SADS-CoV N gene RNA at 1.0×10^8, 1.0×10^7, 1.0×10^6, 1.0×10^5, 1.0×10^4, 1.0×10^3, 1.0×10^2 and 1.0×10^1 copies/μL. Our results obtained from three experiments showed intra coefficients of variation were less than 2.5% and inter coefficients of variation were less than 6.8% (Supplementary Table 1). This indicated that the method was repeatable.

3.2. Clinical sample testing

Our laboratory has previously tested 24 clinical samples from pigs with diarrheal symptoms. The real time RT-PCR tests indicated that

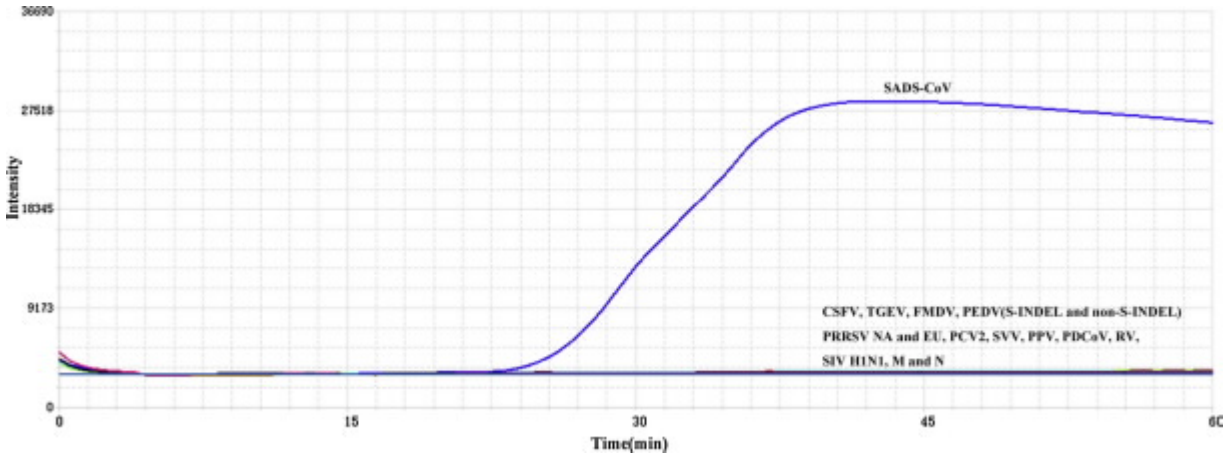


Fig. 1. Specificity of the real time RT-LAMP assay for SADV-CoV. The SADV-CoV RNA was amplified by the assay, but not other swine viruses including classical swine fever virus (CSFV), porcine and respiratory syndrome virus (PRRSV) NA and EU, transmissible gastroenteritis corona virus (TGEV), foot and mouth disease virus (FMDV), porcine epidemic diarrhea virus (PEDV) (S-INDEL and non-S-INDEL), porcine circovirus type 2 (PCV2), senecavalley virus(SVV), porcine parvovirus (PPV), porcine deltacoronavirus (PDCoV), rotavirus (RV), and swine influenza virus (H1N1). Vero cell nucleic acid was used a mock control (M). Nuclease free water was used as a negative control (N). The assay was repeated thrice.

twenty of these samples contained SADS-CoV (Zhou et al., 2018a). These 24 samples were again tested with the real-time RT-LAMP method. The real-time RT-LAMP assay could distinguish between the positive and negative samples (Fig. 3). Therefore, real-time LAMP was sufficiently accurate and reliable for the detection of SADS-CoV.

4. Discussion

SADS-CoV is a novel swine enteric alphacoronavirus identified in Guangdong province of China in 2017 (Gong et al., 2017; Pan et al., 2017). This disease has already caused huge economic losses in the Chinese swine industry. However, its detection was problematic since the disease symptoms are similar to other viruses (Jung et al., 2016; Pensaert and de Bouck, 1978; Stevenson et al., 2013). Although a TaqMan-based real-time RT-PCR assay had already been developed for SADS-CoV, this method required expensive equipment and supplies. Therefore, a simple and rapid method was needed for field studies of SADS-CoV infections. In this study, a real-time RT-LAMP assay was successfully developed using SADS-CoV N gene specific primers. The assay is suitable for SADS-CoV detection in less well-equipped laboratories.

LAMP technology is currently used for the nucleic acid detection of a number of animal pathogens (Niessen et al., 2013; Sahoo et al., 2016). The assay offers multiple advantages compared to conventional PCR including a shorter turnaround time (30–60 min) and a sensitivity 1–2 orders of magnitude higher (Huang et al., 2016; Yu et al., 2015). In the present study, the real-time RT-LAMP method was used to detect SADS-CoV and did not cross-react with other porcine pathogens. SADS-CoV N gene was specifically amplified with a detection limit of 1.0×10^1 copies/μL and had good reproducibility. The sensitivity of the LAMP assay is similar to real-time RT-PCR (Yu et al., 2015). In this study, the RT-LAMP correctly identified 20 out of 24 samples as positive, showing similar diagnostic sensitivity comparable to the real time RT-PCR. Together our data indicates that the real-time RT-LAMP assay was accurate and sensitive.

In conclusion, a novel real-time RT-LAMP method was developed for the detection of SADS-CoV in pigs. The assay was specific, sensitive and reproducible and will be helpful for the diagnosis, surveillance and pathogenesis studies of SADS-CoV infections.

Conflict of interest

Authors declare no competing interests.

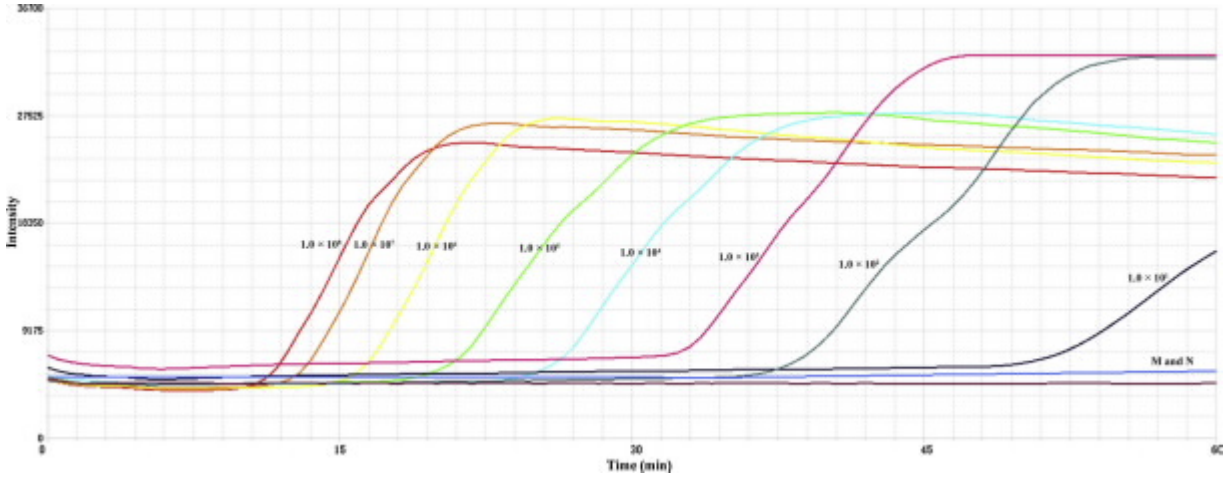


Fig. 2. Sensitivity of the real time RT-LAMP assay for SADS-CoV. Serial dilutions of in vitro transcribed copies of the cloned SADS-CoV N gene were tested in the assay. The detection limit of this assay was 1.0×10^1 copies. Vero cell nucleic acid was used a mock control (M). Nuclease free water was used as a negative control (N). The assay was repeated thrice.

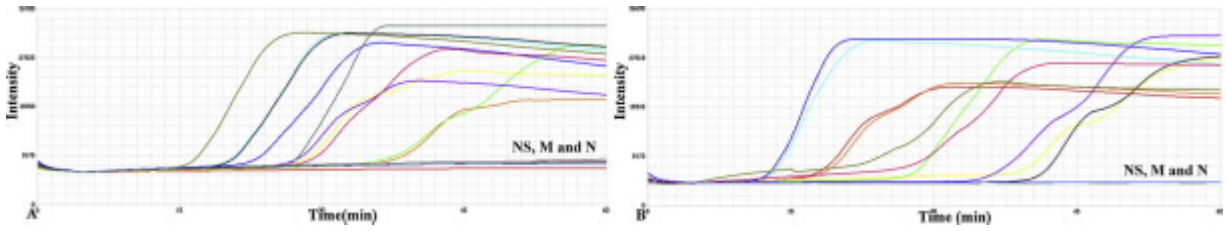


Fig. 3. Detection of the SADS-CoVRNA from clinical samples by real time RT-LAMP assay. (A) and (B), Amplification of 20 SADS-CoVpositive and 4 negative samplespreviously assayedusing real-time RT-PCRwere divided into two groups. NS, negative samples;M, mock control (vero cell nucleic acid), N, negative control (nuclease free water). Each group contained 10 positive samples, 2 negative samples, a mock control and a negative control. This assaywas repeated thrice.

Acknowledgments

The study was supported by the Science and Technology Research Program (2017A070702001, 2017B030314171, 2016A040403060 and 2016201604030059) of the Science and Technology Department of Guangdong Province and Guangzhou.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.06.010 .

References

de Souza, D.K., Quaye, C., Mosi, L., Addo, P., Boakye, D.A., 2012. A quick and cost effective method for the diagnosis of mycobacterium ulcerans infection. BMC Infect. Dis. 12, 8.

Gong, L., Li, J., Zhou, Q.F., Xu, Z.C., Chen, L., Zhang, Y., Xue, C.Y., Wen, Z.F., Cao, Y.C., 2017. A New Bat-HKU2-like coronavirus in swine, China, 2017. Emerg. Infect. Dis. 23, 1607–1609.

Huang, C., Wen, F., Yue, L., Chen, R., Zhou, W., Hu, L., Chen, M., Wang, S., 2016. Exploration of fluorescence-based real-time loop-mediated isothermal amplification (LAMP) assayfor detection of Isospora suis oocysts. Exp. Parasitol. 165, 1–6.

Jung, K., Hu, H., Saif, L.J., 2016. Porcine deltacoronavirus infection: etiology, cell culture for virus isolation and propagation, molecular epidemiology and pathogenesis.Virus Res. 226, 50–59.

Kurosaki, Y., Martins, D.B.G., Kimura, M., Catena, A.D.S., Borba, M., Mattos, S.D.S., Abe, H., Yoshikawa, R., de Lima Filho, J.L., Yasuda, J., 2017. Development and evaluation of a rapid molecular diagnostic test for Zika virus infection by reverse transcription loop-mediated isothermal amplification. Sci. Rep. 7, 13503.

Li, B., Ma, J.J., Xiao, S.B., Zhang, X.H., Wen, L.B., Mao, L., Ni, Y.X., Guo, R.L., Zhou, J.M., Lv, L.X., He, K.W., 2012. Development of a loop-mediated isothermal amplification method for rapid detection of porcine boca-like virus. J. Virol. Methods 179, 390–395.

Niessen, L., Luo, J., Denschlag, C., Vogel, R.F., 2013. The application of loop-mediated isothermal amplification (LAMP) in food testing for bacterial pathogens and fungal contaminants. Food Microbiol. 36, 191–206.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, E63.

Oscorbin, I.P., Belousova, E.A., Zakabunin, A.I., Boyarskikh, U.A., Filipenko, M.L., 2016. Comparison of fluorescent intercalating dyes for quantitative loop-mediated isothermal amplification (qLAMP). Biotechniques 61, 20–25.

Pan, Y.F., Tian, X.Y., Qin, P., Wang, B., Zhao, P.W., Yang, Y.L., Wang, L.X., Wang, D.D., Song, Y.H., Zhang, X.B., Huang, Y.W., 2017. Discovery of a novel swine enteric alphacoronavirus (SeACoV)in southern China. Vet. Microbiol. 211, 15–21.

Parida, M., Shukla, J., Sharma, S., Ranghia Santhosh, S., Ravi, V., Mani, R., Thomas, M., Khare, S., Rai, A., Kant Ratho, R., Pujari, S., Mishra, B., Lakshmana Rao, P.V., Vijayaraghavan, R., 2011. Development and evaluation of reverse transcription loop-mediated isothermal amplification assayfor rapid and real-time detection of the swine-origin influenza a H1N1 virus. J. Mol. Diagnostics JMD 13, 100–107.

Pensaert, M.B., de Bouck, P., 1978. A new coronavirus-like particle associatedwith diarrhea in swine. Arch. Virol. 58, 243–247.

Sahoo, P.R., Sethy, K., Mohapatra, S., Panda, D., 2016. Loop mediated isothermal amplification: an innovative gene amplification technique for animal diseases.Vet. World 9, 465–469.

Stevenson, G.W., Hoang, H., Schwartz, K.J., Burrough, E.R., Sun, D., Madson, D., Cooper, V.L., Pillatzki, A., Gauger, P., Schmitt, B.J., Koster, L.G., Killian, M.L., Yoon, K.J., 2013. Emergenceof porcine epidemic diarrhea virus in the United States:clinical signs, lesions, and viral genomic sequences.J. Vet. Diagn. Invest. 25, 649–654.

Yu, X.W., Shi, L., Lv, X.P., Yao, W., Cao, M.H., Yu, H.X., Wang, X.R., Zheng, S.M., 2015. Development of a real-time reverse transcription loop-mediated isothermal amplification method for the rapid detection of porcine epidemic diarrhea virus. Virol. J. 12.

Yuan, W.Z., Wang, J.C., Zheng, Y.S., Li, L.M., Zhang, X.Y., Sun, J.G., 2014. Rapid detection of encephalomyocarditis virus by one-step reverse transcription loop-mediated isothermal amplification method. Virus Res. 189, 75–78.

Zhao, K., Shi, W., Han, F.T., Xu, Y., Zhu, L.L., Zou, Y., Wu, X.A., Zhu, H., Tan, F.R., Tao, S.R., Tang, X.M., 2011. Specific, simple and rapid detection of porcine circovirus type 2 using the loop-mediated isothermal amplification method. Virol. J. 8.

Zhou, L., Sun, Y., Wu, J.L., Mai, K.J., Chen, G.H., Wu, Z.X., Bai, Y., Li, D., Zhou, Z.H., Cheng, J., Wu, R.T., Zhang, X.B., Ma, J.Y., 2018a. Development of a TaqMan-based real-time RT-PCRassayfor the detection of SADS-CoVassociatedwith severe diarrhea diseasein pigs. J. Virol. Methods 255, 66–70.

Zhou, P., Fan, H., Lan, T., Yang, X.L., Shi, W.F., Zhang, W., Zhu, Y., Zhang, Y.W., Xie, Q.M., Mani, S., Zheng, X.S., Li, B., Li, J.M., Guo, H., Pei, G.Q., An, X.P., Chen, J.W., Zhou, L., Mai, K.J., Wu, Z.X., Li, D., Anderson, D.E., Zhang, L.B., Li, S.Y., Mi, Z.Q., He, T.T., Cong, F., Guo, P.J., Huang, R., Luo, Y., Liu, X.L., Chen, J., Huang, Y., Sun, Q., Zhang, X.L.L., Wang, Y.Y., Xing, S.Z., Chen, Y.S., Sun, Y., Li, J., Daszak, P., Wang, L.F., Shi, Z.L., Tong, Y.G., Ma, J.Y., 2018b. Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. Nature 556 255-+ .

Journal of Virological Methods 255 (2018) 66–70

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development of a TaqMan-based real-time RT-PCR assay for the detection of SADS-CoV associated with severe diarrhea disease in pigs

Ling Zhou[a,b], Yuan Sun[a,b], Jiao-ling Wu[a,b], Kai-jie Mai[a,b], Gui-hua Chen[a,b], Zi-xian Wu[d], Yang Bai[a,b], Di Li[a,b], Zhi-hai Zhou[a,b], Jian Cheng[a,b], Rui-ting Wu[a,b], Xiang-bin Zhang[a,b,c,*], Jing-yun Ma[a,b,*]

[a] College of Animal Science, South China Agricultural University, Guangzhou, China
[b] Key Laboratory of Animal Health Aquaculture and Environmental Control, Guangzhou, Guangdong, China
[c] Hog Production Division, Guangdong Wen's Foodstuffs Group Co., Ltd., Xinxing, 527439, China
[d] Guangdong Wen's Foodstuff Group Co., Ltd., Yanjiang Street, Xinxing, 527400, Guangdong, China

ARTICLE INFO

Keywords:
SADS-CoV
TaqMan-based real-time RT-PCR
Diagnosis
Quantification

ABSTRACT

Swine acute diarrhea syndrome coronavirus (SADS-CoV) is a novel coronavirus which was first reported in southern China in 2017. It can cause severe diarrhea disease in pigs. In order to detect this new emerging virus rapidly and reliably, a TaqMan-based real-time RT-PCR assay was established in this study. Specific primers and probe were designed and synthesized based on the conserved region within the N gene of the viral genome. Results showed that the lowest limit of detection was 3.0×10^1 copies/μL. This approach was specific for SADS-CoV, and there were no cross-reaction observed against other 15 swine viruses. It was 10 times more sensitive than the conventional PCR and gave higher SADS-CoV positive detection rate (70.69%, 123/174) than the conventional PCR (51.15%, 89/174) from clinical samples. These data indicated that the TaqMan-based real-time RT-PCR assay established here was an effective method with high sensitivity, specificity and reproducibility for faster and more accurate detection and quantification of SADS-CoV.

1. Introduction

Swine acute diarrhea syndrome coronavirus (SADS-CoV) is a newly discovered virus, which is an enveloped, positive and single-stranded sense RNA virus and belongs to the order Nidovirales, family Coronaviridae and genus Alphacoronavirus (Zhou et al., 2018; Pan et al., 2017; Woo et al., 2012). SADS-CoV can cause severe and acute diarrhea and rapid weight loss in piglets less than 6-day-old. From January 2017 to May, the outbreak of SADS-CoV has led to the death of almost 25,000 piglets in southern China and resulted in significant economic losses (data unpublished). Considering the clinical symptoms of this new emerging virus that are similar to those caused by other known swine enteric coronaviruese in China, there is an urgent need to establish a faster and reliable diagnostic technique for identification and quantification of SADS-CoV (Dong et al., 2015; Sun et al., 2016).

The real-time PCR using the fluorogenic probe system has been widely used to quantify virus nucleic acids in recent years (Li et al., 2011; Gava et al., 2015; Niu et al., 2016; Dall Agnol et al., 2017). Compared to the conventional PCR, it allows the accurate and reproducible quantitation of viral gene copies, and has advantages in rapidity, accuracy, reproducibility, and high sensitivity (Li et al., 2016; Fowler et al., 2016). So far, there is no diagnostic method have been established for the detection of SADS-CoV. In this study, a TaqMan-based real-time RT-PCR was designed for SADS-CoV diagnosis and quantification in clinical swine tissue samples.

2. Material and methods

2.1. Virus and samples collection

SADS-CoV virus used in this study was isolated from clinical samples with diarrheal symptoms from pig herds in Guangdong Province of China. Specimens from piglets with signs of severe diarrhea and high mortality were collected from February to August 2017 in four SADS-CoV positive farms.

2.2. Nucleic acid extraction and reverse transcription

Specimens were homogenized in phosphate-buffered saline (PBS) (20% w/v), and frozen and thawed three times, and then centrifuged

* Corresponding authors at: College of Animal Science, South China Agricultural University, Tianhe District, Wushan Road 483, Guangzhou 510642, China.
E-mail addresses: Zhangxb@scau.edu.cn (X.-b. Zhang), majy2400@scau.edu.cn (J.-y. Ma).

https://doi.org/10.1016/j.jviromet.2018.02.002
Received 12 November 2017; Received in revised form 21 January 2018; Accepted 2 February 2018
Available online 07 February 2018
0166-0934/ © 2018 Elsevier B.V. All rights reserved.

for 10 min at 10,000 × g. Viral nucleic acid was extracted following the manufacturer's recommendations of AxyPrep™ Body Fluid Viral DNA/RNA Miniprep Kit (Axygen Scientific, Inc). Reverse transcription was carried out according to the manufacturer's protocol of Prime Script™ RT Reagent Kit with gDNA Eraser (Takara, Dalian, China), the first step was performed in a total volume of 10 μL of a mixture containing 2 μL 5 × gDNA Eraser Buffer, 1 μL gDNA Eraser, 1 μL total RNA and 6 μL RNase Free dH2O at the condition of 42 ℃ for 2 min to remove residual genomic DNA, and then, ten microliters of mixture was used in a reaction volume of 20 μL containing 1 μL PrimeScript RT Enzyme Mix I, 1 μL RT Primer Mix, 4 μL 5 × PrimeScript Buffer 2, 4 μL RNase Free dH_2O, reactions were conducted with a program that included 37 °C for 15 min, 87 °C for 5 s. All DNA and cDNA templates were stored at - 80 °C until use.

2.3. Design of primers and probe for the real-time RT-PCR

The genome sequences of SADS-CoV (Accession number: MF094681-MF094684) were retrieved from GenBank. Conserved regions of the N gene were identified by nucleotide sequence alignments with MegAlign7.1.0 (DNAStar, USA). Primers and probe were selected and designed using Primer premers 5.0 to generate a 449 bp amplicon for plasmid and 155 bp real-time RT-PCR product. The probe was labeled with 5-carboxyflourescein(FAM) at the 5′-end and N, N, N' N'-tetramethyyl-6-carboxyrhodamine(TAMRA) at the 3′-end. Nucleotide information about primers and probe are summarized in Table 1.

2.4. Construction of standard plasmids DNA for the real-time RT-PCR

The target fragment was obtained with SADS-CoV-N-F/R by PCR and then cloned into pMD19-T vector (TaKaRa, Dalian, China). PCR product was transformed into DH5α Escherichia coli cells following the manufacture's recommendations (TaKaRa, Dalian, China) and purified by Plasmid Mini Kit 2 (Omega Bio-tek, USA). The positive recombinant plasmid was confirmed by sequencing (BGI, Shenzhen, China). Subsequently, the SADS-CoV plasmid (pSADS-CoV-N) was quantified by spectrophotometric analysis (NanoDrop ND-1000) and converted into copy number. The plasmid DNA was stored at - 80 ℃ in aliquots until use.

2.5. TaqMan-based real-time RT-PCR assay

The optimization of the real-time RT-PCR reaction components and cycling conditions was performed using DNA standards. Concentrations of primers, probe and cDNA were selected based on the greatest fluorescence obtained and the lowest threshold cycle. The optimized real-time RT-PCR assays were carried out in 20 μL reaction mixture containing 10 μL of Premix Ex Taq (Probe qPCR) (2×), 0.8 μL of qSADS-CoV-N-F (10 μM), 0.8 μL of qSADS-CoV-N-R (10 μM), 0.8 μL (10 μM) of probe, 1 μL of cDNA template, and 6.6 μL of ddH_2O. The PCR amplification was performed with a CFX96 Real-Time PCR Detection System (BIO-RAD) under the following conditions: 95 ℃ for 30 s for initial denaturation followed by 45 cycles of 95 ℃ for 5 s and 62 ℃ for 30 s. Ten-fold serial dilutions of pSADS-CoV-N, ranging from 10^9 to 10^2 copies/μL, were tested in five replicates with real-time RT-PCR to generate the standard curve.

Table 1
Primers and probe for SADS-CoV detection.

primers	Sequence(5′-3′)	amplicon
SADS-N-F	CAGGTCTTGGTGTTCGCAATCG	449 bp
SADS-N-R	ACCGTGCTGAACGAGGTCACT	
qSADS-N-F	CTGACTGTTGTTGAGGTTAC	155 bp
qSADS-N-R	TCTGCCAAAGCTTGTTTAAC	
probe	5, -FAM -TCACAGTCTCGTTCTCGCAATCA-TAMRA-3,	

2.6. Sensitivity and specificity of the real-time RT-PCR

The concentration of the plasmid DNA was 196.455 ng/μL, equivalent to 3.0×10^{10} copies/μL before dilution. To examine the detection limit of the real-time RT-PCR, plasmid DNA was diluted serially 10-fold with RNase Free ddH_2O (10^9–10^2 copies/μL) as templates. Briefly, conventional RT-PCR was performed by using 1 μL of diluted cDNA template and 10 μmol of primers in a 20 μL reaction volume with the following thermal profile: 50 °C for 30 min, 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final step of 72 °C for 5 min. And then the detection limit between the real-time RT-PCR and conventional PCR were compared.

The specificity of the TaqMan-based real-time RT-PCR assay was demonstrated with other porcine viruses, including classical swine fever virus (CSFV), foot-and-mouth disease virus (FMDV), porcine circovirus 3 (PCV3), porcine rotavirus (RV), porcine deltacoronavirus (PDCoV), porcine epidemic diarrhea virus (PEDV), porcine kobuvirus (PKV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine sapelovirus (PSV), porcine bocavirus (PBoV), swine vesicular disease virus (SVDV), senecavalley virus (SVA), swine influenza virus (SIV), transmissible gastroenteritis virus (TGEV) and staphylococcus aureus, which were stored at our laboratory. Negative control (RNase Free ddH_2O) were also contained in the run.

2.7. Reproducibility of the real-time RT-PCR

Ten-fold serial plasmid dilutions ranging from 3.0×10^2 to 3.0×10^9 copies/μL were used to assess the coefficients of variation of the real-time RT-PCR. Intra-and inter-assay for Cq values were both included in three different times and quintuplicate.

2.8. Detection of the clinical tissues samples

In order to evaluate the real-time RT-PCR assay, a total of 174 clinical tissue samples with diarrheal symptoms, including heart, liver, spleen, lung, kidney, jejunum, ileum, duodenum, tonsil, lymphoglandulae mesentericae and lymphonodi abdominals were collected and quantified by the developed method. The samples amplified before 40 cycles and showed the typical amplification curves were considered to be SADS-CoV positive. Conventional PCR was preformed simultaneously.

3. Results

3.1. Standard curve and dynamic range

Ten-fold serial dilutions of DNA plasmids were used to establish a standard curve by plotting the logarithm of the plasmid copy number against the measured Cq values. The standard curve generated had a wide dynamic range of 10^9–10^2 copies/μL with a linear correlation (R^2) of 0.999 between the Cq value and the logarithm of the plasmid copy number (Fig. 1).

3.2. Specificity of the real-time RT-PCR

The real-time RT-PCR gave positive results for the standard plasmid of SADS-CoV strain (3.0×10^7 copies/μL) and negative results (Cq > 40) for other porcine viruses, including PEDV, PDCoV, RV, PRRSV, PKV, PSV, PBoV, FMDV, SVDV, SVA, SIV, CSFV, PCV3, TGEV, SA and negative control (RNase Free ddH_2O) (Fig. 2, Table 2).

3.3. Sensitivity of the real-time RT-PCR

To compare the sensitivity of the TaqMan-based real-time RT-PCR

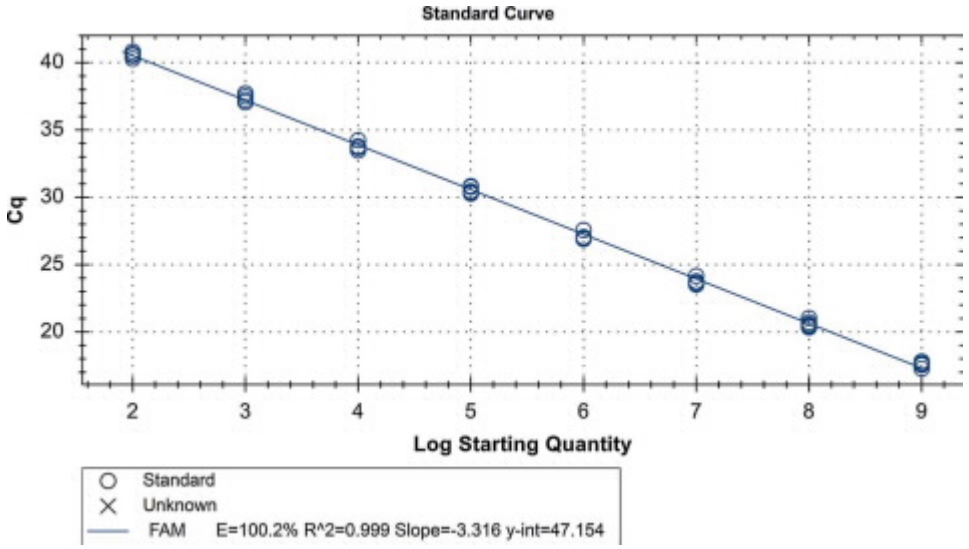


Fig. 1. Standard curve of TaqMan-based real-time RT-PCR assay. The assay was conducted using duplicated ten-fold serial dilutions (3.0×10^9–3.0×10^2 copies/μL) of the plasmid DNA; y = - 3.316x + 47.154.

and the conventional PCR, ten-fold serial dilutions of the plasmid pSADS-N were analyzed. The detection limits for the TaqMan-based real-time RT-PCR and the conventional PCR assays were 3.0×10^1 and 3.0×10^2 DNA copies/μL, respectively (Fig. 3).

3.4. Reproducibility of the TaqMan-based real-time RT-PCR assay

The coefficient of variation (C.V) in the intra-assay and inter-assay was evaluated. The intra-assay C.Vs of real-time PCR ranged from 0.31% to 1.93%, while the inter-assay C.Vs ranged from 0.14% to 1.86% (Table 3).

3.5. Detection of the clinical tissues samples

The developed TaqMan-based real-time RT-PCR assay was evaluated with 174 clinical samples. 123 out of 174 samples were positive for SADS-CoV by the TaqMan-based real-time RT-PCR. When these samples were detected by the conventional PCR, only 89 out of 174 (51.15%) were positive. The established technique increased the positive detection positive rated by 20%. Samples that were tested positively by the conventional PCR were also positive by the real-time RT-PCR. 34 samples were detected negatively by the conventional PCR, while using the TaqMan-based real-time RT-PCR, they were actually positive for SADS-CoV (Table 4).

Table 2
The samples' information of specificity assay for SADS-CoV Taqman-baes real-time RT-PCR. N/A: negative.

Number	Samples	Type	Cq Mean
1	SADS-CoV	isolate strain	16.07
2	PEDV	isolate strain	41.49
3	TGEV	vaccine strain	N/A
4	RV	vaccine strain	N/A
5	PDCoV	isolate strain	N/A
6	FMDV	isolate strain	45.37
7	PRRSV	vaccine strain	N/A
8	CSFV	vaccine strain	N/A
9	SVA	isolate strain	N/A
10	PCV3	wild strain	40.06
11	SVDV	isolate strain	40.57
12	SIV	vaccine strain	N/A
13	PKV	wild strain	N/A
14	PBoV	wild strain	N/A
15	PSV	wild strain	N/A
16	SA	isolate strain	N/A
17	ddH2O	negative control	N/A

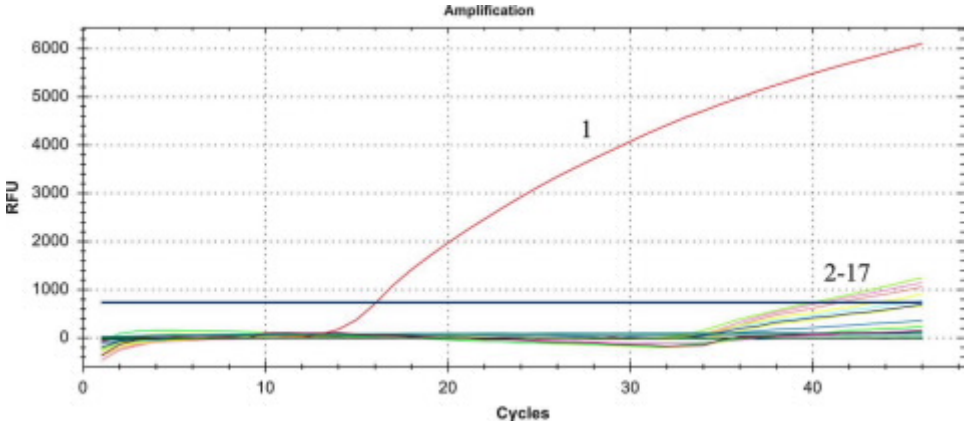


Fig. 2. Specificity of the real-time RT-PCR assay. 1: SADS-CoV 2-17: PEDV, PDCoV, RV, PRRSV, PKV, PBoV, PSV, FMDV, SVDV, SVA, SIV, CSFV, PCV3, TGEV, Staphylococcus aureus and negative control.

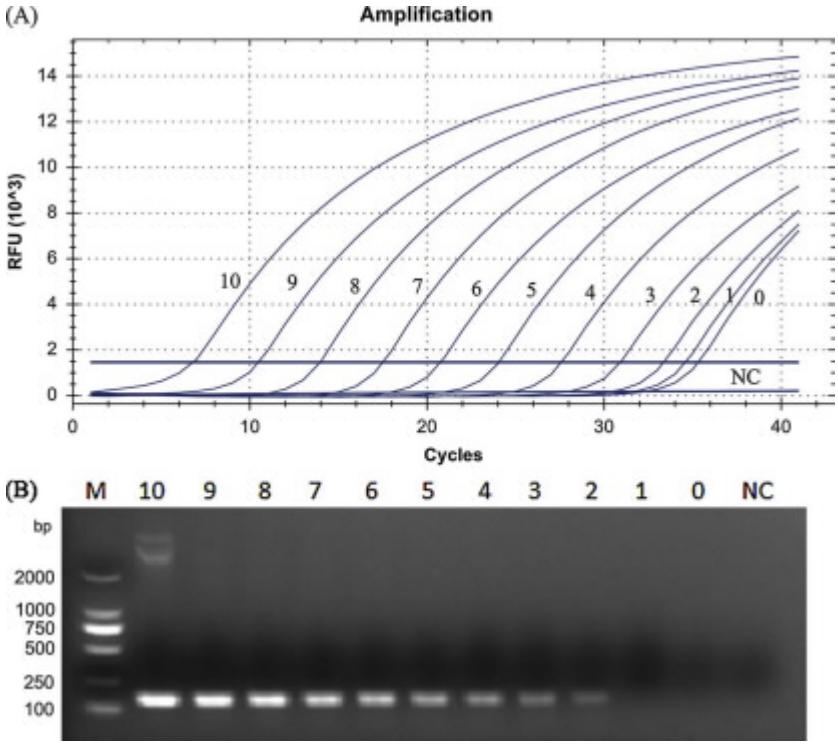


Fig. 3. (A) Sensitivity of the TaqMan-basedreal-time RT-PCRassayfor SADS-CoVdetection. (B) Sensitivity of the conventional PCRfor SADS-CoVdetection. 10- 0: a serial of ten-fold dilutions plasmid DNA (3.0×10^{10}–3.0×10^{0} copies/μL); NC: negative control; M: DL 2000 marker.

Table 3
Reproducibility of intra-assay and inter-assay TaqMan-basedreal-time RT-PCRfor SADS-CoV detection. SD= Standard deviation; C.V (%) = Percentageof coefficient variation.

SADS-CoVclone	Intra-assay			Inter-assay		
(copies/μL)	Cq Mean	SD	C.V(%)	Cq Mean	SD	C.V(%)
3.0×10^{9}	8.77	0.12	1.33%	8.68	0.16	1.86%
3.0×10^{8}	12.99	0.25	1.93%	13.00	0.02	0.16%
3.0×10^{7}	16.68	0.09	0.56%	16.78	0.09	0.53%
3.0×10^{6}	20.09	0.07	0.33%	20.08	0.05	0.23%
3.0×10^{5}	23.25	0.12	0.54%	23.28	0.04	0.19%
3.0×10^{4}	26.52	0.12	0.45%	26.51	0.04	0.14%
3.0×10^{3}	30.17	0.09	0.31%	30.09	0.16	0.52%
3.0×10^{2}	32.64	0.21	0.63%	32.84	0.16	0.48%

Table 4
Comparative results of clinical samplestested by conventional PCRand TaqMan-based real-time RT-PCR for SADS-CoV detection. LA: Lymphonodi abdominals; LM: Lymphoglandulaemesentericae.

samples	Number of samples	Number of SADS-CoVpositive samples	
		Conventional PCR	Real-time RT-PCR
Heart	18	8	11
Liver	18	8	12
Spleen	18	9	13
Lung	18	9	13
Kidney	18	8	14
Jejunum	17	13	15
Ileum	18	17	17
Duodenum	18	11	13
Tonsil	13	1	5
LA	9	1	2
LM	9	4	8
Total	174	89	123

4. Discussion

SADS-CoV is a newly identified virus in south China in 2017. Previous methods for the detection of this novel virus have relied on qualitative rather than quantitative PCR(Zhou et al., 2018; Pan et al., 2017; Gong et al., 2017). The conventional PCRis time-consuming, and it couldn't estimate the amount of viral gene copies, which could be crucial for virus diagnosis and correlations with clinical diseases (Mackay et al., 2002).

In this study, we described the development and validation of a TaqMan-based real-time RT-PCR assay for SADS-CoV diagnosis and quantification in 174 clinical samples from piglets affected by severe diarrhea in southern China. The hydrolysis probe employed in this assaywas located in the conservedregion within the N geneof the viral genome. Resultsshowed that the correlation coefficient of the standard curve in this study was 0.999, much closing to the R^2 of 1 in theory. No cross-reaction signals were observed for fifteen other porcine diseases. The lowest limit of detection was 3.0×10^{1} copies/μL, 10 times more sensitive than the conventional PCR.The intra-assay C.Vs were equal or lessthan 1.93% and the inter-assay C.Vswere equal or lessthan 1.86%, indicating the good reproducibility of this assay.The positive rate determined by the new technique was 70.69%, whereas that of the conventional PCR was merely 51.15%, further demonstrating the higher sensitivity of the former method. All these data showed that the TaqMan-basedreal-time RT-PCRapproach was reliable and sensitive.

In conclusion, the TaqMan-based real-time RT-PCRassay was established in present study for detection and quantification of SADS-CoV in pigs. Becauseof it remarkable sensitivity, reproducibility and rapidity compared to the conventional PCR,this assaymay not only help to resolve diagnosis investigation, but also help to provide knowledge on epidemiology and pathogenesis studies of SADS-CoV.

Conflict of interest

The authors declare no conflict of interests with any organization.

Acknowledgments

This work was supported by the National Key Research and Development Program, Grant/Award Number: 2016YFD0501304. We would acknowledge Guangdong Wen's Foodstuffs Group Co., Ltd. China, for providing us with piglets' tissue samples.

References

Dall Agnol, A.M., Otonel, R.A., Leme, R.A., Alfieri, A.A., Alfieri, A.F., 2017. A TaqMan-based qRT-PCR assay for SenecavirusA detection in tissue samples of neonatal piglets. Mol. Cell. Probes 33, 28–31.

Dong, N., Fang, L., Zeng, S., Sun, Q., Chen, H., Xiao, S., 2015. Porcine deltacoronavirus in mainland China. Emerg. Infect. Dis. 21, 2254–2255.

Fowler, V.L., Ransburgh, R.H., Poulsen, E.G., Wadsworth, J., King, D.P., Mioulet, V., Knowles, N.J., Williamson, S., Liu, X., Anderson, G.A., 2016. Development of a novel real-time RT-PCR assay to detect SenecaValley virus associated with emerging cases of vesicular disease sin pigs. J. Virol. Methods 239, 34.

Gava, D., Souza, C.K., Schaefer, R., Vincent, A.L., Cantao, M.E., Coldebella, A., Ciacci-Zanella, J.R., 2015. A TaqMan-based real-time PCR for detection and quantification of porcine parvovirus 4. J. Virol. Methods 219, 14–17.

Gong, L., Li, J., Zhou, Q., Xu, Z., Chen, L., Zhang, Y., Xue, C., Wen, Z., Cao, Y., 2017. A new Bat-HKU2–like Coronavirus in Swine, China. Emerg. Infect. Dis. 23, 1607–1609.

Li, B., Xiao, S., Ma, J., Liu, Y., Mao, L., Wen, L., Mao, A., Zhang, X., Ni, Y., Guo, R., Zhou, J., Yu, Z., Lv, L., Wang, X., Fang, L., Chen, H., He, K., 2011. Development of a novel TaqMan-based real-time PCR assay for the detection of porcine boca-like virus (PbolikeV). Virol. J. 8, 357.

Li, J., Li, W., Mao, L., Hao, F., Yang, L., Zhang, W., Jiang, J., 2016. Rapid detection of novel caprine parainfluenza virus type 3 (CPIV3) using a TaqMan-based RT-qPCR J. Virol. Methods 236, 126–131.

Mackay, I.M., Arden, K.E., Nitsche, A., 2002. Real-time PCR in virology. Nucleic Acids Res. 30, 1292–1305.

Niu, X., Chen, Yang, Yu, X., Ti, J., Wang, A., Diao, Y., 2016. Development of a TaqMan-based real-time PCR assay for the detection of Novel GPV. J. Virol. Methods 237, 32–37.

Pan, Y., Tian, X., Qin, P., Wang, B., Zhao, P., Yang, Y., Wang, L., Wang, D., Song, Y., Zhang, X., Huang, Y., 2017. Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. Vet. Microbiol. 211, 15–21.

Sun, D., Wang, X., Wei, Chen, Feng, J., 2016. Epidemiology and vaccine of porcine epidemic diarrhea virus in China: a mini-review. J. Vet. Med. Sci. 78, 355–363.

Woo, P.C., Lau, S.K., Lam, C.S., Lau, C.C., Tsang, A.K., Lau, J.H., Bai, Teng, J.L, Tsang, C.C, Wang, Zheng, B., Chan, K.H., Yuen, K.Y., 2012. Discovery of seven novel Mammalian and Avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and Avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. J. Virol. 86, 3995–4008.

Zhou, P., Fan, H., Lan, T., Yang, X.L., Zhang, W., Zhu, Y., et al., 2018. Fatal swine acute diarrhea syndrome caused by an HKU2-related coronavirus of bat origin. Nature (Accepted for publication).

JVI Accepted Manuscript Posted Online 25 September 2019
J. Virol. doi:10.1128/JVI.01448-19
Copyright © 2019 American Society for Microbiology. All Rights Reserved.

Accepted Manuscript Posted Online
Journal of Virology
JVI
Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Broad cross-species infection of cultured cells by the bat HKU2-related swine acute diarrhea syndrome coronavirus (SADS-CoV) and identification of its replication in murine dendritic cells *in vivo* highlight its potential for diverse interspecies transmission

Yong-Le Yang,[a]* Pan Qin,[a]* Bin Wang,[a] Yan Liu,[a] Guo-Han Xu,[a] Lei Peng,[a] Jiyong Zhou,[a] Shu Jeffrey Zhu,[a]# Yao-Wei Huang[a]#

[a]Key Laboratory of Animal Virology of Ministry of Agriculture and Institute of Preventive Veterinary Medicine, Department of Veterinary Medicine, Zhejiang University, Hangzhou 310058, Zhejiang, China.

*These authors contributed equally to this work.

#Address correspondence to

Dr. Yao-Wei Huang, Zhejiang University, yhuang@zju.edu.cn

Dr. Shu Jeffrey Zhu, Zhejiang Univeristy, shuzhu@zju.edu.cn

Running title: Broad species tropism and a mouse model of SADS-CoV

Word counts: manuscript text (5,908 words); abstract (250 words); importance (139 words).

Figure number: 6; **Table number**: 1

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

ABSTRACT

Outbreaks of severe diarrhea in neonatal piglets in Guangdong, China in 2017 resulted in isolation and discovery of a novel swine enteric alphacoronavirus (SeACoV) derived from the species *Rhinolophus bat coronavirus HKU2* (Vet Microbiol, 2017, 211:15-21). SeACoV was later referred to as swine acute diarrhea syndrome (SADS) CoV by another group (Nature, 2018, 556:255-258). The present study was set up to investigate potential species barriers of SADS-CoV *in vitro* and *in vivo*. We first demonstrated that SADS-CoV possesses a broad species tropism and is able to infect cell lines from diverse species including bats, mice, rats, gerbils, hamsters, pigs, chickens, nonhuman primates and humans. Trypsin contributes to, but is not essential for SADS-CoV propagation *in vitro*. Furthermore, C57BL/6J mice were inoculated with the virus via oral or intraperitoneal routes. Although the mice exhibited only subclinical infection, they supported viral replication and prolonged infection in the spleen. SADS-CoV nonstructural proteins and double-stranded RNA were detected in splenocytes of the marginal zone on the edge of lymphatic follicles, indicating active replication of SADS-CoV in the mouse model. We identified that splenic dendritic cells (DCs) are the major targets of virus infection by immunofluorescence and flow cytometry approaches. Finally, we demonstrated that SADS-CoV does not utilize known CoV receptors for cellular entry. The ability of SADS-CoV to replicate in various cells lines from a broad range of species and the unexpected tropism for murine DCs provide important insights into the biology of this bat-origin CoV, highlighting its possible ability to cross interspecies barriers.

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

43 **IMPORTANCE**

44 Infections with bat-origin CoVs (SARS-CoV and MERS-CoV) have caused severe illness
45 in humans after "host jump" events. Recently, a novel bat-HKU2-like CoV named swine acute
46 diarrhea syndrome CoV (SADS-CoV) has emerged in southern China, causing a lethal diarrhea
47 in newborn piglets. It is important to assess the species barriers of SADS-CoV infection since the
48 animal hosts (other than pigs and bats) and zoonotic potential are still unknown. An *in vitro*
49 susceptibility study revealed a broad species tropism of SADS-CoV, including various rodent
50 and human cell lines. We established a mouse model of SADS-CoV infection, identifying its
51 active replication in splenic dendritic cells, which suggests that SADS-CoV has the potential to
52 infect rodents. These findings highlight the potential cross-species transmissibility of SADS-CoV,
53 though further surveillance in other animal populations is needed to fully understand the ecology
54 of this bat-HKU2-origin CoV.

55

56 **Keywords:** Interspecies transmission; Coronavirus; SADS-CoV; Mouse infection model

57

58

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

INTRODUCTION

The spread of zoonotic pathogens remains among the leading threats to global public health . Coronaviruses (CoVs) can infect a wide variety of animals and humans, resulting in several diseases with respiratory, enteric, and neurological pathologies of varying severity (1-4). Because of the various routes of infection and extensive phagocytosis in tissues, close contact between humans and animals provides potential scenarios for adaptive mutation and interspecies transmission (5).

The source of the severe acute respiratory syndrome (SARS)-CoV was traced to civets in animal markets and ultimately to bats, leading to more than 8,000 human infections and 774 deaths after its emergence in 2002 (5-7). The emergence of Middle East respiratory syndrome (MERS)-CoV in 2012 (2) resulted in more than 1,000 clinical cases with a mortality rate of 35%, making it the second marked threatening CoV of the 21st century (8, 9). Although camels can be infected with MERS-CoV, bats are also thought to be the original host of MERS-CoV (5). Both SARS-CoV and MERS-CoV originated in bats, illustrating the damage caused by CoVs during interspecies transmission events, and highlighting the need for increased global vigilance of CoV-associated disease (1, 5, 10).

In February 2017, outbreaks of severe diarrhea of suckling piglets occurred in swine herds in Guangdong Province, China (11). Clinical signs consisted of acute vomiting and watery diarrhea, but porcine viruses commonly associated with diarrhea including porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV) and porcine deltacoronavirus (PDCoV) were not detected in any of the clinical samples (11). The new enteric pathogen of

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

commercial pigs that was isolated was finally identified as a new porcine CoV belonging to the species *Rhinolophus bat coronavirus HKU2* (11-13). Our research group tentatively designated this newly emerged virus as swine enteric alphacoronavirus (SeACoV) (11), and it was later named swine acute diarrhea syndrome (SADS)-CoV by Zhou *et al.* (14). It is also known by other names, such as porcine enteric alphacoronavirus (PEAV) (13). For purposes of unity, SADS-CoV is the name used to refer to this new virus in the current study. The expanded host range of bat-origin HKU2 to pigs indicates that bats play an important role in the ecology and evolution of SADS-CoV, though the mechanism of bat-to-swine transmission remains unclear. In view of the damage caused by SARS and MERS for both animal and public health, careful attention must be paid to the prevalence of CoV-associated disease among humans and domestic animals (15).

Therefore, there is an urgent need for more information on the details of SADS-CoV infection. It is critically important to assess potential species barriers of SADS-CoV transmission since the animal hosts (other than pigs and bats) and zoonotic potential are still unknown. In the present study, we demonstrated that SADS-CoV possesses a very broad species tropism *in vitro* and is able to infect cell lines from diverse species including rodents and humans. Furthermore, *in vivo* evidence from experimental infection of mice with SADS-CoV identified splenic dendritic cells (DCs) as the major site of SADS-CoV replication in mice. Finally, we demonstrated that SADS-CoV does not utilize known CoV protein receptors for cellular entry. These results present the possibility that rodents are among the susceptible hosts of SADS-CoV, highlighting the potential cross-species transmissibility of SADS-CoV.

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

101

102 **MATERIALS AND METHODS**

103 **Virus stocks and viral antibodies.** The SADS-CoV isolate CH/GD-01/2017 at passage 10
104 was used in all experiments and cultured in Vero cells (16). The virus was passaged serially using
105 the culture supernatant to infect fresh Vero cells at a multiplicity of infection (MOI) of 0.1, and
106 viral titers were determined in Vero cells by endpoint dilution as the 50% tissue culture infective
107 dose 50% ($TCID_{50}$). Rabbit polyclonal antibodies (pAb) against the membrane (M),
108 nucleocapsid (N) and the nonstructural protein 3 (Nsp3) acidic domain (AC) of SADS-CoV were
109 generated in-house, and validated in SADS-CoV-infected Vero cells (16). A mouse
110 anti-SADS-CoV-N pAb was also produced to allow double staining when mixed with the rabbit
111 pAb. A monoclonal antibody (MAb) against dsRNA (anti-dsRNA mAb J2, Cat# J2-1702,
112 SCICONS, Hungary) was used to specifically detect viral replication of SADS -CoV.

113 **Cell lines and cell culture.** Twenty-four cell lines derived from tissues of different species
114 were used (Table 1), including human (Huh-7, HepG2/C3A, 293T, A549 and HeLa), monkey
115 (Marc-145, Cos-7, BSC-40, Vero), swine [ST, PK15, LLC-PK1, IPEC-J2 (17)], bat [BFK (18),
116 Tb-1], canine (MDCK), mouse (NIH/3T3, RAW 264.7), hamster (BHK -21, CHO), rat (BRL-3A,
117 NRK-52E), chicken (DF-1) cell lines and a primary kidney cell line from Mongolian gerbils
118 (prepared in-house). The BFK cell line was a generous gift from Dr. Changchun Tu at the
119 Institute of Military Veterinary Medicine, Changchun, China. Each cell line was cultured in
120 Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented wit h 10% (v/v) fetal
121 bovine serum (FBS, Biological Industries), 100 U/m1 penicillin and 100 U/ml streptomycin at

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

122 37 °C, 5% CO_2, and water-saturated humidity conditions.

123 To determine viral susceptibility, each cell line was cultured at 70% confluence in 12-well
124 plates with maintenance medium (MM) containing DMEM, 0.3% tryptose phosphate broth
125 (TPB), and 1% penicillin/streptomycin, or MM with addition of 5 μg/ml trypsin (MMT) (Sigma,
126 Cat#T7186-50TAB, St Louis, MO, USA). After washing with phosphate buffered saline (PBS),
127 cells were inoculated with SADS-CoV diluted in MM or MMT at an MOI of 0.01 for 2 h.
128 Non-attached viruses were removed by washing the cells three times with DMEM, and cell
129 monolayers were subsequently incubated in MM or MMT at 37 °C for 5 days. To determine the
130 effect of trypsin on viral entry, cell monolayers were infected by SADS-CoV in three conditions:
131 1) no trypsin treatment, infected with SADS-CoV diluted in MM, subsequently incubated in MM;
132 2) pre-trypsin treatment, inoculated with SADS-CoV diluted in MMT, subsequently incubated in
133 MM; and 3) double-trypsin treatment, inoculated with SADS-CoV in MMT, subsequently
134 incubated in MMT. Supernatants from cells were collected at 12, 24, 36, 48, 72, and 120 hours
135 post-infection (hpi) for one-step quantitative RT-PCR analysis. Cell cultures were examined for
136 cytopathic effects (CPE) and immunofluorescence assay at 48-72 hpi.

137 **Immunofluorescence assay (IFA) for cell line susceptibility.** Different cells infected with
138 SADS-CoV in 12-well plates were washed twice with PBS and fixed in 4% paraformaldehyde in
139 PBS and then permeabilized with 0.1% Triton X-100 in PBS. Cells were then incubated with the
140 rabbit anti-SADS-CoV-M pAb at 1:5000 dilution for 1 h at 37 °C, washed with PBS and stained
141 with the Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Thermo Fisher
142 Scientific, USA) at 1:1000 dilution. After incubation for 1 h at 37 °C, the cells were washed with

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

143 PBS, stained with 4′,6-diamidino-2-phenylindole (DAPI) at 1:1000 dilution and visualized on a
144 fluorescence microscope.

145 **One-step quantitative RT-PCR analysis targeting the N gene.** The full-length SADS-CoV
146 N gene was inserted into an appropriately digested pET-28a vector using two unique restriction
147 sites, *Nde*I and *Xho*I, and then linearized with *Xho*I. The N gene was *in vitro* transcribed using
148 the T7 High Efficiency Transcription Kit (TransGen Biotech Co., LTD, Beijing China). Standard
149 curves were generated using dilutions of known quantity of N gene RNA to allow absolute
150 quantitation of SADS-CoV RNA copy numbers in samples.

151 Total RNA was extracted from culture supernatants or tissue homogenates using Trizol
152 (ThermoFisher Scientific, USA) following the manufacturer's instructions. SADS-CoV RNA
153 titer was determined by one-step qRT-PCR (TOYOBO Co., LTD) targeting the N gene with the
154 primers: 5'-CTAAAACTAGCCCCACAGGTC-3' and 5'-TGATTGCGAGAACGAGACTG-3',
155 and the probe FAM-GAAACCCAAACTGAGGTGTAGCAGG-TAMRA. Samples with a cycle
156 threshold value <35 were considered positive based upon validation data using the RNA
157 standards.

158 **Mouse infections, tissue harvest and viral load determination.** Wild-type C57BL/6J mice
159 (Jackson no. 000664) were purchased from the Model Animal Research Center of Nanjing
160 University and housed in animal facilities at the Zhejiang University under
161 specific-pathogen-free condition. For SADS-CoV infections, 6- to 8-week-old female and male
162 mice were inoculated with 5×10^5 $TCID_{50}$ (equal to 6×10^8 genome copies) of SADS-CoV, either
163 per oral infection (p.o.) with 25 μl inoculum (2×10^7 $TCID_{50}$/ml) or intraperitoneal infection (i.p.)

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

with 200 µl inoculum (2.5×10^6 $TCID_{50}$/ml). For viral load determination in specific tissues, mice were euthanized at 1, 3, 5, 7, 14, 21, and 28 days post-infection (dpi), and tissues were harvested including stomach, duodenum, jejunum, ileum, cecum, colon, mesenteric lymph nodes, spleen, kidney, liver, heart, lung, blood and feces. Tissues were weighed and homogenized in medium (DMEM contained 2% FBS) by bead beating using sterile zirconium oxide beads (Cat# ZrOB20 MidSci). Total RNA was extracted from tissue homogenates and tested by quantitative RT-PCR analysis targeting the SADS-CoV N gene, as described above. Blood samples were collected from the heart and serum was separated for virus-specific antibody detection.

Enzyme-linked immunosorbent assay (ELISA). SADS-CoV virus particles were purified from infected cell culture supernatants by sucrose density gradient centrifugation, and protein concentration was determined by the BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China). The optimal dilution of antigen was determined by square titration. The IgG antibodies contained in serum at a 1:100 dilution were detected in wells coated with purified SADS-CoV virus particles (6.25 ng/well) as antigen.

Histopathology, immunohistochemistry and immunofluorescence assay for murine spleen. Mice were infected i.p. with SADS-CoV and at 3 dpi, spleens were harvested and fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Tissue sections were then deparaffinized and rehydrated in three changes of xylene, 15 min each, dehydrated in two changes of pure ethanol for 5 min, followed by rehydration in an ethanol gradient of 85% and 75% ethanol. After washing in distilled water, tissues were subjected to hematoxylin and eosin staining for histopathological examinations.

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

185 For antigen retrieval, deparaffinized and rehydrated sections were immersed in sodium
186 citrate antigen retrieval solution (pH 6.0) and maintained at a sub-boiling temperature for 8 min,
187 let stand at 98 °C for 8 min and then incubated again at sub-boiling temperature for 7 min. After
188 allowing to cool to room temperature (RT) and washing three times with PBS (pH 7.4),
189 endogenous peroxidase was blocked by immersion in 3% hydrogen peroxide at RT for 30 min
190 and again washed with PBS. Tissue sections were blocked in 3% BSA at RT for 30 min, then
191 incubated with 1:500 dilution of each primary antibody (anti-dsRNA MAb, anti-SADS-CoV-M
192 pAb or anti-SADS-CoV-AC pAb) overnight at 4 °C. After washing slides three times with PBS
193 (pH 7.4), they were stained with appropriate secondary antibodies labeled with horseradish
194 peroxidase at RT for 50 min. Freshly prepared diaminobenzidine chromogenic reagent was added
195 and counterstained with hematoxylin, then dehydrated and visualized on a light microscope.
196 Spontaneous fluorescence quenching reagent (Wuhan servicebio technology Co., Ltd,
197 Wuhan, China) was added to the tissue sections and incubated for 5 min after antigen retrieval.
198 The sections were then washed in running water, followed with blocking and antibody staining
199 as described above. In addition, the primary antibody was supplement with a CD11c antibody
200 (Wuhan servicebio technology Co., Ltd, Wuhan, China) at a 1:200 dilution, then stained with
201 appropriate secondary antibodies. Finally, DAPI was added and sections were visualized on a
202 fluorescence microscope; nuclei labeled with DAPI appear blue, positive cells are green by
203 labeling with CD11/c or red by labeling with virus-specific antibody.
204 **Preparation of murine splenocytes and flow cytometry.** Mice infected with SADS-CoV
205 were euthanized at 3 dpi, and spleens were removed and placed in 5 ml complete DMEM. After

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

grinding the excised spleen through a 100-μm cell strainer using the plunger end of a 5-ml syringe, cells were washed with an excess of DMEM and centrifuged at 200 ×g for 5 min. After resuspending cells in 3 ml of red blood cell lysis buffer (Solarbio Life Sciences, Beijing, China) and incubation at RT for 10 min, 5 ml of DMEM was added and cells were passed through another strainer to remove clumps. After centrifugation at 200 ×g for 5 min, the supernatant was discarded and cells were resuspended in 10 ml fresh DMEM for cell counting and viability checks using trypan blue and a hemocytometer.

For flow cytometry, cultured cells were resuspended in Fc Block buffer (containing anti-mouse CD16 Fc Block Antibody at 1:500 dilution) and incubated on ice. Cells in Fc Block buffer were added to 96-well plates at 1×10^6 cells/well. After 30 min incubation, cells were centrifuged at 200 ×g for 10 min, the supernatant was discarded and pellets resuspended in 100 μl Cytofix/Cytoperm solution (Cytofix/Cytoperm Soln Kit; BD Biosciences, San Jose, CA, USA) to fix cells. After incubation on ice for 20 min protected from light , cells were centrifuged at 800 ×g for 5 min at 4 °C, supernatant was removed without disturbing cell pellets and cells were washed twice in 150 μl of 1x Perm/Wash buffer. After addition of 50 μl virus-specific primary antibody (anti-dsRNA MAb, anti-SADS-CoV-N pAb or anti-SADS-CoV-AC pAb) diluted in 1× Perm/Wash buffer with 3% BSA and incubation at 4 °C for 30 min, cells were centrifuged at 200 ×g for 10 min. Cells were washed twice in 150 μl of 1× Perm/Wash buffer followed by staining with appropriate secondary antibodies conjugated to Alexa Fluor 488 (Thermo Fisher Scientific, USA) at 4 °C for 30 min. After centrifuging at 800 ×g for 5 min at 4 °C and washing with 1× Perm/Wash buffer, pellets were resuspended in 0.2 ml FACS buffer and analyzed by flow

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

227 cytometry.

228 **Infection of splenocytes *in vitro*.** To detect replication of SADS-CoV in mouse splenic cells
229 *in vitro*, splenocytes were extracted from naïve mice, plated in 100- or 35-mm dishes and
230 infected with SADS-CoV at an MOI of 0.1. At 48 hpi, splenocytes were harvested and placed in
231 a 15-ml tube, centrifuged at 200 ×g for 10 min at 4 °C, and analyzed by flow cytometry as
232 described above. Infected mouse splenic cells in 35-mm dishes were detected by
233 immunofluorescence assay with anti-SADS-CoV-N antibodies, and infection supernatants were
234 collected at 0, 12, 24, 36, 48 and 72 hpi for one-step quantitative RT-PCR analysis.

235 **FACS analysis of splenocytes with cell marker staining.** Mice infected with SADS-CoV
236 were euthanized at 3 dpi, and splenocytes were prepared for flow cytometry by staining with
237 appropriate antibodies: anti-SADS-CoV-AC following secondary antibodies conjugated to Alexa
238 Fluor 647 (Thermo Fisher Scientific, USA); anti-CD19-FITC (eBioscience, Catalog no.4318813)
239 for B cells; anti-CD4-PE (eBioscience, Catalog no.4329629) for T cells; anti-CD11/c-PE-Cy7
240 (BD Bioscience, Catalog no.561022) for DCs; and anti-F4/80-PE/Cy5 (Biolegend, Catalog
241 no.123111) for macrophages. Stained cells were resuspended in 0.2 ml FACS buffer and
242 analyzed by flow cytometry.

243 **Production and transduction of S protein-pseudotyped lentiviruses.** Pseudovirions with
244 various CoV spike proteins were produced as described previously (19). Briefly, each of the
245 plasmids encoding TGEV, SARS-CoV, MERS-CoV, and mouse hepatitis virus (MHV) S proteins
246 were cotransfected into 293T cells with pLenti-Luc-green fluorescent protein (GFP) and psPAX2
247 plasmids (kindly provided by Dr. Zhaohui Qian, Chinese Academy of Medical Sciences &

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

248 Peking Union Medical College) at a molar ratio of 1:1:1 by using polyethylenimine (PEI). The
249 cells were fed with fresh medium in the next 24 h and the supernatant media containing
250 pseudovirions were then collected and centrifuged at 800 ×g for 5 min to remove debris. To
251 quantify S protein-mediated entry of pseudovirions, MDCK cells were seeded at about 25-30%
252 confluency in 24-well plates and transfected with either pAPN-Flag, hDPP4-Flag,
253 mCEACAM1a-Flag, hACE2-GFP (kindly provided by Dr. Zhaohui Qian) (19) or the control
254 backbone vector by using Lipofectamine 3000 (Thermo Fisher). The MDCK cells
255 overexpressing each receptor were inoculated with 500 µl of 1:1 diluted corresponding
256 pseudovirions at 24 h post-transfection. At 40 hpi, cells were lysed at room temperature with 110
257 µl of medium with an equal volume of Steady-Glo (Promega, Madison, WI). The cell lysates
258 were also used to confirm the expression of each receptor by using western blotting.
259 Transduction efficiency was monitored by quantitation of luciferase activity using a Modulus II
260 microplate reader (Turner Biosystems, Sunnyvale, CA). On the other hand, the MDCK cells
261 overexpressing each receptor were inoculated with SADS-CoV (MOI=1) at 24 h
262 post-transfection. IFA was performed to test for SADS-CoV susceptibility using anti-N pAb. The
263 replication competency of SADS-CoV in MDCK cells was further determined by a reverse
264 genetics system. Development of a DNA-launched SADS-CoV (SeACoV) infectious cDNA
265 clone (named pSEA) and rescue of SADS-CoV by transfection of cultured cells with pSEA
266 followed by passaging on Vero cells have been described recently by our lab (16).

267 **Ethics statement.** All animal experiments were performed in strict accordance with the
268 Experimental Animal Ethics Committee of Zhejiang University (approval no. ZJU20170026).

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

RESULTS

SADS-CoV can infect cell lines originating from various species. Previously, we reported that SADS-CoV was isolated in Vero cells supplemented with trypsin (11). Since exogenous trypsin is essential for propagation of PEDV isolates *in vitro* (20), likely by mediating activation of membrane fusion by S glycoprotein proteolysis (21), we were interesting to know whether it is also required for SADS-CoV growth in cell culture. A total of 24 cell lines originating in various tissues of humans and different animal species were tested for susceptibility to SADS-CoV treated with or without trypsin (Table 1). In brief summary of the results, 21 of the 24 cell lines showed significant susceptibility to SADS-CoV infection, defined by efficient viral replication, antigen expression and the appearance of CPE. The three cell lines that were not infected by SADS-CoV were MDCK, BFK and RAW 264.7.

First, CPE was examined by inverted light microscopy at 48 hpi, and scores are shown in Table 1. As the 293T, NIH/3T3, CHO, BRL-3A and NRK-52E cell lines were sensitive to trypsin, they couldn't be tested for SADS-CoV infection in MMT. Apart from that, CPE was visible in Vero, ST and BRL-3A cell lines without trypsin, and prominent CPE appeared or was enhanced with trypsin in Marc-145, Cos-7, BSC-40, Vero, ST, PK15, LLC-PK1 and BHK-21 cell lines (Table 1).

As some cells did not display CPE after SADS-CoV infection, all cell lines were subsequently tested for viral M protein expression by IFA (Fig. 1), revealing the same range as seen by CPE in the different cell lines (data not shown). Syncytia formation was prominent in

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

290 Huh-7, Vero and BHK-21 cells, whereas in MDCK, BFK and RAW 264.7 cells the antigen
291 expression was much less prominent than in the other cell lines (Figs. 1A, 1C and 1I). Most cell
292 lines tested showed evidence of productive infection as indicated by expression of the M protein,
293 while the inefficient antigen expression in Marc-145, LLC-PK1 and IPEC-J2 cells suggested
294 only a limited infection.

295 Next, viral load in the culture supernatants was detected over 5 dpi by quantitative RT-PCR
296 (Fig. 2A-2C). A higher mean viral load was detected by qRT-PCR after trypsin treatment in
297 HepG2, HeLa, Marc-145, Cos-7, BSC-40, Vero, LLC-PK1, IPEC-J2, BHK-21 and DF-1 cells.
298 Therefore, trypsin contributes to, but is not essential for SADS-CoV propagation in these cell
299 lines. There was no difference after trypsin treatment in the other cell lines, though Huh-7 and
300 Tb-1 cells had high levels of SADS-CoV RNA regardless of trypsin treatment.

301 The progressive release of infectious SADS-CoV into the culture medium of six
302 representative cell lines infected with SADS-CoV was determined by titration of supernatants in
303 Vero cells (Fig. 2D). Unlike in MDCK cells, SADS-CoV infection of HeLa, Vero, Tb-1,
304 BHK-21 and PK-15 cells was productive, with HeLa cells showing the greatest susceptibility
305 (Fig. 2D).

306 **Wild-type C57BL/6J mice can be infected by SADS-CoV via oral and intraperitoneal**
307 **routes.** With the observation that SADS-CoV could infect diverse rodent cell lines (from mice,
308 rats and hamsters as well as gerbil primary kidney cells), we hypothesized that mice may be
309 susceptible to SADS-CoV. To test this, we inoculated 6- to 8-week-old wild-type B6 mice with
310 5×10^5 $TCID_{50}$ of SADS-CoV by the p.o. or i.p. route and monitored them for 28 days for clinical

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

311 symptoms. The mice did not succumb to the infection nor did they develop diarrhea or
312 experience weight loss during the incubation period (data not shown).

313 To determine whether SADS-CoV infected the animals asymptomatically, tissue and fecal
314 samples from inoculated mice were collected at 1, 3, 5, 7, 14, 21 and 28 dpi to determine viral
315 growth kinetics and shedding. Analysis of tissue samples by qRT-PCR suggested that
316 SADS-CoV replicated modestly in the stomach early after i.p. or p.o. infection, declining and
317 reaching undetectable levels at 7 or 14 dpi and thereafter (Fig. 3A). A very limited viral
318 replication was observed in each region of the small intestine, with the ileum via i.p. infection
319 showing continuous and decent detectable viral RNA (Fig. 3B). In the large intestine, i.p.
320 infection also resulted in viral RNA loads slightly above the limit of detection at each time point
321 in the ceca, whereas it led to higher viral RNA levels at 1-3 dpi, and much lower viral RNA at
322 21-28 dpi in the colon compared to the p.o. route (Fig. 3C). However, this replication in the large
323 intestine did not translate into higher shedding, as hardly any viral genomes were detected even
324 at 1 dpi in the fecal samples collected from i.p.-infected mice (Fig. 3F). On the contrary,
325 significantly more virus was detected in the feces of p.o.-infected mice at 1 and 3 dpi, indicating
326 that i.p. inoculation does not lead to higher virus shedding.

327 Finally, SADS-CoV replicated more efficiently in the spleen following the i.p. route, with
328 significantly higher viral RNA loads at 21 dpi (Fig. 3D). More importantly, the virus was not
329 cleared from this tissue by 28 dpi in the i.p.-infected group and by 14 dpi in the p.o.-infected
330 group, suggestive of a SADS-CoV prolonged infection in the spleen independent of inoculation
331 route. In contrast to the spleen, only very low levels of viral RNA were detected in the local

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

lymphoid tissue of mesenteric lymph nodes (MLNs) at 1-3 dpi, and no virus was detectable at later time points (Fig. 3D). We also looked for virus in other extraintestinal sites including the heart, lungs, liver, kidneys and blood, but they were all negative or had extremely low levels (Fig. 3E). IgG antibody levels after 7 days detected by SADS-CoV virion-based ELISA showed that the i.p. route could effectively elicit host immune responses (Fig. 3G).

Splenocytes support SADS-CoV replication. With the mouse infection model described above, our next step was to determine the cell tropism of SADS-CoV *in vivo*. Thus, we performed immunohistochemistry (IHC) on sections of small and large intestine and spleen from mice infected i.p. with 5×10^5 $TCID_{50}$ of SADS-CoV at 3 dpi. A monoclonal antibody against dsRNA was used to identify cells that supported active virus replication, as dsRNA is an intermediate that only exists during intracellular viral replication. SADS-CoV dsRNA signals were observed in the splenic white pulp in the marginal zone on the edge of lymphatic follicles, and in the margins of the periarteriolar lymphocyte sheath (Fig. 4A). Staining of tissue sections from mock-infected mice were used as a control (Fig. 4B). In addition to dsRNA, we also used rabbit pAbs to detect expression of viral structural protein (M) or nonstructural protein (Nsp3-AC). At 3 dpi, anti-M or anti-AC staining was observed in the white pulp around the lymphatic nodules (Fig. 4C), similar to the localization of dsRNA staining (Fig. 4A). Tissue sections from SADS-CoV or mock-infected mice probed with preimmune sera were negative, indicating the specificity of the SADS-CoV antibody. Unfortunately, neither viral proteins (structural or nonstructural) nor dsRNA were detected in the intestine of infected mice, consistent with the detection of only very low levels of viral RNA in these tissues by qRT-PCR

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

353 (Fig. 3).

354 Next, SADS-CoV infection was quantified in the spleen using flow cytometry. We
355 inoculated B6 wild-type mice with 5×10^5 $TCID_{50}$ of virus either i.p. or p.o., and extracted the
356 bulk immune cells from the spleen of infected animals at 3 dpi. The flow cytometry method was
357 first validated in Vero cells infected with SADS-CoV at an MOI of 0.01 followed by staining
358 with pAb against the N or AC protein at 24 hpi (Fig. 4D). As the anti-AC pAb exhibited optimal
359 intracellular staining for viral signals (Fig. 4D), it was used to determine the percentage of
360 infected splenocytes. Approximately 1.5- and 2.5-fold increase of total splenocytes were positive
361 for virus replication after p.o. and i.p. inoculation, respectively (Fig. 4E, the left panel; Fig. 4F),
362 with a significant increase in the total number of AC-positive splenocytes in i.p.-infected mice
363 compared to p.o. (Fig. 4E, the right panel). This data is consistent with the significantly lower
364 viral loads in the spleen at 1 and 3 dpi in p.o.-inoculated mice (Fig. 3D), suggesting better virus
365 dissemination and replication and escape from mucosal immune clearance.

366 We then evaluated the growth characteristics of SADS-CoV in splenocytes by assessing
367 antigen production and replication kinetics *ex vitro*. Splenocytes were first extracted from naïve
368 mice, plated in 100 mm dishes and infected with 1×10^5 $TCID_{50}$ of SADS-CoV. We observed
369 clusters of infected cells that appeared to have been engulfed by phagocytes (Fig. 4G, the middle
370 panel), and the structural N protein was shown in the cytoplasm of infected cells by confocal
371 microscopy (Fig. 4G, the middle and right panels). The percentage of infected cells was
372 quantified by flow cytometry using anti-AC pAb, revealing that nearly 2-fold increase of the
373 splenocytes were positive for viral signals (Fig. 4H), very similar to the percentage of infection

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

374 observed *in vivo*. To further characterize the growth kinetic of SADS-CoV in primary
375 splenocytes, cells were infected with 1×10^5 $TCID_{50}$ of SADS-CoV, and culture supernatants
376 were harvested at 0, 12, 24, 48 and 72 hpi. Active viral replication was confirmed, with a 1.5-log
377 time-dependent increase in genomic RNA equivalents, plateauing from 24 to 72 hpi (Fig. 4I).
378 This data suggests that although only ~2% of splenocytes were infected, these cells supported a
379 decent level of viral replication. Together, these results indicate that SADS-CoV productively
380 infects mouse splenocytes.

381 **Splenic DCs support SADS-CoV replication.** Splenocytes were harvested from
382 i.p.-infected mice at 3 dpi, and the extracted cells were co-stained with antibodies against
383 SADS-CoV-AC and each of four cell surface markers (anti-CD19 for B cells; anti-CD4 for T
384 cells; anti-$CD11/c^+$ for DCs and anti-$F4/80^+$ for macrophages) using flow cytometry (Fig. 5A).
385 The percentage of infected $CD11/c^+$ cells was significantly higher than the other cell subgroups,
386 indicating that DCs are the major targets of SADS-CoV infection in the spleen.

387 The phenotype was further confirmed by double-staining IFA with anti-dsRNA, anti-M or
388 anti-AC antibody plus anti-$CD11/c^+$ in splenic sections. As expected, dsRNA staining
389 overlapped with the CD11/c surface marker on the edges of lymphatic follicles (Fig. 5B),
390 whereas no viral signals were seen in the mock-infected control (Fig. 5C). Similar patterns of
391 co-staining were detected by M and AC antibodies (Fig. 5D). To gain insight into the relative
392 quantity of DCs compared to other undefined target cells, cells positive for dsRNA and CD11/c
393 were counted in 10-15 different microscope fields of spleens from 3 infected mice (Fig. 5E),
394 showing that 61.76% of SADS-CoV-infected cells were DCs (Fig. 5F).

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

395 **SADS-CoV does not utilize known CoV protein receptors for cellular entry.** To our
396 knowledge, these results reveal the most extensive cell tropism among known CoVs, suggesting
397 the functional receptor(s) for SADS-CoV is likely to be a very common molecule. In order to test
398 this hypothesis, it was first necessary to find a cell line that was refractory to infection only at the
399 internalization step. MDCK cells, which showed undetectable virus production in early infection
400 tests (Fig. 1 and Fig. 2), were chosen as a potential candidate. There are four known types of
401 functional CoV protein receptors, including angiotensin converting enzyme 2 (ACE2) for
402 SARS-CoV (22), dipeptidyl peptidase 4 (DPP4) for MERS-CoV (23), aminopeptidase N (APN)
403 for TGEV (24) and PDCoV (25, 26), and mouse carcinoembryonic antigen-related cell adhesion
404 molecule 1a (mCEACAM1a) for MHV (27). To test whether one of these molecules serves as the
405 SADS-CoV receptor, we attempted to inoculate non-susceptible MDCK cells overexpressing
406 porcine APN, human DPP4, mouse CEACAM1a, or human ACE2 with SADS-CoV, but none of
407 them allowed infection as staining with anti-SADS-CoV-N pAb was negative (Fig. 6A).
408 Meanwhile, the expression of each receptor in MDCK cells was confirmed by IFA (Fig. 6A) and
409 western blot analysis (Fig. 6B) using antibodies against the tags fused to the receptors. As
410 positive controls, we confirmed that lentiviruses pseudotyped with TGEV, SARS-CoV,
411 MERS-CoV or MHV spike (i.e., pseudoviruses) efficiently entered MDCK cells exogenously
412 expressing the respective receptors (Fig. 6C).

413 Next, we demonstrated that MDCK cells can confer SADS-CoV replication competency by
414 transfection of a SADS-CoV/SeACoV infectious cDNA clone established recently (16), as
415 simultaneous expression of Nsp3-AC and N proteins were clearly detected by IFA (Fig. 6D).

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Moreover, passaging of supernatants from pSEA-transfected MDCK cells onto fresh Vero cells resulted in progeny SADS-CoV infection, as evidenced by expression of the N protein (Fig. 6E), indicating that MDCK cells can also support infectious SADS-CoV production without cell-to-cell spread. Therefore, SADS-CoV apparently does not utilize any of the known CoV receptors for cellular entry. The same conclusion was reached using HeLa cells overexpressing each of the four classical CoV receptors followed by SADS-CoV inoculation by Zhou et al (14); however, the HeLa cell line itself was most susceptible to SADS-CoV infection in the present study (Fig. 2D).

DISCUSSION

In order to assess the potential species barriers of SADS-CoV infection, a cell line susceptibility study was first conducted using 24 different cell lines. As SADS-CoV probably originated from a bat SADSr-CoV (14) derived from HKU2-CoV identified in *Rhinolophus sinicus* (Chinese horseshoe bats) (12), we commenced testing viral susceptibility in two available bat cell lines, BFK from *Myotis daubentonii* (18) and Tb-1 from *Tadarida brasiliensis*. Although BFK cells did not support SADS-CoV replication, it replicated efficiently in Tb-1 cells (Fig. 1A and Fig. 2), suggesting that other bat species in addition to horseshoe bats are likely susceptible to SADS-CoV infection.

Interestingly, SADS-CoV protein expression was detected in almost all of the rodent cells (hamster, gerbil, mouse and rat) including BHK-21, which is not susceptible to other known human CoVs such as SARS-CoV and MERS-CoV (28, 29) as well as three swine enteric CoVs,

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

437 PEDV, PDCoV and TGEV (25). Given the fact that SADS-CoV infects both primary and
438 passaged or primary cell lines originating from rodents, we hypothesized that rodents may be
439 susceptible to SADS-CoV infection. To explore this possibility, we challenged wild-type B6
440 mice with SADS-CoV by two different inoculation routes.

441 The challenged animals neither succumbed to infection nor manifested any signs of
442 gastroenteritis. In fact, experimental infection of neonatal piglets with a higher dose of purified
443 SADS-CoV in our laboratory only resulted in mild diarrheal signs or subclinical infection (11).
444 Also, there was a lack of robust viral replication in the intestines during infection, and no tissue
445 damage was detected throughout the intestines (Fig. 3B and 3C), reflecting the suboptimal
446 infection by SADS-CoV in immunocompetent wild-type mice. On the contrary, the virus had
447 more efficient replication within the spleen, reflected by a continuous detection of viral genomic
448 RNA in the immune cells at all time points over a 28-day period (Fig. 3D). The phenotype was
449 also consistent with the replication kinetics in extracted splenocytes *in vitro*, in which viral
450 genomic RNA peaked and plateaued at 72 hpi (Fig. 4G and 4I). This data collectively led to
451 speculation that SADS-CoV favors splenic cells over other tissues. The most logical explanation
452 for these tissue-specific discrepancies in virus replication is: i) target cells are more concentrated
453 in the spleen and more sporadic in the intestine; or ii) splenic immune cells have enhanced
454 expression of the unknown receptor(s) over intestinal cells. The animals were more susceptible
455 to i.p. infection, resulting in higher virus replication in the distal section of the small intestine,
456 large intestine and spleen, and perhaps a delayed clearance of viral infection in the cecum (Fig.
457 3B to 3E), suggesting the important role of mucosal immunity for controlling early infection in

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

458 SADS-CoV in mice. It should be note that mice (C57BL/6J mice in this study) may not be the
459 optimal rodent species for SADS-CoV infection, as wild rats are more commonly seen in
460 Chinese pig farms. In addition, other transmission routes may be considered. Recently, PDCoV
461 has been shown to possibly spread via the respiratory route in addition to fecal-oral transmission
462 (30). Therefore, it will be interesting to try intranasal route for inoculation in rats or the other
463 rodent species to mimic SADS-CoV natural transmission in future studies.

464 More interestingly, we identified DCs to be the precise cell population that supported
465 SADS-CoV replication (Fig. 5). There have been a few reports of immune cell tropism for CoVs.
466 Macrophages are susceptible to MHV infection, representing the largest group of innate immune
467 cells that infiltrate the central nervous system after infection with neurotropic MHV strains (31).
468 In addition, based on the fact that SARS-CoV spike-pseudotyped HIV-based vectors can
469 efficiently transduce human DCs, Kobinger *et al*. hypothesized that SARS-CoV infection in
470 immature DCs contributes to viral pathogenesis (32). Yang *et al*. demonstrated that SARS-CoV
471 can infect myeloid DCs via S glycoprotein-associated cell entry, and DC infection mediated viral
472 transmission to other cells *in vivo* (33). These previous evidences support our present results,
473 showing that SADS-CoV can efficiently replicate in DCs.

474 Furthermore, this study gives us a novel inspiration that rodents may potentially serve as
475 susceptible hosts for SADS-CoV in addition to bats and pigs. Of note, the species *Rhinolophus*
476 *bat α-CoV HKU2*, including SADS-CoV, possesses unique S genes closely related to the
477 betacoronavirus (β-CoV), in a manner similar to some globally distributed rodent α-CoVs (11, 34,
478 35), implying an unknown evolutionary connection between the bat α-CoV HKU2 and rodents

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

479 α-CoVs. In the field conditions of China, direct contact between pigs and flying bats is a low
480 probability; however, rodents (especially rats) are frequently visible in the swine industry,
481 causing great nuisance due to feed loss. It is possible that as bats prey on insects near pig
482 facilities, they leave feces containing HKU2-like CoVs that contaminate pig feed, which is then
483 eaten by pig and rodents that subsequently become carriers of SADS-CoV. Rats and mice are
484 increasingly implicated as external vectors for a wide range of different pig pathogens, such as *L.*
485 *intracellularis* (36). Rodents not only spread pathogens, but also harm the practitioners of the
486 swine industry, as they are thought to be the major source of leptospirosis in pigs and piggery
487 workers (37). Future study on identifying SADS-CoV-positive samples in rodents near pig farms
488 are warranted to test this hypothesis.

489 In addition to rodents, we also measured the SADS-CoV susceptibility of cell lines from
490 humans, monkeys, chickens and dogs, revealing a remarkably broad spectrum of tropism (Table
491 1 and Fig. 1). As for the ability of SADS-CoV to grow efficiently in human cell lines, we should
492 not underestimate the risk that this bat-origin CoV may 'jump' from pigs to humans. It is
493 noteworthy that camel workers with high rates of exposure to camel nasal and oral secretions had
494 evidence of MERS-CoV infection (38). Considering that SARS-CoV and MERS-CoV originated
495 from bats and spread from one species to another through intermediate hosts (civets and camels,
496 respectively), SADS-CoV may pose a similar risk to human health through transmission from
497 pigs or other susceptible hosts.

498 The cell susceptibility study and testing of overexpression of four known CoV receptors in
499 non-susceptible MDCK cells (Fig. 6) demonstrated that SADS-CoV might use a new receptor

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

molecule that is conserved in bats, pigs, rodents, chickens, monkeys and humans, indicating a low barrier to cross-species transmission. This is in line with the unusual feature of SADS-CoV's apparently broad species tropism.

In summary, these results provide important insights into the ecology of this bat-origin CoV, highlighting the possibility of its jumping interspecies barriers and the potential role of rodents as susceptible hosts in the field. Identification of the unknown SADS-CoV cellular receptor and further surveillance of other animal populations are needed to fully understand the biology of SADS-CoV.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program of China (2016YFD0500102), the National Natural Science Foundation of China (31872488) and the Fundamental Research Funds for the Central Universities of China (2019FZA6014). The professional editing service NB Revisions was used for technical preparation of the text prior to submission.

References

1. **Graham RL, Baric RS.** 2010. Recombination, reservoirs, and the modular spike: mechanisms of coronavirus cross-species transmission. J Virol **84:**3134-3146.

2. **van Boheemen S, de Graaf M, Lauber C, Bestebroer TM, Raj VS, Zaki AM, Osterhaus AD, Haagmans BL, Gorbalenya AE, Snijder EJ, Fouchier RA.** 2012. Genomic characterization of a newly

Accepted Manuscript Posted Online

discovered coronavirus associated with acute respiratory distress syndrome in humans. MBio **3:**e00473-00412.

3. **Soma T, Saito N, Kawaguchi M, Sasai K.** 2018. Feline coronavirus antibody titer in cerebrospinal fluid from cats with neurological signs. J Vet Med Sci **80:**59-62.

4. **Huang YW, Dickerman AW, Pineyro P, Li L, Fang L, Kiehne R, Opriessnig T, Meng XJ.** 2013. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. MBio **4:**e00737-00713.

5. **Lu G, Wang Q, Gao GF.** 2015. Bat-to-human: spike features determining 'host jump' of coronaviruses SARS-CoV, MERS-CoV, and beyond. Trends Microbiol **23:**468-478.

6. **Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, Tong S, Urbani C, Comer JA, Lim W, Rollin PE, Dowell SF, Ling AE, Humphrey CD, Shieh WJ, Guarner J, Paddock CD, Rota P, Fields B, DeRisi J, Yang JY, Cox N, Hughes JM, LeDuc JW, Bellini WJ, Anderson LJ, Group SW.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med **348:**1953-1966.

7. **Drexler JF, Corman VM, Drosten C.** 2014. Ecology, evolution and classification of bat coronaviruses in the aftermath of SARS. Antiviral Res **101:**45-56.

8. **Chan JF, Lau SK, To KK, Cheng VC, Woo PC, Yuen KY.** 2015. Middle East respiratory syndrome coronavirus: another zoonotic betacoronavirus causing SARS-like disease. Clin Microbiol Rev **28:**465-522.

9. **Yin Y, Wunderink RG.** 2018. MERS, SARS and other coronaviruses as causes of pneumonia. Respirology **23:**130-137.

10. **Hulswit RJ, de Haan CA, Bosch BJ.** 2016. Coronavirus Spike Protein and Tropism Changes. Adv Virus

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Accepted Manuscript Posted Online

Res **96:**29-57.

11. **Pan Y, Tian X, Qin P, Wang B, Zhao P, Yang YL, Wang L, Wang D, Song Y, Zhang X, Huang YW.** 2017. Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. Vet Microbiol **211:**15-21.

12. **Lau SK, Woo PC, Li KS, Huang Y, Wang M, Lam CS, Xu H, Guo R, Chan KH, Zheng BJ, Yuen KY.** 2007. Complete genome sequence of bat coronavirus HKU2 from Chinese horseshoe bats revealed a much smaller spike gene with a different evolutionary lineage from the rest of the genome. Virology **367:**428-439.

13. **Gong L, Li J, Zhou Q, Xu Z, Chen L, Zhang Y, Xue C, Wen Z, Cao Y.** 2017. A New Bat-HKU2-like Coronavirus in Swine, China, 2017. Emerg Infect Dis **23:**1607-1609.

14. **Zhou P, Fan H, Lan T, Yang XL, Shi WF, Zhang W, Zhu Y, Zhang YW, Xie QM, Mani S, Zheng XS, Li B, Li JM, Guo H, Pei GQ, An XP, Chen JW, Zhou L, Mai KJ, Wu ZX, Li D, Anderson DE, Zhang LB, Li SY, Mi ZQ, He TT, Cong F, Guo PJ, Huang R, Luo Y, Liu XL, Chen J, Huang Y, Sun Q, Zhang XL, Wang YY, Xing SZ, Chen YS, Sun Y, Li J, Daszak P, Wang LF, Shi ZL, Tong YG, Ma JY.** 2018. Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. Nature **556:**255-258.

15. **Wang L, Su S, Bi Y, Wong G, Gao GF.** 2018. Bat-Origin Coronaviruses Expand Their Host Range to Pigs. Trends Microbiol **26:**466-470.

16. **Yang YL, Liang QZ, Xu SY, Mazing E, Xu GH, Peng L, Qin P, Wang B, Huang YW.** 2019. Characterization of a novel bat-HKU2-like swine enteric alphacoronavirus (SeACoV) infection in cultured cells and development of a SeACoV infectious clone. Virology **536:**110-118.

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Accepted Manuscript Posted Online

563 17. **Ji CM, Wang B, Zhou J, Huang YW.** 2018. Aminopeptidase-N-independent entry of porcine epidemic
564 diarrhea virus into Vero or porcine small intestine epithelial cells. Virology **517:**16-23.
565 18. **He B, Yang F, Yang W, Zhang Y, Feng Y, Zhou J, Xie J, Feng Y, Bao X, Guo H, Li Y, Xia L, Li N,**
566 **Matthijnssens J, Zhang H, Tu C.** 2013. Characterization of a novel G3P[3] rotavirus isolated from a
567 lesser horseshoe bat: a distant relative of feline/canine rotaviruses. J Virol **87:**12357-12366.
568 19. **Ou XY, Zheng WL, Shan YW, Mu ZX, Dominguez SR, Holmes KV, Qian ZH.** 2016. Identification of
569 the Fusion Peptide-Containing Region in Betacoronavirus Spike Glycoproteins. Journal of Virology
570 **90:**5586-5600.
571 20. **Hofmann M, Wyler R.** 1988. Propagation of the virus of porcine epidemic diarrhea in cell culture. J Clin
572 Microbiol **26:**2235-2239.
573 21. **Wicht O, Li W, Willems L, Meuleman TJ, Wubbolts RW, van Kuppeveld FJ, Rottier PJ, Bosch BJ.**
574 2014. Proteolytic activation of the porcine epidemic diarrhea coronavirus spike fusion protein by trypsin in
575 cell culture. J Virol **88:**7952-7961.
576 22. **Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga**
577 **K, Greenough TC, Choe H, Farzan M.** 2003. Angiotensin-converting enzyme 2 is a functional receptor
578 for the SARS coronavirus. Nature **426:**450-454.
579 23. **Raj VS, Mou H, Smits SL, Dekkers DH, Muller MA, Dijkman R, Muth D, Demmers JA, Zaki A,**
580 **Fouchier RA, Thiel V, Drosten C, Rottier PJ, Osterhaus AD, Bosch BJ, Haagmans BL.** 2013.
581 Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. Nature
582 **495:**251-254.
583 24. **Delmas B, Gelfi J, L'Haridon R, Vogel LK, Sjostrom H, Noren O, Laude H.** 1992. Aminopeptidase N

Journal of Virology
JVI
Journal of Virology
Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Accepted Manuscript Posted Online

is a major receptor for the entero-pathogenic coronavirus TGEV. Nature **357:**417-420.

25. **Wang B, Liu Y, Ji CM, Yang YL, Liang QZ, Zhao P, Xu LD, Lei XM, Luo WT, Qin P, Zhou J, Huang YW.** 2018. Porcine deltacoronavirus engages the transmissible gastroenteritis virus functional receptor porcine aminopeptidase N for infectious cellular entry. J Virol **92:**e00318-00318.

26. **Li W, Hulswit RJG, Kenney SP, Widjaja I, Jung K, Alhamo MA, van Dieren B, van Kuppeveld FJM, Saif LJ, Bosch BJ.** 2018. Broad receptor engagement of an emerging global coronavirus may potentiate its diverse cross-species transmissibility. Proc Natl Acad Sci U S A **115:**E5135-E5143.

27. **Williams RK, Jiang GS, Holmes KV.** 1991. Receptor for mouse hepatitis virus is a member of the carcinoembryonic antigen family of glycoproteins. Proc Natl Acad Sci U S A **88:**5533-5536.

28. **Chan JF, Chan KH, Choi GK, To KK, Tse H, Cai JP, Yeung ML, Cheng VC, Chen H, Che XY, Lau SK, Woo PC, Yuen KY.** 2013. Differential cell line susceptibility to the emerging novel human betacoronavirus 2c EMC/2012: implications for disease pathogenesis and clinical manifestation. J Infect Dis **207:**1743-1752.

29. **Muller MA, Raj VS, Muth D, Meyer B, Kallies S, Smits SL, Wollny R, Bestebroer TM, Specht S, Suliman T, Zimmermann K, Binger T, Eckerle I, Tschapka M, Zaki AM, Osterhaus AD, Fouchier RA, Haagmans BL, Drosten C.** 2012. Human coronavirus EMC does not require the SARS-coronavirus receptor and maintains broad replicative capability in mammalian cell lines. MBio **3:**e00515-00512.

30. **Woo PC, Lau SK, Tsang CC, Lau CC, Wong PC, Chow FW, Fong JY, Yuen KY.** 2017. Coronavirus HKU15 in respiratory tract of pigs and first discovery of coronavirus quasispecies in 5'-untranslated region. Emerg Microbes Infect **6:**e53.

31. **Mazaleuskaya L, Veltrop R, Ikpeze N, Martin-Garcia J, Navas-Martin S.** 2012. Protective role of

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Journal of Virology JVI Journal of Virology

Accepted Manuscript Posted Online

605 Toll-like Receptor 3-induced type I interferon in murine coronavirus infection of macrophages. Viruses
606 **4:**901-923.

607 32. **Kobinger GP, Limberis MP, Somanathan S, Schumer G, Bell P, Wilson JM.** 2007. Human
608 immunodeficiency viral vector pseudotyped with the spike envelope of severe acute respiratory syndrome
609 coronavirus transduces human airway epithelial cells and dendritic cells. Hum Gene Ther **18:**413-422.

610 33. **Yang ZY, Huang Y, Ganesh L, Leung K, Kong WP, Schwartz O, Subbarao K, Nabel GJ.** 2004.
611 pH-dependent entry of severe acute respiratory syndrome coronavirus is mediated by the spike glycoprotein
612 and enhanced by dendritic cell transfer through DC-SIGN. J Virol **78:**5642-5650.

613 34. **Tsoleridis T, Chappell JG, Onianwa O, Marston DA, Fooks AR, Monchatre-Leroy E, Umhang G,**
614 **Muller MA, Drexler JF, Drosten C, Tarlinton RE, McClure CP, Holmes EC, Ball JK.** 2019. Shared
615 Common Ancestry of Rodent Alphacoronaviruses Sampled Globally. Viruses **11**.

616 35. **Wang W, Lin XD, Guo WP, Zhou RH, Wang MR, Wang CQ, Ge S, Mei SH, Li MH, Shi M, Holmes**
617 **EC, Zhang YZ.** 2015. Discovery, diversity and evolution of novel coronaviruses sampled from rodents in
618 China. Virology **474:**19-27.

619 36. **Collins AM, Fell S, Pearson H, Toribio JA.** 2011. Colonisation and shedding of Lawsonia intracellularis
620 in experimentally inoculated rodents and in wild rodents on pig farms. Vet Microbiol **150:**384-388.

621 37. **Everard CO, Ferdinand GA, Butcher LV, Everard JD.** 1989. Leptospirosis in piggery workers on
622 Trinidad. J Trop Med Hyg **92:**253-258.

623 38. **Alshukairi AN, Zheng J, Zhao J, Nehdi A, Baharoon SA, Layqah L, Bokhari A, Al Johani SM,**
624 **Samman N, Boudjelal M, Ten Eyck P, Al-Mozaini MA, Zhao J, Perlman S, Alagaili AN.** 2018. High
625 Prevalence of MERS-CoV Infection in Camel Workers in Saudi Arabia. MBio **9:**e01985-01918.

Journal of Virology
JVI
Journal of Virology
Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

626

627 # FIGURE LEGENDS

628

629 **Figure 1. Immunofluorescence assay showing susceptibility of different cell lines to**
630 **SADS-CoV infection.** Immunofluorescence assay of cells infected with SADS-CoV at an
631 MOI=0.01 was performed using rabbit anti-SADS-CoV-M polyclonal Ab (200× magnification)
632 and Alexa Fluor 488-conjugated anti-rabbit IgG as secondary antibody, with DAPI for
633 visualization of cell nuclei. Mock-infected cells were treated with the same procedures as
634 appropriate. Cells were tested from different species of origin, including: **(A)** Bats (BFK and
635 Tb-1); **(B)** Hamsters (CHO and BHK-21); **(C)** Mice (NIH/3T3 and RAW264.7); **(D)** Rats
636 (BRL-3A and NRK-52E); **(E)** Humans (Huh-7, HepG2, 293T, A549, and HeLa); **(F)** Monkeys
637 (Marc-145, Cos-7, BSC-40, and Vero); **(G)** Pigs (ST, PK15, LLC-PK1, and IPEC-J2); **(H)**
638 Chickens (DF-1); **(I)** Dogs (MDCK); and **(J)** Gerbil primary kidney cells.

639

640 **Figure 2. Growth of SADS-CoV in different cell lines through five days post-infection.** To
641 determine the effect of trypsin on SADS-CoV infection, each cell line was infected in three
642 conditions: **(A)** "No trypsin" treatment: inoculated with SADS-CoV diluted in maintenance
643 medium (MM) for 2 h, and subsequently replaced with MM; **(B)** "Pre-trypsin" treatment:
644 inoculated with SADS-CoV diluted in MM containing 5 μg/ml trypsin (MMT) for 2 h, and
645 subsequently replaced with MM; and **(C)** "Double-trypsin" treatment: inoculated with
646 SADS-CoV in MMT, and subsequently replaced with MMT. Infection supernatants were

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

collected at 12, 24, 36, 48, 72 and 120 hpi for viral load detection by a qRT-PCR assay targeting the viral N gene. Data is expressed as the mean viral load (log_{10} copies/µl) ± standard deviation (SD), and all experiments were performed in triplicate. The 293T, CHO, BRL-3A and NRK-52E cell lines did not survive in the presence of trypsin. **(D)** Infectious titers ($TCID_{50}$/ml) of SADS-CoV secreted from HeLa, Vero, Tb-1, BHK-21 PK-15 and MDCK cells were determined on Vero cells.

Figure 3. SADS-CoV infection of mice. C57BL/6J WT mouse were infected per orally (p.o; black) or intraperitoneally (i.p; red) with 5×10^5 $TCID_{50}$ of purified SADS-CoV. Viral loads in different tissue samples including **(A)** stomach, **(B)** small intestinal segments, **(C)** large intestinal segments, **(D)** lymphoid tissues, **(E)** the other organs (liver, kidney, heart and lung) and **(F)** feces collected at 1, 3, 5, 7, 14, 21, and 28 days post-infection (dpi) were determined by qRT-PCR; MLN: Mesenteric lymph nodes. Data are from three independent experiments, and each symbol represents titers from an individual sample (*: $p<0.05$). The limit of detection was 1×10^2 genome copies/mg. **(G)** SADS-CoV IgG antibodies were detected in serum samples collected at euthanasia, using an ELISA based on purified SADS-CoV virus particles.

Figure 4. SADS-CoV replication in mouse splenocytes. (A) Hematoxylin & eosin staining (HE) and immunohistochemistry (IHC) were performed on sections of spleen from intraperitoneally infected mice and **(B)** Mock-infected mice at 3 dpi, using anti-dsRNA antibodies to identify splenic cells that support active virus replication. **(C)** SADS-CoV infection could also be

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

668 detected by IHC using SADS-CoV nonstructural protein antibody (anti-AC) and structural
669 protein antibody (anti-M). Scale bars=50 μm, except for magnified fields shown on the right side,
670 with scale bars=10 μm. **(D)** SADS-CoV antibody validation in Vero cells for developing the flow
671 cytometry assay. Flow cytometry plots of Vero cells infected with SADS-CoV (MOI=0.1) at 24
672 hpi, staining with anti-N or anti-AC. **(E)** Flow cytometry detection of Nsp3-AC antigens of
673 SADS-CoV in splenocytes from infected mice using anti-AC antibody at 3 dpi. The data are
674 presented as the fold-increase in staining splenocytes from infected mice relative to the
675 mock-infected group for statistical purposes (left panel); *: $p<0.05$. **(F)** Representative
676 FACS plots of panel (E). The solid-line frame gated anti-AC positive splenocytes from p.o. or i.p.
677 inoculated mice. The plot of mock-infected cells stained with secondary antibody only was also
678 shown. **(G)** Isolated mouse splenocytes were infected with SADS-CoV at an MOI=1, and
679 SADS-CoV N protein expression was detected by IFA with anti-N antibody. **(H)** Flow cytometry
680 detection of nonstructural protein antigens of SADS-CoV in infected splenocytes at 48 hpi using
681 anti-AC antibody. The data are presented as the fold-increase in staining cells relative to the
682 mock-infected cells for statistical purposes (left panel); *: $p<0.05$. **(I)** Growth of SADS-CoV in
683 *ex vivo* splenocytes was monitored over 72 hpi by qRT-PCR targeting the SADS-CoV N gene.
684
685 **Figure 5. SADS-CoV infection of dendritic cells in the spleen of mice inoculated via i.p.**
686 **route. (A)** Splenocytes were extracted from infected mice at 3 dpi, and flow cytometry was used
687 to detect nonstructural antigen AC of SADS-CoV with immune cell markers on splenocytes
688 including B cells ($CD19^+$), T cells ($CD4^+$), macrophages ($F4/80^+$), and dendritic cells (DCs,

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

689 $CD11c^{+}$). The data are presented as the fold-increase in positive staining cells from infected mice
690 relative to the mock-infected cells for statistical purposes; ***: $p<0.001$. **(B)**
691 Immunofluorescence assay of SADS-CoV dsRNA and DC marker CD11c in sections of spleen
692 from intraperitoneally (i.p.) infected mice and **(C)** mock-infected mice at 3 dpi. **(D)** SADS-CoV
693 infection could also be detected by IFA using anti-AC and anti-M antibodies. **(E)** The numbers of
694 SADS-CoV-positive DCs in i.p.-infected mice were counted and averaged from 10-15 different
695 visual fields, and **(F)** the proportion of DCs in infected cells was presented with a Venn diagram.
696 Scale bars = 50 μm, except for magnified fields shown on the right side, with scale bars =10 μm.
697
698 **Figure 6. SADS-CoV utilizes an unknown receptor for cellular entry. (A)** MDCK cells
699 overexpressing each of the four known CoV receptors fused with detectable tags (pAPN-Flag,
700 hDPP4-Flag, mCEACAM1a-Flag, or hACE2-GFP) did not confer SADS-CoV infection at 24 h
701 post-transfection of the expression plasmids. At 48 h, SADS-CoV-inoculated cells transfected
702 with pAPN-Flag, hDPP4-Flag, or mCEACAM1a-Flag were co-stained with a mouse anti-FLAG
703 MAb and a rabbit anti-SADS-CoV-N pAb. Alexa Fluor 488-conjugated anti-mouse IgG and
704 Alexa Fluor 594-conjugated anti-rabbit IgG were co-stained for secondary antibody detection,
705 followed by DAPI incubation. For challenged cells transfected with hACE2-GFP,
706 anti-SADS-CoV-N pAb and Alexa Fluor 594-conjugated anti-rabbit IgG were used;
707 magnification=200×. **(B)** Western blot analysis also confirmed the expression of CoV receptors
708 in transfected MDCK cells. **(C)** TGEV-, SARS-CoV-, MERS-CoV- or MHV-spike-mediated
709 pseudovirus entry into MDCK cells overexpressing the corresponding receptor. The pseudovirus

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

entry efficiency was characterized as luciferase activity accompanying the entry. Cells transfected with the empty backbone vector were used as controls. **(D)** Rescue of SADS-CoV in MDCK cells transfected with a SeACoV infectious cDNA clone. Detection of expression of Nsp3-AC and N proteins of SADS-CoV was conducted at 72 h post-transfection by co-staining with a rabbit anti-AC pAb and a mouse anti-N pAb (magnification = 200×). **(E)** Infection of fresh Vero cells with progeny SADS-CoV rescued in MDCK cells. The expression of SADS-CoV N protein was detected by staining with anti-N pAb at 36 hpi.

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Table 1. Summary of human and animal cell lines and their susceptibility to SADS-CoV infection as determined by cytopathic effect (CPE) and IFA.

Cell lines			Without Trypsin		With Trypsin	
Species and/or tissue origin	Name	ATCC® Number	IFA	CPE	IFA	CPE
Human						
Hepatocellular carcinoma	Huh-7	N/A	++	-	++	-
Hepatocellular carcinoma	HepG2/C3A	HB-8065	+	-	++	-
Embryonic kidney	293T	CRL-11268	+	-	N/D	N/D
Lung carcinoma	A549	CCL-185EMT	+	-	++	-
Cervix adenocarcinoma	HeLa	CCL-2	+	-	+	-
Monkey						
African green monkey kidney	Marc-145	N/A	+	-	++	+
African green monkey kidney	Cos-7	CRL-1651	++	-	+++	++
African green monkey kidney	BSC-40	CRL-2761	++	-	+++	++
African green monkey kidney	Vero	CRL-1586	++	+	+++	+++
Swine						
Testis	ST	CRL-1746	+	+	++	++
Kidney	PK15	CCL-33	+	-	++	+
Kidney	LLC-PK1	CL-101	+	-	++	+
Small intestinal epithelium	IPEC-J2	N/A	+	-	+	-
Bat						
Myotis petax, fetal kidney	BFK	N/A	-	-	-	-
Tadarida brasiliensis, lung	Tb-1	CCL-88	+	-	++	-
Canine						
Kidney	MDCK	CCL-34	-	-	-	-
Mouse						
Embryo fibroblasts	NIH/3T3	CRL-1658	+	-	N/D	N/D
Monocyte/macrophage	RAW 264.7	TIB-71	-	-	-	-
Hamster						
Syrian golden hamster, kidney	BHK-21	CCL-10	+	-	+++	+
Chinese hamster, ovary	CHO	CCL-61	+	-	N/D	N/D
Rat						
Liver	BRL 3A	CRL-1442	++	+	N/D	N/D
Kidney	NRK-52E	CRL-1571	+	-	N/D	N/D
Gerbil						
Primary kidney cells		N/A	++	-	N/D	N/D
Chicken						
Embryo fibroblasts	DF-1	CRL-12203	+	-	N/D	N/D

Degree of infection as determined by IFA or CPE (-: No infection or obvious lesion≤1%; +: ≤25%; ++: ≤50%; +++: ≤75%; ++++: ≤100%);

N/A: Not available; N/D: Not detected due to cell sensitivity to trypsin.

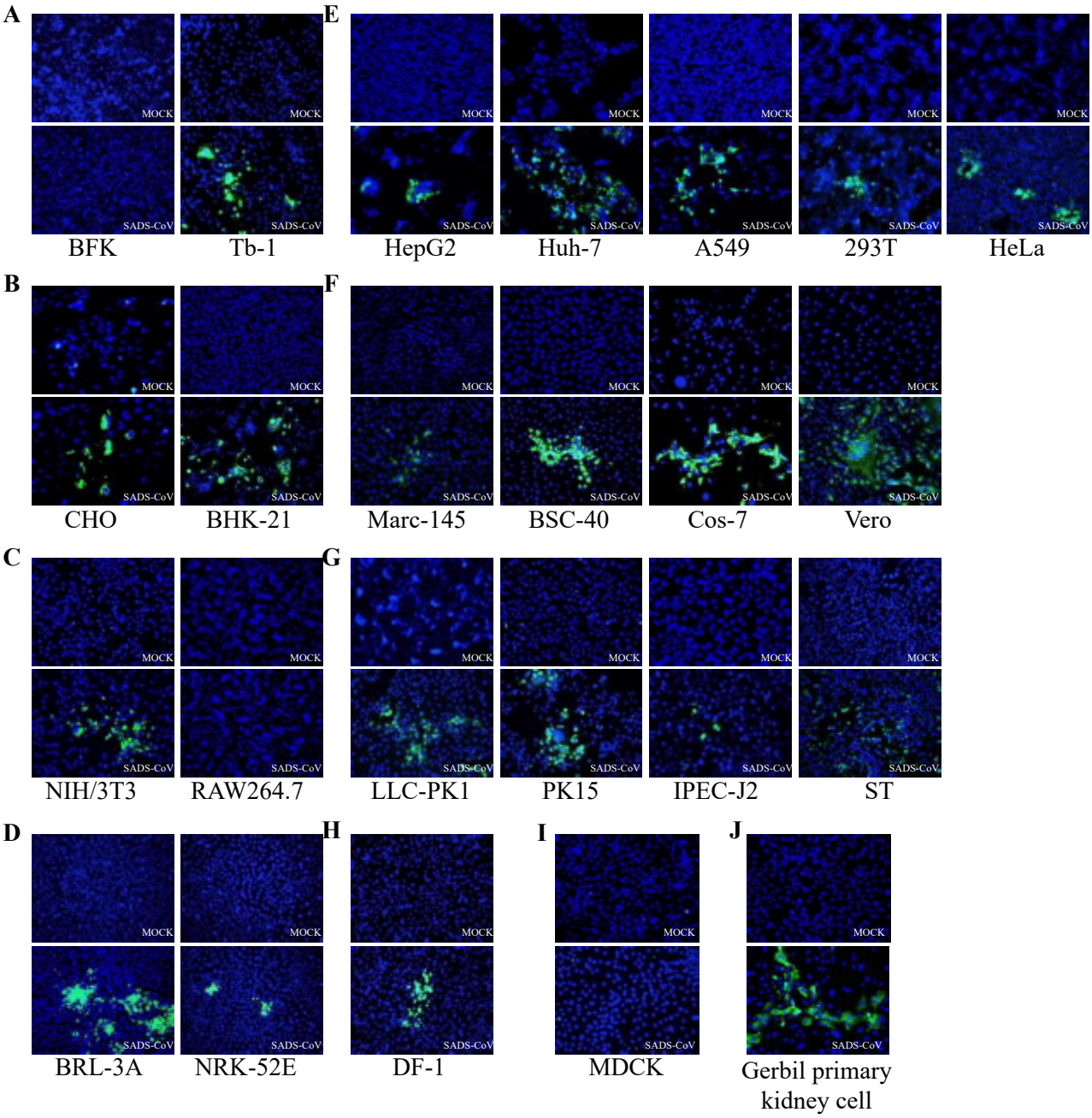
Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Figure 1



Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

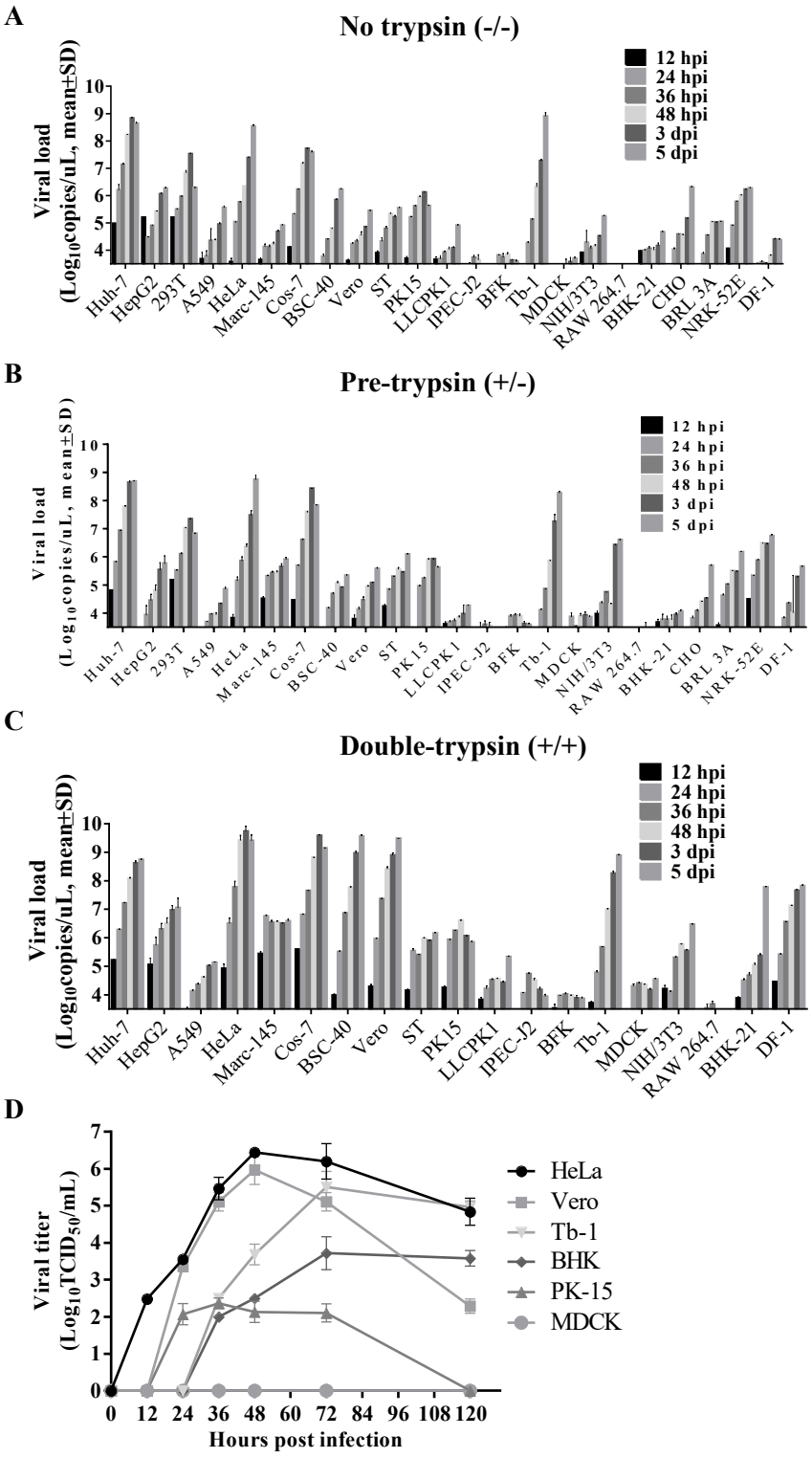
Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

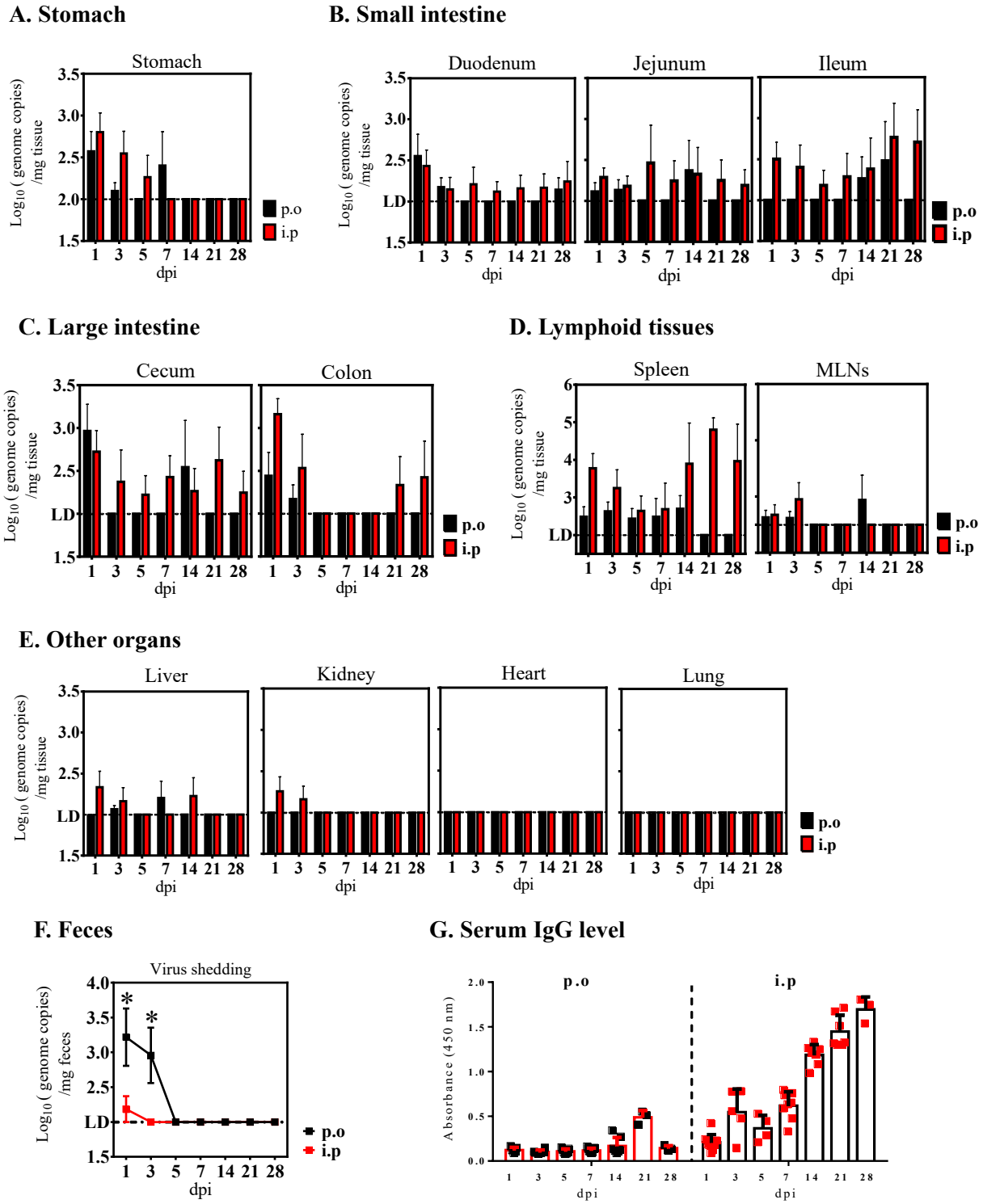
Figure 2



Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Accepted Manuscript Posted Online
Journal of Virology
JVI
Journal of Virology

Figure 3



Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

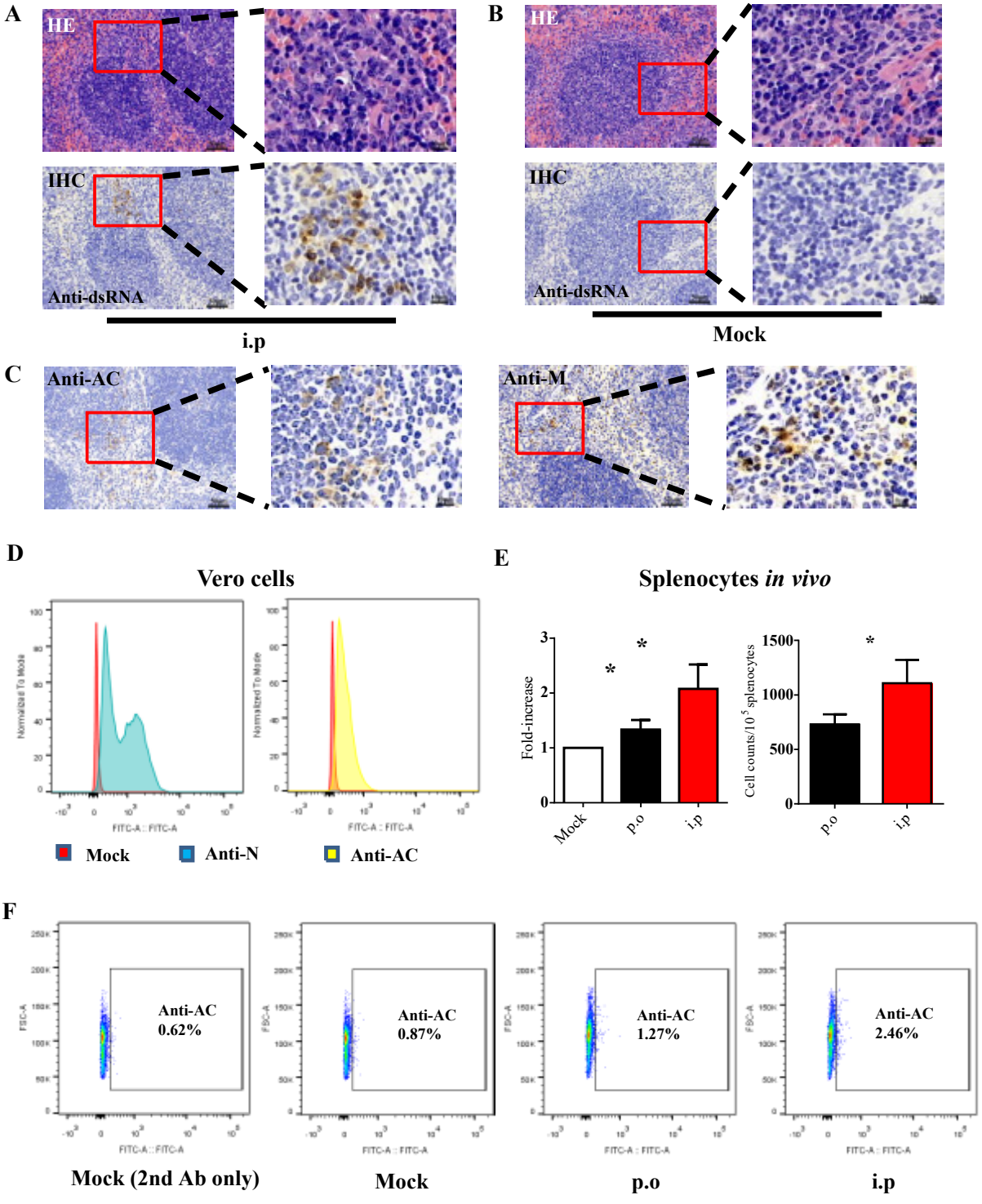
Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

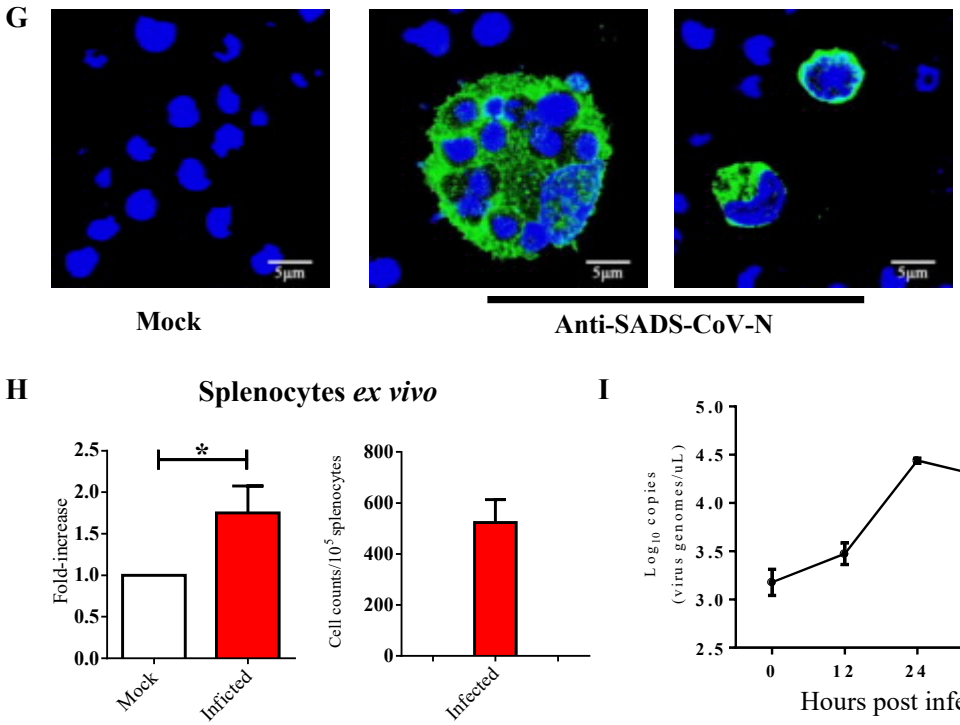
Figure 4



Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Accepted Manuscript Posted Online

Figure 4 (continued)



Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Journal of Virology

JVI

Journal of Virology

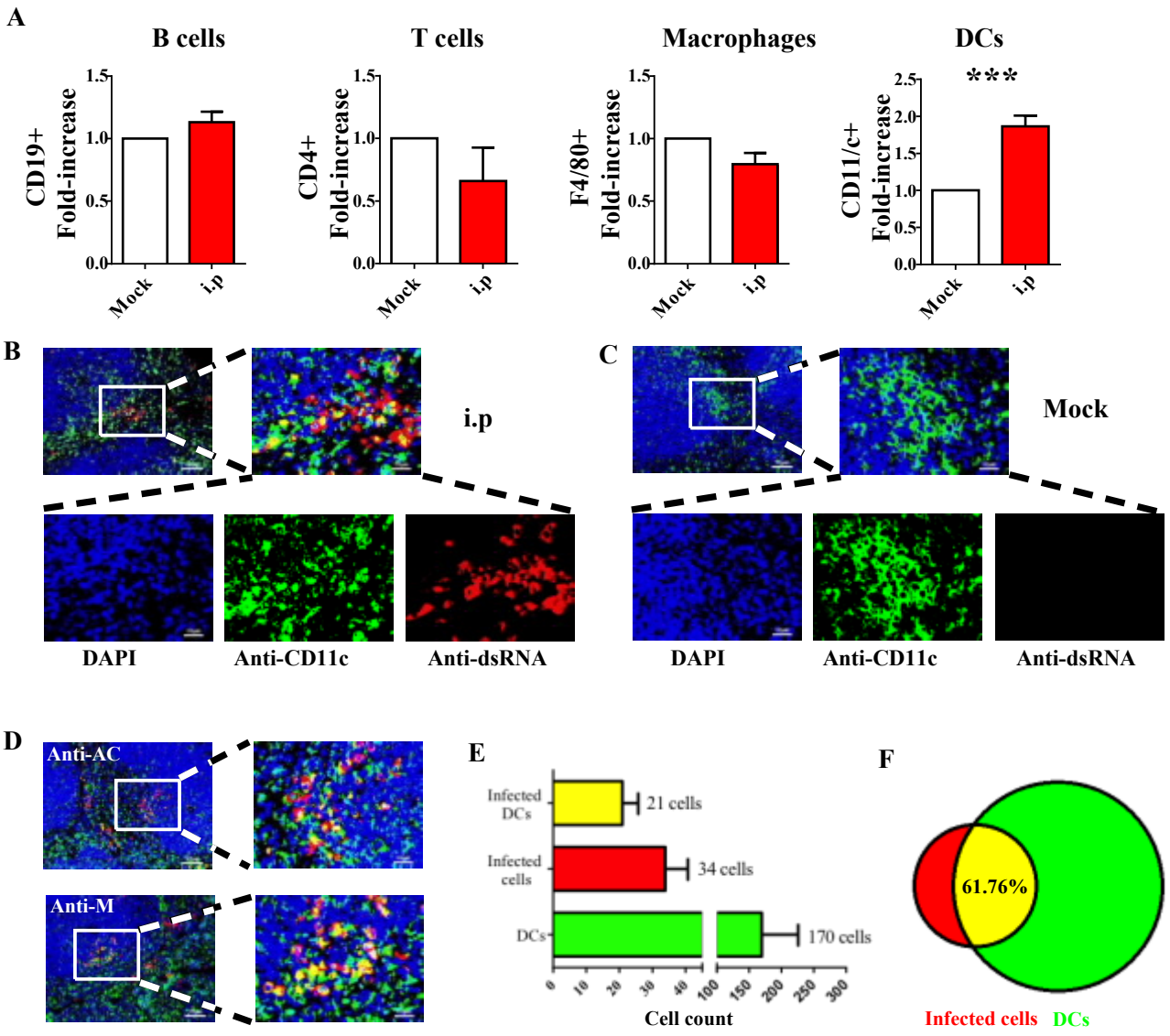
Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Figure 5



Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

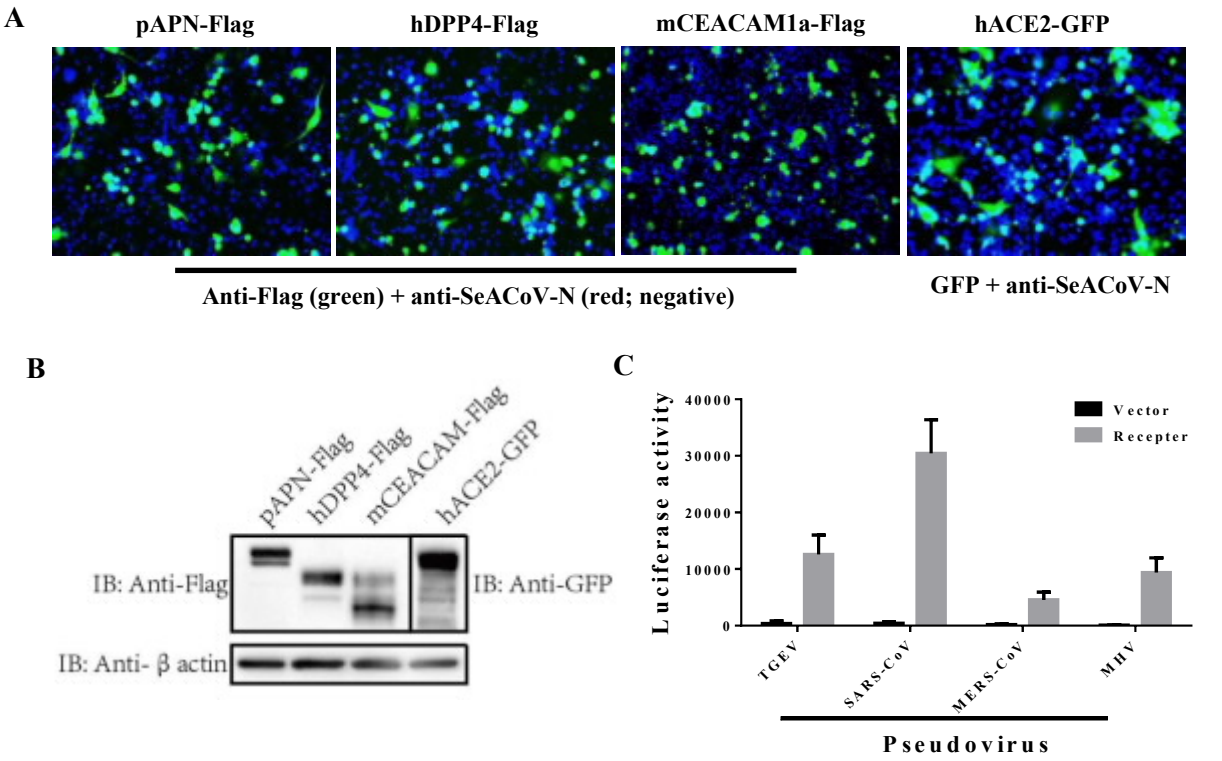
Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Figure 6



Downloaded from http://jvi.asm.org/ on October 22, 2019 at Columbia University Libraries

Received: 25 March 2019 | Revised: 26 May 2019 | Accepted: 11 June 2019

DOI: 10.1111/tbed.13270

Check for updates

SHORT COMMUNICATION

Transboundary and Emerging Diseases WILEY

The re-emerging of SADS-CoV infection in pig herds in Southern China

Ling Zhou[1,2] | Qian N. Li[1,2] | Jiang N. Su[1] | Gui H. Chen[1] | Zi X. Wu[1] | Ying Luo[1] | Rui T. Wu[1,2] | Yuan Sun[1,2] | Tian Lan[1] | Jing Y. Ma[1,2]

[1]College of Animal Science, South China Agricultural University, Guangzhou, China

[2]Key Laboratory of Animal Health Aquaculture and Environmental Control, Guangzhou, China

Correspondence
Jing Y. Ma, College of Animal Science, South China Agricultural University, Tianhe District, Wushan Road 483, Guangzhou 510642, China.
Email: majy2400@scau.edu.cn

Funding information
National Key Research and Development Program of China, Grant/Award Number: 2016YFD0501304; Science and Technology Program of Guangzhou City of China, Grant/Award Number: 201904010433

Abstract

A new highly virulent swine acute diarrhoea syndrome coronavirus (SADS-CoV) emerged in Guangdong province in 2017 followed by fatal diarrhoea that involved the death of 24,693 piglets. And yet from May 2017 to January 2019, there were no new SADS cases arising in pig herds in Guangdong. In this study, we reported the recent diarrhoea outbreak of SADS-CoV in Southern China on February 2019. Intestinal samples collected from diarrhoeal piglets were detected for common swine virus and confirmed that SADS-CoV was responsible for the diarrhoea case. Meanwhile, serological investigation of sows' sera implied that SADS-CoV has existed in the farm and PEDV antibody may not directly contribute to the amplification of SADS-CoV. Homology and phylogenetic analysis of the whole genome showed that the re-emerging SADS-CoV strain shared high sequence identities with existing SADS-CoV strains and all strains clustered together in Alpha coronavirus. All in all, the report herein emphasized the re-emerging of SADS-CoV and highlights continuous monitoring for this virus.

KEYWORDS
phylogenetic analysis, re-emerging, SADS-CoV, Southern China

1 | INTRODUCTION

Swine acute diarrhoea syndrome (SADS) was first identified in South China in January 2017 as a disease causing high mortality in neonatal piglets (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018). It is quickly demonstrated that the causative agent of SADS is a novel HKU2-related coronavirus, swine acute diarrhoea syndrome coronavirus (SADS-CoV) which originated from the same genus of horseshoe bats (*Rhinolophus*) as severe acute respiratory syndrome coronavirus (SARS-CoV), but the mechanism of transmission of bat-originated coronavirus in pigs needs further illumination (Cui, Li, & Shi, 2019; Zhou et al., 2018). Based on the analysis of genomes, SADS-CoV is classified into the genus *Alphacoronavirus* within the subfamily *Orthocoronavirinae* of the family *Coronaviridae* (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018). So far, besides porcine epidemic diarrhoea virus (PEDV), transmissible gastroenteritis virus (TGEV) and porcine deltacoronavirus (PDCoV), SADS-CoV is the fourth porcine enteric coronavirus that circulated in pigs in China (Dong et al., 2015; Xuan et al., 1984). A retrospective investigation showed that SADS-CoV emerged in China at least since August 2016 (Zhou et al., 2019). Li et al. (2018) reported one SADS-CoV strain that was isolated from fecal and small intestinal samples collected from Fujian province and suggested that SADS-CoV might have spread from Guangdong to Fujian province.

In the first outbreak of SADS-CoV occurring between January and May 2017, in order to reduce mortality in suckling pigs, the minced intestines from acutely infected piglets were filtered, centrifuged and inactivated before feeding back to pregnant sows to allow maternal antibodies to be obtained in neonatal piglets. Hence, after May 2017, the epidemic was controlled. And with a period of quiescence, SADS-CoV seemed to have disappeared from pig herds in South China. While on February 2019, there was a large-scale

 © 2019 Blackwell Verlag GmbH

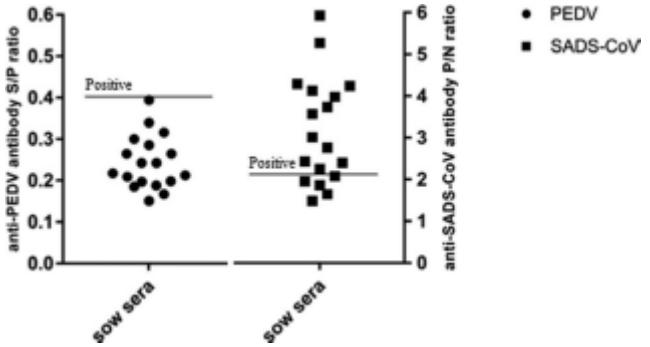


FIGURE 1 The results of ELISA test, *n* = 18 sows sera from outbreak farm. Detection of PEDV antibodies, sample S/P ratios ≥ 0.4 were considered positive. Detection of SADS-CoV antibodies, P/N ratios ≥ 2.1 were considered positive

outbreak of approximately 2000 piglets dying of diarrhoea in one farm, which was near the origin of the SADS pandemic in 2017. The extensive aetiological investigation for common swine viruses suggested that the causative agent of this recent diarrhoea outbreak was SADS-CoV. In this study, we compared the infections of piglets with the original and re-emerging SADS-CoV strains by clinical signs, epidemiological investigation, serological investigation and phylogenetic analysis. The results highlight the significance of SADS-CoV and the disease it causes.

At the beginning of the latest outbreak on February 2019, infected suckling piglets developed acute diarrhoea, vomiting and died after 2–6 days of the diarrhoea, which were consistent with the clinical signs reported in SAD-CoV outbreaking farms (Zhou et al., 2018). Two weeks after the onset, mortality did not increase due to the effective inactivated SADS-CoV vaccine which was propagated in vitro (data unpublished). Clinical small intestine samples were collected rapidly from piglets, and intestinal samples were homogenized, centrifuged and filtered for virus isolate. Viral nucleic acid was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instruction. The potential swine viral pathogens were examined, including SADS-CoV, PEDV, PDCoV, porcine rotavirus (RV), TGEV, reovirus (REO), senecavirus A (SVA), foot and mouth disease virus (FMDV), porcine pseudorabies virus (PRV), porcine circovirus 2 (PCV2), porcine circovirus 3 (PCV3), porcine reproductive

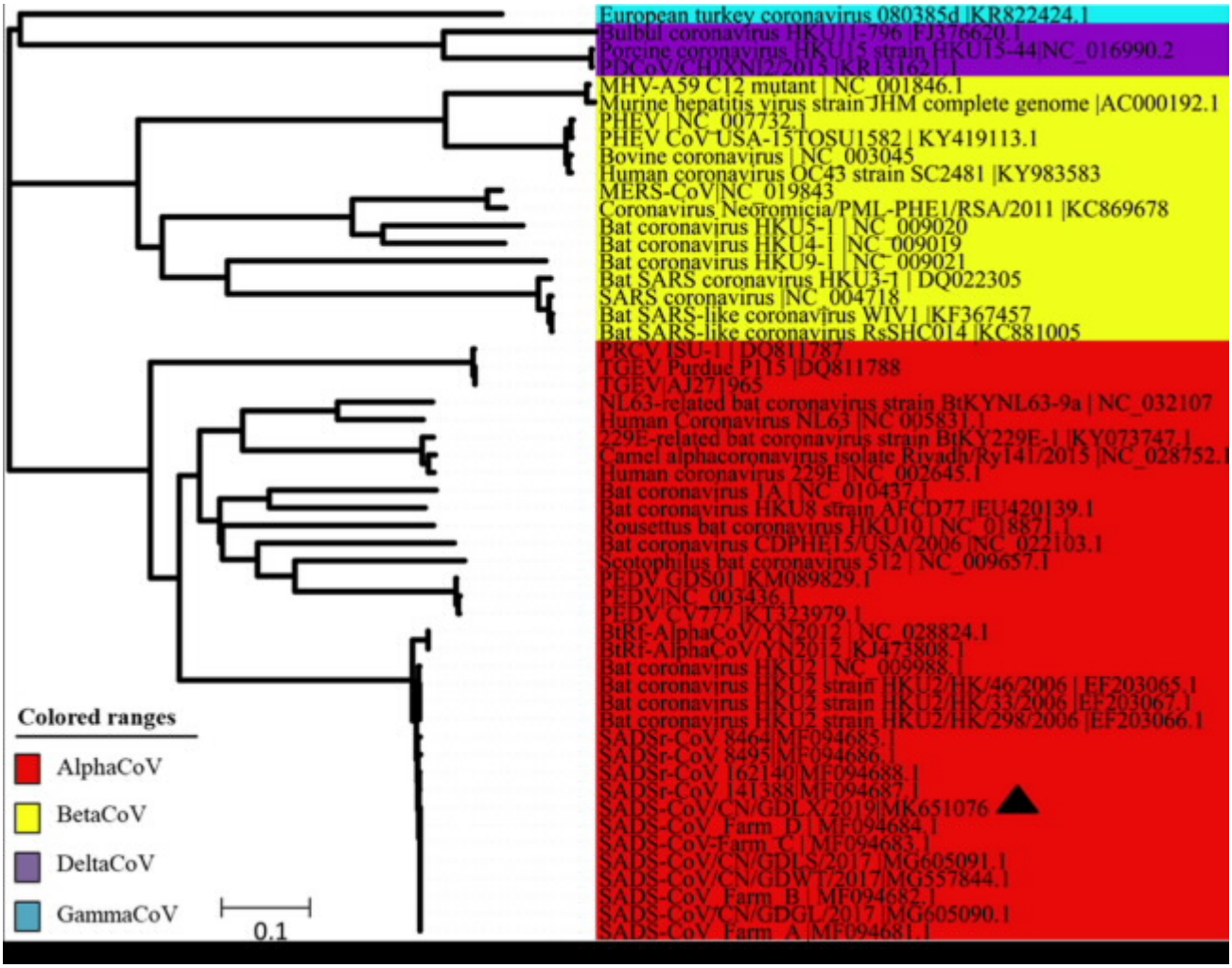


FIGURE 2 Phylogenetic analysis of the complete genome of SADS-CoV and reference coronavirus species. The tree was constructed using MEGA 7.0 software with neighbour-joining methods and 1,000 replicate sets on bootstrap analysis [Colour figure can be viewed at wileyonlinelibrary.com]

and respiratory syndrome virus (PRRSV), porcine parainfluenza virus 1 (PPIV1), swine influenza virus (SIV), atypical porcine pestivirus (APPV), haemophilus parasuis (HPS), hog cholera (HC), porcine parvovirus (PPV), porcine bocavirus (PBoV), porcine sapelovirus (PSV) and porcine kobuvirus (PKV) by PCR or RT-PCR following the established protocol of Key Laboratory of Animal Health Aquaculture and Environmental Control. The extensive aetiological investigations for common swine viruses showed that only SADS-CoV was detected positively, which suggested that the causative agent of the most recent diarrhoea outbreak on February was SADS-CoV.

Furthermore, a total of 18 blood samples were simultaneously collected from healthy sows for serological investigation. Sera samples were obtained after coagulation and centrifugation (2,000 *g*, 10 min) and stored at −20°C until ELISA implemented. The PEDV antibody production was detected by means of commercial ELISA test kit (ZhenRui, China), of which, sample S/P ratios with ≥0.4 were considered positive and <0.4 were considered negative. And for SADS-CoV, ELISA was conducted following the modified procedures established in Key Laboratory of Animal Health Aquaculture and Environmental Control (P/N ≥ 2.1 considered positive). The absorbance at 450 nm wavelength was measured (Multiskan™ FC, Thermo Fisher). The results of ELISA assay revealed that all sera (100%) were negative for the PEDV and 13 of 18 (72.2%) were positive for SADS-CoV (Figure 1). In our previously clinical investigations, PEDV had caused prior outbreaks at most farms with SADS-CoV outbreaks (Zhou et al., 2018). It is possible that the presence of PEDV antibody may have somehow facilitated the amplification of the SADS-CoV. However, our results showed that PEDV antibodies were undetectable in all SADS-CoV antibody-positive sera samples, suggesting that it is SADS-CoV that caused the lethal infection in pigs on February 2019. Collectively, the re-emergence of SADS-CoV in pig herds in South China was confirmed by ELISA test and the association between PEDV and SADS-CoV infection requires further validation.

Subsequently, one SADS-CoV genome was amplified as previously described (Zhou et al., 2019). The novel SADS-CoV strain was named as SADS-CoV/CN/GDLX/2019 (accession number: MK651076). Sequence alignment and phylogenetic analysis were performed with Lasergene coresuite 10 (DNASTAR Inc.). Phylogenetic tree was constructed using the neighbour-joining method with MEGA 7.0 software (Kumar, Stecher, & Tamura, 2016). Bootstrap analysis was carried out on 1,000 replicate data sets

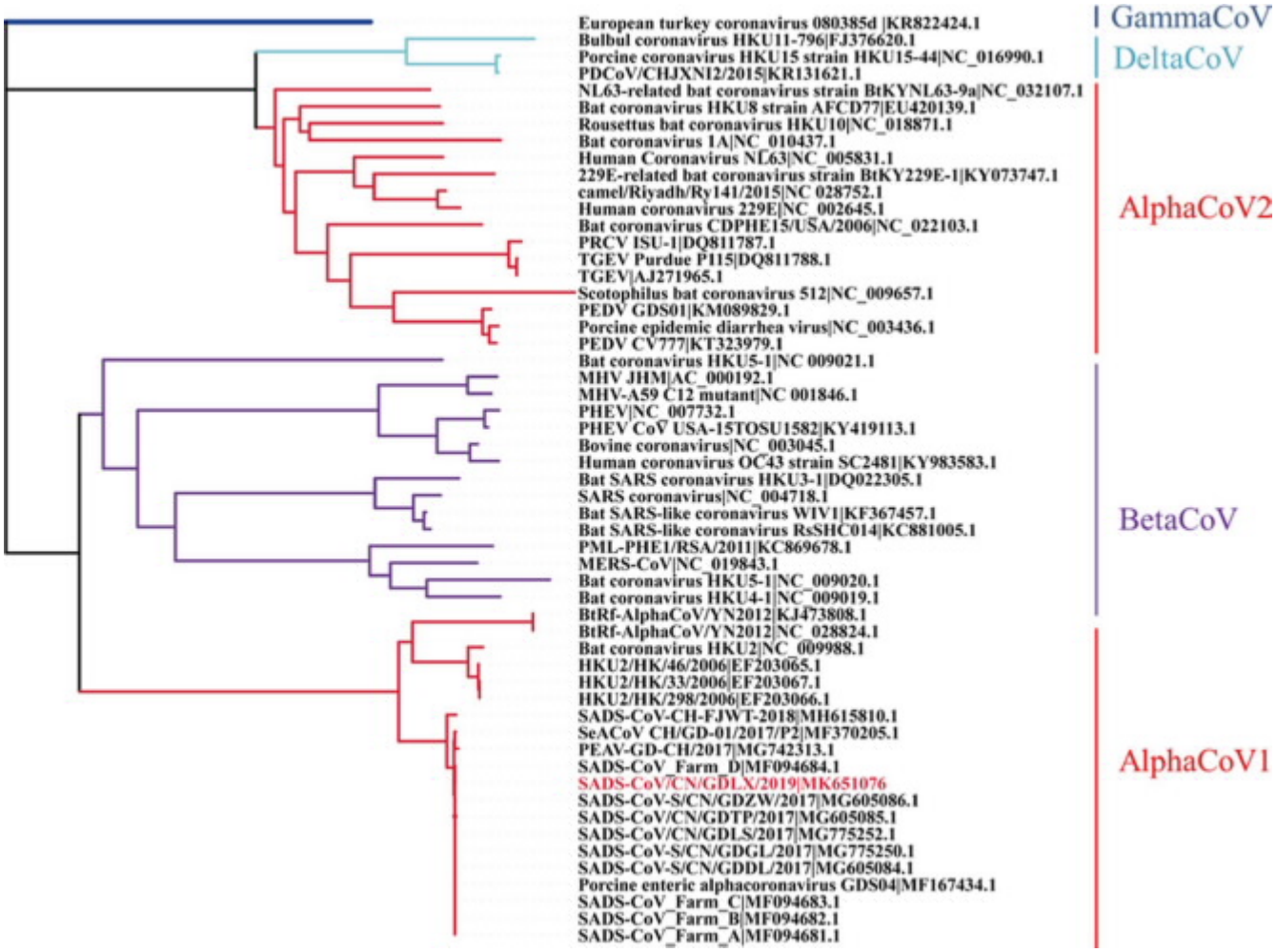


FIGURE 3 Phylogenetic analysis of the S gene of SADS-CoV and reference coronavirus species. The tree was constructed using MEGA 7.0 software with neighbour-joining methods and 1,000 replicate sets on bootstrap analysis [Colour figure can be viewed at wileyonlinelibrary.com]

(Felsenstein, 1985). For the whole genomes, sequence alignment results revealed that the nucleotide identities between SADS-CoV/CN/GDLX/2019 and previously reported SADS-CoV strains ranged from 99.3% to 100%. Moreover, the phylogenetic tree indicated that the novel SADS-CoV complete genome clustered together with other reported SADS-CoV strains and located in the Alpha coronavirus branch (Figure 2). Phylogenetic analysis based on S genes was also conducted, and the result showed that all SADS-CoV, bat HKU2 and BtRf-AlphaCoV strains were separated from other AlphaCoVs and clustered within BetaCoVs (Figure 3), which is consistent with previous studies (Pan et al., 2017; Xu et al., 2019; Zhou et al., 2019). Notably, the S gene of SADS-CoV/CN/GDLX/2019 had high identities of 99.2%–99.9% with other SADS-CoV strains detected in Guangdong and lowest identities of 97.5% with SADS-CoV-CH-FJWT-2018 reported from Fujian. Additionally, the comparative analysis of amino acids encoded by S gene revealed that the S protein of the novel strain had a two-amino-acid mutation (F306 and I882) compared with that of other SADS-CoV strains. It is well known that the S protein of coronavirus play an important role in cellular entry and determining the immunogenicity (Li, Frank, He, Peter, & Berend-Jan, 2016). In view of the lowest identities of Spike gene between the strains from Guangdong and Fujian, continuous monitoring of SADS-CoV genetic evolution is important for better understanding its epidemiology and establishing efficient strategies against its further circulating in pigs in China.

Here, we reported a new case of acute diarrhoea outbreak from a swine farm in Guangdong province on February 2019. Pathogenic and serologic analysis determined that SADS-CoV re-emerged in Guangdong and was responsible for the recent acute diarrhoea outbreak. Phylogenetic analysis based on whole genomes showed that SADS-CoV/CN/GDLX/2019 shared high nucleotide identities with previously reported SADS-CoV strains. Taken together, our study highlighted the significant threat of SADS-CoV and the potential outbreak of SADS epidemic in following months in Guangdong. Therefore, the persistent surveillance and vaccination countermeasures will be necessary to control the spread of SADS-CoV.

ACKNOWLEDGEMENTS

This work was supported by the National Key Research and Development Program of China (No. 2016YFD0501304). The Research and Extension of Major Animal Epidemic Prevention and Control Technologies in the Strategic Project of Rural Revitalization of Guangdong Agricultural Department of China (Building Modern Agricultural System) (2018–2020) and the Science and Technology Program of Guangzhou City of China (No. 201904010433).

CONFLICT OF INTEREST

The authors declare no conflict of interests with any organization.

REFERENCES

Cui, J., Li, F., & Shi, Z. (2019). Origin and evolution of pathogenic coronavirus. *Nat Rew Microbiol*, *17*, 181–192.

Dong, N., Fang, L., Zeng, S., Sun, Q., Chen, H., & Xiao, S. (2015). Porcine Deltacoronavirus in mainland China. *Emerging Infectious Diseases*, *21*, 2254–2255. https://doi.org/10.3201/eid2112.150283

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, *39*, 783–791.

Gong, L., Li, J., Zhou, Q., Xu, Z., Chen, L., Zhang, Y., … Cao, Y. (2017). A new bat-HKU–like coronavirus in swine, China, 2017. *Emerging Infectious Diseases*, *23*, 1607–1609.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* , *33*, 1870–1874.

Li, K., Li, H., Bi, Z., Gu, J., Gong, W., Luo, S., … Tang, Y. (2018). Complete genome sequence of a novel swine acute diarrhea syndrome coronavirus, CH/FJWT/2018, isolated in fujian, china, in 2018. *Microbiol Resour Announc*, *7*(22), e01259–18.

Li, W., Frank, J., He, Q., Peter, J., & Berend-Jan, B. (2016). Cellular entry of the porcine epidemic diarrhea virus. *Virus Research*, *226*, 117–127. https://doi.org/10.1016/j.virusres.2016.05.031

Pan, Y., Tian, X., Qin, P., Wang, B., Zhao, P., Yang, Y.-L., … Huang, Y.-W. (2017). Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. *Veterinary Microbiology*, *211*, 15–21. https://doi.org/10.1016/j.vetmic.2017.09.020

Xu, Z., Zhang, Y., Gong, L., Huang, L., Lin, Y., Qin, J., … Cao, Y. (2019). Isolation and characterization of a highly pathogenic strain of Porcine enteric alphacoronavirus causing watery diarrhoea and high mortality in newborn piglets. *Transboundary and Emerging Diseases*, *66*, 119–130.

Xuan, H., Xing, D., Wang, D., Zhu, W., Zhao, F., Gong, H., & Fei, S. (1984). Study on the culture of porcine epidemic diarrhea virus adapted to fetal porcine intestine primary cell monolayer. *Chin. J. Vet. Sci.*, *4*, 202–208.

Zhou, L., Sun, Y., Lan, T., Wu, R., Chen, J., Wu, Z., … Ma, J. (2019). Retrospective detection and phylogenetic analysis of swine acute diarrhoea syndrome coronavirus in pigs in southern China. *Transboundary and Emerging Diseases*, *66*, 687–695. https://doi.org/10.1111/tbed.13008

Zhou, P., Fan, H., Lan, T., Yang, X.-L., Shi, W.-F., Zhang, W., … Ma, J.-Y. (2018). Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. *Nature*, *556*, 255–258. https://doi.org/10.1038/s41586-018-0010-9

How to cite this article: Zhou L, Li QN, Su JN, et al. The re-emerging of SADS-CoV infection in pig herds in Southern China. *Transbound Emerg Dis*. 2019;66:2180–2183. https://doi.org/10.1111/tbed.13270

MEETING ROSTER
National Institute of Allergy and Infectious Diseases Special Emphasis Panel
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
Emerging Infectious Diseases Research Centers (U01 Clinical Trial Not Allowed)
ZAI1 EC-M (J2)
11/04/2019 - 11/05/2019

Notice of NIH Policy to All Applicants: Meeting rosters are provided for information purposes only. Applicant investigators and institutional officials must not communicate directly with study section members about an application before or after the review. Failure to observe this policy will create a serious breach of integrity in the peer review process, and may lead to actions outlined in NOT-OD-14-073 and NOT-OD-15-106, including removal of the application from immediate review.

CHAIRPERSON(S)

BERGMAN, LAWRENCE W., PHD
PROFESSOR
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
DREXEL COLLEGE OF MEDICINE
DREXEL UNIVERSITY
PHILADELPHIA, PA 19129

MEMBERS

ABIMIKU, ALASH'LE G., PHD
PROFESSOR
DEPARTMENTS OF MEDICINE, EPIDEMIOLOGY
AND PUBLIC HEALTH
INSTITUTE OF HUMAN VIROLOGY NIGERIA &
UNIVERSITY OF MARYLAND SCHOOL OF MEDICINE
BALTIMORE, MD 21201

ADELMAN, ZACH N., PHD
PROFESSOR
DEPARTMENT OF ENTOMOLOGY
TEXAS A&M UNIVERSITY
COLLEGE STATION, TX 77843

AGUILAR-CARRENO, HECTOR, PHD
ASSOCIATE PROFESSOR
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
COLLEGE OF VETERINARY MEDICINE
CORNELL UNIVERSITY
ITHACA, NY 14853

ALIOTA, MATTHEW T, PHD
ASSISTANT PROFESSOR
DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES
UNIVERSITY OF MINNESOTA, TWIN CITIES
ST. PAUL, MN 55108

ARONOW, BRUCE J, PHD
PROFESSOR
DEPARTMENT OF COMPUTATIONAL MEDICINE
CINCINNATI CHILDREN'S HOSPITAL MEDICAL CENTER
UNIVERSITY OF CINCINNATI
CINCINNATI, OH 45229

BERMUDEZ, LUIZ EDUARDO, MD
PROFESSOR
DEPARTMENT OF BIOMEDICAL SCIENCES
OREGON STATE UNIVERSITY
CORVALLIS, OR 97331

BROADHURST, JANA, PHD
ASSISTANT PROFESSOR
DEPARTMENT OF PATHOLOGY AND MICROBIOLOGY
UNIVERSITY OF NEBRASKA MEDICAL CENTER
OMAHA, NE 68198

BUCHMEIER, MICHAEL JOSEPH, PHD
PROFESSOR
DEPARTMENT OF MOLECULAR BIOLOGY, BIOCHEMISTRY,
MEDICINE, MICROBIOLOGY AND MOLECULAR GENETICS
DIVISION OF INFECTIOUS DISEASE
UNIVERSITY OF CALIFORNIA, IRVINE
IRVINE, CA 92697

CARSON, GAIL, PHD
PROFESSOR
CENTRE FOR TROPICAL MEDICINE & GLOBAL HEATLH
UNIVERSITY OF OXFORD
UNITED KINGDOM

COHEN, CRAIG R, MD
PROFESSOR
DEPARTMENT OF OBSTETRICS AND GYNECOLOGY
SCHOOL OF MEDICINE
UNIVERSITY OF CALIFORNIA SAN FRANCISCO
SAN FRANCISCO, CA 94105

DEL POETA, MAURIZIO, MD
PROFESSOR
DEPARTMENT OF MOLECULAR GENETICS AND
MICROBIOLOGY
SCHOOL OF MEDICINE
STONY BROOK UNIVERSITY
STONY BROOK, NY 11794

GRIBSKOV, MICHAEL, BS, PHD
PROFESSOR
DEPARTMENT OF BIOLOGICAL SCIENCES
PURDUE UNIVERSITY
WEST LAFAYETTE, IN 47907

HSIAO, WILLIAM WEI-LIANG, PHD
CLINICAL ASSISTANT PROFESSOR / SENIOR SCIENTIST
BC CENTRE FOR DISEASE CONTROL
VANCOUVER
CANADA

JIMENEZ, VERONICA, PHD
ASSOCIATE PROFESSOR
DEPARTMENT OF BIOLOGICAL SCIENCE
HEALTH PROMOTION RESEARCH INSTITUTE
CALIFORNIA STATE UNIVERSITY, FULLERTON
FULLERTON, CA 92831

MEETING ROSTER
National Institute of Allergy and Infectious Diseases Special Emphasis Panel
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
Emerging Infectious Diseases Research Centers (U01 Clinical Trial Not Allowed)
ZAI1 EC-M (J2)
11/04/2019 - 11/05/2019

Notice of NIH Policy to All Applicants: Meeting rosters are provided for information purposes only. Applicant investigators and institutional officials must not communicate directly with study section members about an application before or after the review. Failure to observe this policy will create a serious breach of integrity in the peer review process, and may lead to actions outlined in NOT-OD-14-073 and NOT-OD-15-106, including removal of the application from immediate review.

JULIANO, JONATHAN JAMES, MD
ASSOCIATE PROFESSOR
DEPARTMENT OF MEDICINE
DIVISION OF INFECTIOUS DISEASES
UNIVERSITY OF NORTH CAROLINA CHAPEL HILL
CHAPEL HILL, NC 27599

KARIM, SHAHID, PHD
PROFESSOR
DEPARTMENT OF BIOLOGICAL SCIENCES
THE UNIVERSITY OF SOUTHERN MISSISSIPPI
HATTIESBURG, MS 39406

KOMAR, NICHOLAS, DSC
BIOLOGIST
DIVISION OF VECTOR-BORNE DISEASES
CENTERS FOR DISEASE CONTROL AND PREVENTION
FORT COLLINS, CO 80521

LARSEN, CHRIS N., PHD
SENIOR SCIENTIST
DEPARTMENT OF PATHOGEN INFORMATICS
VECNA TECHNOLOGIES, INC.
COLLEGE PARK, MD 20740

LAURENS, MATTHEW BRENT, MD
ASSOCIATE PROFESSOR
DIVISION OF MALARIA RESEARCH,
CENTER FOR VACCINE DEVELOPMENT AND GLOBAL HEALTH
UNIVERSITY OF MARYLAND SCHOOL OF MEDICINE
BALTIMORE, MD 21201

MARTIN, EMILY TOTH, PHD
ASSOCIATE PROFESSOR
DEPARTMENT OF EPIDEMIOLOGY
SCHOOL OF PUBLIC HEALTH
UNIVERSITY OF MICHIGAN
ANN ARBOR, MI 48109

MITTAL, SURESH K, PHD, DVM
DISTINGUISHED PROFESSOR OF VIROLOGY
DEPARTMENT OF COMPARATIVE PATHOBIOLOGY
COLLEGE OF VETERINARY MEDICINE
PURDUE UNIVERSITY
WEST LAFAYETTE, IN 47907

MORES, CHRISTOPHER, SCD
PROFESSOR
DEPARTMENT OF GLOBAL HEALTH
MILKEN INSTITUTE SCHOOL OF PUBLIC HEALTH
GEORGE WASHINGTON UNIVERSITY
WASHINGTON, DC 20052

MURPHY, ROBERT L, MD
PROFESSOR
INSTITUTE FOR GLOBAL HEALTH
DEPARTMENT OF MEDICINE AND BIOMEDICAL ENGINEERING
NORTHWESTERN UNIVERSITY
CHICAGO, IL 60611

PALESE, PETER, PHD
CHAIR AND PROFESSOR
DEPARTMENT OF MICROBIOLOGY
MOUNT SINAI SCHOOL OF MEDICINE
NEW YORK, NY 10029

PEREZ, DANIEL R, PHD
PROFESSOR OF VIROLOGY
DEPARTMENT OF POPULATION HEALTH
COLLEGE OF VETERINARY MEDICINE
UNIVERSITY OF GEORGIA
ATHENS, GA 30602

PETERSON, TOWNSEND A., PHD
UNIVERSITY DISTINGUISHED PROFESSOR
BIODIVERSITY INSTITUTE
UNIVERSITY OF KANSAS
LAWRENCE, KS 66045

REILLY, CAVAN S., PHD
PROFESSOR
COORDINATING CENTERS FOR BIOMETRIC RESEARCH
DIVISION OF BIOSTATISTICS
UNIVERSITY OF MINNESOTA
MINNEAPOLIS, MN 55455

RIMOIN, ANNE WALSH, PHD
PROFESSOR
DEPARTMENT OF EPIDEMIOLOGY
SCHOOL OF PUBLIC HEALTH
UNIVERSITY OF CALIFORNIA LOS ANGELES
LOS ANGELES, CA 90095

STEVENS, LORI, PHD
PROFESSOR
DEPARTMENT OF BIOLOGY
UNIVERSITY OF VERMONT
BURLINGTON, VT 05405-0086

THIEL, VOLKER, PHD
PROFESSOR OF VIROLOGY
INSTITUTE FOR VIROLOGY AND IMMUNOLOGY
UNIVERSITY OF BERN
BERN
SWITZERLAND

MEETING ROSTER
National Institute of Allergy and Infectious Diseases Special Emphasis Panel
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
Emerging Infectious Diseases Research Centers (U01 Clinical Trial Not Allowed)
ZAI1 EC-M (J2)
11/04/2019 - 11/05/2019

Notice of NIH Policy to All Applicants: Meeting rosters are provided for information purposes only. Applicant investigators and institutional officials must not communicate directly with study section members about an application before or after the review. Failure to observe this policy will create a serious breach of integrity in the peer review process, and may lead to actions outlined in NOT-OD-14-073 and NOT-OD-15-106, including removal of the application from immediate review.

TRAN, TUAN, MD, PHD
ASSISTANT PROFESSOR OF MEDICINE
INDIANA UNIVERSITY SCHOOL OF MEDICINE
INDIANA UNIVERSITY
NDIANAPOLIS, IN 46202

USSERY, DAVID WAYNE, PHD
PROFESSOR
BIOSCIENCES DIVISION
UNIVERSITY OF ARKANSAS FOR MEDICAL SCIENCES
LITTLE ROCK, AR 72205

WANG, YUFENG, PHD
PROFESSOR
DEPARTMENT OF BIOLOGY
UNIVERSITY OF TEXAS AT SAN ANTONIO
SAN ANTONIO, TX 78249

SCIENTIFIC REVIEW OFFICER

COHEN, ELEAZAR, PHD
SCIENTIFIC REVIEW OFFICER
SCIENTIFIC REVIEW PROGRAM
DIVISION OF EXTRAMURAL ACTIVITIES
NATIONAL INSTITUTES OF HEALTH/NIAID
BETHESDA, MD 20892

PROGRAM REPRESENTATIVE

PATTERSON, L. JEAN, PHD
PROGRAM OFFICER
VIROLOGY BRANCH
DIVISION OF MICROBIOLOGY AND INFECTIOUS DISEASES
NATIONAL INSTITUTES OF HEALTH
ROCKVILLE, MD 20892

GRANTS MANAGEMENT REPRESENTATIVE

KHURANA, DHANA
GRANTS MANAGEMENT SPECIALIST
GRANTS MANAGMENT OFFICE
DIVISION OF EXTRAMURAL ACTIVITIES
NATIONAL INSTITUTES OF HEALTH/NIAID
ROCKVILLE, MD 20892

OTHER REVIEW STAFF

KABIROU, ASHA K., MS
CONTRACT PEER REVIEW SPECIALIST
SCIENTIFIC REVIEW PROGRAM
DIVISION OF EXTRAMURAL ACTIVITIES
NATIONAL INSTITUTES OF HEALTH, NIAID
ROCKVILLE, MD 20892

Consultants are required to absent themselves from the room during the review of any application if their presence would constitute or appear to constitute a conflict of interest.

Commons ID	Senior/Key Personnel (Y/N)	Name	Degree(s)	Role	Person Months		
					Cal	Aca.	Sum.

OTHER SUPPORT

DOE, JANE

ACTIVE

5R01AIXXXXXX-03	07/01/18 – 06/30/23	0.24 Cal
NIH/NIAID (PI: Doe)	$325,678	

Plant Anti-HIV Agents

The major goal of this project is to discover new anti-HIV clinical trial candidates based on natural products from higher plants.

Domestic

Gifts (directly used to subsidize expenses related to your NIH funded project)
- Type of Gift
- Name of Gift giver

Appointments
- Name of Affiliate
- Appointment Type: (honorary, visiting or adjunct)

In-kind Lab or Office Space (*Please defer to application Letters of Support, IAB's, EAB's, OSC)*
- Name of Institution
- Lab name
  (Ex: UNC Children's Hospital, Dr. Seuss)
  Includes use of facilities or instrumentation

Scientific Materials (In-kind or Dollar amount)
- Name of Institution
- Material Name
- In-Kind or Dollar amount

Collaborations
- Collaborators Name and Institution (OSC)

Foreign Component

Gifts (directly used to subsidize expenses related to your NIH funded project)
- Type of Gift
- Name of Gift giver

Appointments
- Name of Affiliate
- Appointment Type: (honorary, visiting or adjunct)

In-kind Lab or Office Space (Please defer to application Letters of Support, IAB's, EAB's, OSC)
- Name of Institution
- Lab name
  (Ex: UNC Children's Hospital, Dr. Seuss)
  Includes use of facilities or instrumentation

Scientific Materials (In-kind or Dollar amount)
- Name of Institution

- Material Name
- In-Kind or Dollar amount

Collaborations
- Collaborators Name and Institution (OSC)
- Including anticipated to result in co-authorship

Visiting faculty/scholars/scientists/post-docs supported from foreign resources who are participating in your NIH project.
- Supported from foreign resources: *Yes or No*
- # for each category (Ex: Scholars – Zero; Post-Docs – 2)

Affiliations with foreign entities or governments, including talent programs.
- Institutional Name
- Type: Honorary, Visiting or Adjunct

Scientific, Financial and Effort Commitment Overlap Statement:

PENDING

1R01-AIXXXXXX-02	04/01/18-03/31/21	0.60 Cal
NIH/Duke University (PI: Moe)	$31,878 (Doe lab)	

Small molecule HIV-1 entry inhibitor with novel mechanisms of action

The major goal of this project is the discovery and identification of optimized aloperine derivatives with low anti-HIV-1 entry activity for further drug development against persistent HIV-1 infection.

Domestic

Gifts (directly used to subsidize expenses related to your NIH funded project)
- Type of Gift
- Name of Gift giver

Appointments
- Name of Affiliate
- Appointment Type: (honorary, visiting or adjunct)

In-kind Lab or Office Space (*Please defer to application Letters of Support, IAB's, EAB's, OSC)*
- Name of Institution
- Lab name
  (Ex: UNC Children's Hospital, Dr. Seuss)
  Includes use of facilities or instrumentation

Scientific Materials (In-kind or Dollar amount)
- Name of Institution
- Material Name
- In-Kind or Dollar amount

Collaborations
- Collaborators Name and Institution (OSC)

Foreign Component

Gifts (directly used to subsidize expenses related to your NIH funded project)

- Type of Gift
- Name of Gift giver

Appointments
- Name of Affiliate
- Appointment Type: (honorary, visiting or adjunct)

In-kind Lab or Office Space (Please defer to application Letters of Support, IAB's, EAB's, OSC)
- Name of Institution
- Lab name
  (Ex: UNC Children's Hospital, Dr. Seuss)
  Includes use of facilities or instrumentation

Scientific Materials (In-kind or Dollar amount)
- Name of Institution
- Material Name
- In-Kind or Dollar amount

Collaborations
- Collaborators Name and Institution (OSC)
- Including anticipated to result in co-authorship

Visiting faculty/scholars/scientists/post-docs supported from foreign resources who are participating in your NIH project.
- Supported from foreign resources: *Yes or No*
- # for each category (Ex: Scholars – Zero; Post-Docs – 2)

Affiliations with foreign entities or governments, including talent programs.
- Institutional Name
- Type: Honorary, Visiting or Adjunct

Scientific, Financial and Effort Commitment Overlap Statement:

Novel Coronavirus (2019-nCoV) Sample Needs

Agency/Division/ Lab	Description of activity/gap for which physical samples are needed	Priority of activity (if multiple activities listed)	Type of samples needed	Minimum volume of sample needed for each sample type	Will the outputs of the proposed activity (e.g., reagents developed) be made available to partners? If yes, will they be made available to domestic partners, international, or both.	Capabilities your lab possesses and can share with potential collaborators for responding to the 2019-nCoV	Point of contact		
							Name	Phone #	Email

Vital Surveillances

The Epidemiological Characteristics of an Outbreak of 2019 Novel Coronavirus Diseases (COVID-19) — China, 2020

The Novel Coronavirus Pneumonia Emergency Response Epidemiology Team

Abstract

Background: An outbreak of 2019 novel coronavirus diseases (COVID-19) in Wuhan, Hubei Province, China has spread quickly nationwide. Here, we report results of a descriptive, exploratory analysis of all cases diagnosed as of February 11, 2020.

Methods: All COVID-19 cases reported through February 11, 2020 were extracted from China's Infectious Disease Information System. Analyses included the following: 1) summary of patient characteristics; 2) examination of age distributions and sex ratios; 3) calculation of case fatality and mortality rates; 4) geo-temporal analysis of viral spread; 5) epidemiological curve construction; and 6) subgroup analysis.

Results: A total of 72,314 patient records—44,672 (61.8%) confirmed cases, 16,186 (22.4%) suspected cases, 10,567 (14.6%) clinically diagnosed cases (Hubei Province only), and 889 asymptomatic cases (1.2%)—contributed data for the analysis. Among confirmed cases, most were aged 30–79 years (86.6%), diagnosed in Hubei (74.7%), and considered mild (80.9%). A total of 1,023 deaths occurred among confirmed cases for an overall case fatality rate of 2.3%. The COVID-19 spread outward from Hubei Province sometime after December 2019, and by February 11, 2020, 1,386 counties across all 31 provinces were affected. The epidemic curve of onset of symptoms peaked around January 23–26, then began to decline, leading up to February 11. A total of 1,716 health workers have become infected and 5 have died (0.3%).

Conclusions: COVID-19 epidemic has spread very quickly taking only 30 days to expand from Hubei to the rest of Mainland China. With many people returning from a long holiday, China needs to prepare for the possible rebound of the epidemic.

Introduction

A cluster of pneumonia cases of unknown origin in Wuhan, China caused concern among health officials in late December 2019. On December 31, an alert was issued by the Wuhan Municipal Health Commission, a rapid response team was sent to Wuhan by the Chinese Center for Disease Control and Prevention (China CDC), and a notification was made to the World Health Organization (WHO) (*1*–*4*). Likely potential causes including influenza, avian influenza, adenovirus, severe acute respiratory syndrome coronavirus (SARS-CoV), and Middle East respiratory syndrome coronavirus (MERS-CoV) were ruled out. Epidemiological investigation implicated Wuhan's Huanan Seafood Wholesale Market, which was shut down and disinfected, and active case finding was initiated and vigorously pursued (*2*,*4*–*5*).

On January 7, 2020, the causative pathogen was identified as a novel coronavirus, and genomic characterization and test method development ensued (*2*–*6*). Now named 2019-nCoV, the virus is distinct from both SARS-CoV and MERS-CoV, yet closely related (*5*,*7*). Early cases suggested that COVID-19 (i.e. the new name for disease caused by the novel coronavirus) may be less severe than SARS and MERS. However, illness onset among rapidly increasing numbers of people and mounting evidence of human-to-human transmission suggests that 2019-nCoV is more contagious than both SARS-CoV and MERS-CoV (*3*,*8*–*11*).

On January 20, China's "*National Infectious Diseases Law*" was amended to make 2019-novel coronavirus diseases (COVID-19) a Class B notifiable disease and its "*Frontier Health and Quarantine Law*" was amended to support the COVID-19 outbreak response effort. Then, on January 23, the Chinese Government began to limit movement of people in and out of Wuhan, and two days later, it announced its highest-level commitment and mobilized all sectors to respond to the epidemic and prevent further spread of COVID-19. Characterization of the epidemiological features of COVID-19 is crucial for the development and implementation of effective control strategies. Here, we report the results of a descriptive, exploratory analysis of all cases found through February 11, 2020.

Method

Study Design

This study was a descriptive, exploratory analysis of all cases of COVID-19 diagnosed nationwide in China as of the end of February 11, 2020. As such, it in some respects uses a cross-sectional study design and hence, we have used the STROBE Guidelines (www.equator-network.org) to aid our thorough reporting of this observational study.

A public health emergency was declared, and a formal investigation began on December 31, 2019, supported by city (Wuhan Municipal Health Commission and Wuhan CDC), provincial (Health Commission of Hubei Province and Hubei Provincial CDC), and national (National Health Commission and China CDC) authorities and resources. This study was reviewed by the China CDC Institutional Review Board via a fast-track mechanism. Although individual informed consent was not required for this study, all data were handled as a deidentified set to protect patient privacy and confidentiality.

Data Source

By categorizing COVID-19 as a Class B notifiable disease, Chinese law required all cases to be immediately reported to China's Infectious Disease Information System. Entry of each case into the system was performed by local epidemiologists and public health workers who investigated and collected information on possible exposures. All case records contain national identification numbers, and therefore, all cases have records in the system and no records are duplicated. All data contained in all COVID-19 case records in the Infectious Disease Information System through the end of February 11, 2020 were extracted from the system as a single dataset and were then stripped of all personal identifying information. No sampling was done to achieve a predetermined study size and no eligibility criteria were used—all cases were included.

Variables

Patient characteristics were collected at baseline, meaning the time of diagnosis, epidemiological investigation, and entry into the Infectious Disease Information System. Patients were categorized as health workers for the occupation variable if they had active employment of any kind in a health facility (i.e., this category did not just include physicians and nurses). Patients were categorized as having a Wuhan-related exposure if they had recently resided in or visited Wuhan or if they had close contact with someone who had. The comorbid conditions variable was determined upon epidemiological investigation by patient self-reported medical history, which was not independently verified using medical records for all cases. The severity of symptoms variable was categorized as mild, severe, or critical. Mild included non-pneumonia and mild pneumonia cases. Severe was characterized by dyspnea, respiratory frequency ≥ 30/minute, blood oxygen saturation ≤93%, PaO_2/FiO_2 ratio <300, and/or lung infiltrates >50% within 24–48 hours. Critical cases were those that exhibited respiratory failure, septic shock, and/or multiple organ dysfunction/failure.

As some variables of interest (i.e., Wuhan-related exposure, comorbid condition, and case severity) are not required fields when creating records in the Infectious Disease Information System, some records have missing data for these variables.

For construction of epidemiological curves, date of onset was defined as the date on which patients self-reported the start of either fever or cough during epidemiological investigation. Cases were categorized as confirmed, suspected, clinically diagnosed (Hubei Province only), or asymptomatic. Confirmed cases were diagnosed based on positive viral nucleic acid test results on throat swab samples (some samples were tested retrospectively). Suspected cases were diagnosed clinically based on symptoms and exposures. Clinically diagnosed cases were suspected cases with lung imaging features consistent with coronavirus pneumonia. Asymptomatic cases were diagnosed based on positive viral nucleic acid test results but without any COVID-19 symptoms (e.g., fever, dry cough). The date of positive viral nucleic acid test result is used as onset date for asymptomatic cases

Analysis

For confirmed cases, demographic and clinical characteristics were summarized using descriptive statistics. Age distribution graphs were constructed using patient age at baseline for confirmed cases diagnosed in Wuhan, Hubei Province (including Wuhan), and China (including Hubei Province). Sex ratio (i.e., male:female [M:F] ratio) was also calculated. Case fatality rates were calculated as the total number of deaths (numerator) divided by the total number of cases (denominator), expressed as a percent. Observed time was summarized using person-days (PD) and

mortality was calculated as the number of deaths (numerator) divided by the total observed time (denominator), expressed per 10 PD.

For geo-temporal analysis, the county-level location of each case at time of diagnosis was used to build color-coded maps of China to indicate the numbers of cases in each province on December 31, 2019; January 10, 2020; January 31, 2020; and February 11, 2020. This analysis was performed using ArcGIS Desktop software (version 10.6; Esri; Redlands, California, USA).

The epidemiological curve for all cases was constructed by plotting the number of cases (y-axis) versus self-reported date of symptom onset (x-axis). Date of symptom onset for confirmed, suspected, clinically diagnosed, and asymptomatic cases were stacked to show total cases over time. The epidemiological curve for confirmed cases was also overlaid with the number of cases versus date of diagnosis to show the delay between onset of symptoms and diagnosis of disease.

Two subgroups were also analyzed separately using epidemiological curves: confirmed cases diagnosed outside of Hubei Province (with and without Wuhan-related exposure) and all cases diagnosed among health workers (confirmed, suspected, clinically diagnosed, and asymptomatic).

Results

Patients

A total of 72,314 unique records were extracted and data from all records were included in the analysis. Thus, all 72,314 individuals diagnosed with COVID-19 as of February 11, 2020, were included in the analysis. Among them, 44,672 cases (61.8%) were confirmed, 16,186 cases (22.4%) were suspected, 10,567 cases (14.6%) were clinically diagnosed, and 889 cases (1.2%) were asymptomatic.

Baseline characteristics of confirmed cases (n=44,672) are presented in Table 1. A majority were aged 30–69 years (77.8%), male (51.4%), farmers or laborers (22.0%), and diagnosed in Hubei Province (74.7%). Most patients reported Wuhan-related exposures (85.8%) and were classified as mild cases (80.9%).

TABLE 1. Patients, deaths, and case fatality rates, as well as observed time and mortality for n=44,672 confirmed COVID-19 cases in Mainland China as of February 11, 2020.

Baseline characteristics	Confirmed cases, N (%)	Deaths, N (%)	Case fatality rate, %	Observed time, PD	Mortality, per 10 PD
Overall	44,672	1,023	2.3	661,609	0.015
Age, years					
0–9	416 (0.9)	–	–	4,383	–
10–19	549 (1.2)	1 (0.1)	0.2	6,625	0.002
20–29	3,619 (8.1)	7 (0.7)	0.2	53,953	0.001
30–39	7,600 (17.0)	18 (1.8)	0.2	114,550	0.002
40–49	8,571 (19.2)	38 (3.7)	0.4	128,448	0.003
50–59	10,008 (22.4)	130 (12.7)	1.3	151,059	0.009
60–69	8,583 (19.2)	309 (30.2)	3.6	128,088	0.024
70–79	3,918 (8.8)	312 (30.5)	8.0	55,832	0.056
≥80	1,408 (3.2)	208 (20.3)	14.8	18,671	0.111
Sex					
Male	22,981 (51.4)	653 (63.8)	2.8	342,063	0.019
Female	21,691 (48.6)	370 (36.2)	1.7	319,546	0.012
Occupation					
Service industry	3,449 (7.7)	23 (2.2)	0.7	54,484	0.004
Farmer/laborer	9,811 (22.0)	139 (13.6)	1.4	137,992	0.010
Health worker	1,716 (3.8)	5 (0.5)	0.3	28,069	0.002
Retiree	9,193 (20.6)	472 (46.1)	5.1	137,118	0.034
Other/none	20,503 (45.9)	384 (37.5)	1.9	303,946	0.013

TABLE 1. (continued)

Baseline characteristics	Confirmed cases, N (%)	Deaths, N (%)	Case fatality rate, %	Observed time, PD	Mortality, per 10 PD
Province					
Hubei	33,367 (74.7)	979 (95.7)	2.9	496,523	0.020
Other	11,305 (25.3)	44 (4.3)	0.4	165,086	0.003
Wuhan-related exposure*					
Yes	31,974 (85.8)	853 (92.8)	2.7	486,612	0.018
No	5,295 (14.2)	66 (7.2)	1.2	71,201	0.009
Missing	7,403	104	2.8	103,796	0.010
Comorbid condition†					
Hypertension	2,683 (12.8)	161 (39.7)	6.0	42,603	0.038
Diabetes	1,102 (5.3)	80 (19.7)	7.3	17,940	0.045
Cardiovascular disease	873 (4.2)	92 (22.7)	10.5	13,533	0.068
Chronic respiratory disease	511 (2.4)	32 (7.9)	6.3	8,083	0.040
Cancer (any)	107 (0.5)	6 (1.5)	5.6	1,690	0.036
None	15,536 (74.0)	133 (32.8)	0.9	242,948	0.005
Missing	23,690 (53.0)	617 (60.3)	2.6	331,843	0.019
Case severity§					
Mild	36,160 (80.9)	–	–	–	–
Severe	6,168 (13.8)	–	–	–	–
Critical	2,087 (4.7)	1,023 (100)	49.0	31,456	0.325
Missing	257 (0.6)	–	–	–	–
Period (by date of onset)					
Before Dec 31, 2019	104 (0.2)	15 (1.5)	14.4	5,142	0.029
Jan 1–10, 2020	653 (1.5)	102 (10.0)	15.6	21,687	0.047
Jan 11–20, 2020	5,417 (12.1)	310 (30.3)	5.7	130,972	0.024
Jan 21–31, 2020	26,468 (59.2)	494 (48.3)	1.9	416,009	0.012
After Feb 1, 2020	12,030 (26.9)	102 (10.0)	0.8	87,799	0.012

Abreviation: PD, person-days. -, not applicable.

* The Wuhan-related exposure variable, only includes a total of 37,269 patients and 919 deaths and these values were used to calculate percentages in the confirmed cases and deaths columns.

† The comorbid condition variable, only includes a total of 20,812 patients and 504 deaths and these values were used to calculate percentages in the confirmed cases and deaths columns.

§ The case severity variable, only includes a total of 44,415 patients and 1,023 deaths and these values were used to calculate percentages in the confirmed cases and deaths columns.

Deaths, Case Fatality Rates, and Mortality

As shown in Table 1, a total of 1,023 deaths have occurred among 44,672 confirmed cases for an overall case fatality rate of 2.3%. Additionally, these 1,023 deaths occurred during 661,609 PD of observed time, for a mortality rate of 0.015/10 PD.

The ≥80 age group had the highest case fatality rate of all age groups at 14.8%. Case fatality rate for males was 2.8% and for females was 1.7%. By occupation, patients who reported being retirees had the highest case fatality rate at 5.1%, and patients in Hubei Province had a >7-fold higher case fatality rate at 2.9% compared to patients in other provinces (0.4%). While patients who reported no comorbid conditions had a case fatality rate of 0.9%, patients with comorbid conditions had much higher rates—10.5% for those with cardiovascular disease, 7.3% for diabetes, 6.3% for chronic respiratory disease, 6.0% for hypertension, and 5.6% for cancer. Case fatality rate was also very high for cases categorized as critical at 49.0%.

Age Distribution and Sex Ratio

The age distribution of cases in Wuhan only, in Hubei Province overall, and in China overall are

presented in Figure 1. The proportion of confirmed cases 30–79 years of age at baseline (i.e., date of diagnosis) was 89.8% for cases in Wuhan city versus 88.6% in Hubei overall (which includes Wuhan) and 86.6% in China overall (which includes Hubei Province and all 30 other provincial-level administrative divisions, or PLADs). The male-to-female ratio was 0.99:1 in Wuhan, 1.04:1 in Hubei, and 1.06:1 in China overall.

Geo-Temporal Findings

On January 19, 2020, National Health Commission of the People's Republic of China confirmed that Guangdong Provincial CDC reported first imported cases of COVID-19, via the Chinese Infectious Diseases Reporting System. This was the first time COVID-19 had been reported outside of Hubei Province via the System. As of January 22, 2020, a total of 301 confirmed COVID-19 cases were reported from 83 counties in 23 provinces. On January 30, 2020, Xizang Autonomous Region (Tibet) reported its first confirmed COVID-19 case coming from Hubei Province. Thus, COVID-19 cases have been reported from all 31 PLADs (Figure 2).

As of February 11, 2020, a total 44,672 confirmed cases were reported from 1,386 counties of 31 provinces, autonomous regions, and municipalities and Hubei Province accounted for 74.7% (Figure 2E). Among them, 0.2% of cases had onset of illness before December 31, 2019 and all were from Hubei Province (Figure 2A); 1.7% had onset of illness during January 1–10, 2020, distributed in 113 counties of 22 PLADs and Hubei Province accounted for 88.5% (Figure 2B); 13.8% had onset of illness during January 11–20, 2020, distributed in 627 counties of 30 PLADs and Hubei Province accounted for 77.6% (Figure 2C); 73.1% had onset of illness during January 21–31, 2020, distributed in 1310 counties of 31 PLADs and Hubei Province accounted for 74.7% (Figure 2D).

Epidemiological Curve

Figure 3A shows the COVID-19 epidemic curve with number of cases plotted by date of patient onset of symptoms from December 8, 2019 to February 11, 2020. Confirmed, suspected, clinically diagnosed, and asymptomatic cases are stacked to show total daily cases by date of symptom onset. The inset shows that in December 2019 only 0–22 cases/day began to experience symptoms. The peak onset of symptoms for all cases overall occurred on February 1, 2020. Since then, onset of illness has declined.

Figure 3B shows the same COVID-19 epidemic curve for confirmed cases only with number of cases plotted by date of patients' onset of symptoms from December 8, 2019 to February 11, 2020. These data are overlaid with confirmed cases plotted by date of diagnosis to show the lag between the time patients fall ill and the time they actually are diagnosed and are reported to the Infectious Disease Information System. Although for confirmed cases onset of illness peaked around January 23–27, diagnosis of infection by nucleic acid testing of throat swabs did not peak until February 4.

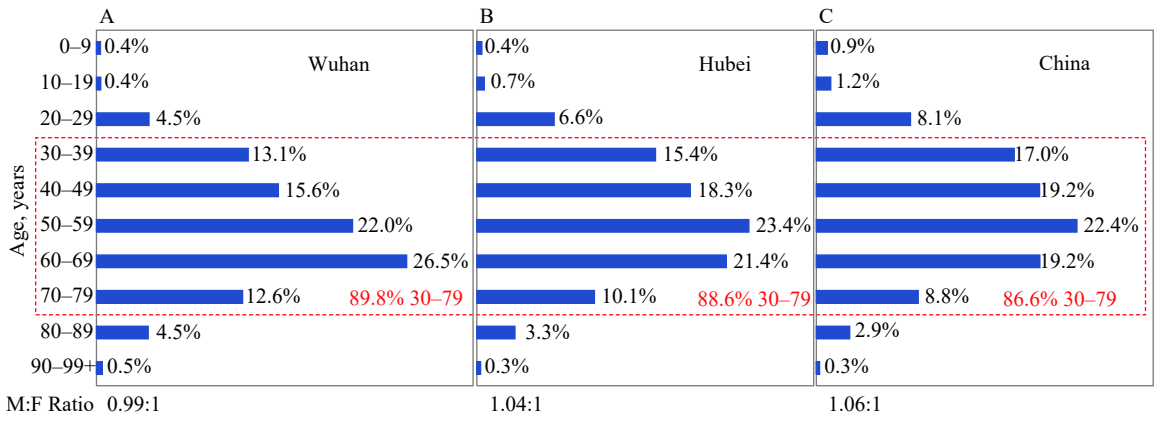


FIGURE 1. Age distribution and sex ratio of all confirmed COVID-19 cases in China through February 11, 2020. (A) patients diagnosed in the city of Wuhan only; (B) patients diagnosed in Hubei Province, which includes Wuhan as its capital city; and (C) patients diagnosed in China overall, including Hubei Province and all 30 other provincial-level administrative divisions (PLADs). Dashed red line highlights the proportion of patients in the 30–79 years age range. Sex ratio (i.e. male-to-female [M:F] ratio) is shown below each graph.

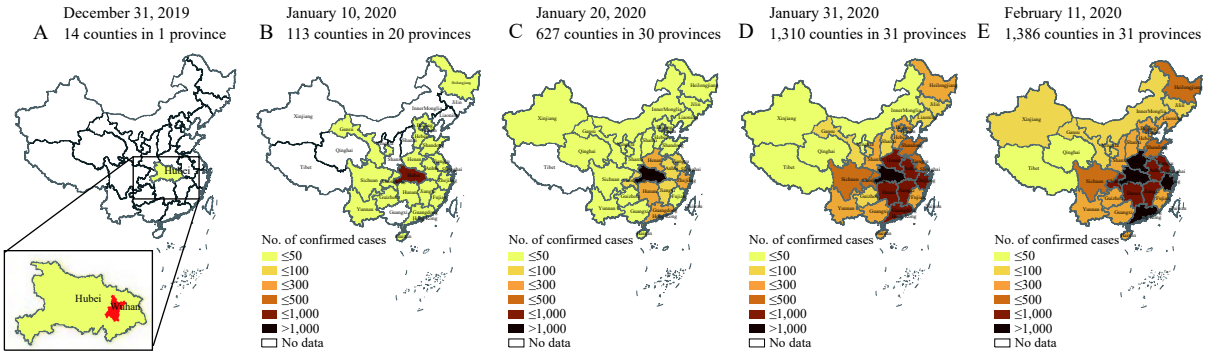


FIGURE 2. Geo-temporal spread of COVID-19 in China through February 11, 2020. (A) a total of 14 county-level administrative areas (hereafter counties) in Hubei Province only (inset) had reported cases as of December 31, 2019; (B) by January 10, 2020, 113 counties in 20 PLADs had reported cases with the highest prevalence still in Hubei Province; (C) nine days later, on January 20, 627 counties in 30 PLADs had reported cases and PLADs neighboring Hubei Province observed increasing prevalence; (D) by the end of January 31, 1,310 counties across all 31 PLADs were affected and prevalence in the central, south, and south-central regions had risen dramatically; (E) by the end of February 11, 1,386 counties nationwide were affected and prevalence in the south-central PLADs had risen to the level of Hubei.

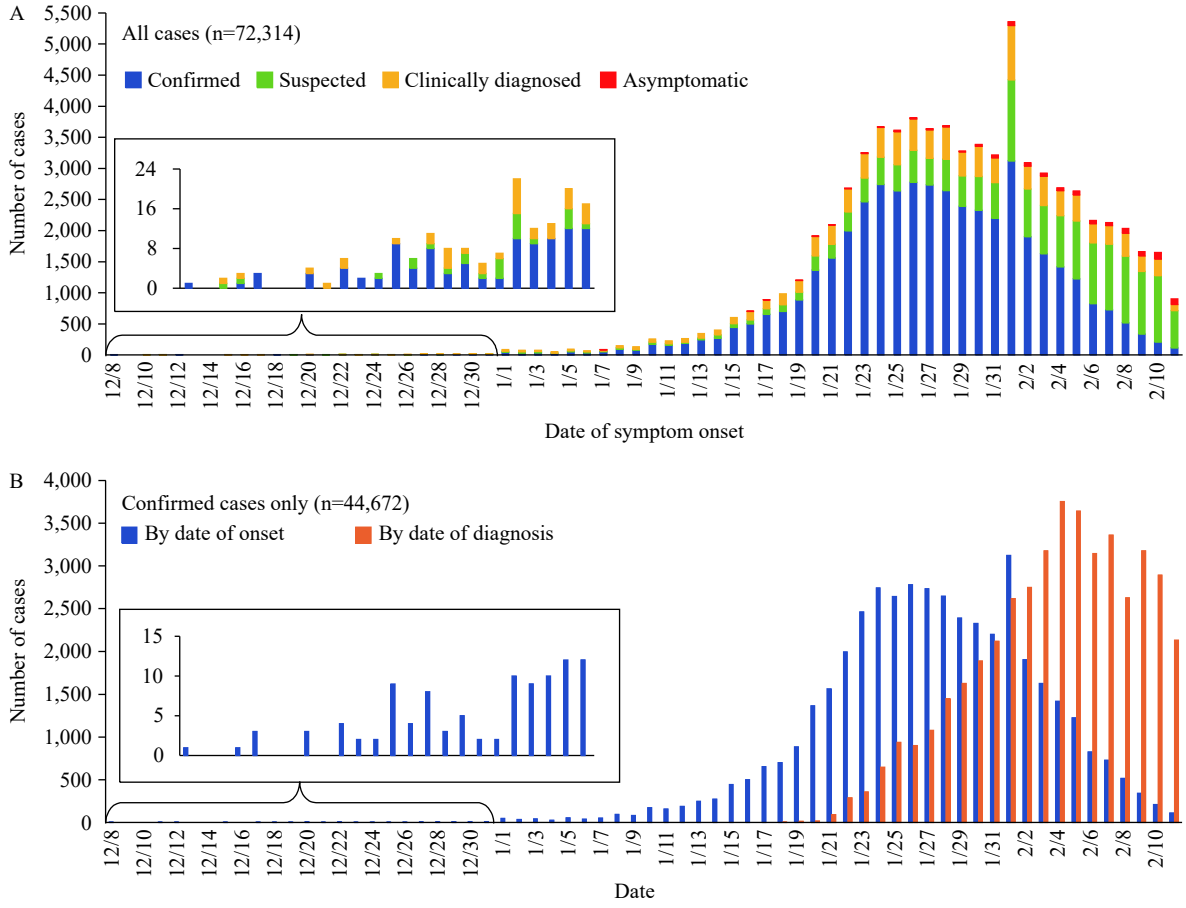


FIGURE 3. Epidemiological curves of COVID-19 in China through February 11, 2020. (A) the epidemiological curve shows the progression of illness in the outbreak over time from December 8, 2019 to February 11, 2020. A total of 72,314 cases are shown and confirmed cases (blue) are compared to suspected cases (green), clinically diagnosed cases (yellow), and asymptomatic cases (red). The inset shows a zoomed-in view of all days in December, when total daily count remained below 24 cases; (B) the epidemiological curve shows the progression of illness in the outbreak over time from December 8, 2019 to February 11, 2020 for confirmed cases only (blue). The number of cases diagnosed each day is also shown for confirmed cases only (orange). The inset shows a zoomed-in view of all days in December, when total daily count remained below 15 cases.

Subgroup Findings

Figure 4 shows the COVID-19 epidemic curve with the number of cases plotted by date of onset of symptoms from December 18, 2019 to February 11, 2020 for two subgroups—confirmed cases found outside of Hubei Province (Figure 4A) and all cases among health workers nationwide (Figure 4B). Peak timing of onset of symptoms among cases outside of Hubei Province occurred on January 27. Most of these cases (85.8%) reported having recently resided in or visited Wuhan or having had close contact with an infected individual from Wuhan. Peak timing of onset of symptoms among health worker cases occurred on February 1. In the 422 medical facilities serving COVID-19 patients, a total of 3,019 health workers have been infected (1,716 confirmed cases), and 5 have died.

Confirmed cases, case severity, and case fatality rates among health workers in different areas of China and different time periods are presented in Table 2. A total of 1,080 confirmed cases among health workers have been found in Wuhan, accounting for 64.0% of

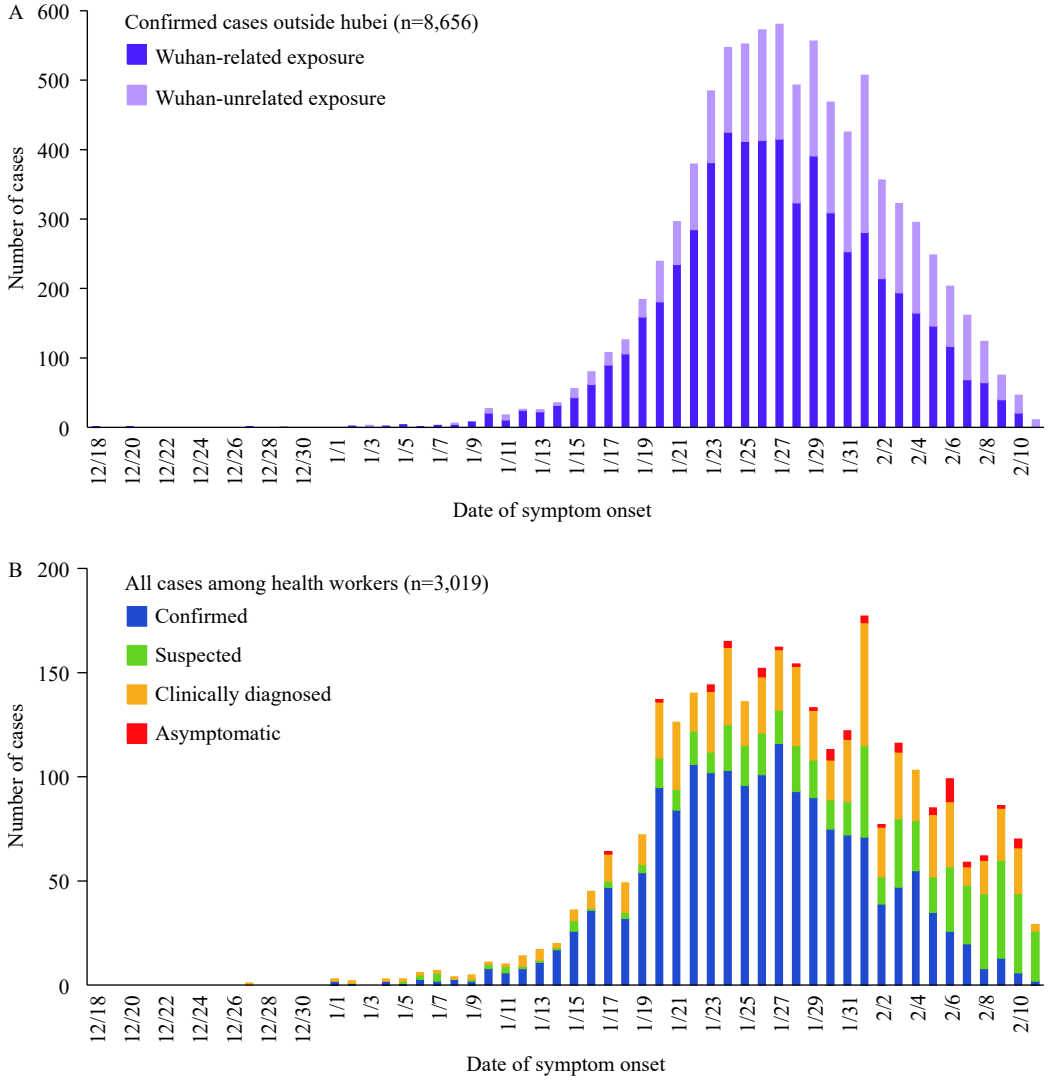


FIGURE 4. Subgroup epidemiological curves of COVID-19 in China through February 11, 2020. (A) subgroup analysis of confirmed cases discovered outside of Hubei Province only. The epidemiological curve shows the progression of illness in the outbreak over time from the onset of symptoms of the first case outside Hubei Province on December 18, 2019 through the end of February 11, 2020. Total confirmed cases outside Hubei Province, and Wuhan-related exposure (dark purple) versus Wuhan-unrelated exposure (light purple), are shown; (B) subgroup analysis of all cases among health workers only. The epidemiological curve shows the progression of illness in the outbreak over time from the onset of symptoms of the first health worker case on December 27, 2019 through the end of February 11, 2020. Total cases among health workers and confirmed (blue) versus suspected (green), clinically diagnosed (yellow), and asymptomatic (red) cases are shown.

national total. An additional 394 health worker cases (23.3%) were found in Hubei Province (excluding Wuhan), and 214 cases (12.7%) were found in the other 30 PLADs. The proportion of health worker cases that were severe or critical was 17.7% in Wuhan, 10.4% in Hubei Province, 7.0% in the remaining 30 PLADs, and 14.6% overall. The proportion of health worker cases in Wuhan classified as severe or critical declined from 38.9% in early January to 12.7% in early February. In China overall, the severe or critical cases among health workers also declined—from 45.0% in early January to 8.7% in early February.

Discussion

A main finding of this characterization and exploratory analysis of the first 72,314 cases of COVID-19 found in China in the 40 days between first recognition of the outbreak of pneumonia with unknown etiology on December 31, 2019 to the end of the study period on February 11, 2020 is that this novel coronavirus is highly contagious. It has spread extremely rapidly from a single city to the entire country within only about 30 days. Moreover, it has achieved such far-reaching effects even in the face of extreme response measures including the complete shutdown and isolation of whole cities, cancellation of Chinese New Year celebrations, prohibition of attendance at school and work, massive mobilization of health and public health personnel as well as military medical units, and rapid construction of entire hospitals.

In light of this rapid spread, it is fortunate that COVID-19 has been mild for 81% of patients and has a very low overall case fatality rate of 2.3%. Among the 1,023 deaths, a majority have been ≥60 years of age and/or have had pre-existing, comorbid conditions such as hypertension, cardiovascular disease, and diabetes. Moreover, the case fatality rate is surprisingly highest among critical cases at 49%, and no deaths have occurred among those with mild or even severe symptoms (Table 1).

A major contribution of our study is a first description of the COVID-19 epidemic curves. We interpret the overall curve (Figure 3A) as having a mixed outbreak pattern—the data appear to indicate a continuous common source pattern of spread in December and then from early January through February 11, 2020, the data appear to have a propagated source pattern. This mixed outbreak time trend is consistent with the working theory that perhaps several zoonotic events occurred at Huanan Seafood Wholesale Market in Wuhan allowed 2019-nCoV to be transmitted from a still-unknown animal into humans and, due to its high mutation and recombination rates, it adapted to become capable of and then increasingly efficient at human-to-human transmission (*3*,*8*).

The early days of the outbreak have been reminiscent of SARS and MERS, and indeed, the discovery that the causative agent was a closely-related, never-before-described coronavirus predicted potential for nosocomial transmission and so-called "super-spreader" events (*8*). Unfortunately, 2019-nCoV did indeed infect health workers in China via nosocomial transmission. Here we offer a first description of the 1,716 confirmed cases among health workers. Overall, they also display a likely mixed outbreak pattern—perhaps the data are characterized by a point source curve beginning in late December 2019, which

TABLE 2. Confirmed cases, case severity, and case fatality rates among health workers in different areas of China by time period.

Period (by date of onset)	Wuhan			Hubei (outside Wuhan)			China (outside Hubei)			China (overall)		
	Confirmed cases, N	Severe + critical, N (%)	Deaths, N (CFR*, %)	Confirmed cases, N	Severe + critical, N (%)	Deaths, N (CFR*, %)	Confirmed cases, N	Severe + critical, N (%)	Deaths, N (CFR*, %)	Confirmed cases, N	Severe + critical, N (%)	Deaths, N (CFR*, %)
Before Dec 31, 2019	0	0	0	0	0	0	0	0	0	0	0	0
Jan 1–10, 2020	18	7 (38.9)	1 (5.6)	1	1 (100)	0	1	1 (100)	0	20	9 (45.0)	1 (5.0)
Jan 11–20, 2020	233	52 (22.3)	1 (0.4)	48	8 (16.7)	0	29	1 (3.4)	0	310	61 (19.7)	1 (0.3)
Jan 21–31, 2020	656	110 (16.8)	0	250	29 (11.6)	2 (0.8)	130	10 (7.7)	0	1,036	149 (14.4)	2 (0.2)
After Feb 1, 2020	173	22 (12.7)	1 (0.6)	95	3 (3.2)	0	54	3 (5.6)	0	322	28 (8.7)	1 (0.3)
Total	1,080	191 (17.7)	3 (0.3)	394	41 (10.4)	2 (0.5)	214	15 (7.0)	0	1,688	247 (14.6)	5 (0.3)

Abreviation: CFR, case-fatality rate.

* CFR presented here was calculated as number of deaths (numerator) divided by total number of confirmed cases in the row (denominator), expressed as a percent.

was eclipsed by a higher magnitude continuous source curve beginning on January 20, 2020. To date, there is no evidence of a super-spreader event occurring in any of the Chinese health facilities serving COVID-19 patients. However, we do not know whether this is due to the nature of the virus itself or whether these events have been successfully prevented.

It is these authors' sincere hope and intent that this new analysis, on what has become a "public health emergency of international concern," (*12*) helps to inform health and public health workers preparing for or perhaps already experiencing COVID-19 in their populations. This study provides important insight into several crucial open questions on this epidemic and how to design strategies to effectively control it (*3*). For instance, the downward trend in the overall epidemic curve suggests that perhaps isolation of whole cities, broadcast of critical information (e.g., promoting hand washing, mask wearing, and care seeking) with high frequency through multiple channels, and mobilization of a multi-sector rapid response teams is helping to curb the epidemic.

China's response is certainly an echo of lessons learned during SARS and is a tribute to the work China and other low- and middle-income countries have been doing, with the much-needed help of international partners, over the past few decades to build infectious disease surveillance systems and public health infrastructure capable of catching outbreaks early and responding swiftly using evidence-based best practices. The 2019-nCoV and other coronaviruses may continue to adapt over time to become more virulent (*3*), and zoonosis is not going to stop. We must remain vigilant, hone our skills, fund our defenses, and practice our responses, and we must help our neighbors to do the same.

The very large number of cases included in our study was a major strength. Nevertheless, our study did have some important limitations. Firstly, a large proportion of cases included in our analysis (37%) were not confirmed by nucleic acid testing since this process is slow, labor intensive, and requires specialized equipment and skilled technicians. Yet all 72,314 cases were at least diagnosed clinically and investigated by trained epidemiologists. Secondly, some records did have missing data for a few important variables of interest—Wuhan-related exposure, comorbid conditions, and case severity—which limits our ability to draw conclusions from the data.

In conclusion, the present descriptive, exploratory analysis of the first 72,314 cases of COVID-19 reported through February 11, 2020 offers important new information to the international community on the epidemic in China. In particular, this analysis chronicles the extremely rapid spread of the novel coronavirus despite extreme efforts to contain it. However, important questions remain including identification of the animal reservoir, determination of infectiousness period, identification of transmission routes, and effective treatment and prevention methods including further test development, drug development, and vaccine development (*3*–*4*,*8*–*9*). As an international community, we must all be responsible partners in surveillance, communication, response, research, and implementation of evidence-based public health and clinical practice. The massive vigorous actions taken by the Chinese government have slowed down the epidemic in China and curbed spread to the rest of the world. Although the epidemic appears to be in decline in the lead up to February 11, 2020, we may yet face more challenges. Huge numbers of people will soon be returning to work and school after the extended New Year holiday. We need to prepare for a possible rebound of the COVID-19 epidemic in the coming weeks and months.

Acknowledgements

This work was supported by National Science and Technology Foundation of China (2018ZX10201002-008-002), and by National Nature Science Foundation of China (NSFC, 71934002). The funder had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

The team thanks all local health workers for their contributions in providing testing, treatment, and care to COVID-19 patients in China.

Disclaimer: The opinions expressed herein reflect the collective views of the co-authors and do not necessarily represent the official position of the National Center for AIDS/STD Control and Prevention of the Chinese Center for Disease Control and Prevention.

In order to share the results of epidemiological characteristics of COVID-19 domestically and internationally, the Chinese Version is jointly published on the *Chinese Journal of Epidemiology*.

Author Group & Contributions: The Novel Coronavirus Pneumonia Emergency Response EpidemiologyTeam includesZijian Feng, Qun Li, Zunyou Wu, Xiaoping Dong, Huilai Ma, DapengYin, Ke Lyu, Dayan Wang, Lei Zhou, Ruiqi Ren, Chao Li, Yali Wang, Dan Ni, Jing Zhao, Bin Li, Rui Wang, Yan Niu, XiaohuaWang, Lijie Zhang, Jingfang Sun, Boxi Liu, Zhiqiang Deng, Zhitao Ma, Yang Yang, Hui Liu, Ge Shao, Huan Li, Yuan Liu, Hangjie Zhang, ShuquanQu, Wei Lou, Dou Shan, YuehuaHu, Lei Hou, Zhenping Zhao, Jiangmei Liu, Hongyuan Wang, Yuanjie Pang, Yuting Han, Qiuyue Ma, Yujia Ma, Shi Chen, Wei Li, Routong Yang, Zhewu Li, Yingnan Guo, Xinran Liu, BahabaikeJiangtulu, ZhaoxueYin, Juan Xu, Shuo Wang, Lin Xiao, Tao Xu, Limin Wang, Xiao Qi, Guoqing Shi, Wenxiao Tu, Xiaomin Shi, Xuemei Su, Zhongjie Li, Huiming Luo, Jiaqi Ma, JenniferM. McGoogan. All Team members jointly conceptualized the study, analyzed and interpreted the data, wrote and revisedthe manuscript,and decidedto submit for publication.

#Corresponding author: Yanping Zhang, zhangyp@chinacdc.cn.

Submitted: February 14, 2020; Accepted: February 14, 2020

References

1. Wuhan Municipal Health Commission. Report of clustering pneumoniaof unknown etiology in Wuhan City. Wuhan, China: Wuhan Municipal Health Commission. http://wjw.wuhan.gov.cn/front/web/showDetail/2019123108989. [2019-12-31]. (In Chinese).
2. World Health Organization.Novel coronavirus– China. Geneva, Switzerland:World Health Organization. https://www.who.int/csr/don/12-january-2020-novel-coronavirus-china/en/. [2020-01-12].
3. Wang C, Hornby PW, Hayden FG, Gao GF. A novel coronavirus outbreak of global health concern. Lancet. http://dx.doi.org/10.1016/S0140-6736(20)30185-9. [2020-01-24].
4. Hui DS, Azhar EI, Madani TA, Ntoumi F, Kock R, Dar O, et al. The continuing 2019-nCoV epidemic threat of novel coronaviruses to global health - the latest 2019 novel coronavirus outbreak in Wuhan, China. Int J Infect Dis 2020;91(2020):264 − 6http://dx.doi.org/10.1016/j.ijid.2020.01.009.
5. Zhu N, Zhang D, Wang W, Li XW, Yang B, Song JD, et al. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. http://dx.doi.org/10.1056/NEJMoa2001017. [2020-01-24].
6. Chan JFW, Kok KH, Zhu Z, Chu H, To KKW, Yuan SF, et al. Genomic characterizationof the 2019 novel human-pathogenic coronavirusisolatedfrom a patient with atypical pneumoniaafter visiting Wuhan. Emerg Microbs Infect. http://dx.doi.org/10.1080/22221751.2020.1719902. [2020-01-24].
7. Tan WJ, Zhao X, Ma XJ, Wang WL, Niu PH, Xu WB, et al. A novel coronavirus genome identified in a cluster of pneumonia cases—Wuhan, China 2019−2020. China CDC Weekly 2020;2(4): 61-2. http://weekly.chinacdc.cn/en/article/ccdcw/2020/4/61.
8. Paules CI, Marston HD, Fauci AS. Coronavirus infection—more than just the common cold. JAMA. http://dx.doi.org/10.1001/jama.2020.0757. [2020-01-23].
9. Munster VJ, Koopmans M, van Doremalen N, van Riel D, de Wit E. A novel coronavirusemergingin China – key questionsfor impact assessment. N Engl J Med. http://dx.doi.org/10.1056/NEJMp2000929. [2020-01-24].
10. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patientsinfectedwith 2019 novel coronavirusin Wuhan, China. Lancet. http://dx.doi.org/10.1016/S0140-6736(20)30183-5. [2020-01-24]
11. Chan JFW, Yuan S, Kok KH, To KKW, Chu H, Yang J, et al. A familial cluster of pneumonia associatedwith the 2019 novel coronavirusindicating person-to-person transmission:a study of a family cluster. Lancet. http://dx.doi.org/10.1016/S0140-6736(20)30154-9. [2020-01-24].
12. World Health Organization. Statement on the second meeting of the International Health Regulations (2005) Emergency Committee regardingthe outbreakof novel coronavirus(2019-nCoV). Geneva, Switzerland:World Health Organization.https://www.who.int/news-room/detail/30-01-2020-statement-on-the-second-meeting-of-the-international-health-regulations-(2005)-emergency-committee-regarding-the-outbreak-of-novel-coronavirus-(2019-ncov). [2020-01-30].

JAMA | Original Investigation

N95 Respirators vs Medical Masks for Preventing Influenza Among Health Care Personnel
A Randomized Clinical Trial

Lewis J. Radonovich Jr, MD; Michael S. Simberkoff, MD; Mary T. Bessesen, MD; Alexandria C. Brown, PhD; Derek A. T. Cummings, PhD; Charlotte A. Gaydos, MD; Jenna G. Los, MLA; Amanda E. Krosche, BS; Cynthia L. Gibert, MD; Geoffrey J. Gorse, MD; Ann-Christine Nyquist, MD; Nicholas G. Reich, PhD; Maria C. Rodriguez-Barradas, MD; Connie Savor Price, MD; Trish M. Perl, MD; for the ResPECT investigators

IMPORTANCE Clinical studies have been inconclusive about the effectiveness of N95 respirators and medical masks in preventing health care personnel (HCP) from acquiring workplace viral respiratory infections.

OBJECTIVE To compare the effect of N95 respirators vs medical masks for prevention of influenza and other viral respiratory infections among HCP.

DESIGN, SETTING, AND PARTICIPANTS A cluster randomized pragmatic effectiveness study conducted at 137 outpatient study sites at 7 US medical centers between September 2011 and May 2015, with final follow-up in June 2016. Each year for 4 years, during the 12-week period of peak viral respiratory illness, pairs of outpatient sites (clusters) within each center were matched and randomly assigned to the N95 respirator or medical mask groups.

INTERVENTIONS Overall, 1993 participants in 189 clusters were randomly assigned to wear N95 respirators (2512 HCP-seasons of observation) and 2058 in 191 clusters were randomly assigned to wear medical masks (2668 HCP-seasons) when near patients with respiratory illness.

MAIN OUTCOMES AND MEASURES The primary outcome was the incidence of laboratory-confirmed influenza. Secondary outcomes included incidence of acute respiratory illness, laboratory-detected respiratory infections, laboratory-confirmed respiratory illness, and influenzalike illness. Adherence to interventions was assessed.

RESULTS Among 2862 randomized participants (mean [SD] age, 43 [11.5] years; 2369 [82.8%]) women), 2371 completed the study and accounted for 5180 HCP-seasons. There were 207 laboratory-confirmed influenza infection events (8.2% of HCP-seasons) in the N95 respirator group and 193 (7.2% of HCP-seasons) in the medical mask group (difference, 1.0%, [95% CI, −0.5% to 2.5%]; $P = .18$) (adjusted odds ratio [OR], 1.18 [95% CI, 0.95-1.45]). There were 1556 acute respiratory illness events in the respirator group vs 1711 in the mask group (difference, −21.9 per 1000 HCP-seasons [95% CI, −48.2 to 4.4]; $P = .10$); 679 laboratory-detected respiratory infections in the respirator group vs 745 in the mask group (difference, −8.9 per 1000 HCP-seasons, [95% CI, −33.3 to 15.4]; $P = .47$); 371 laboratory-confirmed respiratory illness events in the respirator group vs 417 in the mask group (difference, −8.6 per 1000 HCP-seasons [95% CI, −28.2 to 10.9]; $P = .39$); and 128 influenzalike illness events in the respirator group vs 166 in the mask group (difference, −11.3 per 1000 HCP-seasons [95% CI, −23.8 to 1.3]; $P = .08$). In the respirator group, 89.4% of participants reported "always" or "sometimes" wearing their assigned devices vs 90.2% in the mask group.

CONCLUSIONS AND RELEVANCE Among outpatient health care personnel, N95 respirators vs medical masks as worn by participants in this trial resulted in no significant difference in the incidence of laboratory-confirmed influenza.

TRIAL REGISTRATION ClinicalTrials.gov Identifier: NCT01249625

Visual Abstract
Editorial page 817
Supplemental content

Author Affiliations: Author affiliations are listed at the end of this article.

Group Information: The ResPECT investigators appear at the end of the article.

Corresponding Author: Lewis J. Radonovich Jr, MD, National Personal Protective Technology Laboratory, Centers for Disease Control and Prevention, 626 Cochrans Mill Rd, B141, R108, Pittsburgh, PA 15236 (mto5@cdc.gov).

JAMA. 2019;322(9):824-833. doi:10.1001/jama.2019.11645

© 2019 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Health Services Research Library User on 02/27/2020

Health care personnel (HCP) who are routinely exposed to viral respiratory infections in the workplace[1] may transmit infection to others. It is widely recognized that HCP, as a group, incompletely adhere to infection prevention recommendations and practice standards. Inpatient respiratory protection studies suggest adherence rates vary from 10% to 84%.[2-4] While laboratory studies designed to achieve 100% intervention adherence have shown that N95 filtering facepiece respirators are more efficacious than medical masks at reducing exposure to aerosols,[5] comparative clinical effectiveness studies have been inconclusive.[3,4,6] Some experts argue that N95 respirators and medical masks are equivalent in clinical settings.[2,7] Pragmatic effectiveness trials are increasingly recognized as an essential component of medical evidence, in part because efficacy studies may overestimate effectiveness and true adherence.[8]

Disposable N95 respirators and medical masks are both worn by HCP for self-protection; however, these masks have different intended uses. N95 respirators are designed to prevent the wearer from inhaling small airborne particles,[9] must meet filtration requirements,[10] and fit tightly to the wearer's face, limiting facial seal leakage. Medical masks, frequently called surgical masks, are intended to prevent microorganism transmission from the wearer to the patient. Medical masks fit the face loosely and do not reliably prevent inhalation of small airborne particles. However, medical masks prevent hand-to-face contact and facial contact with large droplets and sprays.[11]

Clinical evidence is inconclusive regarding whether N95 respirators are more effective than medical masks for preventing viral respiratory infection among HCP, including influenza,[3,4,6,12] accounting for differing practices[2] and positions held by clinical,[7] public health,[13,14] and regulatory organizations.[15] The objective of this study was to compare[13] the effectiveness of N95 respirators vs medical masks worn by HCP in clinical practice for prevention of workplace-acquired influenza and other viral respiratory infections in geographically diverse, high-exposure, outpatient settings.

Key Points

Question Is the use of N95 respirators or medical masks more effective in preventing influenza infection among outpatient health care personnel in close contact with patients with suspected respiratory illness?

Findings In this pragmatic, cluster randomized clinical trial involving 2862 health care personnel, there was no significant difference in the incidence of laboratory-confirmed influenza among health care personnel with the use of N95 respirators (8.2%) vs medical masks (7.2%).

Meaning As worn by health care personnel in this trial, use of N95 respirators, compared with medical masks, in the outpatient setting resulted in no significant difference in the rates of laboratory-confirmed influenza.

Methods

Study Sites and Institutional Review Boards

The Respiratory Protection Effectiveness Clinical Trial (ResPECT) was approved by the human subjects research board at the National Institute for Occupational Safety and Health (protocol #10-NPPTL-05XP) and the institutional review boards (IRBs) at the 7 participating health systems, as previously described,[16] and approved or exempted by IRBs at the analysis and sample storage sites. All participants were permitted to participate for 1 or more years and gave written consent for each year of participation. Study intervention sites included outpatient settings at the Children's Hospital Colorado (Aurora), Denver Health Medical Center (Denver, Colorado), Johns Hopkins Health System (Baltimore, Maryland), Michael E. DeBakey Veterans Affairs (VA) Medical Center (Houston, Texas), VA Eastern Colorado Healthcare System (Denver), Washington DC VA Medical Center, and VA New York Harbor Healthcare System (New York). Sample storage and data analysis sites were the VA St Louis Healthcare System and St Louis University (St Louis, Missouri), University of Florida (Gainesville), University of Massachusetts (Amherst), and University of Texas Southwestern Medical Center (Dallas).

Design and Oversight

This cluster randomized, multicenter, pragmatic effectiveness trial[16] conducted between September 2011 and May 2015, with final follow-up on June 28, 2016, compared the effect of N95 respirators, used as recommended during the 2009 H1N1 pandemic,[13] and medical masks, used as recommended to prevent seasonal influenza[17,18] and other viral respiratory infections and illnesses, among HCP.[17] The investigators were blinded to the randomization until completion of the study and analysis. An independent data and safety monitoring board assessed the data. Additional details are included in Supplement 1, including the statistical analysis plan and the full protocol that was previously published in an abridged format.[16]

Participants and Setting

This trial was conducted in diverse outpatient settings serving adult and pediatric patients with a high prevalence of acute respiratory illness, including primary care facilities, dental clinics, adult and pediatric clinics, dialysis units, urgent care facilities and emergency departments, and emergency transport services.

All participants in a cluster worked in the same outpatient clinic or outpatient setting. A cluster randomized design was used to improve adherence and increase indirect effects associated with participants in a cluster using the same intervention. Participants were aged at least 18 years, employed at one of the 7 participating health systems, and self-identified as routinely positioned within 6 feet (1.83 m) of patients. Participants were full-time employees (defined as direct patient care for approximately ≥24 hours weekly) and worked primarily at the study site (defined as ≥75% of working hours). Exclusion criteria were medical conditions precluding safe participation or anatomic features that

© 2019 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Health Services Research Library User on 02/27/2020

Box 1. Criteria for Acute Respiratory Illness[a]

Signs
Coryza
Fever (temperature >37.8 °C)
Lymphadenopathy
Tachypnea (respiratory rate >25/min)
Symptoms
Arthralgias/myalgias/body aches
Chills
Cough
Diarrhea
Dyspnea
Fatigue
Headache
Malaise
Other gastrointestinal systems
Sore throat
Sputum production
Sweats
Vomiting/nausea

[a] An acute respiratory illness was defined as the presence of at least 1 sign or 2 symptoms listed, representing a change from baseline.

could interfere with respirator fit, such as facial hair or third-trimester pregnancy. Participants self-identified race and sex using fixed categories; these variables were collected because facial anthropometrics related to race and sex may influence N95 respirator fit.

Participants kept diaries that included signs and symptoms of respiratory illness, annual influenza vaccination status, and exposure to household and community members with respiratory illness. Participants also recorded their participation in aerosol-generating procedures and exposure to patients, coworkers, or both with respiratory illness daily. Participants were categorized for exposure risk by occupational roles.

Procedures, Interventions, and Group Allocation

Each year, participating sites were cluster randomized to have participants wear N95 respirators[13] or medical masks,[17,18] as previously described.[16] N95 respirator models studied were the 3M Corporation 1860, 1860S, and 1870 (St Paul, Minnesota) and the Kimberly Clark Technol Fluidshield PFR95-270, PFR95-274 (Dallas, Texas); medical mask models were the Precept 15320 (Arden, North Carolina) and Kimberly Clark Technol Fluidshield 47107 (Dallas, Texas).

Within each medical center, for each study year, pairs of clusters (clinics and other settings) were matched by the number of participants, health services delivered, patient population served, and additional personal protective equipment. One cluster was randomly assigned to the medical mask group and one to the N95 respirator group. Random allocation of clusters required using constrained randomization, a process that maintains random assignment and balance between groups.[19] Computer-generated random sequences of group assignments were generated by an individual not involved in the study implementation and data analyses. Random sequences of assignment assured that every participant in each season had an equal probability of being assigned to the N95 respirator and medical mask groups and allowed participants to switch groups between seasons. Occupational Safety and Health Administration–accepted fit testing[15] of N95 respirators was conducted annually for all study participants.

Participants were instructed to wear their assigned protective devices (ie, N95 respirators or medical masks) during the 12-week period (the intervention period) during which the incidence of viral respiratory illness and infections was expected to be highest that year, as predicted by the ALERT algorithm[20] developed for this trial. Participants were instructed to put on a new device whenever they were positioned within 6 feet (1.83 m) of patients with suspected or confirmed respiratory illness. Hand hygiene was recommended to all participants in accordance with Centers for Disease Control and Prevention guidelines.[13,17,18] Infection prevention policies were followed at each study site. Participants volunteered to participate for up to 12 weeks each intervention period, for a total of 48 weeks of intervention spanning 4 consecutive viral respiratory seasons.

Surveillance, Outcomes, and Measures of Effectiveness

Study personnel obtained swabs of the anterior nares and oropharynx[21] (FLOQSwabs UTM, Diagnostic Hybrids) from participants who self-reported symptoms of respiratory illness (**Box 1**). Symptomatic swabs were collected within 24 hours of self-report, and again if signs or symptoms persisted beyond 7 days. If symptomatic participants were not at work, samples were self-obtained using a structured process and shipped to the study laboratory. During each 12-week intervention period, 2 random swabs were obtained from all participants, typically while asymptomatic. Additionally, each year, paired serum samples obtained from all participants were assayed for influenza hemagglutinin levels before and after peak viral respiratory season.

The prespecified primary outcome was the incidence of laboratory-confirmed influenza, defined as detection of influenza A or B virus by reverse-transcription polymerase chain reaction[22] in an upper respiratory specimen collected within 7 days of symptom onset; detection of influenza from a randomly obtained swab from an asymptomatic participant; or influenza seroconversion (symptomatic or asymptomatic), defined as at least a 4-fold rise in hemagglutination inhibition antibody titers to influenza A or B virus between preseason and postseason serological samples deemed not attributable to vaccination. Individuals experiencing seroconversion were not required to have a detected symptomatic illness to meet the defined outcome. Influenza reagents used in the hemagglutination inhibition antibody assays were obtained from the International Reagent Resource Program, established by the Centers for Disease Control and Prevention.

© 2019 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Health Services Research Library User on 02/27/2020

Secondary outcome measures were the incidence of 4 measures of viral respiratory illness and infection: (1) acute respiratory illness (Box 1) with or without laboratory confirmation; (2) laboratory-detected respiratory infection, defined as detection of a respiratory pathogen by polymerase chain reaction or serological evidence of infection with a respiratory pathogen during the study surveillance period(s), which was added to the protocol prior to data analysis; (3) laboratory-confirmed respiratory illness, identified as previously described,[23] defined as self-reported acute respiratory illness plus the presence of at least 1 polymerase chain reaction-confirmed viral pathogen (**Box 2**) in a specimen collected from the upper respiratory tract within 7 days of the reported symptoms and/or at least a 4-fold rise from preintervention to postintervention serum antibody titers to influenza A or B virus; and (4) influenzalike illness, defined as temperature of at least 100°F (37.8°C) plus cough and/or a sore throat, with or without laboratory confirmation.

Adherence to Group Assignment and Infection Prevention and Control Practices

Participants were reminded to adhere to protective device and hand hygiene instructions by signage posted at study sites, email, and by study personnel in person. Adherence to assigned devices were reported daily by participants as "always," "sometimes," "never," or "did not recall." In addition, study personnel observed participants' device-wearing behaviors as they entered and exited patient care rooms by conducting unannounced, inconspicuous visits to randomly selected study sites throughout the intervention period. However, to preserve patient confidentiality, monitors were not permitted to enter patient care rooms.

Statistical Analyses

Although we identified no standard definition of a "clinically significant difference," this study[16] was designed to detect a 25% relative reduction in the incidence of laboratory-confirmed influenza or respiratory illness, based on expert opinion, rather than an absolute reduction, which has been described in a previous study.[6] The total sample size required to provide 80% power to show a 25% reduction in the incidence of laboratory-confirmed influenza in the N95 respirator group compared with the medical mask group, with a type I error rate of .05, was 10 024 participant-sessions, and the sample size needed to provide 80% power to show a 25% reduction in the incidence of laboratory-confirmed respiratory illness was 5104 participant-seasons.

Comparative effects of the interventions were estimated for the primary and secondary outcomes by calculating odds ratios (ORs; for binary outcomes) and incidence rate ratios (IRRs; for count outcomes) between participant clusters randomly assigned to wear N95 respirators or medical masks. Laboratory-confirmed influenza was modeled using logistic regression and viral respiratory infection and illness outcomes were modeled using Poisson regression. Unadjusted and adjusted analyses (both prespecified) were conducted according to the statistical analysis plan (Supplement 2). The primary outcome was an adjusted analysis, as specified in the statistical analysis plan. Prespecified covariates used in adjusted analyses included age, sex, race, number of household members younger than 5 years, occupation risk level (defined as low, medium, or high), binary season-specific influenza vaccination status, the proportion of daily exposures to others with respiratory illness, categorical self-reported adherence to hand hygiene, and intervention group assignment. Prespecified adherence rates were calculated as the proportion of reports of adherence in each group reporting "always," "sometimes," "never," or "did not recall." Comparison of proportions between groups were done using χ^2 statistics and comparisons of binomial proportions. Analyses included random effects to account for correlation of outcomes at site-level and individual-level random effects to account for correlation of outcomes at the individual level for participants who participated for multiple seasons.

The primary analysis used available data on all randomized participants for the primary comparison of the intervention. A per-protocol analysis, conducted at the same time as the primary analysis, included only individuals who completed at least 8 weeks of study participation.

A sensitivity analysis was conducted using imputation to assign outcomes to participants who did not complete the study. Missing outcomes were imputed using standard multiple imputation techniques, creating multiple imputed data sets with no missing values for each analysis.[23] Details of this analysis are described in Supplement 2. Intervention group withdrawal rates and time to withdrawal were compared to assess for potential bias. In an additional sensitivity analysis, observed and self-reported exposures and adherence were compared using Pearson χ^2 tests. Mean workplace and household rates of exposure to respiratory illness were compared using mixed-effects logistic regression. For

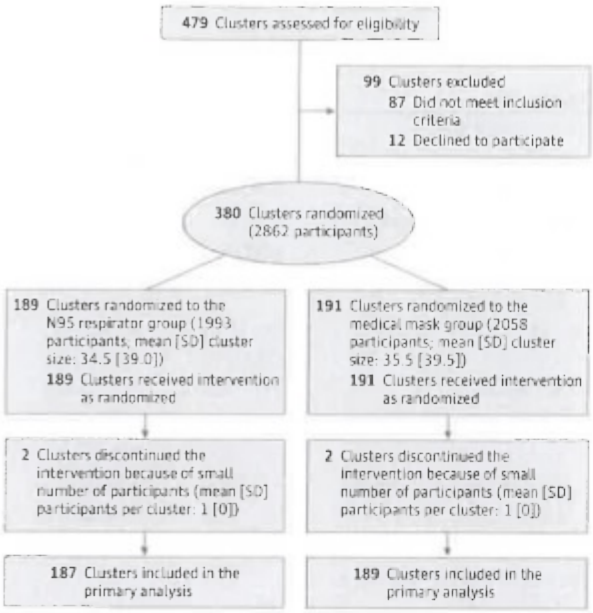
Box 2. Respiratory Pathogens Assayed by Polymerase Chain Reaction

- Adenoviruses
- Coxsackie/echoviruses
- Coronavirus HKU1
- Coronavirus NL63
- Coronavirus OC43
- Coronavirus 229E
- Human metapneumovirus
- Human rhinovirus
- Influenza A
- Influenza B
- Parainfluenza virus type 1
- Parainfluenza virus type 2
- Parainfluenza virus type 3
- Parainfluenza virus type 4a
- Parainfluenza virus type 4b
- Respiratory syncytial virus type A
- Respiratory syncytial virus type B

© 2019 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Health Services Research Library User on 02/27/2020

Figure 1. Study Site Enrollment, Randomization, Follow-up, and Analysis in a Study of the Effect of N95 Respirators vs Medical Masks for Preventing Laboratory-Confirmed Influenza Among Health Care Personnel



all calculations, a 2-sided type I error probability of .05 was used. Because of the potential for type I error due to multiple comparisons, findings for analyses of secondary end points should be interpreted as exploratory. All statistical analyses were performed in R version 3.3.3 (R Foundation).

Results

Participants

The study sites were randomized to provide 380 cluster-seasons of observation over 4 consecutive intervention periods. Of the 2862 participants, 1416 participated for more than 1 year or intervention period. Among 2862 unique randomized participants (mean [SD] age, 43 [11.5] years; 2369 [82.8%] women), 2371 completed the ResPECT protocol over the course of 48 weeks of intervention spanning 4 years. Among these individuals, 1446 participated in one 12-week intervention period, 723 participated in two 12-week intervention periods, and 693 participated in 3 or more 12-week intervention periods, accounting for 5180 HCP-seasons enrolled and randomized from 137 medical centers. Following randomization, 491 participants withdrew or were excluded because the cluster size was below a preestablished threshold of 2. Overall, 4689 HCP-seasons were included in the per-protocol analysis (2243 in the N95 respirator group and 2446 in the medical mask group; **Figure 1**). Some members of the primary analytic cohort did not complete all weeks of the study and were missing serological outcomes. Data were missing because of early withdrawal in 189 of 2512 participants (7.5%) in the N95 respirator group and 145 of 2668 (5.4%) in the medical mask group. In the per-protocol analysis, data were missing from 16 of 2243 participants (0.7%) in the N95 respirator group and 28 of 2446 (1.1%) in the medical mask group.

Baseline characteristics of the participants in the N95 respirator and medical mask groups were similar (**Table 1**). Daily workplace exposure to respiratory illness was reported 22.5% of the time in the N95 group and 21.6% of the time in the medical mask group, while weekly household exposure to respiratory illness was reported 3.6% of the time in the N95 respirator group and 3.4% of the time in the medical mask group (Table 1).

Illness Surveillance and Effectiveness

In the primary analysis, the incidence of laboratory-confirmed influenza infection events occurred in 207 of 2512 HCP-seasons (8.2%) in the N95 respirator group and 193 of 2668 HCP-seasons (7.2%) in the medical mask group, (difference, 1.0% [95% CI, −0.5% to 2.5%]; P = .18) (adjusted OR, 1.18 [95% CI, 0.95-1.45]).

Regarding secondary outcomes, there were 1556 acute respiratory illness events in the N95 respirator group (incidence rate [IR], 619.4 per 1000 HCP-seasons) vs 1711 in the medical mask group (IR, 641.3 per 1000 HCP-seasons) (difference, −21.9 per 1000 HCP-seasons [95% CI, −48.2 to 4.4]; P = .10; adjusted IRR, 0.99 [95% CI, 0.92-1.06]). There were 679 laboratory-detected respiratory infection events in the N95 respirator group (IR, 270.3 per 1000 HCP-seasons) vs 745 in the medical mask group (IR, 279.2 per 1000 HCP-seasons) (difference, −8.9 per 1000 HCP-seasons [95% CI, −33.3 to 15.4]; P = .47; adjusted IRR, 0.99 [95% CI, 0.89-1.09]) (**Table 2** and **Figure 2**). Overall, 371 laboratory-confirmed respiratory illness events occurred in the N95 respirator group (IR, 147.7 per 1000 HCP-seasons) vs 417 in the medical mask group (IR, 156.3 per 1000 HCP-seasons) (difference, −8.6 per 1000 HCP-seasons [95% CI, −28.2 to 10.9]; P = .39; adjusted IRR, 0.96 [95% CI, 0.83-1.11]). There were 128 influenzalike illness events in the N95 respirator group (IR, 51.0 per 1000 HCP-seasons) vs 166 in the medical mask group (IR, 62.2 per 1000 HCP-seasons) (difference, −11.3 per 1000 HCP-seasons [95% CI, −23.8 to 1.3]; P = .08; adjusted IRR, 0.86 [95% CI, 0.68-1.10]). Results were similar in the adjusted primary analysis and per-protocol analyses (Figure 2).

Intervention, Adherence, and Adverse Events

Adherence was reported on daily surveys 22 330 times in the N95 respirator group and 23 315 times in the medical mask group. "Always" was reported 14 566 (65.2%) times in the N95 respirator group and 15 186 (65.1%) times in the medical mask group; "sometimes," 5407 (24.2%) times in the N95 respirator group and 5853 (25.1%) times in the medical mask group; "never," 2272 (10.2%) times in the N95 respirator group and 2207 (9.5%) times in the medical mask group; and "did not recall," 85 (0.4%) times in the N95 respirator group and 69 (0.3%) times in the medical mask group. Participant-reported adherence could not be assessed in 784 participants (31.2%) in the N95 respirator group and 822 (30.8%) in the medical mask group (P = .84)

© 2019 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Health Services Research Library User on 02/27/2020

Table 1. Health Care Personnel (HCP) Demographic Characteristics, Risk Factors, and Site Enrollment in a Study of the Effect of N95 Respirators vs Medical Masks for Preventing Laboratory-Confirmed Influenza

Characteristic	No. (%) N95 Respirator (n = 2512 HCP-Seasons)[a]	No. (%) Medical Mask (n = 2668 HCP-Seasons)[a]
Age, mean (SD), y	43 (11.5)	43 (11.6)
Sex		
Men	378 (15.0)	420 (15.7)
Women	2134 (85.0)	2248 (84.3)
Ethnicity		
Hispanic or Latino	397 (15.8)	427 (16)
Race	(n = 2447)	(n = 2600)
White	1282 (52.4)	1334 (51.3)
Black	720 (29.4)	782 (30.1)
Other	232 (9.5)	252 (9.7)
Asian	195 (8.0)	210 (8.1)
American Indian or Alaska Native	14 (0.6)	13 (0.5)
Native Hawaiian or other Pacific Islander	4 (0.2)	9 (0.3)
Occupation		
Nurse/nursing trainee	1049 (41.8)	1085 (40.7)
Clinical care support staff[b]	574 (22.9)	627 (23.5)
Administrative/clerical	332 (13.2)	337 (12.6)
Other occupation	213 (8.5)	224 (8.4)
Physician/advanced practitioner/ physician trainee	207 (8.2)	240 (9.0)
Registration/clerical reception	94 (3.7)	106 (4.0)
Social worker/pastoral care	35 (1.4)	29 (1.1)
Environmental services/ housekeeping	8 (0.3)	19 (0.7)
Occupational risk[c]		
High	1492 (59.4)	1594 (59.7)
Medium	295 (11.7)	318 (11.9)
Low	724 (28.8)	755 (28.3)
Patient population		
Adult	1409 (56.1)	1486 (55.7)
Pediatric	573 (22.8)	557 (20.9)
Adult and pediatric	530 (21.1)	625 (23.4)
Clinic type		
Primary care	1734 (69.0)	1881 (70.5)
Emergent/urgent care	665 (26.5)	700 (26.2)
Emergency transport	42 (1.7)	33 (1.2)
Specialty care	40 (1.6)	29 (1.1)
Dental/dialysis	31 (1.2)	25 (0.9)
Site		
Johns Hopkins Health System	882 (35.1)	859 (32.2)
Denver Health	534 (21.3)	521 (19.5)
VA New York Harbor Healthcare System	375 (14.9)	433 (16.2)
The Michael E. DeBakey VA Medical Center	233 (9.3)	287 (10.8)
Washington DC VA Medical Center	183 (7.3)	204 (7.6)
VA Eastern Colorado Healthcare System	177 (7.0)	211 (7.9)
Children's Hospital Colorado	128 (5.1)	153 (5.7)
Comorbid conditions		
Asthma	255 (10.2)	284 (10.6)
Other systemic disease	104 (4.1)	118 (4.4)
Other respiratory disease	49 (2.0)	37 (1.4)
Cardiac disease	41 (1.6)	34 (1.3)
Chronic obstructive pulmonary disease	6 (0.2)	6 (0.2)
Influenza vaccination status	(n = 2444)	(n = 2598)
Vaccinated	1993 (79.3)	2048 (76.8)
Not vaccinated	451 (18.0)	550 (20.6)
Other risk factors		
Eyeglasses wearer	960 (38.2)	999 (37.4)
Household members aged <5 y	606 (24.1)	630 (23.6)
Contact lens wearer	371 (14.8)	349 (13.1)
Tobacco smoker	210 (8.4)	234 (8.8)
Exposure to respiratory illness, %		
Daily workplace	22.5	21.6
Weekly household	3.6	3.4

Abbreviation: VA, veterans affairs.

[a] Unless otherwise specified.

[b] Staff who have direct patient contact, such as clinical medical assistants and clinical technicians.

[c] Occupational risk based on direct patient contact, such as physical examination and/or performance of high-risk procedures (intubation, airway suctioning, nebulizer treatments, nasopharyngeal aspiration) for high risk, direct patient contact for medium risk, and no or minimal direct patient contact for low risk.

because of lack of response to surveys or lack of adherence opportunities (ie, participants did not encounter an individual with respiratory signs or symptoms).

Analyzed post hoc, participant adherence was reported as always or sometimes 89.4% of the time in the N95 respirator group and 90.2% of the time in the medical mask group. Additional details about adherence are included in Supplement 1. No serious study-related adverse events were reported. Nineteen participants reported skin irritation or worsening acne during years 3 and 4 at one study site in the N95 respirator group.

Per-Protocol Analysis and Sensitivity Analysis

Results of the per-protocol analysis can be seen in Figure 2. A sensitivity analysis assessed whether there was evidence for bias in self-reported outcomes based on group assignment. In a prespecified multiple-imputation analysis, the rates of laboratory-confirmed influenza infection events were 204 of 2243 HCP seasons (9.1%) in the N95 respirator group and 190 of 2446 HCP-seasons (7.8%) in the medical mask group. Quantitative data are available in Supplement 3.

© 2019 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Health Services Research Library User on 02/27/2020

Table 2. Primary and Secondary Outcomes in a Study of the Effect of N95 Respirators vs Medical Masks for Preventing Laboratory-Confirmed Influenza Among Health Care Personnel

Primary and Secondary Outcome Events	No.									
	2011-2012		2012-2013		2013-2014		2014-2015		Totals	
	N95 Respirator	Medical Mask	N95 Respirator	Medical Mask	N95 Respirator	Medical Mask	N95 Respirator	Medical Mask	N95 Respirator	Medical Mask
Influenza (primary outcome)										
Polymerase chain reaction–detected										
Influenza A	2	3	19	19	8	12	37	28	66	62
Influenza B	0	3	8	11	2	1	1	4	11	19
Hemagglutination inhibition assay–detected										
Influenza A	5	9	30	23	38	38	55	47	128	117
Influenza B	0	2	10	11	12	13	14	10	36	36
All events[a]										
Influenza A	6	10	43	37	46	42	85	65	180	154
Influenza B	0	5	15	18	12	14	15	13	42	50
All influenza	6	15	58	55	58	56	100	78	222	204
Laboratory-confirmed influenza	6	13	52	52	55	51	94	77	207	193
Secondary Outcomes										
Acute respiratory illness	235	234	354	446	398	519	569	512	1556	1711
Laboratory-detected respiratory infection[b]	47	71	165	201	217	260	250	213	679	745
Laboratory-confirmed respiratory illness[b]	26	31	91	116	111	150	143	120	371	417
Influenzalike illness	13	10	30	45	22	50	63	61	128	166

[a] Influenza events were defined as the number of influenza infections attributed to the combination of polymerase chain reaction detection and hemagglutination inhibition assay serologies. Instances in which polymerase chain reaction and hemagglutination inhibition assay were both positive counted as 1 event.

[b] All respiratory viruses assayed, including influenza.

Discussion

In this pragmatic, cluster randomized trial that involved multiple outpatient sites at 7 health care delivery systems across a wide geographic area over 4 seasons of peak viral respiratory illness, there was no significant difference between the effectiveness of N95 respirators and medical masks in preventing laboratory-confirmed influenza among participants routinely exposed to respiratory illnesses in the workplace. In addition, there were no significant differences between N95 respirators and medical masks in the rates of acute respiratory illness, laboratory-detected respiratory infections, laboratory-confirmed respiratory illness, and influenzalike illness among participants. A sensitivity analysis suggested that the primary analysis reported was fairly robust to the missing outcome data with quantitative outcomes varying by less than 5%. This supports the finding that neither N95 respirators nor medical masks were more effective in preventing laboratory-confirmed influenza or other viral respiratory infection or illness among participants when worn in a fashion consistent with current US clinical practice.

Respiratory viruses are primarily transmitted by large droplets. Because a fraction of respiratory viruses may be transmitted by aerosol, N95 respirators have been presumed to provide better protection than medical masks against viral respiratory infections in health care settings.[2] However, definitive evidence of greater clinical effectiveness of N95 respirators is lacking. A well-designed trial[6] found the effectiveness of medical masks to be noninferior to N95 respirators, but the trial was stopped prematurely and was limited by small sample size. Two additional studies[3,4] (and a pooled analysis[12]) concluded that N95 respirators may be more effective than medical masks; however, these studies were limited by uncertain clinical significance of end points.[24] The current study was undertaken because of remaining uncertainty based on previous studies, which made it challenging for infection control clinicians to effectively implement respiratory protection programs in health care settings.[2,7,13,18,24,25]

This trial was designed to assess clinical effectiveness, taking into account many challenges of working in outpatient health care settings. This study had several strengths, including the pragmatic design; wide US geographic and climatic distribution; varied adult and pediatric outpatient settings, including emergency departments; and enrollment spanning 4 seasons of peak viral respiratory illness. Respiratory samples were obtained from symptomatic and asymptomatic participants to determine the incidence of viral respiratory infection, including individuals that were subclinical but still potentially transmissible. Influenza vaccination status information was collected. This trial was cluster randomized to avoid mixing of interventions in each clinic and clinical setting and to minimize cross-contamination from

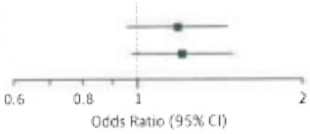
© 2019 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Health Services Research Library User on 02/27/2020

Figure 2. Primary and Secondary Outcomes of Influenza and Respiratory Illnesses and Adjusted Risk Estimates Among Health Care Personnel in the N95 Respirator Group vs the Medical Mask Group

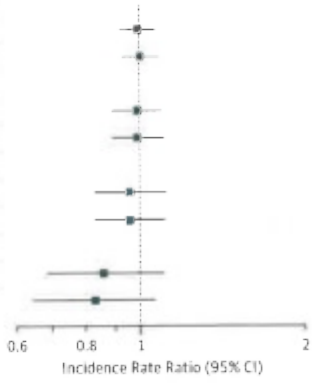
A Primary outcome

	N95 Respirator Events/Seasons	Medical Mask Events/Seasons	Incidence Rate Ratio (95% CI)
Laboratory-confirmed influenza			
ITT cohort	207/2512	193/2668	1.18 (0.95-1.45)
PP cohort	204/2243	190/2446	1.20 (0.97-1.48)



B All secondary outcomes

	N95 Respirator Events/Seasons	Medical Mask Events/Seasons	Incidence Rate Ratio (95% CI)
Acute respiratory illness			
ITT cohort	1556/2512	1711/2668	0.99 (0.92-1.06)
PP cohort	1512/2243	1656/2446	1.00 (0.93-1.08)
Laboratory-detected respiratory infection			
ITT cohort	679/2512	745/2668	0.99 (0.89-1.09)
PP cohort	664/2243	733/2446	0.99 (0.89-1.10)
Laboratory-confirmed respiratory illness			
ITT cohort	371/2512	417/2668	0.96 (0.83-1.11)
PP cohort	361/2243	406/2446	0.96 (0.83-1.11)
Influenzalike illness			
ITT cohort	128/2512	166/2668	0.86 (0.68-1.10)
PP cohort	121/2243	161/2446	0.83 (0.64-1.06)



The adjusted relative risks for the N95 respirator and medical mask groups for both the intention-to-treat (ITT) and per-protocol (PP) groups for the primary outcome and the other predetermined secondary outcomes. Values above 1 indicate higher relative odds or risk in the N95 respirator group compared with the medical mask group.

different HCP behaviors, conducted at 7 medical centers among frontline HCP in varied clinical settings with high exposure risk, and sufficiently powered to detect the predefined difference in laboratory-confirmed respiratory illness. Previous effectiveness studies[3,4,6,12,26-28] have met some, but not all, of these characteristics and have been inconclusive, contributing to the uncertainty and controversy among experts determining public health guidance, regulatory requirements, and health care delivery practices.[2,7,14,17,29] In the current study, findings were consistent across all laboratory-based outcomes and clinical syndromes. Results for the primary and secondary outcomes were in opposite directions (ie, one IRR was associated with increased risk and the other with decreased risk), although the differences were nonsignificant, further supporting a finding of no significant difference in the effectiveness of N95 respirators vs medical masks for prevention of influenza or other respiratory illness.

Limitations

This study has several limitations. First, the criteria for viral polymerase chain reaction testing may have missed participants who were infected but asymptomatic. Unrecognized infections may have increased the probability of finding no difference between interventions, even if a difference existed. Second, self-reporting of symptoms in daily diaries likely underestimated illness among HCP who often work while ill.[30] Third, despite being intentionally conducted as a pragmatic effectiveness trial,[8] incomplete participant adherence to assigned protective devices could have contributed to more unprotected exposures, increasing the probability of finding no difference between interventions even if a difference existed. However, participant-reported data indicates this did not differ by study group. Fourth, participants were not instructed to wear protective devices outside the workplace, which may have biased the results toward finding no difference between groups, although the rates of adherence did not differ by study group and household exposure was reported as much lower than workplace exposure. Fifth, only 2 N95 respirator and medical mask models were studied, limiting the ability to generalize about the protectiveness of other models. Sixth, the sample size required to definitively determine whether N95 respirators or medical masks are more effective for protection from laboratory-confirmed influenza in the health care setting required approximately 10 000 participant-seasons, which was not feasible with the available funding or resources. However, the morbidity and mortality associated with a wide range of viral respiratory infections, including novel and emerging pathogens, renders a secondary outcome in this study, laboratory-confirmed respiratory illness, important.

Conclusions

Among outpatient HCP, N95 respirators vs medical masks as worn by participants in this trial resulted in no significant difference in the incidence of laboratory-confirmed influenza.

© 2019 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Health Services Research Library User on 02/27/2020

ARTICLE INFORMATION

Accepted for Publication: July 25, 2019.

Author Affiliations: National Personal Protective Technology Laboratory, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Pittsburgh, Pennsylvania (Radonovich); Veterans Affairs New York Harbor Healthcare System, New York (Simberkoff); New York University School of Medicine, New York (Simberkoff); Veterans Affairs Eastern Colorado Healthcare System, Denver (Bessesen); University of Colorado School of Medicine, Aurora (Bessesen, Nyquist, Price); University of Massachusetts, Amherst (Brown, Reich); University of Florida, Gainesville (Cummings); Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland (Cummings, Perl); Johns Hopkins School of Medicine, Baltimore, Maryland (Gaydos, Los, Krosche); Weill Cornell Medicine, New York, New York (Krosche); Veterans Affairs Medical Center, Washington, DC (Gibert); George Washington University School of Medical and Health Sciences, Washington, DC (Gibert); Veterans Affairs St Louis Healthcare System, St Louis, Missouri (Gorse); St Louis University School of Medicine, St Louis, Missouri (Gorse); Children's Hospital Colorado, Aurora (Nyquist); Michael E. DeBakey Veterans Affairs Medical Center, Houston, Texas (Rodriguez-Barradas); Baylor College of Medicine, Houston, Texas (Rodriguez-Barradas); Denver Health Medical Center, Denver, Colorado (Price); University of Texas Southwestern Medical Center, Dallas (Perl).

Author Contributions: Drs Perl and Radonovich had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
Concept and design: Radonovich, Simberkoff, Cummings, Gaydos, Gorse, Reich, Perl.
Acquisition, analysis, or interpretation of data: All authors.
Drafting of the manuscript: Radonovich, Simberkoff, Cummings, Gaydos, Nyquist, Reich, Perl.
Critical revision of the manuscript for important intellectual content: Radonovich, Bessesen, Brown, Cummings, Gaydos, Los, Krosche, Gibert, Gorse, Nyquist, Reich, Rodriguez-Barradas, Price, Perl.
Statistical analysis: Brown, Cummings, Reich.
Obtained funding: Cummings, Perl.
Administrative, technical, or material support: Radonovich, Simberkoff, Bessesen, Cummings, Gaydos, Los, Krosche, Gorse, Nyquist, Rodriguez-Barradas, Price, Perl.
Supervision: Radonovich, Simberkoff, Cummings, Los, Nyquist, Reich, Price, Perl.
Other - execution of study design; data management and monitoring: Los.
Other - Site principal investigator for conduct of study and also contributed ongoing input on conduct and analysis of study: Gibert.
Other - laboratory testing support: Gaydos.
Other - recruiting patients: Price.

Conflict of Interest Disclosures: Dr Bessesen reported receiving grants from the Department of Veterans Affairs during the conduct of the study. Dr Brown reported receiving grants from the US Department of Veterans Affairs during the conduct of the study. Dr Cummings reported receiving grants from the Centers for Disease Control and Prevention, the National Institutes of Health, and MedImmune outside the submitted work and the Biomedical Advanced Research and Development Authority during the conduct of the study. Ms Los reported receiving grants from Centers for Disease Control and Prevention, the Veterans Health Administration, and the Biodefense Advanced Research and Development Agency during the conduct of the study. Dr Gibert reported receiving financial support for the conduct of the study, including research personnel, from the Veterans Health Administration during the conduct of the study. Dr Gorse reported receiving grants from the US Department of Veterans Affairs during the conduct of the study. Dr Nyquist reported receiving grants from the Centers for Disease Control and Prevention/Division of Healthcare Quality Promotion, the National Institute for Occupational Safety and Health, and the Veterans Health Administration during the conduct of the study; personal fees and nonfinancial support from Sequirus outside the submitted work; and serving on a policy making committee regarding infectious disease for the American Academy of Pediatrics Committee on Infectious Diseases. Dr Reich reported receiving grants from Veterans Health Administration during the conduct of the study. Dr Rodriguez-Barradas reported receiving grants from Veterans Affairs Central Office during the conduct of the study. Dr Perl reported receiving grants from the Centers for Disease Control and Prevention and Biomedical Advanced Research and Development Authority during the conduct of the study and grants from MedImmune outside the submitted work. No other disclosures were reported.

Funding/Support: This trial was funded by the US Centers for Disease Control and Prevention, Veterans Health Administration, and the Biodefense Advanced Research and Development Agency.

Role of the Funder/Sponsor: The sponsors were not involved in data collection, analysis, or interpretation; writing of the manuscript; or the decision to submit the manuscript. The sponsors reviewed and made technical comments about the study protocol prior to enrollment and the final manuscript prior to submission for publication.

Group Information: For the ResPECT Team (Contributors): The Johns Hopkins University and Health System (Baltimore, MD): Trish M. Perl, MD, MSc; Justin Getka, BA; Tina Hoang, MS; Rose Kajih, PharmD; Amanda Krosche, BS; Meghan Kubala, MS, MD; Jenna Los, MLA; Liandra Presser, MD; Kathleen Pulice, MS; Margaret Spach, DDS. VA New York Harbor Healthcare System (New York, NY): Michael S. Simberkoff, MD; Cynthia Akagbosu, BA, MA; Madeline Dansky, BA; Benedict J. Frederick, BA; Marilyn Last, RN; Scott Laverie, RN; Courtney Pike, BA; Shefali Rikhi, BS; Nicole Spector, RN; Christine A. Reel-Brander, RN. Denver Health & Hospital Authority (Denver, CO): Connie Price, MD; Katie Gorman, BS; Amy Irwin, DNP, RN; Sean O'Malley; Kevin Silva, BS. UT Southwestern Medical Center (Dallas, TX) Trish M. Perl, MD, MSc; Deepa Raj, MPH; VA Eastern Colorado Healthcare System (Aurora, CO): Mary Bessesen, MD; Jill C. Adams BSN, BA; Shannon Kingery, BS; Stefanie Tuder, BS; Erron Fritchman-Palmer, MPH. Children's Hospital Colorado (Denver, CO): Ann-Christine Nyquist, MD, MSPH; Megan Gorski, BA. VA Washington DC Medical Center (Washington, DC): Cynthia Gibert, MD, MSc; Laura Chopko, BA; Kathy Haines, MSW, MPH; Caitlin Langhorne, MPH; Dana Silver, BA; Courtney Southard, MPH. VA Michael C. DeBakey Medical Center (Houston, TX): Maria C. Rodriguez-Barradas MD; Barbara Kertz, MS; Mahwish Mushtaq, MD, MPH; Blanca Vargas, MD. Centers for Disease Control and Prevention, National Institute for Occupational Safety & Health (Pittsburgh, PA): Edward Fisher, MS; Ronald Shaffer, PhD; Lewis J. Radonovich, MD. Veteran's Health Administration Office of Public Health (Gainesville, FL): Aaron Eagan, MPH, RN; Lewis J. Radonovich, MD. HandyMetrics Corporation (Toronto, Ontario, Canada): Melanie Lipka, BS; Michael Tsang, PhD. Laboratory Core at Johns Hopkins University (Baltimore, MD): Charlotte Gaydos, DrPH, Jeffrey Holden, MA; Alexandra Valsamakis, MD, PhD. Laboratory Core at VA St Louis Healthcare System and St Louis University School of Medicine (St Louis, MO): Geoffrey J. Gorse, MD; Michelle Mitchell, BS; Gira B. Patel, MS; Yinyi Yu, BS. REDCap Core at Johns Hopkins University (Baltimore, MD): Andre Hackman, BA, Michael Sherman, BS. Statistical and Epidemiologic Core (University of Florida, Gainesville): Brooke A. Borgert MS., Derek A.T. Cummings, PhD, MPH, MSc.; Susan Rattigan (University of Massachusetts, Amherst): Alexandria C. Brown, PhD; Nicholas G. Reich, PhD (Johns Hopkins University, Baltimore, MD); Justin Lessler, PhD, MHS, MS.

Disclaimer: The findings and conclusions in this article are the authors' own and do not necessarily represent the views of the National Institute for Occupational Safety and Health, the Centers for Disease Control and Prevention, the Department of Veterans Affairs, or other affiliates. Mention of product names does not imply endorsement. All information and materials in this article are original.

Data Sharing Statement: See Supplement 4.

Additional Contributions: We thank the members of the data and safety monitoring board, Daniel Morgan, MD (University of Maryland, Baltimore), Elizabeth Colantuoni, PhD (Johns Hopkins Bloomberg School of Public Health, Baltimore), and Tia Powell, MD (Albert Einstein College of Medicine, Bronx). We also thank David Weissman, MD (National Institute for Occupational Safety and Health) and Michael Hodgson, MD (Occupational Safety and Health Administration). None of our consults were financially compensated for their expertise, support, and guidance throughout the study. We are immensely grateful to the study personnel and coordinators, the supporting clinical staff, and, most importantly, the participants.

REFERENCES

1. Goins WP, Talbot HK, Talbot TR. Health care-acquired viral respiratory diseases. *Infect Dis Clin North Am*. 2011;25(1):227-244. doi:10.1016/j.idc.2010.11.010

2. Institute of Medicine. *Preparing for an Influenza Pandemic: Personal Protective Equipment for Healthcare Workers*. Washington, DC: National Academies Press; 2008.

3. MacIntyre CR, Wang Q, Cauchemez S, et al. A cluster randomized clinical trial comparing fit-tested and non-fit-tested N95 respirators to medical masks to prevent respiratory virus infection in health care workers. *Influenza Other*

© 2019 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Health Services Research Library User on 02/27/2020

Respir Viruses. 2011;5(3):170-179. doi:10.1111/j.1750-2659.2011.00198.x

4. MacIntyre CR, Wang Q, Seale H, et al. A randomized clinical trial of three options for N95 respirators and medical masks in health workers. *Am J Respir Crit Care Med*. 2013;187(9):960-966. doi:10.1164/rccm.201207-1164OC

5. Noti JD, Lindsley WG, Blachere FM, et al. Detection of infectious influenza virus in cough aerosols generated in a simulated patient examination room. *Clin Infect Dis*. 2012;54(11):1569-1577. doi:10.1093/cid/cis237

6. Loeb M, Dafoe N, Mahony J, et al. Surgical mask vs N95 respirator for preventing influenza among health care workers: a randomized trial. *JAMA*. 2009;302(17):1865-1871. doi:10.1001/jama.2009.1466

7. Rupp ME, Whitley R, Nutty C. *Letter on Federal PPE Guidance*. Washington, DC: Infectious Diseases Society of America/Society for Healthcare Epidemiology of America/Association of Professionals in Infection Control and Epidemiology. https://www.idsociety.org/globalassets/idsa/policy--advocacy/current_topics_and_issues/infection-prevention--control/comments/1100509-shea-idsa-apic-letter-re-ppe-guidance.pdf. Published November 5, 2009. Accessed March 28, 2019.

8. Ford I, Norrie J. Pragmatic trials. *N Engl J Med*. 2016;375(5):454-463. doi:10.1056/NEJMra1510059

9. Janssen L, Ettinger H, Graham S, Shaffer R, Zhuang Z. The use of respirators to reduce inhalation of airborne biological agents. *J Occup Environ Hyg*. 2013;10(8):D97-D103. doi:10.1080/15459624.2013.799964

10. Determination of particulate filter efficiency level of N95 series filters against solid particulates for non-powered, air-purifying respirators standard testing procedure. Pittsburgh, PA: National Institute for Occupational Safety and Health; 2016.https://www.cdc.gov/niosh/npptl/stps/pdfs/TEB-APR-STP-0059-508.pdf.

11. Lipp A, Edwards P. Disposable surgical face masks for preventing surgical wound infection in clean surgery. The Cochrane Library website. http://www.cochranelibrary.com/. Published April 26, 2016. Accessed March 28, 2019.

12. MacIntyre CR, Chughtai AA, Rahman B, et al. The efficacy of medical masks and respirators against respiratory infection in healthcare workers. *Influenza Other Respir Viruses*. 2017;11(6):511-517. doi:10.1111/irv.12474

13. Interim recommendations for facemask and respirator use to reduce 2009 influenza A (H1N1) virus transmission. Centers for Disease Control and Prevention website. https://www.cdc.gov/h1n1flu/masks.htm. Published September 24, 2009. Accessed December 6, 2016.

14. Infection control webinar. World Health Organization website. http://www.who.int/gpsc/5may/news/webinars/infection_control_webinar_20100413.ppt. Published April 10, 2010. Accessed March 28, 2019.

15. US Department of Labor. 1910.134 -Respiratory protection standard. Washington, DC: Occupational Safety and Health Administration. https://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=standards&p_id=12716. Accessed March 28, 2019.

16. Radonovich LJ Jr, Bessesen MT, Cummings DA, et al. The respiratory protection effectiveness clinical trial (ResPECT): a cluster-randomized comparison of respirator and medical mask effectiveness against respiratory infections in healthcare personnel. *BMC Infect Dis*. 2016;16:243. doi:10.1186/s12879-016-1494-2

17. Siegel JD, Rhinehart E, Jackson M, Chiarello L; Health Care Infection Control Practices Advisory Committee. 2007 Guideline for isolation precautions: preventing transmission of infectious agents in health care settings. *Am J Infect Control*. 2007;35(10)(suppl 2):S65-S164. doi:10.1016/j.ajic.2007.10.007

18. Centers for Disease Control and Prevention. Updated guidance: prevention strategies for seasonal influenza in healthcare setting. Government Publishing Office website. https://www.gpo.gov/fdsys/pkg/FR-2010-06-22/html/2010-15015.htm. Published June 22, 2010. Accessed March 28, 2019.

19. Moulton LH. Covariate-based constrained randomization of group-randomized trials. *Clin Trials*. 2004;1(3):297-305. doi:10.1191/1740774504cn024oa

20. Reich NG, Cummings DA, Lauer SA, et al. Triggering interventions for influenza: the ALERT algorithm. *Clin Infect Dis*. 2015;60(4):499-504. doi:10.1093/cid/ciu749

21. de la Tabla VO, Masiá M, Antequera P, et al. Comparison of combined nose-throat swabs with nasopharyngeal aspirates for detection of pandemic influenza A/H1N1 2009 virus by real-time reverse transcriptase PCR. *J Clin Microbiol*. 2010;48(10):3492-3495. doi:10.1128/JCM.01105-10

22. Tang YW, Lowery KS, Valsamakis A, et al. Clinical accuracy of a PLEX-ID flu device for simultaneous detection and identification of influenza viruses A and B. *J Clin Microbiol*. 2013;51(1):40-45. doi:10.1128/JCM.01978-12

23. Azur MJ, Stuart EA, Frangakis C, Leaf PJ. Multiple imputation by chained equations: what is it and how does it work? *Int J Methods Psychiatr Res*. 2011;20(1):40-49. doi:10.1002/mpr.329

24. Bessesen MT, Savor-Price C, Simberkoff M, Reich NG, Pavia AT, Radonovich LJ. N95 respirators or surgical masks to protect healthcare workers against respiratory infections: are we there yet? *Am J Respir Crit Care Med*. 2013;187(9):904-905. doi:10.1164/rccm.201303-0581ED

25. Sartor C, Zandotti C, Romain F, et al. Disruption of services in an internal medicine unit due to a nosocomial influenza outbreak. *Infect Control Hosp Epidemiol*. 2002;23(10):615-619. doi:10.1086/501981

26. Jacobs JL, Ohde S, Takahashi O, Tokuda Y, Omata F, Fukui T. Use of surgical face masks to reduce the incidence of the common cold among health care workers in Japan: a randomized controlled trial. *Am J Infect Control*. 2009;37(5):417-419. doi:10.1016/j.ajic.2008.11.002

27. Offeddu V, Yung CF, Low MSF, Tam CC. Effectiveness of masks and respirators against respiratory infections in healthcare workers: a systematic review and meta-analysis. *Clin Infect Dis*. 2017;65(11):1934-1942. doi:10.1093/cid/cix681

28. Smith JD, MacDougall CC, Johnstone J, Copes RA, Schwartz B, Garber GE. Effectiveness of N95 respirators versus surgical masks in protecting health care workers from acute respiratory infection: a systematic review and meta-analysis. *CMAJ*. 2016;188(8):567-574. doi:10.1503/cmaj.150835

29. Peterson K, Novak D, Stradtman L, Wilson D, Couzens L. Hospital respiratory protection practices in 6 U.S. states: a public health evaluation study. *Am J Infect Control*. 2015;43(1):63-71. doi:10.1016/j.ajic.2014.10.008

30. Chiu S, Black CL, Yue X, et al. Working with influenza-like illness: presenteeism among US health care personnel during the 2014-2015 influenza season. *Am J Infect Control*. 2017;45(11):1254-1258. doi:10.1016/j.ajic.2017.04.008

© 2019 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Health Services Research Library User on 02/27/2020

EDITORIAL

Respiratory Protection of Health Care Personnel to Prevent Respiratory Viral Transmission

Thomas R. Talbot, MD, MPH; Hilary M. Babcock, MD, MPH

Influenza and other respiratory viruses continue to cause patient morbidity and mortality, including health care utilization for outpatient visits and inpatient and intensive care unit care. Occupational health and infection-prevention programs partner to prevent these infections among health care personnel (HCP) and their patients through a multifaceted approach that includes comprehensive strategies to achieve high rates of influenza vaccination, rational sick leave and attendance policies that do not reward coming to work while ill (presenteeism), early identification and isolation of possibly infected patients, and appropriate use of personal protective equipment (PPE). While influenza still gets the most attention, these efforts also protect HCP from other respiratory infections.

Related article page 824

Questions have been raised about the best PPE to limit HCP exposure to respiratory viral pathogens, leading to a debate between those who suggest use of medical masks and those who suggest use of particulate respirators. While official recommendations for most encounters still support the use of medical masks,[1] some concerns remain about the risk posed by the potential for small particle aerosol transmission of respiratory viruses, particularly influenza.[2] This risk has been demonstrated primarily in laboratory simulation settings[3,4] or inferred from transmission events occurring on airplanes[5] or despite PPE use.[6] As a result, particulate respirators, or even powered air-purifying respirators, have been suggested for improved protection.[7] Respirators provide filtration of smaller particles than medical masks, and the correct use of a fit-tested respirator also prevents particles of any size getting around the mask to the user's nose or mouth. However, it remains unclear if respirators provide benefits beyond medical masks in clinical settings. Prior studies attempting to address this important question have reached differing conclusions,[8-11] but have also used different study designs.

For many years, medical masks have been used to protect HCP from respiratory viral infections. In 2009, facing the first influenza pandemic in decades, "an abundance of caution" led to a recommendation for respirator use when caring for any patient infected with the emerging H1N1 pandemic influenza strain[12] until it was clear whether usual transmission routes had been altered in the new strain. The recommendation also recognized that a vaccine against the pandemic strain was not yet available and, therefore, HCP were more susceptible to infection in the event of exposure. Later, when it appeared that the transmission of pandemic H1N1 was not different from seasonal influenza, the recommendation for medical masks in most settings was reinstated. However, based on the same abundance of caution, respirators were (and are still) recommended for HCP when performing potentially aerosol-generating procedures (such as bronchoscopy), and the question of whether respirators would provide better protection during all encounters with patients infected with influenza and other respiratory virus infections has persisted.

To help address this question, in this issue of *JAMA*, Radonovich et al report the results of the ResPECT study,[13] a pragmatic, cluster randomized trial of medical masks vs respirators (fit-tested N95 masks) worn by HCP while within 6 feet (1.83 m) of a symptomatic patient. The study included HCP who worked in multiple outpatient settings, including adult and pediatric ambulatory clinics, dental practices, urgent care centers, hemodialysis units, emergency departments, and emergency medical services, over 4 seasons of peak respiratory viral illness. Participants self-reported symptoms of acute respiratory illness, which prompted polymerase chain reaction testing for a wide range of respiratory viruses. In addition, nares and oropharynx swabs were performed twice per participant per season, when participants were asymptomatic, and influenza hemagglutination inhibition titers were measured at the beginning and end of each season. Randomization to wear medical masks or N95 respirators was at the level of the cluster or clinical site and occurred anew each year so that no one site or participant used the same type of PPE for all 4 seasons. Analyses of prespecified clinically relevant outcomes occurred at the level of the participant-season, and were adjusted for age, race, sex, vaccination status, occupational exposure risk, self-reported hand hygiene adherence, and the number of children younger than 5 years at home.

The primary outcome was laboratory-confirmed influenza by either polymerase chain reaction or serology, for which the study was underpowered. The authors note that it would have required more than 10 000 HCP-seasons to have sufficient power for that outcome to detect the 25% relative reduction selected as the target, even though they did amass an impressive near 5000 HCP-seasons. Secondary outcomes included 4 other measures of respiratory illness among the participants: symptomatic acute respiratory illness with or without laboratory pathogen detection; influenzalike illness with or without influenza detection; acute respiratory illness with pathogen detection (laboratory-confirmed respiratory illness); and detection of a pathogen with or without reported symptoms (laboratory-detected respiratory infection).

© 2019 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Health Services Research Library User on 02/27/2020

The study included 1993 participants in 189 clusters who were randomized to wear N95 respirators (accounting for 2512 HCP-seasons of observation) and 2058 participants in 191 clusters who were randomized to wear medical masks (2668 HCP-seasons of observation). For all outcomes, illness with or without laboratory detection of a pathogen and pathogen detection with or without reported symptoms, there were no statistically significant differences between participants wearing N95 respirators and those wearing medical masks. For the primary outcome of laboratory-confirmed influenza, 207 laboratory-confirmed influenza infection events (8.2% of 2512 HCP-seasons) occurred in the N95 respirator group and 193 (7.2% of 2668 HCP-seasons) occurred in the medical mask group (difference, 1.0%, [95% CI, −0.5% to 2.5%]; $P = .18$).

In many ways, the ResPECT study was specifically designed to address limitations of earlier studies. The cohorts that were randomized to use medical masks and N95 respirators were similar, with no significant between-group differences in job title, vaccination status, patient population served, smoking status, use of glasses or contact lenses, and presence of children at home, minimizing the risk of bias through differential exposure risk. The design included serologic testing for influenza hemagglutination inhibition titers and random polymerase chain reaction testing of asymptomatic participants to minimize the chances of missing asymptomatic or clinically mild infections. The symptoms used as criteria for an acute respiratory illness were broad and fairly nonspecific to avoid excluding participants with atypical presentations. The process developed for participants to self-collect samples decreased the risk of laboratory testing being missed because of timing or study staff access. The study included testing for other viruses in addition to influenza because influenza is not the only respiratory virus from which HCP need protection.

The study also had several limitations. One limitation was that the study was underpowered for its prespecified primary outcome. Laboratory-confirmed infections can be missed because of under-reporting of symptoms, limitations in specimen collection technique (self-collection), and delayed specimen collection (allowed up to 7 days after symptom onset). Inclusion of influenza hemagglutination inhibition testing mitigates the risk of missed infections somewhat, although only for influenza. Symptom-based outcomes are also problematic because most symptoms are not specific to the respiratory viral infections, potentially diluting the effectiveness of the intervention. To address these issues, the authors chose to include multiple outcomes, both symptom-based and laboratory-based. The finding of no difference across all of the reported outcomes is reassuring that medical masks provide comparable protection for HCP against respiratory viruses.

The ResPECT study did not directly address inpatient settings and particularly the appropriate protection for potentially aerosol-generating procedures. Although it seems unlikely that most basic principles of viral transmission differ between outpatient and inpatient settings, some differences may potentially affect transmission in inpatient settings, including higher viral burden among more symptomatic patient populations, a higher proportion of immunocompromised patients, and longer and closer interactions between HCP and patients while providing care (eg, bathing patients). It is unclear whether the effect of those possible differences would be affected by the use of respirators. For example, a 2008 study found that in usual use conditions (ie, wearing a mask while caring for possibly infected patients) there was no difference in outcomes between participants wearing medical masks vs N95 respirators in inpatient settings.[8] Additional pragmatic studies of HCP outcomes based on PPE use would still be helpful in these settings; however, such a study among inpatient HCP would require extensive funding support and likely would be quite expensive. Other studies defining changes in particle sizes and risk for pathogen transmission during patient procedures may also provide some guidance for those situations.[14]

Occasionally, the desire for an abundance of caution gets ahead of the available data and leads to changing recommended levels of protection just to be safe. These changes, which can be politically challenging to reverse, may not lead to better protection, but instead only create additional burdens on busy HCP and health care systems. The ResPECT trial provides useful data on appropriate protection against respiratory viral infections for HCP in outpatient settings. In addition to the findings reported in the current manuscript, the authors have previously noted through the ResPECT cohort the association between mandatory HCP influenza vaccination policies on vaccination rates and subsequent reductions in HCP symptomatic absenteeism.[15]

The pragmatic design of the ResPECT study reflects the conditions that many hospital epidemiologists are accustomed to: HCP may or may not wear the PPE they are supposed to, may or may not wear it correctly if they do wear it, and may self-report higher levels of adherence to hand hygiene and PPE use than noted by independent observers. Even though some may consider these elements of the study as weaknesses, these aspects increase the generalizability of the findings outside of a research study and provide reassurance to those making programmatic and policy decisions that, in outpatient settings under usual use conditions, there are no significant differences between medical masks and respirators for protection of HCP against respiratory viruses.

ARTICLE INFORMATION

Author Affiliations: Vanderbilt University School of Medicine, Nashville, Tennessee (Talbot); Washington University School of Medicine in St Louis, St Louis, Missouri (Babcock).

Corresponding Author: Hilary M. Babcock, MD, MPH, Infectious Disease Division, Washington University School of Medicine in St Louis, Campus Box 8051, 4523 Clayton Ave, St Louis, MO 63110 (hbabcock@wustl.edu).

Conflict of Interest Disclosures: None reported.

REFERENCES

1. Prevention strategies for seasonal influenza in healthcare settings: guidelines and recommendations. Centers for Disease Control and Prevention website. https://www.cdc.gov/flu/professionals/infectioncontrol/healthcaresettings.htm#fundamental. Accessed July 18, 2019.

2. Shiu EYC, Leung NHL, Cowling BJ. Controversy around airborne versus droplet transmission of respiratory viruses: implication for infection prevention. *Curr Opin Infect Dis*. 2019;32(4):372-379.

© 2019 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Health Services Research Library User on 02/27/2020

3. Zhou J, Wei J, Choy K. Defining the sizes of airborne particles that mediate influenza transmission in ferrets. *Proc Natl Acad Sci USA*. 2018;115(10):E2386-E2392. doi:10.1073/pnas.1716771115

4. Yan J, Grantham M, Pantelic J, et al; EMIT Consortium. Infectious virus in exhaled breath of symptomatic seasonal influenza cases from a college community. *Proc Natl Acad Sci USA*. 2018;115(5):1081-1086. doi:10.1073/pnas.1716561115

5. Leitmeyer K, Adlhoch C. Review article: influenza transmission on aircraft: a systematic literature review. *Epidemiology*. 2016;27(5):743-751. doi:10.1097/EDE.0000000000000438

6. Christian MD, Loutfy M, McDonald LC, et al; SARS Investigation Team. Possible SARS coronavirus transmission during cardiopulmonary resuscitation. *Emerg Infect Dis*. 2004;10(2):287-293. doi:10.3201/eid1002.030700

7. Bischoff WE, Turner J, Russell G, Blevins M, Missaiel E, Stehle J. How well do N95 respirators protect healthcare providers against aerosolized influenza virus? *Infect Control Hosp Epidemiol*. 2018;40(2):1-3. doi:10.1017/ice.2018.326

8. Loeb M, Dafoe N, Mahony J, et al. Surgical mask vs N95 respirator for preventing influenza among health care workers: a randomized trial. *JAMA*. 2009;302(17):1865-1871. doi:10.1001/jama.2009.1466

9. MacIntyre CR, Wang Q, Seale H, et al. A randomized clinical trial of three options for N95 respirators and medical masks in health workers. *Am J Respir Crit Care Med*. 2013;187(9):960-966. doi:10.1164/rccm.201207-1164OC

10. MacIntyre CR, Wang Q, Cauchemez S, et al. A cluster randomized clinical trial comparing fit-tested and non-fit-tested N95 respirators to medical masks to prevent respiratory virus infection in health care workers. *Influenza Other Respir Viruses*. 2011;5(3):170-179. doi:10.1111/j.1750-2659.2011.00198.x

11. Smith JD, MacDougall CC, Johnstone J, Copes RA, Schwartz B, Garber GE. Effectiveness of N95 respirators versus surgical masks in protecting health care workers from acute respiratory infection: a systematic review and meta-analysis. *CMAJ*. 2016;188(8):567-574. doi:10.1503/cmaj.150835

12. Interim guidance on infection control measures for 2009 H1N1 influenza in healthcare settings, including protection of healthcare personnel. Centers for Disease Control and Prevention website. https://www.cdc.gov/h1n1flu/guidelines_infection_control.htm. Published July 15, 2010. Accessed July 18, 2019.

13. Radonovich L Jr, Simberkoff M, Bessesen M, et al. N95 respirators vs medical masks for preventing influenza among health care personnel: a randomized clinical trial [published September 3, 2019]. *JAMA*. doi:10.1001/jama.2019.11645

14. O'Neil CA, Li J, Leavey A, et al; Centers for Disease Control and Prevention Epicenters Program. Characterization of aerosols generated during patient care activities. *Clin Infect Dis*. 2017;65(8):1335-1341.

15. Frederick J, Brown AC, Cummings DA, et al. Protecting healthcare personnel in outpatient settings: the influence of mandatory versus nonmandatory influenza vaccination policies on workplace absenteeism during multiple respiratory virus seasons. *Infect Control Hosp Epidemiol*. 2018;39(4):452-461. doi:10.1017/ice.2018.9

Evolving Priorities for Imaging Utilization Research
From Outputs to Outcomes

Deborah Levine, MD; Richard Duszak Jr, MD

In this issue of *JAMA*, Smith-Bindman et al[1] report findings from a study of advanced imaging utilization in 3 US integrated health care programs, in 4 US mixed-model insurance plans, and in individuals living in Ontario, Canada. Using electronic health record data from 2000 to 2016, the authors retrospectively characterized more than 135 million medical imaging examinations for 16 million to 21 million individuals annually, including segmenting utilization by both imaging mode and body region.

Related article page 843

Smith-Bindman et al found that among all adults, imaging rates increased significantly from 2000 to 2016 for all imaging tests other than nuclear medicine. For example, among older adults (aged ≥65 years) in US health care systems, rates of imaging with computed tomography (CT) in 2000 and 2016 were 204 per 1000 person-years compared with 428 per 1000 person-years, and in Ontario, were 161 per 1000 person-years compared with 409 per 1000 person-years. For magnetic resonance imaging (MRI), the US rates were 62 per 1000 person-years in 2000 and 139 per 1000 person-years in 2016, and the rates in Ontario were 13 per 1000 person-years in 2000 and 89 per 1000 person-years in 2016. For ultrasound, the rates in 2000 and 2016, respectively, were 324 per 1000 person-years and 495 per 1000 person-years in the United States and 332 per 1000 person-years vs 580 per 1000 person-years in Ontario. The notable exceptions to increased utilization were for nuclear medicine, which declined over time among older adults (from 94 per 1000 person-years in 2000 to 64 per 1000 person-years in 2016 in the United States and from 87 per 1000 person-years in 2000 to 74 per 1000 person-years in 2016 in Ontario) and for CT in children, which declined after 2009 in the United States and after 2006 in Ontario. Additional analyses also found a general slowing in the increase in imaging in the 3 to 5 years up to 2016.

The study findings are both interesting and provocative, but without information about discrete episodes of care, they are not immediately actionable. Without knowing who ordered this imaging (eg, physicians vs an expanding pool of nonphysician practitioners), for what indications (eg, new diagnoses or follow-up of incidental findings), and in what settings (eg, expanding emergency departments vs contracting outpatient imaging centers), more work will be necessary to translate this information into improved patient outcomes. In addition, with passage and implementation in the United States of the Affordable Care Act during the study interval, the contributing role of broader national policy on health care service utilization overall is unclear (for instance, it is not possible to determine whether the addition of a large previously underserved population to health systems may, at least in part, have contributed to these findings).

Nonetheless, utilization research remains important in guiding meaningful health policy, and imaging is associated with both substantial cost and some risk. Such work informs ongoing research and guidelines to optimize imaging

© 2019 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Health Services Research Library User on 02/27/2020

APL Bioengineering

AAV-mediated gene therapy targeting TRPV4 mechanotransduction for inhibition of pulmonary vascular leakage

Cite as: APL Bioeng. 3, 046103 (2019); https://doi.org/10.1063/1.5122967
Submitted: 02 August 2019 . Accepted: 28 October 2019 . Published Online: 02 December 2019

Juan Li, Amy M. Wen, Ratnakar Potla, Ezekiel Benshirim, Ariel Seebarran, Maximilian A. Benz, Olivier Y. F. Henry, Benjamin D. Matthews, Rachelle Prantil-Baun, Sarah E. Gilpin, Oren Levy, and Donald E. Ingber

COLLECTIONS

Paper published as part of the special topic on Microphysiological Systems
Note: This paper is part of the special topic on Microphysiological Systems.

This paper was selected as Featured

View Online Export Citation CrossMark

ARTICLES YOU MAY BE INTERESTED IN

Microphysiological systems modeling acute respiratory distress syndrome that capture mechanical force-induced injury-inflammation-repair
APL Bioengineering 3, 041503 (2019); https://doi.org/10.1063/1.5111549

Balance of interstitial flow magnitude and vascular endothelial growth factor concentration modulates three-dimensional microvascular network formation
APL Bioengineering 3, 036102 (2019); https://doi.org/10.1063/1.5094735

Scalable microphysiological system to model three-dimensional blood vessels
APL Bioengineering 3, 026105 (2019); https://doi.org/10.1063/1.5090986

AIP Conference Proceedings
FLASH WINTER SALE!
50% OFF ALL PRINT PROCEEDINGS
ENTER CODE 50DEC19 AT CHECKOUT

© Author(s).

APL Bioengineering ARTICLE scitation.org/journal/apb

AAV-mediated gene therapy targeting TRPV4 mechanotransduction for inhibition of pulmonary vascular leakage

Cite as: APL Bioeng. 3, 046103 (2019); doi: 10.1063/1.5122967
Submitted: 2 August 2019 · Accepted: 28 October 2019 ·
Published Online: 2 December 2019

Juan Li,[1,a] Amy M. Wen,[1,a] Ratnakar Potla,[1,2] Ezekiel Benshirim,[1,3] Ariel Seebarran,[1,4] Maximilian A. Benz,[1] Olivier Y. F. Henry,[1] Benjamin D. Matthews,[2,5] Rachelle Prantil-Baun,[1] Sarah E. Gilpin,[1] Oren Levy,[1] and Donald E. Ingber[1,2,6,b]

AFFILIATIONS

[1]Wyss Institute for Biologically Inspired Engineering at Harvard University, Boston, Massachusetts 02115, USA
[2]Vascular Biology Program, Boston Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115, USA
[3]Department of Integrative Biology, Harvard College, Cambridge, Massachusetts 02138, USA
[4]Department of Materials Science and Engineering, University of Toronto, Toronto, Ontario M5S 3E4, Canada
[5]Department of Medicine, Boston Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115, USA
[6]Harvard School of Engineering and Applied Sciences, Cambridge, Massachusetts 02139, USA

Note: This paper is part of the special topic on Microphysiological Systems.

[a]Contributions: J. Li and A. M. Wen contributed equally to this work.
[b]Author to whom correspondence should be addressed: don.ingber@wyss.harvard.edu . Tel.: 617-432-7044. Fax: 617-432-7828.

ABSTRACT

Enhanced vascular permeability in the lungs can lead to pulmonary edema, impaired gas exchange, and ultimately respiratory failure. While oxygen delivery, mechanical ventilation, and pressure-reducing medications help alleviate these symptoms, they do not treat the underlying disease. Mechanical activation of transient receptor potential vanilloid 4 (TRPV4) ion channels contributes to the development of pulmonary vascular disease, and overexpression of the high homology (HH) domain of the TRPV4-associated transmembrane protein CD98 has been shown to inhibit this pathway. Here, we describe the development of an adeno-associated virus (AAV) vector encoding the CD98 HH domain in which the AAV serotypes and promoters have been optimized for efficient and specific delivery to pulmonary cells. AAV-mediated gene delivery of the CD98 HH domain inhibited TRPV4 mechanotransduction in a specific manner and protected against pulmonary vascular leakage in a human lung Alveolus-on-a-Chip model. As AAV has been used clinically to deliver other gene therapies, these data raise the possibility of using this type of targeted approach to develop mechanotherapeutics that target the TRPV4 pathway for treatment of pulmonary edema in the future.

© 2019 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/). https://doi.org/10.1063/1.5122967

INTRODUCTION

Pulmonary edema is a life-threatening condition characterized by abnormal accumulation of intravascular fluid in alveolar air spaces and interstitial tissues of the lungs due to vascular leakage across the alveolar-capillary barrier.[1–4] Currently, there are no specific therapies to improve vascular permeability, and clinical management relies on providing supportive measures, including diuretics, vasoactive medications, maintenance of adequate nutrition, hemodynamic monitoring, and mechanical ventilation if necessary.[1] While mechanical ventilation is usually required for the survival of patients with severely compromised lung function, these artificial breathing motions can be detrimental and further compromise the pulmonary vascular barrier as a result of overinflation of the alveoli, a form of "barotrauma" called ventilator-induced lung injury.[5] Thus, a major challenge in pulmonary medicine is to identify molecular targets unique to lung cells that, if blocked, could prevent the increase in pulmonary vascular permeability, particularly that induced by mechanical distortion.

Transient receptor potential vanilloid 4 (TRPV4) is a promising target for the treatment of pulmonary edema due to its mechanosensitive nature,[6] along with its roles in regulating endothelial permeability,[7] epithelial barrier function,[8] lung myogenic tone,[9] and lung vascular remodeling in response to hypoxia.[10–12] TRPV4 ion channels can be activated within 4 ms after mechanical forces are transmitted across cell surface receptors, and mechanical activation of these channels, such as associated with breathing motions or vascular pressure, has been shown to contribute to pulmonary edema progression.[6,13] While chemical inhibitors of TRPV4 channel activity are known and have been shown to prevent pulmonary vascular leakage,[13,14] TRPV4 plays a ubiquitous role and is involved in the regulation of diverse bodily functions, including control of serum osmolarity,[15–22] nociception,[23–26] bone formation and remodeling,[27–30] and bladder tone.[31–34] Therefore, to reduce adverse effects and dose-limiting toxicities from off-target effects of systemic administration of TRPV4 inhibitors,[35] we explored the possibility of developing a more selective inhibitor of pulmonary vascular leakage that preferentially targets the mechanical signaling mechanism by which physical forces activate TRPV4. We have previously shown that mechanical forces that activate TRPV4 are transferred to it from integrin b1 via the transmembrane protein CD98.[6] In addition, overexpression of the high homology (HH) domain of CD98 by transfection exerted a dominant negative effect that specifically inhibited mechanical, but not chemical, activation of TRPV4.[36] However, developing this mechanotransduction-targeted approach into a therapeutic strategy requires a more clinically relevant delivery method.

Adeno-associated virus (AAV) vectors have been used for delivery of gene therapies in the clinic because they provide many advantages, including favorable safety profiles, tailorable tissue tropism, and long-term gene expression,[37] and their efficacy has been demonstrated in wide-ranging clinical trials, from hemophilia B[38] to Parkinson's disease.[39] Thus, we set out to explore whether AAV gene delivery vectors can be used to deliver a gene encoding the CD98 HH domain to demonstrate the feasibility of targeting this mechanotransduction pathway as a way to inhibit pulmonary vascular leakage. We first investigated how AAV serotype and different promoters affect the efficiency of AAV-mediated gene transfer to human pulmonary alveolar epithelial cells (HpAECs) and human primary lung microvascular endothelial cells (HpMVECs) and optimized the transduction efficiency of AAV for these cells. The delivery of the CD98 HH domain with the optimized vectors inhibited mechanical strain-induced activation of TRPV4-dependent responses, including calcium influx and cell realignment. As a proof-of-concept in a more complex biomimetic model, we demonstrated that selective inhibition of mechanical signaling through TRPV4 also suppressed pulmonary barrier leakage in a human Lung Alveolus Chip (Alveolus Chip) that has been previously shown to model multiple human pulmonary diseases with high fidelity, including pulmonary edema and pulmonary thrombosis.[13,40,41]

RESULTS

Optimization of AAV vectors for pulmonary cell transduction

Different serotypes of AAV have been reported to have differential tissue-targeting tropisms.[42–45] While no AAV vectors have been specifically optimized to target human lung alveolar epithelial cells or microvascular endothelial cells, AAV2.5T and AAV2/2 have been previously reported to have enhanced targeting efficiency to human lung airway cells[46] and other types of human endothelial cells,[47,48] respectively. AAV2.5T is an AAV chimera resulting from the combination of AAV2 (aa1-128) and AAV5 (aa129-725) with one point mutation (A581T),[40] while AAV2/2 has the AAV2 genome packaged in an AAV2 capsid.[49] Thus, we first screened these two AAV vectors using recombinant EYFP-expressing constructs and compared their transduction efficiency in HpAECs and HpMVECs. These primary donor-derived cells contain mixed populations, with HpAECs being composed of both type I and type II alveolar epithelial cells and HpMVECs containing pulmonary endothelial cells from both blood and lymphatic sources.

When added to HpAECs differentiated under air-liquid interface (ALI) culture conditions in Transwells, AAV2.5T (MOI ¼ 10 000) exhibited the highest gene delivery efficiency, with 60%–70% being transduced and stable expression sustained for at least 5 days after transduction, whereas only 4%–8% of these alveolar cells were transduced with AAV2/2 [Fig. 1(a)]. Conversely, a higher (25–30%) transduction efficiency was achieved in HpMVECs with AAV2/2 (MOI ¼ 10 000), and this expression was again sustained for at least 5 days, while only 8%–10% of these endothelial cells were transduced with AAV2.5T [Fig. 1(b)]. When these results in primary respiratory cells were benchmarked against the more commonly investigated human umbilical vein endothelial cells (HUVECs), a high gene transfer efficiency of approximately 70% for AAV2/2 (MOI ¼ 10 000) was observed after 3 days, which is consistent with previous literature results.[48] AAV2.5T was less efficient; however, it still achieved a relatively high transduction efficiency of 45% in HUVECs [Fig. 1(c)].

To further optimize transgene expression, a panel of luciferase-expressing vectors was constructed with several commonly used constitutive promoters (CMV, EF1a, and CASI), and their luciferase activities were compared two days after transduction. While the serotype affects the percentage of cells transduced, once a cell is transduced, it is the promoter that determines the amount of gene expression. From initial testing, it was found that AAV2.5T (MOI ¼ 7000) transduction of HpAECs resulted in stronger transgene expression using either EF1a or CASI promoters, while CMV, the most common promoter used in previous AAV studies, had the lowest gene expression with levels less than 1% of the other promoters [Fig. 1(d)]. Moreover, comparable results were obtained in a second type of human lung alveolar epithelial cells (A549 cells) [Fig. 1(e)]. Again, EF1a and CASI yielded the highest levels of expression, with CASI driving approximately 4-fold higher levels than EF1a. An additional commonly used promoter, CAG, was also investigated with the A549 cells, and although the activity levels were on par with EF1a, it did not result in an improvement over levels observed with EF1a or CASI [Fig. 1(e)].

We therefore proceeded to engineer EYFP-expressing AAV vectors using EF1a and CASI promoters. As we found that AAV2.5T can transduce HpMVECs as well as HpAECs under baseline conditions (albeit at approximately 3-fold lower efficiency) [Fig. 1(b)], we investigated whether integrating EF1a or CASI promoters into this vector could improve this efficiency. When transduced with these new EYFP-expressing AAV2.5T vectors, we found that the two promoters resulted in almost identical transgene expression in HpAECs (MOI ¼ 10 000) when analyzed by flow cytometry 3 days after transduction [Fig. 1(f)]. Interestingly, similar to what we observed in A549 cells, the CASI promoter was 3-fold stronger than the EF1a promoter in HpMVECs (MOI was increased to 30 000 to counterbalance the lower

© Author(s) 2019

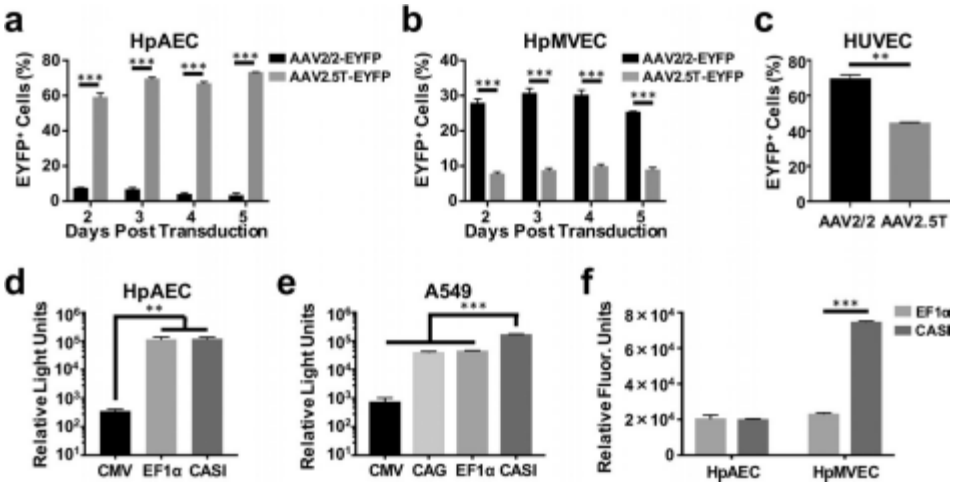


FIG. 1. AAV serotype and promoter affect viral transduction efficiency and transgene expression, respectively. AAV2/2 and AAV2.5T that carry EYFP genes were added to HpAECs (a), HpMVECs (b), or HUVECs (c) at MOI 10 000. Cells were collected 2–5 days after transduction, and the percentage of EYFP-positive cells was analyzed using flow cytometry ($n = 2$, $p < 0.01$, and $p < 0.001$). AAV2.5T-luciferase with different promoters was added to A549 (d) and HpAECs (e) at MOI 7000. Cells were collected at day two of transduction to measure luciferase activity ($n = 3$, $p < 0.01$, and $p < 0.001$). (f) AAV2.5T-EYFP with promoters EF1α and CASI was added to HpAECs and HpMVECs at MOI 10 000 and 30 000, respectively. Cells were collected at day three, and the relative fluorescent units of EYFP-positive cells were analyzed by flow cytometry ($n = 2$, $p < 0.001$).

efficiency observed in our initial studies). Due to the combination of the stronger promoter and the higher MOI used, higher EYFP expression in HpMVECs was found, despite lower transduction efficiency of AAV2.5T for these cells relative to HpAECs. Based on these results, the CASI promotor was used for all subsequent studies.

Disruption of mechanotransduction with AAV-mediated CD98 HH domain delivery

Mechanical activation of TRPV4 ion channels has been implicated in enhanced vascular permeability and pulmonary edema,[13,14] and overexpression of the CD98 HH domain by transfection with CD98 HH can inhibit the force transfer from b1-integrin to TRPV4 that mediates this response.[36] As the CD98 HH domain exerts its dominant negative effects on the cytoplasmand transmembrane delivery of large proteins is difficult to pursue clinically, we investigated whether our engineered AAV vectors with high transduction efficiency in human lung cells could be leveraged to deliver the CD98 HH domain and specifically inhibit TRPV4-mediated mechanotransduction.

Regulation of endothelial cell reorientation under cyclic mechanical strain is mediated by mechanical activation of TRPV4,[50] and thus, we used this as an initial readout of mechanotransduction inhibition. Under baseline conditions, approximately 70% of these endothelial cells and their actin cytoskeleton realigned perpendicular to the direction of the applied mechanical strain (15% cyclic strain, 1 Hz) when cultured on flexible extracellular matrix (ECM)-coated culture substrates [Figs. 2(a) and 2(b)]. In contrast, when AAV2.5T vectors encoding the CASI promotor and both the CD98 HH domain and the fluorescent marker EYFP (AAV2.5T-EYFP-CD98HH) were constructed and transduced (MOI ¼ 10 000) in HpMVECs, this strain-induced cell realignment was almost completely prevented, whereas transduction of the control AAV2.5T-EYFP vector had no detectable effect [Figs. 2a and 2(b)].

We then examined the effect of transduction of HpMVECs with AAV2.5T-EYFP-CD98HH on mechanical strain-induced activation of TRPV4-dependent calcium influx, which mediates the cellular reorientation response.[50] We did not observe an effect of CD98 HH overexpression on the resting level of the Ca^{2+} concentration in the cytosol. Furthermore, individual cells that expressed higher levels of CD98 HH (quantified by the level of EYFP expression) displayed complete inhibition of mechanically activated calcium signaling, as measured by quantifying Rhod-3 calcium dye influx [Figs. 2(c) and 2(d)]. Overexpression of CD98 HH did not interfere directly with the TRPV4 function, as the addition of the TRPV4 chemical agonist GSK1016790A was able to stimulate calcium influx in these cells (supplementary Fig. S1). Thus, overexpression of the CD98 HH domain through delivery by an AAV vector allows for specific suppression of TRPV4-dependent mechanical signaling pathways in human lung endothelial cells.

Inhibition of pulmonary vascular leakage in a human Lung Alveolus Chip

An experimental model that has been shown to mimic pulmonary vascular leakage and edema development with high fidelity in vitro is the human Lung Alveolus Chip, a 2-channel microfluidic culture device lined with alveolar epithelial cells cultured under ALI, which are interfaced with underlying endothelial cells while experiencing simulated breathing motions and fluid flow mimicking the in vivo alveolar microenvironment.[13,51] While the original Lung Chip utilized A549 lung alveolar epithelial cells and HUVECs, we used an improved model that contains primary HpAECs and HpMVECs[40,41] in our studies to more directly explore whether AAV vectors encoding the CD98 HH domain could serve as potential mechanotherapeutics for treatment of pulmonary edema in humans.

© Author(s) 2019

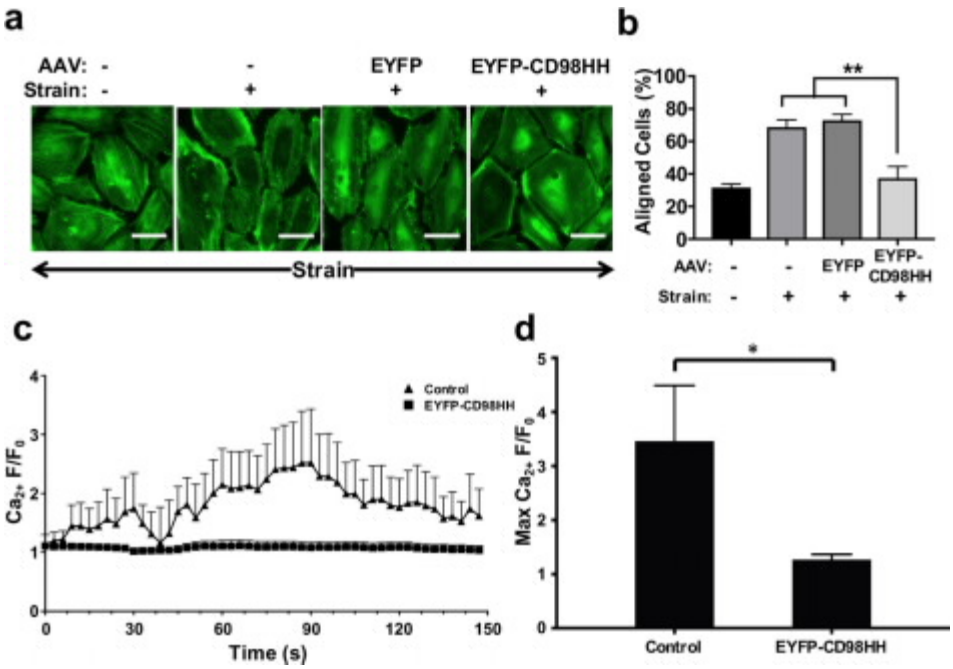


FIG. 2. AAV-mediated expression of CD98 HH disrupts cyclic strain-induced cell realignment and calcium response in human pulmonary endothelial cells. (a) Fluorescence images of HpMVECs stained for F-actin after one day of exposure to 15% cyclic strain (1 Hz) in the horizontal direction; cells were transduced with or without AAV2.5T (MOI 10 000) 2 days earlier (bar, 50 l m). (b) Percentage of cells with actin filaments aligned at 90° 6 30° with respect to the direction of cyclic strain (p < 0.01). (c) Relative change in cytosolic calcium (Ca_{2p} F/F_0) in Rhod-3-loaded HUVECs overexpressing high (bottom) or low (top) levels of CD98 HH in response to static strain (12% for 15 s, arrow). (d) Average maximum relative change in cytosolic calcium (Max Ca_{2p} F/F0) in cells described in c (p < 0.05).

We first elucidated how the route of delivery influences gene expression in the primary human lung epithelium and endothelium by comparing AAV administration through either the airway or circulatory channels. After culturing the cells on the Alveolus Chip and allowing them to mature following established protocols and timelines[40] [supplementary Fig. S2(a)], we infused a droplet of medium containing either AAV2.5T-EYFP (MOI ¼ 10000) to the apical, epithelial channel or AAV2/2-EYFP (MOI ¼ 10000) to the basal endothelium-lined channel, while exposing the cultures continuously to cyclic strain (10%, 0.2 Hz) to mimic breathing motions. After delivery of the vectors and restoration of the ALI, the entire Alveolus Chip was fed by flowing medium through the endothelium-lined vascular channel to mimic pulmonary capillary perfusion in vivo. When we fixed the chips 6 days later and analyzed EYFP expression using confocal microscopy, we observed that gene expression levels were high and sustained for at least 6 days (Fig. 3). Delivery by the apical route was more efficient, with transduction of both HpAECs (12%) and HpMVECs (3%) compared to delivery by the basal route where only transduction of HpMVECs (4%) was achieved. We therefore only administered the vectors apically in subsequent studies.

Fluid accumulation in the lung alveoli and airways is known to result in the loss of lung surfactant, increased permeability of the alveolar–capillary interface, and decreased lung compliance in vivo,[52] which can influence mechanical signals conveyed across the ECM and integrins that feedback to further promote pulmonary edema. To model this physiologically relevant physical stimulus, we infused the apical channel with medium for 4 h under static conditions on day 8 of Alveolus Chip maturation. For these studies, we used chips that had electrodes embedded into the top and bottom of the channels[53,54] [Fig. 4(a)], which allowed us to carry out transepithelial electrical resistance (TEER) measurements to quantitatively assess the pulmonary barrier function in real-time. Studies of Alveolus Chip maturation over time confirmed that the TEER chips can sense to changes in barrier integrity. We measured a rise in TEER values on day 5, which is consistent with a significant improvement in the barrier function after the lung epithelium was placed under an ALI, which has been previously shown to promote epithelial differentiation [supplementary Fig. S2(b)]. The TEER levels and hence the barrier were also maintained for at least 2 days after cyclic mechanical strain (10%) was initiated 2 days after shifting the cells to an ALI [supplementary Fig. S2(b)].

After perfusing the epithelial channel with medium, alone, with AAV2.5T-EYFP-CD98HH, or with the control vector AAV2.5T-EYFP (all MOI ¼ 50 000), while maintaining perfusion through the vascular channel, the ALI was restored to the epithelial channel and cyclic strain application was reinitiated. An increase in vascular leakage through the pulmonary barrier was detected by the following day in response to this physical perturbation, as indicated by a decrease in TEER values by 200–225 Ohms (X) compared to control chips that did not experience fluid immersion [Fig. 4(b); supplementary Fig. S3]. In general, TEER values were 800–1000 X at maturity, which is in line with what was previously measured for airway epithelial cells that form a pseudostratified epithelium (2000 X).[53] Importantly, transduction of the cells with AAV-EYFP-CD98HH encoding the CD98 HH domain significantly inhibited this compromise of the pulmonary barrier function ($p < 0.01$), while the AAV-EYFP control had no protective response [Fig. 4(b)]. It is important to note that fluorescence microscopic imaging of EYFP expression [Fig. 4(c)] and flow cytometry analysis [Fig. 4(d)] revealed that only 30% of HpAECs and 10% of HpMVECs were successfully transduced with the AAV vectors under these conditions. Thus, the scale of this protective response is notable given that only a subset of cells expressed the CD98 HH domain.

In addition to the TEER measurements, ZO-1 and VE-cadherin immunofluorescence staining studies were performed for the epithelial

∀ Author(s) 2019

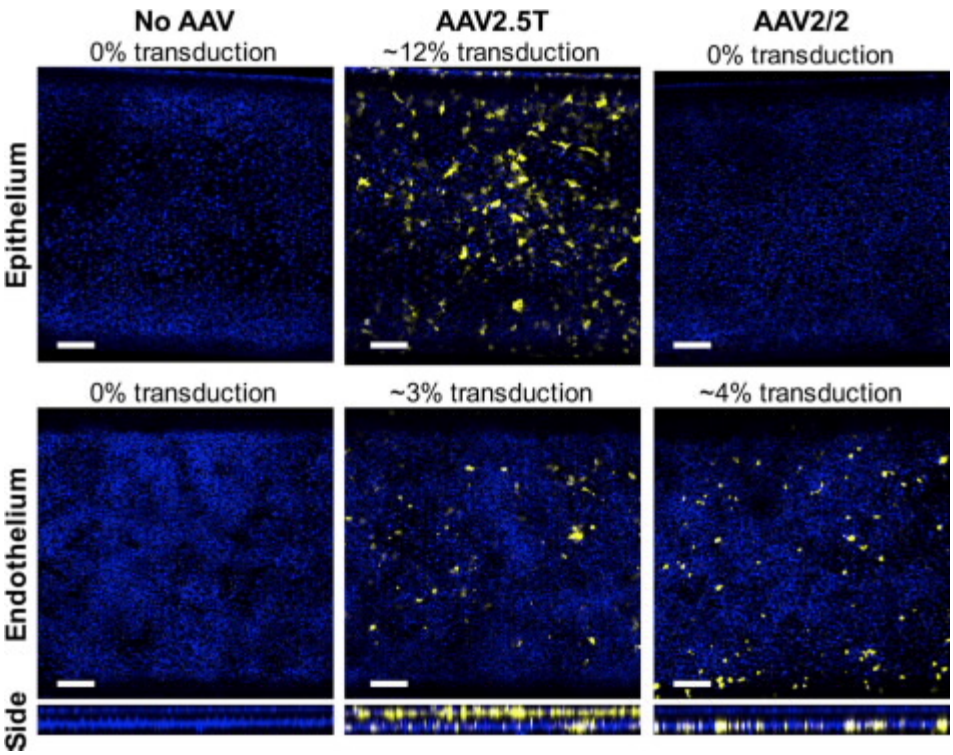


FIG. 3. AAV transduction efficiency in Alveolus Chip AAV-EYFP transduction efficiencies was investigated when AAV2.5T-EYFP (MOI ¼ 10000) was delivered to HpAECs in the top channel of the chip compared to when AAV2/2-EYFP (MOI ¼ 10000) was delivered to HpMVECs in the bottom channel of the human Alveolus Chip (left, control; middle, AAV2.5T-EYFP; right, AAV2/2-EYFP). Confocal images were taken at day 6 of AAV transduction.

and endothelial cells, respectively. For the medium alone and AAV2.5T-EYFP groups, occasional small breaks in cell-cell junctions could be detected, confirming the low-level injury induced by fluid submersion that was measured by TEER [Fig. 4(e)]. Interestingly, although the initial injury was to the epithelium, there was evidence of crosstalk between the two cell populations as previously described,[41] as there were detectable disruptions in the endothelial junctions as well as those in the epithelium [Fig. 4(e)]. Most importantly, fluorescence imaging independently validated that overexpression of CD98 HH protects against this disruption of cell-cell junctions [Fig. 4(e)], which helps to explain its ability to suppress the development of pulmonary vascular leakage as detected by TEER [Fig. 4(b)].

DISCUSSION

AAV2 is the most commonly used viral vector and was first utilized in Phase I/II clinical trials to target a human lung disease (cystic fibrosis).[55,56] These studies established safety profiles for AAV2 in humans, but they did not accomplish therapeutic goals due to the low level of gene transfer in lung epithelial cells. Different serotypes of AAV have different tropisms, and it was later discovered that AAV5, rather than AAV2, binds preferentially to the apical surfaces of airway epithelia.[57] Additionally, AAV5-mediated expression has been reported to persist for at least 12 months in mouse lungs without inducing inflammatory, mechanical, or morphometric changes in the lungs.[58,59]

Here, we demonstrated that AAV2.5T (MOI ¼ 10 000) reached 60% transduction of fully differentiated HpAECs. We further optimized AAV2.5T performance by using the CASI promoter to enhance transgene expression, which increased by more than 100-fold compared to that of the original CMV promoter. Based on previous reports that AAV2 is able to transduce HUVECs and brain endothelial cells,[47,48] we also constructed an endothelium-specific AAV2/2-EYFP vector with a CASI promoter that was able to achieve 30%–60% transduction efficiency in two different types of human endothelial cells (HpMVECs and HUVECs).

We then explored whether we could selectively inhibit TRPV4 mechanotransduction by targeting expression of the CD98 HH domain as a therapeutic for pulmonary vascular disease. Using the optimized AAV vectors, we investigated whether the delivery of CD98 HH could be used to rescue the compromised pulmonary barrier function in a human Lung Alveolus Chip, which has been previously shown to model multiple physiological and pathophysiological features of the human lungs, including pulmonary edema.[13,41] Permeability of the alveolar-capillary interface can be measured in vitro by quantifying the passage of fluorescent tracers or directly measuring TEER, as we have previously demonstrated in previous studies using various Organ Chips, including Lung Chips.[13,53,60] We used TEER measured with embedded electrodes to monitor barrier integrity over time because the TEER method is much more sensitive than the tracer approach (supplementary Fig. S4), and the use of tracer compounds for barrier function measurements can affect transport processes and barrier integrity, especially in Alveolus Chips where the ALI would have to be disrupted for extended periods.[61]

Importantly, by using TEER measurements in the Alveolus Chip, we were able to detect disruption of the pulmonary barrier when the ALI was submerged in liquid medium for 4 h, which is believed to result in a mild mechanical injury due to changes in lung surface tension and compliance.[52] While AAV delivery of EYFP had no effect,

∀ Author(s) 2019

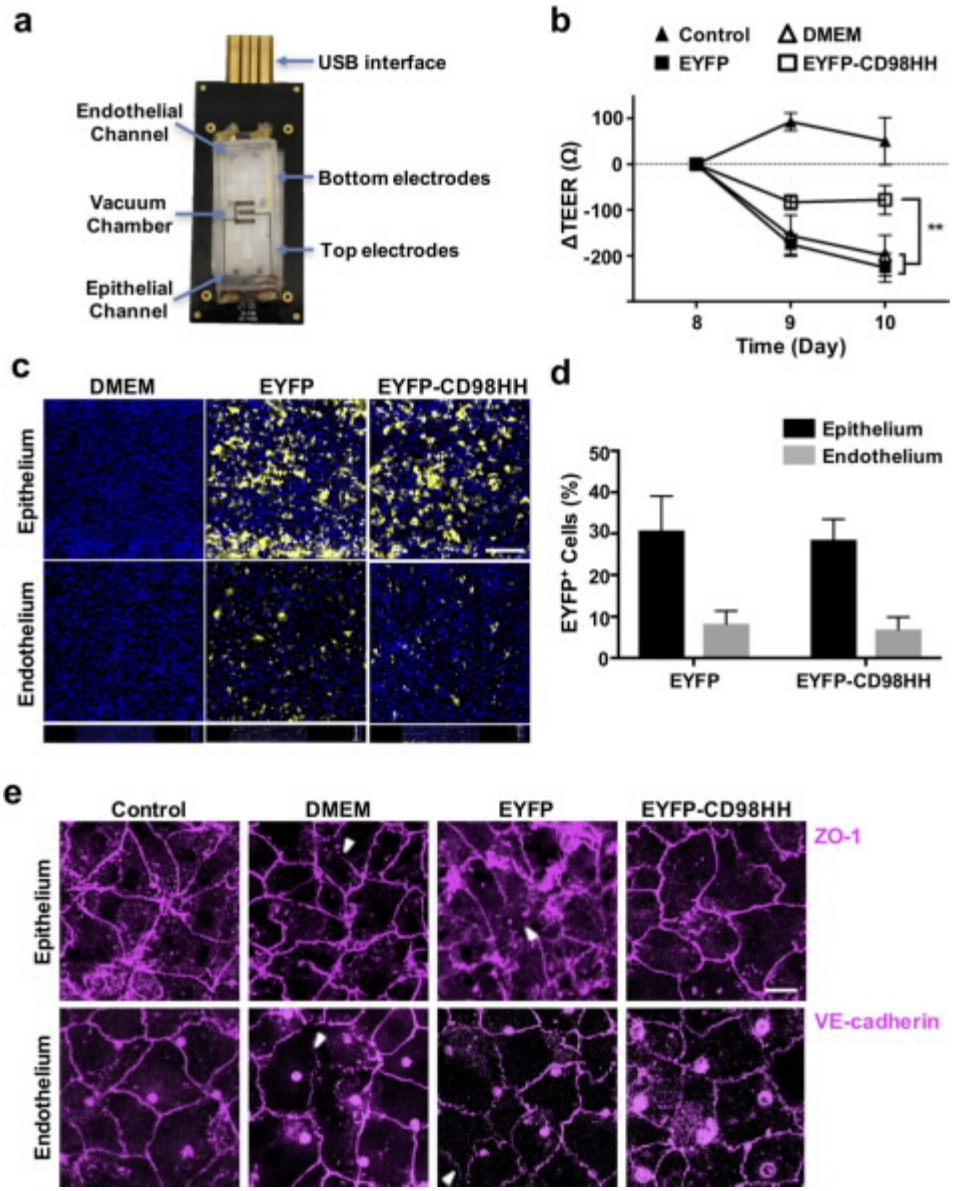


FIG. 4. CD98 inhibition prevents pulmonary vascular leakage in a primary human Alveolus Chip (a) Photograph of the TEER Alveolus Chip setup. (b) Lung barrier permeability reported as changes in TEER in response to mechanical flooding of the apical chamber on day 8 ($p < 0.01$; Control, $n = 5$; DMEM, $n = 9$; AAV2.5T-EYFP, $n = 9$; AAV2.5T-EYFP-CD98HH, $n = 10$). (c) Fluorescence micrographs of AAV transduction efficiency of primary epithelial and endothelial cells 3 days after apical delivery for 4 h (MOI = 50000), displaying EYFP signals from transduced cells (yellow) and DAPI counterstaining (blue) (bar, 200 μm). (d) Percent of cells transduced as quantified by flow cytometry ($n = 3$). (e) Immunostaining of primary epithelial and endothelial cells for ZO-1 (top) and VE-cadherin (bottom), respectively, to visualize cell-cell junctions 3 days after exposure to mechanical flooding and 10% cyclic strain (bar, 20 μm; arrows indicate the breaks in cell-cell junctions).

significant protection (> 65%) against barrier disruption was conferred when the cells were transduced with CD98 HH. The magnitude of recovery is especially remarkable given that less than 30% of the lung cells were transduced with the AAV vectors based on flow cytometric analysis. The MOI of 50 000 that was used was at the upper limit of the maximum achievable AAV concentration. It would be an interesting avenue for the future to investigate if enhanced therapeutic efficacy could be achieved as AAV serotypes with greater affinity for pulmonary cells are engineered or discovered.

There are chemical inhibitors of TRPV4 activity that are currently in Phase II clinical trials for treatment of pulmonary edema in humans,[62] which demonstrates the clinical relevance of this target. However, TRPV4 can mediate a plethora of cellular responses, many of which are activated by chemical signals as well as mechanical cues. Thus, it is important to note the significance of the potential to deliver the CD98HH domain as a highly precise mechanotherapeutic inhibitor that uncouples mechanical activation of TRPV4 from its chemical activation. Using this approach, our results demonstrate that we can minimize the damage to the endothelial-epithelial barrier that occurs from fluid immersion of the airways without affecting TRPV4's chemical signaling function.

Mechanotherapeutic approaches are especially important in the case of patients with acute respiratory distress syndrome (ARDS), who require ventilator support, as fluid accumulation coupled to the stress from the mechanical ventilation can contribute to massive alveolar collapse and perpetuate pulmonary injury.[63] By targeting the

© Author(s) 2019

mechanotransduction mechanism that leads to TRPV4 activation in patients on respirators, rather than generically blocking all TRPV4 channel activity, this exacerbation of injury could potentially be avoided without also introducing the possibility of triggering other adverse events from off-target effects of a generalized channel inhibitor. This targeted mechanotherapeutic design strategy also has the potential to protect against additional mechanical injuries that can be induced by other rescue therapies, such as extracorporeal membrane oxygenation (ECMO).[64] As similar TRPV4-dependent calcium signaling and mechanotransduction have been demonstrated in other cell types (e.g., bovine capillary endothelial cells and human dermal microvascular endothelial cells),[6] it should be possible to extend this mechanotherapeutic design approach to other cell types and applications. Additionally, one caveat of the present study is that TRPV4 may not be the only channel whose mechanical activation is blocked by CD98 HH; however, this suggests the possibility of identifying other non-chemical inhibitors of such channels, which could lead to additional mechanotherapeutics with greater specificity than direct channel blockers.

METHODS

Plasmids and recombinant AAV vector production

Ethics approval was not required. The tested promoters were obtained from the following sources: cytomegalovirus immediate-early promoter (CMV) from pAAV-CMV-luciferase (University of California, Berkeley), chicken b-actin promoter coupled with CMV early enhancer (CAG) from AAV pCAG-FLEX-EGFP-WPRE as a gift from Hongkui Zeng (Addgene Plasmid #51502),[65] human elongation factor 1a (EF1a) from pAAV-EF1a-EGFP (Wyss Institute at Harvard University), and the CASI promoter, which consists of a CMV enhancer, a chicken b-actin promoter, and a ubiquitin enhancer region,[66] from pAAV-CASI-Luc2 (Ragon Institute of MGH, MIT, and Harvard). Luciferase and EYFP genes were cloned into the AAV backbone plasmids with different promoters. As described previously,[66,67] to generate recombinant AAV vectors carrying the transgenes (supplementary Fig. S5), HEK293T cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U ml^{-1} penicillin, and the cells were co-transfected with a three-plasmid system: the coding region containing the backbone plasmid, the helper plasmid pHELP (Applied Viromics), and the seed vector pAAV2.5T (University of California, Berkeley) or pAAV2/2 (Massachusetts Eye and Ear) at a ratio of 1:4:8 using the BioT transfection reagent (Bioland Scientific). The culture supernatant was collected, pooled, and filtered through a 0.2 l m filter, and then a 40% polyethylene glycol (PEG) in 2.5 M NaCl solution was added to the supernatant at a volume ratio of 1:4 and gently mixed at 4 ! C overnight to precipitate the AAV. The precipitated virus was spelleted at 4000 g for 30 min and resuspended in 10 ml of DMEM. To remove the PEG residue and concentrate the virus, the solution was loaded onto 100 kDa MWCO centrifugal filters (Millipore) and spun at 3220 g at 4 ! C until 1 ml of retentate remained. Fresh DMEM was added to the filter, and this process was repeated two more times. The final virus solution was about 2 ml total and stored at " 80 ! C.

Cell culture

HpAECs and HpMVECs (Cell Biologics) were expanded in tissue culture flasks coated with 0.1% (w/v) gelatin (Sigma) using complete human epithelial/endothelial cell medium (Cell Biologics), respectively, with the FBS concentration reduced to 5% (v/v) for the epithelial cells. HUVECs (Lonza) were cultured in endothelial growth medium supplemented with 5% (v/v) FBS and growth factors (EGM-2; Lonza), while A549 cells (ATCC) were cultured in DMEM/F12 (Gibco) with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. The cells were all maintained in an incubator at 37 ! C and 5% CO_2.

AAV transduction in vitro

For plate assays, HpMVECs, HUVECs, and A549 cells were seeded in 6- or 24-well plates, while HpAECs were cultured in 12- or 24-well Transwell plates, treated with 1 l M dexamethasone for 2 days, and introduced to ALI for 4 days before AAV transduction. For chip assays, chips were also allowed to mature at ALI for 4 days (2 days strain) before AAV transduction (see the chip culture section below). AAV vectors were diluted with DMEM and added to cells at MOI ranging from 7000 to 50 000 at 37 ! C for 4 h. After removing the virus solution, epithelial cells were washed once with fresh medium before being returned to ALI, while endothelial cells were replaced with fresh medium, and then the cells were cultured for the desired time.

Flow cytometry

Sample cells were collected by trypsinization and washed with DPBS. After fixing with 2% paraformaldehyde (Electron Microscopy Sciences), cells were stored in 100% methanol at " 20 ! C for future analysis. On the day of analysis, cells were removed from methanol, washed once with wash buffer (DPBS with 1% normal goat serum and 5 mM NaN_3), and finally analyzed in wash buffer using a BD LSRFortessa™ flow cytometer.

Luciferase assay

Two days after AAV transduction, cells were washed with DPBS once and lysed with Glo Lysis Buffer (Promega) at room temperature for five minutes. After transferring to a 96-well plate, the cell lysate was mixed with the Bright-Glo™ Luciferase Assay Reagent (Promega) at a 1:1 volume ratio. Luminescence was then measured using a microplate reader (BioTek).

Cell realignment and calcium imaging for CD98 HH overexpression experiments

HpMVECs and HUVECs were seeded on collagen IV-coated 6-well UniFlex plates (for cell realignment) or StageFlexer membranes (for calcium imaging) (Flexcell) and transduced 24 h after seeding with AAV2/2-EYFP, AAV2/2-EYFP-CD98HH, or nothing (MOI ¼ 10000). 1 day post transduction, cells were exposed to uniaxial cyclic strain (15% strain, 1 Hz) for a further 24 h before fixation with 4% paraformaldehyde. F-actin was stained with Alexa Fluor 647 phalloidin (Invitrogen; diluted 1:40) and visualized by confocal microscopy. The percentage of cells aligned 90 ! 6 30 ! with respect to the direction of cyclic strain was determined using ImageJ. For calcium imaging, cells were loaded with Rhod-3 calcium dye according to the manufacturer's recommendations, mounted on a StageFlexer device, and imaged using Zeiss Axio Imager 2 during and following 12% static strain for 15 s. 100 cells per membrane were sorted based on EYFP expression into five distinct, equally spaced bins, and analysis was

∀ Author(s) 2019

performed comparing intensities from group 5 (high expressers) to group 2 (low expressers). For the group with the TRPV4 chemical agonist GSK1016790A (Tocris), 100 nM GSK1016790A was added 100 s after the start of imaging to induce calcium signaling.

Confocal imaging

Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 5 min. After blocking with 10% goat serum and 0.1% Triton X-100 in DPBS, HpAECs were immunostained with a primary mouse anti-ZO-1 monoclonal antibody (Invitrogen; 1:200) and a secondary goat anti-mouse IgG1 Alexa Fluor 647 (Invitrogen; 1:1000), while HpMVECs were stained with a primary mouse anti-VE-cadherin antibody (BD Biosciences; 1:200) and a secondary goat anti-mouse IgG1 Alexa Fluor 594 (Invitrogen; 1:1000) in an incubation buffer consisting of DPBS with 1% goat serum and 0.1% Triton X-100. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) in DPBS for 5 min (0.5 μg ml^{-1}). AAV transduction was visualized by EYFP. Images were obtained using a Zeiss TIRF/LSM710 confocal microscope and analyzed using ImageJ (NIH) and Imaris (Bitplane).

Chip cell culture

For microfluidic chip cell culture, polydimethylsiloxane (PDMS) chips (Emulate) or TEER chips (see below) were first treated for extracellular matrix (ECM) immobilization using a sulfosuccinimidyl 6-(4′-azido-2′-nitrophenylamino)hexanoate (sulfo-SANPAH) crosslinker (ThermoFisher Scientific). To do so, channels were sequentially washed with 70% ethanol, water, and 50 mM HEPES pH 8.0 before incubation with 0.5 mg/ml sulfo-SANPAH in 50 mM HEPES and pH 8.0 with 0.5% DMSO. The sulfo-SANPAH was then photoactivated with a 36 W UV lamp (NailStar) for 30 min. The channels were washed with 50 mM HEPES, pH 8.0 followed by three additional washes with ice-cold Dulbecco's phosphate-buffered saline (DPBS) before incubation with an ECM coating solution (100 μg ml^{-1} human collagen type I (Advanced BioMatrix), 50 μg ml^{-1} human fibronectin (Corning), and 50 μg ml^{-1} laminin nonapeptide [EMD Millipore)] overnight at 4 °C. The following day, the channels were washed once with respective media before seeding of HpAECs (passage 4) and HpMVECs (passage 5) to the apical and basal channels, respectively. Cell seeding was accomplished by first incubating the basal channel with HpMVECs (4 × 10^6 cells ml^{-1}), flipping the devices upside-down, and incubating the chips for 30 min to allow cell attachment. The chips were then returned upright, HpAECs (4 × 10^6 cells ml^{-1}) were introduced to the apical channel, and the devices were cultured in an incubator overnight. The following two days, endothelial growth medium was replaced in the basal channels and 1 μM dexamethasone in epithelial growth medium was introduced in the apical channels to promote tight junction formation and surfactant production.[13,40,68] ALI was then established by removing the medium from the apical channels, and the basal channels were subjected to continuous perfusion at 60 μl h^{-1} using a peristaltic pump (Ismatec). The perfusion medium for longer-term survival of the cells[40] was composed of phenol red-free endothelial medium without endothelial cell growth supplement (ECGS) and with reduced FBS (0.4%), reduced EGF (0.01%), and additional $CaCl_2$ (1 mM). After 2 days at ALI, physiological breathing motion was initiated by applying vacuum suction to the side chambers of the devices to implement cyclical strain (10% strain, 0.2 Hz).

TEER chip fabrication

Stretchable polydimethylsiloxane (PDMS) microfluidic chips integrated with electrodes for measuring transepithelial electrical resistance (TEER) were fabricated following protocols similar to those reported in the literature.[53] In brief, two parallel microchannels served as the air and microvascular channels (800 μm and 200 μm height, respectively; 1 mm width, and 16.7 mm length in the overlapping region), with a 50 μm PDMS membrane in between allowing for epithelial/endothelial cell-cell interactions through 7 μm pores. Electrodes were positioned above and below the channels to allow for a 4-point impedance measurement [see Fig. 4(a)].

TEER measurements

A PGstat128N potentiostat/galvanostat (Autolab) was used to record four-point impedance spectra in galvanostatic mode over the two-week culture period. The TEER chips were first allowed to equilibrate to room temperature for at least 10 min prior to any measurements. For chips at ALI, 40 μl of room temperature perfusion medium was briefly introduced to the apical channels for the 2 min TEER measurement time before the immediate removal of the medium after each measurement. For each chip, impedance spectra were obtained from 10 Hz to 100 kHz. In the high frequency range (> 10 kHz), the impedance curves are dominated by the resistance of the medium, whereas in the low frequency range (< 100 Hz), the impedance is dominated by the TEER.[61] Therefore, reported TEER data were calculated by subtracted medium resistance at 100 kHz from TEER measured at 21.2 Hz.

Statistical Analysis

All values are expressed as the mean ± standard error of the mean (SEM). All experiments were repeated at least 3 times. Statistical comparisons were performed using Student's *t*-test for experiments with two conditions or ANOVA for experiments with more than two conditions using GraphPad Prism.

SUPPLEMENTARY MATERIAL

See the supplementary material for figures of the response of CD98 overexpressing cells to a TRPV4 chemical agonist, Alveolus Chip maturation over time, and the AAV transgene vector map.

ACKNOWLEDGMENTS

This study was supported by the National Institute for Health research Grant (No. RO1-EB020004 to D.E.I.) and fellowship (No. F32-HL146070), and Wyss Institute for Biologically Inspired Engineering at Harvard University. We thank David V. Schaffer (University of California, Berkeley) for providing pAAV2.5T, Luk H. Vandenberghe and Ru Xiao (Massachusetts Eye and Ear) for providing pAAV2/2, Alejandro Balazs (Ragon Institute of MGH, MIT, and Harvard) for providing pAAV-CASI-Luc2, and George M. Church and Noah Davidsohn (Wyss Institute at Harvard University) for providing pAAV-EF1a-EGFP. We thank Thomas Ferrante for assistance with microscopy and Carlos F. Ng and Elizabeth L. Calamari for assistance with TEER chip fabrication.

© Author(s) 2019

D.E.I. holds equity in Emulate, Inc. and chairs its Scientific Advisory Board.

REFERENCES

[1] L. B. Ware and M. A. Matthay, "Clinical practice Acute pulmonary edema," N. Engl. J. Med. 353(26), 2788–2796 (2005).
[2] J. V. Hurley, "Current views on the mechanisms of pulmonary oedema," J. Pathol. 125(2), 59–79 (1978).
[3] E. D. Robin, C. E. Cross, and R. Zelis, "Pulmonary edema 2," N. Engl. J. Med. 288(6), 292–304 (1973).
[4] E. D. Robin, C. E. Cross, and R. Zelis, "Pulmonary edema 1," N. Engl. J. Med. 288(5), 239–246 (1973).
[5] Acute Respiratory Distress Syndrome Network, R. G. Brower, M. A. Matthay, A. Morris, D. Schoenfeld, B. T. Thompson, and A. Wheeler, "Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome," N. Engl. J. Med. 342(18), 1301–1308 (2000).
[6] B. D. Matthews, C. K. Thodeti, J. D. Tytell, A. Mammoto, D. R. Overby, and D. E. Ingber, "Ultra-rapid activation of TRPV4 ion channels by mechanical forces applied to cell surface beta 1 integrins," Integr. Biol. 2(9), 435–442 (2010).
[7] R. E. Morty and W. M. Kuebler, "TRPV4: An exciting new target to promote alveolocapillary barrier function," Am. J. Physiol. Lung Cell Mol. Physiol. 307(11), L817–821 (2014).
[8] V. K. Sidhaye, K. S. Schweitzer, M. J. Caterina, L. Shimoda, and L. S. King, "Shear stress regulates aquaporin-5 and airway epithelial barrier function," Proc. Natl. Acad. Sci. U. S. A. 105(9), 3345–3350 (2008).
[9] M. A. Mcalexander, M. A. Luttmann, G. E. Hunsberger, and B. J. Undem, "Transient receptor potential vanilloid 4 activation constricts the human bronchus via the release of cysteinyl leukotrienes," J. Pharmacol Exp. Ther. 349(1), 118–125 (2014).
[10] D. Dahan, T. Ducret, J. F. Quignard, R. Marthan, J. P. Savineau, and E. Esteve, "Implication of the ryanodine receptor in TRPV4-induced calcium response in pulmonary arterial smooth muscle cells from normoxic and chronically hypoxic rats," Am. J. Physiol Lung Cell Mol. Physiol 303(9), L824–L833 (2012).
[11] X. R. Yang, A. H. Lin, J. M. Hughes, N. A. Flavahan, Y. N. Cao, W. Liedtke, and J. S. Sham, "Upregulation of osmo-mechanosensitive TRPV4 channel facilitates chronic hypoxia-induced myogenic tone and pulmonary hypertension," Am. J. Physiol Lung Cell Mol. Physiol 302(6), L555–L568 (2012).
[12] H. Y. Yoo, S. J. Park, E. Y. Seo, K. S. Park, J. A. Han, K. S. Kim, D. H. Shin, Y. E. Earm, Y. H. Zhang, and S. J. Kim, "Role of thromboxane A(2)-activated non-selective cation channels in hypoxic pulmonary vasoconstriction of rat," Am. J. Physiol. Cell Physiol. 302(1), C307–C317 (2012).
[13] D. Huh, D. C. Leslie, B. D. Matthews, J. P. Fraser, S. Jurek, G. A. Hamilton, K. S. Thorneloe, M. A. Mcalexander, and D. E. Ingber, "A Human disease model of drug toxicity–induced pulmonary edema in a lung-on-a-chip microdevice," Sci. Transl. Med. 4(159), 159ra147 (2012).
[14] K. S. Thorneloe, M. Cheung, W. Bao, H. Alsaid, S. Lenhard, M. Y. Jian, M. Costell, K. Maniscalco-Hauk, J. A. Krawiec, A. Olzinski, E. Gordon, I. Lozinskaya, L. Elefante, P. Qin, D. S. Matasic, C. James, J. Tunstead, B. Donovan, L. Kallal, A. Waszkiewicz, K. Vaidya, E. A. Davenport, J. Larkin, M. Burgert, L. N. Casillas, R. W. Marquis, G. Ye, H. S. Eidam, K. B. Goodman, J. R. Toomey, T. J. Roethke, B. M. Jucker, C. G. Schnackenberg, M. I. Townsley, J. J. Lepore, and R. N. Willette, "An orally active TRPV4 channel blocker prevents and resolves pulmonary edema induced by heart failure," Sci. Transl. Med. 4(159), 159ra148 (2012).
[15] W. Liedtke, "TRPV4 as osmosensor: A transgenic approach," Pflugers Arch. 451(1), 176–180 (2005).
[16] W. Liedtke and J. M. Friedman, "Abnormal osmotic regulation in TRPV4-/- mice," Proc. Natl. Acad. Sci. U. S. A. 100(23), 13698–13703 (2003).
[17] R. Strotmann, C. Harteneck, K. Nunnenmacher, G. Schultz, and T. D. Plant, "OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity," Nat. Cell Biol. 2(10), 695–702 (2000).
[18] W. Liedtke, Y. Choe, M. A. Marti-Renom, A. M. Bell, C. S. Denis, A. Sali, A. J. Hudspeth, J. M. Friedman, and S. Heller, "Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor," Cell 103(3), 525–535 (2000).
[19] A. Mizuno, N. Matsumoto, M. Imai, and M. Suzuki, "Impaired osmotic sensation in mice lacking TRPV4," Am. J. Physiol. Cell Physiol. 285(1), C96–C101 (2003).
[20] M. Jin, J. Berrout, L. Chen, and R. G. O'neil, "Hypotonicity-induced TRPV4 function in renal collecting duct cells: Modulation by progressive cross-talk with Ca2p -activated Kp channels," Cell Calcium 51(2), 131–139 (2012).
[21] L. Chen, C. Liu, and L. Liu, "Osmolality-induced tuning of action potentials in trigeminal ganglion neurons," Neurosci Lett. 452(1), 79–83 (2009).
[22] J. Li, M. H. Wang, L. Wang, Y. Tian, Y. Q. Duan, H. Y. Luo, X. W. Hu, J. Hescheler, and M. Tang, "Role of transient receptor potential vanilloid 4 in the effect of osmotic pressure on myocardial contractility in rat," Shengli Xuebao 60(2), 181–188 (2008).
[23] Y. Zhang, Y. H. Wang, H. Y. Ge, L. Arendt-Nielsen, R. Wang, and S. W. Yue, "A transient receptor potential vanilloid 4 contributes to mechanical allodynia following chronic compression of dorsal root ganglion in rats," Neurosci Lett. 432(3), 222–227 (2008).
[24] N. Alessandri-Haber, J. J. Yeh, A. E. Boyd, C. A. Parada, X. Chen, D. B. Reichling, and J. D. Levine, "Hypotonicity induces TRPV4-mediated nociception in rat," Neuron 39(3), 497–511 (2003).
[25] S. Bang, T. J. Yang, S. Yoo, T. H. Heo, and S. W. Hwang, "Inhibition of sensory neuronal TRPs contributes to anti-nociception by butamben," Neurosci. Lett. 506(2), 297–302 (2012).
[26] T. T. Liu, H. S. Bi, S. Y. Lv, X. R. Wang, and S. W. Yue, "Inhibition of the expression and function of TRPV4 by RNA interference in dorsal root ganglion," Neurol. Res 32(5), 466–471 (2010).
[27] F. Mizoguchi, A. Mizuno, T. Hayata, K. Nakashima, S. Heller, T. Ushida, M. Sokabe, N. Miyasaka, M. Suzuki, Y. Ezura, and M. Noda, "Transient receptor potential vanilloid 4 deficiency suppresses unloading-induced bone loss," J. Cell Physiol 216(1), 47–53 (2008).
[28] M. J. Rock, J. Prenen, V. A. Funari, T. L. Funari, B. Merriman, S. F. Nelson, R. S. Lachman, W. R. Wilcox, S. Reyno, R. Quadrelli, A. Vaglio, G. Owsianik, A. Janssens, T. Voets, S. Ikegawa, T. Nagai, D. L. Rimoin, B. Nilius, and D. H. Cohn, "Gain-of-function mutations in TRPV4 cause autosomal dominant brachyolmia," Nat. Genet 40(8), 999–1003 (2008).
[29] D. Krakow, J. Vriens, N. Camacho, P. Luong, H. Deixler, T. L. Funari, C. A. Bacino, M. B. Irons, I. A. Holm, L. Sadler, E. B. Okenfuss, A. Janssens, T. Voets, D. L. Rimoin, R. S. Lachman, B. Nilius, and D. H. Cohn, "Mutations in the gene encoding the calcium-permeable ion channel TRPV4 produces spondylometaphyseal dysplasia, Kozlowski type and metatropic dysplasia," Am. J. Hum. Genet 84(3), 307–315 (2009).
[30] S. Muramatsu, M. Wakabayashi, T. Ohno, K. Amano, R. Ooishi, T. Sugahara, S. Shiojiri, K. Tashiro, Y. Suzuki, R. Nishimura, S. Kuhara, S. Sugano, T. Yoneda, and A. Matsuda, "Functional gene screening system identified TRPV4 as a regulator of chondrogenic differentiation," J. Biol. Chem. 282(44), 32158–32167 (2007).
[31] L. Birder, F. A. Kullmann, H. Lee, S. Barrick, W. De Groat, A. Kanai, and M. Caterina, "Activation of urothelial transient receptor potential vanilloid 4 by 4alpha-phorbol 12,13-didecanoate contributes to altered bladder reflexes in the rat," J. Pharmacol Exp. Ther. 323(1), 227–235 (2007).
[32] T. Gevaert, J. Vriens, A. Segal, W. Everaerts, T. Roskams, K. Talavera, G. Owsianik, W. Liedtke, D. Daelemans, L. Dewachter, F. Van Leuven, T. Voets, D. De Ridder, and B. Nilius, "Deletion of the transient receptor potential cation channel TRPV4 impairs murine bladder voiding," J. Clin. Invest. 117(11), 3453–3462 (2007).
[33] K. S. Thorneloe, A. C. Sulpizio, Z. Lin, D. J. Figueroa, A. K. Clouse, G. P. Mccafferty, T. P. Chendrimada, E. S. Lashinger, E. Gordon, L. Evans, B. A. Misajet, D. J. Demarini, J. H. Nation, L. N. Casillas, R. W. Marquis, B. J. Votta, S. A. Sheardown, X. Xu, D. P. Brooks, N. J. Laping, and T. D. Westfall, "N-((1S)-1-{[4-((2S)-2-{[(2,4-dichlorophenyl)sulfonyl]amino}-3-hydroxypropanoyl)-1-piperazinyl]carbonyl}-3-methylbutyl)-1-benzothiophene-2-carboxamide (GSK1016790A) a novel and potent transient receptor potential vanilloid 4 channel agonist induces urinary bladder contraction and hyperactivity: Part I," J. Pharmacol Exp. Ther. 326(2), 432–442 (2008).
[34] X. Xu, E. Gordon, Z. Lin, I. M. Lozinskaya, Y. Chen, and K. S. Thorneloe, "Functional TRPV4 channels and an absence of capsaicin-evoked currents in freshly-isolated guinea-pig urothelial cells," Channels 3(3), 156–160 (2009).

∀ Author(s) 2019

[35] F. Vincent and M. A. Duncton, "TRPV4 agonists and antagonists," Curr. Top. Med. Chem. 11(17), 2216–2226 (2011).
[36] R. Potla, M. Hirano-Kobayashi, H. Wu, H. Chen, A. Mammoto, B. D. Matthews, and D. E. Ingber, "Molecular mapping of transmembrane mechano-transduction through the b1 integrin-CD98hc-TRPV4 axis," bioRxiv:617092
[37] B. J. Carter, "Adeno-associated virus vectors in clinical trials," Hum. Gene Ther. 16(5), 541–550 (2005).
[38] A. C. Nathwani, U. M. Reiss, E. G. Tuddenham, C. Rosales, P. Chowdary, J. Mcintosh, M. Della Peruta, E. Lheriteau, N. Patel, D. Raj, A. Riddell, J. Pie, S. Rangarajan, D. Bevan, M. Recht, Y. M. Shen, K. G. Halka, E. Basner-Tschakarjan, F. Mingozzi, K. A. High, J. Allay, M. A. Kay, C. Y. Ng, J. Zhou, M. Cancio, C. L. Morton, J. T. Gray, D. Srivastava, A. W. Nienhuis, and A. M. Davidoff, "Long-term safety and efficacy of factor IX gene therapy in hemophilia B," N. Engl. J. Med. 371(21), 1994–2004 (2014).
[39] P. A. Lewitt, A. R. Rezai, M. A. Leehey, S. G. Ojemann, A. W. Flaherty, E. N. Eskandar, S. K. Kostyk, K. Thomas, A. Sarkar, M. S. Siddiqui, S. B. Tatter, J. M. Schwalb, K. L. Poston, J. M. Henderson, R. M. Kurlan, I. H. Richard, L. Van Meter, C. V. Sapan, M. J. During, M. G. Kaplitt, and A. Feigin, "AAV2-GAD gene therapy for advanced Parkinson's disease: A double-blind, sham-surgery controlled, randomised trial," Lancet Neurol. 10(4), 309–319 (2011).
[40] B. A. Hassell, G. Goyal, E. Lee, A. Sontheimer-Phelps, O. Levy, C. S. Chen, and D. E. Ingber, "Human organ chip models recapitulate orthotopic lung cancer growth, therapeutic responses, and tumor dormancy in vitro," Cell Rep. 21, 508–516 (2017).
[41] A. Jain, R. Barrile, A. D. Van Der Meer, A. Mammoto, T. Mammoto, K. De Ceunynck, O. Aisiku, M. A. Otieno, C. S. Louden, G. A. Hamilton, R. Flaumenhaft, and D. E. Ingber, "Primary human lung alveolus-on-a-chip model of intravascular thrombosis for assessment of therapeutics," Clin. Pharmacol. Ther. 103(2), 332–340 (2018).
[42] G. Gao, L. H. Vandenberghe, M. R. Alvira, Y. Lu, R. Calcedo, X. Zhou, and J. M. Wilson, "Clades of adeno-associated viruses are widely disseminated in human tissues," J. Virol. 78(12), 6381–6388 (2004).
[43] M. P. Limberis, L. H. Vandenberghe, L. Zhang, R. J. Pickles, and J. M. Wilson, "Transduction efficiencies of novel AAV vectors in mouse airway epithelium in vivo and human ciliated airway epithelium in vitro," Mol. Ther. 17(2), 294–301 (2009).
[44] A. Srivastava, "In vivo tissue-tropism of adeno-associated viral vectors," Curr. Opin. Virol. 21, 75–80 (2016).
[45] C. Zincarelli, S. Soltys, G. Rengo, and J. E. Rabinowitz, "Analysis of AAV serotypes 1–9 mediated gene expression and tropism in mice after systemic injection," Mol. Ther. 16(6), 1073–1080 (2008).
[46] K. J. Excoffon, J. T. Koerber, D. D. Dickey, M. Murtha, S. Keshavjee, B. K. Kaspar, J. Zabner, and D. V. Schaffer, "Directed evolution of adeno-associated virus to an infectious respiratory virus," Proc. Natl. Acad. Sci. U. S. A. 106(10), 3865–3870 (2009).
[47] S. F. Merkel, A. M. Andrews, E. M. Lutton, D. Mu, E. Hudry, B. T. Hyman, C. A. Maguire, and S. H. Ramirez, "Trafficking of adeno-associated virus vectors across a model of the blood-brain barrier; a comparative study of transcytosis and transduction using primary human brain endothelial cells," J. Neurochem. 140(2), 216–230 (2017).
[48] B. L. Ellis, M. L. Hirsch, J. C. Barker, J. P. Connelly, R. J. Steininger, 3rd, and M. H. Porteus, "A survey of ex vivo/in vitro transduction efficiency of mammalian primary cells and cell lines with nine natural adeno-associated virus (AAV1-9) and one engineered adeno-associated virus serotype," Virol. J. 10, 74 (2013).
[49] C. Burger, O. S. Gorbatyuk, M. J. Velardo, C. S. Peden, P. Williams, S. Zolotukhin, P. J. Reier, R. J. Mandel, and N. Muzyczka, "Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system," Mol. Ther. 10(2), 302–317 (2004).
[50] C. K. Thodeti, B. Matthews, A. Ravi, A. Mammoto, K. Ghosh, A. L. Bracha, and D. E. Ingber, "TRPV4 channels mediate cyclic strain-induced endothelial cell reorientation through integrin-to-integrin signaling," Circ. Res. 104(9), 1123–1130 (2009).
[51] D. Huh, B. D. Matthews, A. Mammoto, M. Montoya-Zavala, H. Y. Hsin, and D. E. Ingber, "Reconstituting organ-level lung functions on a chip," Science 328(5986), 1662–1668 (2010).
[52] J. P. Orlowski, M. M. Abulleil, and J. M. Phillips, "The hemodynamic and cardiovascular effects of near-drowning in hypotonic, isotonic, or hypertonic solutions," Ann. Emerg. Med. 18, 1044–1049 (1989).
[53] O. Y. F. Henry, R. Villenave, M. J. Cronce, W. D. Leineweber, M. A. Benz, and D. E. Ingber, "Organs-on-chips with integrated electrodes for trans-epithelial electrical resistance (TEER) measurements of human epithelial barrier function," Lab-on-a-Chip 17, 2264–2271 (2017).
[54] M. Odijk, A. D. Van Der Meer, D. Levner, H. J. Kim, M. W. Van Der Helm, L. I. Segerink, J-P. Frimat, G. A. Hamilton, D. E. Ingber, and A. Van Den Berg, "Measuring direct current trans-epithelial electrical resistance in organ-on-a-chip microsystems," Lab-on-a-Chip 15, 745–752 (2015).
[55] T. Flotte, B. Carter, C. Conrad, W. Guggino, T. Reynolds, B. Rosenstein, G. Taylor, S. Walden, and R. Wetzel, "A phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with mild lung disease," Hum. Gene Ther. 7(9), 1145–1159 (1996).
[56] T. R. Flotte, P. L. Zeitlin, T. C. Reynolds, A. E. Heald, P. Pedersen, S. Beck, C. K. Conrad, L. Brass-Ernst, M. Humphries, K. Sullivan, R. Wetzel, G. Taylor, B. J. Carter, and W. B. Guggino, "Phase I trial of intranasal and endobronchial administration of a recombinant adeno-associated virus serotype 2 (rAAV2)-CFTR vector in adult cystic fibrosis patients: A two-part clinical study," Hum. Gene Ther. 14(11), 1079–1088 (2003).
[57] J. Zabner, M. Seiler, R. Walters, R. M. Kotin, W. Fulgeras, B. L. Davidson, and J. A. Chiorini, "Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer," J. Virol. 74(8), 3852–3858 (2000).
[58] S. V. Martini, S. S. Fagundes, A. C. Schmidt, M. Avila, D. S. Ornellas, V. T. Ribas, H. Petrs-Silva, R. Linden, D. S. Faffe, S. E. Guggino, P. R. Rocco, W. A. Zin, and M. M. Morales, "Does the use of recombinant AAV5 in pulmonary gene therapy lead to lung damage?," Respir. Physiol. Neurobiol. 168(3), 203–209 (2009).
[59] S. G. Sumner-Jones, L. A. Davies, A. Varathalingam, D. R. Gill, and S. C. Hyde, "Long-term persistence of gene expression from adeno-associated virus serotype 5 in the mouse airways," Gene Ther. 13(24), 1703 (2006).
[60] T. E. Park, N. Mustafaoglu, A. Herland, R. Hasselkus, R. Mannix, E. A. Fitzgerald, R. Prantil-Baun, A. Watters, O. Henry, M. Benz, H. Sanchez, H. J. Mccrea, L. C. Goumnerova, H. W. Song, S. P. Palecek, E. Shusta, and D. E. Ingber, "Hypoxia-enhanced blood-brain barrier chip recapitulates human barrier function and shuttling of drugs and antibodies," Nat. Commun. 10(1), 2621 (2019).
[61] B. Srinivasan, A. R. Kolli, M. B. Esch, H. E. Abaci, M. L. Shuler, and J. J. Hickman, "TEER measurement techniques for in vitro barrier model systems," J. Lab. Autom. 20, 107–126 (2015).
[62] N. Goyal, P. Skrdla, R. Schroyer, S. Kumar, D. Fernando, A. Oughton, N. Norton, D. L. Sprecher, and J. Cheriyan, "Clinical pharmacokinetics, safety, and tolerability of a novel, first-in-class TRPV4 ion channel inhibitor, GSK2798745, in healthy and heart failure subjects," Am. J. Cardiovasc. Drugs 19(3), 335–342 (2019).
[63] M. B. Amato, C. S. Barbas, D. M. Medeiros, R. B. Magaldi, G. P. Schettino, G. Lorenzi-Filho, R. A. Kairalla, D. Deheinzelin, C. Munoz, R. Oliveira, T. Y. Takagaki, and C. R. Carvalho, "Effect of a protective-ventilation strategy on mortality in the acute respiratory distress syndrome," N. Engl. J. Med. 338(6), 347–354 (1998).
[64] A. Combes, D. Hajage, G. Capellier, A. Demoule, S. Lavoue, C. Guervilly, D. Da Silva, L. Zafrani, P. Tirot, B. Veber, E. Maury, B. Levy, Y. Cohen, C. Richard, P. Kalfon, L. Bouadma, H. Mehdaoui, G. Beduneau, G. Lebreton, L. Brochard, N. D. Ferguson, E. Fan, A. S. Slutsky, D. Brodie, A. Mercat, R. Eolia, Trial Group, and ECMONet, "Extracorporeal membrane oxygenation for severe acute respiratory distress syndrome," N. Engl. J. Med. 378(21), 1965–1975 (2018).
[65] S. W. Oh, J. A. Harris, L. Ng, B. Winslow, N. Cain, S. Mihalas, Q. Wang, C. Lau, L. Kuan, A. M. Henry, M. T. Mortrud, B. Ouellette, T. N. Nguyen, S. A. Sorensen, C. R. Slaughterbeck, W. Wakeman, Y. Li, D. Feng, A. Ho, E.

© Author(s) 2019

Nicholas, K. E. Hirokawa, P. Bohn, K. M. Joines, H. Peng, M. J. Hawrylycz, J. W. Phillips, J. G. Hohmann, P. Wohnoutka, C. R. Gerfen, C. Koch, A. Bernard, C. Dang, A. R. Jones, and H. Zeng, "A mesoscale connectome of the mouse brain," Nature 508(7495), 207 (2014).
[66] A. B. Balazs, J. Chen, C. M. Hong, D. S. Rao, L. Yang, and D. Baltimore, "Antibody-based protection against HIV infection by vectored immunoprophylaxis," Nature 481(7379), 81 (2012).
[67] J. Li, A. I. Olvera, O. S. Akbari, A. Moradian, M. J. Sweredoski, S. Hess, and B. A. Hay, "Vectored antibody gene delivery mediates long-term contraception," Curr. Biol. 25(19), R820–822 (2015).
[68] M. I. Hermanns, R. E. Unger, K. Kehe, K. Peters, and C. J. Kirkpatrick, "Lung epithelial cell lines in coculture with human pulmonary microvascular endothelial cells: Development of an alveolo-capillary barrier in vitro," Lab. Invest. 84, 736–752 (2004).

© Author(s) 2019

EXTENDED PDF FORMAT
SPONSORED BY
Measuring Cytokines?
Don't Sacrifice on Quality
New ELISA Solutions
>Learn More
RD
www.rndsystems.com

Reconstituting Organ-Level Lung Functions on a Chip

Dongeun Huh *et al.*

Science **328**, 1662 (2010);
DOI: 10.1126/science.1188302

This copy is for your personal, non-commercial use only.

If you wish to distribute this article to others, you can order high-quality copies for your colleagues, clients, or customers by clicking here.

Permission to republish or repurpose articles or portions of articles can be obtained by following the guidelines here.

The following resources related to this article are available online at www.sciencemag.org (this information is current as of December 18, 2013):

Updated information and services, including high-resolution figures, can be found in the online version of this article at:
http://www.sciencemag.org/content/328/5986/1662.full.html

Supporting Online Material can be found at:
http://www.sciencemag.org/content/suppl/2010/06/25/328.5986.1662.DC1.html

A list of selected additional articles on the Science Web sites **related to this article** can be found at:
http://www.sciencemag.org/content/328/5986/1662.full.html#related

This article **cites 41 articles**, 9 of which can be accessed free:
http://www.sciencemag.org/content/328/5986/1662.full.html#ref-list-1

This article has been **cited by** 25 articles hosted by HighWire Press; see:
http://www.sciencemag.org/content/328/5986/1662.full.html#related-urls

This article appears in the following **subject collections:**
Medicine, Diseases
http://www.sciencemag.org/cgi/collection/medicine

Downloaded from www.sciencemag.org on December 18, 2013

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.Copyright 2010 by the American Association for the Advancement of Science; all rights reserved. The title *Science* is a registered trademark of AAAS.

24. The relative phases of interfering partial waves are accessible to measurement because they determine angular photoelectron distributions; still, this information is not sufficient to retrieve either the energy dependence of a phase in a particular photoionization channel or the relative phase between channels that do not interfere.
25. F. T. Smith, Phys. Rev. 118, 349 (1960).
26. A. L. Cavalieri et al., N. J. Phys. 9, 242 (2007).
27. Y. Mairesse, F. Quéré, Phys. Rev. A 71, 011401 (2005).
28. J. Gagnon, E. Goulielmakis, V. S. Yakovlev, Appl. Phys. B 92, 25 (2008).
29. Information on technology and methods is available as supporting material on Science Online.
30. J. Gagnon, V. S. Yakovlev, Opt. Express 17, 17678 (2009).
31. C. A. Nicolaides, Th. Mercouris, Y. Komninos, J. Phys. At. Mol. Opt. Phys. 35, L271 (2002).
32. Th. Mercouris, Y. Komninos, C. A. Nicolaides, Phys. Rev. A 76, 033417 (2007).
33. J. S. Parker, B. J. S. Doherty, K. J. Meharg, K. T. Taylor, J. Phys. At. Mol. Opt. Phys. 36, L393 (2003).
34. Supported by the Max Planck Society and the Deutsche Forschungsgemeinschaft Cluster of Excellence Munich Centre for Advanced Photonics (www.munich-photonics.de) and the King Saud University–Max-Planck-Institut für Quantenoptik collaboration. Supported in part by NSF through TeraGrid resources provided by the National Institute for Computational Sciences and Texas Advanced Computing Center under grant TG-PHY090003. R.P., S.N., and J.B. acknowledge support from the Austrian Science Fund (FWF) under grant FWF-SFB016. F. acknowledges support by NSR through a grant to ITAMP. E.G. acknowledges a Marie-Curie Reintegration grant (MERG-CT-2007-208647). K. acknowledges support from the Sofia Kovalevskaja award of the Alexander von Humboldt Foundation and an ERC starting grant. We thank A. Maquet and R. Taieb for fruitful discussions.

Supporting Online Material
www.sciencemag.org/cgi/content/full/328/5986/1658/DC1
SOM Text
Fig. S1
References

10 March 2010; accepted 10 May 2010
10.1126/science.1189401

Reconstituting Organ-Level Lung Functions on a Chip

Dongeun Huh,[1,2] Benjamin D. Matthews,[2,3] Akiko Mammoto,[2] Martín Montoya-Zavala,[1,2] Hong Yuan Hsin,[2] Donald E. Ingber[1,2,4]*

Here, we describe a biomimetic microsystem that reconstitutes the critical functional alveolar-capillary interface of the human lung. This bioinspired microdevice reproduces complex integrated organ-level responses to bacteria and inflammatory cytokines introduced into the alveolar space. In nanotoxicology studies, this lung mimic revealed that cyclic mechanical strain accentuates toxic and inflammatory responses of the lung to silica nanoparticles. Mechanical strain also enhances epithelial and endothelial uptake of nanoparticulates and stimulates their transport into the underlying microvascular channel. Similar effects of physiological breathing on nanoparticle absorption are observed in whole mouse lung. Mechanically active "organ-on-a-chip" microdevices that reconstitute tissue-tissue interfaces critical to organ function may therefore expand the capabilities of cell culture models and provide low-cost alternatives to animal and clinical studies for drug screening and toxicology applications.

One of the causes of the high cost of pharmaceuticals and the major obstacles to rapidly identifying new environmental toxins is the lack of experimental model systems that can replace costly and time-consuming animal studies. Although considerable advances have been made in the development of cell culture models as surrogates of tissues and organs for these types of studies (1), cultured cells commonly fail to maintain differentiation and expression of tissue-specific functions. Improved tissue organization can be promoted by growing cells in three-dimensional extracellular matrix (ECM) gels (2); however, these methods still fail to reconstitute structural and mechanical features of whole living organs that are central to their function. In particular, existing model systems do not recreate the active tissue-tissue interface between the microvascular endothelium and neighboring parenchymal tissues where critical transport of fluids, nutrients, immune cells, and other regulatory factors occur, nor do they permit application of dynamic mechanical forces (e.g., breathing movements in lung, shear in blood vessels, peristalsis in gut, tension in skin, and the like) that are critical for the development and function of living organs (3).

Microscale engineering technologies first developed to create microchips, such as microfabrication and microfluidics, enable unprecedented capabilities to control the cellular microenvironment with high spatiotemporal precision and to present cells with mechanical and biochemical signals in a more physiologically relevant context (4–7). This approach has made it possible to microfabricate models of blood vessels (8, 9), muscles (10), bones (11), airways (12), liver (13–16), brain (17, 18), gut (19), and kidney (20, 21). However, it has not yet been possible to engineer integrated microsystems that replicate the complex physiological functionality of living organs by incorporating multiple tissues, including active vascular conduits, and placing them in a dynamic and mechanically relevant organ-specific microenvironment.

To provide the proof of principle for a biomimetic microsystems approach, we developed a multifunctional microdevice that reproduces key structural, functional, and mechanical properties of the human alveolar-capillary interface, which is the fundamental functional unit of the living lung. This was accomplished by microfabricating a microfluidic system containing two closely apposed microchannels separated by a thin (10 μm), porous, flexible membrane made of poly(dimethylsiloxane) (PDMS). The intervening membrane was coated with ECM (fibronectin or collagen), and human alveolar epithelial cells and human pulmonary microvascular endothelial cells were cultured on opposite sides of the membrane (Fig. 1A). Once the cells were grown to confluence, air was introduced into the epithelial compartment to create an air-liquid interface and more precisely mimic the lining of the alveolar air space. The compartmentalized channel configuration of the microdevice makes it possible to manipulate fluid flow, as well as delivery of cells and nutrients, to the epithelium and endothelium independently.

During normal inspiration, intrapleural pressure decreases, causing the alveoli to expand; this pulls air into the lungs, resulting in stretching of the alveolar epithelium and the closely apposed endothelium in adjacent capillaries (Fig. 1B). We mimicked this subatmospheric, pressure-driven stretching by incorporating two larger, lateral microchambers into the device design. When vacuum is applied to these chambers, it produces elastic deformation of the thin wall that separates the cell-containing microchannels from the side chambers; this causes stretching of the attached PDMS membrane and the adherent tissue layers (Fig. 1A, right versus left). When the vacuum is released, elastic recoil of PDMS causes the membrane and adherent cells to relax to their original size. This design replicates dynamic mechanical distortion of the alveolar-capillary interface caused by breathing movements.

These hollow microchannels were fabricated by soft lithography in conjunction with a new method that uses chemical etching of PDMS (22) to form the vacuum chambers. Fabrication begins with alignment and permanent bonding of a 10-mm-thick porous PDMS membrane (containing 10-μm-wide pentagonal pores) and two PDMS layers containing recessed microchannels (Fig. 1C). A PDMS etching solution composed of tetrabutyl-

[1]Wyss Institute for Biologically Inspired Engineering at Harvard University, Boston, MA 02115, USA. [2]Vascular Biology Program, Departments of Pathology and Surgery, Children's Hospital Boston and Harvard Medical School, Boston, MA 02115, USA. [3]Department of Medicine, Children's Hospital Boston, Boston, MA 02115, USA. [4]School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138, USA.

*To whom correspondence should be addressed. E-mail: don.ingber@wyss.harvard.edu

ammonium fluoride and N-methylpyrrolidinone is then pumped through the side channels (Fig. 1D). Within a few minutes, PDMS etchant completely dissolves away portions of the membrane in the side channels, creating two large chambers directly adjacent to the culture microchannels. The entire integrated device is only 1 to 2 cm in length, with the central channels only millimeters in width (Fig. 1E), and thus, it is fully amenable to high-density integration into a highly multiplexed microdevice in the future.

Reconstitution of a functional alveolar-capillary interface. When human alveolar epithelial cells and microvascular endothelial cells were introduced into their respective channels, they attached to opposite surfaces of the ECM-coated membrane and formed intact monolayers composed of cells linked by continuous junctional complexes containing the epithelial and endothelial junctional proteins, occludin and vascular endothelial cadherin (VE-cadherin), respectively (Fig. 2A). These cells remained viable for prolonged periods (>2 weeks) after air was introduced into the epithelial microchannel and the alveolar cells were maintained at an air-liquid interface (fig. S1). Addition of air to the upper channel resulted in increased surfactant production by the epithelium (Fig. 2B and fig. S1), which stabilizes the thin liquid layer in vitro as it does in whole lung in vivo, such that no drying was observed. This was also accompanied by an increase in electrical resistance across the tissue layers (Fig. 2C) and enhanced molecular barrier function relative to cells cultured under liquid medium (Fig. 2D). These differences in barrier integrity and permeability likely result from strengthening of intercellular junctions in air-liquid interface culture (12), and from the observed changes in pulmonary surfactant production that can influence alveolar-capillary barrier function (23). Moreover, the low level of protein permeability (2.1%/hour for fluorescently labeled albumin) exhibited by cells cultured at the air-liquid interface (Fig. 2D) closely approximated that observed in vivo (1 to ~2%/hour) (24). It should be noted that although we mimic the alveolar microenvironment by generating an air-liquid interface in contact with the apical surface of epithelium in our microdevice, there is likely a large variation in the microscale properties of this interface in vivo. Thus, it would be difficult to interpret these results in any way other than functional measures at this time. Our results clearly show that the epithelial cells not only remained viable but also increased their surfactant production, enhanced their structural integrity, and restored normal barrier permeability in this biomimetic microsystem.

The microfluidic device was then integrated with computer-controlled vacuum to produce cyclic stretching of the tissue-tissue interface to mimic physiological breathing movements (movie S1). The level of applied strain ranged from 5% to 15% to match normal levels of strain observed in alveoli within whole lung in vivo, as previously described (25). Vacuum application generated uniform, unidirectional mechanical strain across the channel length, as demonstrated by measured displacements of fluorescent quantum dots immobilized on the PDMS membrane (Fig. 2E and movie S2). Membrane stretching also resulted in cell shape distortion, as visualized by concomitant increases in the projected area and length of the adherent cells in the direction of applied tension (Fig. 2F and movie S3).

The permeability of the alveolar-capillary barrier to fluorescent albumin remained unchanged during cyclic stretching over 4 hours with physiological levels of strain (5 to ~15%) or after preconditioning with 10% strain for varying amounts of time (fig. S1). Application of physiological cyclic strain (10% at 0.2 Hz) also induced cell alignment in the endothelial cells in the lower compartment (fig. S2 and movie S4) and, hence, mimicked physiological responses previously observed in cultured endothelium and in living blood vessels in vivo (26, 27). Cyclic stretching caused some pulsatility in the fluid flow, but this unsteady effect was negligible due to the small channel size and low stretching frequency (see SOM text). Thus, this microdevice extends far beyond previously described cell stretching systems by permitting the application of cyclic stretch and fluid shear stress to two opposing cell layers

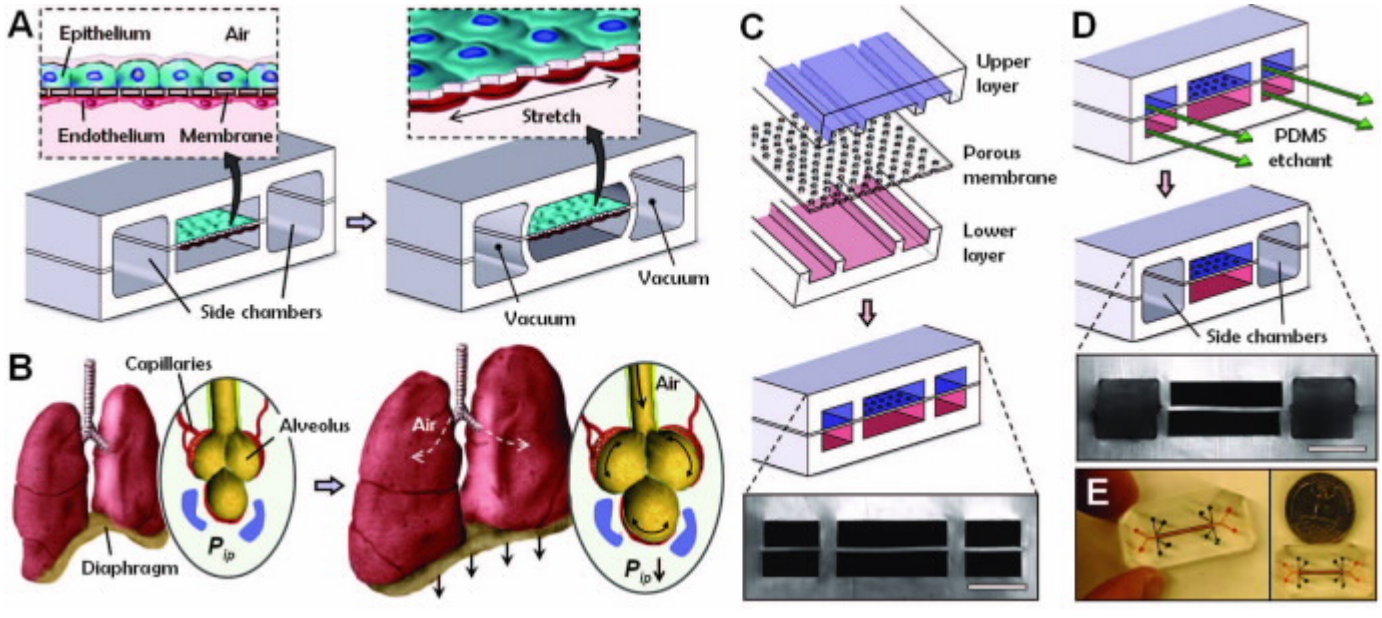


Fig. 1. Biologically inspired design of a human breathing lung-on-a-chip microdevice (A) The microfabricated lung mimic device uses compartmentalized PDMS microchannels to form an alveolar-capillary barrier on a thin, porous, flexible PDMS membrane coated with ECM. The device recreates physiological breathing movements by applying vacuum to the side chambers and causing mechanical stretching of the PDMS membrane forming the alveolar-capillary barrier. (B) During inhalation in the living lung, contraction of the diaphragm causes a reduction in intrapleural pressure (P_{ip}), leading to distension of the alveoli and physical stretching of the alveolar-capillary interface (C) Three PDMS layers are aligned and irreversibly bonded to form two sets of three parallel microchannels separated by a 10-mm-thick PDMS membrane containing an array of through-holes with an effective diameter of 10 mm. Scale bar, 200 mm. (D) After permanent bonding, PDMS etchant is flowed through the side channels. Selective etching of the membrane layers in these channels produces two large side chambers to which vacuum is applied to cause mechanical stretching. Scale bar, 200 mm. (E) Images of an actual lung-on-a-chip microfluidic device viewed from above.

separated by a permeable and flexible ECM, while simultaneously enabling analysis of tissue barrier permeability and transport.

Recapitulation of whole-organ responses. We then explored whether this microsystem could recapitulate more complex integrated organ-level responses observed in whole living lung, such as pulmonary inflammation, by incorporating blood-borne immune cells in the fluid flowing through the vascular channel. Pulmonary inflammatory responses involve a highly coordinated multistep cascade including epithelial production and release of early-response cytokines, activation of vascular endothelium through up-regulation of leukocyte adhesion molecules [e.g., intercellular adhesion molecule-1 (ICAM-1)], and subsequent leukocyte infiltration into the alveolar space from the pulmonary microcirculation (28–30). Because cytokines are produced by cells of the lung parenchyma, we simulated this process by introducing medium containing the potent proinflammatory mediator, tumor necrosis factor–a (TNF-a), into the alveolar microchannel in the presence of physiological mechanical strain and examined activation of the underlying microvascular endothelium by measuring ICAM-1 expression.

TNF-a stimulation of the epithelium substantially increased endothelial expression of ICAM-1 within 5 hours after addition (Fig. 3A and fig. S3), whereas physiological cyclic strain had no effect on ICAM-1 expression. The activated endothelium also promoted firm adhesion of fluorescently labeled human neutrophils flowing in the vascular microchannel, which do not adhere to the endothelium without TNF-a stimulation (Fig. 3B and movies S5 and S6). Real-time, high-resolution, fluorescent microscopic visualization revealed that soon after adhering, the neutrophils flattened (fig. S3) and migrated over the apical surface of the endothelium until they found cell-cell junctions, where they underwent diapedesis and transmigrated across the capillary-alveolar barrier through the membrane pores over the period of several minutes (Fig. 3C and movie S7). Phase-contrast microscopic visualization on the opposite side of the membrane revealed neutrophils crawling up through the spaces between neighboring cells and emerging on the surface of the overlying alveolar epithelium (Fig. 3D), where they remained adherent despite active fluid flow

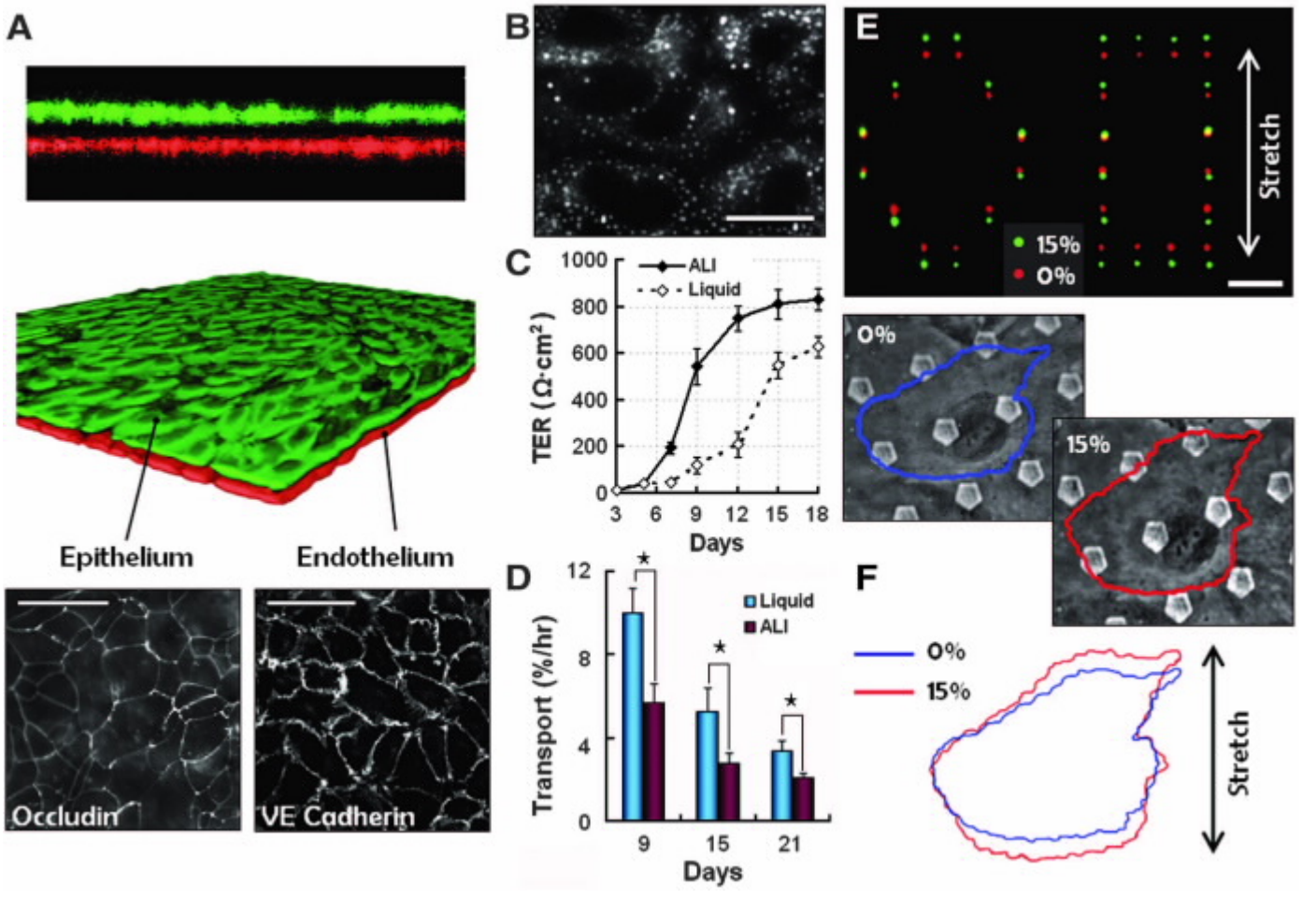


Fig. 2. On-chip formation and mechanical stretching of an alveolar-capillary interface. (A) Long-term microfluidic coculture produces a tissue-tissue interface consisting of a single layer of the alveolar epithelium (epithelium, stained with CellTracker Green) closely apposed to a monolayer of the microvascular endothelium (endothelium, stained with CellTracker Red), both of which express intercellular junctional structures stained with antibodies to occludin or VE-cadherin, separated by a flexible ECM-coated PDMS membrane. Scale bar, 50 mm. (B) Surfactant production by the alveolar epithelium during air-liquid interface culture in our device detected by cellular uptake of the fluorescent dye quinacrine that labels lamellar bodies (white dots). Scale bar, 25 mm. (C) Air-liquid interface (ALI) culture leads to a greater increase in transbilayer electrical resistance (TER) and produces tighter alveolar-capillary barriers with higher TER (>800 Ω·cm^2), as compared with the tissue layers formed under submerged liquid culture conditions. (D) Alveolar barrier permeability, measured by quantitating the rate of fluorescent albumin transport, is significantly reduced in ALI cultures compared with liquid cultures (*$P < 0.001$). Data in (C) and (D) represent the mean ± SEM from three separate experiments. (E) Membrane stretching–induced mechanical strain visualized by the displacements of individual fluorescent quantum dots that were immobilized on the membrane in hexagonal and rectangular patterns before (red) and after (green) stretching. Scale bar, 100 mm. (F) Membrane stretching exerts tension on the cells and causes them to distort in the direction of the applied force, as illustrated by the overlaid outlines of a single cell before (blue) and after (red) application of 15% strain. The pentagons in the micrographs represent microfabricated pores in the membrane. Endothelial cells were used for visualization of cell stretching.

and cyclic stretching. Similar adhesion and transmigration of neutrophils were observed in response to alveolar stimulation with other proinflammatory cytokines, such as interleukin-8. These sequential events successfully replicate the entire process of neutrophil recruitment from the microvasculature to the alveolar compartment, which is a hallmark of lung inflammation.

We also demonstrated that this system could mimic the innate cellular response to pulmonary infection of bacterial origin. Living Escherichia coli bacteria constitutively expressing green fluorescent protein (GFP) were added to the alveolar microchannel. The presence of these pathogens on the apical surface of the alveolar epithelium for 5 hours was sufficient to activate the underlying endothelium, as indicated by capture of circulating neutrophils and their transmigration into the alveolar microchannel. Upon reaching the alveolar surface, the neutrophils displayed directional movement toward the bacteria, which they then engulfed over a period of a few minutes (Fig. 3E and movies S8 and S9), and the phagocytic activity of the neutrophils continued until most bacteria were cleared from the observation area. We specifically excluded the air-liquid interface in the experiments shown in Fig. 3 because inflammatory cytokines are produced by the epithelial tissue, and alveoli often become filled with fluid exudate during early stages of lung infections. These results show that this bioinspired microdevice can effectively recapitulate the normal integrated cellular immune response to microbial infection in human lung alveoli. It also can be used to visualize these cellular responses in real time by high-resolution microscopic imaging during mechanical stimulation, which has been difficult in most existing cell stretching systems.

Identification of novel mechanosensitive responses to nanoparticulates. We also explored the potential value of this lung-on-a-chip system for toxicology applications by investigating the pulmonary response to nanoparticulates delivered to the epithelial compartment. Despite the widespread use of nanomaterials, much remains to be learned about their risks to health and the environment (31–33). Existing toxicology methods rely on oversimplified in vitro models or lengthy and expensive animal testing.

When nanoparticles are delivered to the alveoli of the lung in an aerosol, they are deposited in a thin fluid layer supported by surfactant on the surface of the epithelium. To mimic delivery of airborne nanoparticles into the lung using our microdevice, we injected nanoparticle solution into the alveolar microchannel and then gently aspirated the solution to leave a thin liquid layer containing nanoparticles covering the epithelial surface. When alveolar epithelial cells were exposed for 5 hours to 12-nm silica nanoparticles that are commonly used to model the toxic effects of ultrafine airborne particles (34–36), the underlying endothelium in the microvascular channel became activated and exhibited high levels of ICAM-1 expression (Fig. 4A). Although physiological breathing movements (~10% cyclic strain) had no effect on ICAM-1 expression on their own, mechanical stretching significantly augmented endothelial expression of ICAM-1 induced by the silica nanoparticles (Fig. 4A). Moreover, this effect was sufficient to induce endothelial capture

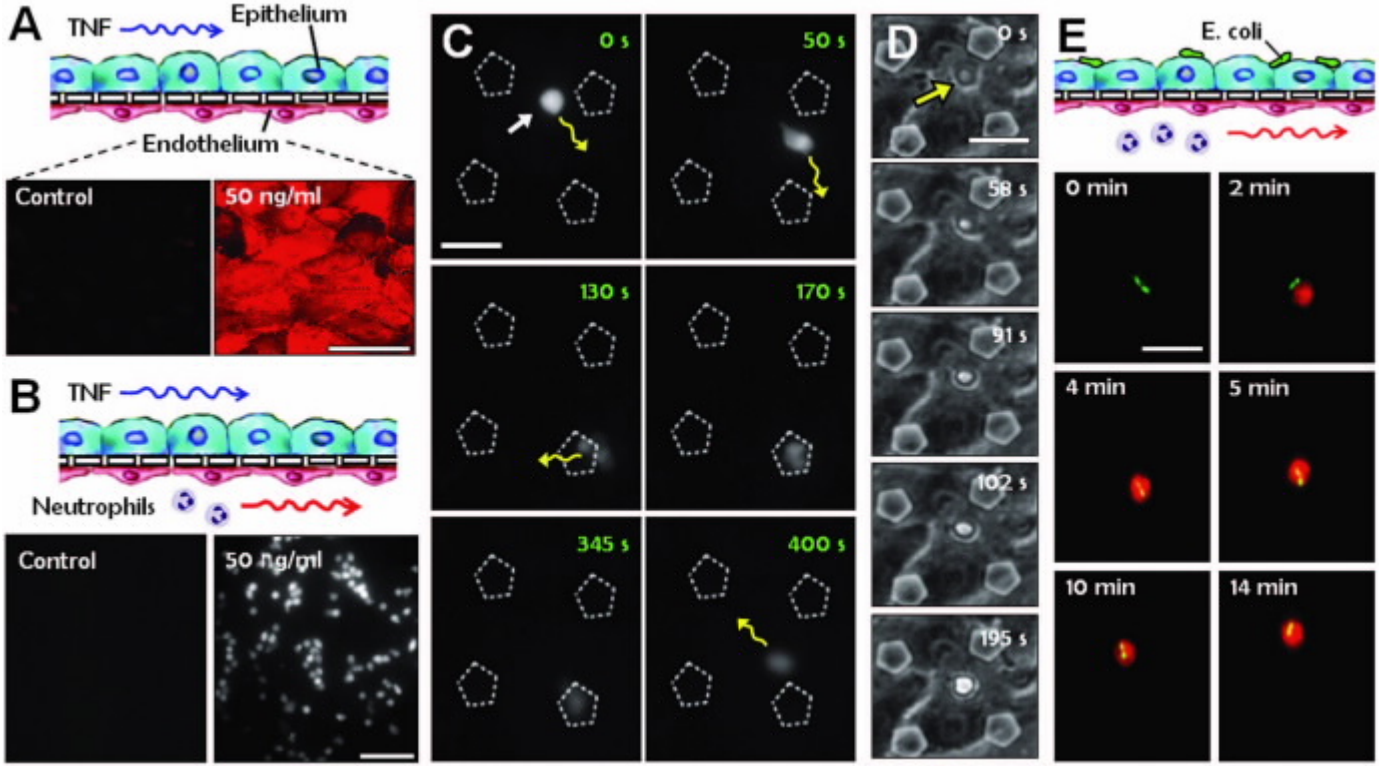


Fig. 3. Reconstitution and direct visualization of complex organ-level responses involved in pulmonary inflammation and infection in the lung-on-a-chip device. (A) Epithelial stimulation with TNFα (50 ng/ml) up-regulates ICAM-1 expression (red) on the endothelium; controls show lack of ICAM-1 expression in the absence of TNFα treatment. Cells were stretched with 10% strain at 0.2 Hz in both cases. (B) Fluorescently labeled human neutrophils (white dots) adhere avidly to the activated endothelium within 1 min after introduction into the vascular channel. (C) Time-lapse microscopic images showing a captured neutrophil (white arrow) that spreads by firm adhesion and then crawls over the apical surface of the activated endothelium (not visible in this view; direction indicated by yellow arrows), until it forces itself through the cell-cell boundary within about 2 min after adhesion (times indicated in seconds). During the following 3 to 4 min, the neutrophil transmigrates through the alveolar-capillary barrier by passing through a pentagonal pore in the PDMS membrane, and then it moves away from the focal plane, causing it to appear blurry in the micrographs. (D) Phase-contrast microscopic images show a neutrophil (arrow) emerging from the apical surface of the alveolar epithelium at the end of its transmigration over a period of ~3 min; thus, complete passage takes approximately 9 min in total. (E) Time-lapse fluorescence microscopic images showing phagocytosis of two GFP-expressing E. coli (green) bacteria on the epithelial surface by a neutrophil (red) that transmigrated from the vascular microchannel to the alveolar compartment. Scale bar, 50 mm in (A) and (B), 20 mm in (C) to (E).

of circulating neutrophils (Fig. 4A) and to promote their transmigration across the tissue-tissue interface and their accumulation on the epithelial surface (fig. S4).

Nanoparticles have been previously shown to induce lung inflammation by stimulating pulmonary epithelial cells to produce proinflammatory cytokines and by causing endothelial cells to express ICAM-1 and recruit circulating leukocytes (31–33, 37). However, our results extend this observation by providing evidence to suggest that breathing motions might greatly accentuate the proinflammatory activities of silica nanoparticles and contribute substantially to the development of acute lung inflammation. This finding also could be relevant for the design of artificial lung motions in positive-pressure lung ventilator devices, which can sometimes induce dangerous inflammatory responses in the clinic. This effect of mechanical stress would never have been detected in conventional culture models based on static cultures, including Transwell culture systems, that fail to incorporate mechanical force regimens.

Next, various nanomaterials were added to the alveolar microchannel, and the cellular oxidative stress response was quantitated by measuring intracellular production of reactive oxygen species (ROS) using microfluorimetry (31). When the ultrafine (12 nm) silica nanoparticles were added to the alveolar epithelium in the absence of mechanical distortion, there was little or no ROS production. However, when the cells were subjected to physiological levels of cyclic strain (10% at 0.2 Hz), the same nanoparticles induced a steady increase in ROS production that increased by a factor of more than 4 within 2 hours (Fig. 4B), and this response could be inhibited with the free radical scavenger *N*-acetylcysteine (NAC) (fig. S5). ROS levels in the underlying endothelium also increased by a factor of almost 3 over 2 hours, but initiation of this response was delayed by about 1 hour compared with the epithelium (fig. S5). Experiments with carboxylated Cd/Se quantum dots (16 nm) produced similar results (fig. S6), whereas cyclic strain alone had no effect on ROS even when applied for 24 hours (fig. S7). Nanomaterial-induced ROS production also increased in direct proportion to the level of applied strain (Fig. 4C). In contrast, exposure of alveolar epithelial cells to 50-nm superparamagnetic iron nanoparticles under the same conditions only exhibited a small transient increase in ROS production (Fig. 4D).

Indeed, this mechanical strain–induced oxidative response appeared to be specific for the silica nanoparticles and carboxylated quantum dots, because it was not induced by treatment with various other nanomaterials, including single-walled carbon nanotubes, gold nanoparticles, polystyrene nanoparticles, or polyethylene glycol–coated quantum dots (fig. S8 and table S1). On the other hand, we found that longer exposure to silica nanoparticles alone for 24 hours induced similar

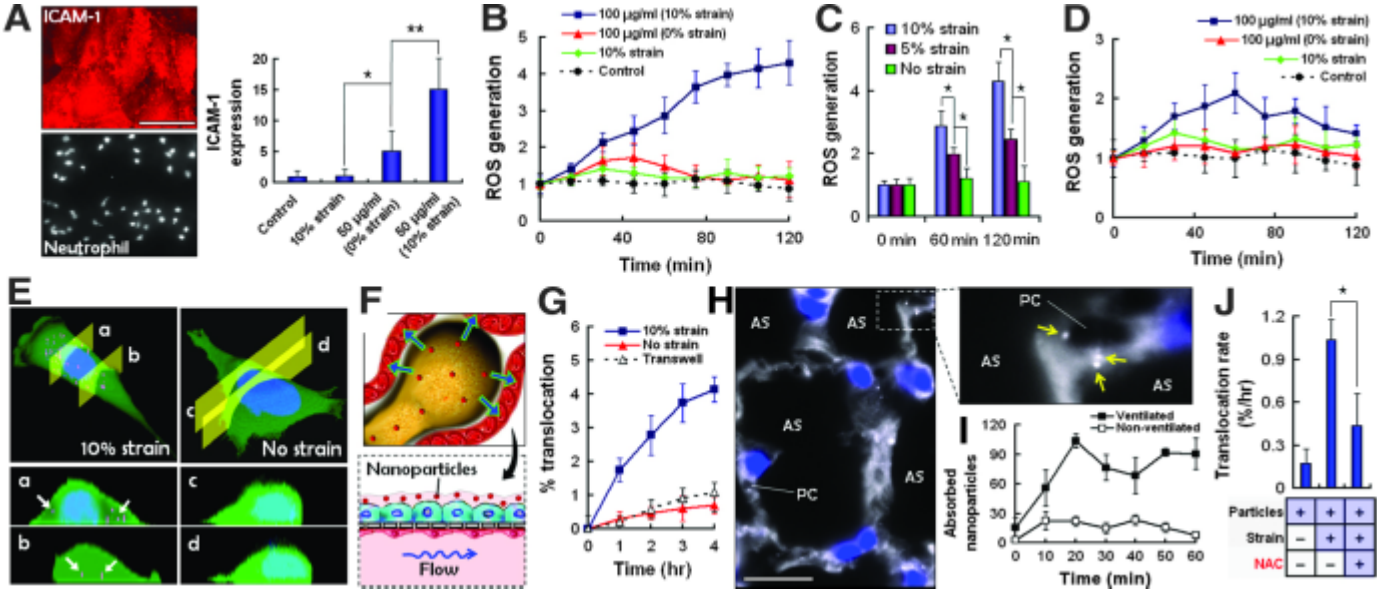


Fig. 4. Microengineered model of pulmonary nanotoxicology. (A) Ultrafine silica nanoparticles introduced through an air-liquid interface overlying the alveolar epithelium induce ICAM-1 expression (red) in the underlying endothelium and adhesion of circulating neutrophils (white dots) in the lower channel. Scale bar, 50 mm. Graph shows that physiological mechanical strain and silica nanoparticles synergistically up-regulated ICAM-1 expression (*$P < 0.005$; **$P < 0.001$). (B) Alveolar epithelial cells increase ROS production when exposed to silica nanoparticles (100 mg/ml) in conjunction with 10% cyclic strain (square) ($P < 0.0005$), whereas nanoparticles (triangle) or strain (diamond) alone had no effect on intracellular ROS levels relative to control cells (circle). ROS generation was normalized to the mean ROS value at time 0. (C) The alveolar epithelium responds to silica nanoparticles in a strain-dependent manner (*$P < 0.001$). (D) Addition of 50-nm superparamagnetic nanoparticles produced only a transient elevation of ROS in the epithelial cells subjected to 10% cyclic strain ($P < 0.0005$). (E) Application of physiological mechanical strain (10%) promotes increased cellular uptake of 100-nm polystyrene nanoparticles (magenta) relative to static cells, as illustrated by representative sections (a to d) through fluorescent confocal images. Internalized nanoparticles are indicated with arrows; green and blue show cytoplasmic and nuclear staining, respectively. (F) Transport of nanomaterials across the alveolar-capillary interface of the lung is simulated by nanoparticle transport from the alveolar chamber to the vascular channel of the lung mimic device. (G) Application of 10% mechanical strain (closed square) significantly increased the rate of nanoparticle translocation across the alveolar-capillary interface compared with static controls in this device (closed triangle) or in a Transwell culture system (open triangle) ($P < 0.0005$). (H) Fluorescence micrographs of a histological section of the whole lung showing 20-nm fluorescent nanoparticles (white dots, indicated with arrows in the inset at upper right that shows the region enclosed by the dashed square at higher magnification) present in the lung after intratracheal injection of nebulized nanoparticles and ex vivo ventilation in the mouse lung model. Nanoparticles cross the alveolar-capillary interface and are found on the surface of the alveolar epithelium, in the interstitial space, and on the capillary endothelium. PC, pulmonary capillary; AS, alveolar space; blue, epithelial nucleus; scale bar, 20 mm. (I) Physiological cyclic breathing generated by mechanical ventilation in whole mouse lung produces an increase by a factor of more than 5-fold in nanoparticle absorption into the blood perfusate when compared to lungs without lung ventilation ($P < 0.0005$). The graph indicates the number of nanoparticles detected in the pulmonary blood perfusate over time, as measured by drying the blood (1 ml) on glass and quantitating the number of particles per unit area (0.5 mm^2). (J) The rate of nanoparticle translocation was significantly reduced by adding NAC to scavenge free radicals (*$P < 0.001$).

high levels of ROS production even in the absence of mechanical strain (fig. S7), confirming findings of past pulmonary nanotoxicology studies that analyzed the effects of ultrafine silica nanoparticles in static cell cultures (34). Taken together, these data suggest that physiological mechanical stresses due to breathing might act in synergy with nanoparticles to exert early toxic effects or accelerate nanoparticle toxicity in the lung.

Mechanical strain enhanced cellular uptake of nanoparticulates in the alveolar epithelium and underlying endothelium. For example, confocal microscopic analysis revealed that the number of fluorescent nanoparticles (100 nm in diameter) or their aggregates present within the intracellular compartment of alveolar epithelial cells was much greater for the first hour after exposure when cells were subjected to physiological breathing movements (Fig. 4E). More than 70% of cells within mechanically active epithelium and endothelium internalized nanoparticles, whereas this fraction was lower by a factor of 10 in the absence of strain (fig. S9).

Modeling nanoparticle transport from alveoli into the lung vasculature. Increasing in vivo evidence suggests that nanomaterials introduced into the alveolar space have the capacity to cross the alveolar-capillary barrier and enter the pulmonary circulation, potentially affecting other organs and causing systemic toxicity (31, 33). When we introduced fluorescent nanoparticles (20 nm in diameter) into the alveolar microchannel and monitored nanoparticle translocation across the alveolar-capillary barrier in air-liquid interface culture by measuring the fraction of particles retrieved from the underlying microvascular channel by continuous fluid flow (Fig. 4F), we observed only a low level of nanoparticle absorption under static conditions. The level was similar to that measured with a static Transwell culture system containing a rigid, porous, ECM-coated membrane lined with opposing layers of alveolar epithelium and capillary endothelium (Fig. 4G). In contrast, there was an increase by more than a factor of 4 in nanoparticle transport into the vascular compartment when our microdevice experienced physiological breathing motions (10% strain at 0.2 Hz) (Fig. 4G). This was striking given that transport of fluorescent albumin remained unchanged under similar loading conditions (fig. S1). Thus, strain-induced increases in nanoparticle absorption across the alveolar-capillary barrier are not due to physical disruption of cell-cell junctions and simple convective transport. Instead, they more likely result from altered transcellular translocation or from a change in paracellular transport that somehow selectively permits passage of these nanoparticulates while restricting movement of small molecules, such as albumin. The presence of an air-liquid interface on the epithelial surface did not appear to produce substantially higher mechanical stresses or change nanoparticle absorption across the alveolar-capillary barrier in our microdevice. An air-liquid interface has been previously shown to exert higher stress on cells (12, 38); however, those studies focused mainly on the deleterious effects of a moving air-liquid interface (e.g., as might occur with a mucus plug), which generates much larger forces that are more relevant to small-airway closure and reopening.

To determine the physiological relevance of these observations, we conducted similar studies in a whole mouse lung ventilation-perfusion model, which enables intratracheal injection of nebulized nanoparticles and monitoring of nanoparticle uptake into the pulmonary vasculature ex vivo (fig. S10). When 20-nm nanoparticles were injected into whole breathing mouse lung, they were delivered into the deep lung, and histological analysis revealed that they reached the surface of the alveolar epithelium, as well as the underlying interstitial space and microvasculature (Fig. 4H). These findings confirm that these nanoparticles are retransported across the alveolar-capillary barrier in vivo, as we observed in vitro. When we monitored the number of absorbed nanoparticles collected from the pulmonary venous circulation after nanoparticle injection into the whole perfused lung, we found that nanoparticle transport from the alveoli into the microvasculature was significantly increased in the presence of cyclic breathing in vivo (Fig. 4I), just as we observed in the lung mimic device in vitro (Fig. 4G). Similar studies carried out with Transwell systems that represent the state of the art for in vitro analysis of tissue barrier permeability only exhibited low levels of nanoparticle translocation (Fig. 4G).

This mechanical force–induced increase in nanoparticle translocation was reduced significantly when the cells were incubated with the antioxidant NAC (Fig. 4J). This finding implies that elevated intracellular production of ROS due to a combination of mechanical strain and nanoparticle exposure might be responsible for the observed increase in barrier permeability to these nanoparticles. We also found that preconditioning of endothelial cells with physiological levels of shear stress (15 dyne/cm^2) slightly increased the rate of nanoparticle translocation in the presence of mechanical stretch (fig. S11), presumably due to a shear-induced increase in endothelial permeability, as previously demonstrated by others (39). Taken together, these results highlight the advantages of our microengineered device over existing in vitro model systems and provide evidence that the inherent mechanical activity of the living lung may contribute substantially to the transport of airborne nanoparticulates from the alveolar space into the bloodstream.

Biomimetic microsystems as replacements for animal testing. Development of cell-based biochips that reproduce complex, integrated organ-level physiological and pathological responses could revolutionize many fields, including toxicology and development of pharmaceuticals and cosmetics that rely on animal testing and clinical trials. This human breathing lung-on-a-chip microdevice provides a proof of principle for this novel biomimetic strategy that is inspired by the integrated chemical, biological, and mechanical structures and functions of the living lung. This versatile system enables direct visualization and quantitative analysis of diverse biological processes of the intact lung organ in ways that have not been possible in traditional cell culture or animal models.

There are still differences between our lung mimic device and the alveolar-capillary barrier in vivo (e.g., barrier thickness, cellular composition, lack of alveolar macrophages, and changes in air pressure and flow), and the transformed lung cells used in our study might not fully reproduce the responses of native alveolar epithelial cells. However, our data clearly demonstrate that this biomimetic microsystem can reconstitute multiple physiological functions observed in the whole breathing lung. Specifically, the lung mimic device reconstitutes the microarchitecture of the alveolar-capillary unit, maintains alveolar epithelial cells at an air-liquid interface, exerts physiologically relevant mechanical forces to the entire structure, and enables analysis of the influence of these forces on various physiological and pathological lung functions, including interactions with immune cells and pathogens, epithelial and endothelial barrier functions, and toxicity and absorption of nanoparticulates across this critical tissue-tissue interface. Existing in vitro mechanical stimulation models fail to offer any of these integrated capabilities, and the fact that our system allows one to study all of these complex physiological phenomena in a single device makes it even more novel. Moreover, this microdevice represents an innovative and low-cost screening platform that could potentially replace in vivo assays or substantially improve the outcome of animal and clinical studies, by enhancing the predictive power of in vitro or in silico computational models.

Microengineering approaches developed in this research also might offer new opportunities to more accurately model critical tissue-tissue interfaces and specialized physical microenvironments found in other organs, such as the gut, kidney, skin, and bone marrow, as well as in human cancers. These biomimetic microsystems are miniaturized (Fig. 1E) and can be easily multiplexed and automated. Hence, with simplified designs and careful choice of biocompatible device materials, they might be useful for high-throughput analysis and screening of cellular responses to drugs, chemicals, particulates, toxins, pathogens, or other environmental stimuli relevant to pharmaceutical, cosmetic, and environmental applications. Furthermore, these microengineering approaches might open the possibility of integrating multiple miniaturized organ model systems into a single device to recapitulate interactions between different organs and enable more realistic in vitro assays of the whole body's response to drugs and toxins (19, 40, 41).

References and Notes

1. J. C. Davila, R. J. Rodriguez, R. B. Melchert, D. Acosta Jr., Annu. Rev. Pharmacol. Toxicol. 38, 63 (1998).

2. F. Pampaloni, E. G. Reynaud, E. H. K. Stelzer, Nat. Rev. Mol. Cell Biol. 8, 839 (2007).
3. D. E. Ingber, FASEB J. 20, 811 (2006).
4. G. M. Whitesides, E. Ostuni, S. Takayama, X. Y. Jiang, D. E. Ingber, Annu. Rev. Biomed. Eng. 3, 335 (2001).
5. A. Khademhosseini, R. Langer, J. Borenstein, J. P. Vacanti, Proc. Natl. Acad. Sci. U.S.A. 103, 2480 (2006).
6. J. El-Ali, P. K. Sorger, K. F. Jensen, Nature 442, 403 (2006).
7. I. Meyvantsson, D. J. Beebe, Annu. Rev. Anal. Chem. 1, 423 (2008).
8. M. Shin et al., Biomed. Microdevices 6, 269 (2004).
9. J. W. Song et al., Anal. Chem. 77, 3993 (2005).
10. M. T. Lam, Y. C. Huang, R. K. Birla, S. Takayama, Biomaterials 30, 1150 (2009).
11. K. Jang, K. Sato, K. Igawa, U. I. Chung, T. Kitamori, Anal. Bioanal. Chem. 390, 825 (2008).
12. D. Huh et al., Proc. Natl. Acad. Sci. U.S.A. 104, 18886 (2007).
13. A. Carraro et al., Biomed. Microdevices 10, 795 (2008).
14. P. J. Lee, P. J. Hung, L. P. Lee, Biotechnol. Bioeng. 97, 1340 (2007).
15. M. J. Powers et al., Biotechnol. Bioeng. 78, 257 (2002).
16. S. R. Khetani, S. N. Bhatia, Nat. Biotechnol. 26, 120 (2008).
17. J. W. Park, B. Vahidi, A. M. Taylor, S. W. Rhee, N. L. Jeon, Nat. Protoc. 1, 2128 (2006).
18. S. G. Harris, M. L. Shuler, Biotechnol. Bioprocess Eng. 8, 246 (2003).
19. G. J. Mahler, M. B. Esch, R. P. Glahn, M. L. Shuler, Biotechnol. Bioeng. 104, 193 (2009).
20. R. Baudoin, L. Griscom, M. Monge, C. Legallais, E. Leclerc, Biotechnol. Prog. 23, 1245 (2007).
21. K. J. Jang, K. Y. Suh, Lab Chip 10, 36 (2010).
22. S. Takayama et al., Adv. Mater. 13, 570 (2001).
23. I. Frerking, A. Günther, W. Seeger, U. Pison, Intensive Care Med. 27, 1699 (2001).
24. K. J. Kim, A. B. Malik, Am. J. Physiol. Lung Cell. Mol. Physiol. 284, L247 (2003).
25. K. G. Birukov et al., Am. J. Physiol. Lung Cell. Mol. Physiol. 285, L785 (2003).
26. T. Iba, B. E. Sumpio, Microvasc. Res. 42, 245 (1991).
27. C. K. Thodeti et al., Circ. Res. 104, 1123 (2009).
28. J. G. Wagner, R. A. Roth, Pharmacol. Rev. 52, 349 (2000).
29. M. I. Hermann et al., Cell Tissue Res. 336, 91 (2009).
30. D. Wong, K. Dorovini-Zis, J. Neuroimmunol. 89, 11 (1992).
31. A. Nel, T. Xia, L. Mädler, N. Li, Science 311, 622 (2006).
32. V. L. Colvin, Nat. Biotechnol. 21, 1166 (2003).
33. G. Oberdörster, E. Oberdörster, J. Oberdörster, Environ. Health Perspect. 113, 823 (2005).
34. W. S. Lin, Y. W. Huang, X. D. Zhou, Y. F. Ma, Toxicol. Appl. Pharmacol. 217, 252 (2006).
35. D. Napierska et al., Small 5, 846 (2009).
36. R. Merget et al., Arch. Toxicol. 75, 625 (2002).
37. N. L. Mills et al., Nat. Clin. Pract. Cardiovasc. Med. 6, 36 (2009).
38. A. M. Bilek, K. C. Dee, D. P. Gaver 3rd, J. Appl. Physiol. 94, 770 (2003).
39. H. W. Sill et al., Am. J. Physiol. 268, H535 (1995).
40. A. Sin et al., Biotechnol. Prog. 20, 338 (2004).
41. C. Zhang, Z. Q. Zhao, N. A. Abdul Rahim, D. van Noort, H. Yu, Lab Chip 9, 3185 (2009).
42. We thank C. K. Thodeti for his help with ROS assays; G. M. Whitesides and P. Cherukuri for providing carbon nanotubes, gold nanoparticles, and helpful comments; N. Korin for helpful discussions; R. Ruch for providing alveolar epithelial cells; R. Mannix for his imaging assistance; and M. Butte for help with neutrophil isolation. This work was supported by grants from NIH (R01-ES016665), the American Heart Association (0835618D), the Department of Defense (W81XWH-05-1-0115), and the Wyss Institute for Biologically Inspired Engineering at Harvard University. D. H. is a recipient of a Wyss Technology Development Fellowship, and D. E. I. is a recipient of a Department of Defense Breast Cancer Innovator Award. A patent on the device described is pending.

Supporting Online Material
www.sciencemag.org/cgi/content/full/328/5986/1662/DC1
Materials and Methods
SOM Text
Figs. S1 to S11
Table S1
References
Movies S1 to S9

16 February 2010; accepted 7 May 2010
10.1126/science.1188302

REPORTS

4D Electron Tomography

Oh-Hoon Kwon and Ahmed H. Zewail*

Electron tomography provides three-dimensional (3D) imaging of noncrystalline and crystalline equilibrium structures, as well as elemental volume composition, of materials and biological specimens, including those of viruses and cells. We report the development of 4D electron tomography by integrating the fourth dimension (time resolution) with the 3D spatial resolution obtained from a complete tilt series of 2D projections of an object. The different time frames of tomograms constitute a movie of the object in motion, thus enabling studies of nonequilibrium structures and transient processes. The method was demonstrated using carbon nanotubes of a bracelet-like ring structure for which 4D tomograms display different modes of motion, such as breathing and wiggling, with resonance frequencies up to 30 megahertz. Applications can now make use of the full space-time range with the nanometer-femtosecond resolution of ultrafast electron tomography.

For nearly a century, the determination of three-dimensional (3D) structures of a crystalline specimen, with redundancy in the repeating units of the architecture, has primarily depended on x-ray and electron-based methods. Recently, with the combination of electron microscopy techniques and fast computation methods, electron tomography has become a powerful tool for 3D structural determination of nanoscopic noncrystalline materials (1, 2) and biological assemblies such as viruses, bacteria, and cells (3–5). Historically, several initial advances were made (5), but the first examples of the approach were published with different variants in the 1960s: reconstruction of 3D biostructures (of high symmetry) from one or more projections (6, 7), reconstruction of an asymmetric protein structure from a sufficient number of projections (8), and average reprojection calculated from a tilt series of images (9). However, decades of advances in computation methods were required to bring the field into the state of the art in high-resolution electron tomography with applications in many fields (10–12), which include the impressive methods of energy-filtering for mapping a specific element or site (13–16) and the use of the high-angular annular dark field (HAADF) for suppressing the diffraction contrast of crystalline materials (17, 18). In all of these studies, the tomograms obtained are those of a static object representing the time-averaged equilibrium state of the structure.

To visualize the dynamics, the dimension of time must be integrated into any electron tomogram that spans a whole tilt series. Furthermore, the time resolution in every step must be high enough to capture the motion of the object, ideally reduced to the atomic scale. This simultaneous real-space and real-time resolution can be obtained using ultrafast electron microscopy (UEM) (11, 19). However, in UEM a snapshot represents a time frame of the 2D projection of the object under investigation, making invisible the spatial information in the dimension along the optical axis of the microscope. This is because of the large focus depth in the specimen.

Here, we report the development of 4D electron tomography. The tomograms are reconstructed from nearly 4000 projections for a whole series of tilt angles and time steps. The methodology is demonstrated in the imaging of multiwalled carbon nanotubes (MWNTs), with the time resolution being independent of the video camera response time of milliseconds, thus enabling the visualization of non-equilibrium, fleeting structures on the femtosecond to millisecond time scale. For the nanotubes, the 4D tomograms in a movie display the mechanical motions and morphological dynamics of the object, a

Physical Biology Center for Ultrafast Science and Technology, Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, CA 91125, USA.

*To whom correspondence should be addressed. E-mail: zewail@caltech.edu

small airway-on-a-chip enables analysis of human lung inflammation and drug responses *in vitro*

Kambez H Benam[1,8], Remi Villenave[1,8], Carolina Lucchesi[1,7], Antonio Varone[1,7], Cedric Hubeau[2], Hyun-Hee Lee[3], Stephen E Alves[3], Michael Salmon[3], Thomas C Ferrante[1], James C Weaver[1,4], Anthony Bahinski[1], Geraldine A Hamilton[1,7] & Donald E Ingber[1,4–6]

here we describe the development of a human lung 'small airway-on-a-chip' containing a differentiated, mucociliary bronchiolar epithelium and an underlying microvascular endothelium that experiences fluid flow, which allows for analysis of organ-level lung pathophysiology *in vitro*. exposure of the epithelium to interleukin-13 (il-13) reconstituted the goblet cell hyperplasia, cytokine hypersecretion and decreased ciliary function of asthmatics. small airway chips lined with epithelial cells from individuals with chronic obstructive pulmonary disease recapitulated features of the disease such as selective cytokine hypersecretion, increased neutrophil recruitment and clinical exacerbation by exposure to viral and bacterial infections. With this robust *in vitro* method for modeling human lung inflammatory disorders, it is possible to detect synergistic effects of lung endothelium and epithelium on cytokine secretion, identify new biomarkers of disease exacerbation and measure responses to anti-inflammatory compounds that inhibit cytokine-induced recruitment of circulating neutrophils under flow.

© 2016 Nature America, Inc. All rights reserved.

The development of new therapeutics for chronic respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD), which pose a huge public health burden[1], has been hindered by the inability to study organ-level complexities of lung inflammation *in vitro*. Although hospitalization and mortality due to these diseases are often consequences of exacerbations triggered by pathogens[2,3], there is currently no way to study these processes in human lung outside of the clinical setting. Animal models of asthma and COPD exist; however, their clinical relevance is questionable because the anatomy, immune system and inflammatory responses of animal lungs differ greatly from those in humans[4–6]. For example, mucin-producing cells, which are central to the development of asthma, are less frequent in the respiratory trees of mice and rats than in those of humans[6]. Neutrophils, which increase in number dramatically in the lungs of patients with COPD and severe asthma[7–9], constitute only 10–25% of circulating leukocytes in mice, whereas they represent 50–70% in humans[5]. Because many animal models do not accurately predict drug activities in humans, the pharmaceutical and biotechnology industries strive to replace or minimize the use of animal models for drug testing whenever possible[10].

Airway inflammatory diseases have been modeled *in vitro* using cultures of primary or immortalized human epithelial cells, sometimes positioned at an air-liquid interface to induce epithelial differentiation[11], or using cocultures of airway epithelium and tissue-resident immune cells (for example, macrophages or dendritic cells)[12]. However, lung inflammation is mediated by organ-level responses involving complex tissue-tissue interactions between the lung airway epithelium and the underlying microvascular endothelium that modulate immune reactions to respiratory pathogens and allergens[13–15] and alter the vascular cell adhesion molecular machinery that recruits circulating immune cells, such as neutrophils. This is important because neutrophil accumulation in the lung is associated with enhanced severity of airflow limitation in people with COPD[7], and it has a critical role in severe asthma as well[8]. Unfortunately, it is not possible to study complex interactions among airway epithelium, endothelium and circulating neutrophils using existing *in vitro* lung models because most fail to recapitulate normal functional coupling between epithelium and endothelium, and none enables analysis of the recruitment of circulating immune cells under active fluid flow. The latter point is crucial because neutrophil adhesion to inflamed endothelium involves initial E-selectin–mediated rolling along the luminal surface of endothelium, which is followed by firm adhesion to ICAM-1 (ref. 16), and this dynamic shear stress–dependent response cannot be studied in a physiologically relevant way using static cell cultures.

Advances in microsystems engineering have recently made it possible to create biomimetic microfluidic cell-culture devices, known as organs-on-chips, that contain continuously perfused microchannels lined by living human cells and which recapitulate the multicellular architectures, tissue-tissue interfaces, physicochemical microenvironments and vascular perfusion of the body[17], potentially offering new opportunities for disease

[1]Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, Massachusetts, USA. [2]Pfizer, Cambridge, Massachusetts, USA. [3]Merck Research Laboratories, Boston, Massachusetts, USA. [4]Harvard John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, Massachusetts, USA. [5]Vascular Biology Program, Boston Children's Hospital, Boston, Massachusetts, USA. [6]Harvard Medical School, Harvard University, Boston, Massachusetts, USA. [7]Present address: Emulate Inc., Cambridge, Massachusetts, USA. [8]These authors contributed equally to this work. Correspondence should be addressed to D.E.I. (donald.ingber@wyss.harvard.edu).

Received 30 July; accepted 21 November; published online 21 December 2015; doi:10.1038/nmeth.3697

modeling and drug-efficacy assessment[18]. We previously used this approach to successfully reconstitute the alveolar-capillary interface of the human lung air sac and associated inflammatory responses *in vitro*[19,20]. Here we adapted this approach to micro engineer a human lung small airway-on-a-chip that supports full differentiation of a columnar, pseudostratified, mucociliary bronchiolar epithelium composed of cells isolated from healthy individuals or people with COPD and underlined by a functional microvascular endothelium.

results

on-chip reconstitution of a functional small airway

To construct the human small airway-on-a-chip, we used soft lithography to create a microfluidic device made of poly(dimethylsiloxane) (PDMS) containing an upper channel with a height and width of 1 mm (similar to the radius of a human bronchiole) separated from a parallel lower microvascular channel (0.2 mm high × 1 mm wide) by a thin (100 μm), porous (0.4 μm pores), polyester membrane coated on both sides with type I collagen (**Fig. 1a**). We cultured primary human airway epithelial cells (hAECs) on top of the membrane until confluent with medium flowing (60 μl h^{-1}) in both channels. To trigger the differentiation of lung airway epithelial cells, we removed the apical medium after 5 d and introduced air to create an air-liquid interface (ALI). We added retinoic acid (1 μg ml^{-1}) to the medium flowing in the lower channel to prevent the development of a squamous phenotype. Three to five weeks later, we seeded primary human lung microvascular endothelial cells on the opposite side of the porous membrane and cultured them at the same flow rate until confluent to create a tissue-tissue interface (**Fig. 1a,b**).

Immunofluorescence confocal microscopic analysis showed that these culture conditions resulted in the formation of a pseudostratified, mucociliary airway epithelium on one side of an extracellular matrix–coated membrane and a planar microvascular endothelium on the opposite side (**Fig. 1c–f**, **Supplementary Fig. 1a** and **Supplementary Videos 1** and **2**). The epithelial cells were linked by a continuous band of ZO1-containing tight junctions along the lateral borders of their apical surfaces (**Fig. 1d**), whereas endothelial cells were joined by adherens junctions containing PECAM-1 (**Fig. 1e**). The epithelium formed from cells isolated from the small airway epithelium of lungs from both healthy humans (**Fig. 1f,g** and **Supplementary Video 2**) and people with COPD (**Supplementary Fig. 1a**) contained many ciliated epithelial cells as well as mucus-producing goblet cells, club cells (**Supplementary Fig. 1b**) and basal cells with proportions strikingly similar to those found in normal human lung (**Table 1**). Immunodetection of tight junctions was accompanied by the formation of a robust epithelial barrier that restricted the passage of fluorescent inulin (4 kDa) and dextrans (10 and 70 kDa) between the parallel channels during analysis

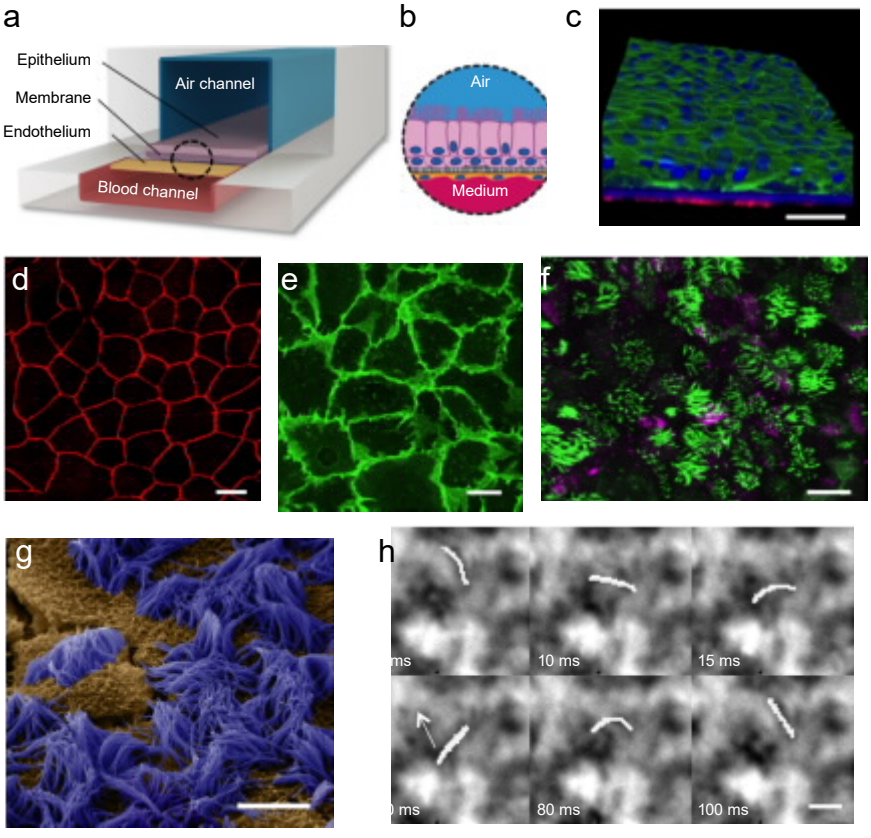


Figure 1 | The human small airway-on-a-chip. (**a**) Schematic diagram of a cross-section through the small airway-on-a-chip. The dashed circle highlights the area depicted in **b**. (**b**) Diagram of the tissue-tissue interface that forms on-chip, showing differentiated airway epithelium (pink cells) cultured on top of a porous collagen-coated membrane at an air-liquid interface in the upper channel and the endothelium below (orange cells) with flowing medium that feeds both tissue layers. (**c**) A 3D reconstruction showing fully differentiated, pseudostratified airway epithelium formed on-chip by cultured hAECs (green, F-actin) with human pulmonary microvascular endothelial cells (red, F-actin) on the opposite side of the membrane. Blue denotes DAPI-stained nuclei; scale bar, 30 μm. (**d**) The differentiated human small airway epithelium exhibited continuous tight junctional connections on-chip, as demonstrated by ZO1 staining (red). Scale bar, 20 μm. (**e**) The human (lung blood microvascular) endothelial monolayer formed on-chip also contained continuous adherens junctions between adjacent cells, as indicated by PECAM-1 staining (green). Scale bar, 20 μm. (**f**) Well-differentiated human airway epithelium formed on-chip using hAECs derived from healthy donors demonstrating the presence of high numbers of ciliated cells labeled for β-tubulin IV (green) and goblet cells stained with anti-MUC5AC (magenta). Scale bar, 20 μm. (**g**) Scanning electron micrograph of cilia (blue) on the apical surface of the differentiated airway epithelium formed on-chip (nonciliated cells are in brown). Scale bar, 10 μm. (**h**) Sequential frames of a video of the apical surface of differentiated epithelium recorded over 100 ms showing cilia beating at a frequency of ~10 Hz. A single cilium is highlighted in white in each frame; time stamps and the white arrow indicate the duration and direction of one forward and return stroke (**Supplementary Video 4** shows the full recording). Scale bar, 5 μm (applies to all images in panel). All images are representative of three to six independent experiments performed on cells from three to six different donors.

© 2016 Nature America, Inc. All rights reserved.

table 1 | Comparison of structure and function between living human airway and human small airway-on-a-chip

	human airway	small airway-on-a-chip
Cell type		
Ciliated cells (%)	~30	~20–30
Goblet cells (%)	~10–15	~10–20
Club cells (%)	~11–44	~25
Basal cells (%)	~6–30	~20
Cilia		
Structure	9 + 2 structure	9 + 2 structure
Length	~6 μm	~6 μm
Beating frequency	9–20 Hz	9–20 Hz
Transport velocity	40–150μm s^{-1}	40–100μm s^{-1}

Human airway cell composition characteristics and properties of pulmonary cilia were obtained from other publications[21,23,48,49].

over 24 h, mimicking the barrier function of living lung mucosa (**Supplementary Fig. 1c**). Electron microscopic analysis of the normal lung small airway-on-a-chip confirmed that the cilia on the apical surface of the polarized epithelium had the same structure (9+2 microtubule organization) and length (~6 μm) (**Supplementary Fig. 1d**) as healthy cilia found in living human lung *in vivo*[21,22]. High-speed microscopic imaging confirmed that these cilia beat actively at a frequency of 9–20 Hz (**Fig. 1h** and **Supplementary Video 3**), which again is nearly the same as that observed in human airways[21,23] (**Table 1**). Moreover, when we measured mucociliary transport by introducing fluorescent polystyrene microbeads into the top channel, we observed rapid coordinated movement of the beads along the surface of the epithelium (**Supplementary Video 4**) as a result of active synchronized cilia beating; again, the particle velocity measured (50–100μm s^{-1}) was nearly identical to that observed in healthy human lung airways (**Table 1**). Thus, the human lung small airway-on-a-chip effectively recapitulated many of the structures and functions of healthy lung bronchioles and sustained them for weeks *in vitro*.

modeling asthma and lung inflammation on-chip

IL-13 has a pivotal role in asthma, as it is necessary and sufficient to induce all features of allergic asthma in animal models of the disease[24]. IL-13 has a direct effect on human airway epithelium[25] and participates in airway inflammation, goblet cell hyperplasia and mucus hypersecretion, as well as subepithelial fibrosis and airway hyper-responsiveness[24]. When we treated the small airway-on-a-chip with IL-13 (100 ng ml^{-1}) for 8 d, we observed a significant increase in the number of goblet cells (**Fig. 2a,b**), in line with other *in vitro* models[26]; higher production and secretion of the inflammatory cytokines G-CSF and GM-CSF into the vascular channel, as measured in the fluid effluent at days 2 and 8, respectively (**Fig. 2c**); and a decrease in cilia beating frequency (**Fig. 2d**) similar to that observed in the airway mucosa of individuals with asthma[27–29].

As mucosal inflammation and its exacerbation by viral infections are major components of both asthma and COPD, and because it has been challenging to recapitulate these clinically relevant responses *in vitro*, we analyzed the effects of exposing the airway epithelium to the viral mimic polyinosinic-polycytidylic acid (poly(I:C)) (10 μg ml^{-1}). Poly(I:C) is an analogue of the double-stranded RNA that is produced in infected cells during viral replication by respiratory viruses[30]. The synthetic nature of our engineered lung airway model enabled us to analyze both independent and collective responses of the lung epithelium and endothelium to poly(I:C) challenge, which is not possible in human subjects. Stimulation with poly(I:C) for 3 h triggered a proinflammatory response in the chips similar to that in human subjects with acute severe asthma exacerbations[8], including large increases in the basal secretion of RANTES, IL-6 and interferon-γ–inducible protein 10 (IP-10) (**Fig. 2e**) that were threefold to sixfold higher when underlying endothelium was present relative to when the endothelium was absent. This effect seemed to be specific for these cytokines in that removal of the endothelium had no effect on the secretion

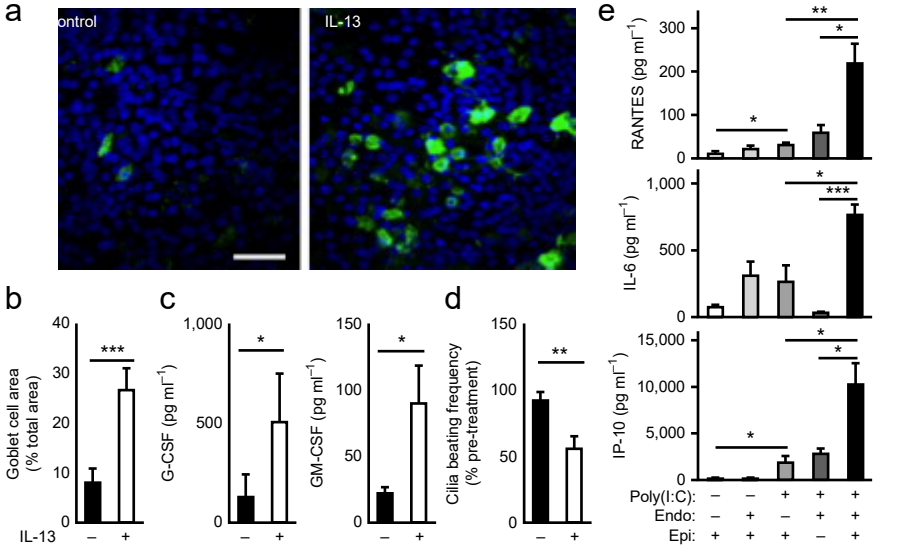


Figure 2 | Modeling asthma and lung inflammation on-chip. (**a**) Immunofluorescence micrographic views of differentiated airway epithelium cultured on-chip for 4–6 weeks at the air-liquid interface in the absence (left) or presence (right) of IL-13 showing epithelium stained for the goblet cell marker MUC5AC (green) and with DAPI (blue). Scale bar, 50 μm (applies to both views); images are representative of three independent experiments performed on cells from three different donors. (**b**–**d**) Total culture area covered by goblet cells (**b**), production of the cytokines G-CSF and GM-CSF (**c**) and cilia beating frequency (**d**) under the conditions described in **a**. Data represent mean and s.e.m. (compared to unstimulated controls) of cells from three healthy donors, with one or two biological replicates per donor; for goblet cell analysis, three to five representative areas per condition were used for quantification; $n = 3–8$. (**e**) Production of the cytokines RANTES, IL-6 and IP-10 by the small airway-on-a-chip with (+) or without (−) a differentiated bronchiolar epithelium (Epi), an umbilical vein endothelium (Endo) or both in the presence or absence of poly(I:C) (10 μg ml^{-1}) stimulation; data represent mean and s.e.m. (compared to unstimulated epithelium or stimulated coculture) of cells from three or four healthy donors, with one biological replicate per donor; $n = 3–4$. Significance determined by unpaired Student's *t*-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

© 2016 Nature America, Inc. All rights reserved.

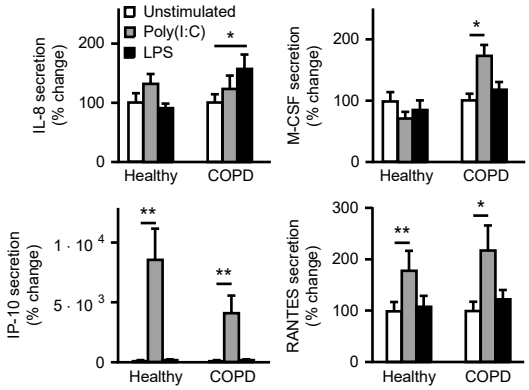


Figure 3 | Modeling COPD exacerbations in the small airway-on-a-chip. The graphs show effects on production of the cytokines IL-8, M-CSF, IP-10 and RANTES by Toll-like receptor stimulation with LPS (10 μg ml^{-1}) or poly(I:C) (10 μg ml^{-1}) in small airway chips lined by 3–5-week-old hAECs obtained from either healthy or COPD subjects (all the COPD subjects had a history of smoking). Note that Toll-like receptor activation by these simulants of bacterial and viral infection significantly induced IL-8 and M-CSF release only in COPD chips, whereas IP-10 and RANTES release increased in both healthy and COPD epithelia (unpaired Student's *t*-test; * $P < 0.05$, ** $P < 0.01$). Data represent mean and s.e.m. (compared to unstimulated controls) of cells from four different healthy and four different COPD donors, with one to three biological replicates (chips) per donor; $n = 5–10$. The variability between biological replicates for a given donor was minimal (s.e.m. <5% of mean).

of GRO-α or IL-8 (**Supplementary Fig. 2a**). We found that this effect was synergistic, as stimulated endothelial cells alone secreted similar or lower levels of cytokines compared with the isolated epithelium (**Fig. 2e**). Notably, although previous studies have investigated airway epithelium homeostasis using cocultures of endothelium and lung basal cells[31,32], inflammatory responses in fully differentiated human small airway epithelium have not been demonstrated previously *in vitro*. In addition, poly(I:C) induced upregulation of E-selectin and VCAM-1 expression in the underlying endothelium (**Supplementary Fig. 2b**); these proteins are crucial for the initial adhesion and rolling of neutrophils under flow in microvessels in inflamed living tissues[33]. In fact, this upregulation resulted in enhanced adhesion, rolling and total recruitment of circulating human neutrophils in the small airway chip when primary human neutrophils were flowed through the lower channel under physiological flow and shear stress (1 dyn cm^{-2}) (**Supplementary Video 5**).

Thus, these findings reveal intrinsic cross-talk between human epithelium and endothelium during poly(I:C)-induced inflammation and corroborate past animal studies suggesting that the pulmonary endothelium contributes significantly to viral-induced inflammation by acting as a central regulator of the cytokine storm[15]. Also, when we compared responses to poly(I:C) stimulation in bronchiolar versus bronchial epithelial cells, we found that the two types of cells displayed nearly identical secretion of inflammatory cytokines (**Supplementary Fig. 2c**).

Modeling COPD exacerbation on-chip

As pathogenic infections are a major cause of COPD exacerbation in patients, we next explored whether the lung small airway-on-a-chip could be used to model these inflammatory responses *in vitro*. We stimulated small airway chips lined by either normal or COPD epithelial cells with the viral mimic poly(I:C) or with lipopolysaccharide endotoxin (LPS), which is a bacterial wall–derived component that stimulates cytokine production and has been widely used to simulate bacterial infection *in vitro*[34]. We analyzed secretion of the cytokine M-CSF because it promotes the differentiation and survival of macrophages, and we studied the production of IL-8 because it attracts neutrophils; these are two major immune cell types that accumulate in the airway tissue of individuals with COPD[35,36]. Stimulation with LPS and poly(I:C) respectively upregulated the secretion of IL-8 and M-CSF in the COPD chips, but these infection mimics did not produce any significant change in chips lined by healthy airway epithelial cells (**Fig. 3**). These results are consistent with findings from past studies showing that bronchial epithelial spheroids from subjects

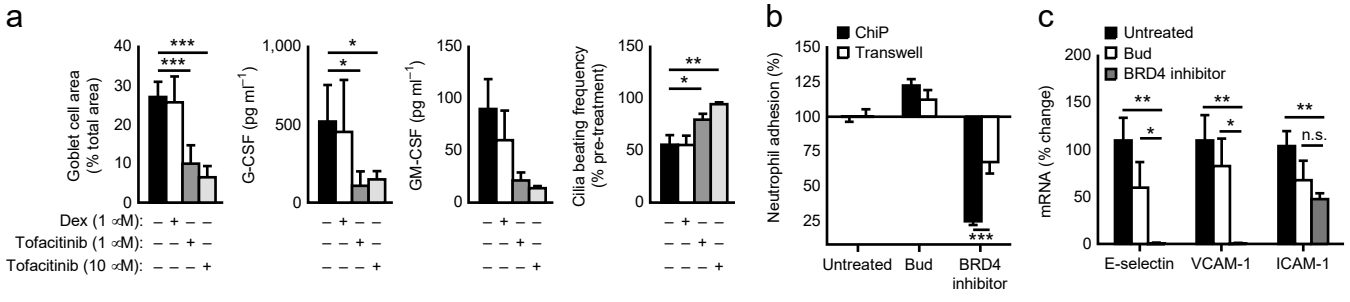


Figure 4 | Pharmacological modulation of IL-13 induced asthmatic phenotype and COPD exacerbation–associated inflammation in the small airway chip. (**a**) Total area covered by goblet cells, effects on production of the cytokines G-CSF and GM-CSF, and cilia beating frequency after exposure of the epithelium to IL-13 with or without dexamethasone (Dex) or tofacitinib for 8 d (skewed cytokine data were log transformed before comparison; data represent mean and s.e.m. (compared to IL-13–treated condition) of cells from three healthy donors, with one or two biological replicates per donor; for goblet cell analysis, three to five representative areas per condition were used for quantification; $n = 3–9$). (**b**) Neutrophil-adhesion results compared with neutrophil-recruitment data obtained when similar studies were carried out in static Transwell cultures. Data represent mean and s.e.m. (chip versus Transwell) of cells from two to four different COPD donors, with one to four biological replicates per donor and three to ten independent fields of view per chip or Transwell; $n = 15–59$. Bud, budesonide; BRD4, bromodomain-containing protein 4. (**c**) Effects of (10 nM) budesonide and (500 nM) BRD4 inhibitor on expression of genes encoding the endothelial cell adhesion molecules E-selectin, VCAM-1 and ICAM-1, compared to the untreated (0.1% DMSO) group, as measured using real-time PCR; human pulmonary blood microvascular cells were studied (n.s., not significant; data represent mean and s.e.m. of cells from one of the donors studied, in three or four biological replicates per condition; $n = 3–4$). Significance determined by unpaired Student's *t*-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n.s., not significant.

© 2016 Nature America, Inc. All rights reserved.

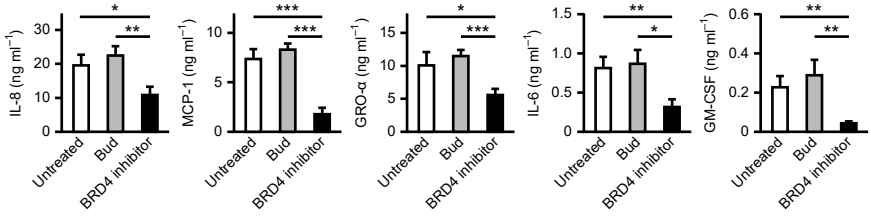


Figure 5 | Therapeutic modulation of inflammatory cytokine and chemokine production in the COPD small airway chip. Analysis of the effects of budesonide (Bud) and BRD4 inhibitor on the secretion of IL-8, MCP-1, GROα, IL-6 and GM-CSF into the microvascular channel of chips lined by COPD epithelium cultured at the air-liquid interface for 3–5 weeks when challenged with poly(I:C) (unpaired Student's *t*-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; data represent mean and s.e.m. (compared to untreated controls) of cells from three different COPD epithelial and two different lung blood microvascular endothelial cell donors, with one to four biological replicates per donor, $n = 6–8$).

with COPD exhibit greater LPS-induced IL-8 release than those from healthy controls[37], and the enhanced levels of M-CSF that we detected might partly explain the accumulation of macrophages observed in the lungs of people with COPD[9]. In contrast, stimulation with poly(I:C) produced large increases in secretion of the cytokines IP-10 and RANTES in both healthy and COPD chips (**Fig. 3**). These results are consistent with the finding that serum IP-10 is an excellent clinical marker for acute viral exacerbations in COPD patients[2]. Our finding that M-CSF levels specifically increased only in poly(I:C)-treated small airway chips (**Fig. 3**) suggests that M-CSF could be a new biomarker for COPD exacerbations induced by respiratory viruses.

evaluation of therapeutic responses in small airway chips

Having established these microfluidic models of human asthmatic and COPD airways, we then asked whether this new *in vitro* method could be useful for drug discovery. As IL-13 signals through the JAK-STAT pathway, we investigated whether tofacitinib, a potent inhibitor of JAK1, -2 and -3 used for patients with rheumatoid arthritis[38], could reverse the IL-13–induced phenotype. When we exposed normal airway epithelium on-chip to IL-13 to produce asthmatic changes in the epithelium and then treated with high doses of tofacitinib (1 and 10 μM) to inhibit JAK signaling, we observed suppressed goblet cell hyperplasia, decreased secretion of G-CSF and GM-CSF, and restoration of normal cilia beating frequency (**Fig. 4a**). In contrast, when similar chips exposed to IL-13 were treated with the corticosteroid dexamethasone, it was found to be ineffective, which is consistent with the clinical finding that although patients with moderate to severe asthma receive dexamethasone inhalation therapy, they often fail to respond to the treatment[39]. These results also validate recent reports suggesting that immunosuppressant JAK inhibitors may represent potential new therapeutics for allergic airway diseases[40].

To test the breadth and robustness of our platform, we also explored whether we could use the human small airway-on-a-chip model of COPD exacerbation by microbial infection to identify pharmacological agents able to suppress the associated inflammation, specifically by inhibiting the recruitment of neutrophils circulating under flow. When we treated COPD epithelial cells that had been stimulated with poly(I:C) on-chip with the glucocorticoid drug budesonide, there was no significant change in neutrophil adhesion (**Fig. 4b**), which again is consistent with the drug's noted lack of activity in many COPD patients[41]. We then tested a new experimental anti-inflammatory drug, 2-methoxy-N-(3-methyl-2-oxo-1,2-dihydroquinolin-6-yl) benzenesulfonamide[42], which is a bromodomain-containing protein 4 (BRD4) inhibitor of NFκB signaling. We chose this drug because it belongs to an emerging class of new anti-inflammatory drugs that were recently shown to alleviate lung inflammation in an LPS-challenged mouse model[43]. When the BRD4 inhibitor was added to the inflamed human small airway chip, it suppressed neutrophil adhesion under flow by almost 75% (**Fig. 4b**). However, when we tested these same drugs on similarly differentiated airway epithelium maintained with an underlying endothelium in static Transwell cultures, we found that the inhibitory effect of the BRD4 inhibitor on neutrophil recruitment was reduced by one-third, whereas the results obtained with budesonide were similar (**Fig. 4b**). This differential response to the BRD4 inhibitor under static versus dynamic flow conditions suggests that its mechanism of action might involve preferential inhibition of early adhesion and rolling responses that are required for initiation of the neutrophil-recruitment cascade because they occur only under dynamic flow conditions[44]. In fact, our studies showed that treatment of inflamed endothelial cells with the BRD4 inhibitor resulted in a more than 50-fold reduction in expression levels of E-selectin and VCAM-1, as well as a 50% reduction in ICAM-1, whereas budesonide treatment did not significantly decrease the expression of either of these vascular cell adhesion molecules (**Fig. 4c**).

Consistent with its ability to suppress neutrophil adhesion, the BRD4 inhibitor also proved greatly superior to the corticosteroid in downregulating expression of the genes encoding IL-8, MCP-1, GRO-α and IL-6 in human lung microvascular endothelial cells (**Supplementary Fig. 3**), as well as suppressing secretion of these cytokines into the lower microvascular channel (**Fig. 5**). These are important findings because these cytokines are key chemoattractants for neutrophils *in vivo*. The BRD4 inhibitor also significantly suppressed the production of GM-CSF (**Fig. 5**), which is a neutrophil chemokine that enhances the functionality of neutrophils[45], in addition to supporting bone marrow cell proliferation and the survival of granulocytic precursors. Although budesonide slightly decreased the expression of selected cytokine genes (**Supplementary Fig. 3**), it did not significantly suppress cytokine secretion (**Fig. 5**), which is the most critical determinant of the inflammatory response; these findings again mimic results from animal models of COPD and lung inflammation[43,46]. Thus, reconstitution of the COPD inflammatory phenotype on-chip not only enabled testing for the efficacy of new experimental therapeutics but also allowed dissection of the mechanism of drug action at the molecular level in a human organ context *in vitro*.

discussion

Although attempts have been made in the past to culture human airway epithelium in microfluidic devices along with endothelium and fibroblasts[47], the small airway-on-a-chip described here achieved greater robustness and fidelity in mimicking the

© 2016 Nature America, Inc. All rights reserved.

structure and functions of the human lung airway, as well as in modeling of pulmonary diseases and recapitulation of lung inflammatory responses *in vitro*. Because the organ-on-chip approach essentially represents 'synthetic biology' at the organ level, we also were able to independently control and vary virtually all system parameters, including the presence or absence of different cell types, vascular flow conditions and soluble factors, while simultaneously analyzing human organ-level responses in real time with molecular-scale resolution. This microengineered lung small airway-on-a-chip model also faithfully recapitulated *in vivo* organ-level responses to therapy, and thus it offers a powerful complement to animal models for both analyzing human pathophysiology and carrying out preclinical drug evaluations (more details can be found in the **Supplementary Discussion**).

methods

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

Acknowledgments

Funding was provided by Pfizer, Merck, Wyss Institute for Biologically Inspired Engineering at Harvard University and the Defense Advanced Research Projects Agency (DARPA) under Cooperative Agreement Number W911NF-12-2-0036. We thank K. Karalis for helpful discussions, and B. Hassell and M. Mazur for technical assistance.

Author contributions

K.H.B., R.V., C.H., H.-H.L., S.E.A., M.S., G.A.H. and D.E.I. designed the research; K.H.B. and R.V. developed the basic small airway chip model; R.V. and A.V. conducted the asthma work; K.H.B. and C.L. conducted the COPD studies; K.H.B. optimized and performed leukocyte-recruitment studies; J.C.W. performed scanning electron microscopy imaging; T.C.F. helped with confocal microscopy imaging; K.H.B. and R.V. prepared the manuscript; G.A.H. and A.B. commented on the manuscript; and D.E.I. critically revised the manuscript.

competing financial interests

The authors declare competing financial interests: details are available in the online version of the paper.

reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. World Health Organization. Top 10 causes of death. http://www.who.int/mediacentre/factsheets/fs310/en/ (2014).
2. Bafadhel, M. *et al.* Acute exacerbations of chronic obstructive pulmonary disease: identification of biologic clusters and their biomarkers. *Am. J. Respir. Crit. Care Med.* **184**, 662–671 (2011).
3. Jackson, D.J., Sykes, A., Mallia, P. & Johnston, S.L. Asthma exacerbations: origin, effect, and prevention. *J. Allergy Clin. Immunol.* **128**, 1165–1174 (2011).
4. Hyde, D.M., Hamid, Q. & Irvin, C.G. Anatomy, pathology, and physiology of the tracheobronchial tree: emphasis on the distal airways. *J. Allergy Clin. Immunol.* **124** (suppl. 6), S72–S77 (2009).
5. Kolaczkowska, E. & Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* **13**, 159–175 (2013).
6. Wright, J.L., Cosio, M. & Churg, A. Animal models of chronic obstructive pulmonary disease. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **295**, L1–L15 (2008).
7. Di Stefano, A. *et al.* Severity of airflow limitation is associated with severity of airway inflammation in smokers. *Am. J. Respir. Crit. Care Med.* **158**, 1277–1285 (1998).
8. Fahy, J.V. Eosinophilic and neutrophilic inflammation in asthma: insights from clinical studies. *Proc. Am. Thorac. Soc.* **6**, 256–259 (2009).
9. Hogg, J.C. *et al.* The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N. Engl. J. Med.* **350**, 2645–2653 (2004).
10. Bueters, T., Ploeger, B.A. & Visser, S.A. The virtue of translational PKPD modeling in drug discovery: selecting the right clinical candidate while sparing animal lives. *Drug Discov. Today* **18**, 853–862 (2013).
11. Fulcher, M.L., Gabriel, S., Burns, K.A., Yankaskas, J.R. & Randell, S.H. Well-differentiated human airway epithelial cell cultures. *Methods Mol. Med.* **107**, 183–206 (2005).
12. Ugonna, K., Bingle, C.D., Plant, K., Wilson, K. & Everard, M.L. Macrophages are required for dendritic cell uptake of respiratory syncytial virus from an infected epithelium. *PLoS ONE* **9**, e91855 (2014).
13. Didierlaurent, A. *et al.* Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. *J. Exp. Med.* **205**, 323–329 (2008).
14. Hammad, H. *et al.* House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* **15**, 410–416 (2009).
15. Teijaro, J.R. *et al.* Endothelial cells are central orchestrators of cytokine amplification during influenza virus infection. *Cell* **146**, 980–991 (2011).
16. Langer, H.F. & Chavakis, T. Leukocyte-endothelial interactions in inflammation. *J. Cell. Mol. Med.* **13**, 1211–1220 (2009).
17. Bhatia, S.N. & Ingber, D.E. Microfluidic organs-on-chips. *Nat. Biotechnol.* **32**, 760–772 (2014).
18. Esch, E.W., Bahinski, A. & Huh, D. Organs-on-chips at the frontiers of drug discovery. *Nat. Rev. Drug Discov.* **14**, 248–260 (2015).
19. Huh, D. *et al.* A human disease model of drug toxicity-induced pulmonary edema in a lung-on-a-chip microdevice. *Sci. Transl. Med.* **4**, 159ra147 (2012).
20. Huh, D. *et al.* Reconstituting organ-level lung functions on a chip. *Science* **328**, 1662–1668 (2010).
21. Wanner, A., Salathe, M. & O'Riordan, T.G. Mucociliary clearance in the airways. *Am. J. Respir. Crit. Care Med.* **154**, 1868–1902 (1996).
22. Fliegauf, M., Benzing, T. & Omran, H. When cilia go bad: cilia defects and ciliopathies. *Nat. Rev. Mol. Cell Biol.* **8**, 880–893 (2007).
23. Livraghi, A. & Randell, S.H. Cystic fibrosis and other respiratory diseases of impaired mucus clearance. *Toxicol. Pathol.* **35**, 116–129 (2007).
24. Wills-Karp, M. Interleukin-13 in asthma pathogenesis. *Immunol. Rev.* **202**, 175–190 (2004).
25. Kuperman, D.A. *et al.* Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat. Med.* **8**, 885–889 (2002).
26. Danahay, H. *et al.* Notch2 is required for inflammatory cytokine-driven goblet cell metaplasia in the lung. *Cell Rep.* **10**, 239–252 (2015).
27. Sousa, A.R., Poston, R.N., Lane, S.J., Nakhosteen, J.A. & Lee, T.H. Detection of GM-CSF in asthmatic bronchial epithelium and decrease by inhaled corticosteroids. *Am. Rev. Respir. Dis.* **147**, 1557–1561 (1993).
28. Ordoñez, C.L. *et al.* Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *Am. J. Respir. Crit. Care Med.* **163**, 517–523 (2001).
29. Thomas, B. *et al.* Ciliary dysfunction and ultrastructural abnormalities are features of severe asthma. *J. Allergy Clin. Immunol.* **126**, 722–729 (2010).
30. Hewson, C.A., Jardine, A., Edwards, M.R., Laza-Stanca, V. & Johnston, S.L. Toll-like receptor 3 is induced by and mediates antiviral activity against rhinovirus infection of human bronchial epithelial cells. *J. Virol.* **79**, 12273–12279 (2005).
31. Curradi, G. *et al.* Airway basal cell vascular endothelial growth factor-mediated cross-talk regulates endothelial cell-dependent growth support of human airway basal cells. *Cell. Mol. Life Sci.* **69**, 2217–2231 (2012).
32. Ding, B.S., Gomi, K., Rafii, S., Crystal, R.G. & Walters, M.S. Endothelial MMP14 is required for endothelial-dependent growth support of human airway basal cells. *J. Cell Sci.* **128**, 2983–2988 (2015).
33. Lawrence, M.B. & Springer, T.A. Neutrophils roll on E-selectin. *J. Immunol.* **151**, 6338–6346 (1993).
34. Mayer, A.K. *et al.* Differential recognition of TLR-dependent microbial ligands in human bronchial epithelial cells. *J. Immunol.* **178**, 3134–3142 (2007).
35. O'Shaughnessy, T.C., Ansari, T.W., Barnes, N.C. & Jeffery, P.K. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. *Am. J. Respir. Crit. Care Med.* **155**, 852–857 (1997).
36. Papi, A. *et al.* Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. *Am. J. Respir. Crit. Care Med.* **173**, 1114–1121 (2006).
37. Deslee, G. *et al.* Bronchial epithelial spheroids: an alternative culture model to investigate epithelium inflammation-mediated COPD. *Respir. Res.* **8**, 86 (2007).
38. Fleischmann, R. *et al.* Placebo-controlled trial of tofacitinib monotherapy in rheumatoid arthritis. *N. Engl. J. Med.* **367**, 495–507 (2012).
39. Bush, A. *et al.* Severe childhood asthma: a common international approach? *Lancet* **372**, 1019–1021 (2008).

© 2016 Nature America, Inc. All rights reserved.

40. Kudlacz, E., Conklyn, M., Andresen, C., Whitney-Pickett, C. & Changelian, P. The JAK-3 inhibitor CP-690550 is a potent anti-inflammatory agent in a murine model of pulmonary eosinophilia. *Eur. J. Pharmacol.* **582**, 154–161 (2008).
41. Ammit, A.J. Glucocorticoid insensitivity as a source of drug targets for respiratory disease. *Curr. Opin. Pharmacol.* **13**, 370–376 (2013).
42. Wu, J. *et al.* Design and chemoproteomic functional characterization of a chemical probe targeted to bromodomains of BET family proteins. *Medchemcomm.* **5**, 1871–1878 (2014).
43. Belkina, A.C., Nikolajczyk, B.S. & Denis, G.V. BET protein function is required for inflammation: Brd2 genetic disruption and BET inhibitor JQ1 impair mouse macrophage inflammatory responses. *J. Immunol.* **190**, 3670–3678 (2013).
44. Abbassi, O., Kishimoto, T.K., McIntire, L.V., Anderson, D.C. & Smith, C.W. E-selectin supports neutrophil rolling in vitro under conditions of flow. *J. Clin. Invest.* **92**, 2719–2730 (1993).
45. Gomez-Cambronero, J., Horn, J., Paul, C.C. & Baumann, M.A. Granulocyte-macrophage colony-stimulating factor is a chemoattractant cytokine for human neutrophils: involvement of the ribosomal p70 S6 kinase signaling pathway. *J. Immunol.* **171**, 6846–6855 (2003).
46. Hubeau, C., Kubera, J.E., Masek-Hammerman, K. & Williams, C.M. Interleukin-6 neutralization alleviates pulmonary inflammation in mice exposed to cigarette smoke and poly(I:C). *Clin. Sci. (Lond.)* **125**, 483–493 (2013).
47. Sellgren, K.L., Butala, E.J., Gilmour, B.P., Randell, S.H. & Grego, S. A biomimetic multicellular model of the airways using primary human cells. *Lab Chip* **14**, 3349–3358 (2014).
48. Boers, J.E., Ambergen, A.W. & Thunnissen, F.B. Number and proliferation of clara cells in normal human airway epithelium. *Am. J. Respir. Crit. Care Med.* **159**, 1585–1591 (1999).
49. Mercer, R.R., Russell, M.L., Roggli, V.L. & Crapo, J.D. Cell number and distribution in human and rat airways. *Am. J. Respir. Cell Mol. Biol.* **10**, 613–624 (1994).

© 2016 Nature America, Inc. All rights reserved.

online methods

Device fabrication. We created the upper and lower layers of the microfluidic device (**Fig. 1a**) by casting PDMS pre-polymer on molds prepared using stereolithography (Fineline, USA). Curing (10:1 PDMS base to curing agent, wt/wt) was carried out overnight at 60 °C to produce devices containing two adjacent parallel microchannels (top channel, 1,000 μm wide × 1,000 μm high; bottom channel, 1,000 μm wide × 200 μm high; length of overlapping channels, 16.7 mm), which were used to form the airway lumen and the microvascular channel, respectively. The channels were separated by a thin (~1 mm) semiporous polyester membrane (0.4 μm pores) that was purchased from Maine Manufacturing (USA). The membrane was laser cut, plasma treated using Plasma Etcher PE-100 (Plasma Etch, USA) with one cycle of 50 watts of oxygen gas (15 standard cm^3 min^{-1}) for 15 s, sandwiched between the carefully aligned top and bottom channels, and treated with ultraviolet light in a UVO-Cleaner (Model 342, Jelight, USA) for 30 min before a collagen coating was applied.

Microfluidic cell culture. Primary hAECs obtained from commercial suppliers (Promocell, Germany; Lonza, USA; and Epithelix, Switzerland) were expanded in 75-cm^2 tissue culture flasks using airway epithelial growth medium (BEBM; catalog no. CC-3171) supplemented with growth factors and/or supplements (BulletKit Supplements; Lonza) until 70–80% confluent. We isolated the lung epithelial cells, on the basis of information provided by the vendors, by dissecting airways from whole lungs or lung biopsies, subjecting them to enzymatic digestion and subsequently culturing them in a selective growth medium. The age and sex of healthy and COPD donors are presented in **Supplementary Table 1**. All of the COPD subjects whose epithelial cells were studied in the experiment described in **Figure 3** had a history of smoking. Cells were randomly checked with a mycoplasma biochemical detection kit (Lonza, USA) and were found to be negative.

Before cell culture, the porous membrane of the device was coated on both sides with rat tail collagen type I (300 μg ml^{-1}; Corning, USA) at 37 °C for 24 h. The hAECs were then trypsinized and seeded onto the collagen-coated porous membrane in the upper channel of the device at a concentration of 2×10^6 to 10×10^6 cells ml^{-1} (2×10^5 to 5×10^5 cells cm^{-2}, or 4×10^4 to 10×10^4 cells per chip) and allowed to attach under static conditions. Three hours later, the cell monolayer was washed with fresh medium, and the cultures were maintained in a submerged state until fully confluent (typically 4–5 d after seeding) under constant flow (60 μl h^{-1}) using an IPC-N series peristaltic pump (Ismatec, Switzerland). The chips were cultured in an incubator containing 5% CO_2 and 16–18% O_2 at 85–95% humidity.

When cultures were confluent, the apical medium was removed and an ALI was generated to trigger differentiation. The hAECs were maintained at the ALI for 3–5 weeks with a constant flow of growth medium in the bottom channel, which was sufficient to support epithelial cell viability and function for weeks in culture. The apical surface of the epithelium was rinsed once weekly with growth medium to remove debris. To control for variability in our studies, we ensured that all cultures reached a similar level of morphological and functional differentiation before we carried out any experiments. In our initial characterization studies, we determined that the primary human epithelial cells formed a stable and terminally differentiated ciliated epithelium within 3–5 weeks after being placed under an ALI, as defined using noninvasive microscopic imaging to confirm the formation of a confluent epithelial layer expressing actively beating surface cilia, and chips that failed to reach this standard by 5 weeks were discarded. We did not observe any notable difference in the differentiation responses of cells from different donors in our studies. Mucociliary transport was visualized using 1-μm-diameter fluorescent microbeads (Life Technologies, USA) diluted in phosphate-buffered saline (PBS), injected in the upper channel of a fully differentiated small airway-on-a-chip and imaged using a high-speed Hamamastu ORCA-Flash 4.0 camera mounted on a Zeiss AxioObserver Z1 microscope. For permeability measurements we flowed inulin (4 kDa)–FITC, dextran (10 kDa)–Cascade blue or dextran (70 kDa)–Texas red diluted in growth medium (100 μg ml^{-1}) at 60 μL h^{-1} in the top channel for 24 h and measured the fluorescence intensity of medium flowing out of the top and bottom channels in three individual small airway chips. The apparent permeability was calculated using the following formula:

$$P_{app} = \frac{J}{A \times \Delta C}$$

where P_{app} is the apparent permeability, J is the molecular flux, A is the total area of diffusion, and ΔC is the average gradient (~1 because of the low flow rate).

Once differentiation of the hAECs was accomplished, human lung microvascular endothelial cells (Lonza; purchased as 'lung blood microvascular' endothelial cells before the supplier switched to the 'lymphatic + vascular' mix) were used for analysis of neutrophil adhesion and rolling, drug-efficacy testing and experiments involving COPD chips. We performed flow cytometric analysis and confirmed that our endothelial cells were >98% pure blood microvascular endothelial cells free of cells expressing lymphatic antigens (lymphatic vessel endothelial receptor 1 and podoplanin). Human umbilical vein endothelial cells (Angioproteomie, USA, or Lonza) were used for synergistic cytokine secretion studies; both types of endothelial cells were seeded onto the lower side of the porous membrane through the bottom channel at a density of 2×10^7 cells ml^{-1} (2×10^5 cells cm^{-2} or 4×10^4 cells per chip). Endothelial cells were seeded in Lonza EBM-2 endothelial cell basal growth medium (catalog no. CC-3156) supplemented with EGM-2MV SingleQuot Kit growth factors and/or supplements under static conditions with the chip oriented upside down to allow cell adhesion to the undersurface of the porous membrane. Two hours later, flowing of growth medium (60 μL h^{-1}) was resumed until cells were fully confluent (usually 3–6 d after seeding). We found no significant difference in the cell densities in the epithelial and endothelial layers when we compared epithelium cultured alone to that cultured in the presence of endothelium.

Immunofluorescence microscopy. For immunofluorescence staining, we washed cells by flowing PBS through the top and bottom channels, fixed them with 4% paraformaldehyde (Electron Microscopy Sciences, USA) for 15 min without flow, and washed them gently by flowing additional PBS before storing them at 4 °C until use. The cells cultured on-chip

© 2016 Nature America, Inc. All rights reserved.

doi:10.1038/nmeth.3697

were washed again with PBS, permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 2 h and exposed to blocking buffer composed of PBS containing 1% BSA (Sigma) and 5% FBS (Life Technologies, USA) for 30 min at room temperature. Cultures were stained for ciliated cells (anti–β-tubulin IV, 1:100, clone ONS.1A6; Genetex, Taiwan), goblet cells (anti-Mucin5AC, clone H-160, Santa Cruz Biotechnology, USA), basal cells (anti-Cytokeratin 5, Abcam, USA), tight junctions (anti-ZO1, clone 1A12; Life Technologies, USA) or PECAM-1 (clone WM-59; eBioscience, USA). When cell-adhesion molecules were stained on endothelial cells, the cells were not permeabilized to visualize surface antigens. In all studies, antibodies diluted in blocking buffer were introduced in the channels and incubated for 1 h at room temperature or overnight at 4 °C, and cultures were washed with PBS three times each for 5 min at room temperature. Secondary antibodies (Life Technologies, USA) were incubated for 45 min at room temperature and washed three times with PBS. In some studies, TO-PRO-3 (Life Technologies, USA) was used to label nuclei after secondary antibody staining. Devices were then cut using a razor blade, and membranes were delicately lifted from the PDMS and mounted on microscopy slides in mounting medium (Vectashield; Vector Laboratories) with or without DAPI. Fluorescence imaging was carried out using confocal laser-scanning microscopy (SP5 X MP DMI-6000, Germany). Image processing and three-dimensional *Z*-stack reconstruction were done using Imaris (Bitplane, Switzerland) and ImageJ software (downloaded from http://imagej.nih.gov/ij/). When two primary antibodies of the same species were used, the Zenon antibody labeling kit (Life Technologies, USA) was used to preconjugate the antibodies separately before addition to fixed cells. For IL-13 experiments, cultures were treated with IL-13 (Peprotech, USA), dexamethasone (Sigma-Aldrich, USA) or tofacitinib; fixed after 8 d of treatment; and stained for MUC5AC and DAPI. Tofacitinib was purchased commercially (CP-690550, Selleckchem, USA) and was not obtained from Pfizer, and the doses were optimized in-house. We quantified goblet cell hyperplasia by measuring the percentage of the projected area covered by goblet cells in three to five different fields for each condition.

Scanning and transmission electron microscopy. For electron microscopic analysis, the porous membranes supporting the cell cultures were excised from the devices with a razor blade, and cells were fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences, USA) in 1% sodium cacodylate (Sigma) for 1 h at room temperature. Fixed cells were rinsed with 1% sodium cacodylate and post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences, USA) in 1% sodium cacodylate for 90 min in a fume hood. Cells were dehydrated sequentially in ethanol gradients; rinsed in hexamethyldisilazane; air-dried overnight in a desiccator at room temperature; and then mounted on a conductive carbon support, coated with gold and imaged with a VEGA II scanning electron microscope (Tescan, Czech Republic).

For transmission electron microscopy, fixed cells were embedded in Taab 812 resin (Marivac Ltd., Nova Scotia, Canada) and incubated at 60 °C for 1 d. Samples were cut in 80-nm sections with a Leica ultracut microtome, picked up on 300-mesh formvar-carbon–coated copper grids, stained with 0.2% lead citrate, and viewed and imaged under a Philips Technai BioTwin Spirit electron microscope.

Analysis of ciliated, goblet and basal cells and of mucociliary transport. Cilia beating frequency was measured using a high-speed Hamamatsu ORCA-Flash 4.0 camera mounted on a Zeiss AxioObserver Z1 microscope. The epithelium was washed with warm PBS before analysis to avoid mucus accumulation and slowing of cilia beating. High-speed videos of beating cilia were recorded at approximately 200 frames per second and played at 10 frames per second for 1 s to allow manual counting. Four to five random areas of each chip were counted, and cilia-beating frequencies were averaged for each condition. For IL-13 treatment experiments, cilia beating frequency was recorded and measured after 8 d of treatment ($n = 3$–4 chips). Epithelial cell types were quantified as previously described[50]. Briefly, for ciliated cells and basal cells, fully differentiated epithelia from three small airway chips were trypsinized, centrifuged (1,000 *g*, 2 min), air-dried for 1 h at room temperature, fixed with 100% ice-cold acetone, rinsed with PBS, stained for β-tubulin IV (ciliated cell marker) or Cytokeratin 5 (basal cell marker) and counterstained with DAPI. Ciliated cells and basal cells were counted in ten fields per chip, and percentages were calculated. For goblet cells, fully differentiated epithelia from four chips were fixed with 4% paraformaldehyde and stained for MUC5AC (goblet cell marker). We quantified goblet cells by measuring the percentage of the projected area covered by MUC5AC staining in five different fields for each condition. We evaluated mucociliary transport by measuring the displacement of 1-μm polystyrene fluorescent beads diluted in PBS and introduced in the top channel of the small airway chip for 1 s as shown in **Supplementary Video 4**

Gene expression analysis. Total RNA was extracted from differentiated epithelial or endothelial cells *in situ* on-chip with the RNeasy mini kit (Qiagen, USA). The RNA was incubated with DNase I (Qiagen, USA) for 15 min at room temperature to remove residual contaminating genomic DNA, enzyme heat-inactivated at 65 °C for 5 min, and then reverse transcribed into cDNA using the SuperScript Reverse Transcriptase III kit (Invitrogen, USA). We primed cDNA synthesis by mixing 1 μl of oligodT (50 μM) and 1 μl of dNTP mix (10 mM) with up to 500 ng total RNA. Real-time PCR was carried out using a CFX-96 real-time PCR system (Bio-Rad, USA). Reactions contained 1 μl of cDNA, 10 μl of 2× universal SYBR Green Supermix (Bio-Rad, USA), 3 μl of each forward and reverse primer (300 nM final concentration), and 2 μl of molecular biology-grade water, and results were quantified using the $2^{-\Delta\Delta Ct}$ method[51]. Primers were either designed using the online Primer3 application (http://frodo.wi.mit.edu/primer3/) or used from previous reports[52], and sequences are presented in **Supplementary Table 2**

Analysis of chemokines and cytokines. The effluent of flowing medium was analyzed for a panel of cytokines and chemokines (IL-8, IP-10, RANTES, IL-6, M-CSF, G-CSF, GM-CSF and GRO) using custom Milliplex assay kits (Millipore, USA). Analyte concentrations were determined according to the manufacturer's instructions using a LuminexFlexMap 3D system coupled with Luminex XPONENT software (Luminex, USA). For endothelium depletion experiments, basal secretions were collected for each condition, and amounts of RANTES, IL-6 and IP-10 were measured at 24 h after poly(I:C) (InvivoGen, USA) treatment. For Toll-like receptor stimulation experiments, COPD and healthy

© 2016 Nature America, Inc. All rights reserved.

epithelia were challenged with LPS (10 μg ml^{-1}) or poly(I:C) (10 μg ml^{-1}) for 1 h, and amounts of secreted IL-8, M-CSF, IP-10 and RANTES were measured 24 h after treatment. For COPD drug studies, in chips containing cocultures of differentiated COPD epithelium and microvascular pulmonary endothelium, the endothelial cells were treated with 10 nM budesonide (Sigma), 500 nM BRD4 inhibitor (provided by Pfizer) or 0.1% DMSO diluent (Sigma) under flow (60 μL h^{-1}) through the vascular channel for 24 h before poly(I:C) (10 μg ml^{-1}) was delivered into the airway channel for 6 h. The vascular effluents were then collected for cytokine and chemokine analysis.

Analysis of neutrophil adhesion. Neutrophils were isolated from fresh human blood by two-step gradient sedimentation. The peripheral blood mononuclear cells were removed from the polymorphonuclear cell population using Ficoll (Stem Cell Technologies, Canada) density centrifugation, and then the polymorphonuclear cells were isolated using a modified Percoll (Sigma) protocol[53]. Flow cytometry analysis for CD15 and CD16 expression confirmed that the purity of the neutrophil population was >93%. Isolated neutrophils were then live-stained for 30 min at 37 °C using CellTracker red or Hoechst (Life Technologies, USA), resuspended in RPMI medium containing 10% FBS (vol/vol) (Life Technologies, USA) and used within 3 h for recruitment and adhesion assays in chips. The chips were flipped upside down, and neutrophils (1 × 10^{7} cells ml^{-1}) were flowed (2.7 ml h^{-1}; 1 dyn cm^{-2}) through the microvascular channel of the device to mimic the physiological hemodynamic conditions in human postcapillary venules[33]. After 10 min, unbound neutrophils were washed away by flowing cell-free RPMI–10% FBS medium for 5 min, and micrographs of four or five random areas were taken for subsequent counting; we carried out quantification by counting attached neutrophils using ImageJ (http://imagej.nih.gov/ij/) and CellProfiler (http://www.cellprofiler.org) software. For experiments using Transwells, the inserts were placed upside down, and 50 μl of neutrophil suspension (1 × 10^{7} cells ml^{-1}) was added gently to endothelium on the undersurface of each insert and incubated for 10 min at 37 °C. The inserts were then reverted to the upright position in cell culture medium for 5 min to remove unbound neutrophils and subsequently imaged and analyzed as chips. Micrographs of three to ten random areas were taken to count adherent neutrophils.

Statistical analysis. All results and error bars are presented as mean and s.e.m. Data were analyzed with an unpaired Student's *t*-test using Graphpad Prism (GraphPad Software Inc., San Diego, CA, USA) or Excel software (Microsoft, USA). Differences between groups were considered statistically significant when $P < 0.05$. Variability between biological replicates (for example, in **Fig. 3**) for a given donor was minimal (s.e.m. < 5% of mean).

50. Villenave, R. *et al.* In vitro modeling of respiratory syncytial virus infection of pediatric bronchial epithelium, the primary target of infection in vivo. *Proc. Natl. Acad. Sci. USA* **109**, 5040–5045 (2012).
51. Livak, K.J. & Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402–408 (2001).
52. Benam, K.H., Kok, W.L., McMichael, A.J. & Ho, L.P. Alternative spliced CD1d transcripts in human bronchial epithelial cells. *PLoS ONE* **6**, e22726 (2011).
53. Kitagawa, S., Takaku, F. & Sakamoto, S. Evidence that proteases are involved in superoxide production by human polymorphonuclear leukocytes and monocytes. *J. Clin. Invest.* **65**, 74–81 (1980).

© 2016 Nature America, Inc. All rights reserved.

doi:10.1038/nmeth.3697

JCI The Journal of Clinical Investigation

The convalescent sera option for containing COVID-19

Arturo Casadevall, Liise-anne Pirofski

J Clin Invest. 2020. https://doi.org/10.1172/JCI138003.

Viewpoint

As of early 2020, humanity is confronting a pandemic in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 causes coronavirus disease, abbreviated as COVID-19. At the time of this writing, SARS-CoV-2 is spreading in multiple countries, threatening a pandemic that will affect billions of people. This virus appears to be a new human pathogen. Currently there are no vaccines, monoclonal antibodies (mAbs), or drugs available for SARS-CoV-2, although many are in rapid development and some may be available in a short time. This Viewpoint argues that human convalescent serum is an option for prevention and treatment of COVID-19 disease that could be rapidly available when there are sufficient numbers of people who have recovered and can donate immunoglobulin-containing serum. Passive antibody therapy Passive antibody therapy involves the administration of antibodies against a given agent to a susceptible individual for the purpose of preventing or treating an infectious disease due to that agent. In contrast, active vaccination requires the induction of an immune response that takes time to develop and varies depending on the vaccine recipient. Thus, passive antibody administration is the only means of providing immediate immunity to susceptible persons. Passive antibody therapy has a storied history going back to the 1890s and was the only means of treating certain infectious diseases prior to the development of antimicrobial therapy in […]

Find the latest version:

https://jci.me/138003/pdf

The convalescent sera option for containing COVID-19

Arturo Casadevall[1] and Liise-anne Pirofski[2]

[1]Department of Molecular Microbiology and Immunology, Johns Hopkins School of Public Health, Baltimore, Maryland, USA. [2]Division of Infectious Diseases, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York, USA.

As of early 2020, humanity is confronting a pandemic in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 causes coronavirus disease, abbreviated as COVID-19. At the time of this writing, SARS-CoV-2 is spreading in multiple countries, threatening a pandemic that will affect billions of people. This virus appears to be a new human pathogen. Currently there are no vaccines, monoclonal antibodies (mAbs), or drugs available for SARS-CoV-2, although many are in rapid development and some may be available in a short time. This Viewpoint argues that human convalescent serum is an option for prevention and treatment of COVID-19 disease that could be rapidly available when there are sufficient numbers of people who have recovered and can donate immunoglobulin-containing serum.

Passive antibody therapy

Passive antibody therapy involves the administration of antibodies against a given agent to a susceptible individual for the purpose of preventing or treating an infectious disease due to that agent. In contrast, active vaccination requires the induction of an immune response that takes time to develop and varies depending on the vaccine recipient. Thus, passive antibody administration is the only means of providing immediate immunity to susceptible persons. Passive antibody therapy has a storied history going back to the 1890s and was the only means of treating certain infectious diseases prior to the development of antimicrobial therapy in the 1940s (1, 2). Experience from prior outbreaks with other coronaviruses, such as SARS-CoV-1, shows that such convalescent sera contain neutralizing antibodies to the relevant virus (3).

In the case of SARS-CoV-2, the anticipated mechanism of action by which passive antibody therapy would mediate protection is viral neutralization. However, other mechanisms may be possible, such as antibody-dependent cellular cytotoxicity and/or phagocytosis. Possible sources of antibody for SARS-CoV-2 are human convalescent sera from individuals who have recovered from COVID-19, mAbs, or preparations generated in certain animal hosts, such as genetically engineered cows that produce human antibody (4). Although many types of preparations are or will soon be under development, the only antibody type that is currently available for immediate use is that found in human convalescent sera (Figure 1). As more individuals contract COVID-19 and recover, the number of potential donors will continue to increase.

A general principle of passive antibody therapy is that it is more effective when used for prophylaxis than for treatment of disease. When used for therapy, antibody is most effective when administered shortly after the onset of symptoms. The reason for temporal variation in efficacy is not well understood but could reflect that passive antibody works by neutralizing the initial inoculum, which is likely to be much smaller than that of established disease (5). Another explanation is that antibody works by modifying the inflammatory response, which is also more easily achieved during the initial immune response, a stage that may be asymptomatic (6). As an example, passive antibody therapy for pneumococcal pneumonia was most effective when administered shortly after the onset of symptoms, and there was no benefit if antibody administration was delayed past the third day of disease (7).

For passive antibody therapy to be effective, a sufficient amount of antibody must be administered. When given to a susceptible person, this antibody will circulate in the blood, reach tissues, and provide protection against infection. Depending on the antibody amount and composition, the protection conferred by the transferred immunoglobulin can last from weeks to months.

Historical precedents

In the early twentieth century convalescent sera was used to stem outbreaks of viral diseases such as poliomyelitis (8), measles (9, 10), mumps (11), and influenza (12). A retrospective meta-analysis of eight studies on the use of convalescent sera involving 1703 patients during the 1918 H1N1 influenza virus pandemic suggested that those who received serum had lower mortality (13). Although the efficacy of convalescent sera varied with the virus and the study, there was consensus at the time that this intervention was useful, and it was used in numerous outbreaks. It is noteworthy that historically, convalescent sera were developed and used in many cases without the means to measure antibody titers or knowledge about viral serotypes, and in clinical studies that did not meet modern criteria for randomization or blinding.

More recently, convalescent serum was used during viral epidemics. In the 2009–2010 H1N1 influenza virus pandemic, convalescent serum antibody preparations obtained by apheresis were used to treat individuals with severe H1N1 2009 infection requiring intensive care (14). Serum-treated individuals manifested reduced respiratory viral burden, serum cytokine responses, and mortality (14). Convalescent serum was also used in the 2013 West African Ebola epidemic. A small nonrandomized study in Sierra Leone revealed significantly longer survival for those treated with convalescent whole blood relative to those who received standard treatment (15). Two patients transferred to the United States and treated with a combination of convalescent serum and an experimental drug also survived (16). There is anecdotal evidence from the H5N1 (17, 18) and H7N9 (19) avian flu out-

Conflict of interest: The authors have declared that no conflict of interest exists.
Copyright: © 2020, American Society for Clinical Investigation.
Reference information: *J Clin Invest*. https://doi.org/10.1172/JCI138003.

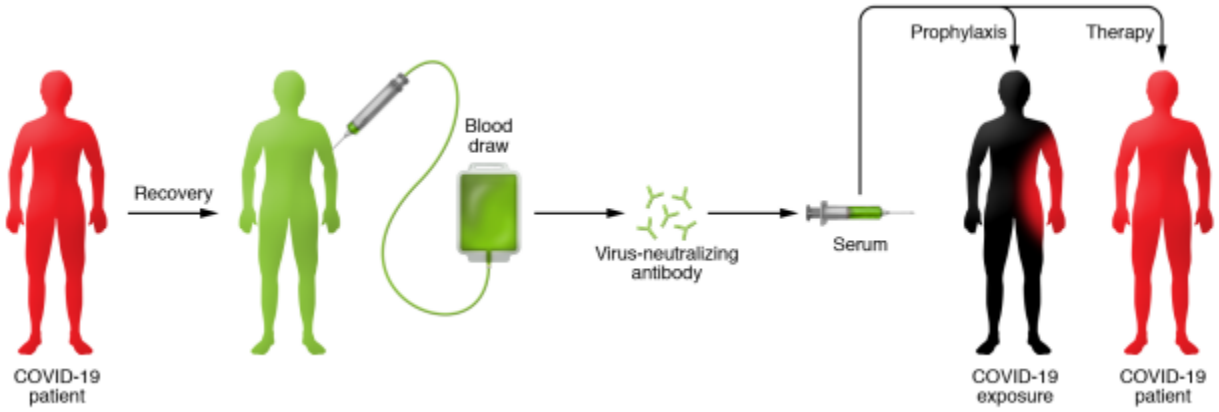


Figure 1. Schematic of the use of convalescent sera for COVID-19. An individual who is sick with COVID-19 and recovers has blood drawn and screened virus-neutralizing antibodies. Following identification of those with high titers of neutralizing antibody, serum containing these virus-neutralizing antibodies can be administered in a prophylactic manner to prevent infection in high-risk cases, such as vulnerable individuals with underlying medical conditions, health care providers, and individuals with exposure to confirmed cases of COVID-19. Additionally, convalescent serum could potentially be used in individuals with clinical disease to reduce symptoms and mortality. The efficacy of these approaches is not known, but historical experience suggests that convalescent sera may be more effective in preventing disease than in the treatment of established disease.

breaks that use of convalescent sera was effective, with all patients surviving.

Although every viral disease and epidemic is different, these experiences provide important historical precedents that are both reassuring and useful as humanity now confronts the COVID-19 epidemic.

Experience with the use of convalescent sera against coronavirus diseases

In the twenty-first century, there have been two other epidemics with coronaviruses that were associated with high mortality, SARS in 2003 and Middle East respiratory syndrome (MERS) in 2012. The SARS epidemic was contained, but MERS became endemic in the Middle East and triggered a secondary major outbreak in South Korea. In both outbreaks, the high mortality and absence of effective therapies led to the use of convalescent serum. The largest study involved the treatment of 80 patients with SARS in Hong Kong (20). Patients treated before day 14 had improved prognosis defined by discharge from hospital before day 22, consistent with the notion that earlier administration is more likely to be effective (20). In addition, those who were PCR positive and seronegative for coronavirus at the time of therapy had improved prognosis (20). There is also some anecdotal information on the use of convalescent serum in seriously ill individuals. Three patients with SARS in Taiwan were treated with 500 mL convalescent serum, resulting in a reduction in serum virus titer, and each survived (21). Three patients with MERS in South Korea were treated with convalescent serum, but only two of the recipients had neutralizing antibody in their serum (22). The latter study highlights a challenge in using convalescent sera, namely, that some who recover from viral disease may not have high titers of neutralizing antibody (23). Consistent with this point, an analysis of 99 samples of convalescent sera from patients with SARS showed that 87 had neutralizing antibody, with a geometric mean titer of 1:61(3). This suggests that antibody declines with time and/or that few patients make high-titer responses. It is also possible that non-neutralizing antibodies are produced that contribute to protection and recovery, as described for other viral diseases (24–26). There are reports that convalescent serum was used for therapy of patients with COVID-19 in China during the current outbreak (27). Although few details are available from the epidemic in China and published studies involved small numbers of patients, the available information suggests that convalescent serum administration reduced viral load and was safe.

Risks and benefits

COVID-19 convalescent sera can be used for either prophylaxis of infection or treatment of disease. In a prophylactic mode, the benefit of convalescent serum administration is that it can prevent infection and subsequent disease in those who are at high risk for disease, such as vulnerable individuals with underlying medical conditions, health care providers, and those with exposure to confirmed cases of COVID-19. Passive antibody administration to prevent disease is already used in clinical practice. For example, patients exposed to hepatitis B and rabies viruses are treated with hepatitis B immune globulin (HBIG) and human rabies immune globulin (HRIG), respectively. In addition, passive antibody is used for the prevention of severe respiratory syncytial virus (RSV) disease in high-risk infants. Until recently, a polyclonal hyperimmune globulin (RSV-IG) prepared from samples of donors with high serum titers of RSV neutralizing antibody was used, but these preparations have now been replaced by palivizumab, a humanized murine mAb. Used therapeutically, convalescent serum would be administered to those with clinical disease in an effort to reduce their symptoms and mortality. The efficacy of these approaches cannot be inferred without carrying out a controlled clinical trial. Based on the historical experience with antibody administration, it can be anticipated that antibody administration would be more effective in preventing disease than in the treatment of established disease (12).

Risks of passive administration of convalescent sera fall into two categories, known and theoretical. Known risks are

those associated with transfer of blood substances, which include inadvertent infection with another infectious disease agent and reactions to serum constituents, including immunological reactions such as serum sickness. With modern blood banking techniques that screen for blood-borne pathogens and match the blood type of donors and recipients, the risks of inadvertently transferring known infectious agents or triggering transfusion reactions are low. However, convalescent sera used in a therapeutic mode would likely be administered to individuals with pulmonary disease, in whom plasma infusion carries some risk for transfusion-related acute lung injury (TRALI) (28), and this should be a consideration in the risk-benefit assessment. The theoretical risk involves the phenomenon of antibody-dependent enhancement of infection (ADE). ADE can occur in several viral diseases and involves an enhancement of disease in the presence of certain antibodies. For coronaviruses, several mechanisms for ADE have been described, and there is the theoretical concern that antibodies to one type of coronavirus could enhance infection to another viral strain (29). It may be possible to predict the risk of ADE of SARS-CoV-2 experimentally, as proposed for MERS (29). Since the proposed use of convalescent sera in the COVID-19 epidemic would rely on preparations with high titers of neutralizing antibody against the same virus, SARS2-CoV-2, ADE may be unlikely. The available evidence from the use of convalescent sera in patients with SARS1 and MERS (30), and anecdotal evidence from its use in 245 patients with COVID-19 (27), suggest it is safe. Nevertheless, in convalescent serum trials, caution and vigilance to identify any evidence of enhanced infection will be required.

Another theoretical risk is that antibody administration to those exposed to SARS-CoV-2 may prevent disease in a manner that attenuates the immune response, leaving such individuals vulnerable to subsequent reinfection. In this regard, passive antibody administration before vaccination with respiratory syncytial virus was reported to attenuate humoral but not cellular immunity (31). This concern could be investigated as part of a clinical trial by measuring immune responses in those exposed and treated with convalescent sera to prevent disease. If the risk proved real, these individuals could be vaccinated against COVID-19 when a vaccine becomes available.

Given that historical and current anecdotal data on use of convalescent serum suggest it is safe in coronavirus infection, the high mortality of COVID-19, particularly in elderly and vulnerable persons, suggests that the benefits of its use in those at high risk for or with early disease outweigh the risks. However, for all cases where convalescent serum administration is considered, a risk-benefit assessment must be conducted to assess individual variables. These considerations were invoked recently with the decision to use mAbs in the treatment of Ebola virus disease (32)

Deployment and proposed use

To deploy convalescent serum administration for COVID-19 the following six conditions must be met: (i) availability of a population of donors who have recovered from the disease and can donate convalescent serum; (ii) blood banking facilities to process the serum donations; (iii) availability of assays, including serological assays, to detect SARS-CoV-2 in serum and virological assays to measure viral neutralization; (iv) virology laboratory support to perform these assays; (v) prophylaxis and therapeutic protocols, which should ideally include randomized clinical trials to assess the efficacy of any intervention and measure immune responses; and (vi) regulatory compliance, including institutional review board approval, which may vary depending on location. Ideally, the use of convalescent serum would involve multiple centers, follow randomized control protocols, and have a single center as a governing body. Each of these conditions should be available in developed areas affected by COVID-19. At least one pharmaceutical company, Takeda, is gearing up to generate antibody preparations against SARS2-CoV-2 from COVID-19 convalescent sera (33). Producing highly purified preparations containing a high titer of neutralizing antibodies against SARS2-CoV-2 is preferable to convalescent sera given that these are safer and have higher activity. Unfortunately, such preparations will not be available for many months, whereas locally produced convalescent sera could be available much sooner.

We anticipate that once the necessary regulatory permissions are in place, individuals who recover from COVID-19 can be approached to donate blood for serum preparation or antibody isolation through apheresis. Recovery from COVID-19 will be assessed clinically, and such individuals must be shown to free of SARS-CoV-2, including in their blood by appropriate viral nucleic acid screening. Donated blood products will be screened for infectious agents according to current blood banking practices, and individual sera will be studied for specific antibody content and neutralizing activity to SARS-CoV-2. Depending on the volumes needed and the neutralizing activity of donated convalescent sera, these could be pooled or used individually, and preparations for clinical use would be treated for pathogen attenuation. At this time, we do not know what an effective neutralizing titer would be in a susceptible individual given passive antibody therapy for prophylaxis, and determining this parameter would be part of the study design. Similarly, we do not know what doses would be effective therapeutically. We do know that when convalescent serum was used to prevent measles or mumps the amounts used were in the order of 10–40 cc (10, 11) In contrast, when convalescent serum was used to treat severe disease in soldiers with 1918 influenza, the amounts given were in the hundreds of milliliters (34). These older studies claimed efficacy even though convalescent serum was given without any knowledge of neutralizing titers. Those experiences suggest that even small amounts of antibody may prevent and/or treat infection. Hence, we can anticipate that effective prophylactic doses would be much smaller than therapeutic doses. This makes sense, since the infecting inoculum is likely to be much smaller than the viral burden during severe disease.

COVID-19 convalescent sera could be used to treat individuals with early symptoms and prevent disease in those exposed. Today, nurses, physicians, and first responders exposed to known cases of COVID-19, some of whom have developed disease, are being quarantined, which threatens to collapse the health care system. It is anticipated that convalescent serum will prevent SARS-CoV-2 infection in those to whom it is administered. If this is established, indi-

viduals who receive convalescent sera may be able to avoid a period of quarantine. This could allow them to continue their critical function as health care providers. Convalescent sera could also be used to prevent disease among family members caring for COVID-19 patients at home. Clearly, the use of convalescent serum would be a stopgap measure that could be used in the midst of the current epidemic. However, even local deployment will entail considerable coordination between different entities, such as infectious disease specialists, hematologists, blood banking specialists, and hospital administrators. Hence, as we are in the midst of a worldwide pandemic, we recommend that institutions consider the emergency use of convalescent sera and begin preparations as soon as possible. Time is of the essence.

Acknowledgments

We are grateful to Shmuel Sholam for helpful comments and suggestions. AC was supported in part by NIH grants HL059842, AI052733 and AI152078. LP was supported in part by NIH grants 6R01AG045044-07, AI123654, and AI143453.

Address correspondence to: Arturo Casadevall, 615 N. Wolfe Street, Room E5132, Baltimore, Maryland 21205, USA. Phone: 410.955.3457; Email: acasadevall@jhu.edu

1. Casadevall A, Scharff MD. Return to the past: the case for antibody-based therapies in infectious diseases. *Clin Infect Dis*. 1995;21(1):150–161.
2. Casadevall A, Dadachova E, Pirofski LA. Passive antibody therapy for infectious diseases. *Nat Rev Microbiol*. 2004;2(9):695–703.
3. Zhang JS, et al. A serological survey on neutralizing antibody titer of SARS convalescent sera. *J Med Virol*. 2005;77(2):147–150.
4. Beigel JH, et al. Safety and tolerability of a novel, polyclonal human anti-MERS coronavirus antibody produced from transchromosomic cattle: a phase 1 randomised, double-blind, single-dose-escalation study. *Lancet Infect Dis*. 2018;18(4):410–418.
5. Robbins JB, Schneerson R, Szu SC. Perspective: hypothesis: serum IgG antibody is sufficient to confer protection against infectious diseases by inactivating the inoculum. *J Infect Dis*. 1995;171(6):1387–1398.
6. Casadevall A, Pirofski LA. Antibody-mediated regulation of cellular immunity and the inflammatory response. *Trends Immunol*. 2003;24(9):474–478.
7. Casadevall A, Scharff MD. Serum therapy revisted: animal models of infection and development of passive antibody therapy. *Antimicrob Agents Chemother* 1994; 38(8):1695–1702.
8. Park WH. Therapeutic use of antipoliomyelitis serum in preparalytic cases of poliomyelitis. *JAMA*. 1932;99:1050–1053.
9. Park WH, Freeman RG. The prophylactic use of measles convalescent serum. *JAMA*. 1926;87(8):556–558.
10. Gallagher JR. Use of convalescent measles serum to control measles in a preparatory school. *Am J Public Health Nations Health*. 1935;25(5):595–598.
11. Rambar AC. Mumps; use of convalescent serum in the treatment and prophylaxis of orchitis. *Am J Dis Child*. 1946;71:1–13.
12. Luke TC, Casadevall A, Watowich SJ, Hoffman SL, Beigel JH, Burgess TH. Hark back: passive immunotherapy for influenza and other serious infections. *Crit Care Med*. 2010;38(4 suppl):e66–e73.
13. Luke TC, Kilbane EM, Jackson JL, Hoffman SL. Meta-analysis: convalescent blood products for Spanish influenza pneumonia: a future H5N1 treatment? *Ann Intern Med*. 2006;145(8):599–609.
14. Hung IF, et al. Convalescent plasma treatment reduced mortality in patients with severe pandemic influenza A (H1N1) 2009 virus infection. *Clin Infect Dis*. 2011;52(4):447–456.
15. Sahr F, et al. Evaluation of convalescent whole blood for treating Ebola virus disease in Freetown, Sierra Leone. *J Infect*. 2017;74(3):302–309.
16. Kraft CS, et al. The Use of TKM-100802 and convalescent plasma in 2 patients with Ebola virus disease in the United States. *Clin Infect Dis*. 2015;61(4):496–502.
17. Kong LK, Zhou BP. Successful treatment of avian influenza with convalescent plasma. *Hong Kong Med J*. 2006;12(6):489.
18. Zhou B, Zhong N, Guan Y. Treatment with convalescent plasma for influenza A (H5N1) infection. *N Engl J Med*. 2007;357(14):1450–1451.
19. Wu XX, Gao HN, Wu HB, Peng XM, Ou HL, Li LJ. Successful treatment of avian-origin influenza A (H7N9) infection using convalescent plasma. *Int J Infect Dis*. 2015;41:3–5.
20. Cheng Y, et al. Use of convalescent plasma therapy in SARS patients in Hong Kong. *Eur J Clin Microbiol Infect Dis*. 2005;24(1):44–46.
21. Yeh KM, et al. Experience of using convalescent plasma for severe acute respiratory syndrome among healthcare workers in a Taiwan hospital. *J Antimicrob Chemother* 2005;56(5):919–922.
22. Ko JH, et al. Challenges of convalescent plasma infusion therapy in Middle East respiratory coronavirus infection: a single centre experience. *Antivir Ther (Lond)*. 2018;23(7):617–622.
23. Arabi YM, et al. Feasibility of using convalescent plasma immunotherapy for MERS-CoV infection, Saudi Arabia. *Emerging Infect Dis*. 2016;22(9):1554–1561.
24. van Erp EA, Luytjes W, Ferwerda G, van Kasteren PB. Fc-mediated antibody effector functions during respiratory syncytial virus infection and disease. *Front Immunol*. 2019;10:548.
25. Jenks JA, Goodwin ML, Permar SR. The roles of host and viral antibody fc receptors in herpes simplex virus (HSV) and human cytomegalovirus (HCMV) infections and immunity. *Front Immunol*. 2019;10:2110.
26. Gunn BM, et al. A role for Fc function in therapeutic monoclonal antibody-mediated protection against ebola virus. *Cell Host Microbe*. 2018;24(2):221–233.e5.
27. China puts 245 COVID-19 patients on convalescent plasma therapy. News release. Xinhua. February 28, 2020. Accessed March 10, 2020. http://www.xinhuanet.com/english/2020-02/28/c_138828177.htm.
28. Gajic O, et al. Transfusion-related acute lung injury in the critically ill: prospective nested case-control study. *Am J Respir Crit Care Med*. 2007;176(9):886–891.
29. Wan Y, et al. Molecular mechanism for antibody-dependent enhancement of coronavirus entry. *J Virol*. 2020;94(5):e02015-19.
30. Mair-Jenkins J, et al. The effectiveness of convalescent plasma and hyperimmune immunoglobulin for the treatment of severe acute respiratory infections of viral etiology: a systematic review and exploratory meta-analysis. *J Infect Dis*. 2015;211(1):80–90.
31. Crowe JE, Firestone CY, Murphy BR. Passively acquired antibodies suppress humoral but not cell-mediated immunity in mice immunized with live attenuated respiratory syncytial virus vaccines. *J Immunol*. 2001;167(7):3910–3918.
32. Mulangu S, et al. A randomized, controlled trial of Ebola virus disease therapeutics. *N Engl J Med*. 2019;381(24):2293–2303.
33. Hopkins JS. Drugmaker Takeda Is Working on Coronavirus Drug. *Wall Street Journal*. https://www.wsj.com/articles/drugmaker-takeda-is-working-on-coronavirus-drug-11583301660. Published March 4, 2020. Accessed March 10, 2020.
34. McGuire LW, Redden WR. The use of convalescent human serum in influenza pneumonia-a preliminary report. *Am J Public Health (N Y)*. 1918;8(10):741–744.

MATERIAL TRANSFER AGREEMENT
[THE AGREEMENT IS NOT FOR USE WITH HUMAN MATERIALS]

MTA ID: MO-12665
Date: 02/21/2020

Provider:
The University of North Carolina at Chapel Hill
Office of Technology Development
100 Europa Drive, Suite 430
Chapel Hill, NC 27517-4105
Provider Scientist: Dr. Ralph Baric

Coordinator:
Wuhan Institute of Virology
44 Xiao Hong Shan
Wuhan, Hubei 430071
China
Coordinator scientist: Dr. Zheng-Li Shi

Recipient: Sun Yat-sen University, 132 Wai Huan Dong Road, Guangzhou, China

Recipient Scientist: Xiaojun Xia

Material: transgenic mice expressing human angiotensin converting enzyme 2 from lung cell specific promotors
Purpose: Pathogenesis study of bat SARS-like coronavirus.
Fee: $0

In response to the Recipient's request for the Material the Provider asks that the Recipient agree to the following before the Recipient receives the Material:

1. The above referenced Material (and any progeny, unmodified derivatives, including any Material incorporated in substances/in modifications created by Recipient) is the property of the Provider and is made available as a service to the research community.
2. THIS MATERIAL IS NOT FOR USE IN HUMAN SUBJECTS.
3. The Material will be used for teaching or not-for-profit research purposes only.
4. The Material will not be further distributed to others without the Provider's written consent. The Recipient shall refer any request for the Material to the Provider.
5. The Recipient agrees to acknowledge the source of the Material in any publications reporting use of it. The Recipient agrees to acknowledge the provider scientist and coordinator scientist as the coauthors in the first publication.
6. Any Material delivered pursuant to this Agreement is understood to be experimental in nature and may have hazardous properties. THE PROVIDER MAKES NO REPRESENTATIONS AND EXTENDS NO WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED. THERE ARE NO EXPRESS OR IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR

RECIPIENT INFORMATION and AUTHORIZED SIGNATURE

Authorized Signature
Name: Xiaojun Xia
Title: Investigator

Acknowledged

Recipient Scientist

MATERIAL TRANSFER AGREEMENT
[THE AGREEMENT IS NOT FOR USE WITH HUMAN MATERIALS]

MTA ID: MO-12665
Date: 02/21/2020

Provider:
The University of North Carolina at Chapel Hill
Office of Technology Development
100 Europa Drive, Suite 430
Chapel Hill, NC 27517-4105
Provider Scientist: Dr. Ralph Baric

Coordinator:
Wuhan Institute of Virology
44 Xiao Hong Shan
Wuhan, Hubei 430071
China
Coordinator scientist: Dr. Zheng-Li Shi

Recipient: Sun Yat-sen University, 132 Wai Huan Dong Road, Guangzhou, China

Recipient Scientist: Xiaojun Xia

Material: transgenic mice expressing human angiotensin converting enzyme 2 from lung cell specific promotors
Purpose: Pathogenesis study of bat SARS-like coronavirus.
Fee: $0

In response to the Recipient's request for the Material the Provider asks that the Recipient agree to the following before the Recipient receives the Material:

1. The above referenced Material (and any progeny, unmodified derivatives, including any Material incorporated in substances/in modifications created by Recipient) is the property of the Provider and is made available as a service to the research community.
2. THIS MATERIAL IS NOT FOR USE IN HUMAN SUBJECTS.
3. The Material will be used for teaching or not-for-profit research purposes only.
4. The Material will not be further distributed to others without the Provider's written consent. The Recipient shall refer any request for the Material to the Provider.
5. The Recipient agrees to acknowledge the source of the Material in any publications reporting use of it. The Recipient agrees to acknowledge the provider scientist and coordinator scientist as the coauthors in the first publication.
6. Any Material delivered pursuant to this Agreement is understood to be experimental in nature and may have hazardous properties. THE PROVIDER MAKES NO REPRESENTATIONS AND EXTENDS NO WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED. THERE ARE NO EXPRESS OR IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR

RECIPIENT INFORMATION and AUTHORIZED SIGNATURE

Authorized Signature
Name: Xiaojun Xia
Title: Investigator

Acknowledged

Recipient Scientist

Please wait...

If this message is not eventually replaced by the proper contents of the document, your PDF viewer may not be able to display this type of document.

You can upgrade to the latest version of Adobe Reader for Windows®, Mac, or Linux® by visiting http://www.adobe.com/go/reader_download.

For more assistance with Adobe Reader visit http://www.adobe.com/go/acrreader.

Windows is either a registered trademark or a trademark of Microsoft Corporation in the United States and/or other countries. Mac is a trademark of Apple Inc., registered in the United States and other countries. Linux is the registered trademark of Linus Torvalds in the U.S. and other countries.

Donna M. Ambrosino, MD

EDUCATION AND TRAINING:

1970 - 1974	AB, Harvard University, Cambridge, Massachusetts
1974 - 1977	MD, Dartmouth Medical School, Hanover, New Hampshire

Postdoctoral Training
Internship and Residencies:

1977 - 1978	Intern in Pediatrics, Dartmouth-Hitchcock Medical Center, Hanover, New Hampshire
1978 - 1979	Resident in Pediatrics, Dartmouth-Hitchcock Medical Center, Hanover, New Hampshire

Clinical and Research Fellowships:

1979 - 1980	Clinical Fellow in Infectious Diseases, Dana-Farber Cancer Institute, Beth Israel Hospital, Children's Hospital Medical Center, Boston, Massachusetts
1980 - 1982	Research Fellow in Infectious Diseases, Dana-Farber Cancer Institute, Children's Hospital Medical Center, Boston, Massachusetts

WORK EXPERIENCE:

2019-Present	Co-founder ClearSite Antibodies LLC
2016- 2019	CEO, Nosocomial Vaccine Company, Rockville MD
2014 - 2019	Chief Medical Officer, ClearPath Vaccines Company, Rockville, MD
2012 - 2014	Chief Medical Officer, Visterra Inc, Cambridge MA
1998 - 2011	CEO, MassBiologics, Professor of Pediatrics, University of Massachusetts Medical School
1992	Associate Professor in Pediatrics, Children's Hospital and Dana- Farber Cancer Center, Harvard Medical School
1986	Assistant Professor in Pediatrics, Children's Hospital and Dana- Farber Cancer Center, Harvard Medical School
1982	Instructor in Pediatrics, Children's Hospital and Dana-Farber Cancer Center, Harvard Medical School

LICENSURE AND CERTIFICATION:

1982 - 2017	Massachusetts License Registration No. 47141
1981	American Board of Pediatrics

AWARDS AND HONORS:

1974	Summa Cum Laude, Biology, Harvard University
1974	Phi Beta Kappa, Harvard University
1974	Class Officer and Marshall, Harvard University
1977	M.D. Honors, Dartmouth Medical School, Hanover, New Hampshire
1977	Head Marshall Medical School Class, Dartmouth Medical School, Hanover, New Hampshire
1983	Young Investigator Award, Infectious Disease Society of America
1992	Elected to Harvard Medical School Faculty Council
1995	Nominated as Outstanding Physician Mentor, Harvard Medical School
2006	Manuel Carballo Governor's Award for Excellence in Public Service

PATENTS

US7396914: SARS nucleic acids, proteins, antibodies, and uses thereof. Inventors: **Ambrosino, D.**, Hernandez, D., Greenough, T., Luzuriaga, K., Somasundaran, M., Babcock, G., Thomas, W. and Sullivan, J. Issued: 07/08/2008.
US7625559: Antibodies against *Clostridium difficile* toxins and uses thereof. Inventors: **Ambrosino, D.**, Babcock, G., Broering, T., Graziano, R., Hernandez, H., Lowy, I., Mandell, R., Molrine, D., Thomas, W. and Zhang, H. Issued: 12/01/2009.
US7727532: Antibodies against rabies virus and uses thereof. Inventors: Thomas, W., **Ambrosino, D.**, Mandell, R., Sloan, S., Babcock, G. and Rupprecht, C. Issued 06/01/2010.
US8226952: Antibodies against rabies virus and issues thereof. Inventors: Thomas, W., **Ambrosino, D.**, Mandell, R., Sloan, S., Babcock, G. and Rupprecht, C. Issued 07/24/2012.
US8236311: Antibodies against *Clostridium difficile* toxins and uses thereof. Inventors: **Ambrosino, D.**, Babcock, G., Broering, T., Graziano, R., Hernandez, H., Lowy, I., Mandell, R., Molrine, D., Thomas, W. and Zhang, H. Issued: 08/07/2012.
US8257709: Antibodies against *Clostridium difficile* toxins and uses thereof. Inventors: **Ambrosino, D.**, Babcock, G., Broering, T., Graziano, R., Hernandez, H., Lowy, I., Mandell, R., Molrine, D., Thomas, W. and Zhang, H. Issued: 09/04/2012.
US2008/001922: Antibodies against hepatitis C virus and uses thereof. Inventors: Broering, T., Sloan, S., Babcock, G., **Ambrosino, D.** and Thomas, W.
US2011/057699: Anti-SOD1 antibodies and uses thereof. Inventors: **Ambrosino, D.**, Babcock, G., Broering, T. and Xu, Z.

BIBLIOGRAPHY:

Original Reports

1. Daum R, Glode M, Goldmann D, Halsey N, Ambrosino D, Welborn C, Mather F, Willard J, Sullivan B, Murray M, Johansen T. Rifampin chemoprophylaxis for household contacts of patients with invasive infections due to Haemophilus influenzae type b. J Pediatr 1981;98:485-491.
2. Siber G, Ambrosino D, Gorgone B. Blood group A-like substance in pneumococcal vaccine. Ann Intern Med 1982;96:580-586.
3. Ambrosino D, Schreiber JR, Daum RS, Siber GR. Efficacy of human hyperimmune globulin in prevention of Haemophilus influenzae type b disease in infant rats. Infect Immun 1983;39:709-714.
4. Glode MP, Daum RS, Halsey NA, Johansen TL, Goldmann DA, Ambrosino D, Boies E, Granoff DM. Rifampin alone and in combination with trimethoprim in chemoprophylaxis for infections due to Haemophilus influenzae type b. Rev Infect Dis 1983;5:S549-S555.
5. Daum RS, Glode MP, Ambrosino D, Halsey N, Goldmann DA, Mather FJ, Russell R, Kamon J, Murray M, Band J, Johansen T. Trimethoprim and rifampin in combination for chemoprophylaxis of household contacts of patients with invasive infections due to Haemophilus influenzae type b. Antimicrob Agents Chemother 1983;24:658-662.
6. Siber GR, Ambrosino DM, McIver J, Ervin TJ, Schiffman G, Sallan S, Grady GF. Preparation of human hyperimmune globulin to Haemophilus influenzae type b, Streptococcus pneumoniae, and Neisseria meningitidis. Infect Immun 1984;45:248-254.
7. Ambrosino DM, Glode MP, Williams CL, Siber GR. Antibody response of infants to Haemophilus influenzae type b capsular polysaccharide combined with Bordetella pertussis. J Infect Dis 1985;151:1174.
8. Ambrosino DM, Schiffman G, Gotschlich EC, Schur PH, Rosenberg GA, DeLange GG, van Loghem E, Siber GR. Correlation between G2m(n) immunoglobulin allotype and human antibody response and susceptibility to polysaccharide encapsulated bacteria. J Clin Invest 1985;75:1935-1942.
9. Umetsu DT, Ambrosino DM, Quinti I, Siber GR, Geha RS. Recurrent sinopulmonary infection and impaired antibody response to bacterial capsular polysaccharide antigen in children with selective IgG-subclass deficiency. N Engl J Med 1985;313:1247-1251.
10. Ambrosino DM, Landesman SH, Gorham CC, and Siber GR. Passive immunization against disease due to Haemophilus influenzae type b: concentrations of antibody to capsular polysaccharide in high-risk children. J Infect Dis 1986;153:1-7.
11. Ambrosino DM, Barrus VA, De Lange GG, Siber GR. Correlation of the Km(1) immunoglobulin allotype with anti-polysaccharide antibody in Caucasian adults. J Clin Invest 1986;78:361-365.
12. Ambrosino DM, Siber GR. Simultaneous administration of vaccines for Haemophilus influenzae type b, pneumococci, and meningococci. J Infect Dis 1986;154:893-96.

13. Umetsu DT, Ambrosino DM, Geha RS. Children with selective IgG subclass deficiency and recurrent sinopulmonary infection: impaired response to bacterial capsular polysaccharide antigens. Monogr Allergy 1986;20:57-61.
14. Anderson AB, Ambrosino DM, Siber GR. Haemophilus influenzae type b unsuspected bacteremia. Pediatr Emerg Care 1987;3:82-85.
15. Ambrosino DM, Siber GR, Chilmonczyk BA, Jernberg JB, Finberg RW. An immunodeficiency characterized by impaired antibody responses to polysaccharides. N Engl J Med 1987;316:790-793.
16. Santosham M, Reid R, Ambrosino DM, Wolff MC, Almeido-Hill J, Priehs C, Aspery KM, Garrett S, Croll L, Foster S, Burge G, Page P, Zacher B, Moxon R, Siber GR. Prevention of Haemophilus influenzae type b infections in high-risk infants treated with bacterial polysaccharide immune globulin. N Engl J Med 1987;317:923-929.
17. Shurin PA, Giebink GS, Wegman DL, Ambrosino D, Rholl J, Overman M, Bauer T, Siber GR. Prevention of pneumococcal otitis media in chinchillas with human bacterial polysaccharide immune globulin. J Clin Microbiol 1988;26:755-759.
18. Leung DY, Ambrosino DM, Arbeit RD, Newton JL, Geha RS. Impaired antibody responses in the hyperimmunoglobulin E syndrome. J Allergy Clin Immunol 1988;81:1082-1087.
19. Ambrosino DM, Umetsu DT, Siber GR, Howie G, Goularte TA, Michaels R, Martin P, Schur PH, Noyes J, Schiffman G, Geha RS. Selective defect in the antibody response to Haemophilus influenzae type b in children with recurrent infections and normal serum IgG subclass levels. J Allergy Clin Immunol 1988;81:1175-1179.
20. Ambrosino DM, Morell A, Vassalli G, DeLange GG, Skvaril F, Siber GR. Correlations of G2m(n) and Km(1) allotypes with subclass- and light-chain-specific antibody. Monogr Allergy 1988;23:244-255.
21. Morell A, Vassalli G, de Lange GG, Skvaril F, Ambrosino DM and Siber GR. Class and subclass composition of natural antibodies to group A streptococcal carbohydrate: correlations with serum immunoglobulin concentrations and allotypes. J Immunol 1989;142:2495-2500.
22. Fingeroth JD, Heath ME, Ambrosino DM. Proliferation of resting B cells is modulated by CR2 and CR1. Immunol Letters 1989;21:291-302.
23. Reimer CB, Black CM, Wells TW, Plikaytis BD, Insel RA, Gellin B, Ambrosino DM, McQuillan GM, Keyserling HL, Carlone GM, Phillips DJ, Broome CV. Immunodeficiency and Haemophilus influenzae type b (Hib) capsular polysaccharide vaccine failures. Protides of the Biological Fluids 1989;36:87-96.
24. Ambrosino DM, Delaney N, Shamberger RC. Polysaccharide specific B cells are responsive to pokeweed and IL-6. J Immunol 1990;144:1221-1226.
25. Ambrosino DM, Greif W, Thompson C, Siber GR. Kappa and lambda light chain composition of antibody to the capsular polysaccharide of Haemophilus influenzae type b. J Infect Dis 1990;161:922-925.
26. Meissner C, Reimer CB, Black C, Broome C, Rabson A, Siber GR, Delaney N, Connors M, Ambrosino DM. Interpretation of IgG subclass values: A comparison of two assays. J Pediatr 1990;117:726-731.
27. Ambrosino DM, Kanchana MV, Delaney NR, Finberg RW. Human B cells secrete predominantly l light chains in the absence of heavy chain expression. J Immunol 1991;146:599-602.

28. Ambrosino DM, Black CM, Plikaytis BD, Reimer CB, Lee MC, Evatt BL, Carlone GMK. IgG subclass values differ for healthy black and white children. J Pediatr 1991;119:875-879.
29. Ambrosino DM. Impaired polysaccharide responses in immunodeficient patients: relevance to bone marrow transplant patients. Bone Marrow Transplantation 1991;75(Suppl):48-51.
30. Ambrosino DM, Lee M-YC, Chen D, Shamberger RC. Response to Haemophilus influenzae type B (HIB) conjugate vaccine in children undergoing splenectomy. J Peds Surg 1992;27:1045-1048.
31. Ambrosino DM, Sood SK, Lee MC, Chen D, Collard HR, Bolon DL, Johnson C, Daum RS. IgG1, IgG2 and IgM responses to two Haemophilus influenzae type b conjugate vaccines in young infants. Pediatr Infect Dis J 1992;11:855-859.
32. Ambrosino DM, Bolon D, Collard H, Van Etten R, Kanchana MV, Finberg RW. The effect of Haemophilus influenzae polysaccharide outer membrane protein complex conjugate vaccine (PRP-OMP) on macrophages. J Immunol 1992;149:3978-3983.
33. Chung G-H, Scott MG, Kim KH, Kearney J, Siber GR, Ambrosino DM, Nahm MH. Clonal characterization of the human IgG antibody repertoire to H. influenzae type b polysaccharide: V. in vivo expression of individual antibody clones is dependent on Ig CH haplotypes and the categories of antigen. J Immunol 1993;151:4352-4361.
34. Shurin PA, Rehmus JM, Johnson CE, Marchant CD, Carlin SA, Super DM, Van Hare GF, Jones PK, Ambrosino DM, Siber GR. Bacterial polysaccharide immune globulin for prophylaxis of acute otitis media in high risk children. J Pediatr 1994;123:801-810.
35. Guinan EC, Molrine DC, Antin JH, Lee MC, Weinstein HJ, Sallan SE, Parsons SK, Wheeler C, Gross W, McGarigle C, Blanding P, Schiffman G, Finberg RW, Siber GR, Bolon D, Wang M, Cariati S, Ambrosino DM. Polysaccharide conjugate vaccine responses in bone marrow transplant patients. Transplantation 1994;57(5):677-684.
36. Ambrosino DM, Wang M, Ciamarra A, Chan M, Bolon D, Minn J, Finberg RW. T cells and natural killer cells regulate human IgG subclass concentrations in SCID mice. Cellular Immunology 1994;155:134-143.
37. Soiffer RJ, Murray C, Ritz J, Phillips N, Jacobsohn D, Chariter S, Ambrosino DM. Recombinant Interleukin 2 (r-IL2) infusions and decreased IgG2 subclass concentrations. Blood 1995;85:925-928
38. Molrine DC, Polk DB, Ciamarra A, Phillps N, Ambrosino DM. Impaired human responses to tetanus toxoid in vitamin A deficient SCID-hu-PBL mice. Infect Immun 1995;63:2867-2872
39. Molrine DC, George S, Tarbell N, Mauch P, Diller L, Neuberg D, Shamberger RC, Anderson EL, Phillips NR, Kinsella K, Ambrosino DM. Antibody responses to polysaccharide and polysaccharide-conjugate vaccines following treatment for Hodgkin's Disease. Annals Int Med 1995;123:828-834
40. Molrine DC, Guinan EC, Antin JH, Parsons SK, Weinstein HJ, Wheeler C, McGarigle C, Blanding P, Phillips NR, Kinsella K, Deans K, Ciamarra A, Goorin A, George S, Ambrosino DM. Donor immunization with Haemophilus influenzae type b (HIB)-conjugate vaccine in allogeneic bone marrow transplantation. Blood 1996;87:3012-3018.

41. Chan CY, Molrine DC, George S, Tarbell NJ, Mauch P, Diller L, Shamberger RC, Phillips NR, Goorin A, Ambrosino DM. Pneumococcal conjugate vaccine primes for antibody response to polysaccharide pneumococcal vaccine following treatment of Hodgkin's Disease. J Inf Dis 1996;173:256-8.
42. Molrine DC, Guinan EC, Antin JH, Wheeler C, Parsons SK, Weinstein HJ, McGarigle C, Blanding P, Phillips NR, Ciamarra A, George S, Ambrosino DM. Haemophilus influenzae type b (HIB)-conjugate immunization before bone marrow harvest in autologous bone marrow transplantation. Bone Marrow Transplantation 1996;17:1149-1155
43. Chan CY, Molrine DC, Antin JH, Wheeler C, Guinan EC, Weinstein HJ, Phillips NR, McGarigle C, Harvey S, Schnipper C, and Ambrosino DM. Antibody responses to tetanus toxoid and Haemophilus influenzae tybe b conjugate vaccines following autologous peripheral blood stem cell transplantation(PBSCT). Bone Marrow Transplantation 1997;20:33-38.
44. Vance E, George S, Guinan EC, Wheeler C, Antin JH, Ambrosino DM, Molrine DC. Comparison of multiple immunization schedules for Haemophilus influenzae tybe b-conjugate and tetanus toxoid vaccines following bone marrow transplantation. Bone Marrow Transplantation 1998; 22:735-741
45. Malley R, DeVincenzo J, Ramilo O, Dennehy PH, Meissner HC, Gruber WC, Sanchez PJ, Jafri H, Balsley J, Carlin D, Buckingham S, Vernacchio L and Ambrosino DM. Reduction of Respiratory Syncytial Virus (RSV) in Tracheal Aspirates in Intubated Infants Using Humanized Monoclonal Antibody to RSV F Protein. J Inf Dis 1998;178:1555-1561
46. Karron R and Ambrosino DM. Respiratory Syncytial Vaccines. Ped Inf Dis J 1998;17:919-922.
47. Molrine DC, Siber GR, Samra Y, Shevy DS, MacDonald K, Cerci R and Ambrosino DM. Normal IgG and Impaired IgM Responses to Polysaccharide Vaccines in Asplenic Patients. JID, 1999;179:513-517
48. Malley R, Vernachio L, DeVincenzo J, Ramilo O, Dennehy P, Meissner C, Gruber WC, Jafri HS, Sanchez PJ, MacDonald K, Montana J, Thompson CM, and Ambrosino DM. Enzyme-linked immunosorbent assay to assess respiratory syncytial virus concentration an correlate results with inflammatory mediators in tracheal secretions. Pediatr Infect Dis J, 2000;19:1-7.
49. Malley R and Ambrosino DM. Pneumococcal Diseases in Children: Morbidity, Mortality and Resistance. University of Chicago Children's Hospital Reports on Current Concepts in the Use of Pediatric Vaccines. 1998;1(1): 1-9.
50. Vernacchio L, Romero-Steiner S, Martinez JE, MacDonald K, Barnard S, Pilishvili T, Carlone GM, Ambrosino DM and Molrine DC. Comparison of an opsonophagocytic assay and IgG ELISA to assess responses to pneumococcal polysaccharide and pneumococcal-conjugate vaccines in patients with sickle cell disease. J Infect Dis, 2000;181:1162-1166.
51. Paoletti LC, Rench MA, Kasper DL, Molrine D, Ambrosino D, Baker CJ. Effects of alum adjuvant or a booster dose on immunogenicity during clinical trials of group B streptococcal type III conjugate vaccines. Infect Immun 2001; 69:6696-6701.
52. Vernacchio L, Bernstein H, Pelton S, Allen C, MacDonald K, Dunn J, Duncan D, Tsao G, LaPosta V, Eldridge J, Laussucq S, Ambrosino DM, Molrine DC. Effect of monophosphoryl lipid A (MPL®) on T-helper cells when administered as an adjuvant with pneumococcal-CRM_{197} conjugate vaccine in healthy toddlers Vaccine 2002; 20:3658-3667.

53. Molrine DC, Antin JH, Guinan EC, Soiffer RJ, MacDonald K, Malley R, Malinoski F, Trocciola S, Wilson M, Ambrosino DM. Donor immunization with pneumococcal conjugate vaccine and early protective antibody responses following allogeneic hematopoietic cell transplantation. Blood 2003; 101(3):831-836.
54. Babcock GJ, Esshaki DJ, Thomas, WD Jr., Ambrosino DM. Amino Acids 270-510 of the SARS-CoV Spike Protein are Required for Interaction with Receptor. J Virol 2004; 78(9):4552-4560.
55. Jeffers SA, Tusell SM, Gillim-Ross L, Hemmila EM, Achenbach JE, Babcock GJ, Thomas WD, Jr., Thackray KB, Young MD, Mason RJ, Ambrosino DM, Wentworth DE, DeMartini JC, Holmes KV. CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. PNAS 2004; 101(44):15748-15753.
56. Greenough TC, Babcock GJ, Roberts A, Hernandez HJ, Thomas WD Jr., Coccia JA, Graziano RF, Srinivasan M, Lowy I, Finberg RW, Subbarao K, Vogel L, Somasundaran M, Luzuriaga K, Sullivan JL, Ambrosino DM. Development and Characterization of a Severe Acute Respiratory Syndrome-Associated Coronavirus-Neutralizing Human Monoclonal Antibody That Provides Effective Immunoprophylaxis in Mice. J Inf Dis 2005; 191:507-514.
57. Tripp RA, Haynes LM, Moore D, Anderson B, Tamin A, Harcourt BH, Jones LP, Yilla M, Babcock GJ, Greenough T, Ambrosino DM, Alvarez R, Callaway J, Cavitt S, Kamrud K, Alterson H, Smith J, Harcourt JL, Maio C, Razdan R, Comer JA, Rollin PE, Ksiazek TG, Sanchez A, Rota PA, Bellini WJ, Anderson LJ. Monoclonal Antibodies to SARS-Associated Coronavirus (SARS-CoV): Identification of Neutralizing and Antibodies Reactive to S, N, M and E Viral Proteins. J Virol Methods 2005; 128:21-28.
58. Roberts A, Thomas WD Jr., Guarner J, Lamirand EW, Babcock GJ, Greenough TC, Vogel L, Hayes N, Sullivan JL, Zaki S, Subbarao K, Ambrosino DM. Therapy with a SARS-Coronavirus-Neutralizing Human Monoclonal Antibody Reduces Disease Severity and Lowers Viral Burden in Golden Syrian Hamsters. J Inf Dis. 2006; 193: 686-692.
59. Babcock GJ, Broering TJ, Hernandez HJ, Malsberger RB, Donahue K, Boatright N, Stack AM, Lowy I, Graziano R, Molrine D, Ambrosino DM, Thomas, Jr WD. Human Monoclonal Antibodies Directed Against Toxins A and B Prevent *Clostridium difficile* – Induced Mortality in Hamsters. Infect Immun. 2006; 74(11):6339-47.
60. Sloan SE, Hanlon C, Weldon W, Niezgoda M, Blanton J, Self J, Rowley KJ, Mandell RB, Babcock GJ, Thomas, Jr. WD, Rupprecht CE, Ambrosino DM. Identification and Characterization of a Human Monoclonal Antibody that Potently Neutralizes a Broad Panel of rabies Virus Isolates. Vaccine. 2007; 25(15):2800-10.
61. Haynes LM, Miao C, Harcourt JL, Montgomery JM, Mai LTQ, Dryga S, Kamrud K, Rivers B, Babcock GJ, Reynolds M, Uyeki TM, Bausch D, Thomas Ksiazek, Atlerson H, Smith J, Ambrosino DM, Anderson LJ. Recombinant Protein-based Assays for the Detection of Antibodies to SARS-CoV Spike and Nucleocapsid Proteins. Clin Vaccine Immunol. 2007; 14(3):331-3.
62. Broering T, Garrity K, BoatrightN, Sloan SE, Sandor F, Thomas, Jr WD, Szabo G, FinbergR, Ambrosino DM, Babcock GJ. Identification and Characterization of Broadly Neutralizing Human Monoclonal Antibodies Directed against the E2 Envelope Glycoprotein of Hepatitis C Virus. J Virol 2009; 83(23):12473-12482.
63. Leav BA, Blair B, Leney M, Knauber M, Reilly C, Lowy I, Gerding DN, Kelly CP, Katchar K, Baxter R, Ambrosino DM, Molrine DC. Serum anti-toxin B antibody correlates with protection from recurrent *Clostridium difficile* infection (CDI). Vaccine 2009; doi:10.1016/j.vaccine.2009.10.144.

64. Lowy I, Molrine DC, Leav BA, Blair B, Baxter R, Gerding D, Nichol G, Thomas, Jr WD, Leney M, Sloan SE, Hay C, Ambrosino DM. Treatment with Monoclonal Antibodies against Clostridium difficile Toxins. N Engl J Med 2010: 362(3): 197-205.
65. Wang Y, Rowley KJ, Booth BJ, Sloan SE, Ambrosino DM, and Babcock GJ. G glycoprotein Amino Acid Residues Required for Human Monoclonal Antibody RAB1 Neutralization are conserved in Rabies Virus Street Isolates. Antiviral Res., 2009: 91 (2): 187-94.
66. Morin TM, Broering TJ, Leav BA, Blair BM, Bouucher EN, Wang Y, Cheslock PS, Knauber M, Olsen DB, LudmererSW, Szabo G, Finberg RW, Purcell RH, Lanford RE Ambrosino DM, Molrine DC, and Babcock GJ. PLoS Pathog. 2012: 8 (8): 1-13, e1002895.
67. Chung RT, Gordon FD, Curry MP, Schiano TD, Emre S, Corey K, Markman J, Hertl M, Pomposelli J, Pomfret EA, Florman S, Schilsky M, Broering TJ, Finberg RW, Szabo G, Zamore PD, Khettry U, Babcock GJ, Ambrosino DM, Leav B, Leney M, Smith HL, Molrine, DC. Human Monoclonal Antibody MBL-HCV1 Suppresses Return of HCV Following Liver Transplantation: A Randomized, Controlled Study. Am J Transplant. 2013:13(4): 1047-54.
68. Broering TJ, Wang H, Boatright NK, Wang Y, Baptista K, Shayan G, Garrity KA, Kayatekin C, Bosco DA, Matthews CR, Ambrosino DM, Xu Z, Babcock GJ Identification of human monoclonal antibodies specific for human SOD1 recognizing distinct epitopes and forms of SOD1. PLoS One. 2013: 8(4):e61210.
69. Graham, B and Ambrosino, D. History of Passive Antibody Administration for Prevention and Treatment of Infectious Diseases. Curr Opin HIV AIDS 2015; 10(3):129-34

Proceedings of Meetings:

1. Ambrosino DM. Impaired polysaccharide responses in immunodeficient patients: relevance to bone marrow transplant patients. Proceedings of the International Course on Bone Marrow Transplantation in Children. Bone Marrow Transplantation 1991; 75(3):48-51.
2. Malley R, Ambrosino DM. Pneumococcal Disease in Children: Morbidity, Mortality and Resistance. Pediatr Vaccines 1998; 1:1-11.

Reviews and Educationally Relevant Publications:

1. Molrine DC, Ambrosino DM. Immunizations in Immunocompromised Cancer Patients. Infectious in Medicine 1996:13,259,263-75

Books and Monographs (other than text books):

1. Siber GR, Ambrosino DM. Heavy and light chain restriction of human antibodies to polysaccharide antigens. In: Nydegger U.E., Morell A, eds. Clinical Use of Intravenous Immunoglobulins. London: Academic Press, 1986.
2. Ambrosino DM, Morell A, Vassalli G, De Lange GG, Skvaril F, Siber GR. Correlations of G2m(n) and Km(1) allotypes with subclass and light chain specific antibody. In: Skvaril F and Morell A, eds. Clinical aspects of IgG

subclasses and therapeutic applications. 1987.

3. Siber GR, and Ambrosino DM. Hyperimmune human immunoglobulins: preparation and characterization of H. influenzae b, Pneumococcal and Meningococcal globulin. In: Robbins JB et al, eds. Bacterial Vaccines. New York: Praeger, 1987.
4. Ambrosino DM, and Molrine DC. A critical appraisal of the use of immunization strategies for the prevention of infection in the compromised host. In Pizzo PA et al, eds. Hematology Oncology Clinics of North America. Philadelphia: W.B. Saunders Co., 1993.
5. Ambrosino DM. Bacterial Vaccines. In: Austen F, Burakoff S, Rosen FS, and Strom T eds. Therapeutic Immunology. Boston: Blackwell Science, 1995.

Letters

1. Ambrosino DM, Siber GR, Chilmonczyk BA, Jernberg JA, Finberg RW. Impaired immune response to polysaccharides. N Engl J Med. 1987; 317:837-839.

Abstracts

1. Ambrosino DM, Siber GR, Van Loghem E, Schiffman G. Increased antibody response to immunization with Haemophilus influenzae type b (Hib) and pneumococcal capsular polysaccharide (PS) vaccines in adults with the G2m(n) allotype [Abstract #93l]. Pediatric Research 1982; l6:234A.
2. Schreiber JR, Ambrosino DM, Daum RS, Siber GR. Prevention of H. influenzae type b (Hib) disease in infant rats with human hyperimmune globulin (HIG) [Abstract #1029]. Pediatric Research 1982;l6:250A.
3. Ambrosino DM, Gorham CA, McIver J, Siber GR. Pharmacokinetics of anti-polyribophosphate antibody in human hyperimmune globulin in children [Abstract #1066]. Pediatric Research 1983;17:264A.
4. Ambrosino DM, Rosenberg GE, De Lange G, Siber GR. Association of the G2m(n) immunoglobulin allotype with H. influenzae type b disease., Las Vegas: Interscience Conference on Antimicrobial Agents and Chemotherapy, 1983.
5. Ward JI, Ambrosino D, Burkhart K, Sonne B, Dengrove J, Siber G. Passive protection for Haemophilus influenzae type b (Hib) disease: Antibody levels in infants given hyperimmune globulin [Abstract #1151]. Pediatric Research 1984;18:237A, Presented at Plenary Session.
6. Ambrosino DM, Barrus VA, DeLange GM, Siber GR. Association of the Km(1) immunoglobulin allotype with anti-PRP antibody response in caucasians [Abstract #835]. Washington, DC: Interscience Conference on Antimicrobial Agents and Chemotherapy, 1984:236.
7. Geha R, Ambrosino D, Siber G, Martin P, Howie G, Umetsu D. Children with IgG2 subclass deficiency have an impaired response to the polyribose phosphate (PRP) polysaccharide vaccine.New York: American Academy of Allergy and Immunology, March 1985.
8. O'Rourke E, Martin P, Macone A, Anderson A, Goldmann D, Siber G, Ambrosino D. Pneumococcal antigen detection in children at risk for occult bacteremia [Abstract]. Pediatric Research 1985.
9. Shurin PA, Giebink GS, Siber GR, Ambrosino D, Overman M, Bauer T. Immunoglobulin G (IgG) in mucosal immunity: Prevention of experimental

pneumococcal otitis media (OM) with human bacterial polysaccharide immunoglobulin (BPIG) [Abstract #977]. Pediatric Research 1986; 20:321A .
10. Santosham, M, Reid R, Aspery K, Almeido J, Ambrosino D, Page P, Moxon R, Siber G. Prevention of H. influenzae b (Hib) and pneumococcal infections in high-risk infants with bacterial polysaccharide immune globulin (BPIG). Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, LA, 1986; p 134 [Abstract #213].
11. Letson GW, Santosham M, Reid R, Burns A, Jahnke D, Smith C, Johnson C, Siber G, Ambrosino D, Moxon R. Immunogenicity and safety of Haemophilus influenzae b (Hib) diphtheria toxoid conjugate vaccine given with or without bacterial polysaccharide immune globulin (BPIG) in Navajo infants.New Orleans, LA: Interscience Conference on Antimicrobial Agents and Chemotherapy, 1986; p. 134 [Abstract #214].
12. Ambrosino DM, Umetsu DT, Geha RS, Michaels RS, Noyes J, Goularte TA, Howie G, Siber GR. Impaired antibody response to polysaccharide antigen in children with recurrent sinopulmonary infections and normal IgG subclass concentrations. New Orleans, LA: Interscience Conference on Antimicrobial Agents and Chemotherapy, 1986, p 255.
13. Shamberger RC, Chen D, Lee M, Ambrosino DM. Polysaccharide response to conjugate vaccines in children undergoing splenectomy. American Pediatric Surgical Association. [Abstract] May 1991.
14. Ambrosino DM, Sood S, Lee MC, Chen D, Collard H, Bolon D, Johnson C, Daum RS. IgG1, IgG2 and IgM responses to two HIB conjugate vaccines in young infants. Chicago, IL: Interscience Conference on Antimicrobial Agents and Chemotherapy, September 1991.
15. Molrine DC, Guinan EC, Parsons SK, Weinstein HJ, Antin JH, Wheeler C, Siber GR, Schiffman G, Ambrosino DM. Polysaccharide-conjugate responses in bone marrow transplant patients. Society of Pediatric Research, Washington, DC. May 1993.
16. Wang M, Minn J, Bolon DL, Ciamarra A, Finberg RW, Ambrosino DM. T-cell influences of human IgG subclass concentrations in SCID mice. Washington, DC: Society of Pediatric Research, May 1993.
17. Molrine D, Polk B, Ciamarra A, Ambrosino DM. Impaired human antibody response to tetanus toxoid in vitamin A deficient SCID mice. New Orleans, LA: Interscience Conference on Antimicrobial Agents and Chemotherapy, October 1993.
18. MolrineDC, Guinan EC, Parsons SK, Weinstein HJ, Antin JH, Wheeler C, Phillips NR, Kinsella K, Deans K, George S, Ambrosino DM. Antibody responses to multiple doses of polysaccharide (PS)-conjugate vaccine post bone marrow transplantation. Society of Pediatric Research, Seattle, WA 1994
19. Molrine D, George S, Mauch P, Tarbell N, Diller L, Neuberg D, Shamberger R, Anderson E, Phillips N, Kinsella K, Ambrosino DM. Post treatment antibody responses to polysaccharide-conjugate vaccines in Hodgkin's Disease patients. Society of Pediatric Research, Seattle, WA 1994.
20. Molrine D, Guinan E, Parsons S, Weinstein H, Antin J, Wheeler C, Phillips N, Kinsella K, Deans K, Ciamarra A, Ambrosino DM. Donor immunization and antibody responses to polysaccharide (PS) - conjugate vaccine in bone marrow transplantation. Society of Pediatric Research, San Diego, CA 1995.

21. Chan C, Molrine D, George S, Goorin A, Tarbell N, Mauch P, Diller L, Phillips N, Ambrosino DM. Heptavalent pneumococcal conjugate vaccine (7-OMPC) primes for antibody response to pure polysaccharide pneumococcal vaccine following treatment for Hodgkin's Disease. Society of Pediatric Research, San Diego, CA 1995.
22. Malley R, Montana JB, Huntoon SA, Thompson CM, Ambrosino DM, Kurt-Jones E. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assessment of T helper cell response in a murine model of respiratory syncytial virus (RSV) infection. Society of Pediatric Research, Washington DC, 1997.
23. DeVincenzo JP, Malley R, Ramilo O, Dennehy PH, Meissner HC, Gruber WC, Sanchez P, Jafri H, Balsley J, Carlin D, Buckingham S, Vernacchio L, Ambrosino DM. Viral concentration in upper and lower respiratory secretions from respiratory synctial virus (RSV) infected children treated with RSV monoclonal antibody (Medi-493). Society of Pediatric Research, New Orleans LA, 1998.
24. Vernacchio L, Romero-Steiner S, Martinez JE, MacDonald K, Barnard S, Pilishvilli T, Carlone GM, Ambrosino DM, Molrine DC. Comparison of an opsonophagocytic assay and IgG ELISA to assess responses to pneumococcal vaccines in patients with sickle cell disease. Washington, DC: Pneumococcal Vaccines for the World Conference, October 1998.
25. Ambrosino, DM. Immunizations in Stem Cell Transplantation Patients. Infectious Diseases Society of America, Denver, 1998.
26. Jafri H, Sheeran P, Buckingham S, DeVincenzo J, Sanchez P, Malley R, Ambrosino D, Dennehy P, Meissner HC, Carlin D, Ramilo O. Impact of Palivizumab (Pal) Therapy on Dynamics of Chemokine (Chem) Concentrations (conc) in Respiratory Secretions of Children with Severe Respiratory Syncytial Virus (RSV) Disease. Presented at The American Society for Microbiology, 41st Annual ICAAC Meeting, Chicago, IL. 2001.
27. Molrine D, Antin J, Guinan E, Soiffer R, Ambrosino D, Maldonado J, Wilson M. Pneumococcal Conjugate Vaccine (PCV) Elicits Protective Responses in Allogeneic Bone Marrow Transplant (BMT) Recipients. Presented at The American Society for Microbiology, 41st Annual ICAAC Meeting, Chicago, IL. 2001.
28. Molrine DC, Antin JH, Guinan EC, Soiffer RJ, Avigan D, Martin VJ, Ambrosino DM. Pneumococcal conjugate vaccine (PCV) is immunogenic in autologous hemapoietic stem cell transplant (HCT) patient. Abstract, 41st Annual IDSA Meeting, San Diego, CA 2003.
29. Babcock G, Thomas W, Hernandez H, Greenough T, Luzuriaga K, Somasundaran M, Sullivan J, Finberg R, Lowy I, Ambrosino D. Expression and Immunogenicity of Recombinant SARS Spike Protein. Abstract, 41st Annual IDSA Meeting, San Diego, CA 2003.
30. Babcock GJ, Greenough TC, Hernandez HJ, Thomas WD, Lowy I, Finberg R, Subbarao K, Roberts A, Vogel L, Somasundaran M, Luzuriaga K, Ambrosino D, Sullivan J. Human Monoclonal Antibodies against S Glycoprotein Neutralize SARS Virus in Mice. Abstract, 17th International Conference on Antiviral Research, Tucson, AZ., 2004.
31. Babcock GJ, Mandell RB, Coccia JA, Esshaki DJ, Broering TH, Molrine DC, Lowy I, Graziano R, Ambrosino DM, Thomas WD Jr. Human Monoclonal Antibody against Toxin A Protects Hamsters from *Clostridium difficile* disease. Abstract, 41nd Annual IDSA Meeting, Boston, MA 2004.

32. Ambrosino DM, Roberts A, Vogel L, Thomas WD Jr., Babcock GJ, Greenough TC, Guarner J, Zaki S, Sullivan JL, Subbarao K. Treatment of SARS Infected Hamsters with Human Monoclonal Antibody (HuMab 201). Abstract, 41nd Annual IDSA Meeting, Boston, MA 2004.
33. Mandell RB, Babcock GJ, Hill AC, Coccia JA, Zhang H, Lowy I, Graziano RF, Ambrosino DM, Thomas WD Jr. Neutralizing Human Monoclonal Antibodies Against *Clostridium difficile* Toxin A. Abtract, 44th Annual ICAAC Meeting, Washington, D.C., 2004.
34. Ambrosino DM, Schonhoff SE, Neizgoda M, Mandell RB, Weldon W, Blanton J, Hanlon C, Babcock GJ, Thomas WD Jr., Rupprecht CE. Identification of Human Monoclonal Antibody that Neutralizes a Broad Panel of Rabies Virus Isolates. Presented at the First International Conference on 'Rabies in Europe', Kiev, Ukraine, 2005.
35. Ambrosino DM, Babcock GJ, Thomas WD, Broering TJ, Hernandez HJ, Lowy I, Graziano R, Molrine DC, Gerding DN. Human Monoclonal Antibodies Neutralize Toxins Produced by Epidemic Strains of *Clostridium difficile*. Abstract, 43rd Annual IDSA Meeting, San Francisco, 2005.
36. Taylor CP, Tummala S, Molrine D, Farrell RJ, Lembo A, Hibberd PL, Davidson L, Lowy I, Kelly CP. Open-Label, Dose Escalation Phase I Study in Healthy Volunteers to Evaluate the Safety and Pharmacokinetics of *Clostridium difficile* Toxin A Human Monoclonal Antibody (CDA1). Digestive Disease Week, Washington, D.C., 2006.
37. Broering TJ, Hernandez HJ, Sloan SE, Sandor F, Boatright NK, Giacomozzi BL, Donahue KM, Pierciey FJ, St. Pierre C, Szabo G, Thomas WD, Finberg R, Ambrosino DM, Babcock GJ. Neutralizing human monoclonal antibodies specific for a highly conserved linear epitope of HCV E2 protein. Abstract, Digestive Disease Week, Washington, D.C., 2007.
38. Smith MK, Broering TJ, Castano A, Ambrosino DM, Holmes KV, University of Colorado Health Sciences Center, Aurora, CO 80045, University of Massachusetts Medical School, Jamaica Plain, MA 02130. Antigenicity of the spike glycoprotien of human corona virum NL63. Abstract, 8th International Synposium on Positive Strand RNA Viruses, Washington, D.C., 2007.
39. Babcock GJ**,** Leav BA, Sloan SE, Singh S, Fehlner-Gardiner C, Wandeler A, Kapre SV, Molrine DC, Ambrosino DM. Fully human monoclonal antibody 17C7 neutralizes rabies virus endemic to Asia. Abstract, 19th International Conference on Rabies in the Americas (RITA), Atlanta, GA., 2008.
40. Leav B, Blair B, Leney M, Knauber M, Reilly C, Lowy I, Kohberger R, Gerding D, Kelly C, Katchar K, Baxter R, Ambrosino D. Serum Anti-Toxin B Antibody Correlates with Protection from Recurrent *Clostridium difficile* associated diarrhea (CDAD). IDSA/ICAAC, 2008.
41. Blair BM, Broering TJ, Babcock GJ, Szabo G, Finberg RW, Cheslock PS, Knauber M, Leav BA, Lanford R, Purcell RH, Ambrosino DM, Molrine DC. A Novel Human Monoclonal Antibody Directed Against the E2 Glycoprotein of Hepatitis C Virus (HCV) Prevents Infection in Chimpanzees. EASL, Milan, Italy, 2009.
42. Leav BA, Blair B, Baxter R, Lowy I, Nichol G, Kohberger R, Leney M, Sloan S, Molrine DC, Ambrosino DM. Efficacy of Human Monoclonal Antibody Treatment in Phase II Trial for C. *difficile* Recurrence. Digestive Disease Week, Chicago, 2009.
43. Wang Y, Rowley KJ, Booth BJ, Sloan SE, Fehlner-Gardiner C, Wandeler A, Ambrosino DM, Babcock GJ. G glycoprotein amino acid residues required for RAB1 neutralization

are conserved in rabies virus street isolates. Abstract, 20th International Conference on Rabies in the Americas (RITA), Toronto, Canada, 2009.

44. Blair BM, Leav BA, Cheslock P, Knauber M, Sloan S, Ambrosino DM, Molrine DC. Safety and Pharmacokinetics of a Novel Human Monoclonal Antibody Directed Against the E2 Glycoprotein of Hepatitis C Virus (MBL-HCV1) in Healthy Volunteers. Poster, International Liver Congress 2010 by EASL, Vienna, Austria, 2010.
45. Leav B, Kulkarni P, Gogtay N, Cheslock P, Martin V, Wiggin H, Ambrosino DM, Molrine D, Kapre S. A Human Monoclonal to Rabies Virus Provides Protective Neutralizing Activity: Results of a Phase 1 Study. Poster, ICCAC, Boston, MA 2010.
46. Moore S, Hanlon C, Gardner J, Leav B, Molrine D, Leney M, Kulkarni P, Sharma H, Gogtay N, Kapre S, Ambrosino DM. Measuring Rabies Antibody at the Threshold of Detection and Quantitation. Presentation, RITA, Guadalajara Mexico, 2010.
47. Wang H, Broering T, Babcock G, Boatright N, Wang Y, Song YY, Brady S, Xu Z, Ambrosino DM. Human Monoclonal Antibodies Directed Against SOD1 Extend Survival in a Mouse Model of ALS. Poster, Chicago Biomedical Consortium, Chicago, IL, 2010.
48. Babcock GJ**,** Szretter K, Sloan S, Rowley K, Himansu S, Viswanathan K, Robinson L, Barnard D, Ambrosino D, Shriver Z. (2013) VIS410, a Broadly HA-Targeting Human Antibody, Neutralizes H5 and H7 Isolates with Pandemic Potential. Interscience Conference on Antimicrobial Agents and Chemotherapy, Denver, CO, 2013.
49. Simon R, Broering T, Michon F, Malley R, Tennant S, Sinclair J, Leney M, Amin M, Lu Y, Sycheya L, McCombs J, Cross A, Ambrosino D, Siber G. Novel Multivalent Vaccine for Gram-Negative Bacterial Pathogens, including Multiple Antibiotic-resistant Strains. Word Vaccine Congress, Washington DC, 2017.

Vaccine 38 (2020) 2751–2757

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.else vier.com/locate/vaccine

Conference report

Workshop report: Optimization of animal models to better predict influenza vaccine efficacy

M. Chelsea Lane[a,⇑], Jennifer L. Gordon[a], Chao Jiang[b], Wolfgang W. Leitner[b], Thames E. Pickett[a], Erik Stemmy[a], Brooke A. Bozick[a], Alison Deckhut-Augustine[b], Alan C. Embry[a], Diane J. Post[a]

[a] Division of Microbiology and Infectious Diseases,National Institute of Allergy and Infectious Diseases,Bethesda, MD, USA
[b] Division of Allergy, Immunology, and Transplantation, National Institute of Allergy and Infectious Diseases,Bethesda, MD, USA

article info

Article history:
Received 27 September 2019
Received in revised form 17 January 2020
Accepted 30 January 2020

Keywords:
Animal models
Influenza virus
Universal influenza vaccine

abstract

Animal models that can recapitulate the human immune system are essential for the preclinical development of safe and efficacious vaccines. Development and optimization of representative animal models are key components of the NIAID strategic plan for the development of a universal influenza vaccine. To gain insight into the current landscape of animal model usage in influenza vaccine development, NIAID convened a workshop in Rockville, Maryland that brought together experts from academia, industry and government. Panelists discussed the benefits and limitations of the field's most widely-used animal models, identified currently available and critically needed resources and reagents, and suggested areas for improvement based on inadequacies of existing models. Although appropriately-selected animal models can be useful for evaluating safety, mechanism-of-action, and superiority over existing vaccines, workshop participants concluded that multiple animal models will likely be required to sufficiently test all aspects of a novel vaccine candidate. Refinements are necessary for all current model systems, for example, to better represent special human populations, and will be facilitated by the development and broader availability of new reagents. NIAID continues to support progress towards increasing the predictive value of animal models.

1. Introduction

Seasonal influenza infects millions, causing thousands of hospitalizations and deaths each year. While even healthy people can get very sick, infection can present particularly serious problems for young children, the elderly, pregnant women and people with certain medical conditions such as asthma and heart disease. In addition to supporting research to improve seasonal influenza vaccines, the National Institute of Allergy and Infectious Diseases (NIAID), part of the National Institutes of Health (NIH), is working to prepare for a potential pandemic influenza threat. This effort includes the development of a universal influenza vaccine that ideally provides long-lasting protection against both seasonal and pandemic strains. As emphasized in NIAID's ''Pathway to a Universal Influenza Vaccine" workshop [1] and strategic plan [2], the use of animal models is essential for understanding mechanisms of vaccine-mediated protection against influenza. Animal models are also important for pre-clinical testing and down-selection of universal influenza vaccine candidates. However, there is a need to improve existing animal models or develop novel animal models that more accurately represent influenza immunity in humans, with an emphasis on increasing the predictive value of models for evaluating novel universal influenza vaccines. A workshop organized by the EDUFLUVAC Consortium, a European Union-funded project coordinated by the European Vaccine Initiative, convened on June 23–24, 2016 to discuss the current knowledge and future perspectives for testing broadly reactive influenza vaccines in animal models [3]. From that workshop, several key recommendations were made to the influenza community, including the need for harmonized and standardized pre-clinical models for evaluation of new influenza vaccines. To build upon these discussions, NIAID hosted a workshop on August 7–8, 2018 entitled, ''Optimization of Animal Models to Better Predict Influenza Vaccine Efficacy (VE)". Approximately 100 U.S.and international scientists from academia, industry, and government convened in Rockville, Maryland to review the benefits and limitations of current influenza animal models, address the availability of and need for critical resources and reagents, and establish the steps required

⇑ Corresponding author at: 5601 Fishers Lane, Room 8A19, Bethesda, MD 20892-9826, USA.
E-mail address: lanemc@niaid.nih.gov (M.C. Lane).

https://doi.org/10.1016/j.vaccine.2020.01.101

to address current shortcomings. Anthony S. Fauci, Director of NIAID, provided opening remarks and re-iterated NIAID's commitment to universal influenza vaccine research and development, including research on improving animal models.

The workshop commenced with a keynote presentation by Kanta Subbarao, Director of the World Health Organization (WHO) Collaborating Centre for Reference and Research on Influenza at The Peter Doherty Institute for Infection and Immunity in Melbourne, Australia, who conveyed considerations for improving the predictive value of animal models in efficacy studies of influenza vaccines and biologics. Most importantly, she stressed that an appropriate animal model should be selected based on the specific research question. Dr. Subbarao noted that animal models are critical for studying pandemic viruses and performing pre-clinical testing of therapeutics and vaccines, particularly those directed at strains with pandemic potential. However, current animal models cannot completely recapitulate infection in humans due to several key differences. First, animals are immunologically naïve while humans experience multiple exposures/infections with influenza in their lifetime. This type of acquired immunity is difficult to model in animals. Vaccine preparations used in animals can differ from those used in clinical trials, and challenge infections in animals often use a high dose of virus. Finally, it is difficult to extrapolate the specifics of dose sparing in animal models to humans. To advance current influenza animal models, recommendations include: 1) improving the similarity of animal models to humans by genetic engineering; 2) considering infection history and coinfection status; 3) incorporating controlled variability through methods such as genetic crosses and including both female and male animals as well as those with a diverse microbiome; and 4) identifying biomarkers and correlates of protection through iterative comparative studies between animals and humans.

The workshop was composed of four animal model sessions and two panel discussions. For each animal model session, the utility and limitations of each model were addressed with respect to: (1) examining pre-existing immunity and sequential vaccination/-exposure; (2) estimating influenza vaccine efficacy in humans; (3) ability to stratify vaccine candidates for further evaluation in later stage human challenge studies and clinical trials; (4) availability of immunological reagents for the animal model including antibodies that recognize, activate, or deplete specific immune cell populations; methods for cytokine/chemokine detection; major histocompatibility complex (MHC) tetramers; and (5) cost and other limitations (e.g., space). For the first panel discussion, representatives from NIAID, U.S. Food and Drug Administration (FDA), Biomedical Advanced Research and Development Authority (BARDA), and industry were invited to engage the participants in a discussion of the utility of pre-clinical models for informing influenza vaccine candidate prioritization. To conclude the workshop, a final panel discussion summarized the need for optimized and/or complementary influenza animal models to better predict influenza vaccine efficacy.

2. Mouse and hamster models

Mouse models have been used extensively in mechanistic studies of influenza infection, as well as for the pre-clinical evaluation of vaccine candidates. Despite the advantages of reproducibility, ease of genetic manipulation, controlled environment factors, availability of reagents, and relatively low cost, it was agreed that standard laboratory mice also have limitations related to studying various aspects of influenza. Such limitations include, but are not limited to, the lack of genetic heterogeneity of inbred mouse lines; the need to adapt seasonal influenza virus strains to cause sufficient replication and virulence in mice; the difference of respiratory tract anatomy and influenza receptor distribution between mice and humans; the shorter life span is not optimal for studying long-lasting immune responses; and that mice are not optimal for studying influenza transmission. The session's presentations highlighted recent efforts to overcome some of these limitations.

To date, most mouse studies have been conducted in a limited number of inbred strains, allowing researchers to control for genetic variability. However, each mouse strain only represents one individual genotype; thus, these do not model more complex populations like humans. One way to overcome this issue is to use genetically diverse mouse reference populations, such as the Collaborative Cross (CC). The CC is a collection of recombinant inbred lines derived from initial random crosses of eight genetically-diverse inbred founder strains which include three wild-derived strains [4]. These mice capture 90% of the known genetic variation present in the genome of standard laboratory mice and their genetic diversity is comparable to that of humans. With their diverse genotypes, the CC mice display an increased range of phenotypes compared to standard laboratory strains, and extreme phenotypes have been identified that provide a unique opportunity to pinpoint underlying genetic causes for susceptibility or resistance to pathogens. Mark Heise, Professor of Genetics at the University of North Carolina-Chapel Hill, detailed his work using the CC mice and their genetic derivatives in a non-mouse-adapted influenza A virus (IAV, 2009 H1N1) infection model. Heise and colleagues conducted quantitative trait loci (QTL) mapping and narrowed down the genetic intervals associated with disease phenotype and immune function. They identified candidate genes and discovered novel cell populations that may be involved in the immune response against IAV [5]. In the context of vaccine research, the CC also provides a valuable tool for identifying genes that regulate the breadth and durability of vaccine-induced immune responses as well as gene networks that regulate adjuvant activity, discoveries which will allow for the development of new models for testing vaccine efficacy and safety.

In addition to inherited differences, researchers have noticed that non-heritable parameters like environmental factors can shape the immune system and lead to variations in immune response. While laboratory mice are often housed in a pathogen-free environment and their immune cells are largely "naïve", humans generally have much more antigen experience resulting in more differentiated immune cells. Growing evidence indicates that this difference diminishes the translational ability of mice to mimic human immune responses. In his presentation, Ryan Langlois, Assistant Professor at the University of Minnesota, summarized recent advances in research using "dirty" mice and discussed his work using these mice for influenza infection studies. A dirty mouse colony can be generated by natural microbiome transmission between animals through either co-housing or transfer of fomites as well as by deliberate infection with pathogens that model those which commonly infect humans (such as MHV68, murine cytolomegalovirus and Heligmosomoides polygyrus) [6]. The reconstitution of laboratory mice with a complex microbiome has a profound impact on the cell composition of the innate and adaptive immune systems as well as blood cytokine and antibody profiles, and results in altered immune responses to infection and vaccination [6–8]. The immune signatures of dirty mice resemble those of healthy adult humans more closely than standard laboratory mice. Therefore, it is plausible that dirty mice may serve as improved mouse models to add new dimensions in immunology research and vaccine discovery. While researchers are enthusiastic about the dirty mouse concept and its potential, establishing and maintaining such a colony is challenging [9]. Concerns have also been raised by the scientific community regarding the reproducibility of experiments using dirty mice generated by natural

transmission. The requirement and/or feasibility of standardization of a complex microbiome remains debatable. However, it is well documented that humans have diverse microbial experience that varies among individuals. Even monozygotic twin studies in humans indicate that environmental/epigenetic influences drive a significant degree of human variation in many immune system measurements [10]. Perhaps results that hold true among a majority of diverse dirty mice, including those in different colonies, will be most likely to be recapitulated among diverse humans.

Yoshihiro Kawaoka, Professor of Virology at the University of Wisconsin- Madison, noted that when looking for an animal model to better predict influenza vaccine efficacy, considerations should include efficacy evaluation methods, availability of reagents to measure immune response, susceptibility of the model to recent human influenza viruses, pre-existing immunity, practicality (e.g., husbandry and economics) and feasibility of genetic manipulation. Given these factors, Dr. Kawaoka suggested that hamsters may provide a better animal model than mice for influenza research [11]. First, replication of human influenza viruses in hamsters can occur without adaptation, and alpha 2–6 sialic acid expression in the respiratory system is similar to that of humans [11]. Second, hamsters live longer than mice which provides an opportunity to study sequential infections. Third, hamsters reproduce quickly and the process of establishing inbred lines is faster than in mice. Finally, hamster genomic and transcriptomic information is available, and gene-targeting techniques have been established that enable gene manipulation [12–14]. Dr. Kawaoka concluded that hamsters are an excellent model for evaluating influenza vaccine efficacy and that more reagents need to be developed to increase their use.

3. Ferret models

Ferrets are used extensively in influenza research to model pathogenesis and viral transmission and are also used in efficacy studies of vaccines and therapeutics. Ferrets are an attractive model of influenza infection because they are susceptible to human strains of the virus and several clinical symptoms of influenza infection in ferrets resemble those observed in humans [1]. Additionally, there are similarities between ferrets and humans in lung anatomy, cell biology and sialic acid receptor distribution. Limitations to the ferret model include the lack of species-specific reagents and standardized assays. Availability also may become a limitation, as demand for ferrets may outpace the available supply from the few ferret sources that currently exist. In this session, presentations highlighted promising work in ferret models of influenza.

John Engelhardt, Professor and Chair of the Department of Anatomy and Cell Biology at the University of Iowa, presented his work manipulating the ferret genome to investigate the effects of cystic fibrosis on the lung and pancreas [15]. These genetic approaches provide the opportunity to interrogate the contributions of different gene products to influenza pathogenesis and immunity. Further, advances in genetic engineering in ferret zygotes using CRISPR/Cas9have allowed for the generation of gene knock-out lines [16,17], knock-in lines harboring human mutations [18], and transgenic ferrets carrying a dual-fluorescent Cre reporter system flanked by integrase sites to enable rapid engineering of additional transgenic ferret models [17,19]. Such gene editing approaches may also be useful for generating in vitro airway models allowing more rapid screening of relevant influenza targets, prior to engineering a transgenic ferret model.

Scott Hensley, Associate Professor of Microbiology at the University of Pennsylvania, discussed his work on understanding prior exposure and pre-existing immunity in ferrets. Most ferrets used for influenza virus research have not been previously exposed to influenza, while humans have typically been infected or vaccinated with influenza by 3 or 4 years of age. Initial childhood influenza exposures can leave life-long immunological imprints that shape the specificity of influenza virus antibody responses over the lifespan [20]. Dr. Hensley showed that naïve ferrets and humans produce antibodies of different specificities after exposure to the same influenza strain. Importantly, he showed that ferrets that are previously exposed to older seasonal influenza virus strains produce antibodies that have similar specificities to human antibodies, which suggests that differences in antibody specificity can be linked to prior exposure to influenza virus. This extends beyond influenza infections and suggests that responses to influenza vaccination are also driven by prior exposure to influenza viruses.

Stacey Schultz-Cherry, Full Member (Professor) in the Department of Infectious Diseases and Deputy Director of the WHO Collaborating Centre for Studies on the Ecology of Influenza in Animals and Birds at St Jude Children's Research Hospital, presented ongoing work from a global collaboration aimed at developing validated reagents and assays to improve understanding of ferret innate and adaptive immune responses [21]. With support from NIAID's Centers of Excellence for Influenza Research and Surveillance (CEIRS),Dr. Schultz-Cherry is leading a team working to develop influenza and ferret-specific reagents including antibodies, recombinant proteins, and primers/probes. A repository of ferret lung, spleen, trachea, mediastinal lymph nodes, blood, peripheral blood mononuclear cells, single cell suspension, and paraffin-embedded tissues has been developed to support reagent validation (Supplementary Tables 1–5). Primary cell lines from ferrets including fibroblast, respiratory epithelial cells (nasal, tracheal, alveolar), and soft palate cells also have been established. Reagents developed by the CEIRSnetwork are freely available for distribution. For a complete list of influenza ferret reagents and tissues, see http://www.niaidceirs.org/resources/ceirs-reagents/ . Dr. Schultz-Cherry also summarized the work being done by her laboratory and others to develop models for pregnant, immunocompromised, and diet-induced obese ferrets [21]. These models will allow investigation of influenza infection and vaccination in groups at higher risk for influenza infection-related complications.

4. Swine models

Amy Vincent, Research Veterinary Medical Officer at the USDA National Animal Disease Center, provided an overview of the swine model for influenza [22]. Swine are a natural host for influenza infections, and they experience clinical signs similar to humans when infected with endemic swine influenza strains. With the exception of H1N1pdm09, human seasonal and avian influenza strains do not readily infect or cause disease in swine without adaptive changes. For example, several avian influenza and human seasonal viruses, including H3N2 viruses, do not readily infect swine or cause lung lesions, and the mechanisms of adaptation and establishment of these human lineage influenza viruses in swine populations are not well-understood. Swine share many physiological and anatomical similarities to humans including tracheobronchial tree structure, physiology, and lung size [23–28]. Despite many commonalities between the human and swine immune systems, swine have different immune cell populations (e.g. large populations of cr T cells and CD4 + CD8 + double-positive T cells).

Currently, researchers are developing more broadly protective, durable influenza vaccines for veterinary use and these efforts also could inform universal influenza vaccine development in humans. Renukaradhya J.Gourapura, Professor in the Department of Veterinary Preventative Medicine , Food Animal Health Research Program in the Ohio Agricultrual Research and Development Center at the

Ohio State University, discussed three separate intranasal nanoparticle vaccine formulations to evaluate mucosal responses and immune correlates of cross-protective influenza immunity in swine [29–31] . He also described the growing availability of swine-specific reagents and immunological tools as well as ongoing international efforts to develop the Swine Immune Toolkit, a collaborative activity between U.S. and U.K. veterinary immunologists to generate new immune reagents, mAbs, new assays for swine immune markers, and protocols to better understand immune pathways important in swine infection and vaccination [32,33] .

Inbred pigs provide an additional tool to elucidate immunological responses to influenza infection and vaccination. The large inbred Babraham pig is 85%homozygous across its genome, including across the pig MHC (referred to as the swine leukocyte antigen [SLA]) class I and II regions [34] . Elma Tchilian, Head of Mucosal Immunology at the Pirbright Institute, presented studies using the inbred Babraham pig to characterize local lung cellular immune responses after aerosol delivery of the novel single cycle influenza vaccine, S-FLU, followed by influenza challenge [35,36] . These studies utilized newly-developed immunological tools in swine models, including long-term in vitro T cell cultures and peptide SLA tetramers, to identify influenza T cell epitopes and track antigen-specific CD8+ T cell responses after immunization with S-FLU or influenza infection [37] . Other studies presented during the session demonstrated the feasibility of the swine model to evaluate the effectiveness of candidate influenza vaccines to disrupt transmission.

During discussions, there was consensus that the swine model has the potential to be a valuable tool in evaluating universal influenza vaccines for both clinical and agricultural use. Due to their long life-spans, swine could serve as a good model for evaluating the duration of influenza immune responses and protection against both natural exposure and vaccine-induced immunity. Dr. Vincent also highlighted the potential value of using swine as a model for studying vaccine safety due to the ability to screen influenza vaccine candidates for their potential to induce vaccine-associated enhanced respiratory disease (VAERD). Previous studies showed that VAERD occurs in swine vaccinated with adjuvanted whole-inactivated virus that are subsequently challenged with an antigenically mismatched virus of the same subtype (i.e., H1 or H3) [38] . Although VAERD has not been reported in humans, workshop participants pointed to an overall need for better animal models for vaccine safety studies.

Logistical challenges of the swine model were also discussed. Both outbred and inbred swine models are commercially available and range in size from micro-minipigs (~20 kg) and mini pigs (~40 kg) to traditional sizes (ranging from ~10–100 kg). Recent studies have indicated the genome, distribution of receptors, and sensitivity to influenza infection are roughly equivalent across these swine models [39] . Due to the specialized facilities and technically skilled personnel needed to conduct influenza research in swine, panelists confirmed that these studies can be cost-prohibitive. Studies requiring large animal BSL-3 facilities will likely be inaccessible for many investigators; however, most studies are done at BSL-2 for endemic swine, human seasonal, and non-HPAI avian strains. Another challenge is finding sources of pigs that are negative for influenza virus and antibodies. Panelists strongly encouraged those interested in working with swine not to be deterred by these challenges, noting that agricultural labs are often willing to collaborate to test new vaccine candidates in this model.

5. Non-human primate models

Douglas Reed, Associate Professor of Immunology at the University of Pittsburgh, briefly outlined the history of non-human primate (NHP) influenza model development, which has largely settled on the use of healthy adult rhesus and cynomolgus macaques. He noted that although these NHP species typically develop mild clinical signs following infection with seasonal influenza viruses, they poorly model human infection with H5N1 avian strains. Nearly all NHP influenza models employ intranasal or intratracheal challenge which, due to differences in sialic receptor expression, favors upper respiratory tract infection. The inability to adequately model H5N1 infection in NHPs could be due to the fact that seasonal flu strains preferentially infect the upper respiratory tract, while H5N1 avian strains mainly deposit in the lower respiratory tract. Dr. Reed and colleagues undertook a project to improve the NHP avian flu infection model by inducing a lower respiratory tract infection through small particle aerosol challenge [40] . This study demonstrated that small particle aerosol challenge of cynomolgus macaques with an H5N1 avian strain results in a severe acute infection defined by fever, depressed respiration, evidence of cytokine storm, and rapid uniform lethality. Further studies using varying particle size and prolonging the disease course are needed to refine this model to better simulate human infection with H5N1.

Todd Suscovich, Research Specialist at the Ragon Institute of MGH, MIT and Harvard, spoke about the potential of systems serology approaches to identify immunologic correlates and mechanisms of humoral protection when traditional measures, such as antibody titers and neutralization, are inadequate predictors of protective immunity. He noted that the ability of a vaccine to elicit a high neutralizing titer, although generally accepted as a major indicator of protective efficacy, does not often adequately predict protection. The systems serology approach can be used to comprehensively characterize polyclonal antibody responses through network analysis of antibody features and Fc-mediated functional profiles elicited by a vaccine. This approach offers a potentially powerful tool to compare antibody profiles between different vaccine groups or populations, including between NHPs and humans. A few noted caveats include the limited information available on Fc effector function for NHP species other than rhesus and cynomolgus macaques, key differences in IgG conservation across subclasses, and Fcc receptor diversity in macaques compared with humans.

Kathleen Engelman, Program Director at the University of Massachusetts Medical School, described the NIH-funded NHP Reagent Resource Program [41] which seeks to facilitate the optimal use of NHP models in biomedical research. This resource provides GMP-grade antibodies to academic investigators for use in NHP studies requiring in vitro diagnostics, reference reagents for immune assays, and to selectively block or deplete immune effector function to better characterize immune responses to pathogenic agents or vaccines. This resource has developed antibody platforms including engineered antibodies that can finely modulate immune effector function, bispecific antibodies, and modifications to immunoglobulin glycosylation to enhance effector function. Dr. Engelman indicated that the resource currently features rhesus macaque reagents, but also highlighted NHPRR efforts to develop antibody resources specific for several species of New World primate.

Meeting participants agreed that there are multiple advantages offered by NHP models such as respiratory architecture, susceptibility to infection in the absence of uniform lethality, and the availability of technologies to track upper and lower respiratory tract infection over time in the same animal. Participants noted that there are more immunological reagents available for NHPs than for any species other than mice, allowing for a more profound characterization of immune responses to infection or vaccination. In addition, the lifespan of these animals permits evaluation of age-diverse infection models from neonate to elderly that may more

closely approximate human infection/vaccination in these age groups. Despite these advantages, there are a few important drawbacks that must be considered, in particular cost, availability and care requirements. The purchase price for NHPs is substantially greater than for other relevant models: up to five times higher than swine, more than ten times higher than ferrets and more than one-hundred times higher than small rodent species. Due to current demand, locating and acquiring NHPs of appropriate size, age, and immune status has become increasingly difficult and can present limitations to study design and delays in study initiation. Finally, NHPs have care requirements that exceed those of smaller model species. For example, stress reduction through social housing and enrichment activities is critical to the well-being of NHP species, but can place demands on facility capacity and staff capability that many institutions cannot meet. Participants acknowledged that investigation of viral transmission is warranted in the NHP model, as is further evaluation of the aerosol challenge model.

6. Panel 1 discussion

Following in-depth discussions on the attributes and challenges of each animal model, workshop participants then considered pre-clinical animal models more broadly in the context of influenza vaccine development. Specifically, they discussed how animal models could be optimized to better predict vaccine safety and efficacy, and how this information could be used to inform the prioritization and down-selection of promising candidates.

Hana Golding, Chief of the FDA Laboratory of Retrovirus Research, FDA Center for Biologics Evaluation and Research, reminded workshop participants that the FDA will evaluate new universal influenza vaccine candidates following the same general pathway used for all vaccines. Vaccines must demonstrate safety and efficacy in clinical trials, and researchers must consider similarities between the immune responses measured in their animal model and those of humans. Therefore, product characterization and formulation should be similar to those for the intended human vaccine product. The FDA will additionally consider the following questions: Will the dosing and administration protocol be the same? Will the formulation need to change between animal and human studies? Does an adjuvant work the same in animals as predicted in humans? How does the time interval differ between animal models and optimal immune responses in humans? Dr. Golding also noted that although animal models are available to evaluate general safety concerns, a gap remains in the ability to use current animal models to predict potential rare adverse effects. Finally, new assays measuring biological activities of antibodies will require bridging between animals and humans. In vitro and in vivo assays should be selected carefully to ensure pre-clinical data will accurately inform clinical findings.

Armen Donabedian, Scientific Technical Advisor and Chief of the Vaccine Advanced Development Branch, Influenza and Emerging Diseases Division of BARDA, described BARDA's mission and role in universal influenza vaccine development. BARDA assists in pandemic preparedness by supporting the advanced development and licensure of medical countermeasures against chemical, biological, radiological, and nuclear (CBRN) threats. For influenza vaccines, BARDA must balance investments between efforts to improve the speed and scalability of vaccine manufacturing and efforts to support clinical development of improved, broadly-protective vaccines that could be deployed during a pandemic. BARDA is most interested in animal models that can accurately identify candidates ready and suitable for advanced development. Dr. Donabedian highlighted the opportunity to develop a standardized data package to aid in the prioritization of candidates. The package could include data on safety, efficacy, formulation and mechanism of action. Useful information could also include data on the protection of vaccinated animal models against a defined panel of viruses representing circulating contemporary strains as well as those with pandemic potential. Head-to-head comparisons showing improved benefits of a candidate over other licensed vaccines is also of interest. Like Dr. Golding, Dr. Donabedian emphasized that efficacy data in animal models should reflect the vaccine regimen and route of inoculation proposed for clinical use, and he reemphasized the value of animal models in defining correlates of protection and predicting how candidates will perform in advanced clinical trials.

Mario Barro, Associate Vice President and Head of FluNXT Advanced Research at Sanofi Pasteur, provided an industry perspective. Sanofi Pasteur has established an independent unit called FluNXT to develop advanced analytics and computational structural biology-based approaches for vaccine R&D. FluNXT is using these techniques to integrate clinical and pre-clinical data from animal models to better elucidate complex findings in human immune responses. Individual responses to standard of care vaccination are diverse, and it is important to define precise targets for future vaccine improvements. Their iterative approach allows for refinement of translational studies and better connects animal findings to observations from human subjects.

Although workshop participants agreed that using animal models to predict rare adverse effects is a significant challenge, panelists stated that animal models can be used to identify some safety concerns early in development. For example, tissue tropism can be defined during the early stages of vaccine development. Participants agreed that vaccine development efforts are based on careful risk/benefit considerations and that animal models need to be optimized to help understand these trade-offs. Adjuvants also are an area of opportunity for further animal model development. Adjuvants need to demonstrate a true added benefit to a vaccine formulation and must be tested with the specific vaccine candidate that will eventually be considered for licensure. However, depending on the mode of action and the presence, distribution and specificity of the corresponding innate immune receptor, some adjuvants may not exert the same biological and immunological activities in specific animal model, so care must be taken in both adjuvant and animal model selection to improve predictability of benefit/risk in humans. Influenza animal models also are needed to better understand risks and benefits in specific populations such as pregnant women and immunocompromised individuals.

There was general agreement that any candidate considered for advanced development needs to show clear superiority to licensed vaccines to garner funder and investor interest. However, concern was raised over the possibility that animal models might falsely down-select promising candidates. Dr. Barro responded that, for this reason, it is important to integrate and examine all the available data to assess the full picture. A more holistic evaluation can also help the community to understand why certain approaches may not be superior in certain animal models, to pinpoint where and why candidates are likely to fail, and to design studies to understand and address those findings.

7. Summary

The second panel discussion, led by representatives from each of the animal model sessions, summarized the benefits and limitations of existing influenza animal models, which are noted in each of the animal model sessions above and summarized in a recent comprehensive review on animal models for influenza viruses [42] . The panel concluded by summarizing the ongoing tool development and model refinement efforts for each of the influenza

Table 1
Ongoing tool development & model refinement for existing influenza animal models.

Animal	Ongoing tool development and model refinement
Mice	ff Use of transgenic tools to "humanize" mice by adding human host or immune genes ff Use of CC mice to introduce genetic diversity within inbred mouse lines [5] ff Reconstitution of laboratory mice with a complex microbiome ("dirty" mice)
Hamsters	ff Use of transgenic tools to "humanize" hamsters by adding human host or immune genes ff Use of Syrian hamsters for pathogenicity studies of recent H3N2 viruses and other influenza strains [11]
Ferrets	ff Use of transgenic tools to "humanize" ferrets by adding human host or immune genes ff Concentrated efforts to generate immune reagents [43] ff Development of models for pregnant, immunocompromised, and diet-induced obese ferrets [21]
Swine	ff Use of transgenic tools to "humanize" pigs by adding human host or immune genes ff International efforts to develop a 'Swine Immune Toolkit' that provides new immune reagents, mAbs, assays, and protocols for swine health, vaccine and disease studies for widespread use by the community [32,33] ff Use of the MHC homozygous inbred Babraham pig as a model to characterize local lung cellular immune responses to influenza [34,44]
NHPs	ff Variation of aerosol particle size during infection of cynomolgus macaques to better simulate human infection with H5N1 [40] ff Generation and provision of GMP-grade antibodies for use in NHP studies requiring in vitro diagnostics, reference reagents for immune assays, and to selectively block or deplete immune effector function to better characterize immune responses to pathogenic agents or vaccines [41]

animal models that were discussed at length during the workshop (Table 1).

To conclude the workshop, Alan Embry, Chief of the Respiratory Diseases Branch at NIAID re-emphasized the value of animal models in furthering the development of universal influenza vaccines, a key component of NIAID's universal influenza vaccine strategic plan [2]. He emphasized the importance of: (1) considering the strengths and weaknesses of each model; (2) considering the specific research questions being addressed; (3) assessing the role of pre-existing immunity and the effect of sequential vaccination/infection in animal models; (4) performing iterative studies between humans and animals to evaluate immune mechanisms and improve predictive value of models; and (5) utilizing more than one animal model to address all aspects of a novel vaccine candidates. As an outcome of this workshop, NIAID has published a funding opportunity to solicit applications that aim to improve existing animal models or develop novel animal models that more accurately represent influenza immunity in humans, with an emphasis on increasing the predictive value of models for evaluating novel universal influenza vaccines (https://grants.nih.gov/grants/guide/pa-files/PAR-19–248.html, https://grants.nih.gov/grants/guide/pa-files/PAR-19–247.html). Additionally, NIAID supports the development of validated reagents and assays to improve understanding of ferret innate and adaptive immune responses through a number of resources, including the CEIRSnetwork [21] (Supplementary Tables 1–5), BEI Resources Repository (https://www.niaid.nih.gov/research/bei-resources-repository), and the NIH Tetramer Core Facility (http://tetramer.yerkes.emory.edu/). Lastly, NIH supports the development and refinement of animal models for studying human disease through targeted funding opportunities. Such targeted funding opportunities can be located on NIAID's funding opportunities website (https://www.niaid.nih.gov/grants-contracts/find-funding-opportunity) as well as the NIH Office of Research Infrastructure Programs website (https://orip.nih.gov/funding-opportunities-list), and include PAR-19-247 and PAR-19-248, Research Projects to Improve the Predictive Value of Animal Models in Recapitulating Human Immunity to Influenza Infection and Vaccination.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This report is the summary of the collective views from the meeting participants and does not necessarily reflect the views of NIAID.

Acknowledgements

We thank all speakers for their presentations and manuscript edits, and Amanda Coleman for critical reviews and edits.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2020.01.101.

References

[1] Paules CI, Marston HD, Eisinger RW, Baltimore D, Fauci AS. The pathway to a universal influenza vaccine. Immunity 2017;47:599–603. https://doi.org/10.1016/j.immuni.2017.09.007.
[2] Erbelding EJ,Post DJ,Stemmy EJ,Roberts PC,Augustine AD, Ferguson S,et al. A universal influenza vaccine: the strategic plan for the national institute of allergy and infectious diseases. J Infect Dis 2018;218:347–54. https://doi.org/10.1093/infdis/jiy103.
[3] D'Alessio F, Koopman G, Houard S, Remarque EJ,Stockhofe N, Engelhardt OG. Workshop report: experimental animal models for universal influenza vaccines. Vaccine 2018;36:6895–901. https://doi.org/10.1016/j.vaccine.2018.10.024.
[4] Churchill GA, Airey DC, Allayee H, Angel JM, Attie AD, Beatty J, et al. The collaborative cross, a community resource for the genetic analysis of complex traits. Nat Genet 2004;36:1133–7. https://doi.org/10.1038/ng1104-1133.
[5] Ferris MT, Aylor DL, Bottomly D, Whitmore AC, Aicher LD, Bell TA, et al. Modeling host genetic regulation of influenza pathogenesis in the collaborative cross. PLoS Pathog 2013;9:e1003196. https://doi.org/10.1371/journal.ppat.1003196.
[6] Reese TA, Bi K, Kambal A, Filali-Mouhim A, Beura LK, Bürger MC, et al. Sequential infection with common pathogens promotes human-like immune gene expression and altered vaccine response. Cell Host Microbe 2016;19:713–9. https://doi.org/10.1016/j.chom.2016.04.003.
[7] Rosshart SP, Vassallo BG, Angeletti D, Hutchinson DS, Morgan AP, Takeda K, et al. Wild mouse gut microbiota promotes host fitness and improves disease resistance. Cell 2017;171(1015–1028):. https://doi.org/10.1016/j.cell.2017.09.016 e13.
[8] Beura LK, Hamilton SE, Bi K, Schenkel JM, Odumade OA, Casey KA, et al. Normalizing the environment recapitulates adult human immune traits in laboratory mice. Nature 2016;532:512–6. https://doi.org/10.1038/nature17655.
[9] Masopust D, Sivula CP,Jameson SC.Of Mice, dirty mice, and men: using mice to understand human immunology. J Immunol 2017;199:383–8. https://doi.org/10.4049/jimmunol.1700453.
[10] Brodin P, Jojic V, Gao T, Bhattacharya S,Angel CJL,Furman D, et al. Variation in the human immune system is largely driven by non-heritable influences. Cell 2015;160:37–47. https://doi.org/10.1016/j.cell.2014.12.020.
[11] Iwatsuki-Horimoto K, Nakajima N, Ichiko Y, Sakai-Tagawa Y, Noda T, Hasegawa H, et al. Syrian hamster as an animal model for the study of human influenza virus infection. J Virol 2017. https://doi.org/10.1128/jvi.01693-17. JVI.01693-17.
[12] Tchitchek N, Safronetz D, Rasmussen AL, Martens C, Virtaneva K, Porcella SF, et al. Sequencing, annotation and analysis of the syrian hamster (Mesocricetus auratus) transcriptome. PLoS One 2014;9:e112617. https://doi.org/10.1371/journal.pone.0112617.
[13] Ying B, Toth K, Spencer JF,Aurora R, Wold WSM. Transcriptome sequencing and development of an expression microarray platform for liver infection in adenovirus type 5-infected Syrian golden hamsters. Virology 2015;485:305–12. https://doi.org/10.1016/j.virol.2015.07.024.
[14] Fan Z, Li W, Lee SR,Meng Q, Shi B, Bunch TD, et al. Efficient gene targeting in golden Syrian hamsters by the CRISPR/Cas9 system. PLoS One 2014;9:e109755. https://doi.org/10.1371/journal.pone.0109755.

[15] Rosen BH, Chanson M, Gawenis LR, Liu J, Sofoluwe A, Zoso A, et al. Animal and model systems for studying cystic fibrosis. J Cyst Fibros 2018;17:S28–34. https://doi.org/10.1016/j.jcf.2017.09.001.
[16] Johnson MB, Sun X, Kodani A, Borges-Monroy R, Girskis KM, Ryu SC, et al. Aspm knockout ferret reveals an evolutionary mechanism governing cerebral cortical size. Nature 2018;556:370–5. https://doi.org/10.1038/s41586-018-0035-0.
[17] Sun X, Sui H, Fisher JT, Yan Z, Liu X, Cho H-J, et al. Disease phenotype of a ferret CFTR-knockout model of cystic fibrosis. J Clin Invest 2010;120:3149–60. https://doi.org/10.1172/JCI43052.
[18] Sun X, Yi Y, Yan Z, Rosen BH, Liang B, Winter MC, et al. In utero and postnatal VX-770 administration rescues multiorgan disease in a ferret model of cystic fibrosis. Sci Transl Med 2019;11:eaau7531. https://doi.org/10.1126/scitranslmed.aau7531.
[19] Yu M, Sun X, Tyler SR, Liang B, Swatek AM, Lynch TJ, et al. Highly efficient transgenesis in ferrets using CRISPR/Cas9-mediated homology-independent insertion at the ROSA26 locus. Sci Rep 2019;9:1971. https://doi.org/10.1038/s41598-018-37192-4.
[20] Cobey S, Hensley SE. Immune history and influenza virus susceptibility. Curr Opin Virol 2017;22:105–11. https://doi.org/10.1016/j.coviro.2016.12.004.
[21] Albrecht RA, Liu W-C, Sant AJ, Tompkins SM, Pekosz A, Meliopoulos V, et al. Moving forward: recent developments for the ferret. Biomed Res Model MBio 2018;9. https://doi.org/10.1128/mBio.01113-18.
[22] Rajao DS, Vincent AL. Swine as a model for influenza A virus infection and immunity. ILAR J 2015;56:44–52. https://doi.org/10.1093/ilar/ilv002.
[23] Rothkötter H-J. Anatomical particularities of the porcine immune system–a physician's view. Dev Comp Immunol 2009;33:267–72. https://doi.org/10.1016/j.dci.2008.06.016.
[24] Saalmüller A, Gerner W. The immune system of swine. Encycl Immunobiol 2016:538–48. https://doi.org/10.1016/B978-0-12-374279-7.12019-3. Elsevier.
[25] Mair KH, Sedlak C, Käser T, Pasternak A, Levast B, Gerner W, et al. The porcine innate immune system: an update. Dev Comp Immunol 2014;45:321–43. https://doi.org/10.1016/j.dci.2014.03.022.
[26] Butler JE, Wertz N, Sinkora M. Antibody repertoire development in swine. Annu Rev Anim Biosci 2017;5:255–79. https://doi.org/10.1146/annurev-animal-022516-022818.
[27] Gerner W, Talker SC, Koinig HC, Sedlak C, Mair KH, Saalmüller A. Phenotypic and functional differentiation of porcine αb T cells: current knowledge and available tools. Mol Immunol 2015;66:3–13. https://doi.org/10.1016/j.molimm.2014.10.025.
[28] Sandbulte M, Spickler A, Zaabel P, Roth J. Optimal use of vaccines for control of influenza A virus in swine. Vaccines 2015;3:22–73. https://doi.org/10.3390/vaccines3010022.
[29] Dhakal S, Renu S, Ghimire S, Shaan Lakshmanappa Y, Hogshead BT, Feliciano-Ruiz N, et al. Mucosal immunity and protective efficacy of intranasal inactivated influenza vaccine is improved by chitosan nanoparticle delivery in pigs. Front Immunol 2018;9:934. https://doi.org/10.3389/fimmu.2018.00934.
[30] Hiremath J, Kang K, Xia M, Elaish M, Binjawadagi B, Ouyang K, et al. Entrapment of H1N1 influenza virus derived conserved peptides in PLGA nanoparticles enhances T cell response and vaccine efficacy in pigs. PLoS One 2016;11:e0151922. https://doi.org/10.1371/journal.pone.0151922.
[31] Dhakal S, Goodman J, Bondra K, Lakshmanappa YS, Hiremath J, Shyu D-L, et al. Polyanhydride nanovaccine against swine influenza virus in pigs. Vaccine 2017;35:1124–31. https://doi.org/10.1016/j.vaccine.2017.01.019.
[32] Dawson HD, Lunney JK. Porcine cluster of differentiation (CD) markers 2018 update. Res Vet Sci 2018;118:199–246. https://doi.org/10.1016/j.rvsc.2018.02.007.
[33] US-UK Collaborative: Swine Immune Toolkit: Development of new immune reagents for swine health, vaccine and disease studies – AGRICULTURAL RESEARCH SERVICE; n.d. https://reeis.usda.gov/web/crisprojectpages/1005670-us-uk-collaborative-swine-immune-toolkit-development-of-new-immune-reagents-for-swine-health-vaccine-and-disease-studies.html [accessed December 26, 2018].
[34] Schwartz JC, Hemmink JD, Graham SP, Tchilian E, Charleston B, Hammer SE, et al. The major histocompatibility complex homozygous inbred Babraham pig as a resource for veterinary and translational medicine. HLA 2018;92:40–3. https://doi.org/10.1111/tan.13281.
[35] Powell TJ, Silk JD, Sharps J, Fodor E, Townsend ARM. Pseudotyped influenza A virus as a vaccine for the induction of heterotypic immunity. J Virol 2012;86:13397–406. https://doi.org/10.1128/JVI.01820-12.
[36] Holzer B, Morgan SB, Matsuoka Y, Edmans M, Salguero FJ, Everett H, et al. Comparison of heterosubtypic protection in ferrets and pigs induced by a single-cycle influenza vaccine. J Immunol 2018;200:4068–77. https://doi.org/10.4049/jimmunol.1800142.
[37] Tungatt K, Dolton G, Morgan SB, Attaf M, Fuller A, Whalley T, et al. Induction of influenza-specific local CD8 T-cells in the respiratory tract after aerosol delivery of vaccine antigen or virus in the Babraham inbred pig. PLoS Pathog 2018;14:e1007017. https://doi.org/10.1371/journal.ppat.1007017.
[38] Rajao DS, Anderson TK, Gauger PC, Vincent AL. Pathogenesis and vaccination of influenza A virus in swine. Curr Top Microbiol Immunol 2014;385:307–26. https://doi.org/10.1007/82_2014_391.
[39] Iwatsuki-Horimoto K, Nakajima N, Shibata M, Takahashi K, Sato Y, Kiso M, et al. The microminipig as an animal model for influenza A virus infection. J Virol 2017;91. https://doi.org/10.1128/JVI.01716-16.
[40] Wonderlich ER, Swan ZD, Bissel SJ, Hartman AL, Carney JP, O'Malley KJ, et al. Widespread virus replication in alveoli drives acute respiratory distress syndrome in aerosolized H5N1 influenza infection of macaques. J Immunol 2017;198:1616–26. https://doi.org/10.4049/jimmunol.1601770.
[41] NIH Nonhuman Primate Reagent Resource; n.d. http://www.nhpreagents.org/nhp/default.aspx [accessed January 10, 2020].
[42] Margine I, Krammer F. Animal models for influenza viruses: implications for universal vaccine development. Pathog (Basel, Switzerland) 2014;3:845–74. https://doi.org/10.3390/pathogens3040845.
[43] NIAID CEIRS– CEIRSReagents; n.d. http://www.niaidceirs.org/resources/ceirs-reagents/ [accessed February 19, 2019].
[44] Crank MC, Gordon IJ, Yamshchikov GV, Sitar S, Hu Z, Enama ME, et al. Phase 1 study of pandemic H1 DNA vaccine in healthy adults. PLoS One 2015;10:e0123969. https://doi.org/10.1371/journal.pone.0123969.

Assessing the Risks Posed by SARS CoV-2 in and via North American Bats
A Rapid Decision and Risk Assessment

Introduction to Expert Elicitation for an Expert Panel

Evan Grant*, Mike Runge
U.S. Geological Survey Patuxent Wildlife Research Center
*Correspondence: ehgrant@usgs.gov
09 April 2020

Introduction

The novel betacoronavirus, SARS-CoV-2, that has caused a pandemic disease in humans, arose from a mammalian host, possibly Old-World bats in the family *Rhinolophidae*; the closest known virus discovered in wildlife was found in a horseshoe bat (*Rhinolopus affinis*) from Yunnan province in China (Zhou et al. 2000). No SARS-related betacoronaviruses have yet been identified in New-World bats, but a different type of betacoronavirus has been identified in a New-World bat from Mexico (Anthony et al. 2013). This raises an important question about whether North and South American bats could be vulnerable to infection with SARS-CoV-2, via contact with humans, which in turn raises questions about whether there may be reciprocal spread to humans via a bat reservoir. This inquiry is designed to be a rapid assessment of the risk of transmission of SARS-CoV-2 from humans to North American bats, the management contexts in which this risk might be relevant, and an assessment of possible mitigation actions that may be implemented by those who come into contact with bats or their habitats.

Justification for an expert elicitation

Ideally, we would obtain parameter estimates from empirical data and associated mathematical models. Because these information are unavailable and time is of the essence for decision makers, we aim to use an expert panel to elicit parameter values with associated uncertainty, using techniques of expert judgment that utilize best available scientific information, account for uncertainty, and reduce bias (Morgan 2014, Sutherland and Burgman 2015).

Expert elicitation is a formal, structured process of obtaining expert judgment for specific questions. An expert is someone who possesses substantive information on a particular topic that is not widely known by others. We know that experts have knowledge, often privileged knowledge, that accrues as a result of their research and experience, even about processes for which data have not been collected. The question is how to extract that knowledge accurately and precisely. Expert judgment is a quantitative expression of an expert's belief based on knowledge and experience; it is an informed belief. Expert elicitation can provide improved information over single-expert inquiry when a diverse group of experts is asked to provide estimates, using a facilitated approach with discrete opportunities for information sharing, provision of estimates, and review of summarized information (Martin et al. 2012). Expert elicitation, when conducted with the same level of rigor as the collection and use of empirical data, can result in reliable predictions (e.g., O'Hagan et al. 2006, Speirs-Bridge et al. 2010, Runge et al. 2011, Martin et al. 2012, Adams-Hosking et al. 2016).

An expert elicitation is governed by specific protocols to avoid inherent biases resulting from cognitive traps. These cognitive traps are shortcuts, or heuristics, that serve us well for simple decisions but result in biased estimates for more complex tasks (O'Hagan 2019). These biases include:

- Availability bias (experts will be influenced by evidence or events that are easily recalled)
- Anchoring bias (experts fail to consider possible values far from an initial estimate)
- Overconfidence (experts tend to underestimate their uncertainty, and make forecasts that are too narrow)
- Representativeness bias (a tendency to think of probabilities related to readily-available examples)
- Motivational bias (an innate desire to further our own interests)

When the number of experts is limited, we would additionally be concerned about small-sample bias.

There are additional biases that arise through the behavior of groups. To some extent, these can be collectively referred to as "groupthink", the tendency for groups to converge too quickly on consensus estimates or decisions and to ignore or forget divergent views that are held my members of the group. In this way, groups of experts can be collectively overconfident, or even biased.

So, the methodological challenge of expert judgment is to reliably extract the desired information from each member of a group of experts, without falling into the cognitive and behavioral biases that can undermine such an exercise. The best practices in an expert judgment approach have evolved by considering this challenge, testing approaches via experiments, and recommending a set of protocols for conducting an expert elicitation.

Steps in an elicitation

We are using a protocol based on a modified Delphi method called the IDEA protocol (Hanea et al. 2017), with the four-point elicitation method (Speirs-Bridge et al. 2010). There are six steps in the process:

1) Select experts
2) Calibrate experts (seed questions and sharing available information)
3) Elicitation of parameter values (4-point method)
4) Summary, review, and discussion (aimed at reducing linguistic uncertainty – relating to the instructions – and sharing insights, not to reach consensus)
5) Experts revise their initial values (if desired)
6) Aggregate information across experts

Steps 3-6 comprise a modified Delphi approach (described below).

Selection of experts

Experts are individuals with specific subject-matter experience and knowledge. Experts should have relevant expertise which may come from formal training and be demonstrated by professional accomplishments such as peer-reviewed publications, familiarity with and

knowledge of the system or related systems, willingness to participate fully and impartially in an elicitation process, and good interpersonal and communication skills (Ayyub 2001, Fazey et al., 2006).

Groups of experts have been found to perform as well (in terms of providing information close to the true empirically observed data) as more specific experts (e.g., Burgman et al. 2011). The expert panel should be diverse – possessing knowledge of North American bats, zoonotic disease, and possible mitigation strategies; representing multiple institutions, specialization, and gender. The optimal number of experts for a structured elicitation is between 5 and 12, with decreasing marginal benefit after 12 experts (Hogarth 1978, Hemming et al. 2018).

Calibration questions

Before starting the elicitation concerning the questions of interest, we will provide the expert panel a chance to practice the elicitation methods. We will provide questions that are known (i.e., we have identified values from the literature, but are unlikely to be known precisely by experts). We use these questions to ensure that the instructions are understood by experts, and to allow experts a chance to calibrate their estimates of uncertainty.

Three questions are listed below (see accompanying spreadsheet < BatEE Practice Questions v2.xlsx>). For each question, we ask experts to provide four responses: an estimate that represents your view of the lowest reasonable value; an estimate of the highest reasonable value; an estimate that represents the best central value; and your confidence that the true value lies within the low and high values that you have provided. We have attached a spreadsheet in which you can enter these values; the spreadsheet automatically calculates a probability distribution that represents your uncertainty, as immediate feedback about whether your responses reflect your expert belief. This is a "closed book" exercise (we ask that you do not check this information in books or online). Please return your answers to us; we will use them to provide feedback to the group about your individual and collective accuracy and precision; as a means of allowing you to calibrate your thinking process prior to the elicitation for the questions of central importance.

The calibration questions are:

1) What is the mean forearm length (in centimeters) of an adult little brown bat (*Myotis lucifugus*)?

2) What is the average number of subsequent white-nose syndrome infections resulting from a single infected little brown bat (i.e., R_0)?

3) In a population that has already experienced decline due to WNS, out of 100 adult female little brown bats, how many would you expect to breed in a given year?

Elicitation of parameters using a modified Delphi approach

To generate empirical estimates of each parameter, we use a '4-point' elicitation method. This approach has been shown to reduce overconfidence in experts (Speirs-Bridge et al. 2010) and can generate a quantitative estimate from experts who may be uncomfortable providing estimates. We derive a median and credible interval for each parameter from the following four questions:

1) Realistically, what is the lowest reasonable value for the parameter?
2) Realistically, what is the highest reasonable value for the parameter?
3) Realistically, what is the most likely reasonable value (i.e., your best estimate) for the parameter?
4) How confident are you that the true value is between the lowest and highest values you provided?

We then assume that the most likely value is the median value, and combine the upper and lower estimates and the reported confidence to generate a credible interval.

Experts provide their estimates anonymously, and summaries are provided that maintain anonymity, to avoid biases associated with group thinking and dominant personalities. Experts are encouraged to discuss the information during a facilitated discussion of the summarized data, after which experts have the opportunity to revise any of their estimates.

The modified Delphi sequence (independent-group-independent) is important to preserve the unique insights help by individuals while at the same time allowing the benefit of wisdom to be shared. By asking experts to perform the first estimate independently, their own personal views are captured. By allowing the expert to share and discuss their initial estimates, we can explore whether there is residual linguistic uncertainty that needs to be corrected and we can allow insights to be shared across experts. By allowing the final estimates to be made independently, we guard against dominant voices in the group and retain the diversity of insights among the experts.

Aggregation of information across experts

Following the elicitation, we will aggregate the results to produce a single probability distribution that represents an estimate, with uncertainty, for each parameter. To do this, we will first transform the four-point elicitation results into a probability distribution for each expert. We will then average these probability distributions across experts, with equal weighting. (There are involved methods for weighting experts based on sets of calibration questions, but we are both skeptical of these methods and limited on time).

Literature Cited

Adams-Hosking, C. McBride, M.F., Baxter, G., Burgman, M., de Villiers, D., Kavanagh, R., Lawler, I., Lunney, D., Melzer, A., Menkhorst, P., Molsher, R., Moore, B.D., Phalen, D., Rhodes, J.R., Todd, C., Whisson, D., McAlpine, C.A. (2016) Use of expert knowledge to elicit population trends for the koala (*Phascolarctos cinereus*). *Diversity and Distributions*, 22, 249–262.

Anthony SJ, Ojeda-Flores R, Rico-Chávez O, Navarrete-Macias I, Zambrana-Torrelio CM, Rostal MK, Epstein JH, Tipps T, Liang E, Sanchez-Leon M and others. 2013. Coronaviruses in bats from Mexico. Journal of General Virology 94:1028-1038.

Ayyub, B.M., 2001. Elicitation of expert opinions for uncertainty and risks. CRC press.

Burgman, M. A., McBride, M., Ashton, R., Speirs-Bridge, A., Flander, L., Wintle, B., … Twardy, C. (2011). Expert status and performance. PLoS ONE, 6, 1–7.

Fazey, I., Fazey, J.A., Salisbury, J.G., Lindenmayer, D.B. & Dovers, S. (2006) The nature and role of experiential knowledge for environmental conservation. Environmental Conservation, 33,1–10.

Hanea A, McBride M, Burgman M, Wintle B, Fidler F, Flander L, Twardy C, Manning B, Mascaro S. 2017. I nvestigate D iscuss E stimate A ggregate for structured expert judgement. International Journal of Forecasting 33:267-279.

Hemming, V., T. V. Walshe, A. M. Hanea, F. Fidler, and M. A. Burgman. 2018. Eliciting improved quantitative judgements using the IDEA protocol: a case study in natural resource management. PLoS ONE 13:e0198468.

Hogarth, R. M. 1978. A note on aggregating opinions. Organizational Behavior and Human Performance 21:40–46.

Martin, T. G. et al. Eliciting Expert Knowledge in Conservation Science. Conservation Biology 26, 29–38 (2012).

Morgan, M.G. 2014. Use (and abuse) of expert elicitation in support of decision making for public policy: Proceedings of the National Academy of Sciences of the United States of America 111: 7176–7184.

O'Hagan, A. (2019). Expert Knowledge Elicitation: Subjective but Scientific. Am. Stat., 73, 69–81.

O'Hagan, A., Buck, C. E., Daneshkhah, A., Eiser, J. R., Garthwaite, P. H., Jenkinson, D. J., ... & Rakow, T. (2006). *Uncertain judgements: eliciting experts' probabilities*. John Wiley & Sons.

Runge, M.C., Converse, S.J. & Lyons, J.E. (2011). Which uncertainty? Using expert elicitation and expected value of information to design an adaptive program. *Biol. Conserv.*, 144, 1214–1223.

Speirs-Bridge A, Fidler F, McBride MF, Flander L, Cumming G, Burgman MA. 2010. Reducing overconfidence in the interval judgments of experts. Risk Analysis 30:512-523.

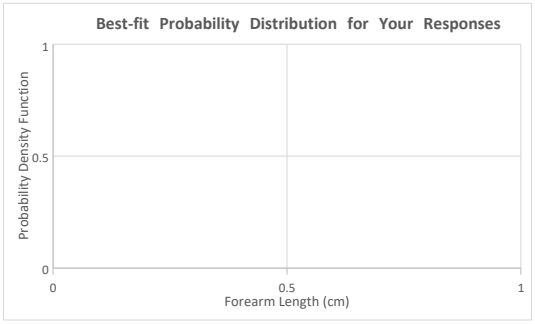
Sutherland WJ, Burgman M. 2015. Policy advice: use experts wisely. Nature 526:317-318.

Zhou P, Yang X-L, Wang X-G, Hu B, Zhang L, Zhang W, Si H-R, Zhu Y, Li B, Huang C-L and others. 2020. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature 579:270-273.

1. What is the mean forearm length (in centimeters) of an adult little brown bat (*Myotis lucifugus*)?

What is the lowest reasonable estimate?
What is the highest reasonable estimate?
What is your central estimate?
How confidence are you that the true mean is between your low and high estimates?
(Confidence should be a number greater than 50 and less than 100)

Error: Confidence should be >50



Assume: normal uncertainty distribution

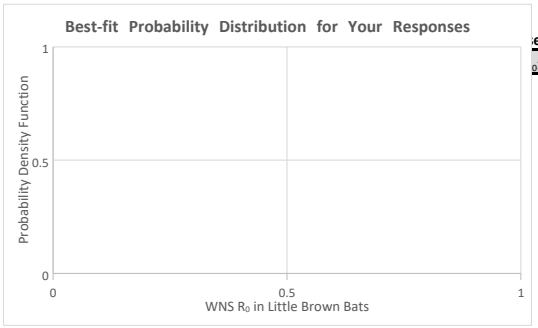
Quantile	IPDF			
0.5	0#DIV/0!	mean	#DIV/0!	
0.5	0#DIV/0!	sd	#DIV/0!	
0.5	0			

x	pdf
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!

2. What is the average number of subsequent white-nose syndrome infections resulting from a single infected little brown bat (i.e., R_0)?

Assume: log-normal uncertainty distribution

		ln(x)	Quantile	IPDF		
What is the lowest reasonable estimate?		Error: R0 must be >0	0.5	0#DIV/0!	mean	#DIV/0!
What is the highest reasonable estimate?		Error: R0 must be >0	0.5	0#DIV/0!	sd	#DIV/0!
What is your central estimate?		Error: R0 must be >0	0.5	0	min	#DIV/0!
How confidence are you that the true mean is between your low and high estimates?		**Error: Confidence should be >50**			max	#DIV/0!
(Confidence should be a number between 50 and 100)					delta	#DIV/0!



ed on your responses:

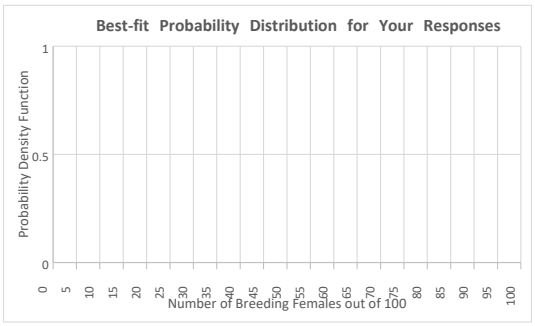
>1)	#DIV/0!

x	ln(x)	pdf
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!

3. In a population that has already experienced decline due to WNS, out of 100 adult female little brown bats, how many would you expect to breed in a given year?

Assume: logit-normal uncertainty distribution

		logit(x)	Quantile	IPDF		
What is the lowest reasonable estimate?		Error: must be between 0 and 100	0.5	0#DIV/0!	mean	#DIV/0!
What is the highest reasonable estimate?		Error: must be between 0 and 100	0.5	0#DIV/0!	sd	#DIV/0!
What is your central estimate?		Error: must be between 0 and 100	0.5	0	min	#DIV/0!
How confidence are you that the true mean is between your low and high estimates?		**Error: Confidence should be >50**			max	#DIV/0!
(Confidence should be a number between 50 and 100)					delta	#DIV/0!



x	logit(x)	pdf
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!

	lo	hi	best	CI	distn	mean	sd
Question 1	0	0	0	0	normal	#DIV/0!	#DIV/0!
Question 2	0	0	0	0	log-normal	#DIV/0!	#DIV/0!
Question 3	0	0	0	0	logit-normal	#DIV/0!	#DIV/0!

Consider a wildlife biologist engaged in research, survey, monitoring, or management (RSM) who is actively shedding SARS-CoV-2 virus (CoV+), performing their routine activities in the absence of any new restrictions, regulations, or protocols.

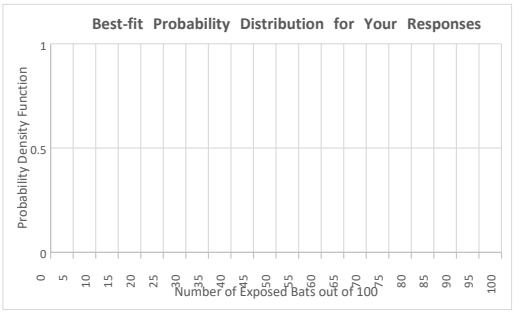
Q1

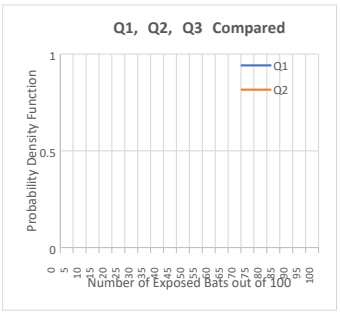
1. If that biologist **directly handles** 100 average little brown bats, how many of those bats do you estimate will be exposed to a sufficient viral dose of SARS-CoV-2 that they could become infected?

	logit(x)
What is the lowest reasonable estimate?	Error: must be between 0 and 100
What is the highest reasonable estimate?	Error: must be between 0 and 100
What is your central estimate?	Error: must be between 0 and 100
How confidence are you that the true mean is between your low and high estimates?	**Error: Confidence should be >50**
(Confidence should be a number between 50 and 100)	

Assume: logit-normal uncertainty distribution

Quantile	IPDF		
0.5	0	#DIV/0!	mean #DIV/0!
0.5	0	#DIV/0!	sd #DIV/0!
0.5	0		min #DIV/0!
			max #DIV/0!
			delta #DIV/0!





x	logit(x)	Npdf	pdf
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Consider a wildlife biologist engaged in research, survey, monitoring, or management (RSM) who is actively shedding SARS-CoV-2 virus (CoV+), performing their routine activities in the absence of any new restrictions, regulations, or protocols.

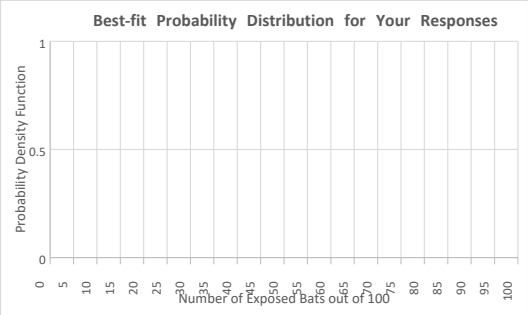
Q2

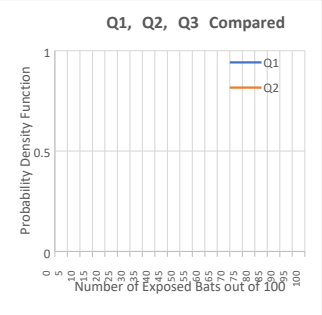
2. If that biologist is **in an enclosed space and within 6 feet** of 100 average little brown bats (but does not handle them), how many of those bats will be exposed to a sufficient viral dose that they could become infected?

		logit(x)
What is the lowest reasonable estimate?		Error: must be between 0 and 100
What is the highest reasonable estimate?		Error: must be between 0 and 100
What is your central estimate?		Error: must be between 0 and 100
How confidence are you that the true mean is between your low and high estimates?		**Error: Confidence should be >50**
(Confidence should be a number between 50 and 100)		

Assume: logit-normal uncertainty distribution

Quantile	IPDF		
0.5	0	#DIV/0!	mean #DIV/0!
0.5	0	#DIV/0!	sd #DIV/0!
0.5	0		min #DIV/0!
			max #DIV/0!
			delta #DIV/0!





x	logit(x)	Npdf	pdf
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Consider a wildlife biologist engaged in research, survey, monitoring, or management (RSM) who is actively shedding SARS-CoV-2 virus (CoV+), performing their routine activities in the absence of any new restrictions, regulations, or protocols.

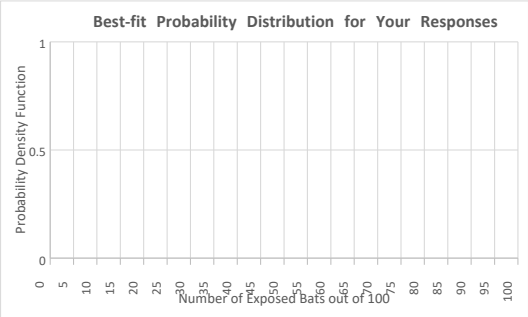
Q3

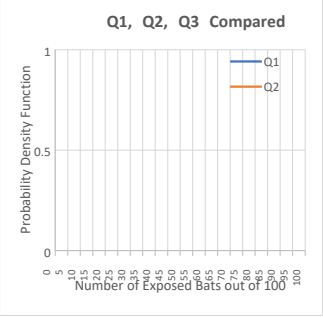
3. If the RSM biologist is **not in an enclosed space but is within a 6-foot proximity** of 100 little brown bats (and does not handle them), how many of those bats will be exposed to a sufficient viral dose that they could become infected?

		logit(x)
What is the lowest reasonable estimate?		Error: must be between 0 and 100
What is the highest reasonable estimate?		Error: must be between 0 and 100
What is your central estimate?		Error: must be between 0 and 100
How confidence are you that the true mean is between your low and high estimates?		**Error: Confidence should be >50**
(Confidence should be a number between 50 and 100)		

Assume: logit-normal uncertainty distribution

Quantile	IPDF		
0.5	0	#DIV/0!	mean #DIV/0!
0.5	0	#DIV/0!	sd #DIV/0!
0.5	0		min #DIV/0!
			max #DIV/0!
			delta #DIV/0!





x	logit(x)	Npdf	pdf
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Now consider a wildlife rehabilitator (WR) who is actively shedding SARS-CoV-2 virus, performing their routine activities in the absence of any new restrictions, regulations, or protocols.

Q4

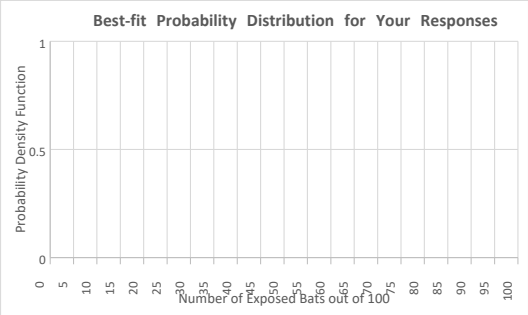
4. If that rehabilitator directly handles 100 average little brown bats, how many of those bats do you estimate will be exposed to a sufficient viral dose of SARS-CoV-2 that they could become infected?

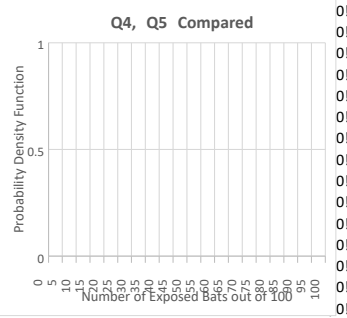
		logit(x)
What is the lowest reasonable estimate?		Error: must be between 0 and 100
What is the highest reasonable estimate?		Error: must be between 0 and 100
What is your central estimate?		Error: must be between 0 and 100
How confidence are you that the true mean is between your low and high estimates?		**Error: Confidence should be >50**
(Confidence should be a number between 50 and 100)		

Assume: logit-normal uncertainty distribution

Quantile	IPDF		
0.5	0	#DIV/0!	
0.5	0	#DIV/0!	
0.5	0		

mean	#DIV/0!
sd	#DIV/0!
min	#DIV/0!
max	#DIV/0!
delta	#DIV/0!





x	logit(x)	Npdf	pdf
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Now consider a wildlife rehabilitator (WR) who is actively shedding SARS-CoV-2 virus, performing their routine activities in the absence of any new restrictions, regulations, or protocols.

Q5

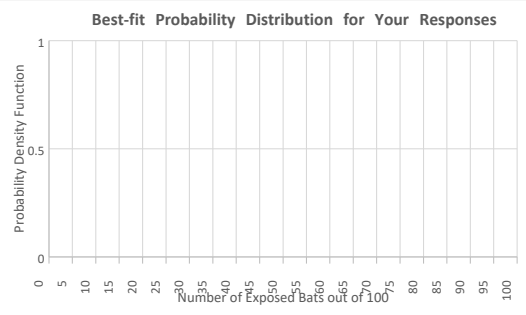
5. If that rehabilitator is **within a 6-foot proximity (whether enclosed or unenclosed)** of 100 average little brown bats but does not handle them, how many of those bats will be exposed to a sufficient viral dose that they could become infected?

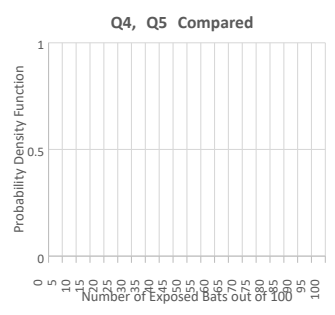
		logit(x)
What is the lowest reasonable estimate?		Error: must be between 0 and 100
What is the highest reasonable estimate?		Error: must be between 0 and 100
What is your central estimate?		Error: must be between 0 and 100
How confidence are you that the true mean is between your low and high estimates?		**Error: Confidence should be >50**
(Confidence should be a number between 50 and 100)		

Assume: logit-normal uncertainty distribution

Quantile	IPDF		
0.5	0	#DIV/0!	
0.5	0	#DIV/0!	
0.5	0		

mean	#DIV/0!
sd	#DIV/0!
min	#DIV/0!
max	#DIV/0!
delta	#DIV/0!





x	logit(x)	Npdf	pdf
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Now consider a wildlife control operator (WC) who is actively shedding SARS-CoV-2 virus, performing their routine activities that involve handling bats, in the absence of any new restrictions, regulations, or protocols. For example, a typical activity might involve capturing bats in a home or trapping and transporting bats from an attic.

Q6

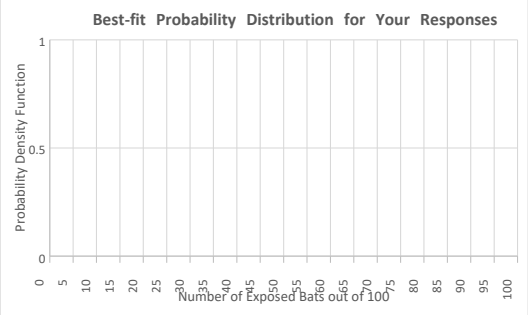
6. If that WC operator **directly handles** 100 average little brown bats, how many of those bats do you estimate will be exposed to a sufficient viral dose of SARS-CoV-2 that they could become infected?

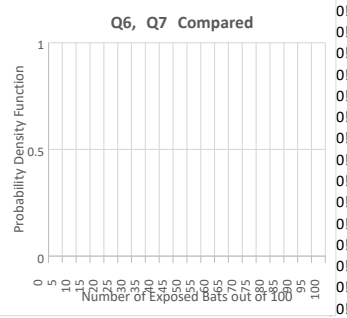
		logit(x)
What is the lowest reasonable estimate?		Error: must be between 0 and 100
What is the highest reasonable estimate?		Error: must be between 0 and 100
What is your central estimate?		Error: must be between 0 and 100
How confidence are you that the true mean is between your low and high estimates?		**Error: Confidence should be >50**
(Confidence should be a number between 50 and 100)		

Assume: logit-normal uncertainty distribution

Quantile	IPDF		
0.5	0	#DIV/0!	
0.5	0	#DIV/0!	
0.5	0		

mean	#DIV/0!
sd	#DIV/0!
min	#DIV/0!
max	#DIV/0!
delta	#DIV/0!





x	logit(x)	Npdf	pdf
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Now consider a wildlife control operator (WC) who is actively shedding SARS-CoV-2 virus, performing their routine activities that involve handling bats, in the absence of any new restrictions, regulations, or protocols. For example, a typical activity might involve capturing bats in a home or trapping and transporting bats from an attic.

Q7

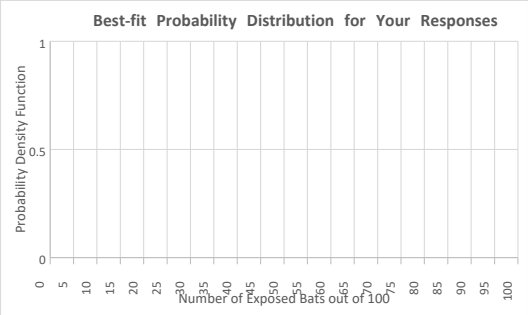
7. If that WC operator is **within a 6-foot proximity (whether enclosed or unenclosed)** of 100 average little brown bats but does not handle them, how many of those bats will be exposed to a sufficient viral dose that they could become infected?

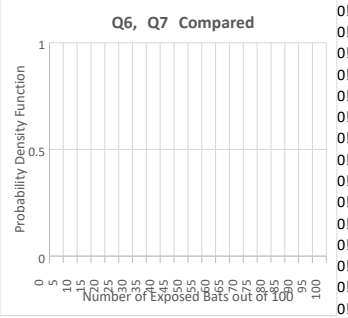
		logit(x)
What is the lowest reasonable estimate?		Error: must be between 0 and 100
What is the highest reasonable estimate?		Error: must be between 0 and 100
What is your central estimate?		Error: must be between 0 and 100
How confidence are you that the true mean is between your low and high estimates? (Confidence should be a number between 50 and 100)		Error: Confidence should be >50

Assume: logit-normal uncertainty distribution

Quantile	IPDF		
0.5	0	#DIV/0!	
0.5	0	#DIV/0!	
0.5	0		

mean	#DIV/0!
sd	#DIV/0!
min	#DIV/0!
max	#DIV/0!
delta	#DIV/0!



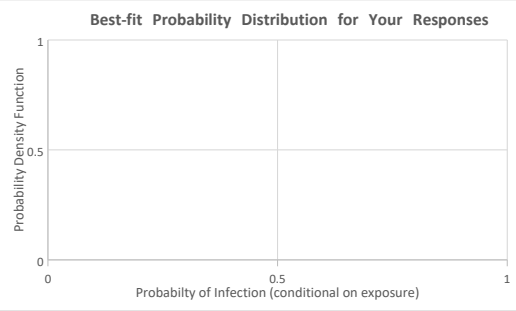


x	logit(x)	Npdf	pdf
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Q8

8. What is the probability that a little brown bat exposed to a sufficient viral dose of SARS-CoV-2 would actually become infected by the virus (that is, sustained viral replication would occur in their tissue)?

		logit(x)
What is the lowest reasonable estimate?		Error: must be between 0 and 1
What is the highest reasonable estimate?		Error: must be between 0 and 1
What is your central estimate?		Error: must be between 0 and 1
How confidence are you that the true mean is between your low and high estimates?		**Error: Confidence should be >50**
(Confidence should be a number between 50 and 100)		



Assume: logit-normal uncertainty distribution

Quantile	IPDF				
0.5	0	#DIV/0!	mean	#DIV/0!	
0.5	0	#DIV/0!	sd	#DIV/0!	
0.5	0		min	#DIV/0!	
			max	#DIV/0!	
			delta	#DIV/0!	

x	logit(x)	Npdf	pdf
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Consider your response to question 1, regarding exposure through handling by RSM scientists. The new guidance and protocols consist of: restriction of fieldwork to people without symptoms and without contact with someone who had symptoms of COVID-19 in the last 14 days; proper training and compliance protocols for the use of PPE; proper use of Tyvek or other dedicated clothing; proper use of an N95 respirator; and proper use of gloves for handling bats. In these questions, please assume that the biologists have proper training, have access to PPE, and are using it appropriately.

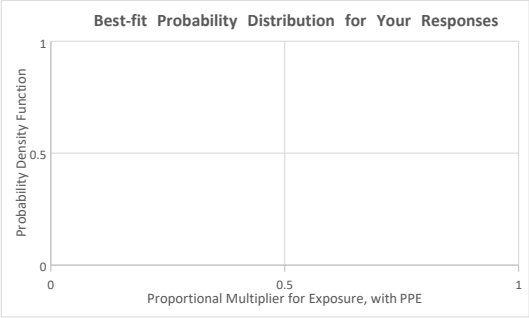
Q9

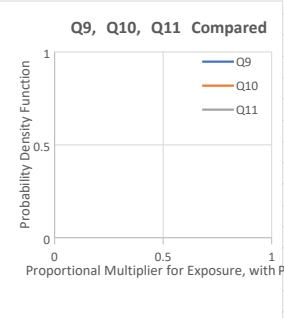
9. By what proportion should this exposure probability be multiplied if the new guidance and protocols are put into place? (Note that a proportion of 1 means there would be no change in exposure probability; a proportion of less than 1 would indicate a reduction in exposure probability; and a proportion of greater than 1 would indicate an increase in exposure probability as a result of such guidance.)

		ln(x)
What is the lowest reasonable estimate?		Error: proportion must be >0
What is the highest reasonable estimate?		Error: proportion must be >0
What is your central estimate?		Error: proportion must be >0
How confidence are you that the true mean is between your low and high estimates?		**Error: Confidence should be >50**
(Confidence should be a number greater than 50 and less than 100)		

Assume: log-normal uncertainty distribution

Quantile	IPDF			
0.5	0	#DIV/0!	mean	#DIV/0!
0.5	0	#DIV/0!	sd	#DIV/0!
0.5	0		min	#DIV/0!
			max	#DIV/0!
			delta	#DIV/0!





x	ln(x)	Npdf	pdf
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Consider your response to question 2, regarding exposure through proximity in an enclosed space by RSM scientists. The new guidance and protocols consist of: restriction of fieldwork to people without symptoms and without contact with someone who had symptoms of COVID-19 in the last 14 days; proper training and compliance protocols for the use of PPE; proper use of Tyvek or other dedicated clothing; proper use of an N95 respirator; and proper use of gloves for handling bats. In these questions, please assume that the biologists have proper training, have access to PPE, and are using it appropriately.

Q10

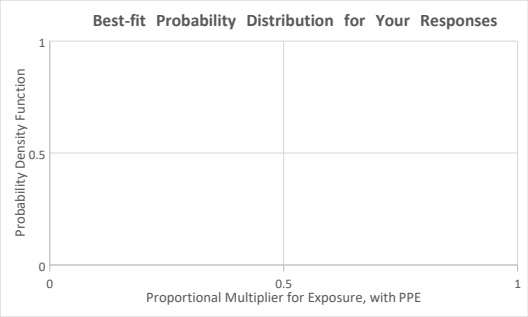
10. By what proportion should this exposure probability be multiplied if the new guidance and protocols are put into place?

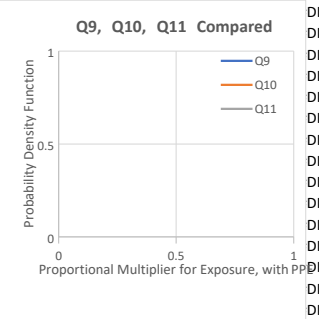
		ln(x)
What is the lowest reasonable estimate?		Error: proportion must be >0
What is the highest reasonable estimate?		Error: proportion must be >0
What is your central estimate?		Error: proportion must be >0
How confidence are you that the true mean is between your low and high estimates?		Error: Confidence should be >50
(Confidence should be a number greater than 50 and less than 100)		

Assume: log-normal uncertainty distribution

Quantile	IPDF		
0.5	0	#DIV/0!	
0.5	0	#DIV/0!	
0.5	0		

mean	#DIV/0!
sd	#DIV/0!
min	#DIV/0!
max	#DIV/0!
delta	#DIV/0!





x	ln(x)	Npdf	pdf
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Consider your response to question 3, regarding exposure through proximity in an *un*enclosed space by RSM scientists. The new guidance and protocols consist of: restriction of fieldwork to people without symptoms and without contact with someone who had symptoms of COVID-19 in the last 14 days; proper training and compliance protocols for the use of PPE; proper use of Tyvek or other dedicated clothing; proper use of an N95 respirator; and proper use of gloves for handling bats. In these questions, please assume that the biologists have proper training, have access to PPE, and are using it appropriately.

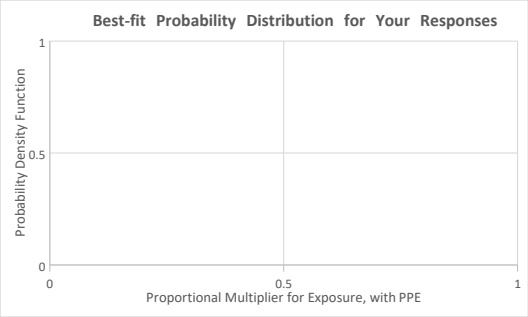
Q11

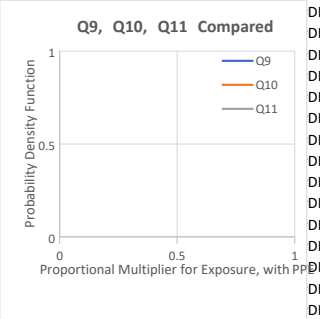
11. By what proportion should this exposure probability be multiplied if the new guidance and protocols are put into place?

		ln(x)
What is the lowest reasonable estimate?		Error: proportion must be >0
What is the highest reasonable estimate?		Error: proportion must be >0
What is your central estimate?		Error: proportion must be >0
How confidence are you that the true mean is between your low and high estimates?		Error: Confidence should be >50
(Confidence should be a number greater than 50 and less than 100)		

Assume: log-normal uncertainty distribution

Quantile	IPDF		
0.5	0	#DIV/0!	mean #DIV/0!
0.5	0	#DIV/0!	sd #DIV/0!
0.5	0		min #DIV/0!
			max #DIV/0!
			delta #DIV/0!





x	ln(x)	Npdf	pdf
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Open-ended response. Are there reasons to believe that the proportional change in the handling and proximity exposure probabilities for wildlife rehabilitators (WR) and wildlife control operators (WC), owing to the same protocol guidance, would be different than for scientists involved in research, survey, and management (RSM)? Explain.

Q13

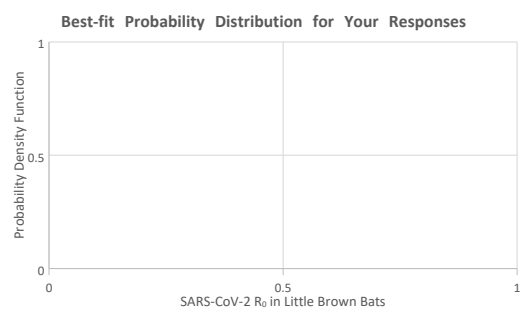
13. What is R_0 for SARS-CoV-2 in little brown bats during the active season? That is, for each infected little brown bat, how many other little brown bats would become infected with the virus? Note that R_0 can be less than 1, in which case you can think of it as the probability that an infected bat will infect one other bat, or it can be greater than 1, in which case each infected bat infects more than one other bat. Note that the spreadsheet calculates from your responses (in cell D13) the probability that R_0 is greater than 1.

		ln(x)
What is the lowest reasonable estimate?		Error: R0 must be >0
What is the highest reasonable estimate?		Error: R0 must be >0
What is your central estimate?		Error: R0 must be >0
How confidence are you that the true mean is between your low and high estimates?		**Error: Confidence should be >50**
(Confidence should be a number between 50 and 100)		

Assume: log-normal uncertainty distribution

Quantile	IPDF		
0.5	0	#DIV/0!	mean
0.5	0	#DIV/0!	sd
0.5	0		min
			max
			delta

#DIV/0!	



Based on your responses:

p(R_0>1)	#DIV/0!

x	ln(x)	Npdf	pdf
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

	lo	hi	best	CI	distn	mean	sd
Question 1	0	0	0	0	logit-normal	#DIV/0!	#DIV/0!
Question 2	0	0	0	0	logit-normal	#DIV/0!	#DIV/0!
Question 3	0	0	0	0	logit-normal	#DIV/0!	#DIV/0!
Question 4	0	0	0	0	logit-normal	#DIV/0!	#DIV/0!
Question 5	0	0	0	0	logit-normal	#DIV/0!	#DIV/0!
Question 6	0	0	0	0	logit-normal	#DIV/0!	#DIV/0!
Question 7	0	0	0	0	logit-normal	#DIV/0!	#DIV/0!
Question 8	0	0	0	0	logit-normal	#DIV/0!	#DIV/0!
Question 9	0	0	0	0	lognormal	#DIV/0!	#DIV/0!
Question 10	0	0	0	0	lognormal	#DIV/0!	#DIV/0!
Question 11	0	0	0	0	lognormal	#DIV/0!	#DIV/0!
Question 13	0	0	0	0	lognormal	#DIV/0!	#DIV/0!

Received: 5 March 2020 | Accepted: 30 March 2020

DOI: 10.1002/jmv.25817

SHORT COMMUNICATION

JOURNAL OF MEDICAL VIROLOGY WILEY

SARS-CoV-2 spike protein favors ACE2 from Bovidae and Cricetidae

Junwen Luan[1] | Xiaolu Jin[1,2] | Yue Lu[1,2] | Leiliang Zhang[1]

[1]Institute of Basic Medicine, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, Shandong, China

[2]School of Medicine and Life Sciences, Shandong Academy of Medical Sciences, University of Jinan, Jinan, Shandong, China

Correspondence
Leiliang Zhang, PhD, Institute of Basic Medicine, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, 250062 Shandong, China.
Email: armzhang@hotmail.com

Funding information
National Key Plan for Research and Development of China, Grant/Award Number: 2016YFD0500300; Shandong Academy of Medical Sciences Grant, Grant/Award Number: 2017-52; Innovation Project of Shandong Academy of Medical Sciences; Academic promotion programme of Shandong First Medical University, Grant/Award Number: 2019LJ001

Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes the recent COVID-19 public health crisis. Bat is the widely believed original host of SARS-CoV-2. However, its intermediate host before transmitting to humans is not clear. Some studies proposed pangolin, snake, or turtle as the intermediate hosts. Angiotensin-converting enzyme 2 (ACE2) is the receptor for SARS-CoV-2, which determines the potential host range for SARS-CoV-2. On the basis of structural information of the complex of human ACE2 and SARS-CoV-2 receptor-binding domain (RBD), we analyzed the affinity to S protein of the 20 key residues in ACE2 from mammal, bird, turtle, and snake. Several ACE2 proteins from Primates, Bovidae, Cricetidae, and Cetacea maintained the majority of key residues in ACE2 for associating with SARS-CoV-2 RBD. The simulated structures indicated that ACE2 proteins from Bovidae and Cricetidae were able to associate with SARS-CoV-2 RBD. We found that nearly half of the key residues in turtle, snake, and bird were changed. The simulated structures showed several key contacts with SARS-CoV-2 RBD in turtle and snake ACE2 were abolished. This study demonstrated that neither snake nor turtle was the intermediate hosts for SARS-CoV-2, which further reinforced the concept that the reptiles are resistant against infection of coronavirus. This study suggested that Bovidae and Cricetidae should be included in the screening of intermediate hosts for SARS-CoV-2.

KEYWORDS

ACE2, Bovidae, Cricetidae, intermediate host, SARS-CoV-2

1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19), which was first reported in Wuhan, Hubei province, China, has caused over 80 422 human infections and more than 2984 deaths (as of 4 March 2020) in China.[1,2] The confirmed cases outside China are increasing, which raised major global concern. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified to be the pathogen of COVID-19. SARS-CoV-2 has joined SARS-CoV and Middle East respiratory syndrome-related coronavirus (MERS-CoV) as another coronavirus that causes severe respiratory disease and human death.[3,4]

The specificity of the interaction between virus and receptor determines its host range for the virus. Spike protein (S) of SARS-CoV-2 has attracted great attention because of its role in receptor binding. Angiotensin-converting enzyme 2 (ACE2) binds to the receptor-binding domain (RBD) of SARS-CoV-2 S protein and functions as a receptor for SARS-CoV-2.[5,6] The origin of SARS-CoV-2 is considered as bat.[6] However, the intermediate host is unknown. Some studies suggest that pangolin is involved in the evolution of SARS-CoV-2.[7,8] Others suggested that snake and turtles are potential intermediate hosts for SARS-CoV-2.[9,10] In this study, we compared the key amino acids (AAs) in ACE2 from different species for the binding ability to RBD. On the basis of

 © 2020 Wiley Periodicals, Inc.

potential interaction between S protein and ACE2, it was speculated that SARSCoV-2 preserved the ability to infect Bovidae and Cricetidae but not snake or turtle.

2 | METHODS

2.1 | Sequence analysis of ACE2

A total of 93 ACE2 protein sequences were selected from 85 mammals, 4 birds, 3 turtles, and 1 snake. These ACEs with their corresponding species are listed as follows: hACE2: Homo sapiens (BAB40370.1), RhiACE2: Rhinopithecus roxellana (XP_010364367.2), MacmACE2: Macaca mulatta (NP_001129168.1), MuseACE2: Mustela erminea (XP_032187679.1), CamdACE2: Camelus dromedarius (XP_031301717.1), PlACE2: Procyon lotor (XP_031301717.1), PcACE2: Paguma larvata (AAX63775.1), RmACE2: Rhinolophus macrotis (ADN93471.1), RfACE2: Rhinolophus ferrumequinum (BAH02663.1), RsACE2: Rhinolophus sinicus (ADN93472.1), RlACE2: Rousettus leschenaultii (BAF50705.1), SsACE2: Sus scrofa (NP_001116542.1), MpfACE2: Mustela putorius furo (BAE53380.1), RatACE2: Rattus norvegicus (Q5EGZ1), MmACE2: Mus musculus (Q3URC9), ClfACE2: Canis lupus familiaris (J9P7Y2), FcACE2: Felis catus (A0A384DV19), MjACE2: Manis javanica (XP_017505752.1), RpACE2: Rhinolophus pearsonii (ABU54053.1), PvACE2: Pteropus vampyrus (XP_011361275.1), PoaACE2: Pongo abelii (NP_001124604.1), EcACE2: Equus caballus (F6V9L3), BtACE2: Bos taurus (Q58DD0), PtACE2: Pan troglodytes (A0A2J8KU96), OraACE2: Ornithorhynchus anatinus (F7FDA2), OvaACE2: Ovis aries (W5PSB6), PanACE2: Papio Anubis (A0A096N4X9), LaACE2: Loxodonta africana (G3T6Q2), SsdACE2: Sus scrofa domesticus (A0A220QT48), EeACE2: Erinaceus europaeus (A0A1S3APE5), OcACE2: Oryctolagus cuniculus (G1TEF4), NpACE2: Nyctereutes procyonoides (B4XEP4), VvACE2: Vulpes vulpes (A0A3Q7-RAT9), PhcACE2: Phodopus campbelli (C7ECU7), MaACE2: Mesocricetus auratus (C7ECV1), CjACE2: Callithrix jacchus (F7CNJ6), SusACE2: Suricata suricatta (XP_029786256.1), HgACE2: Heterocephalus glaber (A0A0N8EUX7), DoACE2: Dipodomys ordii (A0A1S3GHT7), ItACE2: Ictidomys tridecemlineatus (XP_005316051.3), CpACE2: Cavia porcellus (XP_023417808.1), CgACE2: Cricetulus griseus (A0A061HZ66), ChACE2: Capra hircus (A0A452EVJ5); BibtACE2: Bos indicus x Bos taurus (A0A4W2H3A1), BmACE2: Bos mutus (L8I4I4), NlACE2: Nomascus leucogenys (G1RE79); CsACE2: Chlorocebus sabaeus (A0A0D9RQZ0); MfACE2: Macaca fascicularis (A0A2K5X283); PpACE2: Pan paniscus (A0A2R9BKD8); CaACE2: Cercocebus atys (A0A2K5KSD8); MnACE2: Macaca nemestrina (A0A2K6D1N8); MalACE2: Mandrillus leucophaeus (A0A2K5ZV99); TsACE2: Tarsius syrichta (A0A1U7TY97); PrcACE2: Propithecus coquereli (A0A2K6GHW5); UmACE2: Ursus maritimus (A0A452TT30); OgACE2: Otolemur garnettii (H0WMI5); SbbACE2: Saimiri boliviensis boliviensis (A0A2K6SBD4); CciACE2: Cebus capucinus imitator (A0A2K5PYM0); GggACE2: Gorilla gorilla gorilla (G3QWX4); AnACE2: Aotus nancymaae (A0A2K5DQI6); ChaACE2: Chlorocebus aethiops (Q1LZX8); AmACE2: Ailuropoda melanoleuca (G1MC42); VuACE2: Vombatus ursinus (A0A4X2M679); UaACE2: Ursus americanus (A0A452R1Z9); UahACE2: Ursus arctos horribilis (A0A3Q7TE16); PmACE2: Physeter macrocephalus (A0A2Y9S5T9); LvACE2: Lipotes vexillifer (A0A340Y3Y6); BasACE2: Balaenoptera acutorostrata scammoni (A0A452CBT6); DlACE2: Delphinapterus leucas (A0A2Y9M9H3); TtACE2: Tursiops truncatus (A0A2U4AJL3); NaaACE2: Neophocaena asiaeorientalis asiaeorientalis (A0A341BCI8); CuACE2: Callorhinus ursinus (A0A3Q7N3M7); NsACE2: Neomonachus schauinslandi (A0A2Y9GEI9); TmlACE2: Trichechus manatus latirostris (A0A2Y9E393); ElkACE2: Enhydra lutris kenyoni (A0A2Y9KLV0); ClACE2: Chinchilla lanigera (C7ECU0); MdACE2: Monodelphis domestica (F6WXR7); LpACE2: Lynx pardinus (A0A485NF12); PaACE2: Pipistrellus abramus (C7ECT9); MbACE2: Myotis brandtii (S7N573); DrACE2: Desmodus rotundus (K9INV8); RhpACE2: Rhinolophus pusillus (E2DHI9); RaACE2: Rhinolophus alcyone (A0A0N7IQX6); RlACE2(2): Rhinolophus landeri (A0A0P0IB69); MylACE2: Myotis lucifugus (G1PXH7), GgACE2: Gallus gallus (F1NHR4), ApACE2: Anas platyrhynchos (R0LHX5), MgACE2: Meleagris gallopavo (G1NPB8), CaaACE2: Cathartes aura (A0A091MDI4), OhACE2: Ophiophagus hannah (V8NIH2), CpbACE2: Chrysemys picta bellii (XP_023964517.1), CmACE2: Chelonia mydas (XP_007070561.1); and PsACE2: Pelodiscus sinensis (XP_006122891.1). On the basis of known 20 key sites in human ACE2 interacting with SARSCoV-2 RBD,[11] we analyzed whether these sites were conserved on other ACE2 proteins. Phylogenetic and molecular evolutionary analysis of ACE2 protein was conducted using molecular evolutionary genetics analysis version X (MEGA-X).[12] Phylogenetic tree was generated with Jones-Taylor-Thornton evolutionary model using a maximum-likelihood method.

2.2 | Structure simulation of ACE2-RBD complex

On the basis of the structure of hACE2 with SARSCoV-2 S RBD (PDB: 6LZG), the structure of SARSCoV-2 S and ACE2 from Bos taurus, Cricetulus griseus, Pelodiscus sinensis, and Ophiophagus hannah were simulated by SWISSMODEL online server[13] and analyzed by Chimera software version 1.14.[14]

3 | RESULTS

3.1 | Sequence alignment of ACE2

According to the recently resolved structure of the complex of human ACE2 and SARSCoV-2 RBD, there are 20 key AAs in hACE2 for interacting with RBM.[11] We analyzed those AAs of ACE2 protein from a list of mammals, birds, turtles, and snake, as shown in Table 1. Next, a phylogenetic tree for mammalian ACE2 proteins was constructed by MEGA-X software. There were 16 primates ACE2, 5 Bovidae ACE2, 2 Cricetidae ACE2, and 3 Cetacea ACE2 (Table 1 and Figure 1A), possessing at least 90% (18/20) critical AAs. Pangolin ACE2 preserved only 70% (14/20) AAs. Nearly half of the key residues in turtles (CpbACE2, CmACE2, and PsACE2) and snake (OhACE2) were changed (Table 1). ACE2 from Aves, including Gallus gallus, Anas platyrhynchos, Meleagris gallopavo, and Cathartes aura, only matched 10 to 11 AAs (Table 1).

TABLE 1 Analysis of the key AAs in ACE2 for SARSCoV-2 RBD binding

	AA position																			
ACE2	24	27	28	30	31	34	35	37	38	41	42	45	82	83	330	353	354	355	357	393	Matched AA
hACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
RhiACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
MacmACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
PoaACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
PtACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
PanACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
NlACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
CsACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
MfACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
PpACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
CaACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
MnACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
MalACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
GggACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
ChaACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	20
PrcACE2	Q	T	F	D	K	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	19
BtACE2	Q	T	F	E	K	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	18
OvaACE2	Q	T	F	E	K	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	18
MaACE2	Q	T	F	D	K	Q	E	E	D	Y	Q	L	N	Y	N	K	G	D	R	R	18
CgACE2	Q	T	F	D	K	Q	E	E	D	Y	Q	L	N	Y	N	K	G	D	R	R	18
ChACE2	Q	T	F	E	K	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	18
BibtACE2	Q	T	F	E	K	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	18
BmACE2	Q	T	F	E	K	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	18
PmACE2	Q	T	F	Q	K	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	18
DlACE2	Q	T	F	Q	K	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	18
NaaACE2	Q	T	F	Q	K	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	18
PhcACE2	Q	T	F	D	K	Q	E	E	D	Y	Q	L	N	Y	N	K	E	D	R	R	17
HgACE2	Q	T	F	D	K	Q	E	E	D	Y	Q	L	A	Y	N	K	D	D	R	R	17
ItACE2	L	T	F	D	K	Q	E	E	D	Y	Q	L	A	Y	N	K	G	D	R	R	17
BasACE2	Q	T	F	Q	K	H	E	E	D	Y	R	L	T	Y	N	K	G	D	R	R	17
CamdACE2	L	T	F	E	E	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	16
RlACE2	L	T	F	E	K	T	E	E	D	Y	Q	L	T	Y	K	K	G	D	R	R	16
SsACE2	L	T	F	E	K	L	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	16
FcACE2	L	T	F	E	K	H	E	E	E	Y	Q	L	T	Y	N	K	G	D	R	R	16
SsdACE2	L	T	F	E	K	L	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	16
OcACE2	L	T	F	E	K	Q	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	16
CjACE2	Q	T	F	D	K	H	E	E	D	H	E	L	T	Y	N	K	Q	D	R	R	16
DoACE2	L	T	F	D	N	Q	E	E	D	Y	Q	L	I	Y	N	K	G	D	R	R	16
UmACE2	L	T	F	E	K	Y	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	16

(Continues)

TABLE 1 (Continued)

	AA position																				
ACE2	24	27	28	30	31	34	35	37	38	41	42	45	82	83	330	353	354	355	357	393	Matched AA
SbbACE2	Q	T	F	D	K	H	E	E	D	H	E	L	T	Y	N	K	Q	D	R	R	16
CciACE2	Q	T	F	D	K	H	E	E	D	H	E	L	T	Y	N	K	Q	D	R	R	16
AnACE2	Q	T	F	D	K	H	E	E	D	H	E	L	T	Y	N	K	Q	D	R	R	16
AmACE2	L	T	F	E	K	Y	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	16
UaACE2	L	T	F	E	K	Y	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	16
UahACE2	L	T	F	E	K	Y	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	16
LvACE2	R	T	F	Q	K	H	E	E	D	Y	Q	L	T	F	N	K	G	D	R	R	16
TtACE2	R	T	F	Q	K	R	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	16
LpACE2	L	T	F	E	K	H	E	E	E	Y	Q	L	T	Y	N	K	G	D	R	R	16
MpfACE2	L	T	F	E	K	Y	E	E	E	Y	Q	L	T	Y	N	K	R	D	R	R	15
ClfACE2	L	T	F	E	K	Y	E	E	E	Y	Q	L	T	Y	N	K	G	D	R	R	15
RpACE2	R	T	F	D	K	H	E	E	D	H	E	L	D	Y	N	K	D	D	R	R	15
LaACE2	L	T	F	D	T	Q	E	E	D	Y	Q	L	D	F	N	K	G	D	R	R	15
VvACE2	L	T	F	E	K	Y	E	E	E	Y	Q	L	T	Y	N	K	G	D	R	R	15
CpACE2	Q	T	F	D	E	L	K	E	D	Y	Q	L	A	Y	N	K	N	D	R	R	15
TsACE2	Q	T	F	D	K	Q	E	E	D	H	Q	L	S	Y	N	N	S	D	R	R	15
TmlACE2	L	T	F	D	T	Q	E	E	D	Y	Q	L	N	F	N	K	G	D	R	R	15
ClACE2	Q	T	F	D	N	E	K	E	D	Y	Q	L	A	Y	N	K	D	D	R	R	15
MuseACE2	L	T	F	E	K	Y	E	E	E	Y	Q	L	T	Y	N	K	R	D	R	R	14
PlACE2	L	T	F	E	N	N	E	E	E	Y	Q	L	T	Y	N	K	G	D	R	R	14
MjACE2	E	T	F	E	K	S	E	E	E	Y	Q	L	N	Y	N	K	H	D	R	R	14
PvACE2	L	T	F	E	K	T	E	E	D	Y	Q	L	A	Y	K	K	G	D	R	K	14
EcACE2	L	T	F	E	K	S	E	E	E	H	Q	L	T	Y	N	K	G	D	R	R	14
NpACE2	L	T	F	E	K	Y	E	E	E	Y	Q	L	T	Y	N	R	G	D	R	R	14
OgACE2	Q	T	F	D	N	R	E	E	E	H	Q	L	T	Y	N	K	D	D	R	R	14
VuACE2	R	E	F	E	T	K	E	E	E	Y	Q	L	T	F	N	K	G	D	R	R	14
NsACE2	L	T	F	E	K	Y	E	E	E	Y	Q	L	T	Y	N	K	H	D	R	R	14
ElkACE2	P	T	F	E	K	Y	E	E	E	Y	Q	L	T	Y	N	K	R	D	R	R	14
RhpACE2	L	K	F	N	D	S	E	E	D	Y	Q	L	N	Y	N	K	G	D	R	R	14
RmACE2	E	K	F	D	K	S	K	E	D	Y	E	L	N	Y	K	K	G	D	R	R	13
RsACE2	E	I	F	D	K	T	K	E	D	H	Q	L	N	Y	N	K	G	D	R	R	13
RatACE2	K	S	F	N	K	Q	E	E	D	Y	Q	L	N	F	N	H	G	D	R	R	13
MmACE2	N	T	F	N	N	Q	E	E	D	Y	Q	L	S	F	N	H	G	D	R	R	13
CuACE2	L	T	F	E	K	S	E	E	E	Y	Q	F	T	Y	N	K	H	D	R	R	13
RlACE2(2)	L	T	F	D	D	S	A	E	N	Y	Q	L	N	F	N	K	G	D	R	R	13
PcACE2	L	T	F	E	T	Y	E	Q	E	Y	Q	V	T	Y	N	K	G	D	R	R	12
RfACE2	L	K	F	D	D	S	E	E	N	H	Q	L	N	F	N	K	G	D	R	R	12
SusACE2	L	T	F	E	Q	H	E	Q	E	Y	L	V	A	Y	N	K	G	D	R	R	12

TABLE 1 (Continued)

	AA position																			
ACE2	24	27	28	30	31	34	35	37	38	41	42	45	82	83	330	353	354	355	357	393	Matched AA
MdACE2	D	T	F	D	D	A	K	E	E	H	Q	L	T	Y	N	K	N	D	R	R	12
DrACE2	E	T	F	E	N	T	E	E	E	Y	Q	L	T	Y	N	N	K	D	R	R	12
RaACE2	L	I	F	D	N	S	E	E	N	H	Q	L	N	F	N	K	G	D	R	R	12
OraACE2	E	Q	F	T	Q	K	Q	E	D	Y	Q	L	K	F	N	K	N	D	R	R	11
EeACE2	E	K	F	D	D	R	Q	E	N	Y	E	L	N	Y	N	N	G	D	R	R	11
MbACE2	K	I	F	E	N	S	K	E	D	H	E	L	T	Y	N	K	G	D	R	R	11
MylACE2	K	I	F	E	N	S	A	E	D	H	E	L	T	Y	N	K	G	D	R	R	11
GgACE2	E	T	F	A	E	V	R	E	D	Y	E	L	R	F	N	K	N	D	R	R	11
ApACE2	Q	M	F	A	E	V	R	E	D	Y	E	L	N	F	N	K	N	D	R	R	11
MgACE2	E	T	F	A	E	V	R	E	D	Y	E	L	R	F	N	K	N	D	R	R	11
CpbACE2	E	N	F	S	Q	V	R	E	D	Y	A	L	K	Y	N	K	K	D	R	R	11
CmACE2	E	N	F	S	Q	V	R	E	D	Y	A	L	K	Y	N	K	K	D	R	R	11
PsACE2	E	N	F	S	E	V	Q	E	D	Y	A	L	K	Y	N	K	K	D	R	R	11
PaACE2	E	R	F	V	K	H	E	E	N	H	E	L	G	F	D	K	N	D	R	R	10
CaaACE2	Q	I	F	E	E	P	R	E	N	Y	E	L	S	F	N	K	N	D	R	R	10
OhACE2	…	K	F	E	Q	A	R	T	D	Y	N	I	M	F	N	K	E	D	R	R	9

Note: hACE2, Homo sapiens (BAB40370.1), RhiACE2, Rhinopithecus roxellana (XP_010364367.2), MacmACE2, Macacamulatta (NP_001129168.1), MuseACE2, Mustela erminea (XP_032187679.1), CamdACE2, Camelus dromedarius (XP_031301717.1), PlACE2, Procyonlotor (XP_031301717.1), PcACE2, Pagumalarvata (AAX63775.1), RmACE2, Rhinolophus macrotis (ADN93471.1), RfACE2, Rhinolophus ferrumequinum (BAH02663.1), RsACE2, Rhinolophus sinicus (ADN93472.1), RlACE2, Rousettus leschenaultii (BAF50705.1), SsACE2, Susscrofa (NP_001116542.1), MpfACE2, Mustela putorius furo (BAE53380.1), RatACE2, Rattus norvegicus (Q5EGZ1), MmACE2, Mus musculus (Q3URC9), ClfACE2, Canislupus familiaris (J9P7Y2), FcACE2, Feliscatus (A0A384DV19), MjACE2, Manis javanica (XP_017505752.1), RpACE2, Rhinolophus pearsonii (ABU54053.1), PvACE2, Pteropusvampyrus (XP_011361275.1), PoaACE2, Pongoabelii (NP_001124604.1), EcACE2, Equus caballus (F6V9L3), BtACE2, Bostaurus (Q58DD0), PtACE2, Pan troglodytes (A0A2J8KU96), OraACE2, Ornithorhynchus anatinus (F7FDA2), OvaACE2, Ovis aries (W5PSB6), PanACE2, Papio Anubis (A0A096N4X9), LaACE2, Loxodonta africana (G3T6Q2), SsdACE2, Susscrofadomesticus (A0A220QT48), EeACE2, Erinaceus europaeus (A0A1S3APE5), OcACE2, Oryctolagus cuniculus (G1TEF4), NpACE2, Nyctereutes procyonoides (B4XEP4), VvACE2, Vulpesvulpes (A0A3Q7RAT9), PhcACE2, Phodopus campbelli (C7ECU7), MaACE2, Mesocricetus auratus (C7ECV1), CjACE2, Callithrix jacchus (F7CNJ6), SusACE2, Suricatasuricatta (XP_029786256.1), HgACE2, Heterocephalus glaber (A0A0N8EUX7), DoACE2, Dipodomysordii (A0A1S3GHT7), ItACE2, Ictidomystridecemlineatus (XP_005316051.3), CpACE2, Cavia porcellus (XP_023417808.1), CgACE2, Cricetulus griseus (A0A061HZ66), ChACE2, Capra hircus (A0A452EVJ5); BibtACE2, Bos indicus x Bostaurus (A0A4W2H3A1), BmACE2, Bos mutus (L8I4I4), NlACE2, Nomascus leucogenys (G1RE79); CsACE2, Chlorocebus sabaeus (A0A0D9RQZ0); MfACE2, Macacafascicularis (A0A2K5X283); PpACE2, Pan paniscus (A0A2R9BKD8); CaACE2, Cercocebus atys (A0A2K5KSD8); MnACE2, Macacanemestrina (A0A2K6D1N8); MalACE2, Mandrillus leucophaeus (A0A2K5ZV99); TsACE2, Tarsiussyrichta (A0A1U7TY97); PrcACE2, Propithecus coquereli (A0A2K6GHW5); UmACE2, Ursusmaritimus (A0A452TT30); OgACE2, Otolemur garnettii (H0WMI5); SbbACE2, Saimiriboliviensis boliviensis (A0A2K6SBD4); CciACE2, Cebus capucinus imitator (A0A2K5PYM0); GggACE2, Gorilla gorillagorilla (G3QWX4); AnACE2, Aotus nancymaae (A0A2K5DQI6); ChaACE2, Chlorocebus aethiops (Q1LZX8); AmACE2, Ailuropodamelanoleuca (G1MC42); VuACE2, Vombatusursinus (A0A4X2M679); UaACE2, Ursusamericanus (A0A452R1Z9); UahACE2, Ursusarctoshorribilis (A0A3Q7TE16); PmACE2, Physetermacrocephalus (A0A2Y9S5T9); LvACE2, Lipotesvexillifer (A0A340Y3Y6); BasACE2, Balaenoptera acutorostratascammoni (A0A452CBT6); DlACE2, Delphinapterusleucas (A0A2Y9M9H3); TtACE2, Tursiopstruncatus (A0A2U4AJL3); NaaACE2, Neophocaena asiaeorientalis asiaeorientalis (A0A341BCI8); CuACE2, Callorhinusursinus (A0A3Q7N3M7); NsACE2, Neomonachus schauinslandi (A0A2Y9GEI9); TmlACE2, Trichechus manatuslatirostris (A0A2Y9E393); ElkACE2, Enhydralutriskenyoni (A0A2Y9KLV0); ClACE2, Chinchillalanigera (C7ECU0); MdACE2, Monodelphisdomestica (F6WXR7); LpACE2, Lynx pardinus (A0A485NF12); PaACE2, Pipistrellusabramus (C7ECT9); MbACE2, Myotis brandtii (S7N573); DrACE2, Desmodusrotundus (K9INV8); RhpACE2, Rhinolophuspusillus (E2DHI9); RaACE2, Rhinolophusalcyone (A0A0N7IQX6); RlACE2(2), Rhinolophuslanderi (A0A0P0IB69); MylACE2, Myotis lucifugus (G1PXH7), GgACE2, Gallusgallus (F1NHR4), ApACE2, Anas platyrhynchos (R0LHX5), MgACE2, Meleagrisgallopavo (G1NPB8), CaaACE2, Cathartesaura (A0A091MDI4), OhACE2, Ophiophagushannah (V8NIH2), CpbACE2, Chrysemyspicta bellii (XP_023964517.1), CmACE2, Cheloniamydas (XP_007070561.1); and PsACE2, Pelodiscussinensis (XP_006122891.1).
Abbreviations: AA, amino acid; ACE2, angiotensin-converting enzyme 2; RBD, receptor-binding domain; SARSCoV-2, severe acute respiratory syndrome coronavirus 2.

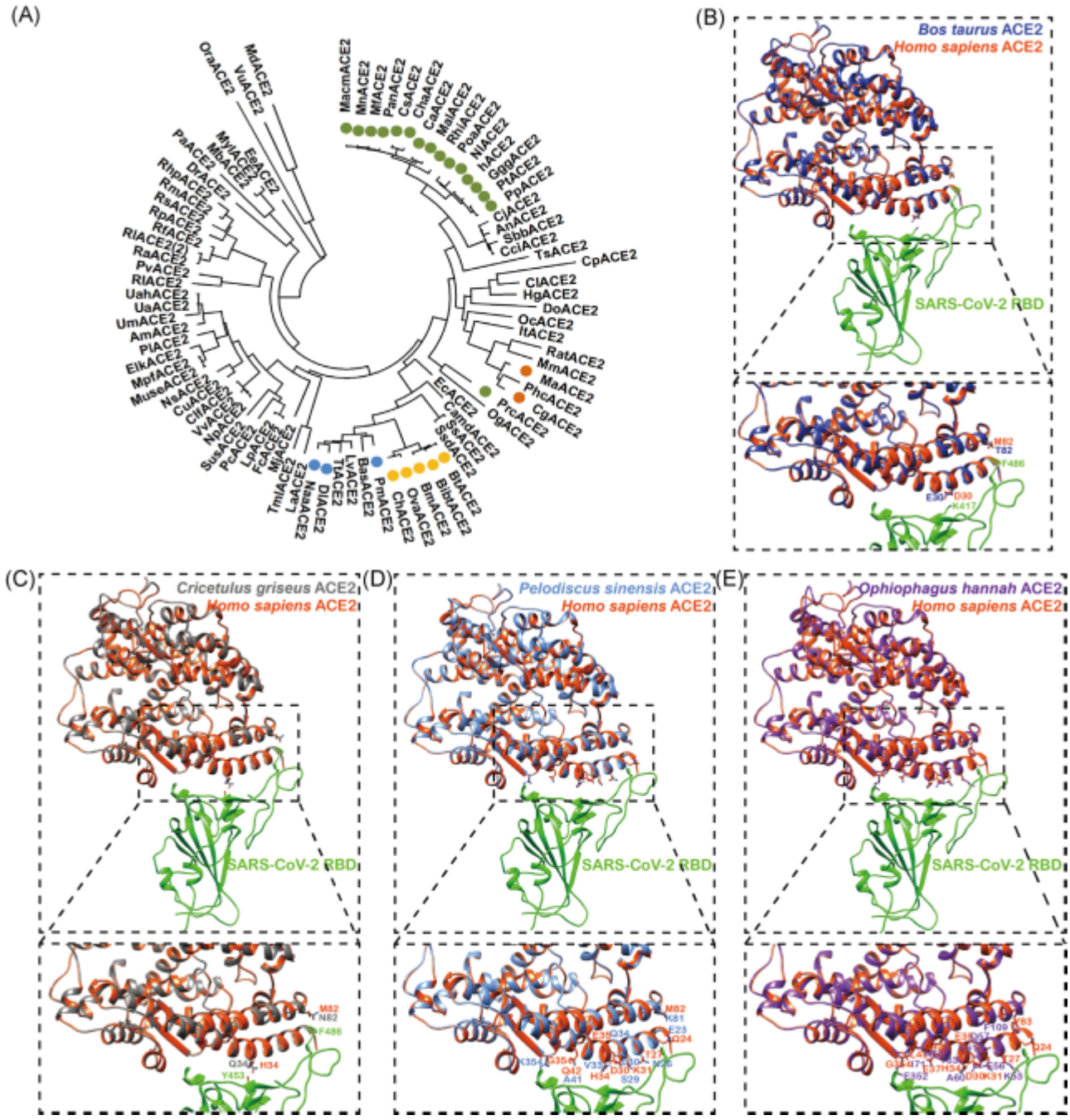


FIGURE 1 Structure simulation of SARSCoV-2 RBD with ACE2 from different species. A, Phylogenetic tree of mammalian ACE2. ACE2 proteins from a total of 85 mammals were analyzed by MEGA-X and the phylogenetic tree was constructed using a maximum-likelihood method. The green, yellow, orange, and blue represent ACE2 from Primates, Bovidae, Cricetidae, and Cetacea, respectively. B, Structural simulation of the protein complex of Bostaurus ACE2 and SARSCoV-2 RBD. Bostaurus ACE2, Homo sapiens ACE2, and SARSCoV-2 RBD are in medium blue, orange red, and green, respectively. C, Structural simulation of the protein complex of Cricetulus griseus ACE2 and SARSCoV-2 RBD. Cricetulus griseus ACE2, Homo sapiens ACE2, and SARSCoV-2 RBD are in dim gray, orange red, and green, respectively. D, Structural simulation of the protein complex of Pelodiscus sinensis ACE2 and SARSCoV-2 RBD. Pelodiscus sinensis ACE2, Homo sapiens ACE2, and SARSCoV-2 RBD are in cornflower blue, orange red, and green, respectively. E, Structural simulation of the protein complex of Ophiophagus hannah ACE2 and SARSCoV-2 RBD. Ophiophagus hannah ACE2, Homo sapiens ACE2, and SARSCoV-2 RBD are in purple, orange red, and green, respectively. ACE2, angiotensin-converting enzyme 2; MEGA-X, Molecular Evolutionary Genetics Analysis version X; RBD, receptor-binding domain; SARSCoV-2, severe acute respiratory syndrome coronavirus 2

3.2 | Structure simulation of the protein complex of SARS-CoV-2 RBD and Bovidae/Cricetidae/turtle/snake ACE2

Recently, the structure of SARS-CoV-2 RBD with human ACE2 has been resolved. To investigate whether Bovidae/Cricetidae ACE2 maintained the binding affinity with SARS-CoV-2 RBD, we simulated the potential structure of the protein complex. T82 and E30 in Bos taurus ACE2 kept the contact to F486 and K417 in SARS-CoV-2 S (Figure 1B). N82 and Q34 in Cricetulus griseus ACE2 maintained the contact to F486 and Y453 in SARS-CoV-2 S (Figure 1C). We concluded that Bovidae/Cricetidae ACE2 could associate with SARS-CoV-2 S (Figure 1B,C).

To investigate the potential association between SARS-CoV-2 and ACE2 from turtle and snake, we simulated the potential structure of turtle/snake ACE2 with SARS-CoV-2 RBD. The AA correlated to hACE2 Q42 is changed to A (A41) in turtle (Figure 1D). We also noticed that the AA correlated to hACE2 H34 is changed to A (A60) in a snake (Figure 1E). When the contact AA was mutated to smaller AA (A), the contact force for protein-protein interaction will be reduced. Moreover, the corresponding AA of K31 was changed to E (E30) in turtle and Q (Q57) in snake ACE2 (Figure 1D). K31 in hACE2 was critical for SARS-CoV RBD binding and ACE2-K31D mutant abolished its association with SARS-CoV RBD.[15] Taken together, turtle and snake ACE2 are unlikely to bind to S protein of SARS-CoV-2.

4 | DISCUSSION

SARS-CoV, MERS-CoV, and SARS-CoV-2 have caused severe human infectious diseases in the last 2 decades. These three human coronaviruses originated from bats, but the intermediate hosts were different. SARS-CoV came from the Paguma larvata,[16] and the intermediate host for MERS-CoV is Camelus dromedaries[17] The new coronavirus SARS-CoV-2 has recently caused a serious pandemic in China and other countries. However, it is not clear which animals are involved in the evolution of SARS-CoV-2 and which animals may be infected by SARS-CoV-2. RBD region in S protein of pangolin coronavirus is similar to that of SARS-CoV-2,[7,8] indicating the involvement of pangolin in the recombination of SARS-CoV-2. By analyzing the codon usage of SARS-CoV-2, people suggested that snake might be a potential host for SARS-CoV-2.[9] Another study indicated that turtle is a potential intermediate host for SARS-CoV-2 based on the key AAs in ACE2 for interacting with SARS-CoV RBD.[10] The late study raised the concerns of SARS-CoV-2 infection in the turtle aquaculture and pet turtle. Most of the coronaviruses hosts are mammals; with a few of exceptions are birds. Considering that all known hosts for coronaviruses are thermostatic animals, it is unlikely that reptiles will be infected with SARS-CoV-2.

There are 20 key AAs in ACE2 critical for binding S protein of SARS-CoV-2.[11] On the basis of these 20 AAs, we analyzed the corresponding AAs in ACE2 from a list of mammal, bird, turtle, and snake. We found that the ACE2 of turtles and snake lost the capability to associate with S protein (Table 1 and Figure 1D,E). These reptiles should be ruled out from the potential host list for SARS-CoV-2. Aves ACE2 was unlikely to associate with SARS-CoV-2 RBD because they lost the critical K corresponding to K31 in human ACE2 (Table 1). Pangolin ACE2 was predicted to recognize SARS-CoV-2 RBD less efficiently because it only preserved 14 of 20 critical AAs (Table 1). Interestingly, we found that ACE2 proteins from Primates, Bovidae, Cricetidae, and Cetacea were capable to recognize RBD of SARS-CoV-2 by maintaining the majority of key residues in ACE2 for associating with SARS-CoV-2 RBD. Swine ACE2 (CpACE2) with 15 of 20 matched critical AAs was shown to support SARS-CoV-2 entry.[6] Bovidae/Cricetidae ACE2 matched more AAs than swine ACE2, thus they should recognize SARS-CoV-2 RBD. It would strengthen our conclusion if we have biochemical evidence for the S-ACE2 interaction analysis for Bovidae/Cricetidae ACE2. On the basis of human ACE2 and SARS-CoV-spike complex structure model (PDB ID: 2AJF), we and others recently predicted that hamster ACE2 could associate with SARS-CoV-2 and hamster might be a candidate small animal model for SARS-CoV-2 infection.[18,19] Indeed, golden Syrian hamster (Mesocricetus auratus) has been established as a model to study the pathogenesis and transmission of COVID-19.[19] One of Cetacea, Neophocaena asiaeorientalis asiaeorientalis (Yangtze finless porpoise), lives in the middle and lower reaches of the Yangtze River and its lakes, where Wuhan located nearby.[20] It will be interesting to investigate whether Yangtze finless porpoise could be infected with SARS-CoV-2 or related coronavirus.

In conclusion, we found that Bovidae/Cricetidae ACE2 but not turtle/snake ACE2 could recognize SARS-CoV-2 RBD. More attention should be paid to Bovidae and Cricetidae in hunting the potential intermediate host for SARS-CoV-2.

ACKNOWLEDGMENTS

The authors would like to thank Dr Shan Gao for the discussion. This study is supported by grants from National Key Plan for Research and Development of China (2016YFD0500300), Shandong Academy of Medical Sciences Grant (2017-52), the Innovation Project of Shandong Academy of Medical Sciences, and Academic Promotion Program of Shandong First Medical University (2019LJ001). Molecular graphics and analyses performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

LZ conceived the work. JL and XJ collected and analyzed the data. JL and YL contributed to graphics processing. LZ wrote the manuscript. All authors approved the final version for publication.

ORCID

Leiliang Zhang http://orcid.org/0000-0002-7015-9661

REFERENCES

1. Zhu N, Zhang D, Wang W, et al. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. 2020;382(8):727-733.
2. Wu F, Zhao S, Yu B, et al. A new coronavirus associated with human respiratory disease in China. Nature. 2020;579(7798):265-269.
3. Zhong N, Zheng B, Li Y, et al. Epidemiology and cause of severe acute respiratory syndrome (SARS) in Guangdong, People's Republic of China, in February, 2003. Lancet 2003;362(9393):1353-1358.
4. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med. 2012;367(19):1814-1820.
5. Letko M, Marzi A, Munster V. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. Nat Microbiol. 2020;5(4):562-569.
6. Zhou P, Yang XL, Wang XG, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020; 579(7798):270-273.
7. Wong MC, Cregeen SJJ, Ajami NJ, Petrosino JF. Evidence of recombination in coronaviruses implicating pangolin origins of nCoV-2019. bioRxiv 2020. https://doi.org/10.1101/2020.02.07.939207
8. Lam TT-Y, Shum MH-H, Zhu H-C, et al. Identification of 2019-nCoV related coronaviruses in Malayan pangolins in southern China. bioRxiv. 2020. https://doi.org/10.1101/2020.02.13.945485
9. Ji W, Wang W, Zhao X, Zai J, Li X. Cross-species transmission of the newly identified coronavirus 2019-nCoV. J Med Virol. 2020;92(4): 433-440.
10. Liu Z, Xiao X, Wei X, et al. Composition and divergence of coronavirus spike proteins and host ACE2 receptors predict potential intermediate hosts of SARS-CoV-2. J Med Virol. 2020. https://doi.org/10.1002/jmv.25726
11. Lan J, Ge J, Yu J, et al. Crystal structure of the 2019-nCoV spike receptor-binding domain bound with the ACE2 receptor. bioRxiv 2020. https://doi.org/10.1101/2020.02.19.956235
12. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol 2018;35(6):1547-1549.
13. Waterhouse A, Bertoni M, Bienert S, et al. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res 2018;46(W1):W296-W303.
14. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera—a visualization system for exploratory research and analysis. J Comput Chem 2004;25(13):1605-1612.
15. Li W, Zhang C, Sui J, et al. Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2. EMBO J. 2005;24(8):1634-1643.
16. Kan B, Wang M, Jing H, et al. Molecular evolution analysis and geographic investigation of severe acute respiratory syndrome coronavirus-like virus in palm civets at an animal market and on farms. J Virol. 2005;79(18):11892-11900.
17. Chan RWY, Hemida MG, Kayali G, et al. Tropism and replication of Middle East respiratory syndrome coronavirus from dromedary camels in the human respiratory tract: an in-vitro and ex-vivo study. Lancet Respir Med. 2014;2(10):813-822.
18. Luan J, Lu Y, Jin X, Zhang L. Spike protein recognition of mammalian ACE2 predicts the host range and an optimized ACE2 for SARS-CoV-2 infection. Biochem Biophys Res Commun 2020. https://doi.org/10.1016/j.bbrc.2020.03.047
19. Chan JFW, Zhang AJ, Yuan S. Simulation of the clinical and pathological manifestations of coronavirus disease 2019 (COVID-19) in golden Syrian hamster model: implications for disease pathogenesis and transmissibility. Clin Infect Dis. 2019. https://doi.org/10.1093/cid/ciaa325
20. Zhou X, Guang X, Sun D, et al. Population genomics of finless porpoises reveal an incipient cetacean species adapted to freshwater. Nat Commun 2018;9(1):1276.

How to cite this article: Luan J, Jin X, Lu Y, Zhang L. SARS-CoV-2 spike protein favors ACE2 from Bovidae and Cricetidae J Med Virol. 2020;1–8.
https://doi.org/10.1002/jmv.25817

nature medicine

Brief CommuniC ation

https://doi.org/10.1038/s41591-020-0843-2

Check for updates

Respiratory virus shedding in exhaled breath and efficacy of face masks

Nancy H. L. Leung[1], Daniel K. W. Chu[1], Eunice Y. C. Shiu[1], Kwok-Hung Chan[2], James J. McDevitt[3], Benien J. P. Hau[4], Hui-Ling Yen[1], Yuguo Li[5], Dennis K. M. Ip[1], J. S. Malik Peiris[2], Wing-Hong Seto[1,6], Gabriel M. Leung[1], Donald K. Milton[7,8] and Benjamin J. Cowling[1,8]✉

We identified seasonal human coronaviruses, influenza viruses and rhinoviruses in exhaled breath and coughs of children and adults with acute respiratory illness. Surgical face masks significantly reduced detection of influenza virus RNA in respiratory droplets and coronavirus RNA in aerosols, with a trend toward reduced detection of coronavirus RNA in respiratory droplets. Our results indicate that surgical face masks could prevent transmission of human coronaviruses and influenza viruses from symptomatic individuals.

Respiratory virus infections cause a broad and overlapping spectrum of symptoms collectively referred to as acute respiratory virus illnesses (ARIs) or more commonly the 'common cold'. Although mostly mild, these ARIs can sometimes cause severe disease and death[1]. These viruses spread between humans through direct or indirect contact, respiratory droplets (including larger droplets that fall rapidly near the source as well as coarse aerosols with aerodynamic diameter >5 µm) and fine-particle aerosols (droplets and droplet nuclei with aerodynamic diameter ≤5 µm)[2,3]. Although hand hygiene and use of face masks primarily targeting contact and respiratory droplet transmission, have been suggested as important mitigation strategies against influenza virus transmission[4], little is known about the relative importance of these modes in the transmission of other common respiratory viruses[3,5]. Uncertainties similarly apply to the modes of transmission of COVID-19 (refs. [6,7]).

Some health authorities recommend that masks be worn by ill individuals to prevent onward transmission (source control)[4,8]. Surgical face masks were originally introduced to protect patients from wound infection and contamination from surgeons (the wearer) during surgical procedures, and were later adopted to protect healthcare workers against acquiring infection from their patients. However, most of the existing evidence on the filtering efficacy of face masks and respirators comes from in vitro experiments with nonbiological particles[9,10], which may not be generalizable to infectious respiratory virus droplets. There is little information on the efficacy of face masks in filtering respiratory viruses and reducing viral release from an individual with respiratory infections[8], and most research has focused on influenza[11,12].

Here we aimed to explore the importance of respiratory droplet and aerosol routes of transmission with a particular focus on coronaviruses, influenza viruses and rhinoviruses, by quantifying the amount of respiratory virus in exhaled breath of participants with medically attended ARIs and determining the potential efficacy of surgical face masks to prevent respiratory virus transmission.

Results

We screened 3,363 individuals in two study phases, ultimately enrolling 246 individuals who provided exhaled breath samples (Extended Data Fig. 1). Among these 246 participants, 122 (50%) participants were randomized to not wearing a face mask during the first exhaled breath collection and 124 (50%) participants were randomized to wearing a face mask. Overall, 49 (20%) voluntarily provided a second exhaled breath collection of the alternate type.

Infections by at least one respiratory virus were confirmed by reverse transcription PCR (RT–PCR) in 123 of 246 (50%) participants. Of these 123 participants, 111 (90%) were infected by human (seasonal) coronavirus (n = 17), influenza virus (n = 43) or rhinovirus (n = 54) (Extended Data Figs. 1 and 2), including one participant co-infected by both coronavirus and influenza virus and another two participants co-infected by both rhinovirus and influenza virus. These 111 participants were the focus of our analyses.

There were some minor differences in characteristics of the 111 participants with the different viruses (Table 1a). Overall, 24% of participants had a measured fever ≥37.8°C, with patients with influenza more than twice as likely than patients infected with coronavirus and rhinovirus to have a measured fever. Coronavirus-infected participants coughed the most with an average of 17 (s.d. = 30) coughs during the 30-min exhaled breath collection. The profiles of the participants randomized to with-mask versus without-mask groups were similar (Supplementary Table 1).

We tested viral shedding (in terms of viral copies per sample) in nasal swabs, throat swabs, respiratory droplet samples and aerosol samples and compared the latter two between samples collected with or without a face mask (Fig. 1). On average, viral shedding was higher in nasal swabs than in throat swabs for each of coronavirus (median 8.1 $\log_{10}$ virus copies per sample versus 3.9), influenza virus (6.7 versus 4.0) and rhinovirus (6.8 versus 3.3), respectively. Viral RNA was identified from respiratory droplets and aerosols for all three viruses, including 30%, 26% and 28% of respiratory droplets and 40%, 35% and 56% of aerosols collected while not wearing a face mask, from coronavirus, influenza virus and rhinovirus-infected participants, respectively (Table 1b). In particular for coronavirus, we identified OC43 and HKU1 from both respiratory

[1]WHO Collaborating Centre for Infectious Disease Epidemiology and Control, School of Public Health, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China. [2]Department of Microbiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China. [3]Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA. [4]Department of Surgery, Queen Mary Hospital, Hong Kong, China. [5]Department of Mechanical Engineering, The University of Hong Kong, Hong Kong, China. [6]Department of Pathology, Hong Kong Baptist Hospital, Hong Kong, China. [7]Maryland Institute for Applied Environmental Health, School of Public Health, University of Maryland, College Park, MD, USA. [8]These authors jointly supervised this work: Donald K. Milton, Benjamin J. Cowling. ✉e-mail: bcowling@hku.hk

Table 1a | Characteristics of individuals with symptomatic coronavirus, influenza virus or rhinovirus infection

	all who provided exhaled breath (*n* = 246)	Coronavirus (*n* = 17)	Influenza virus (*n* = 43)	Rhinovirus (*n* = 54)
	n (%)	*n* (%)	*n* (%)	*n* (%)
Female	144 (59)	13 (76)	22 (51)	30 (56)
age group, years				
11–17	12 (5)	0 (0)	8 (19)	4 (7)
18–34	114 (46)	10 (59)	11 (26)	24 (44)
35–50	79 (32)	2 (12)	16 (37)	18 (33)
51–64	35 (14)	4 (24)	8 (19)	5 (9)
≥ 65	6 (2)	1 (6)	0 (0)	3 (6)
Chronic medical conditions				
Any	49 (20)	5 (29)	5 (12)	10 (19)
Respiratory	18 (7)	0 (0)	4 (9)	3 (6)
Influenza vaccination				
Ever	94 (38)	6 (35)	15 (35)	20 (37)
Current season	23 (9)	2 (12)	1 (2)	4 (7)
Previous season only	71 (29)	4 (24)	14 (33)	16 (30)
Ever smoker	31 (13)	1 (6)	6 (14)	6 (11)
t ime since illness onset, h				
<24	22 (9)	0 (0)	5 (12)	2 (4)
24–48	100 (41)	9 (53)	13 (30)	25 (46)
48–72	85 (35)	8 (47)	18 (42)	20 (37)
72–96	39 (16)	0 (0)	7 (16)	7 (13)
History of measured fever ≥37.8°C	58 (24)	3 (18)	17 (40)	8 (15)
Measured fever ≥37.8°C at presentation	36 (15)	2 (12)	18 (42)	2 (4)
Measured body temperature (°C) at enrollment (mean, s.d.)	36.8 (0.8)	36.9 (0.8)	37.4 (0.9)	36.6 (0.7)
Symptoms at presentation				
Fever	111 (45)	10 (59)	27 (63)	16 (30)
Cough	198 (80)	15 (88)	40 (93)	44 (81)
Sore throat	211 (86)	15 (88)	31 (72)	49 (91)
Runny nose	200 (81)	17 (100)	36 (84)	48 (89)
Headache	186 (76)	13 (76)	30 (70)	38 (70)
Myalgia	176 (72)	12 (71)	31 (72)	34 (63)
Phlegm	176 (72)	9 (53)	34 (79)	41 (76)
Chest tightness	64 (26)	3 (18)	12 (28)	9 (17)
Shortness of breath	103 (42)	6 (35)	14 (33)	25 (46)
Chills	100 (41)	8 (47)	29 (67)	16 (30)
Sweating	95 (39)	5 (29)	18 (42)	20 (37)
Fatigue	218 (89)	16 (94)	38 (88)	48 (89)
Vomiting	19 (8)	2 (12)	5 (12)	2 (4)
Diarrhea	17 (7)	2 (12)	1 (2)	6 (11)
Number of coughs during exhaled breath collection (mean, s.d.)	3 (14)	17 (30)	8 (11)	5 (9)

Seasonal coronavirus (*n* = 17), seasonal influenza virus (*n* = 43) and rhinovirus (*n* = 54) infections were confirmed in individuals with acute respiratory symptoms by RT–PCR in any samples (nasal swab, throat swab, respiratory droplets and aerosols) collected.

dropletsand aerosols,but only identified NL63 from aerosolsand not from respiratorydroplets(SupplementaryTable2 andExtended Data Fig. 3).

We detectedcoronavirusin respiratorydropletsandaerosolsin 3 of 10(30%)and4 of 10(40%)of thesamplescollectedwithout face masks,respectively,but did not detectanyvirusin respiratorydroplets or aerosolscollectedfrom participantswearingfacemasks;this differencewassignificantin aerosolsandshowedatrend toward reduceddetectionin respiratorydroplets(Table1b). For influenza virus,we detectedvirusin 6 of 23(26%)and8 of 23(35%)of the

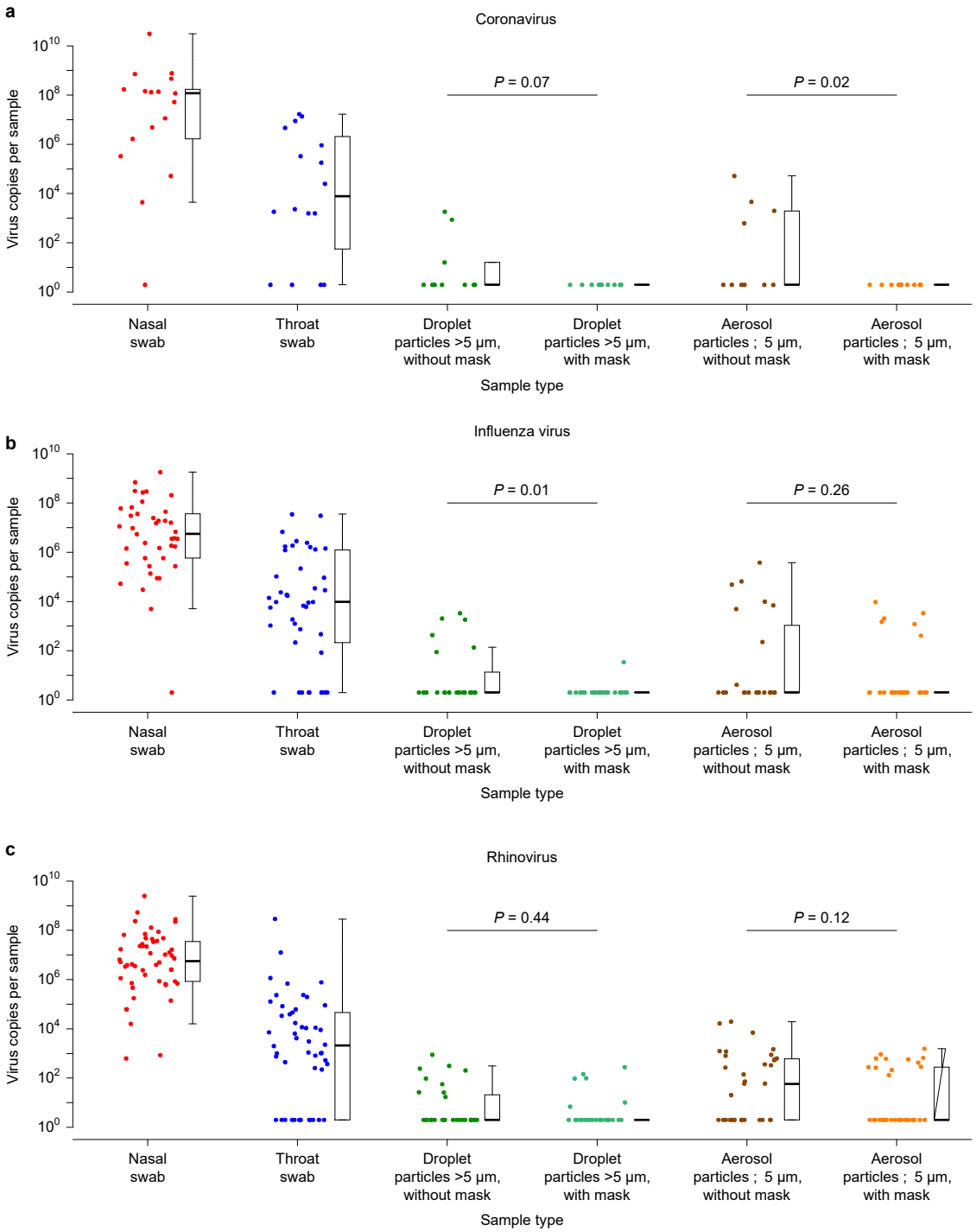


Fig. 1 | Efficacy of surgical face masks in reducing respiratory virus shedding in respiratory droplets and aerosols of symptomatic individuals with coronavirus, influenza virus or rhinovirus infection. a–**c**, Virus copies per sample collected in nasal swab (red), throat swab (blue) and respiratory droplets collected for 30min while not wearing (dark green) or wearing (light green) a surgical face mask, and aerosols collected for 30min while not wearing (brown) or wearing (orange) a face mask, collected from individuals with acute respiratory symptoms who were positive for coronavirus (**a**), influenza virus (**b**) and rhinovirus (**c**), as determined by RT–PCR in any samples. *P* values for mask intervention as predictor of $\log_{10}$ virus copies per sample in an unadjusted univariate Tobit regression model which allowed for censoring at the lower limit of detection of the RT–PCR assay are shown, with significant differences in bold. For nasal swabs and throat swabs, all infected individuals were included (coronavirus, *n*=17; influenza virus, *n*=43; rhinovirus, *n*=54). For respiratory droplets and aerosols, numbers of infected individuals who provided exhaled breath samples while not wearing or wearing a surgical face mask, respectively were: coronavirus (*n*=10 and 11), influenza virus (*n*=23 and 28) and rhinovirus (*n*=36 and 32). A subset of participants provided exhaled breath samples for both mask interventions (coronavirus, *n*=4; influenza virus, *n*=8; rhinovirus, *n*=14). The box plots indicate the median with the interquartile range (lower and upper hinge) and ±1.5×interquartile range from the first and third quartile (lower and upper whiskers).

Table 1b | Efficacy of surgical face masks in reducing respiratory virus frequency of detection and viral shedding in respiratory droplets and aerosols of symptomatic individuals with coronavirus, influenza virus or rhinovirus infection

	Droplet particles >5 µm			aerosol particles ≤5 µm		
Virus type	**Without surgical face mask**	**With surgical face mask**	***P***	**Without surgical face mask**	**With surgical face mask**	***P***
	Detection of virus					
	No. positive/no. total (%)	**No. positive/no. total (%)**		**No. positive/no. total (%)**	**No. positive/no. total (%)**	
Coronavirus	3 of 10 (30)	0 of 11 (0)	0.09	**4 of 10 (40)**	**0 of 11 (0)**	**0.04**
Influenza virus	**6 of 23 (26)**	**1 of 27(4)**	**0.04**	8 of 23 (35)	6 of 27 (22)	0.36
Rhinovirus	9 of 32 (28)	6 of 27 (22)	0.77	19 of 34 (56)	12 of 32 (38)	0.15
	Viral load (log_{10} virus copies per sample)					
	Median (IQR)	**Median (IQR)**		**Median (IQR)**	**Median (IQR)**	
Coronavirus	0.3 (0.3, 1.2)	0.3 (0.3, 0.3)	0.07	**0.3 (0.3, 3.3)**	**0.3 (0.3, 0.3)**	**0.02**
Influenza virus	**0.3 (0.3, 1.1)**	**0.3 (0.3, 0.3)**	**0.01**	0.3 (0.3, 3.0)	0.3 (0.3, 0.3)	0.26
Rhinovirus	0.3 (0.3, 1.3)	0.3 (0.3, 0.3)	0.44	1.8 (0.3, 2.8)	0.3 (0.3, 2.4)	0.12

P values for comparing the frequency of respiratory virus detection between the mask intervention were obtained by two-sided Fisher's exact test and (two-sided) *P* values for mask intervention as predictor of log_{10} virus copies per sample were obtained by an unadjusted univariate Tobit regression model, which allowed for censoring at the lower limit of detection of the RT–PCR assay, with significant differences in bold. Undetectable values were imputed as 0.3 log_{10} virus copies per sample. IQR, interquartile range.

respiratory droplet and aerosol samples collected without face masks, respectively. There was a significant reduction by wearing face masks to 1 of 27 (4%) in detection of influenza virus in respiratory droplets, but no significant reduction in detection in aerosols (Table 1b). Moreover, among the eight participants who had influenza virus detected by RT–PCR from without-mask aerosols, five were tested by viral culture and four were culture-positive. Among the six participants who had influenza virus detected by RT–PCR from with-mask aerosols, four were tested by viral culture and two were culture-positive. For rhinovirus, there were no significant differences between detection of virus with or without face masks, both in respiratory droplets and in aerosols (Table 1b). Conclusions were similar in comparisons of viral shedding (Table 1b). In addition, we found a significant reduction in viral shedding (Supplementary Table 2) in respiratory droplets for OC43 (Extended Data Fig. 4) and influenza B virus (Extended Data Fig. 5) and in aerosols for NL63 (Extended Data Fig. 4).

We identified correlations between viral loads in different samples (Extended Data Figs. 6–8) and some evidence of declines in viral shedding by time since onset for influenza virus but not for coronavirus or rhinovirus (Extended Data Fig. 9). In univariable analyses of factors associated with detection of respiratory viruses in various sample types, we did not identify significant association in viral shedding with days since symptom onset (Supplementary Table 3) for respiratory droplets or aerosols (Supplementary Tables 4–6).

A subset of participants (72 of 246, 29%) did not cough at all during at least one exhaled breath collection, including 37 of 147 (25%) during the without-mask and 42 of 148 (28%) during the with-mask breath collection. In the subset for coronavirus ($n = 4$), we did not detect any virus in respiratory droplets or aerosols from any participants. In the subset for influenza virus ($n = 9$), we detected virus in aerosols but not respiratory droplets from one participant. In the subset for rhinovirus ($n = 17$), we detected virus in respiratory droplets from three participants, and we detected virus in aerosols in five participants.

Discussion

Our results indicate that aerosol transmission is a potential mode of transmission for coronaviruses as well as influenza viruses and rhinoviruses. Published studies detected respiratory viruses[13,14] such as influenza[12,15] and rhinovirus[16] from exhaled breath, and the detection of SARS-CoV[17] and MERS-CoV[18] from air samples (without size fractionation) collected from hospitals treating patients with severe acute respiratory syndrome and Middle East respiratory syndrome, but ours demonstrates detection of human seasonal coronaviruses in exhaled breath, including the detection of OC43 and HKU1 from respiratory droplets and NL63, OC43 and HKU1 from aerosols.

Our findings indicate that surgical masks can efficaciously reduce the emission of influenza virus particles into the environment in respiratory droplets, but not in aerosols[12]. Both the previous and current study used a bioaerosol collecting device, the Gesundheit-II (G-II)[12,15,19], to capture exhaled breath particles and differentiated them into two size fractions, where exhaled breath coarse particles >5 µm (respiratory droplets) were collected by impaction with a 5-µm slit inertial Teflon impactor and the remaining fine particles ≤5 µm (aerosols) were collected by condensation in buffer. We also demonstrated the efficacy of surgical masks to reduce coronavirus detection and viral copies in large respiratory droplets and in aerosols (Table 1b). This has important implications for control of COVID-19, suggesting that surgical face masks could be used by ill people to reduce onward transmission.

Among the samples collected without a face mask, we found that the majority of participants with influenza virus and coronavirus infection did not shed detectable virus in respiratory droplets or aerosols, whereas for rhinovirus we detected virus in aerosols in 19 of 34 (56%) participants (compared to 4 of 10 (40%) for influenza and 8 of 23 (35%) for coronavirus). For those who did shed virus in respiratory droplets and aerosols, viral load in both tended to be low (Fig. 1). Given the high collection efficiency of the G-II (ref. [19]) and given that each exhaled breath collection was conducted for 30 min, this might imply that prolonged close contact would be required for transmission to occur, even if transmission was primarily via aerosols, as has been described for rhinovirus colds[20]. Our results also indicate that there could be considerable heterogeneity in contagiousness of individuals with coronavirus and influenza virus infections.

The major limitation of our study was the large proportion of participants with undetectable viral shedding in exhaled breath for each of the viruses studied. We could have increased the sampling duration beyond 30 min to increase the viral shedding being captured, at the cost of acceptability in some participants. An alternative approach would be to invite participants to perform forced coughs during exhaled breath collection[12]. However, it was the aim of our present study to focus on recovering respiratory

virus in exhaled breath in a real-life situation and we expected that some individuals during an acute respiratory illness would not cough much or at all. Indeed, we identified virus RNA in a small number of participants who did not cough at all during the 30-min exhaled breath collection, which would suggest droplet and aerosol routes of transmission are possible from individuals with no obvious signs or symptoms. Another limitation is that we did not confirm the infectivity of coronavirus or rhinovirus detected in exhaled breath. While the G-II was designed to preserve viability of viruses in aerosols, and in the present study we were able to identify infectious influenza virus in aerosols, we did not attempt to culture coronavirus or rhinovirus from the corresponding aerosol samples.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41591-020-0843-2.

Received: 2 March 2020; Accepted: 20 March 2020;
Published online: 03 April 2020

References

1. Nichols, W. G., Peck Campbell, A. J. & Boeckh, M. Respiratory viruses other than influenza virus: impact and therapeutic advances. *Clin. Microbiol. Rev.* **21**, 274–290 (2008).
2. Shiu, E. Y. C., Leung, N. H. L. & Cowling, B. J. Controversy around airborne versus droplet transmission of respiratory viruses: implication for infection prevention. *Curr. Opin. Infect. Dis.* **32**, 372–379 (2019).
3. Tellier, R., Li, Y., Cowling, B. J. & Tang, J. W. Recognition of aerosol transmission of infectious agents: a commentary. *BMC Infect. Dis.* **19**, 101 (2019).
4. Xiao, J. et al. Nonpharmaceutical measures for pandemic influenza in nonhealthcare settings—personal protective and environmental measures. *Emerg. Infect. Dis.* https://doi.org/10.3201/eid2605.190994 (2020).
5. Kutter, J. S., Spronken, M. I., Fraaij, P. L., Fouchier, R. A. M. & Herfst, S. Transmission routes of respiratory viruses among humans. *Curr. Opin. Virol.* **28**, 142–151 (2018).
6. Cowling, B. J. & Leung, G. M. Epidemiological research priorities for public health control of the ongoing global novel coronavirus (2019-nCoV) outbreak. *Euro Surveill.* https://doi.org/10.2807/1560-7917.ES.2020.25.6.2000110 (2020).
7. Han, Q., Lin, Q., Ni, Z. & You, L. Uncertainties about the transmission routes of 2019 novel coronavirus. *Influenza Other Respir. Viruses* https://doi.org/10.1111/irv.12735 (2020).
8. MacIntyre, C. R. & Chughtai, A. A. Facemasks for the prevention of infection in healthcare and community settings. *BMJ* **350**, h694 (2015).
9. Ha'eri, G. B. & Wiley, A. M. The efficacy of standard surgical facemasks: an investigation using "tracer particles". *Clin. Orthop. Relat. Res.* **148**, 160–162 (1980).
10. Patel, R. B., Skaria, S. D., Mansour, M. M. & Smaldone, G. C. Respiratory source control using a surgical mask: an in vitro study. *J. Occup. Environ. Hyg.* **13**, 569–576 (2016).
11. Johnson, D. F., Druce, J. D., Birch, C. & Grayson, M. L. A quantitative assessment of the efficacy of surgical and N95 masks to filter influenza virus in patients with acute influenza infection. *Clin. Infect. Dis.* **49**, 275–277 (2009).
12. Milton, D. K., Fabian, M. P., Cowling, B. J., Grantham, M. L. & McDevitt, J. J. Influenza virus aerosols in human exhaled breath: particle size, culturability, and effect of surgical masks. *PLoS Pathog.* **9**, e1003205 (2013).
13. Huynh, K. N., Oliver, B. G., Stelzer, S., Rawlinson, W. D. & Tovey, E. R. A new method for sampling and detection of exhaled respiratory virus aerosols. *Clin. Infect. Dis.* **46**, 93–95 (2008).
14. Stelzer-Braid, S. et al. Exhalation of respiratory viruses by breathing, coughing and talking. *J. Med. Virol.* **81**, 1674–1679 (2009).
15. Yan, J. et al. Infectious virus in exhaled breath of symptomatic seasonal influenza cases from a college community. *Proc. Natl Acad. Sci. USA* **115**, 1081–1086 (2018).
16. Tovey, E. R. et al. Rhinoviruses significantly affect day-to-day respiratory symptoms of children with asthma. *J. Allergy Clin. Immunol.* **135**, 663–669 (2015).
17. Booth, T. F. et al. Detection of airborne severe acute respiratory syndrome (SARS) coronavirus and environmental contamination in SARS outbreak units. *J. Infect. Dis.* **191**, 1472–1477 (2005).
18. Kim, S. H. et al. Extensive viable Middle East respiratory syndrome (MERS) coronavirus contamination in air and surrounding environment in MERS isolation wards. *Clin. Infect. Dis.* **63**, 363–369 (2016).
19. McDevitt, J. J. et al. Development and performance evaluation of an exhaled-breath bioaerosol collector for influenza virus. *Aerosol Sci. Technol.* **47**, 444–451 (2013).
20. Jennings, L. C. & Dick, E. C. Transmission and control of rhinovirus colds. *Eur. J. Epidemiol.* **3**, 327–335 (1987).

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature America, Inc. 2020

Methods

Study design. Participants were recruited year-round from March 2013 through May 2016 in a general outpatient clinic of a private hospital in Hong Kong. As routine practice, clinic staff screened all individuals attending the clinics for respiratory and any other symptoms regardless of the purpose of the visit at triage. Study staff then approached immediately those who reported at least one of the following symptoms of ARI for further screening: fever ≥37.8 °C, cough, sore throat, runny nose, headache, myalgia and phlegm. Individuals who reported ARI symptoms, within 3 d of illness onset and ≥11 years of age were eligible to participate. After explaining the study to and obtaining informed consent from the participants, a rapid influenza diagnostic test, the Sofia Influenza A+B Fluorescent Immunoassay Analyzer (cat. no. 20218, Quidel), was used to identify influenza A or B virus infection as an incentive to participate. All participants provided a nasal swab for the rapid test and an additional nasal swab and a separate throat swab for subsequent virologic confirmation at the laboratory. All participants also completed a questionnaire to record basic information including age, sex, symptom severity, medication, medical conditions and smoking history. In the first phase of the study from March 2013 to February 2014 ('Influenza Study'), the result of the rapid test was used to determine eligibility for further participation in the study and exhaled breath collection, whereas in the second phase of the study from March 2014 to May 2016 ('Respiratory Virus Study'), the rapid test did not affect eligibility. Eligible participants were then invited to provide an exhaled breath sample for ≥30 min on the same clinic visit.

Before exhaled breath collection, each participant was randomly allocated in a 1:1 ratio to either wearing a surgical face mask (cat. no. 62356, Kimberly-Clark) or not during the collection. To mimic the real-life situation, under observation by the study staff, participants were asked to attach the surgical mask themselves, but instruction on how to wear the mask properly was given when the participant wore the mask incorrectly. Participants were instructed to breathe as normal during the collection, but (natural) coughing was allowed and the number of coughs was recorded by study staff. Participants were then invited to provide a second exhaled breath sample of the alternate type (for example if the participant was first assigned to wearing a mask they would then provide a second sample without a mask), but most participants did not agree to stay for a second measurement because of time constraints. Participants were compensated for each 30-min exhaled breath collection with a supermarket coupon worth approximately US$30 and all participants were gifted a tympanic thermometer worth approximately US$20.

Ethical approval. Written informed consent was obtained from all participants ≥18 years of age and written informed consent was obtained from parents or legal guardians of participants 11–17 years of age in addition to their own written informed consent. The study protocol was approved by the Institutional Review Board of The University of Hong Kong and the Clinical and Research Ethics Committee of Hong Kong Baptist Hospital.

Collection of swabs and exhaled breath particles. Nasal swabs and throat swabs were collected separately, placed in virus transport medium, stored and transported to the laboratory at 2–8 °C and the virus transport medium was aliquoted and stored at −70 °C until further analysis. Exhaled breath particles were captured and differentiated into two size fractions, the coarse fraction containing particles with aerodynamic diameter >5 μm (referred to here as 'respiratory droplets'), which included droplets up to approximately 100 in diameter and the fine fraction with particles ≤5 μm (referred to here as 'aerosols') by the G-II bioaerosol collecting device[12,13]. In the G-II device, exhaled breath coarse particles (>5 μm) were collected by a 5-μm slit inertial Teflon impactor and the remaining fine particles (≤5 μm) were condensed and collected into approximately 1 ml of 0.1% BSA/PBS. Both the impactor and the condensate were stored and transported to the laboratory at 2–8 °C. The virus on the impactor was recovered into 1 ml and the condensate was concentrated into 0.2 ml 0.1% BSA/PBS, aliquoted and stored at −70 °C until further analysis. In a validation study, the G-II was able to recover over 85% of fine particles 0.05–5 μm in size and had comparable collection efficiency of influenza virus as the SKC BioSampler[13].

Laboratory testing. Samples collected from the two studies were tested at the same time. Nasal swab samples were first tested by a diagnostic-use viral panel, xTAG Respiratory Viral Panel (Abbott Molecular) to qualitatively detect 12 common respiratory viruses and subtypes including coronaviruses (NL63, OC43, 229E and HKU1), influenza A (nonspecific, H1 and H3) and B viruses, respiratory syncytial virus, parainfluenza virus (types 1–4), adenovirus, human metapneumovirus and enterovirus/rhinovirus. After one or more of the candidate respiratory viruses was detected by the viral panel from the nasal swab, all the samples from the same participant (nasal swab, throat swab, respiratory droplets and aerosols) were then tested with RT–PCR specific for the candidate virus(es) for determination of virus concentration in the samples. Infectious influenza virus was identified by viral culture using MDCK cells as described previously[14], whereas viral culture was not performed for coronavirus and rhinovirus.

Statistical analyses. The primary outcome of the study was virus generation rate in tidal breathing of participants infected by different respiratory viruses and the efficacy of face masks in preventing virus dissemination in exhaled breath, separately considering the respiratory droplets and aerosols. The secondary outcomes were correlation between viral shedding in nose swabs, throat swabs, respiratory droplets and aerosols and factors affecting viral shedding in respiratory droplets and aerosols.

We identified three groups of respiratory viruses with the highest frequency of infection as identified by RT–PCR, namely coronavirus (including NL63, OC43, HKU1 and 229E), influenza virus and rhinovirus, for further statistical analyses. We defined viral shedding as $\log_{10}$ virus copies per sample and plotted viral shedding in each sample (nasal swab, throat swab, respiratory droplets and aerosols); the latter two were stratified by mask intervention. As a proxy for the efficacy of face masks in preventing transmission of respiratory viruses via respiratory droplet and aerosol routes, we compared the respiratory virus viral shedding in respiratory droplet and aerosol samples between participants wearing face masks or not, by comparing the frequency of detection with a two-sided Fisher's exact test and by comparing viral load (defined as $\log_{10}$ virus copies per sample) by an unadjusted univariate Tobit regression model, which allowed for censoring at the lower limit of detection of the RT–PCR assay. We also used the unadjusted univariate Tobit regression to investigate factors affecting viral shedding in respiratory droplets and aerosols without mask use, for example age, days since symptom onset, previous influenza vaccination, current medication and number of coughs during exhaled breath collection. We investigated correlations between viral shedding in nasal swab, throat swab, respiratory droplets and aerosols with scatter-plots and calculated the Spearman's rank correlation coefficient between any two types of samples. We imputed 0.3 virus copies ml^{-1} for undetectable values before transformation to $\log_{10}$ virus copies per sample. All analyses were conducted with R v.3.6.0 (ref. [22]) and the VGAM package v.1.1.1 (ref. [23]).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Anonymized raw data and R syntax to reproduce all the analyses, figures, tables and supplementary tables in the published article are available at: https://doi.org/10.5061/dryad.w9ghx3fkt.

References

21. Chan, K. H., Peiris, J. S., Lim, W., Nicholls, J. M. & Chiu, S. S. Comparison of nasopharyngeal flocked swabs and aspirates for rapid diagnosis of respiratory viruses in children. *J. Clin. Virol.* **42**, 65–69 (2008).
22. R: a language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria, 2019).
23. Yee, T. W. *Vector Generalized Linear and Additive Models: with an Implementation in R* (Springer, 2016).

acknowledgements

This work was supported by the General Research Fund of the University Grants Committee (grant no. 765811), the Health and Medical Research Fund (grant no. 13120592) and a commissioned grant of the Food and Health Bureau and the Theme-based Research Scheme (project no. T11-705/14-N) of the Research Grants Council of the Hong Kong SAR Government. We acknowledge colleagues including R. O. P. Fung, A. K. W. Li, T. W. Y. Ng, T. H. C. So, P. Wu and Y. Xie for technical support in preparing and conducting this study and enrolling participants; J. K. M. Chan, S. Y. Ho, Y. Z. Liu and A. Yu for laboratory support; S. Ferguson, W. K. Leung, J. Pantelic, J. Wei and M. Wolfson for technical support in constructing and maintaining the G-II device; V. J. Fang, L. M. Ho and T. T. K. Lui for setting up the database; and C. W. Y. Cheung, L. F. K. Cheung, P. T. Y. Ching, A. C. H. Lai, D. W. Y. Lam, S. S. Y. Lo, A. S. K. Luk and other colleagues at the Outpatient Center and Infection Control Team of Hong Kong Baptist Hospital for facilitating this study.

author contributions

All authors meet the International Committee of Medical Journal Editors criteria for authorship. The study protocol was drafted by N.H.L.L. and B.J.C. Data were collected by N.H.L.L., E.Y.C.S. and B.J.P.H. Laboratory testing was performed by D.K.W.C. and K.-H.C. Statistical analyses were conducted by N.H.L.L. N.H.L.L. and B.J.C. wrote the first draft of the manuscript, and all authors provided critical review and revision of the text and approved the final version.

Competing interests

B.J.C. consults for Roche and Sanofi Pasteur. The authors declare no other competing interests.

additional information

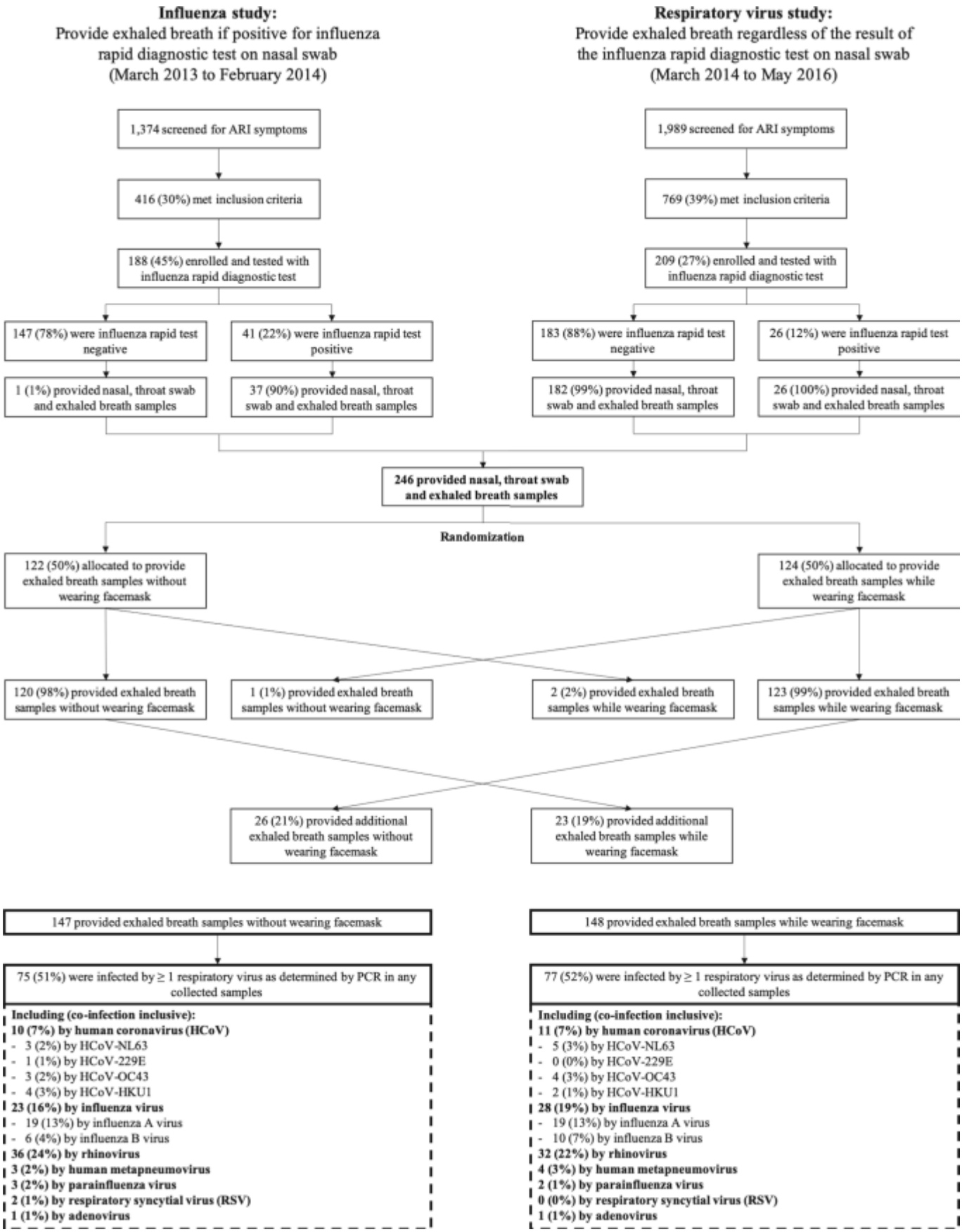
Extended data is available for this paper at https://doi.org/10.1038/s41591-020-0843-2.

Supplementary information is available for this paper at https://doi.org/10.1038/s41591-020-0843-2.

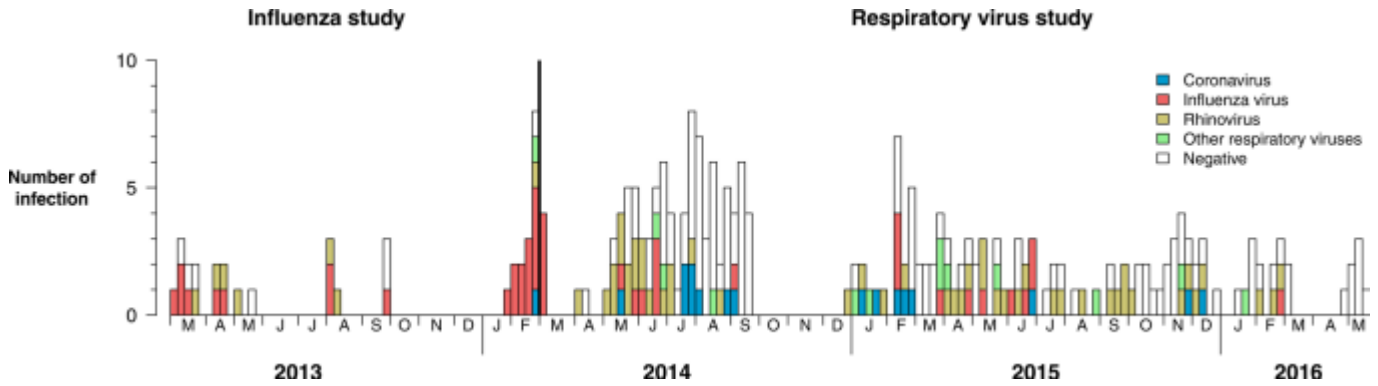
Correspondence and requests for materials should be addressed to B.J.C.

Peer review information Alison Farrell was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

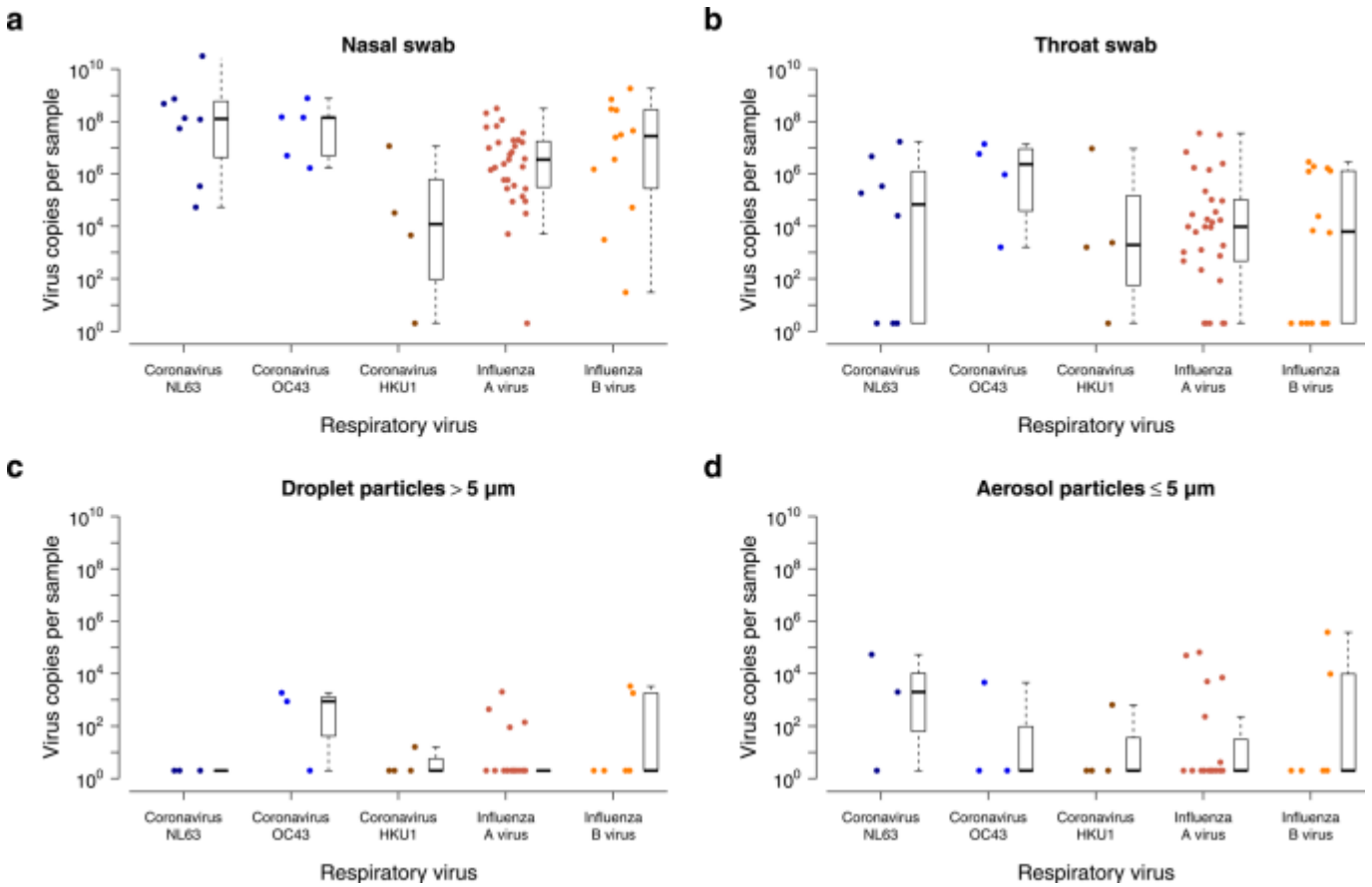
Reprints and permissions information is available at www.nature.com/reprints.



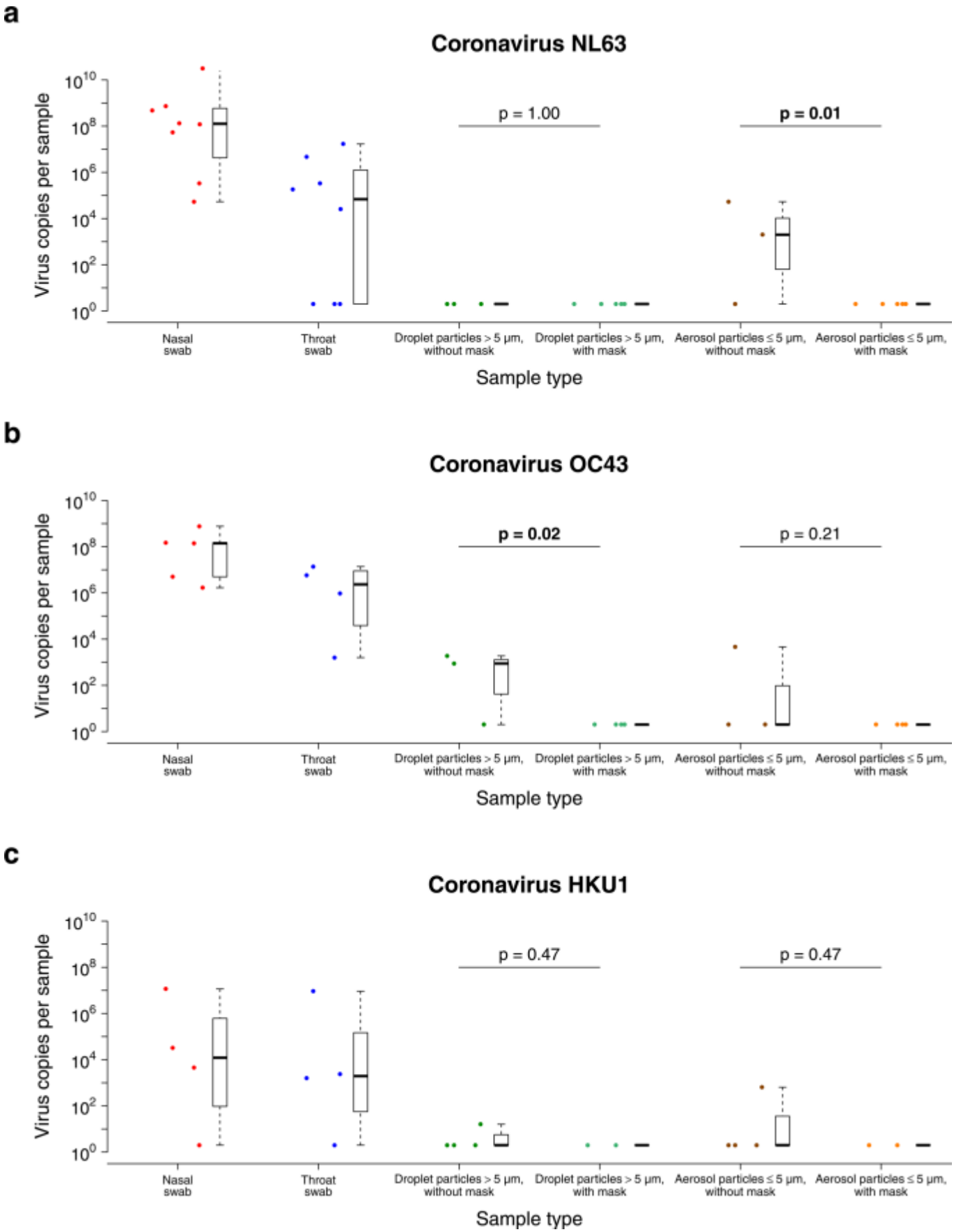
Extended Data Fig. 1 | Participant enrolment, randomization of mask intervention and identification of respiratory virus infection.



Extended Data Fig. 2 | Weekly number of respiratory virus infections identified by RT-PCR in symptomatic individuals who had provided exhaled breath samples (respiratory droplets and aerosols) during the study period. Blue, coronavirus; red, influenza virus; yellow, rhinovirus; green, other respiratory viruses including human metapneumovirus, parainfluenza virus, respiratory syncytial virus and adenovirus; white, no respiratory virus infection identified.

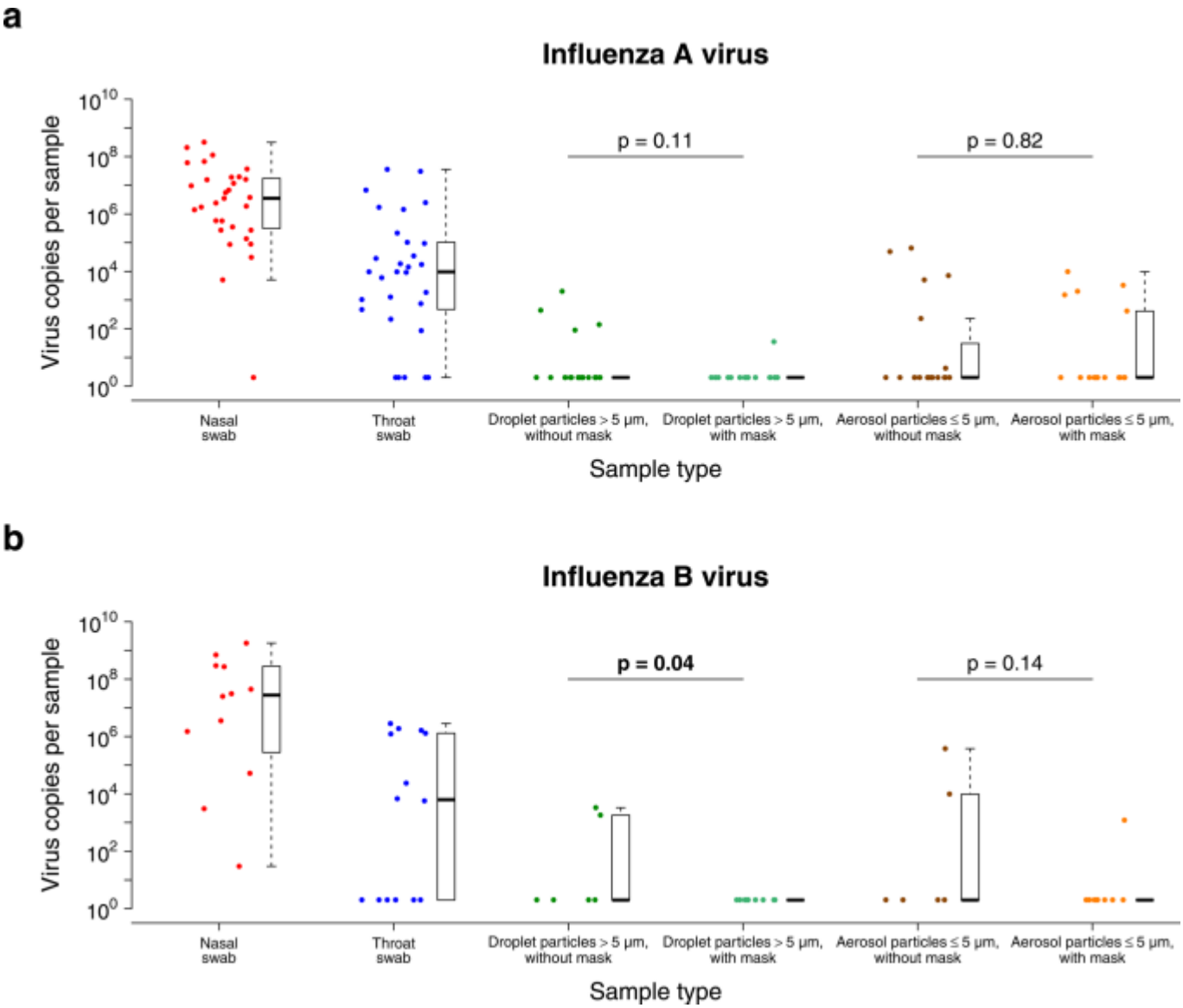


Extended Data Fig. 3 | Respiratory virus shedding in (a) nasal swab, (b) throat swab, (c) respiratory droplets and (d) aerosols in symptomatic individuals with coronavirus NL63, coronavirus OC43, coronavirus HKU1, influenza a and influenza B virus infection. For nasal swabs and throat swabs, all infected individuals identified by RT-PCR in any collected samples were included: coronavirus NL63 (n = 8), coronavirus OC43 (n = 5), coronavirus HKU1 (n = 4), influenza A virus (n = 31) and influenza B virus (n = 14). For respiratory droplets and aerosols, only infected individuals who provided exhaled breath samples while not wearing a surgical face mask were included: coronavirus NL63 (n = 3), coronavirus OC43 (n = 3), coronavirus HKU1 (n = 4), influenza A virus (n = 19) and influenza B virus (n = 6). The box plots indicate the median with the interquartile range (lower and upper hinge) and ± 1.5 × interquartile range from the first and third quartile (lower and upper whisker). Dark blue, coronavirus NL63; light blue, coronavirus OC43; brown, coronavirus HKU1; red, influenza A virus; orange, influenza B virus.

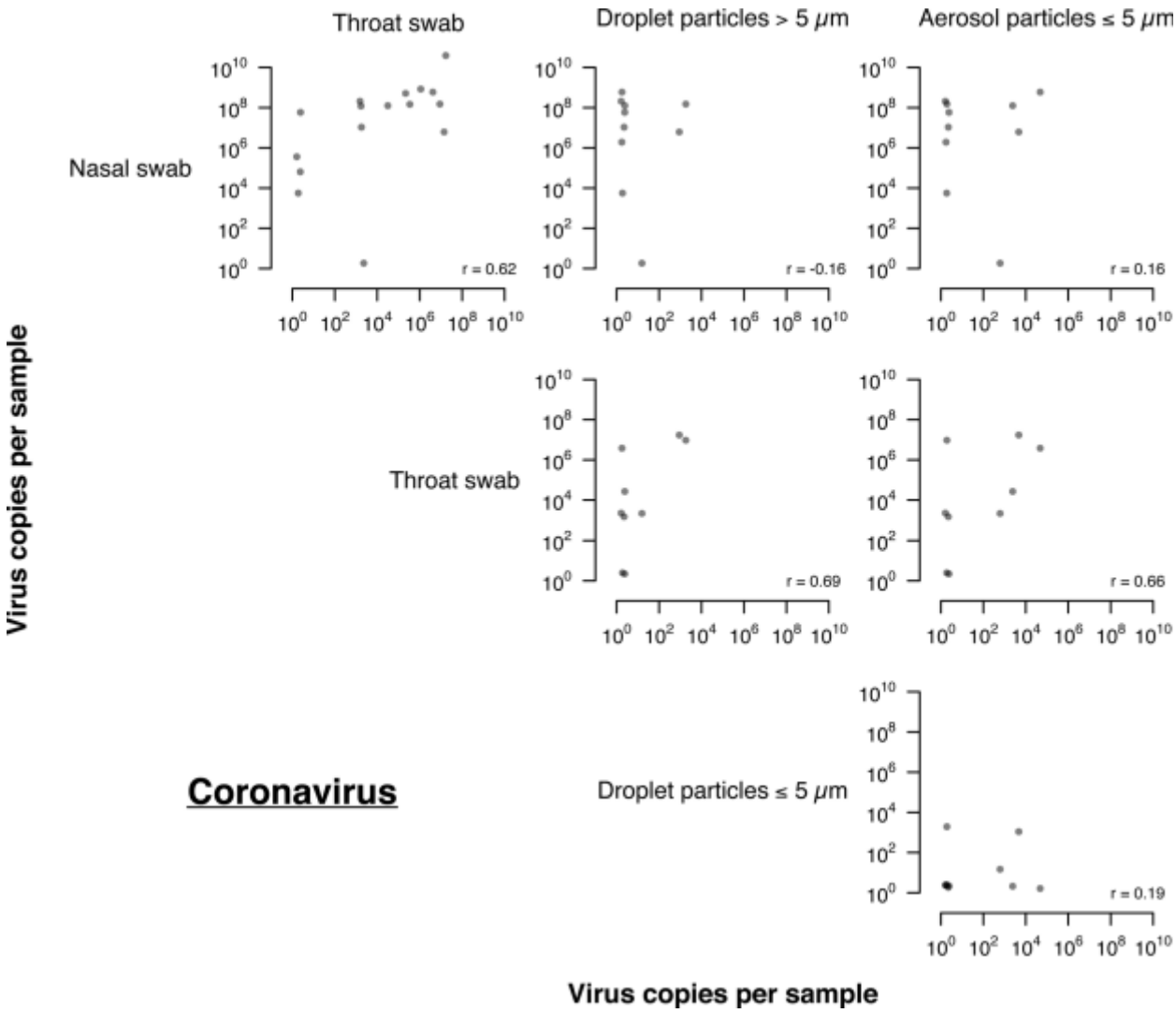


Extended Data Fig. 4 | See next page for caption.

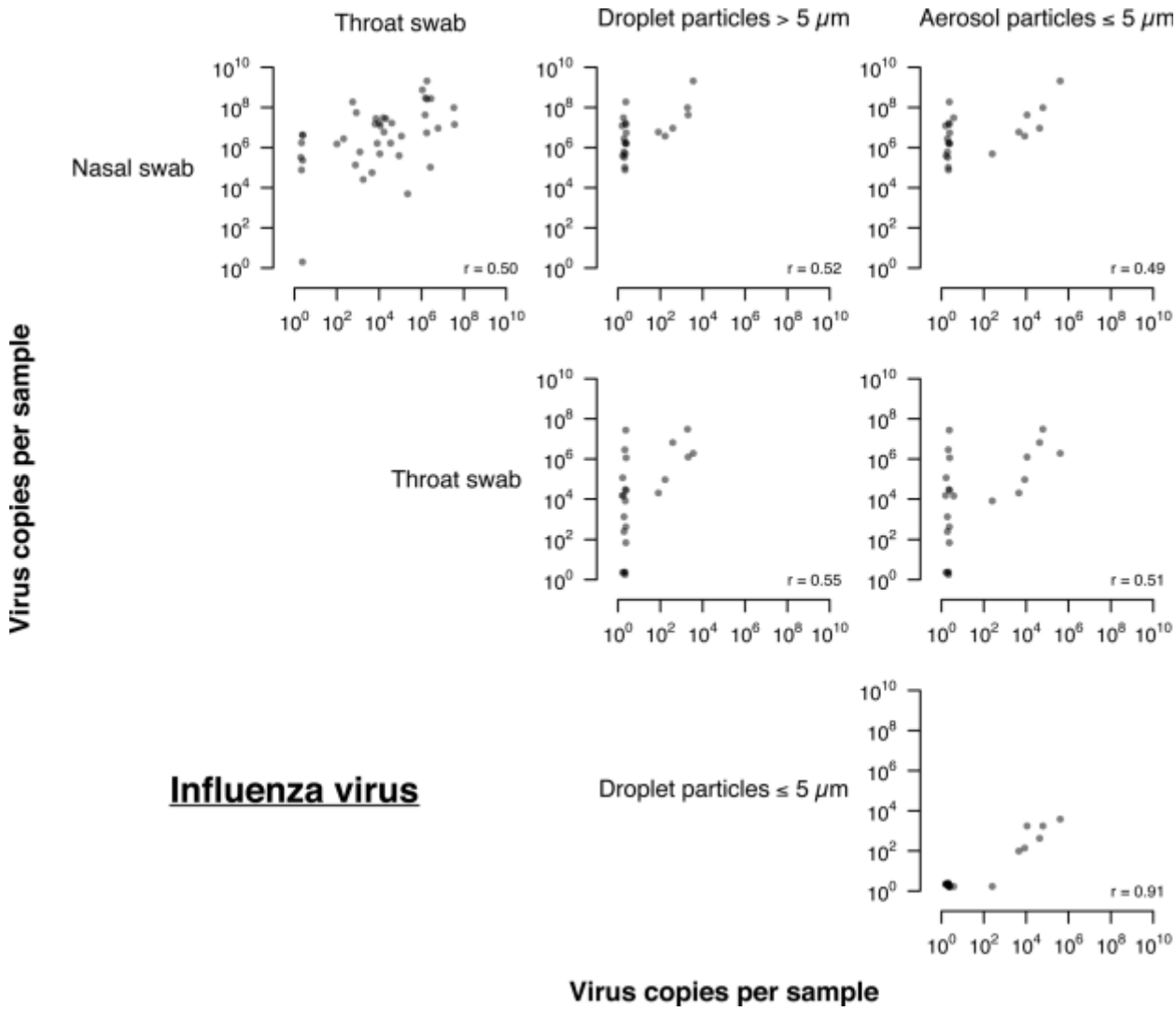
Extended Data Fig. 4 | Efficacy of surgical face masks in reducing respiratory virus shedding in respiratory droplets and aerosols of symptomatic individuals with seasonal coronaviruses including (a) coronavirus NL63, (b) coronavirus OC43 and (c) coronavirus HKU1. This figure shows the virus copies per sample collected in nasal swab (red), throat swab (blue), respiratory droplets collected for 30 min while not wearing (dark green) or wearing (light green) a surgical face mask and aerosols collected for 30 min while not wearing (brown) or wearing (orange) a face mask, collected from individuals with acute respiratory symptoms who were positive for coronavirus NL63, coronavirus OC43 and coronavirus HKU1 as determined by RT-PCR in any samples. *P* values for mask intervention as predictor of $\log_{10}$ virus copies per sample in an unadjusted univariate Tobit regression model which allowed for censoring at the lower limit of detection of the RT-PCR assay are shown, with significant differences in bold. For nasal swabs and throat swabs, all infected individuals were included (coronavirus NL63, n = 8; coronavirus OC43, n = 5; coronavirus HKU1, n = 4). For respiratory droplets and aerosols, numbers of infected individuals who provided exhaled breath samples while not wearing or wearing a surgical face mask, respectively were: coronavirus NL63 (n = 3 and 5), coronavirus OC43 (n = 3 and 4), coronavirus HKU1 (n = 4 and 2). A subset of participants provided exhaled breath samples for both mask interventions (coronavirus NL63, n = 0; coronavirus OC43, n = 2; coronavirus HKU1, n = 2).



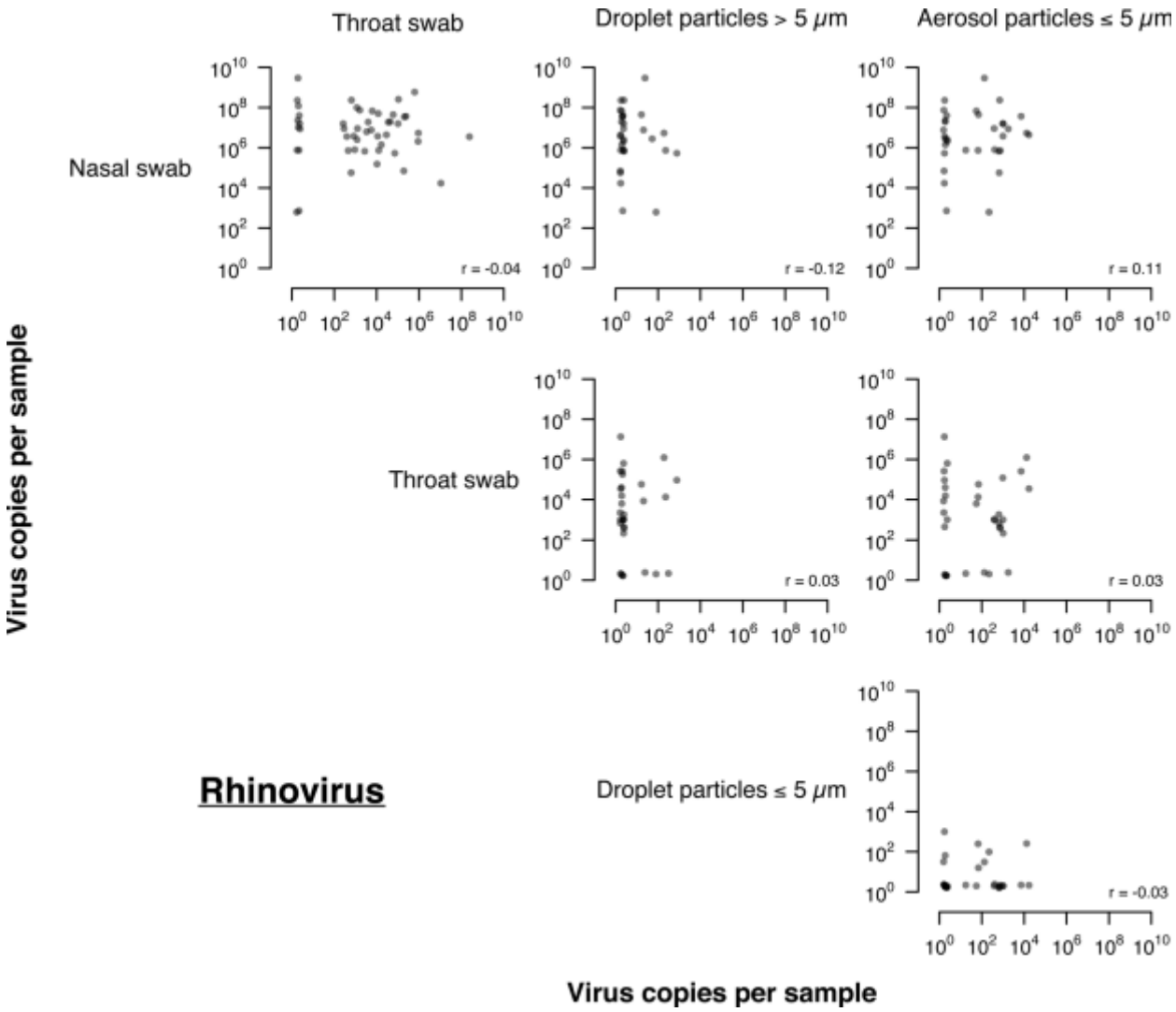
Extended Data Fig. 5 | Efficacy of surgical face masks in reducing respiratory virus shedding in respiratory droplets and aerosols of symptomatic individuals with seasonal influenza viruses including (a) influenza a and (b) influenza B virus. The figure shows the virus copies per sample collected i nasal swab (red), throat swab (blue), respiratory droplets collected for 30 min while not wearing (dark green) or wearing (light green) a surgical face mask and aerosols collected for 30 min while not wearing (brown) or wearing (orange) a face mask, collected from individuals with acute respiratory symptoms who were positive for influenza A and influenza B virus as determined by RT-PCR in any samples. *P* values for mask intervention as predictor of $\log_{10}$ virus log copies per sample in an unadjusted univariate Tobit regression model which allowed for censoring at the lower limit of detection of the RT-PCR assay are shown, with significant differences in bold. For nasal swabs and throat swabs, all infected individuals were included (influenza A virus, n = 31; influenza B virus, n = 14). For respiratory droplets and aerosols, numbers of infected individuals who provided exhaled breath samples while not wearing or wearing a surgical face mask, respectively were: influenza A virus (n = 19 and 19), influenza B virus (n = 6 and 10). A subset of participants provided exhaled breath samples for both mask interventions (influenza A virus, n = 7; influenza B virus, n = 2).



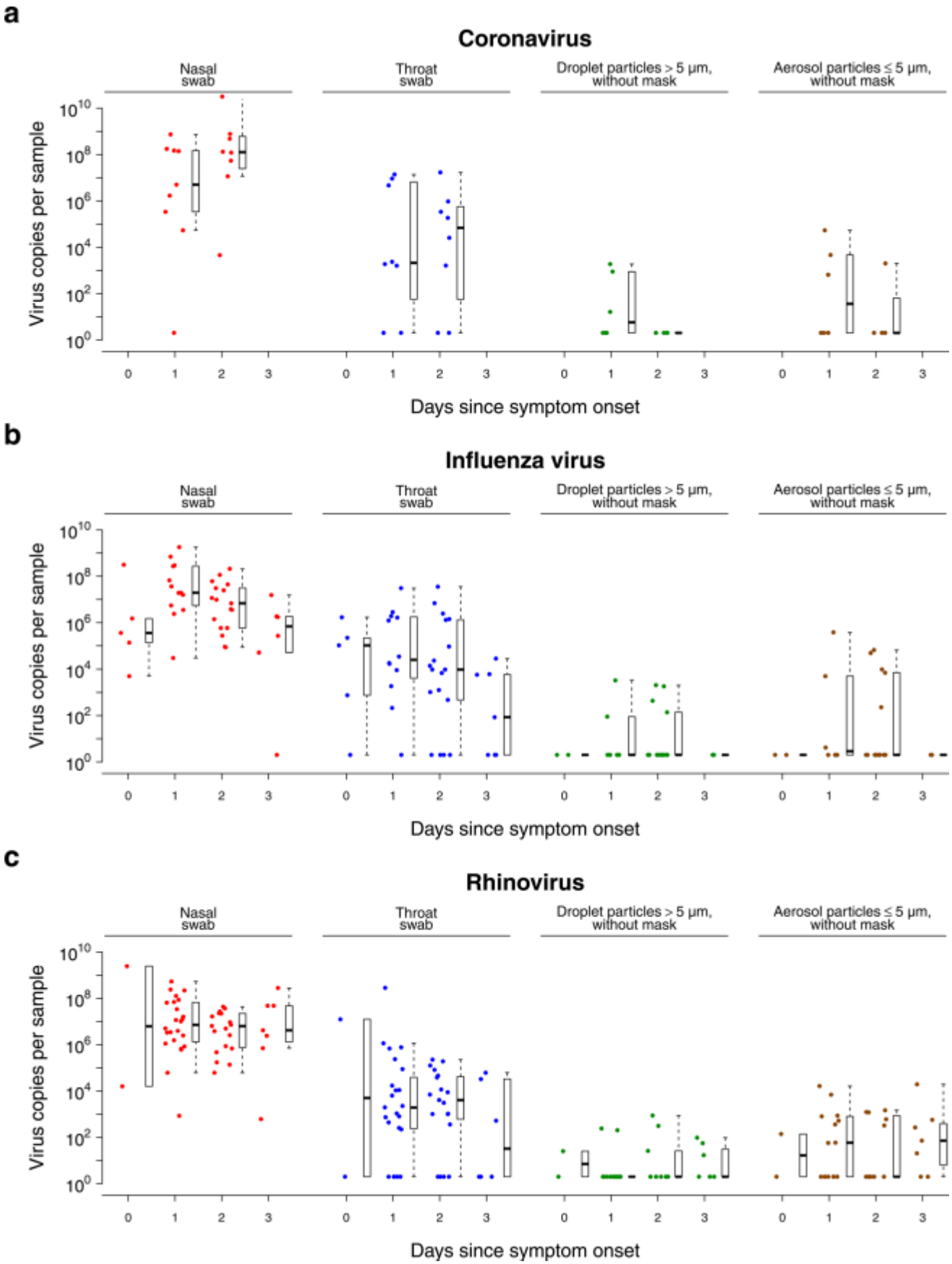
Extended Data Fig. 6 | Correlation of coronavirus viral shedding between different samples (nasal swab, throat swab, respiratory droplets and aerosols) in symptomatic individuals with seasonal coronavirus infection. For nasal swabs and throat swabs, all infected individuals were included (n = 17). For respiratory droplets and aerosols, only infected individuals who provided exhaled breath samples while not wearing a surgical face mask were included (n = 10). r, the Spearman's rank correlation coefficient.



Extended Data Fig. 7 | Correlation of influenza viral shedding between different samples (nasal swab, throat swab, respiratory droplets and aeroso in symptomatic individuals with seasonal influenza infection. For nasal swabs and throat swabs, all infected individuals were included (n = 43). For respiratory droplets and aerosols, only infected individuals who provided exhaled breath samples while not wearing a surgical face mask were included (n = 23). r, the Spearman's rank correlation coefficient.



Extended Data Fig. 8 | Correlation of rhinovirus viral shedding between different samples (nasal swab, throat swab, respiratory droplets and aeros in symptomatic individuals with rhinovirus infection. For nasal swabs and throat swabs, all infected individuals were included (n = 54). For respiratory droplets and aerosols, only infected individuals who provided exhaled breath samples while not wearing a surgical face mask were included (n = 36). r, the Spearman's rank correlation coefficient.



Extended Data Fig. 9 | See next page for caption.

Extended Data Fig. 9 | Respiratory virus shedding in respiratory droplets and aerosols stratified by days from symptom onset for (a) coronavirus, influenza virus or (c) rhinovirus. The figures shows the virus copies per sample collected in nasal swab (red), throat swab (blue), respiratory droplets (dark green) and aerosols (brown) collected for 30 min while not wearing a surgical face mask, stratified by the number of days from symptom onset on which the respiratory droplets and aerosols were collected. For nasal swabs and throat swabs, all infected individuals were included (coronavirus, n = 17; influenza virus, n = 43; rhinovirus, n = 54). For respiratory droplets and aerosols, numbers of infected individuals who provided exhaled breath samples while not wearing or wearing a surgical face mask, respectively were: coronavirus (n = 10 and 11), influenza virus (n = 23 and 28), rhinovirus (n = 36 and 32). A subset of participants provided exhaled breath samples for both mask interventions (coronavirus, n = 4; influenza virus, n = 8; rhinovirus, n = 14). The box plots indicate the median with the interquartile range (lower and upper hinge) and ± 1.5 × interquartile range from the first and third quartile (lower and upper whisker).

nature research

Corresponding author(s): Benjamin John Cowling

Last updated by author(s): Mar 4, 2020

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No software was used.
Data analysis	All analyses were conducted with R version 3.6.0 and the VGAM package 1.1.1.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Anonymized raw data and R syntax to reproduce all the analyses, figures, tables and supplementary tables in the published article are available at: [Dryad link pending].

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	We estimated a priori the sample size to be 300 participants. The primary outcome of the study was the reduction in the exhaled virus concentration of normal tidal breathing by wearing face mask in terms of total virus by RT-PCR as a proxy for infectious virus particle. We expected that a 1-log reduction in exhaled virus particle by face mask intervention would have a clinically relevant effect in reducing the probability of transmission. Except for influenza, there was no quantitative data available from exhaled breath samples from respiratory virus-infected individuals before the present study. If the standard deviation of exhaled virus concentration was 1 log copies/ml (Milton et al., PLoS Pathog 2013), we would detect a difference of >1 log copies/ml in the mask vs control group as long as we have >15 participants with a specific respiratory virus. For example, if our study included 23 participants with rhinovirus detectable in exhaled breath without a mask, we will have 80% power and 0.05 significance level to identify differences in viral shedding in aerosols of 1.28 log10 copies associated with the use of face masks, assuming a standard deviation of 1.54 log10 copies based on data from nasal and throat swab (Lu et al., J Clin Microbiol 2008). We expected from 300 individuals with ARI, at least 150 to have a respiratory virus, and at least 20-30 to have each of rhinovirus, coronavirus, adenovirus and parainfluenza plus small numbers of other respiratory viruses, assuming the Viral Panel would detect respiratory viruses in 60% of participants including 10% by influenza (since we partly recruited during the influenza seasons) and the other 50% made up of rhinovirus, coronavirus, adenovirus and parainfluenza virus.
Data exclusions	As described in the Results section and Supplementary Figure 1, only participants who provided exhaled breath samples and randomized to mask intervention were included; and final analyses were performed only for participants with either coronavirus, influenza virus or rhinovirus infection, which had sufficient sample size for comparison between mask intervention.
Replication	Samples from a subset of participants identified with a coronavirus, influenza or rhinovirus infection were re-tested by RT-PCR with consistent results. R syntax is available to reproduce all the analyses, figures, tables and supplementary tables in the published article
Randomization	Prior to the exhaled breath collection, each participant was randomly allocated in a 1:1 ratio to either wearing a surgical face mask or not during the exhaled breath collection using a computer-generated sequence. The allocation was concealed to the study stuff performing the exhaled breath collection before allocation of the mask intervention.
Blinding	Blinding to the participant and the study stuff for the mask intervention was not possible. The study staff performing the statistical analyses was also involved in the data collection. We expected there would be minimal bias due to unblinding since data collection for questionnaires was done before randomization to mask intervention, and viral load from a sample measured by RT-PCR is an objective measurement

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☐	☒ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	Madin-Darby Canine Kidney (MDCK) cells
Authentication	European Collection of Authenticated Cell Cultures.
Mycoplasma contamination	We confirm that all cell lines tested negative for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	Nil

Human research participants

Policy information about studies involving human research participants

Population characteristics	As described in the Results section, Table 1a and Supplementary Table 1, there were some differences in characteristics of participants with the different viruses. Overall, most participants were younger adults and 5% were age 11-17 years, but there were more children with influenza virus and no children in the subgroup with coronavirus infection. Overall, 59% were female, but there were more females among the subgroup with coronavirus infection. The majority of participants did not have underlying medical conditions and overall 9% had received influenza vaccination for the current season but only 2% among those with influenza virus infection. The majority of participants were sampled within 24–48 or 48–72 hours of illness onset. 24% of participants had a measured fever ≥37.8ºC, with influenza patients more than twice as likely than coronavirus and rhinovirus-infected patients to have a measured fever. Coronavirus-infected participants coughed the most with an average of 17 (SD 30) coughs during the 30-minute exhaled breath collection. The profile of the participants randomized to with-mask vs without-mask groups were similar.
Recruitment	As described in the Methods section, participants were recruited year-round from March 2013 through May 2016 in a general outpatient clinic of a private hospital in Hong Kong. As routine practice, clinic staff screened all individuals attending the clinics for respiratory and any other symptoms regardless of the purpose of the visit at the triage. Study staff then approached immediately those who reported at least one of the following symptoms of acute respiratory illness (ARI) for further screening: fever ≥37.8ºC, cough, sore throat, runny nose, headache, myalgia and phlegm. Individuals who reported ≥2 ARI symptoms, within 3 days of illness onset and ≥11 years of age were eligible to participate.
Ethics oversight	As described in the Methods section, the study protocol was approved by the Institutional Review Board of The University of Hong Kong and the Clinical and Research Ethics Committee of Hong Kong Baptist Hospital.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	The present study was not registered in clinical trials registries, as it was a laboratory-based study of detection of viruses in exhaled breath and the effect of wearing surgical facemasks on virus detection. It was not a Phase II/III clinical trial.
Study protocol	Not available in clinical trials registries (as above). Study protocol will be made available to editors and peer reviewers if requested.
Data collection	As described in the Methods section, participants were recruited year-round from March 2013 through March 2016 in a general outpatient clinic of a private hospital in Hong Kong. Data collection for questionnaires and exhaled breath sample collection was done face-to-face with the participant by trained study staff at the same clinic on the day of participant enrolment.
Outcomes	As pre-specified in the study protocol, the primary outcomes of the study were the virus generation rate in the tidal breathing of participants infected by different respiratory viruses, and the efficacy of face mask in preventing virus dissemination in exhaled breath especially at the aerosol fraction. As pre-specified in the study protocol, one of the secondary outcomes was to provide indirect evidence for relative importance of different transmission routes of influenza and other respiratory viruses. In this regard, in the present manuscript we examined the correlation between viral shedding in nose swabs, throat swabs, respiratory droplets and aerosols, and factors affecting viral shedding in respiratory droplets and aerosols. As described in the Discussion section in the present manuscript about the limitation of our study, there was large proportion of participants with undetectable viral shedding in exhaled breath for each of the viruses studied, and therefore we were unable to examine the exhaled respiratory virus reduction proportion by chi-squared test, nor the exhaled respiratory virus reduction volume (i.e. viral load) by t-test and linear regression as pre-specified in the study protocol. Instead, we have used Fisher's exact test and Tobit regression for the same purposes respectively.

National Wildlife Rehabilitators Association

Position Statement:
Rehabilitating North American Bats during the SARS-CoV2/COVID-19 Pandemic

The newly emerging SARS-CoV2, which causes COVID-19 in humans, is currently a worldwide pandemic and has been diagnosed in people from every state and province in the United States[1] and Canada. A summary of the non-human animal SARS-CoV2 positive individuals as of 4/10/2020 is provided as an appendix at the end of this statement. Zoonotic transmission from these animals back to humans has not been reported.

Currently, it is thought that the newly emerging SARS-CoV2 virus originated from wildlife and possibly from East Asian pangolins, and/or East Asian bats[3]. Details about these theories are summarized at the end of this statement. SARS-CoV2 has not been found naturally in any wild bat. One preliminary study did show the ability to infect Egyptian fruit bats with SARS-CoV2 in a research setting, however, they did not shed enough virus to infect conspecifics[4]. There are over 1400 different species of bats[5], and it is unknown if this virus acts similarly in other species of bats. Many other coronaviruses use bats as a reservoir[6,7] and there is currently no evidence that introduction of SARS-CoV2 to North American bats would or would not cause population declines.

A certain percentage of humans can be asymptomatic shedders of SARS-CoV2[1]. Current expert understanding is that SARS-CoV2 is primarily transmitted person-to-person, and not a current zoonotic concern in any species[8].

Many species of North American bats are incredibly adaptable and live in and around human dwellings (houses, attics, flashing, backyard foliage and trees, etc.), even in dense human populations. Bats come into close proximity of many people every day.

Wildlife rehabilitators across the continent who are trained to work with bats are a valuable public health resource. Members of the public come into contact with bats regularly, and often are directly exposed to bats before contacting professionals. Wildlife rehabilitators direct these individuals to local public health departments, help walk individuals through learning about rabies risk factors and following all public health recommendations, and ultimately act as an advocate for the health of the finder and the bat.

The NWRA Veterinary Committee supports the continued admittance of bats for rehabilitation to wildlife rehabilitation professionals not only to ensure proper bat care, welfare, and conservation, but foremost as part of the solution to public health concerns surrounding bats. Without wildlife rehabilitators, it is highly likely the public will have more human-bat interactions

that lack the involvement of a professional to guide zoonotic concerns and take necessary biosecurity precautions. Wildlife rehabilitators also represent a valuable resource for sample collection for researchers and are encouraged to collaborate whenever possible.

The NWRA Veterinary Committee supports one-health initiatives to improve human health, animal health and environmental health. Furthermore, the NWRA Veterinary Committee supports evidence-based medicine and subsequent actions.

Restrictions on bat rehabilitation and release have massive implications for the thousands of bats that are rehabilitated in the US and Canada each year, and for the adverse human-bat interactions that will inevitably occur as untrained people continue to find bats in need of care. If SARS-CoV2 is able to be transmitted from humans to bats, it has likely already occurred due to the high number of human cases in the United States and the frequency that people interact with bats at their homes.

Furthermore, due to the COVID-19 outbreak, fewer resources are available at municipalities to handle wildlife cases resulting in additional risk for the public who may take it upon themselves to handle a bat, without appropriate PPE, thus potentially having a negative impact on the health and welfare of the bat and the health of the person who handled it. Wildlife rehabilitators' expertise is needed even more during this crisis to protect human and animal health.

RECOMMENDATIONS

1. We recommend that authorized wildlife rehabilitators should be allowed to accept and rehabilitate bats, following appropriate biosecurity and safety measures to prevent human-bat respiratory virus exposures.
2. New bat admissions should be quarantined for 14 days
3. There is no need to euthanize captive bats as a method of disease prevention
4. Limit the number of people who have access to areas where bats are housed and cared for.
5. Rehabilitators showing any clinical signs of Covid-19 such as fever, coughing, or shortness of breath should immediately cease working with bats and consult their doctor for testing and quarantine recommendations. Backup caregivers should be pre-arranged for bats and any other animals undergoing rehabilitation.
6. Working with the local or regional veterinary laboratories and state/provincial public health officials, samples should be collected from rehabilitated bats if feasible, and if/when testing for bats is available, the bats should be tested for Covid-19 prior to release if available. However, bats should not be delayed from appropriate release simply because testing is not available.

Because this is an emerging pathogen, new research may sway directives and this statement will be updated accordingly.

Appendix

Since the outbreak of severe acute respiratory syndrome (SARS) 18 years ago, a large number of SARS-related coronaviruses (SARSr-CoVs) have been discovered in their natural reservoir host, bats, and previous studies have shown that some bat SARSr-CoVs have the potential to infect humans.[6]

The coronaviruses found in the Malayan pangolin (*Manis javanica*) and the *Rhinolophus affinis* bat remain the current closest to SARS-CoV2 across the genome (~96%[6]); some pangolin coronaviruses exhibit strong similarity to SARS-CoV2 in the virus receptor binding domain (RBD), including all six key RBD residues.[3] However, bat coronaviruses are massively understudied[3].

Currently, it is thought that SARS-CoV2 virus originated from wildlife naturally[3], either via natural selection in the species in question before zoonotic transfer or natural selection in humans after zoonotic transfer from the species in question[3]. See the table below for current knowledge regarding positive test results suggestive of human to animal transmission of SARS-CoV2.

Summary below of current non-human animals that have tested positive for SARS-CoV2A[8]:

Species	Location	Antigen test results	Antibody test results	Symptoms	Possible source	Notes
Domestic dog	China	Weak positive	Positive	None	Owner had COVID-19	Elderly, died 3 d after release from unknown causes
Domestic dog[11]	Hong Kong	Positive		None	Owner had COVID-19	German Shepherd
Domestic cat	Belgium	Positive	--	Respiratory, vomiting	Owner had COVID-19	Unknowns around sampling
Domestic cat	China	Positive	--	None	Owner had COVID-19	
Tiger	NY, USA	Positive Confirmed by NVSL		Respiratory	Presumed asymptomatic keeper positive for COVID-19	Several big cats in zoo sick with respiratory signs
Domestic cats[12]	China	Positive (15)	Positive (11)	None? Not clear in report	Owned and stray (community acquired)	Tested 102 cats in Wuhan
Domestic cat	Hong Kong	Positive		None	Owner had COVID-19	

References

1. CDC. Coronavirus Disease 2019 Cases in U.S. doi:10.1056/NEJMoa2001316
2. Parodi SM, Liu VX. From Containment to Mitigation of COVID-19 in the US. *J Am Med Assoc*. 2020;2019:2019-2020. doi:10.1001/jama.2020.3882
3. Andersen KG, Rambaut A, Lipkin WI, Holmes EC, Garry RF. The proximal origin of SARS-CoV-2. *Nat Med*. 2020:2-4. doi:10.1038/s41591-020-0820-9
4. Friedrich-Loeffler-Institut. Novel Coronavirus SARS-CoV-2: Fruit bats and ferrets are susceptible, pigs and chickens are not. Press Release. doi:10.1007/s00403006-0675-z
5. No Title. Department of the Interior. https://www.doi.gov/blog/13facts-about-bats. Accessed July 4, 2020.
6. Zhou P, Yang X Lou, Wang XG, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*. 2020;579(7798):270-273. doi:10.1038/s41586-020-2012-7
7. Banerjee A, Kulcsar K, Misra V, Frieman M, Mossman K. Bats and coronaviruses. *Viruses*. 2019;11(1):7-9. doi:10.3390/v11010041
8. AVMA. SARS-CoV2 in animals, including pets. https://www.avma.org/resources tools/animal-health-and-welfare/covid-19/sars-cov-2-animals-including-pets. Published 2020. Accessed August 4, 2020.
9. Davy CM, Donaldson ME, Subudhi S, et al. Whitenose syndrome is associated with increased replication of a naturally persisting coronaviruses in bats. *Sci Rep*. 2018;8(1):1-12. doi:10.1038/s41598-018-33975-x
10. Wan Y, Shang J, Graham R, Baric RS, Li F. Receptor Recognition by the Novel Coronavirus from Wuhan: an Analysis Based on Decade-Long Structural Studies of SARS Coronavirus. *J Virol*. 2020;94(7):1-9. doi:10.1128/jvi.00127-20
11. Kong G of H. *Pet Dog Tests Positive for COVID-19 Virus*.; 2020. https://www.info.gov.hk/gia/general/202003/19/P2020031900606.htm.
12. Zhang Q, Zhang H, Huang K, et al. SARS-CoV-2 neutralizing serum antibodies in cats: a serological investigation. *bioRxiv*. 2020:2020.04.01.021196. doi:10.1101/2020.04.01.021196

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 April 2020 doi:10.20944/preprints202004.0203.v1

Face Masks Against COVID-19: An Evidence Review

Jeremy Howard[a,c,1], Austin Huang[b], Zhiyuan Li[k], Zeynep Tufekci[m], Vladimir Zdimal[e], Helene-Mari van der Westhuizen[f,g], Arne von Delft[o,g], Amy Price[n], Lex Fridman[d], Lei-Han Tang[i,j], Viola Tang[l], Gregory L. Watson[h], Christina E. Bax[s], Reshama Shaikh[q], Frederik Questier[r], Danny Hernandez[p], Larry F. Chu[n], Christina M. Ramirez[h], and Anne W. Rimoin[t]

[a] fast.ai, 101 Howard St, San Francisco, CA 94105, US; [b] Warren Alpert School of Medicine, Brown University, 222 Richmond St, Providence, RI 02903; [c] Data Institute, University of San Francisco, 101 Howard St, San Francisco, CA 94105, US; [d] Department of Electrical Engineering & Computer Science, Massachusetts Institute of Technology, 77 Massachusetts Ave, Cambridge, MA 02139; [e] Institute of Chemical Process Fundamentals, Czech Academy of Sciences, Rozvojová 135, CZ-165 02 Praha 6, Czech Republic; [f] Department of Primary Health Care Sciences, Woodstock Road, University of Oxford, OX2 6GG, United Kingdom; [g] TB Proof, Cape Town, South Africa; [h] Department of Biostatistics, UCLA Fielding School of Public Health, 650 Charles E Young Drive, Los Angeles, CA 90095; [i] Department of Physics, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR, China; [j] Complex Systems Division, Beijing Computational Science Research Center, Haidian, Beijing 100193, China; [k] Center for Quantitative Biology, Peking University, Haidian, Beijing 100871, China; [l] Department of Information Systems, Business Statistics and Operations Management, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China; [m] University of North Carolina at Chapel Hill; [n] School of Medicine Anesthesia Informatics and Media (AIM) Lab, Stanford University, 300 Pasteur Drive, Grant S268C, Stanford, CA 94305; [o] School of Public Health and Family Medicine, University of Cape Town, Anzio Road, Observatory, 7925, South Africa; [p] OpenAI, 3180 18th St, San Francisco, CA 94110; [q] Data Umbrella, 345 West 145th St, New York, NY 10031; [r] Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium; [s] University of Pennsylvania, 3400 Civic Center Blvd, Philadelphia, PA 19104; [t] Department of Epidemiology, UCLA Fielding School of Public Health, 650 Charles E Young Drive, Los Angeles, CA 90095

This manuscript was compiled on April 12, 2020

The science around the use of masks by the general public to impede COVID-19 transmission is advancing rapidly. Policymakers need guidance on how masks should be used by the general population to combat the COVID-19 pandemic. Here, we synthesize the relevant literature to inform multiple areas: 1) transmission characteristics of COVID-19, 2) filtering characteristics and efficacy of masks, 3) estimated population impacts of widespread community mask use, and 4) sociological considerations for policies concerning mask-wearing. A primary route of transmission of COVID-19 is likely via small respiratory droplets, and is known to be transmissible from presymptomatic and asymptomatic individuals. Reducing disease spread requires two things: first, limit contacts of infected individuals via physical distancing and contact tracing with appropriate quarantine, and second, reduce the transmission probability per contact by wearing masks in public, among other measures. The preponderance of evidence indicates that mask wearing reduces the transmissibility per contact by reducing transmission of infected droplets in both laboratory and clinical contexts. Public mask wearing is most effective at stopping spread of the virus when compliance is high. The decreased transmissibility could substantially reduce the death toll and economic impact while the cost of the intervention is low. Thus we recommend the adoption of public cloth mask wearing, as an effective form of source control, in conjunction with existing hygiene, distancing, and contact tracing strategies. We recommend that public officials and governments strongly encourage the use of widespread face masks in public, including the use of appropriate regulation.

COVID-19 | SARS-CoV-2 | Masks | Pandemic

Policymakers need urgent guidance on the use of masks by the general population as a tool in combating SARS-CoV-2, the respiratory virus that causes COVID-19. Masks have been recommended as a potential tool to tackle the COVID-19 pandemic since the initial outbreak in China (1), although usage during the outbreak varied by time and province (2). Globally, countries are grappling with translating the evidence of public mask wearing to their contexts. These policies are being developed in a complex decision-making environment, with a novel pandemic, rapid generation of new research, and exponential growth in cases and deaths in many areas. There is currently a global shortage of N95 or FFP2 respirators and surgical masks for use in hospitals. Simple cloth masks present a pragmatic solution for use by the public. This has been supported by the United States and European Centres for Disease Control. We present a literature review on the role of simple cloth masks and policies in reducing COVID-19 transmission.

1. Components to Evaluate for Public Mask Wearing

In order to identify whether public mask wearing is an appropriate policy, we need to consider these questions:

1. Do asymptomatic or presymptomatic patients pose a risk of infecting others?
2. Would a face mask likely decrease the number of people infected by an infectious mask wearer?
3. Are there alternative face covers that will not disrupt the medical supply chain, e.g. homemade cloth masks?
4. Will wearing a mask impact the probability of the wearer becoming infected themselves?
5. Does mask use reduce compliance with other recommended strategies, such as physical distancing and quarantine?

Significance Statement

Governments are evaluating the use of non-medical masks in the community amidst conflicting guidelines from health organizations. This review synthesizes available evidence to provide clarity, and advances the use of the 'precautionary principle' as a key consideration in developing policy around use of non-medical masks in public.

Jeremy Howard prepared the initial literature list; Reshama Shaikh prepared the initial literature summaries; Frederik Questier did additional literature searches and summaries; Zhiyuan Li, Violet Tang, Lei-Han Tang, and Danny Hernandez did impact modeling; Zeynep Tufekci provided sociological research and analysis; Helene-Mari van der Westhuizen and Arne von Delft provided analysis of additional impacts; Christina Bax provided review and feedback; All authors contributed to the writing.

Anne W. Rimoin is an editor of the British Medical Journal. Larry F. Chu is a member of the editorial advisory board of the British Medical Journal.

[1] To whom correspondence should be addressed. E-mail: jphoward@usfca.edu

© 2020 by the author(s). Distributed under a Creative Commons CC BY license.

6. Are there any other potential benefits to universal mask wearing such as reducing stigma, signaling solidarity, and increased compliance with other measures?

We will evaluate each consideration in turn.

2. Transmission Characteristics of COVID-19

A primary route of transmission of SARS-CoV-2 is likely via small droplets that are ejected when speaking, coughing or sneezing. The most common droplet size threshold has a minimum at 5 µm to 10 µm (3, 4). There is much debate about whether these droplets should sometimes be considered an aerosol (5). An added complexity is that aerosols are not consistently defined in the literature.

Although earlier studies assumed that droplets were spread mainly through coughing, a more recent analysis has found that transmission through talking may be a key vector, with louder speech creating increasing quantities and sizes of droplets, which are associated with a higher viral load (6).

SARS-CoV-2 is highly transmissible, with a replication number estimated to be approximately 2.4 (7) although estimates vary (8) and will likely change as improved measurements of asymptomatic spread become available. Many COVID-19 patients are asymptomatic, and nearly all have a pre-symptomatic incubation period ranging from 2 to 15 days, with a median length of 5.1 days (9). Patients are most infectious during the initial days of infection (10–15), when symptoms are mildest or not present. This characteristic differentiates SARS-CoV-2 (COVID-19) from SARS-CoV, as replication is activated early in the upper respiratory tract (14, 16). High viral titers of SARS-CoV-2 are reported in the saliva of COVID-19 patients. These titers have been highest at time of patient presentation and viral levels are just as high in asymptomatic or presymptomatic patients (11, 16).

A consequence of these disease characteristics is that any successful intervention policy must properly address transmission due to infectious patients that display few or no symptoms and may not realize that they are infected.

3. Filtering Capability of Masks

Masks can be made of different materials and designs (17) which influence their filtering capability. There are rigorous standards evaluating masks used in healthcare settings but these focus on personal protective equipment (PPE) efficacy, that is, the ability of the mask to protect the wearer from infectious particles. N95 (the American standard; the equivalent in Europe is FFP2) respirators are recommended for health workers conducting aerosol-generating procedures during clinical care of COVID-19 patients. While it has been shown that N95 or FFP2 respirators perform well as PPE, they can become a scarce resource during a pandemic. Toner and Waldhorn (2006) (18) point out that shortages of N95 or FFP2 respirators should be anticipated, and say that if no other masks are available, surgical masks, which will provide droplet protection, should be used. One approach that has been studied for handling N95 or FFP2 respirator shortages is sterilization and re-use, which can be effective (19).

Masks can also be used for source control, which refers to blocking droplets ejected by the wearer, as well as PPE. Although we consider both of these as important, our focus in this paper is on source control, because if everyone is wearing masks to decrease the chance that they themselves are unknowingly infecting someone, everyone ends up being more protected.

Multiple studies show the filtration effects of cloth masks relative to surgical masks. Particle sizes for speech are on the order of 1 µm (20) while typical definitions of droplet size are 5 µm-10 µm (5). Generally available household materials had between a 49% and 86% filtration rate for 0.02 µm exhaled particles whereas surgical masks filtered 89% of those particles (21). In a laboratory setting, household materials had 3% to 60% filtration rate for particles in the relevant size range, finding them comparable to some surgical masks (22). In another laboratory setup, a tea cloth mask was found to filter 60% of particles between 0.02 µm to 1 µm, where surgical masks filtered 75% (23). Dato et al (2006) (24), note that "quality commercial masks are not always accessible." They designed and tested a mask made from heavyweight T-shirts, finding that it "offered substantial protection from the challenge aerosol and showed good fit with minimal leakage". Although cloth and surgical masks are primarily targeted towards droplet particles, some evidence suggests they may have a partial effect in reducing viral aerosol shedding (25).

When considering the relevance of these studies of ingress, it's important to note that they are likely to substantially underestimate effectiveness of masks for source control. When someone is breathing, speaking, or coughing, only a tiny amount of what is coming out of their mouths is already in aerosol form. Nearly all of what is being emitted is droplets. Many of these droplets will then evaporate and turn into aerosolized particles that are 3 to 5-fold smaller. The point of wearing a mask as source control is largely to stop this process from occurring, since big droplets dehydrate to smaller aerosol particles that can float for longer in air (26).

Anfinrud et al (6) used laser light-scattering to sensitively detect droplet emission while speaking. Their analysis showed that virtually no droplets were "expelled" with a homemade mask consisting of a washcloth attached with two rubber bands around the head, while significant levels were expelled without a mask. The authors stated that "wearing any kind of cloth mouth cover in public by every person, as well as strict adherence to distancing and handwashing, could significantly decrease the transmission rate and thereby contain the pandemic until a vaccine becomes available."

An important focus of analysis for public mask wearing is droplet source control. This refers to the effectiveness of blocking droplets from an infectious person, particularly during speech, when droplets are expelled at a lower pressure and are not small enough to squeeze through the weave of a cotton mask. Many recommended cloth mask designs also include a layer of paper towel or coffee filter, which could increase filter effectiveness for PPE, but does not appear to be necessary for blocking droplet emission (6, 27, 28).

In summary, there is laboratory-based evidence that household masks have some filtration capacity in the relevant droplet size range, as well some efficacy in blocking droplets and particles from the wearer (26). That is, these masks help people keep their droplets to themselves.

4. Mask Efficacy Studies

Although no randomized controlled trials (RCT) on the use of masks as source control for SARS-CoV-2 has been published, a number of studies have attempted to indirectly estimate the efficacy of masks. Overall, an evidence review (29) finds "moderate certainty evidence shows that the use of handwashing plus masks probably reduces the spread of respiratory viruses."

The most relevant paper (30), with important implications for public mask wearing during the COVID-19 outbreak, is one that compares the efficacy of surgical masks for source control for seasonal coronavirus, influenza, and rhinovirus. With ten participants, the masks were effective at blocking coronavirus droplets of all sizes for every subject. However, masks were far less effective at blocking rhinovirus droplets of any size, or of blocking small influenza droplets. The results suggest that masks may have a significant role in source control for the current coronavirus outbreak. The study did not use COVID-19 patients, and it is not yet known whether seasonal coronavirus behaves the same as SARS-CoV-2; however, they are of the same genus, so similar behavior is likely.

Another relevant (but under-powered, with n=4) study (31) found that a cotton mask blocked 96% (reported as 1.5 log units or about a 36-fold decrease) of viral load on average, at eight inches away from a cough from a patient infected with COVID-19. If this is replicated in larger studies it would be an important result, because it has been shown (32) that "every 10-fold increase in viral load results in 26% more patient deaths" from "acute infections caused by highly pathogenic viruses".

A comparison of homemade and surgical masks for bacterial and viral aerosols (21) observed that "the median-fit factor of the homemade masks was one-half that of the surgical masks. Both masks significantly reduced the number of microorganisms expelled by volunteers, although the surgical mask was 3 times more effective in blocking transmission than the homemade mask." Research focused on aerosol exposure has found all types of masks are at least somewhat effective at protecting the wearer. Van der Sande et al (33) found that "all types of masks reduced aerosol exposure, relatively stable over time, unaffected by duration of wear or type of activity", and concluded that "any type of general mask use is likely to decrease viral exposure and infection risk on a population level, despite imperfect fit and imperfect adherence". Overall however, analysis of particle filtration is likely to underestimate the effectiveness of masks, since the fraction of particles that are emitted as aerosol (vs. droplet) is quite small (26). Analysis of seasonal coronavirus compared to rhinovirus (30) suggests that filtration of COVID-19 may be much more effective, especially for source control.

The importance of using masks for health care workers has been observed (34) in three Chinese hospitals where, in each hospital, medical staff wearing masks (mainly in quarantine areas) had no COVID-19 infections, despite being around COVID-19 patients far more often, whilst other medical staff had 10 or more infections in each of the three hospitals.

Masks seem to be effective for source control in the controlled setting of an airplane. One case report (35) describes a man who flew from China to Toronto and then tested positive for COVID-19. He was wearing a mask during the flight. The 25 people closest to him on plane/flight attendants were tested and all were negative. Nobody has been reported from that flight as getting COVID-19. Another case study involving a masked influenza patient on an airplane (36) found that "wearing a face mask was associated with a decreased risk for influenza acquisition during this long-duration flight".

Guideline development for health worker personal protective equipment have focused on whether surgical masks or N95 respirators should be recommended. Most of the research in this area focuses on influenza. At this point, it is not known to what extent findings from influenza studies apply to COVID-19 filtration. Wilkes et al (37) found that "filtration performance of pleated hydrophobic membrane filters was demonstrated to be markedly greater than that of electrostatic filters." However, even substantial differences in materials and construction do not seem to impact the transmission of droplet-borne viruses in practice, such as a meta-analysis of N95 respirators compared to surgical masks (38) that found "the use of N95 respirators compared with surgical masks is not associated with a lower risk of laboratory-confirmed influenza." Johnson et al (39) showed that "surgical and N95 masks were equally effective in preventing the spread of PCR-detectable influenza". Radonovich et al (40) found in an outpatient setting that "use of N95 respirators, compared with medical masks... resulted in no significant difference in the rates of laboratory-confirmed influenza."

One of the most frequently mentioned papers evaluating the benefits and harms of cloth masks have been by MacIntyre et al (41). Findings have been misinterpreted, and therefore justify detailed discussion here. The authors "caution against the use of cloth masks" for healthcare professionals compared to the use of surgical masks and regular procedures, based on an analysis of transmission in hospitals in Hanoi. We emphasize the setting of the study - health workers using masks to protect themselves against infection. The study compared a "surgical mask" group which received 2 new masks per day, to a "cloth mask" group that received 5 masks for the entire 4 week period and were required to wear the masks all day, to a "control group" which used masks in compliance with existing hospital protocols, which the authors describe as a "very high level of mask use". It is important to note that the authors did not have a "no mask" control group because it was deemed "unethical to ask participants to not wear a mask." The study does not inform policy pertaining to public mask wearing as compared to the absence of masks in a community setting, since there is not a "no mask" group. The results of the study show that the group with a regular supply of new surgical masks each day had significantly lower infection of rhinovirus than the group that wore a limited supply of cloth masks. This paper lends support to the use of clean, surgical masks by medical staff in hospital settings to avoid rhinovirus infection by the wearer, and is consistent with other studies that show cloth masks provide poor filtration for rhinovirus (30). Its implementation does not inform the effect of using cloth masks versus not using masks in a community setting for source control of SARS-CoV-2, which is of the same genus as seasonal coronavirus, which has been found to be effectively filtered by cloth masks in a source control setting (30).

A. Studies of Impact on Community Transmission. When evaluating the available evidence for the impact of masks on community transmission, it is critical to clarify the setting of the research study (health care facility or community), the res-

piratory illness being evaluated and what reference standard was used (no mask or surgical mask). There are no RCTs that have been done to evaluate the impact of masks on community transmission during a coronavirus pandemic. While there is some evidence from influenza outbreaks, the current global pandemic poses a unique challenge. A review (42) of 67 studies including randomized controlled trials and observational studies found that simple and lowcost interventions would be useful for reducing transmission of epidemic respiratory viruses. The review recommended that "the following effective interventions should be implemented, preferably in a combined fashion, to reduce transmission of viral respiratory disease: 1. frequent handwashing with or without adjunct antiseptics; 2. barrier measures such as gloves, gowns, and masks with filtration apparatus; and 3. suspicion diagnosis with the isolation of likely cases". However, it cautioned that routine longterm implementation of some measures assessed might be difficult without the threat of an epidemic.

Seuesset al conducted an RCT (43)that suggests household transmission of influenza can be reduced by the use of non-pharmaceutical interventions, namely the use of face masks and intensified hand hygiene, when implemented early and used diligently. Concerns about acceptability and tolerability of the interventions should not be a reason against their recommendation (43). Cowling et al (44) investigated hand hygiene and face masks in an RCT that seemed to prevent household transmission of influenza virus when implemented within 36 hours of index patient symptom onset. These findings suggestthat non-pharmaceutical interventions are important for mitigation of pandemic and inter-pandemic influenza.

RCT findings by Aiello et al (45) "suggest that face masks and hand hygiene may reduce respiratory illnesses in shared living settings and mitigate the impact of the influenza A (H1N1) pandemic". A randomized intervention trial (46) found that "face masks and hand hygiene combined may reduce the rate of ILI [influenza-like illness] and confirmed influenza in community settings. These non-pharmaceutical measures should be recommended in crowded settings at the start of an influenza pandemic." The authors noted that their study "demonstrated a significant association between the combined use of face masks and hand hygiene and a substantially reduced incidence of ILI during a seasonalinfluenza outbreak. If masks and hand hygiene have similar impacts on primary incidence of infection with other seasonal and pandemic strains, particularly in crowded, community settings, then transmission of viruses between persons may be significantly decreasedby these interventions."

An observational study in Hong Kong on SARS (47) found "frequent mask use in public venues, frequent hand washing, and disinfecting the living quarters were significant protective factors (OR 0.36 to 0.58)". An important observation was that "members of the case group [infected with SARS] were less likely than members of the control group [not infected] to have frequently worn a face mask in public venues (27.9% vs. 58.7%)".

B. Implementation and Sociolological Considerations. For a novel diseasewhere much is unknown, it is important to examine the context of studies closely and also distinguish "absenceof evidence" from "evidence of absence"(2). We discuss estimates of cloth mask filtering performance in Filtering Capability of Masks and summarize modelling on population impact in Estimating Population Impacts.

Some of the concerns about public mask wearing have not been around primary evidence for the efficacy of source control, but concerns about how they will be used. We present someconsiderations for the translation of evidence about public mask wearing to diverse countries acrossthe globe, outside of the parameters of a controlled research setting:

B.1. Supply chain management of N95 respirators and surgical masks. There has been a global shortage of protective equipment for health workers, with health workers falling ill and dying of occupationally acquired COVID-19 disease(48). Public messaging encouraging mask use and depleting critical supplies have been a major concern. Some regions, like South Korea and Taiwan, have decided to promote surgical mask use on a mass scale and opted to address potential stock issues through rapidly increasing production of surgical masks. In regions where surgical mask supplies are scarce, cloth masks may be a pragmatic temporary alternative to surgical masks for the public.

B.2. Sociological considerations and anticipating population-level behavior changes. It is difficult to predict the behavior change that would accompany regulations encouraging public mask use. One concern around public health messaging promoting the use of face-covering has been that members of the public may use risk compensation behavior and neglect physical distancing based on overvaluing the protection a surgical mask may offer due to an exaggerated or false sense of security (49). Similar arguments have previously been made for HIV prevention strategies (50) (51) and other safety devices and mandates such as motorcycle helmet laws (52) and seat-belts (53). However, research on these topics finds no such increase in adverse outcomes at the population level but rather improvements in safety and well-being, suggesting that even if risk compensation occurs in some individuals, that effect is dwarfed by the increased safety at the population level (53, 54). Further, even for deliberately high-risk recreational activities such as alpine skiing and snowboarding, wearing a helmet was generally associated with risk reduction oriented-behavior (55), suggesting safety devices are both compatible with and perhaps encourage safety-oriented behavior. Even for high-risk recreational activities like alpine skiing and snowboarding, helmet use has greatly reduced injury rates (56).

In general, various forms of risk compensation theories have been proposed for many different safety innovations, but have been not found to have empirical support (57) at the population level. These findings strongly suggestthat, instead of withholding a preventative tool, accompanying it with accurate messagingthat combines different preventative measures would display trust in the general public's ability to act responsibly and empower citizens, and risk compensation is unlikely to undo the positive benefits at the population level (58).

At the height of the 2009 influenza epidemic in Mexico City it was found (59) that mandatory mask requirements increasedcompliance compared to voluntary recommendations. Voluntary compliance was strongly influenced by public perception regarding the effectivenessof the recommended measures.

For many infectious diseases,including, for example, tuberculosis, health authorities recommend masks only for those

infected or people who are taking care of someone infected. However, research shows that many sick people are reluctant to wear a mask if it identifies them as sick, and thus end up not wearing them at all in an effort to avoid the stigma of illness (60, 61). Stigma is a powerful force in human societies, and many illnesses come with stigma for the sick as well as fear of them, and managing the stigma is an important part of the process of controlling epidemics as stigma also leads to people avoiding treatment as well as preventive measures that would "out" their illness (62). Many health authorities have recommended wearing masks for COVID-19 only if people are sick; however, reports of people wearing masks being attacked, shunned and stigmatized have already been observed (63). Having masks worn only by the suspected/confirmed infected also has led to employers in high-risk environments like grocery stores and prisons, and even hospitals, banning employees from wearing one sometimes with the idea that it would scare the customer or the patients (64, 65). Further, in many countries, minorities suffer additional stigma and assumptions of criminality (66). In that vein, black people in the United States have reported that they were reluctant to wear masks in public during this pandemic for fear of being mistaken as criminals (67, 68). Even if it were possible to encourage only infected people to wear masks, given the lack of access to testing in many countries, it is not possible for many people to know for sure if they are infected or not (69). Thus, while this paper has shown the importance of masks for source-control – preventing asymptomatic and presymptomatic people from infecting others – it may not even be possible to have infected/sick people wear masks due to stigma, employer restrictions, or simple lack of knowledge of one's status without mask-wearing becoming universal policy.

Another important benefit of recommending universal mask wearing would be to serve as a visible signal and reminder of the pandemic, and given the importance of ritual and solidarity in human societies (70), it is plausible that visible, public signaling via mask wearing can potentially increase compliance with other health measures as well, such as keeping distance and hand-washing. Health, especially during an epidemic, is a form of public good in that everyone else's health behaviors improve the health odds of everyone else, and that it is non-rivalrous in that one person's health does not diminish the health of anyone else (71, 72). Visible signals play an important role in human societies (73). As such, signaling participation in health behaviors by wearing a mask as well as visible enforcement (for example, shops asking customers to wear masks) can increase compliance (74). Further, historically epidemics are a time of fear, confusion and helplessness (75, 76). Mask-wearing and even mask-making or distribution can provide feelings of empowerment and self-efficacy (77), which would in turn also suggest masks could increase compliance in other health-behaviors as well by increasing self-efficacy. In Hong Kong, for example, a community-driven focus on epidemic prevention started in the early days of COVID-19, and included community activists acquiring and distributing masks especially to those without resources and the elderly, even before it was officially declared a pandemic or before their own government had taken strong steps (78). Currently, Hong Kong has not only a relatively contained epidemic compared with many other countries, but a significant reduction in influenza cases as well which their health authorities attribute, among other factors, to the near-universal mask wearing and strong norms around it (79–81).

C. Universal or near-universal mask wearing. Estimating adherence to regulations for public mask wearing is a key input for modeling the impact of public mask wearing. Telephone surveys during the SARS-CoV-2 outbreak in Hong Kong reported enhanced adherence to public mask wearing as the pandemic progressed over three weeks, with 74.5% self reported mask wearing when going out increasing to 97.5%, without mandatory requirements (82). Similar surveys reported face mask use in Hong Kong during the SARS outbreak in 2003 as 79% (83), and approximately 10% during the influenza A(H1N1) pandemic in 2009 (84). This suggests that the public have enhanced awareness of their risk, and display higher adherence levels to prevention strategies than during other epidemics. Cloth masks could be an additional tool to enhance awareness of the importance of physical distancing in public places, serving as a visual reminder. Should masks be reserved solely for use in symptomatic patients, they become a symbol of illness and could lead to public stigmatization that discourages use, as has been described for patients with tuberculosis (61). Countries like the Czech Republic and Hong Kong offer interesting perspectives on the role of citizen advocacy and on the acceptability of face-covering in public.

D. Balancing potential harm of cloth masks with additional benefits for concurrent epidemic. Based on our detailed discussion above, cloth masks have not been shown to increase the risk of infection in people using them compared to not wearing any mask. While the focus of this article has been on preventing the spread of COVID-19 disease through public mask wearing, many low-middle income countries face concurrent epidemics of diseases like tuberculosis. Tuberculosis kills 1.5 million people globally per year, and in 2018, 10 million people fell ill (85). Face covering has been shown to also reduce the transmission of tuberculosis (86) and offer additional benefits to public mask wearing. Similarly, influenza transmissibility in the community was found to have declined by 44% in Hong Kong after the implementation of changes in population behaviors, including social distancing and increased mask wearing, enforced in most stores, during the COVID-19 outbreak (82).

It has been noted (87) that ensuring compliance with non-pharmaceutical interventions can be challenging: "Mask wearing is a promising non-pharmaceutical intervention to reduce risk of secondary transmission of viral URI [upper respiratory infections], but it is likely that adherence to mask wearing would occur only if there was a major pandemic that resulted in a heightened level of community concern and fear." Many regions have now passed laws to ensure compliance. The first RCT (2008) on mask use (88) "found compliance to be low, but compliance is affected by the perception of risk. In a pandemic, we would expect compliance to improve." The authors noted that "in compliant users, masks were highly efficacious."

5. Estimating Population Impacts

At the national and global scale, effective local interventions are aggregated into epidemiological parameters of disease spread. The standard epidemiological measure of spread is known as the reproduction number R_0 which parameterizes

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 April 2020 doi:10.20944/preprints202004.0203.v1

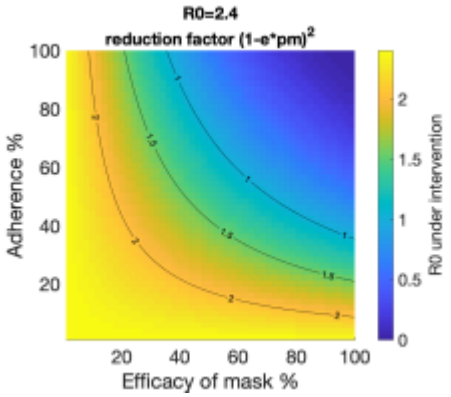


Fig. 1. Impact of public mask wearing under the full range of mask adherence and efficacy scenarios. The color indicates the resulting reproduction number R_0 from an initial R_0 of 2.4 (7).

the number of casesinfected by one case,in a completely susceptible population. R_0 determines the rate of growth, with a superlinear effect. The goal of any related healthcare policy is to have an aggregate effect of reducing R_0 to below 1.0.

Efficacy of face masks within local interventions would have an aggregate effect on the reproduction number of the epidemic. What is the magnitude of such an effect? The HKBU COVID-19 Modelling Group developed a transmission model that incorporated mask wearing and mask efficacy as a factor in the model (89). They estimate reductions in the basic reproduction number R_0 under common intervention measures. For wearing masks, they find that wearing masks reduces R_0 by a factor $(1 - ep_m)^2$, where e is the efficacy of trapping viral particles inside the mask, and p_m is the percentage of the population that wears masks. When combined with contact tracing, the two effects multiply.

A conservative assessmentapplied to the COVID-19 estimated R_0 of 2.4 (7) might posit 50% mask usage and a 50% mask efficacy level, reducing R_0 to 1.35, an order of magnitude impact rendering spread comparable to the reproduction number of seasonalinfluenza. To put this in perspective, 100 cases at the start of a month becomes31,280 casesby the month's end (R_0 = 2.4) vs. only 584 cases(R_0 = 1.35). Such a slowdown in case-loadprotects healthcare capacity and renders a local epidemic amenable to contact tracing interventions that can eliminate the spread entirely.

A full range of efficacy e and adherence p_m is shown with the resulting R_0 in Figure 1, illustrating regimes in which growth is halted entirely (R_0 < 1) as well as pessimistic regimes (e.g. due to poor implementation or population compliance) that nonetheless result in a beneficial effect in suppressing the exponential growth of the pandemic.

Yan et al (90) provide an additional example of an incremental impact assessmentof respiratory protective devices using an augmented variant of a traditional SIR model in the context of influenza with N95 respirators. They showed that a sufficiently high adherence rate (~ 80% of the population) resulted in the elimination of the outbreak with most respiratory protective devices.

Qualitative comparisons of outcomes between countries (91, 92) are suggestive of policy differences leading to differencesin diseasespread of up to three orders of magnitude. Although between-country comparisons do not allow for causal attribution, they suggestmask wearing to be a low-risk measure with a potentially large positive impact, with many countries with widespread use of masks in public keeping deaths below one in a million.

Abaluck et al (93) extend the between-country analyses from a cost perspective, estimating the marginal benefit per cloth mask worn to range from $3,000-$6,000. They also found that "the average daily growth rate of confirmed positives is 18% in countries with no preexisting mask norms and 10% in countries with such norms." and "that the growth rate of deaths is 21% in countries with no mask norms and 11% in countries with such norms."

6. Discussion and Recommendations

Our review of the literature offers evidence in favor of widespread mask use to reduce community transmission: non-medical masks use materials that obstruct droplets of the necessary size; people are most infectious in the initial period post-infection, where it is common to have few or no symptoms (10–16); non-medical masks have been effective in reducing transmission of influenza; non-medical masks have been shown to be effective in small trials at blocking transmission of coronavirus; and places and time periods where mask usage is required or widespread have shown substantially lower community transmission.

The available evidence suggeststhat near-universal adoption of non-medical masks when out in public, in combination with complementary public health measurescould successfully reduce effective-R to below 1.0, thereby stopping community spread. Economic analysis suggeststhat the impact of mask wearing could be thousands of US dollars saved per person per mask (93).

Interventions to reduce COVID-19 spread should be prioritized in order of their expected multiple on effective R divided by their cost. By this criterion experimentation with and deployment of universal masks look particularly promising. When used in conjunction with widespread testing, contact tracing, quarantining of anyone that may be infected, hand washing, and physical distancing, face masks are a valuable tool to reduce community transmission. All of these measures,through their effect on R_0, have the potential to reduce the period of lockdown required. As governments talk about relaxing lockdowns, keeping transmissions low enough to preserve health care capacity will be critical until a vaccine can be developed. Mask wearing may be critical to preventing a second wave of infections from overwhelming the health care system – further researchis urgently neededhere.

UNESCO states that "when human activities may lead to morally unacceptable harm that is scientifically plausible but uncertain, actions shall be taken to avoid or diminish that harm" (94). This is known as the "precautionary principle". The World Charter for Nature, which was adopted by the UN General Assembly in 1982, was the first international endorsement of the precautionary principle. It was implemented in an international treaty in the 1987 Montreal Protocol. The loss of life and economic destruction that has been seen already from COVID-19 is a "morally unacceptable harm". The positive impact of public mask wearing on this is "scientifically plausible but uncertain". This notion is reflected in Figure 1 - while researchersmay reasonably disagree on the magnitude of transmissibility reduction and compliance, seemingly

modest benefits can be massively beneficial in the aggregate due to the exponential character of the transmission process. Therefore, the action of ensuring widespread use of masks in the community should be taken, based on this principle (95).

Models suggest that public mask wearing is most effective at stopping spread of the virus when compliance is high. This is the same situation as we see with vaccines - the more people are vaccinated, the higher the benefit to the whole population including those who cannot be vaccinated like infants or immuno-compromised people. A common policy response to this conundrum is to ensure compliance by using laws and regulations, such as widespread state laws in the US which require vaccinations to attend school. Research shows that the strength of the mandate to vaccinate greatly influences compliance rates for vaccines and that policies that set a higher bar for vaccine exemptions result in higher vaccination rates. (96) The same approach is now being used in many jurisdictions to increase mask wearing compliance, by mandating mask use in a variety of settings (such as public transportation or grocery stores or even at all times outside the home). Early results suggest that these laws are effective at increasing compliance and slowing or stopping the spread of COVID-19 (91). We recommend that mask use requirements are implemented by governments, or when governments do not, by organizations that provide public-facing services, such as transit service providers or stores, as "no mask, no service" rules. Such mandates must be accompanied by measures to ensure access to masks, possibly including distribution and rationing mechanisms so that they do not become discriminatory but remain focused on the public health benefit. Given the value of the source control principle, especially for presymptomatic people, it is not good enough for only employees to wear masks, customers must wear masks as well.

It is also important for health authorities to provide clear guidelines for the production, use and sanitization or re-use of face masks, and consider their distribution as shortages allow. A number of countries have distributed surgical masks (South Korea, Taiwan) from early on while Japan and Singapore are now distributing cloth masks to their whole population. Clear and implementable guidelines can help increase compliance, and bring communities closer to the goal of reducing and ultimately stopping the spread of COVID-19.

Materials and Methods

A community-driven approach was used for building the paper list used in this literature review. A multidisciplinary community of researchers used online tools to review and actively discuss publications related to the question of the effectiveness and policy of public mask wearing.

ACKNOWLEDGMENTS. Thank you to Sylvain Gugger for LaTeX help, and to Cam Woodsum for assistance with preparing bibtex citations.

References

1. Q Wang, C Yu, Letter to editor: Role of masks/respirator protection against 2019-novel coronavirus (COVID-19). *Infect. Control. & Hosp. Epidemiol.*, 1–7 (year?).
2. S Feng, et al., Rational use of face masks in the COVID-19 pandemic. *The Lancet Respir. Medicine* **0** (2020).
3. J Duguid, The size and the duration of air-carriage of respiratory droplets and droplet-nuclei. *Epidemiol. & Infect.* **44**, 471–479 (1946).
4. L Morawska, et al., Size distribution and sites of origin of droplets expelled from the human respiratory tract during expiratory activities. *J. Aerosol Sci.* **40**, 256–269 (2009).
5. L Bourouiba, Turbulent Gas Clouds and Respiratory Pathogen Emissions: Potential Implications for Reducing Transmission of COVID-19. *JAMA* (2020).
6. P Anfinrud, CE Bax, V Stadnytskyi, A Bax, Could sars-cov-2 be transmitted via speech droplets? *medRxiv* (2020).
7. N Ferguson, et al., Report 9: Impact of non-pharmaceutical interventions (npis) to reduce covid19 mortality and healthcare demand (2020).
8. Y Liu, AA Gayle, A Wilder-Smith, J Rocklöv, The reproductive number of covid-19 is higher compared to sars coronavirus. *J. travel medicine* (2020).
9. SA Lauer, et al., The Incubation Period of Coronavirus Disease 2019 (COVID-19) From Publicly Reported Confirmed Cases: Estimation and Application. *Annals Intern. Medicine* (2020).
10. KKW To, et al., Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. *Lancet Infect. Dis.* **0** (2020).
11. L Zou, et al., SARS-CoV-2 Viral Load in Upper Respiratory Specimens of Infected Patients. *New Engl. J. Medicine* **382**, 1177–1179 (2020).
12. Y Bai, et al., Presumed asymptomatic carrier transmission of covid-19. *Jama* (2020).
13. J Zhang, et al., Evolving epidemiology and transmission dynamics of coronavirus disease 2019 outside Hubei province, China: a descriptive and modelling study. *The Lancet Infect. Dis.* **0** (2020).
14. N van Doremalen, et al., Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1. *New Engl. J. Medicine* **0**, null (2020).
15. WE Wei, Presymptomatic Transmission of SARS-CoV-2 â Singapore, January 23âMarch 16, 2020. *MMWR. Morb. Mortal. Wkly. Rep.* **69** (2020).
16. R Wölfel, et al., Virological assessment of hospitalized patients with covid-2019. *Nature*, 1–10 (2020).
17. Brosseau, N95 Respirators and Surgical Masks | | Blogs | CDC (2009).
18. E Toner, R Waldhorn, What Hospitals Should Do to Prepare for the Influenza Pandemic. *Mary Ann Liebert Inc.* **4**, 397–402 (2006).
19. P de Man, et al., Sterilization of disposable face masks by means of standardized dry and steam sterilization processes: an alternative in the fight against mask shortages due to COVID-19 (2020).
20. S Asadi, et al., Aerosol emission and superemission during human speech increase with voice loudness. *Sci. reports* **9**, 1–10 (2019).
21. A Davies, et al., Testing the Efficacy of Homemade Masks: Would They Protect in an Influenza Pandemic? *Disaster Medicine Public Heal. Prep.* **7**, 413–418 (2013).
22. S Rengasamy, B Eimer, RE Shaffer, Simple Respiratory Protection Evaluation of the Filtration Performance of Cloth Masks and Common Fabric Materials Against 201000 nm Size Particles. *The Annals Occup. Hyg.* **54**, 789–798 (2010).
23. Mvd Sande, P Teunis, R Sabel, Professional and Home-Made Face Masks Reduce Exposure to Respiratory Infections among the General Population. *PLOS ONE* **3**, e2618 (2008).
24. VM Dato, D Hostler, ME Hahn, Simple Respiratory Mask. *Emerg. Infect. Dis.* **12**, 1033–1034 (2006).
25. DK Milton, MP Fabian, BJ Cowling, ML Grantham, JJ McDevitt, Influenza Virus Aerosols in Human Exhaled Breath: Particle Size, Culturability, and Effect of Surgical Masks. *PLOS Pathog.* **9**, e1003205 (2013).
26. RS Papineni, FS Rosenthal, The size distribution of droplets in the exhaled breath of healthy human subjects. *J. Aerosol Medicine* **10**, 105–116 (1997).
27. DIY Face Mask – 8 Steps in Making Protective Gear | Consumer Council (2020) [Online; accessed 8. Apr. 2020].
28. Coronavirus Disease 2019 (COVID-19) (2020) [Online; accessed 8. Apr. 2020].
29. J Burch, C Bunt, Can physical interventions help reduce the spread of respiratory viruses? *Cochrane Clin. Answers* (2020).
30. NH Leung, et al., Respiratory virus shedding in exhaled breath and efficacy of face masks. *Nat. Medicine*, 1–5 (2020).
31. S Bae, et al., Effectiveness of Surgical and Cotton Masks in Blocking SARSCoV-2: A Controlled Comparison in 4 Patients. *Annals Intern. Medicine* (2020).
32. SC Jiang, et al., Every 10-fold increase in viral load results in 26% more patient deaths: a correlation analysis. *Int J Clin Exp Med* **12**, 13712–13722 (2019).
33. M van der Sande, P Teunis, R Sabel, Professional and Home-Made Face Masks Reduce Exposure to Respiratory Infections among the General Population. *PLoS ONE* **3** (2008).
34. X Wang, Z Pan, Z Cheng, Association between 2019-nCoV transmission and N95 respirator use. *J. Hosp. Infect.* **0** (2020).
35. KL Schwartz, et al., Lack of COVID-19 Transmission on an International Flight. *CMAJ* (2020).
36. L Zhang, et al., Protection by Face Masks against Influenza A(H1N1)pdm09 Virus on Trans-Pacific Passenger Aircraft, 2009. *Emerg. Infect. Dis.* **19**, 1403–1410 (2013).
37. AR Wilkes, JE Benbough, SE Speight, M Harmer, The bacterial and viral filtration performance of breathing system filters*. *Anaesthesia* **55**, 458–465 (2000).
38. Y Long, et al., Effectiveness of N95 respirators versus surgical masks against influenza: A systematic review and meta-analysis. *J. Evidence-Based Medicine* **n/a** (2020).
39. DF Johnson, JD Druce, C Birch, ML Grayson, A quantitative assessment of the efficacy of surgical and N95 masks to filter influenza virus in patients with acute influenza infection. *Clin. Infect. Dis. An Off. Publ. Infect. Dis. Soc. Am.* **49**, 275–277 (2009).
40. LJ Radonovich, et al., N95 Respirators vs Medical Masks for Preventing Influenza Among Health Care Personnel: A Randomized Clinical Trial. *JAMA* **322**, 824–833 (2019).
41. CR MacIntyre, et al., A cluster randomised trial of cloth masks compared with medical masks in healthcare workers. *BMJ Open* **5**, e006577 (2015).
42. T Jefferson, et al., Physical interventions to interrupt or reduce the spread of respiratory viruses. *The Cochrane Database Syst. Rev.* **2011** (2011).
43. T Suess, et al., The role of facemasks and hand hygiene in the prevention of influenza transmission in households: results from a cluster randomised trial; Berlin, Germany, 2009-2011. *BMC infectious diseases* **12**, 26 (2012).
44. BJ Cowling, et al., Facemasks and hand hygiene to prevent influenza transmission in households: a cluster randomized trial. *Annals Intern. Medicine* **151**, 437–446 (2009).
45. AE Aiello, et al., Mask use, hand hygiene, and seasonal influenza-like illness among young

adults: a randomized intervention trial. *The J. Infect. Dis.* **201**, 491–498 (2010).
46. AE Aiello, et al., Facemasks, Hand Hygiene, and Influenza among Young Adults: A Randomized Intervention Trial. *PLoS ONE* **7** (2012).
47. JT Lau, H Tsui, M Lau, X Yang, SARS Transmission, Risk Factors, and Prevention in Hong Kong. *Emerg. Infect. Dis.* **10**, 587–592 (2004).
48. The Lancet, COVID-19: protecting health-care workers. *The Lancet* **395**, 922 (2020).
49. LM Brosseau, ScD, M Sietsema, P| Apr 01, 2020, COMMENTARY: Masks-for-all for COVID-19 not based on sound data (2020).
50. MM Cassell, DT Halperin, JD Shelton, D Stanton, Risk compensation: the achilles' heel of innovations in hiv prevention? *Bmj* **332**, 605–607 (2006).
51. D Rojas Castro, RM Delabre, JM Molina, Give prep a chance: moving on from the risk compensation concept. *J. Int. AIDS Soc.* **22**, e25351 (2019).
52. JV Ouellet, Helmet use and risk compensation in motorcycle accidents. *Traffic injury prevention* **12**, 71–81 (2011).
53. DJ Houston, LE Richardson, Risk compensation or risk reduction? seatbelts, state laws, and traffic fatalities. *Soc. Sci. Q.* **88**, 913–936 (2007).
54. Y Peng, et al., Universal motorcycle helmet laws to reduce injuries: a community guide systematic review. *Am. journal preventive medicine* **52**, 820–832 (2017).
55. MD Scott, et al., Testing the risk compensation hypothesis for safety helmets in alpine skiing and snowboarding. *Inj. Prev.* **13**, 173–177 (2007).
56. G Ruedl, M Kopp, M Burtscher, Does risk compensation undo the protection of ski helmet use? *Epidemiology* **23**, 936–937 (2012).
57. B Pless, Risk compensation: Revisited and rebutted. *Safety* **2**, 16 (2016).
58. A Burgess, M Horii, Risk, ritual and health responsibilisation: Japans safety blanketof surgical face mask-wearing. *Sociol. health & illness* **34**, 1184–1198 (2012).
59. BJ Condon, T Sinha, Who is that masked person: the use of face masks on mexico city public transportation during the influenza a (h1n1) outbreak. *Heal. Policy* **95**, 50–56 (2010).
60. K Abney, containing tuberculosis, perpetuating stigma: the materiality of n95 respirator masks. *Anthropol. South. Afr.* **41**, 270–283 (2018).
61. E Buregyeya, et al., Acceptability of masking and patient separation to control nosocomial tuberculosis in uganda: a qualitative study. *J. Public Heal.* **20**, 599–606 (2012).
62. G Joachim, S Acorn, Stigma of visible and invisible chronic conditions. *J. advanced nursing* **32**, 243–248 (2000).
63. DK Li, R Abdelkader, Coronavirus hate attack: Woman in face mask allegedly assaulted by man who calls her 'diseased'. *NBC News* (2020).
64. Tampa Bay nurses were told not to wear masks in hallways. Now hospitals are changing the rules. (2020) [Online; accessed 9. Apr. 2020].
65. S Malone, NY Correctional Officers Ordered Not To Wear Masks, Even If They Have Them. *Maven* (2020).
66. D Pager, H Shepherd, The sociology of discrimination: Racial discrimination in employment, housing, credit, and consumer markets. *Annu. Rev. Sociol* **34**, 181–209 (2008).
67. C Fernando Alfonso Iii, Why some people of color say they won't wear homemade masks (2020) [Online; accessed 9. Apr. 2020].
68. T Jan, Two black men say they were kicked out of Walmart for wearing protective masks. Others worry it will happen to them. *Wash. Post* (2020).
69. K Wells, Why cant I get tested? *Atlantic* (2020).
70. RE Watson-Jones, CH Legare, The social functions of group rituals. *Curr. Dir. Psychol. Sci.* **25**, 42–46 (2016).
71. P Illingworth, WE Parmet, Solidarity and health: A public goods justification. *Diametros* **43**, 65–71 (2015).
72. LC Chen, TG Evans, RA Cash, , et al., Health as a global public good. *Glob. public goods*, 284–304 (1999).
73. R BliegeBird, et al., Signaling theory, strategic interaction, and symbolic capital. *Curr. anthropology* **46**, 221–248 (2005).
74. R Van Houten, L Malenfant, B Huitema, R Blomberg, Effects of high-visibility enforcement on driver compliance with pedestrian yield right-of-way laws. *Transp. research record* **2393**, 41–49 (2013).
75. W Van Damme, W Van Lerberghe, Editorial: Epidemics and fear. *Trop. Med. Int. Heal.* **5**, 511–514 (2000).
76. MA Riva, M Benedetti, G Cesana, Pandemic fear and literature: observations from jack londons the scarlet plague. *Emerg. infectious diseases* **20**, 1753 (2014).
77. E Taal, JJ Rasker, ER Seydel, O Wiegman, Health status, adherence with health recommendations, self-efficacy and social support in patients with rheumatoid arthritis. *Patient education counseling* **20**, 63–76 (1993).
78. Coronavirus can travel twice as far as official 'safe distance', study says (2020) [Online; accessed 10. Apr. 2020].
79. CC Leung, TH Lam, KK Cheng, Mass masking in the COVID-19 epidemic: people need guidance. *The Lancet* **395**, 945 (2020).
80. J Lyons, To curb the coronavirus, hong kong tells the world masks work; city embraces widespread use of face coverings alongside other measures to slow spread of disease (2020).
81. N Liu, Hong kongs coronavirus response leads to sharp drop in flu cases. *FT.com* (2020) Name - University of Hong Kong; Chinese University of Hong Kong; Copyright - Copyright The Financial Times Limited Mar 5, 2020; Last updated - 2020-03-23; SubjectsTermNotLitGenreText - China; Hong Kong.
82. BJ Cowling, et al., Impact assessment of non-pharmaceutical interventions against COVID-19 and influenza in Hong Kong: an observational study. *medRxiv* (2020).
83. GM Leung, et al., A tale of two cities: community psychobehavioral surveillance and related impact on outbreak control in hong kong and singapore during the severe acute respiratory syndrome epidemic. *Infect. Control. & Hosp. Epidemiol.* **25**, 1033–1041 (2004).
84. BJ Cowling, et al., Community psychological and behavioral responses through the first wave of the 2009 influenza a (h1n1) pandemic in hong kong. *The J. infectious diseases* **202**, 867–876 (2010).
85. World Health Organization (WHO), Global Tuberculosis Report 2019, (World Health Organization, Geneva), Technical report (2019).
86. AS Dharmadhikari, et al., Surgical face masks worn by patients with multidrug-resistant tuberculosis: impact on infectivity of air on a hospital ward. *Am. journal respiratory critical care medicine* **185**, 1104–1109 (2012).
87. EL Larson, et al., Impact of Non-Pharmaceutical Interventions on URIs and Influenza in Crowded, Urban Households. *Public Heal. Reports* **125**, 178–191 (2010).
88. CR MacIntyre, et al., The First Randomized, Controlled Clinical Trial of Mask Use in Households to Prevent Respiratory Virus Transmission. *Int. J. Infect. Dis.* **12**, e328 (2008).
89. L Tian, et al., Calibrated intervention and containment of the covid-19 pandemic (2020).
90. J Yan, S Guha, P Hariharan, M Myers, Modeling the Effectiveness of Respiratory Protective Devices in Reducing Influenza Outbreak. *Risk Analysis* **39**, 647–661 (2019).
91. C Leffler, E Ing, CA McKeown, D Pratt, A Grzybowski, Country-wide Mortality from the Novel Coronavirus (COVID-19) Pandemic and Notes Regarding Mask Usage by the Public, Technical report (2020).
92. C Kenyon, Widespread use of face masks in public may slow the spread of SARS CoV-2: an ecological study. *medRxiv*, 2020.03.31.20048652 (2020).
93. J Abaluck, et al., The Case for Universal Cloth Mask Adoption and Policies to Increase Supply of Medical Masks for Health Workers, (Social Science Research Network, Rochester, NY), SSRN Scholarly Paper ID 3567438 (2020).
94. WC on the Ethics of Scientific Knowledge, Technology, The precautionary principle (2005).
95. T Greenhalgh, MB Schmid, T Czypionka, D Bassler, L Gruer, Face masks for the public during the covid-19 crisis. *BMJ* **369** (2020).
96. WD Bradford, A Mandich, Some state vaccination laws contribute to greater exemption rates and disease outbreaks in the united states. *Heal. Aff.* **34**, 1383–1390 (2015).

PERSPECTIVE
Host-Microbe Biology

COVID-19: an Immunopathological View

Kamran Kadkhoda[a]

[a]Immunopathology Laboratory, Robert J. Tomsich Pathology & Laboratory Medicine Institute, Cleveland Clinic, Cleveland, Ohio, USA

ABSTRACT Since its emergence in December 2019, it took only a couple of months for an outbreak of the novel coronavirus disease 2019 (COVID-19) to be declared a pandemic by the World Health Organization (WHO). This along with the highly infectious nature of the disease and the associated mortality call for particular attention to the underlying (immuno)pathomechanism(s). The latter will inform case management and vaccine design. Unravelling these mechanisms can assist basic scientists, laboratory medicine practitioners, clinicians, public health practitioners, funding agencies, and health care policymakers in responding to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic.

KEYWORDS COVID-19, SARS-CoV-2, immunopathology

Xu et al. (1) reported on a 50-year-old man with no known underlying conditions who presented with pneumonia. Despite the presence of lymphopenia and absence of neutropenia, all inflammatory cells observed on the lung biopsy specimen were mononuclear cells. Peripheral blood lymphocytes showed high levels of activation markers (HLA-DR and CD38) by flow cytometry. These cells were strongly perforin- and/or granulysin-positive $CD8^{ffi}$ T cells or were inflammatory Th17 cells. This had led to significant damage to the lung tissues as demonstrated by diffuse alveolar damage, indicating acute respiratory distress syndrome (ARDS). This is consistent with high-level surface expression of angiotensin-converting enzyme 2 (ACE2), the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) receptor, on pneumocytes (2). Unfortunately, the patient passed away, but it is important to note that methylprednisolone was started on day 8 after the onset of symptoms, while the catastrophic ARDS cascade was already under way. Interestingly, inhalational alpha interferon (IFN-ffi) was also instituted along with methylprednisolone. Since IFN-ffi can boost antiviral immune responses, it might have further played a role in the tissue damage in this case. On a related note, previous studies with macaques using SARS-CoV had shown that older macaques had stronger innate immune responses (including those related to IFN-ffi) compared with younger macaques. In contrast, expression of IFN-ffi, as an anti-inflammatory cytokine, was reduced in older macaques (3). It has recently been shown that interleukin-6 (IL-6) was also expressed significantly higher in patients who succumbed to coronavirus disease 2019 (COVID-19) than in survivors (4). Observations like this case made by a number of front-line clinicians since the inception of the COVID-19 pandemic have led to pointing fingers at immunopathology as the potential main culprit.

There have been several studies including the large epidemiological joint report by WHO and China (5) stating that the case fatality rate (CFR) is exceedingly low in pediatric patients especially in the very young (CFR of 21.9% for individuals over 80 years of age versus 0% for individuals under 8 years of age). This is a striking finding that further suggests an immunopathological component to this observation. Another observation is the seroprevalence of community-acquired coronaviruses among adults is very high (90 to 100%) (6), but this is not necessarily the case in pediatric patients (7). Additionally, there are antigenic commonalities among coronaviruses (CoVs); for in-

Citation Kadkhoda K. 2020. COVID-19: an immunopathological view. mSphere 5:e00344-20. https://doi.org/10.1128/mSphere.00344-20

Editor Helene F. Rosenberg, National Institute of Allergy and Infectious Diseases

Copyright © 2020 Kadkhoda. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Address correspondence to kadkhok@ccf.org.

Published 22 April 2020

Downloaded from http://msphere.asm.org/ on April 22, 2020 by guest

stance, similar to SARS-CoV-2, CoV-NL63 also uses ACE2 as the receptor (8). As individuals age, the chance of exposure to common community-acquired CoVs (229E, OC43, NL63, and HKU1) increases. As a consequence, with such background anamnesis, once individuals are exposed to novel (zoonotic) CoVs such as SARS-CoV-2, the fast and furious immune response does the damage (the original antigenic sin [OAS]). According to OAS, the initial antigen imprints an immune response so that subsequent exposure to related antigen(s) preferentially chooses the already-existing memory cells. The resultant response, although brisk and apparently strong (a high-titer IgG response would be faster than that of IgM), may be unduly insufficient and inappropriate to the point that it might be initially nonprotective. In the context of COVID-19, since ACE2 is highly expressed in the gastrointestinal (GI) tract (9), shedding the virus in the stool is prolonged (10); however, diarrhea is uncommon likely because virus-specific effector memory T cells typically home to the mucosal surfaces they had previously encountered with an infection with a common CoV, i.e., upper and lower respiratory tract. As a result, despite ARDS in the lungs, almost no significant intestinal damage occurs, if at all. To further confound matters, lungs also express high levels of CD32a (Fc! RIIa, typically on alveolar macrophages), whereas GI tissues express almost no CD32a protein (The Human Protein Atlas [https://www.proteinatlas.org/] [accessed March 2020]). It has recently been shown that SARS-CoV and the Middle East respiratory syndrome (MERS)-CoV take advantage of non- or subneutralizing antibodies and enter cells via surface CD32a receptors (Trojan horse mechanism) (11, 12). Therefore, another even more biologically plausible pathomechanism is the so-called antibody-dependent enhancement (ADE). ADE has also been shown in infection with dengue viruses, Zika virus, Ebola virus, and human immunodeficiency virus. ADE is not only triggered by neutralizing antibodies but also by nonneutralizing antibodies. Additionally, antibody affinity plays an important role in ADE (11, 13). The most important pathomechanism in COVID-19, therefore, could be ADE in which CD32a plays the central role. CD32a is expressed on the surfaces of monocytes and macrophages among other cells such as alveolar macrophages, and once aggregated by several IgG molecules, it transduces its signal through the associated immunoreceptor tyrosine-based activation motif (ITAM). This results in the release of proinflammatory cytokines including IFN-! , tumor necrosis factor alpha, interleukin-1 (IL-1), and IL-6 (14). This IL-6 overproduction is the justification for using IL-6 receptor antagonists such as tocilizumab in COVID-19 clinical trials. It should be noted, however, that activation of ITAM occurs through phosphorylation by certain members of Src family of tyrosine kinases such as Fyn and Syk. Interestingly, agents such as cerdulatinib have dual inhibitory function against Syk as well as against Janus kinase family members (such as JAK1/2) (15), the latter also play key roles in IL-6 receptor signaling (16).

To complete the picture, SARS-CoV nucleoprotein and RNA had previously been found within alveolar macrophages of nonsurvivors (17). These cells also express dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN; CD209 and also CD209L). These surface molecules have been reported to be coreceptors for SARS-CoV entry (18). In this way, alveolar macrophages can function as a local niche for the virus and thereby pass it on to other cells.

Findings similar to the aforementioned epidemiological finding have been made for MERS-CoV in which disease severity increases with age and severe disease has been uncommon among pediatric patients (19). Furthermore, along the lines of ADE, it has been known for decades that immunization of cats with spike protein of feline CoV leads to more severe future infections with feline CoV in cats (11). Therefore, ADE is perhaps more effective when the time intervals between CoV infections are just long enough so antibody titers drop to at least subneutralizing levels. This may explain why association of older age with severe COVID-19 is not always the rule. Infection of alveolar macrophages through ADE may explain their excessive activation and generation of local hyperinflammatory environment and the resultant systemic cytokine storm that may even fulfill criteria for secondary hemophagocytic lymphohistiocytosis and macrophage activation syndrome. The pivotal involvement of macrophages is further highlighted by the fact that very high levels of ferritin are seen in severe

Downloaded from http://msphere.asm.org/ on April 22, 2020 by guest

COVID-19 cases (20). Additionally, the fact that severe COVID-19 is associated with much higher viral load compared with mild cases further suggests that ADE could play an important role in severe cases (21). Another explanation for age-associated severity of COVID-19 may be attributed to C-reactive protein (CRP). It is known that CRP levels increase with age and that serum CRP levels in adults are higher than in children, with the highest median level observed in the elderly (22). Furthermore, it has been demonstrated that CD32a is the major biological receptor for CRP (23).

Furthermore, the course of COVID-19 is in general more severe in those with underlying conditions such as hypertension, poorly controlled diabetes mellitus, and cardiovascular disease among others (4). This may be attributed to the known increased expression of CD32a on monocytes and macrophages in these patients (24). However, it is a common assumption that immunocompromised patients are at increased risk of developing severe COVID-19. To challenge this assumption, a report from Italy (25) showed that 3 out of 111 liver transplant recipients who passed away due to COVID-19 had received their transplants 10 years prior to death. Interestingly, these patients were stable with very low trough concentrations of calcineurin inhibitors in plasma. They, however, were all male, older than 65 years, overweight, diabetic, and on treatment for hypertension. In contrast, in the same study, 3 out of 40 individuals who had recently received liver transplants and were on intense immunosuppression drugs (without the other risk factors mentioned for the former group) and who tested positive for SARS-CoV-2 experienced an uneventful course of COVID-19. The CFR in the former group was 3% in contrast to the overall CFR of ca. 10% in Italy. Abatement of IFN-ffi signaling by calcineurin inhibitors may explain these observations. IFN-ffi can also be largely produced as a result of CD32a activation (26). Similar observations in other immunocompromised patient populations with larger sample sizes will be paramount to further understand the role of immunopathology in COVID-19.

All in all, further corroborative research is urgently needed to find out why advanced age is a major risk factor for COVID-19 and whether immunopathomechanisms can potentially be harnessed early enough to prevent irreversible consequences. This may be ground-breaking and would not only change the current individual patient management but also potentially inform prudent vaccine design and recommendations as was the case with dengue virus vaccination. The implications of ADE will be immense if IgG serology is used to determine immune status for health care workers, as a positive result does not necessarily mean one is immune to COVID-19 (even with a test specificity of 100%). More importantly, ADE may cause harm if plasma from clinically resolved patients is used for treatment. A very careful balance between risks and benefits established through large multicenter randomized controlled trials will provide answers to all these questions.

ACKNOWLEDGMENT

I sincerely thank Stanley Plotkin for reviewing the article.

REFERENCES

1. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, Song J, Xia P, Dong J, Zhao J, Wang FS. 2020. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. Lancet Respir Med 8:420–422. https://doi.org/10.1016/S2213-2600(20)30076-X.
2. Tse GM, To KF, Chan PK, Lo AW, Ng KC, Wu A, Lee N, Wong HC, Mak SM, Chan KF, Hui DS, Sung JJ, Ng HK. 2004. Pulmonary pathological features in coronavirus associated severe acute respiratory syndrome (SARS). J Clin Pathol 57:260–265. https://doi.org/10.1136/jcp.2003.013276.
3. Smits SL, de Lang A, van den Brand JMA, Leijten LM, van IJcken WF, Eijkemans MJC, van Amerongen G, Kuiken T, Andeweg AC, Osterhaus ADME, Haagmans BL. 2010. Exacerbated innate host response to SARS-CoV in aged non-human primates. PLoS Pathog 6:e1000756. https://doi.org/10.1371/journal.ppat.1000756.
4. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, Wu X, Xu J, Tu S, Zhang Y, Chen H, Cao B. 2020. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet 395:1054–1062. https://doi.org/10.1016/S0140-6736(20)30566-3.
5. World Health Organization. 2020. Report of the WHO-China Joint Mission on Coronavirus Disease 2019 (COVID-19). World Health Organization, Geneva, Switzerland. https://www.who.int/docs/default-source/coronaviruse/who-china-joint-mission-on-covid-19-final-report.pdf.
6. Gorse GJ, Patel GB, Vitale JN, O'Connor TZ. 2010. Prevalence of antibodies to four human coronaviruses is lower in nasal secretions than in serum. Clin Vaccine Immunol 17:1875–1880. https://doi.org/10.1128/CVI.00278-10.
7. Prill MM, Iwane MK, Edwards KM, Williams JV, Weinberg GA, Staat MA, Willby MJ, Talbot HK, Hall CB, Szilagyi PG, Griffin MR, Curns AT, Erdman DD, New Vaccine Surveillance Network. 2012. Human coronavirus in young children hospitalized for acute respiratory illness and asymptomatic controls. Pediatr Infect Dis J 31:235–240. https://doi.org/10.1097/INF.0b013e31823e07fe.
8. Wu K, Li W, Peng G, Li F. 2009. Crystal structure of NL63 respiratory

Downloaded from http://msphere.asm.org/ on April 22, 2020 by guest

coronavirus receptor-binding domain complexed with its human receptor. Proc Natl Acad Sci U S A 106:19970–19974. https://doi.org/10.1073/pnas.0908837106.
9. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. 2004. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J Pathol 203:631–637. https://doi.org/10.1002/path.1570.
10. Tang A, Tong ZD, Wang HL, Dai YX, Li KF, Liu JN, Wu WJ, Yuan C, Yu ML, Li P, Yan JB. 9 March 2020. Detection of novel coronavirus by RT-PCR in stool specimen from asymptomatic child, China. Emerg Infect Dis https://doi.org/10.3201/eid2606.200301.
11. Wan Y, Shang J, Sun S, Tai W, Chen J, Geng Q, He L, Chen Y, Wu J, Shi Z, Zhou Y, Du L, Li F. 2019. Molecular mechanism for antibody-dependent enhancement of coronavirus entry. J Virol 94:e02015-19. https://doi.org/10.1128/JVI.02015-19.
12. Walls AC, Xiong X, Park YJ, Tortorici MA, Snijder J, Quispe J, Cameroni E, Gopal R, Dai M, Lanzavecchia A, Zambon M, Rey FA, Corti D, Veesler D. 2019. Unexpected receptor functional mimicry elucidates activation of coronavirus fusion. Cell 176:1026–1039.e15. https://doi.org/10.1016/j.cell.2018.12.028.
13. Li M, Zhao L, Zhang C, Wang X, Hong W, Sun J, Liu R, Yu L, Wang J, Zhang F, Jin X. 2018. Dengue immune sera enhance Zika virus infection in human peripheral blood monocytes through Fc gamma receptors. PLoS One 13:e0200478. https://doi.org/10.1371/journal.pone.0200478.
14. Anania JC, Chenoweth AM, Wines BD, Hogarth PM. 2019. The human Fc! RII (CD32) family of leukocyte FcR in health and disease. Front Immunol 10:464. https://doi.org/10.3389/fimmu.2019.00464.
15. Liu D, Mamorska-Dyga A. 2017. Syk inhibitors in clinical development for hematological malignancies. J Hematol Oncol 10:145. https://doi.org/10.1186/s13045-017-0512-1.
16. Choy EH. 2019. Clinical significance of Janus kinase inhibitor selectivity. Rheumatology (Oxford) 58:1122. https://doi.org/10.1093/rheumatology/kez002.
17. Nicholls JM, Butany J, Poon LL, Chan KH, Beh SL, Poutanen S, Peiris JS, Wong M. 2006. Time course and cellular localization of SARS-CoV nucleoprotein and RNA in lungs from fatal cases of SARS. PLoS Med 3:e27. https://doi.org/10.1371/journal.pmed.0030027.
18. Chen J, Subbarao K. 2007. The immunobiology of SARS. Annu Rev Immunol 25:443–472. https://doi.org/10.1146/annurev.immunol.25.022106.141706.
19. Arabi YM, Balkhy HH, Hayden FG, Bouchama A, Luke T, Baillie JK, Al-Omari A, Hajeer AH, Senga M, Denison MR, Nguyen-Van-Tam JS, Shindo N, Bermingham A, Chappell JD, Van Kerkhove MD, Fowler RA. 2017. Middle East respiratory syndrome. N Engl J Med 376:584–594. https://doi.org/10.1056/NEJMsr1408795.
20. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ. 2020. Covid-19: consider cytokine storm syndrome and immunosuppression. Lancet 395:1033–1034. https://doi.org/10.1016/S0140-6736(20)30628-0.
21. Liu Y, Yan LM, Wan L, Xiang TX, Le A, Liu JM, Peiris M, Poon LLM, Zhang W. 19 March 2020. Viral dynamics in mild and severe cases of COVID-19. Lancet Infect Dis https://doi.org/10.1016/S1473-3099(20)30232-2.
22. Schlenz H, Intemann T, Wolters M, González-Gil EM, Nappo A, Fraterman A, Veidebaum T, Molnar D, Tornaritis M, Sioen I, Mårild S, Iacoviello L, Ahrens W, IDEFICS Consortium. 2014. C-reactive protein reference percentiles among pre-adolescent children in Europe based on the IDEFICS study population. Int J Obes (Lond) 38(Suppl 2):S26–S31. https://doi.org/10.1038/ijo.2014.132.
23. Bharadwaj D, Stein MP, Volzer M, Mold C, Du Clos TW. 1999. The major receptor for C-reactive protein on leukocytes is fcgamma receptor II. J Exp Med 190:585–590. https://doi.org/10.1084/jem.190.4.585.
24. Devaraj S, Chen X, Adams-Huet B, Jialal I. 2013. Increased expression of Fc-! receptors on monocytes in patients with nascent metabolic syndrome. J Clin Endocrinol Metab 98:E1510–E1515. https://doi.org/10.1210/jc.2013-2112.
25. Bhoori S, Rossi RE, Citterio D, Mazzaferro V. 9 April 2020. COVID-19 in long-term liver transplant patients: preliminary experience from an Italian transplant centre in Lombardy. Lancet Gastroenterol Hepatol https://doi.org/10.1016/S2468-1253(20)30116-3.
26. Batteux F, Palmer P, Daëron M, Weill B, Lebon P. 1999. FCgammaRII (CD32)-dependent induction of interferon-alpha by serum from patients with lupus erythematosus. Eur Cytokine Netw 10:509–514.

Downloaded from http://msphere.asm.org/ on April 22, 2020 by guest

JCI insight

Anti–spike IgG causes severe acute lung injury by skewing macrophage responses during acute SARS-CoV infection

Li Liu, … , Kwok-Yung Yuen, Zhiwei Chen

JCI Insight. 2019;4(4):e123158. https://doi.org/10.1172/jci.insight.123158.

Research Article | Infectious disease | Pulmonology

Newly emerging viruses, such as severe acute respiratory syndrome coronavirus (SARS-CoV), Middle Eastern respiratory syndrome CoVs (MERS-CoV), and H7N9, cause fatal acute lung injury (ALI) by driving hypercytokinemia and aggressive inflammation through mechanisms that remain elusive. In SARS-CoV/macaque models, we determined that anti–spike IgG (S-IgG), in productively infected lungs, causes severe ALI by skewing inflammation-resolving response. Alveolar macrophages underwent functional polarization in acutely infected macaques, demonstrating simultaneously both proinflammatory and wound-healing characteristics. The presence of S-IgG prior to viral clearance, however, abrogated wound-healing responses and promoted MCP1 and IL-8 production and proinflammatory monocyte/macrophage recruitment and accumulation. Critically, patients who eventually died of SARS (hereafter referred to as deceased patients) displayed similarly accumulated pulmonary proinflammatory, absence of wound-healing macrophages, and faster neutralizing antibody responses. Their sera enhanced SARS-CoV–induced MCP1 and IL-8 production by human monocyte–derived wound-healing macrophages, whereas blockade of FcγR reduced such effects. Our findings reveal a mechanism responsible for virus-mediated ALI, define a pathological consequence of viral specific antibody response, and provide a potential target for treatment of SARS-CoV or other virus-mediated lung injury.

Find the latest version:

http://jci.me/123158/pdf

JCI insight

RESEARCH ARTICLE

Anti–spike IgG causes severe acute lung injury by skewing macrophage responses during acute SARS-CoV infection

Li Liu,[1,2] Qiang Wei,[3] Qingqing Lin,[1] Jun Fang,[1] Haibo Wang,[1] Hauyee Kwok,[1] Hangying Tang,[1] Kenji Nishiura,[1] Jie Peng,[1] Zhiwu Tan,[1] Tongjin Wu,[1] Ka-Wai Cheung,[1] Kwok-Hung Chan,[1] Xavier Alvarez,[4] Chuan Qin,[3] Andrew Lackner,[4] Stanley Perlman,[5,6] Kwok-Yung Yuen,[1] and Zhiwei Chen[1,2]

[1]AIDS Institute and Department of Microbiology, State Key Laboratory of Emerging Infectious Disease, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China. [2]HKU-AIDS Institute Shenzhen Research Laboratory and AIDS Clinical Research Laboratory, Shenzhen Key Laboratory of Infection and Immunity, Shenzhen Key Clinical Department of Emerging Infectious Diseases, Shenzhen Third People's Hospital, Shenzhen, China. [3]Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College (PUMC), Beijing, China. [4]Division of Comparative Pathology, Tulane National Primate Research Center, Covington, Louisiana, USA. [5]Department of Microbiology and Immunology, University of Iowa, Iowa City, Iowa, USA. [6]State Key Laboratory of Respiratory Disease, National Clinical Research Center for Respiratory Disease, Guangzhou Institute of Respiratory Health, the First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China.

Newly emerging viruses, such as severe acute respiratory syndrome coronavirus (SARS-CoV), Middle Eastern respiratory syndrome CoVs (MERS-CoV), and H7N9, cause fatal acute lung injury (ALI) by driving hypercytokinemia and aggressive inflammation through mechanisms that remain elusive. In SARS-CoV/macaque models, we determined that anti–spike IgG (S-IgG), in productively infected lungs, causes severe ALI by skewing inflammation-resolving response. Alveolar macrophages underwent functional polarization in acutely infected macaques, demonstrating simultaneously both proinflammatory and wound-healing characteristics. The presence of S-IgG prior to viral clearance, however, abrogated wound-healing responses and promoted MCP1 and IL-8 production and proinflammatory monocyte/macrophage recruitment and accumulation. Critically, patients who eventually died of SARS (hereafter referred to as deceased patients) displayed similarly accumulated pulmonary proinflammatory, absence of wound-healing macrophages, and faster neutralizing antibody responses. Their sera enhanced SARS-CoV–induced MCP1 and IL-8 production by human monocyte–derived wound-healing macrophages, whereas blockade of Fcγ reduced such effects. Our findings reveal a mechanism responsible for virus-mediated ALI, define a pathological consequence of viral specific antibody response, and provide a potential target for treatment of SARS-CoV or other virus-mediated lung injury.

Authorship note: AL is decease

Conflict of interest: This work w
financially supported by grants fro
the US NIH RO1HL080211 and HH
TRS T11-706/18-N to ZC, the TNPRC
(base grant RR00164), RO1060699 to
SP, HMRF 16150662, and the University
Development Fund/Li Ka Shing Faculty
of Medicine Matching Fund of the
University of Hong Kong to its AIDS
Institute and Hong Kong RGV.

License: Copyright 2019, American
Society for Clinical Investigation.

Submitted: July 23, 2018
Accepted: January 11, 2019
Published: February 21, 2019

Reference information:
JCI Insight. 2019;4(4):e123158.
https://doi.org/10.1172/jci.
insight.123158.

Introduction

Severe acute respiratory syndrome coronavirus (SARS-CoV) causes fatal human respiratory disease (1–3). Patients with SARS (hereafter referred to as SARS patients) displayed the characteristics of acute lung injury (ALI), including diffuse alveolar damage (DAD), epithelial necrosis, and fibrin and hyaline deposition (4, 5). Most patients who die of SARS develop acute respiratory distress syndrome (ARDS), the most severe form of ALI (6, 7). Recent outbreaks of severe acute respiratory infections of emerging viruses, including Middle Eastern respiratory syndrome CoVs (MERS-CoV), highly pathogenic avian influenza viruses (e.g., H5N1 and H7N9), highlight the need to characterize the mechanisms responsible for virus-mediated ALI or ARDS.

Fundamental to ARDS is the acute onset of lung inflammation, which is intimately tied to monocyte/macrophage polarization and function (7, 8). Lung macrophages are highly plastic and heterogeneous cells that are resident in the lung interstitium and alveoli or recruited upon inflammatory stimuli. Inflammatory monocytes and resident tissue macrophages play critical roles in initiating and maintaining inflammation

during the acute stage of ARDS, as well as in the resolution phages of inflammation and recovery from ARDS. At steady state, resident macrophages are normally quiescent to prevent damaging the alveoli and are critically involved in normal tissue homeostasis. After tissue injury or during infection, resident macrophages become activated. Circulating monocytes can be efficiently recruited to the site of injury. Inflammatory monocytes/macrophages (IMMs) and resident macrophages undergo marked phenotypic and functional changes, and they can be classified into proinflammatory (M1 or classically activated) and inflammatory-resolving (M2, alternatively activated, wound-healing, or antiinflammatory) macrophages, with a continuum of macrophage polarization existing beyond these discrete categories (9). During acute infection, monocytes/macrophages often display a phenotype of classically activated macrophages. These cells mediate host defenses against viruses and also promote lung injury by producing nitric oxide (NO); ROS; IL-1, IL-6, and IL-8; and TNF. Simultaneously, some macrophages may become alternatively activated, exerting antiinflammatory function and regulating wound healing by producing matrix metalloproteinases (MMPs), growth factors, and antiinflammatory cytokines, particularly TGF-β. When the pathogen or inflammatory stimulus is eliminated, proinflammatory macrophages diminish. The predominant macrophage population assumes a wound-healing phenotype. At the final recovery stage, macrophages show a regulatory/suppressive phenotype, secreting increased levels of IL-10, which facilitates the resolution of wound healing and restores homeostasis. When the wound-healing response is well organized and controlled, the inflammatory response resolves quickly, and normal tissue architecture is restored. Disturbances in wound-healing response can lead to uncontrolled production of inflammatory mediators, contributing to a state of persistent injury (9–11). In patients who eventually died of SARS (hereafter referred to as deceased patients) and animal models, extensive lung damage is associated with high initial viral loads, increased IMM accumulation in the lungs, and elevated serum proinflammatory cytokine (IL-1, IL-6, IL-8, CXCL-10, and MCP1) levels (4, 5, 12, 13). While much is known about the terminal phase of SARS, little is known about the early immune events during the acute phase of infection. Studies defining macrophage heterogeneity and function during acute infection and ALI using nonhuman primate and patient specimens are limited, and the factors driving the hypercytokinemia and aggressive inflammation remain elusive.

During the SARS outbreak in Hong Kong, most patients (70%–80%) presented with abnormal chest radiographs, with approximately one-quarter of these individuals progressing to ALI. After 12 days, 80% of these SARS patients developed ARDS, coincident with IgG seroconversion (6). In a detailed analysis of antibody responses against SARS-CoV spike (S) glycoprotein, we reported that the anti-S neutralizing antibody (NAb) response developed significantly faster in deceased patients compared with recovered patients after the onset of clinical symptoms (14). It took an average of 20 days for the recovered patients to reach their peak of NAb activities, as opposed to only 14.7 days for the deceased patients. Moreover, the actual NAb titer is significantly higher in deceased patients compared with that in the recovered patients during the same time period (14). These findings suggest a role of anti-S antibodies in SARS-CoV–mediated ALI during acute infection. Consistently, preexisting serum antibodies against influenza antigens were found to associate with worse clinical severity and poor outcomes in patients during the 2009 influenza pandemic (15, 16). Moreover, multiple vaccine platforms and viral infection appeared to induce SARS-CoV–specific immune memory that enhanced lung inflammation following homologous challenge in mice and African green monkeys (17–19). The mechanism responsible for the immunopathologic reaction remains elusive. Recent studies suggested that T cells play a crucial role in protection of mice against lethal SARS-CoV infection (20–22). Enhanced pulmonary immunopathology in vaccinated and challenged animals reflects an inadequate Th1 response (22, 23). The role of virus-specific antibody response in SARS-CoV–induced lung injury has yet to be clearly defined. Therefore, we used vaccination and anti–S-IgG passive immunization strategies to evaluate the effects of anti-S antibodies on SARS-CoV–induced ALI in Chinese rhesus monkeys (*Macaca mulatta*). Our results now show that, in productively infected lungs, anti–S–IgG causes severe ALI by skewing inflammatory resolving responses during acute infection.

Results

Vaccine-induced S-specific immunity resulted in severe ALI in SARS-CoV–infected Chinese macaques. We initially compared the pathological changes in the lungs of rhesus macaques vaccinated with a modified vaccinia Ankara (MVA) virus encoding full-length SARS-CoV S glycoprotein (ADS-MVA) or control at 7 and 35 days after pathogenic SARS-CoV$_{PUMC}$ (1×10^5 tissue culture infectious dose of 50% [$TCID_{50}$]) challenge (24). Three healthy macaques were included as controls. Sixteen macaques were i.m. immunized twice: 8 animals with

ADS-MVA and another 8 animals with a control MVA (ADC-MVA) (Figure 1A) (24). Vaccination with ADS-MVA induced high levels of anti–SARS-CoV NAbs in all 8 macaques, with sera IC_{50} values ranging from 10,232- to 28,703-fold dilutions (Figure 1B). None of the macaques developed fever during or after vaccination (normal body temperature 37.8°C–38.1°C). The IC_{50} values of these sera were maintained, with an over-2,000–fold reciprocal serum dilution at 4 weeks after the secondary immunization, a time when all of the animals were i.n. challenged (Figure 1B). After viral challenge, all macaques developed fever between 1 and 5 days after inoculation (dpi) ranging from 38.6°C–39.2°C, and sera from control vaccinated macaques showed increased neutralizing activity after 14 dpi, indicating establishment of infection (Figure 1B). SARS-CoV RNA was readily detected on oral swabs from all control macaques examined at multiple time points using real-time reverse transcription PCR (real-time RT-PCR) but only in 3 of the 8 ADS-MVA–vaccinated macaques with lower NAb titers at 2 dpi (SL11, SE13, and SL14) (Figure 1C), suggesting reduced productive viral infection in the immunized macaques through S-specific immunity.

However, histological examination revealed acute DAD with various degrees of severity in 6 ADS-MVA–vaccinated macaques at 7 and 35 dpi, whereas most control macaques in the ADC-MVA group showed minor to moderate inflammation (Figure 1D). To better characterize the pathological changes, we adopted a 6-grade scoring system to describe the severity of the lung damage from least severe to most severe: 0, –; 1, +; 2, ++; 3, +++; 4, ++++; and 5, +++++ (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.123158DS1). In the control vaccinated group, 5 macaques showed minor inflammation with slight septa broadening and inflammatory cells infiltration that we scored as + (CL23, CE25, CE20, CE21, and CL19; Figure 1D, Supplemental Figure 1B, and Supplemental Figure 2). Two macaques showed moderate inflammation with more interstitial mononuclear inflammatory infiltration and were scored as ++ (CL26, CE24; Supplemental Figure 1C and Supplemental Figure 2). Only 1 macaque showed typical symptoms of acute DAD with extensive exudation and cell infiltration at 35 dpi (CL22, scored as ++++; Supplemental Figure 2). In the ADS-MVA–vaccinated group, 4 macaques showed typical symptom of acute DAD with extensive exudation and many-cell infiltration in alveolar cavities (SL11, SE12, SL16, and SL18, scored as ++++; Supplemental Figure 1E and Supplemental Figure 2). One macaque showed severe acute DAD with exudation, hyaline membrane formation along the alveoli, pneumocyte desquamation, and damaged alveolus filled with hemorrhage and inflammatory cells (SL15, scored as +++++; Figure 1D). One macaque showed early symptom of acute DAD (SL14, scored as +++; Supplemental Figure 1D), and the remaining 2 macaques showed moderate inflammation (SE13 and SE17, scored as ++; Supplemental Figure 2). The comparison of lung histopathological scores of 2 groups showed significantly enhanced lung injury in the ADS-MVA–vaccinated group at both 7 and 35 dpi compared with the control ADC-MVA group, suggesting that S-specific— but not MVA-specific — immunity promotes ALI during SARS-CoV infection (Figure 1E). Moreover, there is a moderate correlation between lung pathological scores and sera NAb titers at 0 dpi (Figure 1F), suggesting a role of S-specific antibody in enhancing SARS-CoV–mediated lung injury.

Anti–S-IgG induced severe lung injury during acute SARS-CoV infection. In our previous study, i.n. inoculation of Chinese macaques with $SARS\text{-}CoV_{PUMC}$ (1×10^5 $TCID_{50}$) led to lower respiratory tract infection in all animals within 2 days (25). However, most of them rapidly cleared infection in the lungs, and all of the animals ($n = 8$) exhibited mild lung lesions before 3 dpi (25). Severe lung injury in SARS-CoV–infected Chinese macaques has not been detected until 7 dpi (26, 27). To determine the effect of anti-S antibody on the extent of SARS-CoV–mediated lung injury, we adoptively transferred 5 mg (low dose) and 200 mg (high dose) purified anti–S-IgG from ADS-MVA–vaccinated but unchallenged macaques into 2 groups of unvaccinated macaques ($n = 6$/group) via i.v. injection, and then i.n. challenged recipients with $SARS\text{-}CoV_{PUMC}$ (1×10^5 $TCID_{50}$) (Figure 2A). As controls, another 2 macaques were administered 200 mg of control IgG (C-IgG) derived from ADC-MVA–vaccinated macaques. We sacrificed half of the macaques in each group at 2 dpi to avoid potential disruption by virus infection–induced antibodies against nucleocapsid protein (N) and other viral proteins — and prior to when induction of ALI was typically observed. The remaining half of the macaques in each group was sacrificed at 21 dpi to evaluate long-term impact (Figure 2A). At day 2, sera from macaques that received low- or high-dose S-IgG showed neutralizing activity, with IC_{50} values ranging from 10–260 and 1,000–10,000, corresponding to the dose of sera transfer; macaques that received C-IgG failed to show neutralizing activity (Figure 2B and Supplemental Table 1). While NAb titers in the sera of macaques in the high-dose group declined steadily between 2 and 21 dpi, macaques in the low-dose and control groups displayed increased NAb titers over time (Figure 2B), suggesting active viral replication.

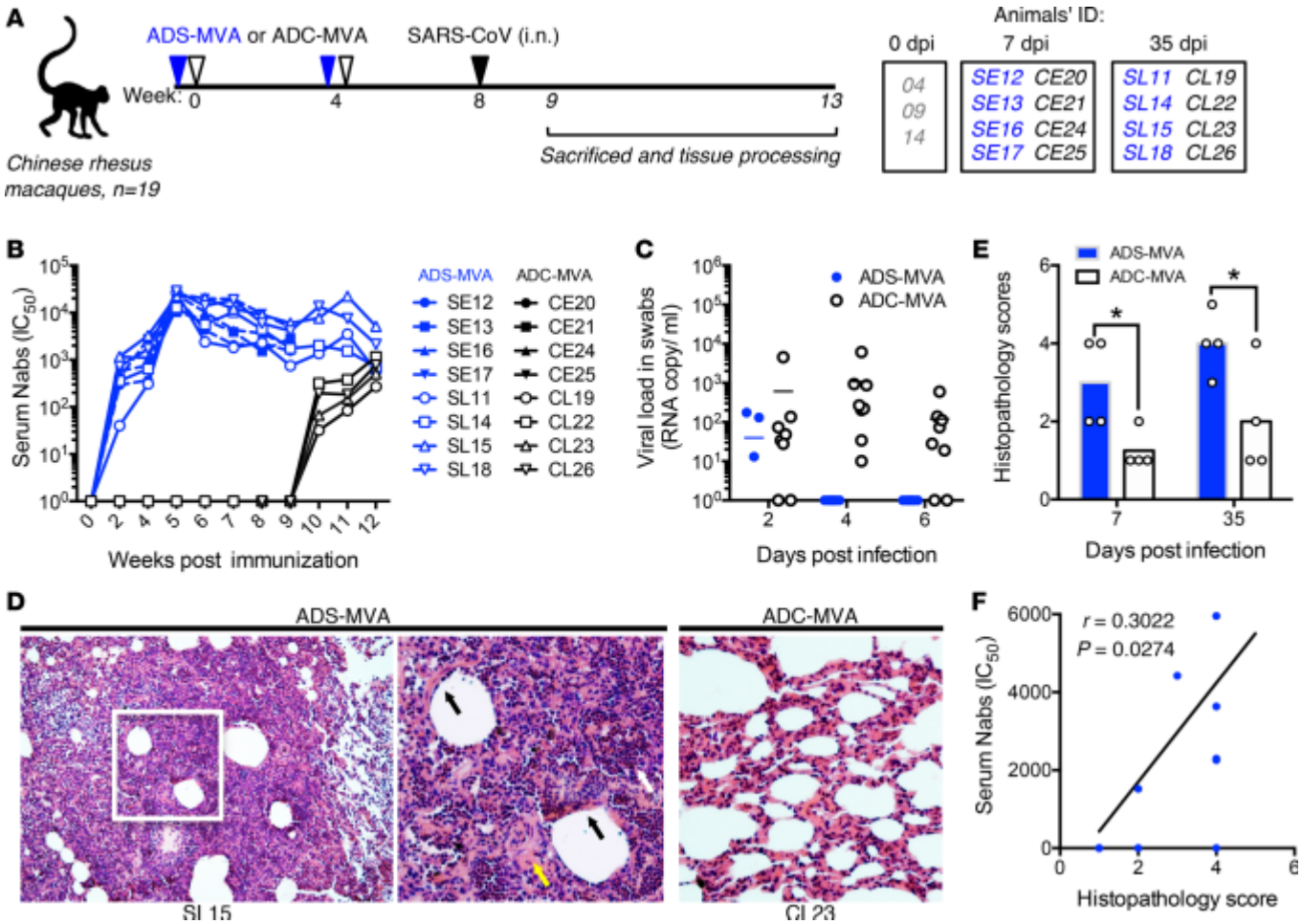


Figure 1. ADS-MVA–induced S-specific immune response enhanced pulmonary pathology in SARS-CoV–infected Chinese rhesus macaques. (**A**) imental design used to investigate the influence of S-specific immunity on SARS-CoV–induced lung injury. Two groups of Chinese rhesus macaques (*n* = 8/group) were subjected to i.m. injections of ADS-MVA or control vaccine ADC-MVA at weeks 0 and 4, followed by i.n. challenge with live pathogenic SARS-CoV_{PUMC} (1×10^5 $TCID_{50}$) at 4 weeks after the second vaccination. Four animals each were sacrificed at 1 and 5 weeks after inoculation. Three healthy macaques were included as controls. (**B**) Serum neutralizing activity. Sera collected from macaques were tested for a capacity to neutralize SARS-CoV pseudotype virus. (**C**) Detection of viral RNA in oral swabs. SARS-CoV RNA was detected by nested RT-PCR in the swabs at the indicated time points relative to infection. (**D**) Pathology changes of the lung tissue. Sections were stained with H&E. **D** shows symptom of acute DAD exhibited in 6 of 8 ADS-MVA–vaccinated macaques with extensive exudation (yellow arrow), hyaline membranes lining the alveolar walls (black arrows), and massive cell infiltration in alveolar cavities (white arrow). Left image shows a low magnification overview (100×). Middle image shows higher magnification of the boxed area in left image (200×). Right image shows minor inflammation observed in 7 macaques received ADC-MVA (*n* = 8) with slight alveolar septa broadening and sparse monocyte infiltration (original magnification, 100×). (**E**) Histopathological scores of the ADS-MVA group, including lung samples collected at both 7 and 35 dpi, were compared against the ADC-MVA control group. See Supplemental Figure 1 for the scoring index based on severity of lung histopathology. Data represent mean ± SEM values. Statistical analysis was undertaken using 2-tailed unpaired Student's *t* test. $^*P < 0.05$, *n* = 4. (**F**) Correlation of lung histopathological scores of all macaques with sera NAb titers at 0 dpi. Solid lines denote the relationship between histopathology scores and serum neutralizing activity. Statistical analysis was performed using Spearman's rank correlation test.

Indeed, SARS-CoV RNA was detected using real-time RT-PCR on oral swabs, and/or virus was recovered from lung tissue in both control animals and 5 of 6 animals (83%) in the low-dose group. Only 2 of 6 animals (33%) in the high-dose group were viral RNA^+ (Figure 2C and Supplemental Table 1), suggesting reduced viral production by high-dose S-IgG.

Consistent with the findings in the control ADC-MVA–vaccinated group, histopathological examination of the lungs indicated minor and moderate inflammation at day 2 and 21 in C-IgG recipients, respectively. In contrast, all S-IgG recipients exhibited symptoms of acute DAD with various degrees of exudation, hyaline membrane formation, and hemorrhage and inflammatory cells within alveolus at both day 2 and 21 (Figure 2, D and E, and Supplemental Table 2). Of note, despite the presence of high titer sera NAbs during the chronic stage of infection (Figure 1B and Figure 2B), only 1 of 5 C-IgG and control

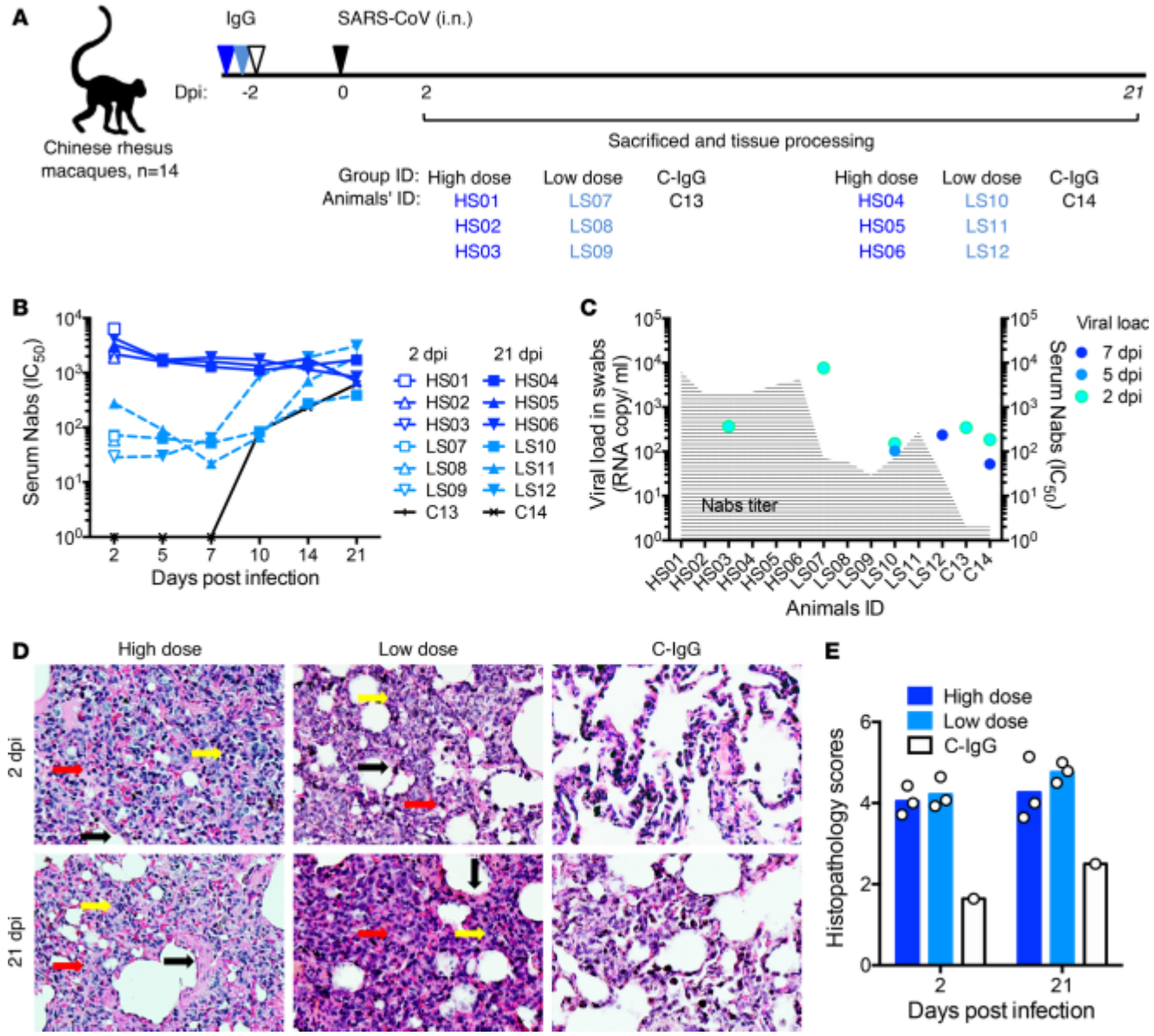


Figure 2. Anti-spike antibodies induced ALI in SARS-CoV–infected Chinese rhesus macaques. (**A**) Experimental design used to investigate the influence of S-IgG on SARS-CoV–induced lung injury. Two groups of Chinese rhesus macaques (*n* = 6/group) were subjected to i.v. injection of high-dose (200 mg) or low-dose (5 mg) purified S-IgG from ADS-MVA–vaccinated but unchallenged macaques. As controls, another 2 macaques were administered 200 mg of C-IgG derived from ADC-MVA–vaccinated macaques. After 2 days, 3 groups of macaques were challenged i.n. with SARS-CoV (10^6 $TCID_{50}$). Half of the animals from each group were sacrificed at 2 and 21 dpi. Sera (**B**) from macaques at the indicated time points were tested for the capacity to neutralize SARS-CoV pseudotype virus. (**C**) Detection of viral RNA in oral swabs. SARS-CoV RNA was detected by nested RT-PCR in the swabs from one of the high-dose S-IgG–treated macaques (*n* = 6), 3 low-dose S-IgG–treated macaques (*n* = 6), and 2 C-IgG–treated macaques (*n* = 2) at the indicated time points. Left *y* axis shows the viral RNA copy number per milliliter swab. Right *y* axis shows the serum NAb titers of each macaque at 2 dpi, which are highlighted by shaded area. (**D**) Pathology changes of the lung tissue (200×). Sections from macaques were stained with H&E. Images show symptom of acute DAD exhibited in macaques received high-dose and low-dose S-IgG (*n* = 2) with extensive exudation (red arrows), hyaline membranes (black arrows), and massive cell infiltration (yellow arrows) at 2 and 21 dpi. Right panel shows minor and moderate inflammation in the macaques received C-IgG with slight alveolar septa broadening and sparse monocyte infiltration at 2 and 21 dpi. (**E**) Histological scores of the high-dose and low-dose S-IgG groups, including lung samples collected at both 2 and 21 dpi, were compared against the C-IgG group. See Supplemental Figure 1 for the scoring index based on severity of lung histopathology.

ADC-MVA vaccine recipients showed acute DAD (Figure 1E and Figure 2E), indicating a time-restricted role for S-IgG, which mostly causes severe ALI in acutely infected macaques. Therefore, we conclude that, despite viral suppression, the presence of S-IgG at the acute stage of SARS-CoV infection caused severe ALI that persists until late stages.

S-IgG failed to prevent SARS-CoV lower respiratory tract infection and amplified IMM infiltration and accumulation in the lungs. To determine the potential cause of S-IgG–enhanced ALI, we measured viral infection and monocytes/macrophages infiltration in the lungs and serum cytokine profile at 2 dpi. We previously found that pulmonary infection of Chinese macaques could be rapidly controlled. Viral RNA$^+$ cells were detectable in the lungs of only 2 of 4 animals at 2 dpi, although all of them were viral nucleoprotein-positive (NP$^+$) in the lungs and the hilar lymph nodes (LNs), where the lymphatics of the lungs drain (25). Therefore, we measured viral RNA$^+$ cells by in situ hybridization (ISH), as well as NP signals in the lungs and the hilar LNs by IHC staining to evaluate the actual pulmonary infection of each macaque. Consistent with the results by viral isolation (Supplemental Table 1), viral RNA was detected in pneumocytes of 1 macaque in the high-dose (HS02, 33%), 2 macaques in the low-dose (LS01, LS02; 66%), and the C-IgG–treated macaque (Figure 3A and Supplemental Table 3), suggesting reduced productive infection by S-IgG. However, an NP antibody identified positive signals in the lungs and hilar LNs of all S-IgG and C-IgG recipients (7 of 7 macaques) but not healthy controls (Figure 3B and Supplemental Table 3), suggesting that S-IgG failed to prevent SARS-CoV entry, and SARS-CoV established lower respiratory tract infection in all of the challenged animals.

Consistent with previous findings in SARS-CoV–inoculated naive Chinese macaques (n = 4) (25), double-immunostaining with the NP antibody and antibodies specific to the stages of macrophage differentiation and inflammation (MAC387, CD68, CD163) revealed significant infiltration of IMMs in the lungs of C-IgG recipients, consisting of newly infiltrated (MAC387$^+$) and inflammatory (CD163$^+$) monocytes/macrophages (Figure 3C and Supplemental Figure 3). The CD163$^+$ cells greatly exceeded the CD68$^+$ population and typically gathered around NP$^+$ cells to form cell clusters (Figure 3C), showing an association between viral infection and IMM infiltration in these macaques.

Compared with C-IgG recipients, more robust signals for MAC387 and CD163 were identified in the lungs of S-IgG recipients (Figure 3C and Supplemental Figure 3), suggesting enhanced IMM infiltration by S-IgG. MAC387$^+$ and CD163$^+$ monocytes/macrophages not only gathered around NP$^+$ cells, but also formed clusters in tissues with little NP signal (Figure 3C), suggesting that infection-induced recruitment from circulation continued after viral clearance and accumulated at the inflamed site. Moreover, more-concentrated CD68$^+$ signals were found, indicating altered macrophages functional response by S-IgG treatment (Figure 3C). Consistently, elevated serum levels of IL-8 were observed in macaques treated with high-dose S-IgG at 2 dpi (Supplemental Figure 3), and the IL-8 levels are strongly correlated with anti–SARS-CoV NAb titers (Supplemental Figure 3). IL-8 is a macrophage-derived inflammatory cytokine important in initiating ALI. These results, therefore, suggest that S-IgG likely altered macrophage functional response in the lungs during acute infection, resulted in increase in IL-8 production, and enhanced ALI and IMM infiltration and accumulation.

Alveolar monocytes/macrophages assumed wound-healing function as early as 2 dpi in macaques not treated with S-IgG. Macrophage activation is dynamic and plastic. Different monocyte/macrophage activation statues have different specialized and critically timed roles, taking part in either the initiation and maintenance or resolution of inflammation (9). A well-organized wound-healing response leads to timely resolution of inflammation, whereas disturbance in wound-healing response results in persistent inflammation and injury. To understand the S-IgG–caused excessive inflammation, we further defined macrophage heterogeneity and activation statues in the lung lesions of C-IgG (n = 1) and S-IgG recipients (n = 3) at 2 dpi. Three healthy macaques and 4 naive Chinese macaques challenged with the same dose of SARS-CoV$_{PUMC}$ and scarified at 2 dpi from a previous study were included as controls (25).

By combining 5 macrophages markers (MAC387, CD68, CD163, HAM56, and CD206) (28–30), 2 subpopulations of resident macrophages were shown in the lungs of Chinese rhesus macaques at steady state as previously described (29). They are interstitial macrophages (IMs; MAC387$^+$HAM56$^-$CD206$^-$) (Figure 4A) and resident alveolar macrophages (AMs; CD68$^+$ HAM56$^-$) expressing low levels of CD163 and CD206, a marker specific to the macrophage wound-healing response, corresponding to their intrinsic antiinflammatory function (Figure 4B) (29). In lung lesions of both C-IgG–treated and naive macaque groups challenged with SARS-CoV$_{PUMC}$, IMMs were further characterized and divided into 2 subpopulations. One subpopulation was the recently recruited MAC387$^+$HAM56$^-$CD68$^-$ monocytes (Figure 4C) (29), which is like IM but is larger in size and primarily coexpressed CD163 (Supplemental Figure 4). The other subpopulation was single CD163$^+$ inflammatory macrophages that had differentiated from newly recruited MAC387$^+$ monocytes/macrophages (Figure 4D and Supplemental Figure 4) (29). Importantly, resident

AMs (CD68$^+$) differentiated into HAM56$^+$ cells, with a slight increase in numbers and increased size, with stronger CD163 and — more importantly — CD206 staining, suggesting that they are alternatively activated (Figure 4D). Strikingly, elevated levels of TGF-β expression were detected in all MAC387$^+$ — and most enlarged CD163$^+$ — as well as CD68$^+$ macrophages in the lungs of both C-IgG–treated and unvaccinated macaques compared with healthy controls (Figure 4, G–I), indicating that many IMMs and activated resident AMs have assumed a wound-healing function in infected lungs within 2 days of infection.

S-IgG treatment skewed wound-healing macrophage response in the lungs during acute SARS-CoV infection. Compared with the macaques not treated with S-IgG, the number of IMMs (MAC387$^+$ and CD163$^+$HAM56$^-$) significantly increased in S-IgG recipients (Figure 4, E and F). Moreover, many recently recruited MAC387$^+$HAM56$^-$ monocytes/macrophages did not coexpress CD163 (Supplemental Figure 4), representing the newest recruited blood monocytes, as a result of enhanced monocytes infiltration by S-IgG. Importantly, the majority of CD163$^+$HAM56$^-$ IMMs coexpressed CD68 (Supplemental Figure 4), suggesting a change of function of this subpopulation by S-IgG. Moreover, resident macrophages (CD68$^+$HAM56$^+$) in the lungs of S-IgG recipients no longer express CD206, which — being surrounded by many CD163$^+$ IMMs (Figure 4F) — suggests a loss of wound-healing function and a role in recruiting blood monocytes. Consistently, S-IgG–treated macaques displayed a loss of TGF-β and high expression of IL-6 in all macrophage subpopulations (Figure 4, G, H, and J). IL-6 favors macrophage accumulation at the site of injury and is important for the development of persistent inflammation and ALI (31, 32). Therefore, we conclude that, while AMs underwent phenotypic and functional changes in acutely infected lungs, many monocytes/macrophages assumed a wound-healing function for inflammation resolution, but the presence of S-IgG skewed the wound-healing response, leading to uncontrolled inflammation and tissue injury.

Onset of an antibody response prior to viral clearance is associated with abrogated wound-healing responses and increased IMM lung infiltration in unvaccinated Chinese rhesus macaques. To understand the mechanism underlying the time-restricted role of S-IgG in promoting ALI, we conducted a temporal analysis of the productive viral infection, antibody response, and macrophage functional changes in unvaccinated Chinese macaques during the first week of infection. Naive macaques were challenged i.n. with 1×10^6 $TCID_{50}$ $SARS\text{-}CoV_{PUMC}$ and sacrificed at 2, 3, and 7 dpi (4 macaques/group) (Figure 5A) (25). Successful lower respiratory tract infection was confirmed by detection of viral NP using IHC staining in the lungs of all macaques (25). Viral RNA$^+$ alveolar pneumocytes were primarily found at 2 dpi (2 of 4 macaques), and infrequently at 3 dpi (0 of 4 macaques; Figure 5B and Supplemental Table 4) (25). At 7 dpi, viral RNA was detectable in the lungs of 2 macaques, AD0516 and AD0518, suggesting productive infection there (Figure 5, B and C). Only few scattered NP$^+$ cells were found in AD0517 (Figure 5D). Neither NP nor viral RNA was found in the lungs or swabs of AD0515, suggesting clearance of productive viral pulmonary infection in this macaque (Figure 5D and Supplemental Table 4). Serum NAb was not observed at 2 and 3 dpi until 7 dpi in 2 of 4 macaques, AD0515 and AD0516 (Supplemental Table 4 and Figure 5E).

In agreement with the findings in the C-IgG group, temporal analysis of macrophage heterogeneity revealed significantly increased number of IMMs (CD163$^+$CD206$^-$) in viral RNA$^+$ lungs at 2 dpi (Figure 5F, right image). The IMMs diminished at 3 dpi (4 of 4 macaques) (Figure 5G) and became nearly completely absent at 7 dpi in macaques without productive pulmonary viral infection (2 of 4 macaques, AD0515 and AD0517) (Figure 5H), indicating that inflammatory response resolved with 7 days following the control of productive infection. Critically, wound-healing macrophages (CD163$^+$CD206$^+$ and CD163$^+$TGF-β$^+$) were observed in the lungs of all macaques at 2 dpi, despite productive pulmonary viral infection in macaques AD0506 and AD0508 (Figure 5F). In contrast, macaque AD0516, with serum NAbs and productive virus infection in the lungs, displayed a significantly enhanced proinflammatory macrophage response and reduced inflammatory-resolving macrophages (Figure 5I), which was not observed in macaques with productive viral infection but undetectable NAb responses (AD0518) or in macaques with detectable NAbs but undetectable viral replication (AD0515) (Figure 5D and Supplemental Table 4). Therefore, persistent viral replication and concomitant NAb detection is associated with increased IMM lung infiltration and reduced wound-healing macrophage numbers.

S-IgG modifies functional response of alternatively but not classically activated macrophages to SARS-CoV infection. To determine whether S-IgG skews wound-healing response through modifying wound-healing macrophage functional response during SARS-CoV infection, we treated in vitro–polarized alternatively activated human monocyte–derived macrophages (MDM) for 20 hours with SARS-CoV pseudovirus and heat-inactivated sera of high-dose S-IgG–treated macaques (S-IgG sera) collected at 2 dpi. We

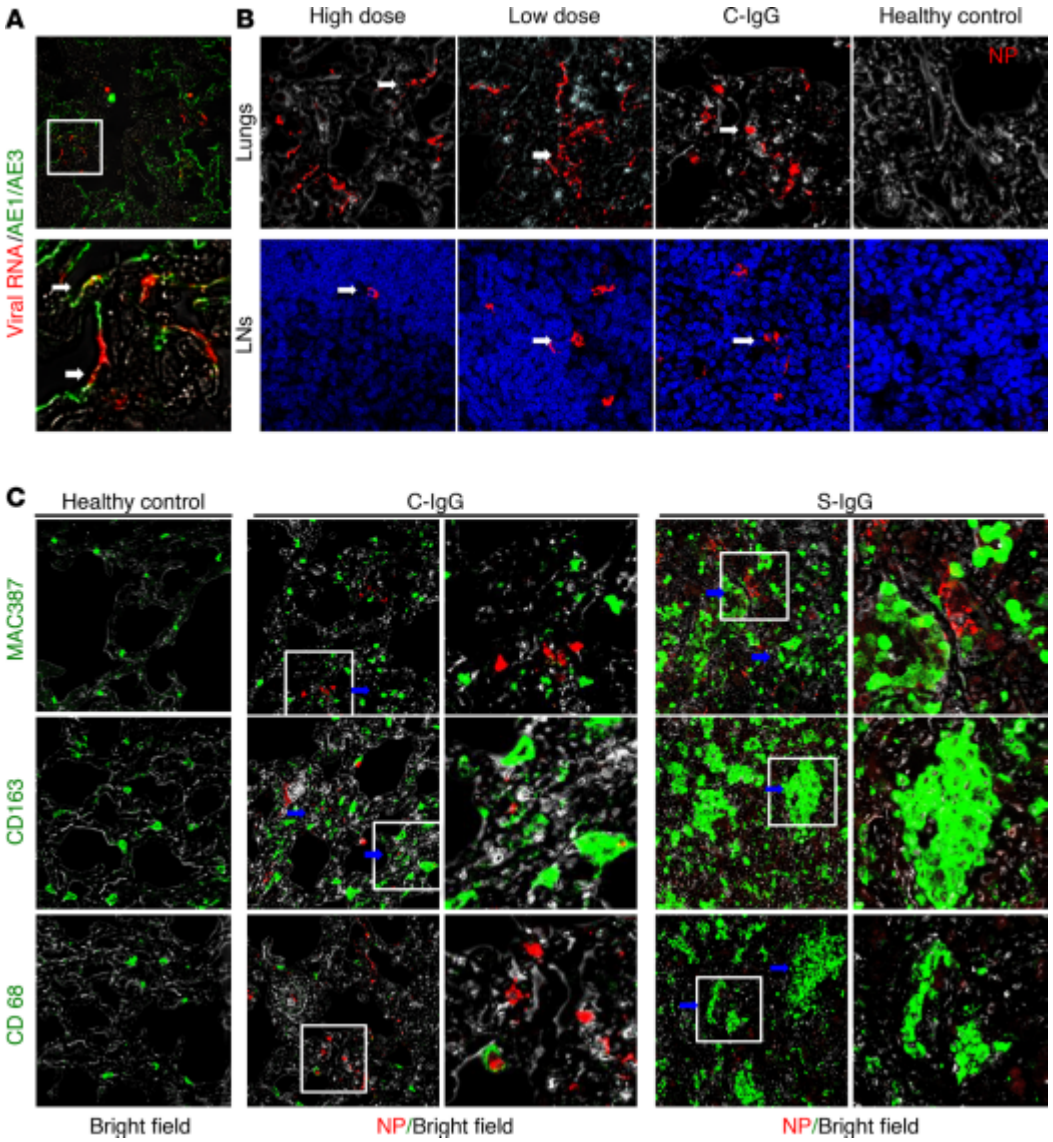


Figure 3. SARS-CoV infection and monocytes/macrophages infiltration in the lungs of C-IgG– and S-IgG–treated macaques at 2 dpi. (**A**) Represen images of SARS-CoV RNA (TRITC) and AE1/AE3 (FITC) cells in the lungs of infected macaques (white arrows). The upper photo shows a low magnification overview (200×); the bottom photo shows the boxed area in the upper photo. (**B**) Representative images of viral protein (NP) immunostaining of the lungs and hilar lymph nodes (LN) of 7 infected and 3 uninfected animals (TRITC, white arrows) (original magnification, 200×). (**C**) Representative images of viral NP and monocytes/macrophages. These sections showed significantly increased IMMs in the lungs of the S-IgG group compared with the C-IgG group (MAC387, CD163, or CD68, blue arrows). Tissue samples are double-immunostained for the SARS-CoV NP (TRITC) and markers for macrophages, including MAC387 (FITC), CD163 (FITC), and CD68 (FITC). Within the panel of representative images for the C-IgG and S-IgG groups, the left panel shows low magnification overview; the right panel shows the boxed area in the left panel (original magnification, 200×).

then measured the concentrations of 13 inflammatory cytokines in cell culture supernatants, including IL-1β, IFN-α, IFN-γ, TNF-α, MCP1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33. Sera from C-IgG recipients or healthy macaques were included as controls. In vitro–polarized classically activated MDM was also studied and compared with alternatively activated MDM in parallel. Consistent with previous report, unstimulated classically activated MDM produced high levels of IL-8 and IL-6; alternatively activated MDM only produced minimal levels of those cytokines (Figure 6A). Addition of SARS-CoV pseudovirus induced IL-8 production in alternatively activated MDM to levels comparable with those found in unstimulated, classically activated MDM, as well as in low-level MCP1 and IL-6 production (Figure 6B). By contrast, SARS-CoV pseudovirus caused less than a 1-fold increase in IL-8 secretion, but not other cytokine secretion, by classically activated MDM (Figure 6C). Strikingly, while none of the sera samples induced cytokine production when they were administered into MDMs alone,

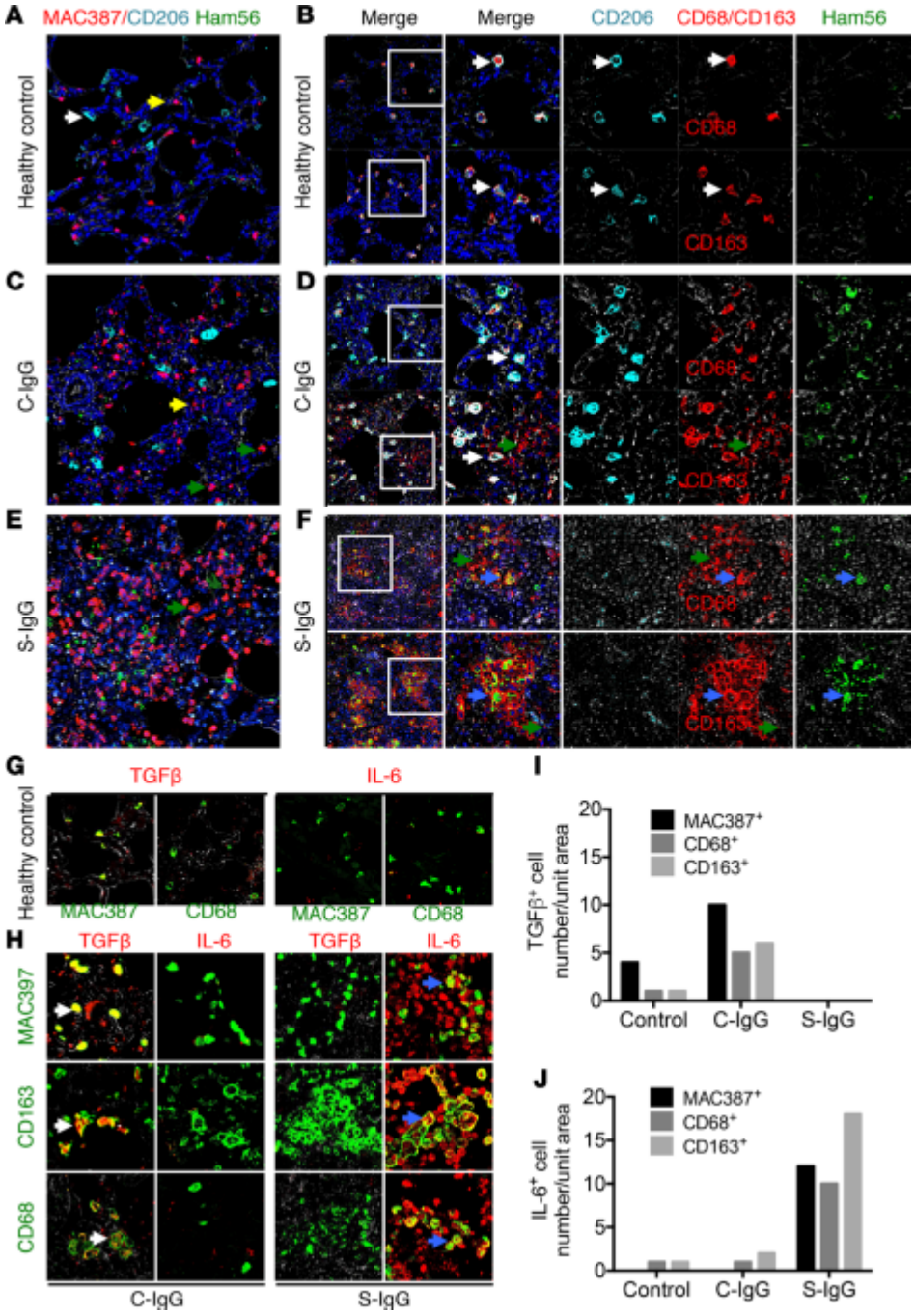


Figure 4. Comparison of monocyte/macrophage phenotype and function in the lungs of S-IgG– and C-IgG–treated macaques. (**A**–**F**) Phenotype of monocytes/macrophages subpopulations in the lungs of healthy, C-IgG–, and S-IgG–treated macaques (original magnification, 200×). These sections were triple-immunostained with antibodies for CD206 (cyan), HAM56 (FITC), and MAC387 (TRITC) (**A**, **C**, and **E**); CD206 (cyan), HAM56 (FITC), and CD68 (TRITC) (upper panel, **B**, **D**, and **F**); or CD163 (TRITC) (lower panel, **B**, **D**, and **F**). **A** and **B** show 2 subpopulations at steady states, including interstitial macrophages (IM) (MAC387$^+$HAM56$^-$CD206$^+$) (yellow arrow), and resident alveolar macrophages (AM) (CD163$^+$CD206$^+$HAM56$^-$) (white arrows). **C** and **D** show the presence of alternatively activated resident AM and accumulated IMMs in the C-IgG group. Alternatively activated AMs are CD206$^+$CD68$^+$CD163$^+$HAM56$^-$ (**D**, white arrows). IM are MAC387$^+$HAM56$^-$CD206$^+$ (**C**, yellow arrow). IMMs include newly infiltrating monocytes/macrophages (MAC387$^+$) (**C**, green arrow); and inflammatory macrophages (CD68$^+$CD206$^-$HAM56$^-$) (**D**, green arrow). **E** and **F** show significantly increased number of IMMs and decreased number of alternatively activated macrophages in the S-IgG group. IMMs include newly infiltrating monocytes/macrophages (MAC387$^+$) (**E**, green arrow) and inflammatory macrophages that are CD68$^+$HAM56$^-$ (green arrow, **F**, upper panel) and CD163$^+$CD206$^+$HAM56$^-$ (green arrow, **F**, lower panel). Resident AMs are no longer expressing CD206$^+$ (CD68$^+$CD163$^+$CD206$^-$) (blue arrow, **F**, upper panel). (**G**–**J**) TGF-β and IL-6 expression in macrophages in the lungs. These sections were double-immunostained with antibodies for TGF-β (TRITC) and MAC387, CD163, or CD68 (FITC). (**G**) shows low expression levels of TGF-β and IL-6 in IMs (MAC387$^+$) and AMs (CD68$^+$) in healthy macaques. **H** shows increased TGF-β expression in the C-IgG group (white arrows) but increased IL-6 in the S-IgG group (blue arrows). (**I** and **J**) show the numbers of TGF-β$^+$ and IL-6$^+$ monocytes/macrophages in 3 groups in a 200× field.

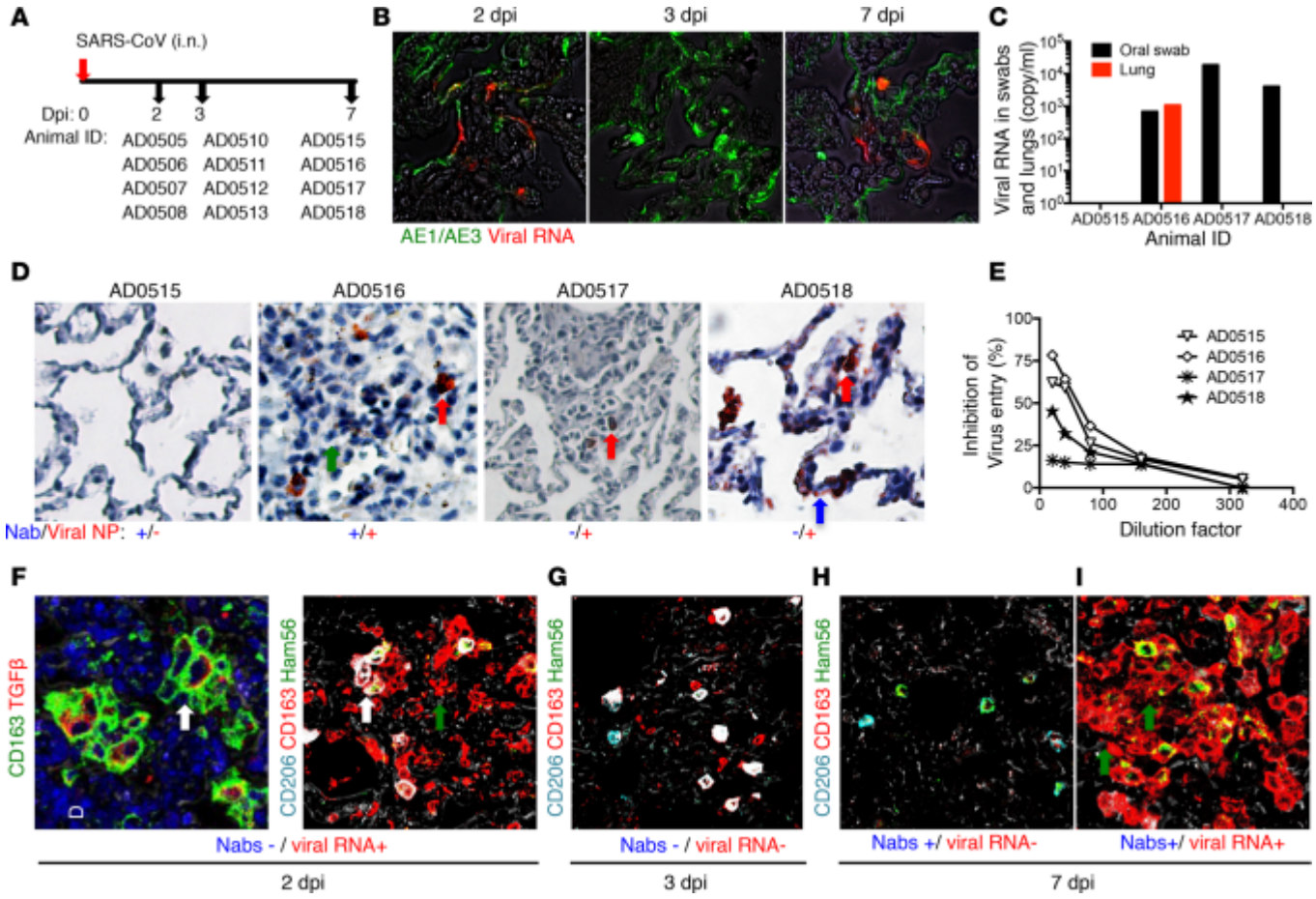


Figure 5. Dynamics of the viral replication, antibody response, and macrophage activation during acute SARS-CoV infection. (**A**) Experimental scheme. A total of 12 Chinese rhesus macaques were challenged i.n. with SARS-CoV ($\times 10^{5}$ $TCID_{50}$). Four animals each were sacrificed at 2, 3, and 7 dpi. (**B**) SARS-CoV RNA detection by ISH in the lungs at 2, 3, and 7 dpi (original magnification, 200 ×). Viral RNA in type I pneumocytes (FITC) was found at 2 dpi ($n = 2$) and 7 dpi ($n = 1$). (**C**) Viral RNA in the swabs and lung homogenates on 7 dpi. SARS-CoV RNA was detected using nested RT-PCR in swabs from 3 macaques (AD0516, AD0517, and AD0518) and lung homogenates from macaque AD0516 and AD0518. (**D**) Viral antigen and inflammatory infiltrates in the lungs at 7 dpi (200×). These sections were stained for SARS-CoV NP (red) and nucleus (blue) by IHC, showing NP signal in macrophage-like cells in AD0516 and AD0517 (red arrows), and type I pneumocytes in AD0518 (blue arrows), but substantial inflammatory infiltrates were only observed in AD0516 (green arrows). (**E**) Serum neutralizing activity. Serum neutralizing activity was detected in macaques AD0515 and AD0516 at 7 dpi. (**F**–**I**) Decreased wound-healing response in macaques with productive pulmonary viral infection and serum neutralizing activity (200×). These sections were double-immunostained for TGF-β (TRITC) and CD163 (FITC) or triple stained for CD206 (cyan), HAM56 (FITC), and CD163 (TRITC). The figure shows that wound-healing response took place within 2 dpi, with alternatively activated monocytes/macrophages (TGF-β+ CD206+ white arrows) and IMMs (CD163+ CD206+ green arrows) coexisting in the lungs (**F**). After viral clearance, IMMs diminish at 3 dpi (**G**), and the homeostasis was restored at 7 dpi (AD0515) (**H**). When pulmonary viral infection persists, IMM (CD163+ CD206+ green arrows) infiltration and accumulation is enhanced, and wound-healing macrophages (CD206+) are reduced in macaques that have faster NAb response (AD0516) (**I**).

administration of S-IgG sera caused a dose-dependent increase in the production of all 3 inflammatory cytokines in SARS-CoV–stimulated alternatively activated MDM, resulted in a greater than 10-fold increase in IL-6, an 8-fold increase in MCP1, and a 5-fold increase in IL-8 (Figure 6, D and E). This enhancement, however, was not observed when S-IgG sera were replaced with sera from C-IgG recipients or healthy macaques (Figure 6, F, G, and H), suggesting that S-IgG, but not other sera components, enhanced SARS-CoV–stimulated cytokine production by alternatively activated MDM. Administration of S-IgG sera had no effect on cytokine production by SARS-CoV–treated classically activated MDM (Figure 6, I, J, and K), indicating that S-IgG do not modulate virus-mediated classically activated macrophage functional response. MCP1 and IL-8 are key cytokines that regulate monocytes/macrophages migration and promote neutrophils infiltration, while IL-6 promotes persistent inflammation and injury. These results suggest that, in response to SARS-CoV infection, wound-healing macrophages produce IL-8 and low amounts of MCP1 to recruit neutrophils and blood monocytes, whereas the presence

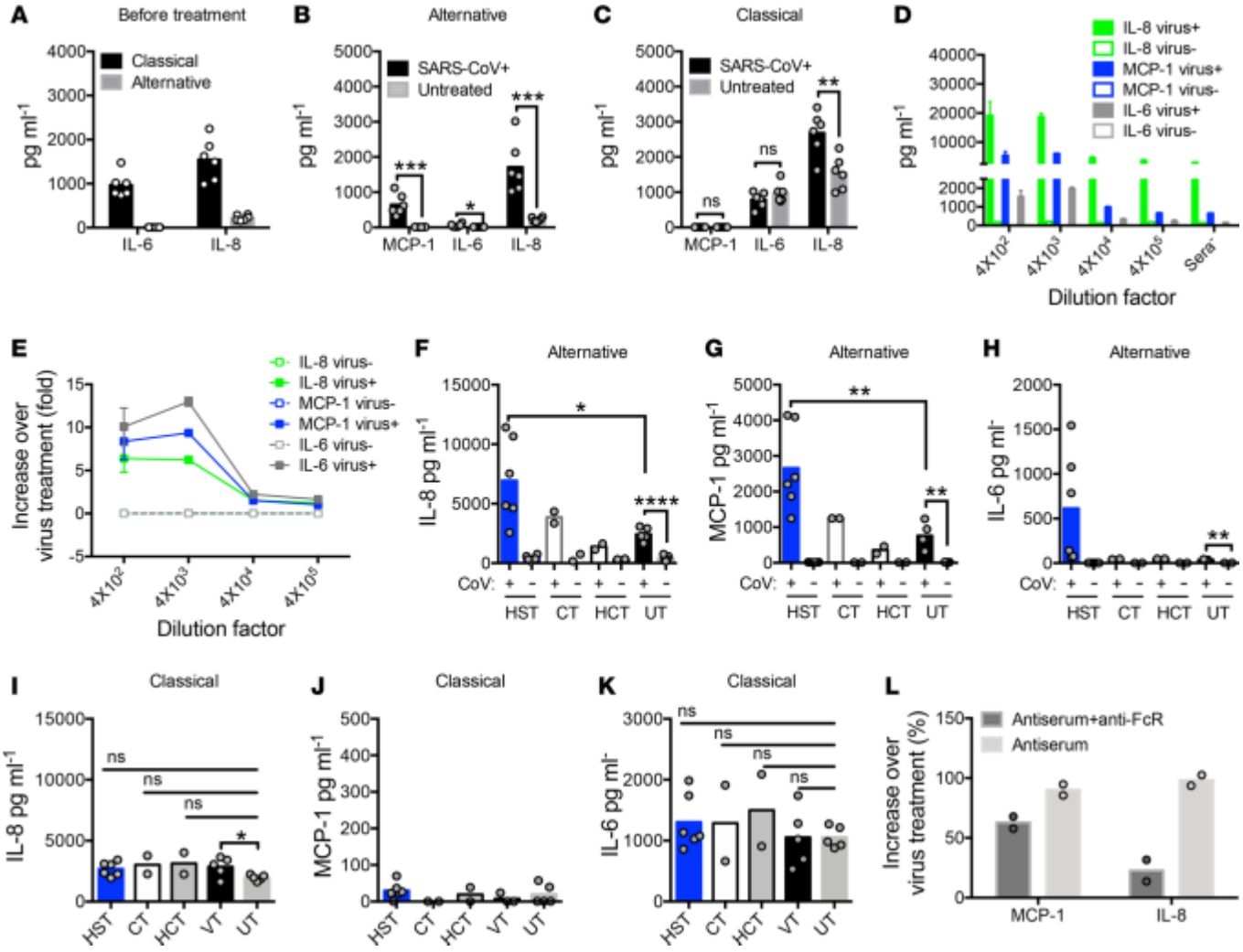


Figure 6. S-IgG significantly amplified proinflammatory cytokine production by SARS-CoV–treated alternatively activated macrophages. In vit polarized alternatively activated macrophages were either left unstimulated or were incubated with SARS-CoV pseudovirus alone or cocultured with SARS-CoV pseudovirus and diluted sera from the high-dose S-IgG group (n = 6) or C-IgG group (n = 2) collected at 2 dpi or from healthy macaques (n = 2) for 20 hours. Secreted cytokine and chemokine levels were measured in the culture supernatant and are shown in data represented as a column graph (**A–D** and **F–K**) and fold or percentage over supernatants from macrophages treated with virus alone and (**E**). **A** shows the levels of IL-8 and IL-6 in the supernatant of classically activated and alternatively activated MDM before treatment and **B** and **C** show that SARS-CoV treatment induced MCP1, IL-8, and very low levels of IL-6 production by alternatively activated MDM, as well as enhanced IL-8 production by classically activated MDM. **D** and **E** show that sera from S-IgG recipients caused dose-dependent increase in cytokine production from SARS-CoV–treated alternatively activat ed MDM. **F–H** show that virus treated alternatively activated MDM (VT), but not sera treated or untreated cells (UT), secreted proinflammatory cytokines IL-8 (**F**), MCP1 (**G**), and very low levels of IL-6 (**H**). Addition of sera (1:4,000 dilutions) from high-dose S-IgG (HST), but not C-IgG treated macaques (CT) or healthy controls (HCT), significantly amplified IL-8 (**F**), MCP1 (**G**), and IL-6 (**H**) production. **I–K** show that addition of sera (1:4,000 diluted) from high-dose S-IgG–treated (HST), C-IgG treated macaques (CT), or healthy controls (HCT) had no effect on MCP-1 (**J**), and IL-6 (**K**) production by SARS-CoV–treated classically activated MDM. **L** shows that blockade of FcγR significantly reduced antiserum-enhanced IL-8 secretion and partially reduced MCP1 secretion. Data represent mean values or mean values ± SEM of at least 3 independent experiments. Two-tailed unpaired Student's t test was used for statistical analysis. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

of S-IgG can result in 5- to 10-fold increases in IL-8, MCP1, and IL-6 production by wound-healing macrophages, leading to significantly enhanced and persistent monocytes infiltration and tissue injury. Critically, in the presence of Fcγ receptor (FcγR) blocking antibody, IL-8 was significantly reduced when cells were treated with SARS-CoV pseudovirus and sera from high-dose S-IgG–treated macaques (diluted 1:4,000), indicating that anti–SARS-CoV antibody modifies wound-healing response partially through FcγRs (Figure 6L). Therefore, we conclude that S-IgG directly modifies functional response of alternatively activated macrophages during acute infection, thus skewing wound-healing response, leading to hypercytokinemia, aggressive inflammation and severe lung injury.

Deceased SARS patients displayed higher levels of serum NAbs, accumulated proinflammatory macrophage infiltration, and absence of wound-healing macrophages in the lungs. To examine the role of S-IgG in promoting ALI in a clinical setting, we investigated antibody responses during the early stage of infection in 2 groups of deceased patients or individuals recovered from SARS and characterized the heterogeneity of monocytes/macrophages in the lungs of 3 deceased SARS patients. H&E staining of lung specimens derived from 3 deceased SARS patients revealed severe ALI (Figure 7A). Consistent with the macaque findings, IHC analysis revealed significant infiltration of $CD163^+$ monocytes/macrophages into the lungs of patients, with little TGF-β expression and loss of the wound-healing marker $CD206^+$, indicating predominantly proinflammatory macrophage activation and reduced inflammatory-resolving response (Figure 7B). Moreover, significantly higher levels of anti-S NAbs were detected in the sera of deceased patients (n = 6) during acute infection compared with recovered patients (n = 8) (Figure 7C). These results, therefore, are consistent with findings in experimental macaques.

Antisera from deceased SARS patients skewed wound-healing response during SARS-CoV infection partially through the FcγR. To examine whether human anti–SARS-CoV antibody modifies wound-healing response during SARS-CoV infection, we treated in vitro–polarized alternatively activated MDM with SARS-CoV pseudovirus for 20 hours in the presence of sera collected from SARS patients during acute infection. Macrophages treated with SARS-CoV pseudovirus or sera alone were included as controls. We then examined the protein levels of cytokines/chemokines in the cell culture supernatant. Consistent with findings in macaques, administration of sera from a deceased SARS patient (D1; Supplemental Table 5) into SARS-CoV–treated alternatively activated MDM caused a dose-dependent increase in the production of cytokines, leading to a 2- to 3-fold increase in MCP1 and IL-8 after 20 hours (Figure 8, A and B). This enhancement, however, was not observed in cells treated with sera alone (Figure 8, A–D). Administration of sera from recovered patients (n = 8; Supplemental Table 5) had no effect on cytokine production except 1 sample (Figure 8, C and D). These differences may be explained, at least in part, by their lower NAb titers, although it is also possible that sera from recovered patients contain different antibody populations. Consistently, the only serum sample — R2, which enhanced chemokine production — has a relatively higher NAb titer among the samples tested (Supplemental Table 5). Moreover, statistical analysis shows that IL-8 production strongly correlates with NAb titer of sera from deceased patients (Figure 8E). Furthermore, IL-8 production was significantly reduced when cells were treated with SARS-CoV pseudovirus and sera from deceased SARS patient (diluted 1:4,000) in the presence of FcγR blocking antibody, indicating that anti–SARS-CoV antibody modifies wound-healing response in humans partially through FcγRs (Figure 8F).

Discussion

Here, we present evidence of a detrimental role of anti–S-IgG in ALI during SARS-CoV infection. Respiratory CoVs infection poses a unique challenge to the immune system: not only must the virus be rapidly eliminated, but lung inflammation must also be controlled to prevent acute respiratory failure (33). In the present study, we show that, despite markedly reducing virus titers, anti–S-IgG caused lung injury during the early stages of infection by abrogating a wound-healing macrophage response and TGF-β production, while promoting proinflammatory cytokine IL-8 and MCP1 production and inflammatory macrophage accumulation. To our knowledge, our data demonstrate a previously unrecognized mechanism underlying virus-mediated ALI and suggest that modulation of the anti-S antibody response or blockage of Fcγ receptors during acute infection might be needed for effective treatment for respiratory CoV infection.

Many studies have demonstrated the role of NAbs induced by the S glycoproteins in protecting viral replication in susceptible hosts, including mice, ferrets, hamsters, and macaques (24, 34, 35). However, the effect of S-specific immunity in protecting against pulmonary immunopathology has been controversial. In some cases, vaccination-induced immunity assists the viral clearance and protects mice or ferrets against lethal challenge (36–39), whereas in many other situations, multiple vaccine platforms appear to induce increased eosinophilic proinflammatory pulmonary response upon challenge (18, 23, 40). These differences may be explained, in part, by the characteristics of the vaccine being studied, the infectious dose, and the viral strain employed, as well as by the type or quality of immune response elicited. Recent studies suggest that $CD8^+$ T cell response plays a crucial role in viral clearance and protection of mice against eosinophilic immunopathology and lethal SARS-CoV infection, whereas immunopathology predominantly reflects an inadequate vaccine-induced Th1 response (20–23).

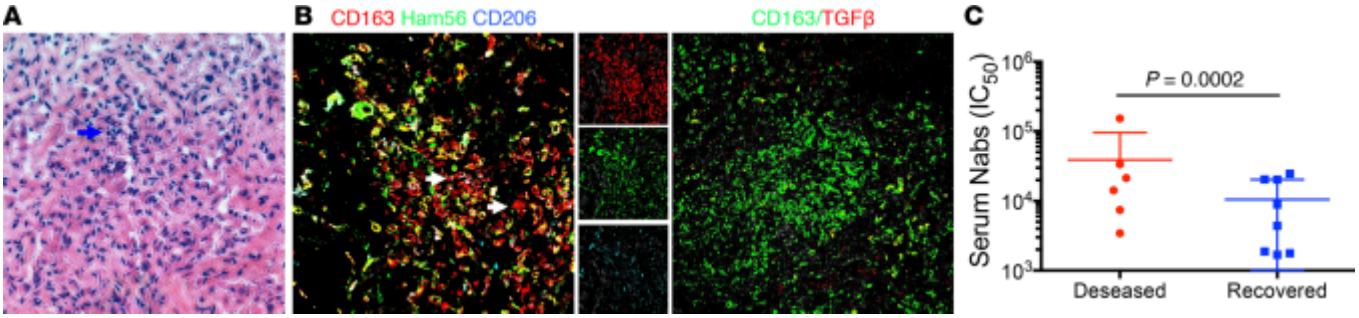


Figure 7. NAb response and phenotype analysis of accumulating monocytes/macrophages in the lungs in deceased SARS patients. (**A**) Pathological changes of the lung tissue. Sections from deceased SARS patients were stained with H&E and are representative of 3 patients (original magnification, 100×), showing symptom of acute DAD with massive cell infiltration in alveolar cavities (blue arrow). (**B**) Massive accumulation of IMMs and absence of wound-healing macrophage response in the lungs of deceased patients. These sections were triple-immunostained with antibodies for CD206 (cyan), HAM56 (FITC), and CD163 (TRITC) or double-immunostained with TGF-β (FITC) and CD163 (FITC) (original magnification, 200×). The right panel in the left image shows single colors from the left image (200×). These representative images show accumulation of IMMs (CD163+HAM56+, white arrows) and the absence of wound-healing response, indicated by loss of the signal for CD206 (cyan, B1) and TGF-β (TRITC, B2) in the lungs. (**C**) Serum-neutralizing activity against SARS-CoV. Sera collected from deceased ($n = 6$), or recovered (blue, $n = 8$) SARS patients at the early stage of infection were tested for the capacity to neutralize SARS-CoV pseudotype virus. Two-tailed unpaired Student's *t* test was used for statistical analysis.

In fact, a protective effect against pulmonary immunopathology mediated by full length S protein–based vaccine in SARS-CoV–infected nonhuman primate models has not been described, to our knowledge. Although several vaccine candidates protected macaques against viral replication in the lungs, pulmonary immunopathology was not evaluated in these studies (24, 41, 42). By contrast, it was shown that SARS-CoV–specific immune memory induced by prior infection enhanced lung inflammation following homologous challenge in African green monkeys (17). Similarly, augmentation of disease by vaccination has also been described in studies of atypical measles and dengue hemorrhagic fever, as well as several respiratory diseases, including respiratory syncytial virus (RSV) and pandemic influenza (16, 43). In the study of RSV vaccine conducted in 1966 and 1967, 80% of RSV vaccinees needed hospitalization, whereas only 5% of RSV-infected children in the control vaccine group required admission, although the underlying mechanisms remain incompletely understood. Using Chinese rhesus macaques, we show that an ADS-MVA–based vaccine protected macaques against viral replication but significantly enhanced acute DAD at both 7 and 35 dpi compared with the control ADC-MVA group, suggesting that S-specific but not MVA-specific immunity promotes ALI (Figure 1). Until now, it remains unknown if SARS vaccine candidates with a focus on the receptor-binding domain (RBD) of S protein can avoid the induction of ALI in nonhuman primates. These vaccines have been shown to induce highly potent NAb responses and elicit long-term protective immunity in immunized small animals (38).

SARS-CoV infection of Chinese macaques is often characterized by the rapid control of viral replication with mild lung lesions in most macaques (25). The mechanisms underlying why Chinese macaques do not often develop ALI, as observed in most SARS patients, are largely unknown. Our data suggest that these effects, in part, reflect the rapid control of viral replication in the lungs, which peaked between 24 and 48 hours after inoculation (hpi) and diminished within 7 days, thus creating an interval between lung inflammation and high titer antibody production in most macaques. Indeed, although SARS-CoV infection resulted in significant infiltration of macrophages in the lungs, the number of macrophages was rapidly reduced following virus clearance at 3 dpi (Figure 5). At 7 dpi, inflammation appeared to be mostly resolved (Figure 5). By contrast, control of virus replication is less efficient in SARS patients, and the peak in viral load coincides with the first appearance of an antibody response, approximately 10 days after the onset of symptoms (6).

The low rate of ALI and interval between lung inflammation resolution and antibody production, however, makes Chinese macaques an ideal animal model for the present study. Using vaccination and passive immunization, we conditioned anti-S antibody titers in macaques during the early stage of infection when ALI was not typically observed. We found that prior administration of anti–S-IgG led to massive accumulation of monocytes/macrophages in the lungs within 2 dpi. This result is consistent with previous reports, in which mice given the SARS vaccine exhibited an immunopathologic lung reaction after the subsequent challenge with SARS-CoV (18, 19). Moreover, in SARS patients, clinical course and outcome are more favorable in children younger than 12 years of age compared with adolescents and adults (44), indicating that prior alternative CoV infection might

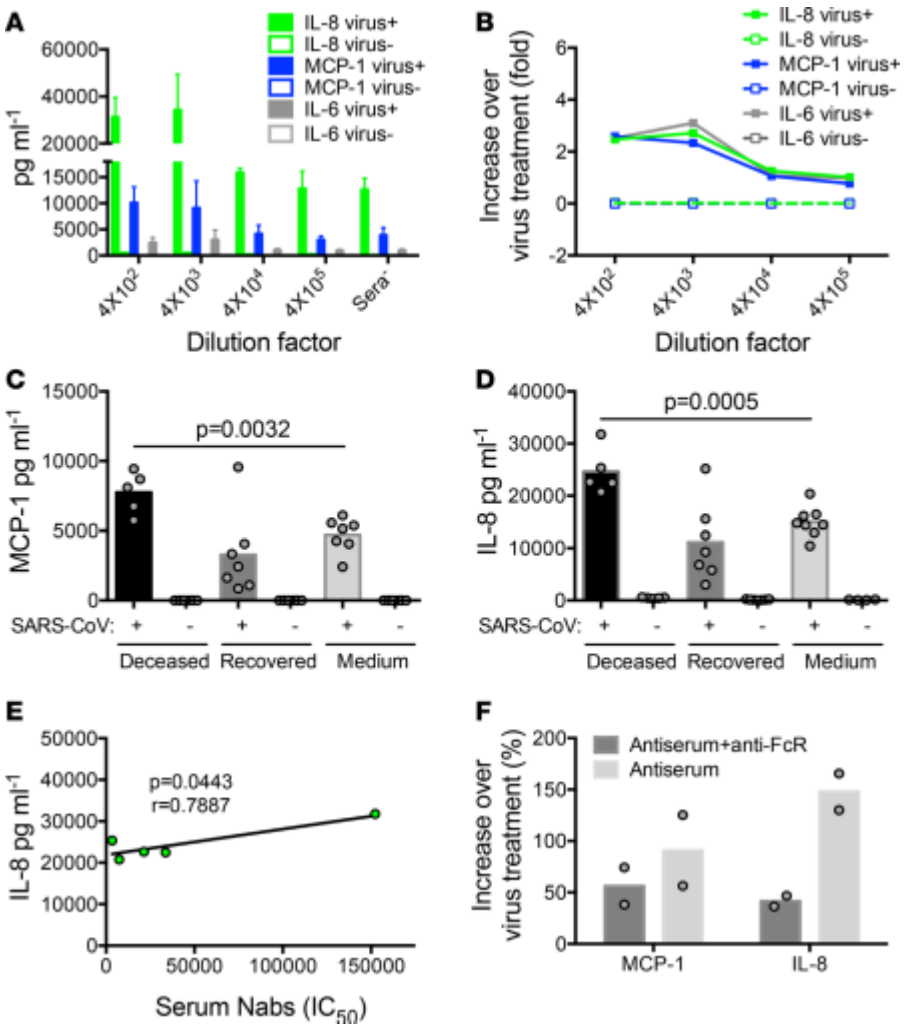


Figure 8. Sera from deceased SARS patients skewed wound-healing macrophage response partially through FcγR. In vitro–polarized alternatively activated MDM were either left unstimulated or were incubated with SARS-CoV pseudovirus alone or cocultured with SARS-CoV pseudovirus and 1–400,000 serials diluted or 1/4,000 diluted sera from deceased (n = 5) or recovered SARS patients (n = 7) for 20 hours. Cells treated with sera from patients alone served as controls. Secreted cytokine and chemokine levels were measured in the culture supernatant and are shown in data represented as a column graph (**A**, **C**, and **D**) and fold or percentage over supernatants from macrophages treated with virus alone (**B** and **F**). **A** and **B** show that addition of sera from deceased patient (D1) dose-dependently enhanced production of IL-8, IL-6, and MCP-1 by SARS-CoV–treated alternatively activated MDM. **C** and **D** show significantly enhanced IL-8 and MCP-1 production by alternatively activated macrophages treated with sera from deceased SARS patients and virus compared with cells treated with virus alone. Sera treatment alone did not induce IL-8 or MCP-1 production. (**E**) Correlation of IL-8 production with NAb titers of sera of deceased patients. Solid lines denote the relationship between histopathology scores and serum neutralizing activity. (**F**) Blockade of human FcγR significantly reduced antiserum-enhanced IL-8 secretion and partially reduced MCP-1 secretion. In vitro–polarized alternatively activated human monocyte–derived macrophages were cocultured with SARS-CoV pseudovirus and 1–4,000 diluted sera from deceased patient (D1) with or without FcγR blocking antibody for 20 hours. Secreted cytokine and chemokine levels were measured in the culture supernatant and are shown in data represented as increase over supernatants from macrophages treated with virus alone. Data represent mean values or mean values ± SEM of at least 3 independent experiments. Two-tailed unpaired Student's t test and Spearman's rank correlation test were used for statistical analysis.

play a role in driving enhanced pulmonary inflammation. A recent study reported that pathogenic immune complexes promoted lung injury during the 2009 H1N1 pandemic (16). It remains unknown whether immune complexes may also play a role in driving ALI during SARS-CoV infection.

Detailed analyses of macrophage heterogeneity at different stages of infection and in lungs with severe injury or mild lesion revealed distinct roles for proinflammatory and wound-healing macrophages in SARS disease progression. We observed that the early transition of macrophage responses from proinflammatory

to wound healing with increased TGF-β expression is associated with inflammation resolution and mild lung injury. By contrast, abrogated wound healing by S-IgG resulted in decreased TGF-β production and prolonged classical macrophage activation, and it promoted severe lung injury. Our findings are primarily based on imaging of formalin-fixed tissues of infected macaques, which may have technical limitations. A detailed flow cytometric analysis would be helpful to further characterize macrophage subpopulations in infected lungs. Unfortunately, we were not allowed to obtain any live cells, including macrophages, for analysis by flow cytometry outside the BSL-3 laboratory in China, which is in line with "WHO biosafety guidelines for handling of SARS specimens" (https://www.who.int/csr/sars/biosafety2003_04_25/en/). Having said this, TGF-β is mainly derived from wound-healing macrophages but not from classically activated macrophages. Therefore, by staining both TGF-β and CD206 (another biomarker of wound-healing macrophages), we believe that the finding of reduction of this type of macrophages in the S-IgG–treated lungs is convincing.

Patients who received convalescent plasma from recovered SARS patients had shorter hospital stays and lower mortality rates without evident adverse effects (45–47). These results might reflect a different quality of antibodies in recovered patients from deceased patients during early infection. Indeed, we previously observed that deceased patients had significantly higher NAbs with a very low level of anti-N antibodies in serum during the early stage of infection (14). In contrast, recovered patients had more rapid anti-N antibody and slower NAb development (14). Consistently, serum from deceased patients — but not SARS survivors during early stages of infection — enhanced IL-6, IL-8, and MCP1 production by wound-healing macrophages (Figure 8). We previously identified epitopes in S protein that elicited NAbs and antibodies that enhanced SARS-CoV infection (48, 49). Further studies that will attempt to identify antibodies and epitopes that mediate prevention or enhancement of ALI might be needed for future vaccine design and immunotherapy.

Lastly, blockade of FcγRs reduced proinflammatory cytokine production, suggesting a potential role of FcγRs for the postulated reprogramming of alternatively activated macrophage FcγRs are functionally divided into activating and inhibitory receptors. Activating FcγRs, such as FcγRI, FcγRIIA, and FcγRIII, triggers production of proinflammatory chemokines and cytokines through an immunoreceptor tyrosine–based activation motif (ITAM) in their intracytoplasmic domain and SRC family kinases and spleen tyrosine kinase (SYK), whereas the inhibitory FcγR, FcγRIIB, has an immunoreceptor tyrosine–based inhibition motif (ITIM) in its intracytoplasmic domain and counteracts the signals that are mediated by activating FcγRs. Based on literature and our experiments, human monocyte–derived wound-healing macrophages express higher levels of CD16 (FcγRIII) and CD32A (FcγRIIA) (50). Therefore, it is likely that S-IgG promoted proinflammatory cytokine production through FcγRI and/or FcγRIIA. S-IgG did not affect classically activated macrophage function in vitro. This difference can be possibly explained by the high baseline level of proinflammatory cytokines before infection and the downregulated expression of FcγRs on classically activated MDM by SARS-CoV treatment.

Methods

Immunization of animals. We vaccinated Chinese rhesus macaques as previously described (51). Briefly, 16 Chinese rhesus monkeys were immunized twice on days 0 and 28. Eight of these animals received ADS-MVA, whereas the other 8 animals received ADC-MVA. ADS-MVA and ADC-MVA vectors encoding SARS-CoV S protein or HCV E1E2 were constructed and produced as previously described (51). The dose for both immunizations was 5×10^8 $TCID_{50}$. The vaccine strain SARS-CoV HKU39849 (AY278491) shares 100% sequence homology of the S gene with the challenge strain $SARS\text{-}CoV_{PUMC}$ (AY350750).

Animals, tissues, and SARS-CoV infection. SARS-CoV–infected animals and control animals were all adult female Chinese rhesus macaques. A total of 42 Chinese rhesus macaques were challenged i.n. with a live pathogenic SARS-CoV (PUM, $TCID_{50}$ = 50) as previously described (25). An additional 3 uninfected macaques were inoculated with PBS as negative controls. Animals were subjected to daily measurement of anal temperature, routine blood assays, and chest radiography. Animals were euthanized at 2, 3, 7, 21, and 35 days after inoculation (Figure 1, Figure 2, and Figure 6). All animals were humanely euthanized with an i.v. overdose of pentobarbital and were immediately necropsied. Lung tissues (left and right cranial, middle and caudal lobes, and right accessory lobe) were collected and fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 5 μm.

Passive transfer of IgG. SARS-CoV S protein–immune serum was generated by vaccination with ADS-MVA vectors. IgG was purified from pooled sera by protein G affinity chromatography to >95% purity. IgG was depyrogenated, concentrated to 20–30 mg protein ml^{-1}, and administered i.v. Recipients received 200 mg or 5 mg of purified IgG per kg body weight at 48 hours before the viral challenge.

Neutralization. A neutralization assay was established to determine the humoral immune responses generated by the vaccine as previously described (51). The neutralizing activity of heat-inactivated animal sera was determined using a pseudotype viral entry assay. The pseudotype virus was generated through cotransfection of 293T cells with 2 plasmids, pcDNA-Sopt9 and pNL4-3Luc_Env_Vpr_, carrying the optimized S gene and a human immunodeficiency virus type 1 backbone, respectively. The serial diluted serum samples were incubated with equal amounts of pseudotype virus at 37°C for 1 hour. The serum-virus mixtures were subsequently added into preseeded HEK 293T-ACE2 cells. After 56 hours, infected cells were lysed to measure luciferase activity.

Nested RT-PCR and isolation of SARS-CoV. Pharyngeal swab samples were collected from infected monkeys on the first day after inoculation and subsequently tested for SARS-CoV using nested RT-PCR as previously described (25). Briefly, RNA was isolated using TRIZOL (Invitrogen). RT-PCR was performed in a 50 μl reaction volume with outer primer pair (5′-GCTGCATTGGTTTGTTATATCGTTA-TGC-3′) and (5′-ATACAGAATACAT-AGATTGCTGTTATCC-3′), inner primer pair (5′-TCACTTGCTTC-CGTTGAGGTAGC-CAGCGTGGT-GGTTCATACAA-3′) and (5′-GGTTTCGGATGTTACAGC-GTCTCCCGG-CAGAAAGCTGTAAGCT-3′). All PCR products were verified by nucleotide sequencing. SARS-CoV was isolated as previously described. Briefly, pharyngeal swab samples from the infected macaques were inoculated onto Vero-E6 cells and cultured in DMEM (Thermo Fisher Scientific). Both cytopathic effect (CPE) and immunofluorescence assay (IFA) (incubation with 1:10 dilution of SARS patient serum) were used to determine the infection status of the animals.

Double and triple immunofluorescence staining on paraffin sections. The samples were deparaffinized and rehydrated. After blocking with normal goat serum for 30 minutes at room temperature, a rabbit anti–SARS-CoV nucleocapsid antibody (ss-006-0100, ENZYME) was applied at 4°C overnight, followed by an Alexa Fluor 448 conjugated goat anti–rabbit IgG antibody for 1 hour at room temperature. Additional immunofluorescence staining was subsequently performed by overnight incubation with primary antibodies, followed by the detection of signals using appropriate fluorescent secondary antibodies (MilliporeSigma) for 1 hour at room temperature.

Primary antibodies for immunofluresence staining. The following primary antibodies were used in this study: SARS-CoV NP (ss-006-0100, ENZYME); MAC387 (clone MAC387, AbD, Serotec); CD68 (clone KPI, DAKO; ab125047, Abcam); CD163 (clone EDHu1, AbD and ab183476, Abcam); HAM56 (clone HAM56, DAKO); CD206 (HPA004114, MilliporeSigma); TGF-β (sc-146, Santa Cruz Biotechnology Inc.); IL-6 (sc-1265, Santa Cruz Biotechnology Inc.); AE1/AE3 (clone AE1/AE3, DAKO).

ISH studies. ISH was developed for the detection of SARS-CoV. DNA fragments of N and S proteins from the SARS-CoV strain from Hong Kong (AY278491) were used as templates to generate RNA probes. The probes were labeled with digoxigenin (GENEWIZ Inc.). After deparaffinization and rehydration, the sections were treated with microwave heating in an 800 W domestic microwave oven (NN-ST556M, Panasonic) at medium power for 10 minutes in 0.01 M sodium citrate buffer. Hybridization was performed at high stringency with 150 ng/ml of denatured probe at 45°C for 16 hours in 50 μl of hybridization mix (50% formamide, 10% dextran sulfate in 2× saline sodium citrate [SSC]) in a moist chamber. Excess probe was removed by washing twice in 2× SSC for 20 minutes at 45°C, followed by 2 washes in 1× SSC and 0.1× SSC for 20 minutes at 45°C. Immunological detection was conducted using the DIG Nuclear Acid Detection Kit (Roche Diagnostics) according to the manufacturer's instructions. Briefly, after incubation with 1% blocking reagent for 30 minutes, the sections were incubated with sheep anti-digoxigenin alkaline phosphatase conjugate (11093274910, Roche Diagnostics) diluted at 1: 500 for 2 hours at room temperature. After 3 separate washes with washing buffer (0.1 M maleic acid, 0.15 M NaCl [pH 7.5], 0.3% Tween 20), the signal was developed using 200 μl of a HNPP/Fast Red TR mix (1758888, Roche Diagnostics) in the dark for 45 minutes. The sections were counterstained with DAPI. Tissue sections incubated with sense RNA probe were used as a negative control.

Confocal microscopy. Confocal microscopy was performed using a Carl Zeiss LSM 700 confocal microscope equipped with 4 lasers (Zeiss). ZEN (Zeiss) was used to assign colors to the 4 channels collected: Alexa Fluor 568 and HNPP/Fast Red, which fluoresces when exposed to a 568-nm wavelength laser and appears red; Alexa Fluor 488 (Molecular Probes), which appears green; Alexa Fluor 647 (Molecular Probes), which appears cyan; and the differential interference contrast (DIC) image, which is in gray scale. The 4 channels were simultaneously collected. To differentiate between individual cells, Hoechst 33258 (nuclear marker; Molecular Probes) was used at 1 μg/ml and incubated for 5 minutes, followed by rinsing with water. The colocalization of antigens was demonstrated by the addition of colors as indicated in the figure legend.

Monocyte isolation, differentiation, and polarization of macrophages. Monocytes were purified from peripheral blood mononuclear cells obtained from blood buffy coats (provided by Red Cross, Hong Kong, China) or monkey peripheral blood, as we previously described (52). Monkey peripheral blood was obtained in heparinized vacutainer collection tubes from healthy female rhesus monkeys. To generate M1 macrophages, monocytes were differentiated in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with GM-CSF (400 IU/ml), 2 mM glutamine, 10% decomplemented FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific) for 4 days, followed by exposure to fresh medium supplemented with 5% FCS and containing GM-CSF and LPS (100 ng/ml) + IFN-γ (20 ng/ml) for an additional 72 hours. To generate M2 macrophages, monocytes were differentiated in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with M-CSF (50 ng/ml), 2 mM glutamine, 10% decomplemented FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin for 4 days, followed by exposure to fresh medium supplemented with 5% FCS and containing M-CSF (50 ng/ml) + IL-4 (20 ng/ml) for an additional 72 hours.

Quantification of cytokine levels. A panel of 13 cytokines, including IL-1β, IFN-α, IFN-γ, TNF-α, MCP1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33 in monkey sera and in culture medium were quantified using multiplexing laser bead technology (BioLegend). To assess the cytokine production of macrophages, in vitro–polarized cells were washed 3 times with PBS and were subsequently activated with SARS-CoV pseudotype virus in RPMI 1640 medium (Thermo Fisher Scientific) with or without administration of highly diluted and heat-inactivated (56°C) sera from macaques or SARS patients. FcγR-specific mouse monoclonal antibody (5 μg, clone 3G8, anti-hCD16, 16016682; clone FLI8.26, anti-hCD32, 16032981; and clone 10.1, anti-hCD64, 16064981 [BD Pharmingen]) was used for blockade of human FcγR. The supernatant of these cultures was recovered after 20 hours of stimulation, and the cytokine levels were measured and analyzed.

Statistics. All statistical analyses were performed using the 2-tailed Student's *t* test. *P* values less than 0.05 were considered statistically significant. Data are presented as the mean values ± SEM of at least 3 independent experiments unless otherwise indicated.

Study approval. The experiment was conducted in a Biosafety Level 3 animal facility. Handling and experimental procedures, including injections, blood collection, virus challenge, and sacrificing were approved and performed according to the animal welfare committee on the Use of Live Animals in Teaching and Research of Institute of Laboratory Animal Science, Beijing, China. All study subjects gave informed consent, and procedures were reviewed and approved by the University of Hong Kong Research Ethics Committee.

Author contributions

ZC, LL, and QW designed the study. LL and QW analyzed the data. LL, ZC, KYY, SP, and AL contributed to drafting the manuscript. ZC is the principal investigator, with overall responsibility for the design of the study. QW and CQ coordinated the autopsy organization and collection of autopsy materials of Chinese macaques. QW was involved in the development and performance of the RT-PCR assay. LL, HT, TW, and KWC performed the neutralization assay. LL, XA, and AL were involved in the development of the H&E, SARS-CoV IHC, and ISH assays. HW performed the ISH assay and the IHC staining of NP and calculation of the mean florescence intensity in the lungs. LL, HK, and KN performed the H&E and double- or triple-IHC staining. JF and HT performed culture of monocyte-derived macrophages. QL, HT, JP, and ZT performed analysis of cytokine production in cell culture supernatant and monkey sera. KHC and KYY coordinated the collection of sera and tissue sample from SARS patients. All authors were involved in the correlative interpretation of the pathological and molecular data.

Acknowledgments

This work was financially supported by grants from the U.S. NIH RO1HL080211 and Hong Kong Research Grants Council TRS T11-706/18-N to ZC, the TNPRC (base grant RR00164), RO1060699 to SP, HMRF 16150662, and the University Development Fund/Li Ka Shing Faculty of Medicine Matching Fund of the University of Hong Kong to its AIDS Institute and Hong Kong RGV. The authors would like to thank D.D. Ho and K. Liu for support and scientific advice, and Wenjie Yu for assistance of S-IgG and C-IgG purification.

Address correspondence to: Zhiwei Chen or Li Liu, AIDS Institute and Department of Microbiology, State Key Laboratory of Emerging Infectious Disease, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong S.A.R., China. Phone: 852.28199831; Email: zchenai@hku.hk (ZC). Phone: 852.39179094; Email: liuli71@hku.hk (LL).

1. Drosten C, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med*. 2003;348(20):1967–1976.
2. Ksiazek TG, et al. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med*. 2003;348(20):1953–1966.
3. Hwang DM, Chamberlain DW, Poutanen SM, Low DE, Asa SL, Butany J. Pulmonary pathology of severe acute respiratory syndrome in Toronto. *Mod Pathol*. 2005;18(1):1–10.
4. Peiris JS, Guan Y. Confronting SARS: a view from Hong Kong. *Philos Trans R Soc Lond, B, Biol Sci*. 2004;359(1447):1075–1079.
5. Nicholls J, Dong XP, Jiang G, Peiris M. SARS: clinical virology and pathogenesis. *Respirology*. 2003;8 Suppl:S6–S8.
6. Peiris JS, et al. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. *Lancet*. 2003;361(9371):1767–1772.
7. Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med*. 2000;342(18):1334–1349.
8. Herold S, Mayer K, Lohmeyer J. Acute lung injury: how macrophages orchestrate resolution of inflammation and tissue repair. *Front Immunol*. 2011;2:65.
9. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol*. 2011;11(11):723–737.
10. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8(12):958–969.
11. Wynn TA, Vannella KM. Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity*. 2016;44(3):450–462.
12. Perlman S, Dandekar AA. Immunopathogenesis of coronavirus infections: implications for SARS. *Nat Rev Immunol*. 2005;5(12):917–927.
13. Channappanavar R, et al. Dysregulated Type I Interferon and Inflammatory Monocyte-Macrophage Responses Cause Lethal Pneumonia in SARS-CoV-Infected Mice. *Cell Host Microbe*. 2016;19(2):181–193.
14. Zhang L, et al. Antibody responses against SARS coronavirus are correlated with disease outcome of infected individuals. *J Med Virol*. 2006;78(1):1–8.
15. To KK, et al. High titer and avidity of nonneutralizing antibodies against influenza vaccine antigen are associated with severe influenza. *Clin Vaccine Immunol*. 2012;19(7):1012–1018.
16. Monsalvo AC, et al. Severe pandemic 2009 H1N1 influenza disease due to pathogenic immune complexes. *Nat Med*. 2011;17(2):195–199.
17. Clay C, et al. Primary severe acute respiratory syndrome coronavirus infection limits replication but not lung inflammation upon homologous rechallenge. *J Virol*. 2012;86(8):4234–4244.
18. Bolles M, et al. A double-inactivated severe acute respiratory syndrome coronavirus vaccine provides incomplete protection in mice and induces increased eosinophilic proinflammatory pulmonary response upon challenge. *J Virol*. 2011;85(23):12201–12215.
19. Tseng CT, et al. Immunization with SARS coronavirus vaccines leads to pulmonary immunopathology on challenge with the SARS virus. *PLoS ONE*. 2012;7(4):e35421.
20. Zhao J, et al. Airway Memory CD4(+) T Cells Mediate Protective Immunity against Emerging Respiratory Coronaviruses. *Immunity*. 2016;44(6):1379–1391.
21. Channappanavar R, Fett C, Zhao J, Meyerholz DK, Perlman S. Virus-specific memory CD8 T cells provide substantial protection from lethal severe acute respiratory syndrome coronavirus infection. *J Virol*. 2014;88(19):11034–11044.
22. Zhao J, Zhao J, Perlman S. T cell responses are required for protection from clinical disease and for virus clearance in severe acute respiratory syndrome coronavirus-infected mice. *J Virol*. 2010;84(18):9318–9325.
23. Honda-Okubo Y, Barnard D, Ong CH, Peng BH, Tseng CT, Petrovsky N. Severe acute respiratory syndrome-associated coronavirus vaccines formulated with delta inulin adjuvants provide enhanced protection while ameliorating lung eosinophilic immunopathology. *J Virol*. 2015;89(6):2995–3007.
24. Chen Z, et al. Recombinant modified vaccinia virus Ankara expressing the spike glycoprotein of severe acute respiratory syndrome coronavirus induces protective neutralizing antibodies primarily targeting the receptor binding region. *J Virol*. 2005;79(5):2678–2688.
25. Liu L, et al. Spatiotemporal interplay of severe acute respiratory syndrome coronavirus and respiratory mucosal cells drives viral dissemination in rhesus macaques. *Mucosal Immunol*. 2016;9(4):1089–1101.
26. Li BJ, et al. Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nat Med*. 2005;11(9):944–951.
27. Qin C, et al. An animal model of SARS produced by infection of Macaca mulatta with SARS coronavirus. *J Pathol*. 2005;206(3):251–259.
28. Soulas C, et al. Recently infiltrating MAC387(+) monocytes/macrophages a third macrophage population involved in SIV and HIV encephalitic lesion formation. *Am J Pathol*. 2011;178(5):2121–2135.
29. Cai Y, Sugimoto C, Arainga M, Alvarez X, Didier ES, Kuroda MJ. In vivo characterization of alveolar and interstitial lung macrophages in rhesus macaques: implications for understanding lung disease in humans. *J Immunol*. 2014;192(6):2821–2829.
30. Murray PJ, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*. 2014;41(1):14–20.
31. Imai Y, et al. Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell*. 2008;133(2):235–249.
32. Atreya R, et al. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med*. 2000;6(5):583–588.
33. Braciale TJ, Sun J, Kim TS. Regulating the adaptive immune response to respiratory virus infection. *Nat Rev Immunol*. 2012;12(4):295–305.

34. Subbarao K, et al. Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome coronavirus in the respiratory tract of mice. *J Virol*. 2004;78(7):3572–3577.
35. Yang ZY, et al. A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. *Nature*. 2004;428(6982):561–564.
36. Du L, et al. Priming with rAAV encoding RBD of SARS-CoV S protein and boosting with RBD-specific peptides for T cell epitopes elevated humoral and cellular immune responses against SARS-CoV infection. *Vaccine*. 2008;26(13):1644–1651.
37. Du L, et al. Intranasal vaccination of recombinant adeno-associated virus encoding receptor-binding domain of severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein induces strong mucosal immune responses and provides long-term protection against SARS-CoV infection. *J Immunol*. 2008;180(2):948–956.
38. Du L, et al. Receptor-binding domain of SARS-CoV spike protein induces long-term protective immunity in an animal model. *Vaccine*. 2007;25(15):2832–2838.
39. Fett C, DeDiego ML, Regla-Nava JA, Enjuanes L, Perlman S. Complete protection against severe acute respiratory syndrome coronavirus-mediated lethal respiratory disease in aged mice by immunization with a mouse-adapted virus lacking E protein. *J Virol*. 2013;87(12):6551–6559.
40. Iwata-Yoshikawa N, et al. Effects of Toll-like receptor stimulation on eosinophilic infiltration in lungs of BALB/c mice immunized with UV-inactivated severe acute respiratory syndrome-related coronavirus vaccine. *J Virol*. 2014;88(15):8597–8614.
41. Qin E, et al. Immunogenicity and protective efficacy in monkeys of purified inactivated Vero-cell SARS vaccine. *Vaccine*. 2006;24(7):1028–1034.
42. Zhou J, et al. Immunogenicity, safety, and protective efficacy of an inactivated SARS-associated coronavirus vaccine in rhesus monkeys. *Vaccine*. 2005;23(24):3202–3209.
43. Openshaw PJ, Tregoning JS. Immune responses and disease enhancement during respiratory syncytial virus infection. *Clin Microbiol Rev*. 2005;18(3):541–555.
44. Stockman LJ, et al. Severe acute respiratory syndrome in children. *Pediatr Infect Dis J*. 2007;26(1):68–74.
45. Mair-Jenkins J, et al. The effectiveness of convalescent plasma and hyperimmune immunoglobulin for the treatment of severe acute respiratory infections of viral etiology: a systematic review and exploratory meta-analysis. *J Infect Dis*. 2015;211(1):80–90.
46. Cheng Y, et al. Use of convalescent plasma therapy in SARS patients in Hong Kong. *Eur J Clin Microbiol Infect Dis*. 2005;24(1):44–46.
47. Soo YO, et al. Retrospective comparison of convalescent plasma with continuing high-dose methylprednisolone treatment in SARS patients. *Clin Microbiol Infect*. 2004;10(7):676–678.
48. Wang Q, et al. Immunodominant SARS Coronavirus Epitopes in Humans Elicited both Enhancing and Neutralizing Effects on Infection in Non-human Primates. *ACS Infect Dis*. 2016;2(5):361–376.
49. Yi CE, Ba L, Zhang L, Ho DD, Chen Z. Single amino acid substitutions in the severe acute respiratory syndrome coronavirus spike glycoprotein determine viral entry and immunogenicity of a major neutralizing domain. *J Virol*. 2005;79(18):11638–11646.
50. Pahl JH, et al. Macrophages inhibit human osteosarcoma cell growth after activation with the bacterial cell wall derivative liposomal muramyl tripeptide in combination with interferon-γ. *J Exp Clin Cancer Res*. 2014;33:27.
51. Kieran J, et al. The relative efficacy of boceprevir and telaprevir in the treatment of hepatitis C virus genotype 1. *Clin Infect Dis*. 2013;56(2):228–235.
52. Cheng L, et al. Monoclonal antibodies specific to human Δ42PD1: A novel immunoregulator potentially involved in HIV-1 and tumor pathogenesis. *MAbs*. 2015;7(3):620–629.

PERSPECTIVE
Host-Microbe Biology

COVID-19: an Immunopathological View

Kamran Kadkhoda[a]

[a]Immunopathology Laboratory, Robert J. Tomsich Pathology & Laboratory Medicine Institute, Cleveland Clinic, Cleveland, Ohio, USA

ABSTRACT Since its emergence in December 2019, it took only a couple of months for an outbreak of the novel coronavirus disease 2019 (COVID-19) to be declared a pandemic by the World Health Organization (WHO). This along with the highly infectious nature of the disease and the associated mortality call for particular attention to the underlying (immuno)pathomechanism(s). The latter will inform case management and vaccine design. Unravelling these mechanisms can assist basic scientists, laboratory medicine practitioners, clinicians, public health practitioners, funding agencies, and health care policymakers in responding to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic.

KEYWORDS COVID-19, SARS-CoV-2, immunopathology

Xu et al. (1) reported on a 50-year-old man with no known underlying conditions who presented with pneumonia. Despite the presence of lymphopenia and absence of neutropenia, all inflammatory cells observed on the lung biopsy specimen were mononuclear cells. Peripheral blood lymphocytes showed high levels of activation markers (HLA-DR and CD38) by flow cytometry. These cells were strongly perforin- and/or granulysin-positive $CD8^{ffi}$ T cells or were inflammatory Th17 cells. This had led to significant damage to the lung tissues as demonstrated by diffuse alveolar damage, indicating acute respiratory distress syndrome (ARDS). This is consistent with high-level surface expression of angiotensin-converting enzyme 2 (ACE2), the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) receptor, on pneumocytes (2). Unfortunately, the patient passed away, but it is important to note that methylprednisolone was started on day 8 after the onset of symptoms, while the catastrophic ARDS cascade was already under way. Interestingly, inhalational alpha interferon (IFN-ffi) was also instituted along with methylprednisolone. Since IFN-ffi can boost antiviral immune responses, it might have further played a role in the tissue damage in this case. On a related note, previous studies with macaques using SARS-CoV had shown that older macaques had stronger innate immune responses (including those related to IFN-ffi) compared with younger macaques. In contrast, expression of IFN-ffi, as an anti-inflammatory cytokine, was reduced in older macaques (3). It has recently been shown that interleukin-6 (IL-6) was also expressed significantly higher in patients who succumbed to coronavirus disease 2019 (COVID-19) than in survivors (4). Observations like this case made by a number of front-line clinicians since the inception of the COVID-19 pandemic have led to pointing fingers at immunopathology as the potential main culprit.

There have been several studies including the large epidemiological joint report by WHO and China (5) stating that the case fatality rate (CFR) is exceedingly low in pediatric patients especially in the very young (CFR of 21.9% for individuals over 80 years of age versus 0% for individuals under 8 years of age). This is a striking finding that further suggests an immunopathological component to this observation. Another observation is the seroprevalence of community-acquired coronaviruses among adults is very high (90 to 100%) (6), but this is not necessarily the case in pediatric patients (7). Additionally, there are antigenic commonalities among coronaviruses (CoVs); for in-

Citation Kadkhoda K. 2020. COVID-19: an immunopathological view. mSphere 5:e00344-20. https://doi.org/10.1128/mSphere.00344-20

Editor Helene F. Rosenberg, National Institute of Allergy and Infectious Diseases

Copyright © 2020 Kadkhoda. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Address correspondence to kadkhok@ccf.org.

Published 22 April 2020

Downloaded from http://msphere.asm.org/ on April 22, 2020 by guest

stance, similar to SARS-CoV-2, CoV-NL63 also uses ACE2 as the receptor (8). As individuals age, the chance of exposure to common community-acquired CoVs (229E, OC43, NL63, and HKU1) increases. As a consequence, with such background anamnesis, once individuals are exposed to novel (zoonotic) CoVs such as SARS-CoV-2, the fast and furious immune response does the damage (the original antigenic sin [OAS]). According to OAS, the initial antigen imprints an immune response so that subsequent exposure to related antigen(s) preferentially chooses the already-existing memory cells. The resultant response, although brisk and apparently strong (a high-titer IgG response would be faster than that of IgM), may be unduly insufficient and inappropriate to the point that it might be initially nonprotective. In the context of COVID-19, since ACE2 is highly expressed in the gastrointestinal (GI) tract (9), shedding the virus in the stool is prolonged (10); however, diarrhea is uncommon likely because virus-specific effector memory T cells typically home to the mucosal surfaces they had previously encountered with an infection with a common CoV, i.e., upper and lower respiratory tract. As a result, despite ARDS in the lungs, almost no significant intestinal damage occurs, if at all. To further confound matters, lungs also express high levels of CD32a (Fc! RIIa, typically on alveolar macrophages), whereas GI tissues express almost no CD32a protein (The Human Protein Atlas [https://www.proteinatlas.org/] [accessed March 2020]). It has recently been shown that SARS-CoV and the Middle East respiratory syndrome (MERS)-CoV take advantage of non- or subneutralizing antibodies and enter cells via surface CD32a receptors (Trojan horse mechanism) (11, 12). Therefore, another even more biologically plausible pathomechanism is the so-called antibody-dependent enhancement (ADE). ADE has also been shown in infection with dengue viruses, Zika virus, Ebola virus, and human immunodeficiency virus. ADE is not only triggered by neutralizing antibodies but also by nonneutralizing antibodies. Additionally, antibody affinity plays an important role in ADE (11, 13). The most important pathomechanism in COVID-19, therefore, could be ADE in which CD32a plays the central role. CD32a is expressed on the surfaces of monocytes and macrophages among other cells such as alveolar macrophages, and once aggregated by several IgG molecules, it transduces its signal through the associated immunoreceptor tyrosine-based activation motif (ITAM). This results in the release of proinflammatory cytokines including IFN-! , tumor necrosis factor alpha, interleukin-1 (IL-1), and IL-6 (14). This IL-6 overproduction is the justification for using IL-6 receptor antagonists such as tocilizumab in COVID-19 clinical trials. It should be noted, however, that activation of ITAM occurs through phosphorylation by certain members of Src family of tyrosine kinases such as Fyn and Syk. Interestingly, agents such as cerdulatinib have dual inhibitory function against Syk as well as against Janus kinase family members (such as JAK1/2) (15), the latter also play key roles in IL-6 receptor signaling (16).

To complete the picture, SARS-CoV nucleoprotein and RNA had previously been found within alveolar macrophages of nonsurvivors (17). These cells also express dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN; CD209 and also CD209L). These surface molecules have been reported to be coreceptors for SARS-CoV entry (18). In this way, alveolar macrophages can function as a local niche for the virus and thereby pass it on to other cells.

Findings similar to the aforementioned epidemiological finding have been made for MERS-CoV in which disease severity increases with age and severe disease has been uncommon among pediatric patients (19). Furthermore, along the lines of ADE, it has been known for decades that immunization of cats with spike protein of feline CoV leads to more severe future infections with feline CoV in cats (11). Therefore, ADE is perhaps more effective when the time intervals between CoV infections are just long enough so antibody titers drop to at least subneutralizing levels. This may explain why association of older age with severe COVID-19 is not always the rule. Infection of alveolar macrophages through ADE may explain their excessive activation and generation of local hyperinflammatory environment and the resultant systemic cytokine storm that may even fulfill criteria for secondary hemophagocytic lymphohistiocytosis and macrophage activation syndrome. The pivotal involvement of macrophages is further highlighted by the fact that very high levels of ferritin are seen in severe

Downloaded from http://msphere.asm.org/ on April 22, 2020 by guest

COVID-19 cases (20). Additionally, the fact that severe COVID-19 is associated with much higher viral load compared with mild cases further suggests that ADE could play an important role in severe cases (21). Another explanation for age-associated severity of COVID-19 may be attributed to C-reactive protein (CRP). It is known that CRP levels increase with age and that serum CRP levels in adults are higher than in children, with the highest median level observed in the elderly (22). Furthermore, it has been demonstrated that CD32a is the major biological receptor for CRP (23).

Furthermore, the course of COVID-19 is in general more severe in those with underlying conditions such as hypertension, poorly controlled diabetes mellitus, and cardiovascular disease among others (4). This may be attributed to the known increased expression of CD32a on monocytes and macrophages in these patients (24). However, it is a common assumption that immunocompromised patients are at increased risk of developing severe COVID-19. To challenge this assumption, a report from Italy (25) showed that 3 out of 111 liver transplant recipients who passed away due to COVID-19 had received their transplants 10 years prior to death. Interestingly, these patients were stable with very low trough concentrations of calcineurin inhibitors in plasma. They, however, were all male, older than 65 years, overweight, diabetic, and on treatment for hypertension. In contrast, in the same study, 3 out of 40 individuals who had recently received liver transplants and were on intense immunosuppression drugs (without the other risk factors mentioned for the former group) and who tested positive for SARS-CoV-2 experienced an uneventful course of COVID-19. The CFR in the former group was 3% in contrast to the overall CFR of ca. 10% in Italy. Abatement of IFN-ffi signaling by calcineurin inhibitors may explain these observations. IFN-ffi can also be largely produced as a result of CD32a activation (26). Similar observations in other immunocompromised patient populations with larger sample sizes will be paramount to further understand the role of immunopathology in COVID-19.

All in all, further corroborative research is urgently needed to find out why advanced age is a major risk factor for COVID-19 and whether immunopathomechanisms can potentially be harnessed early enough to prevent irreversible consequences. This may be ground-breaking and would not only change the current individual patient management but also potentially inform prudent vaccine design and recommendations as was the case with dengue virus vaccination. The implications of ADE will be immense if IgG serology is used to determine immune status for health care workers, as a positive result does not necessarily mean one is immune to COVID-19 (even with a test specificity of 100%). More importantly, ADE may cause harm if plasma from clinically resolved patients is used for treatment. A very careful balance between risks and benefits established through large multicenter randomized controlled trials will provide answers to all these questions.

ACKNOWLEDGMENT

I sincerely thank Stanley Plotkin for reviewing the article.

REFERENCES

1. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, Song J, Xia P, Dong J, Zhao J, Wang FS. 2020. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. Lancet Respir Med 8:420–422. https://doi.org/10.1016/S2213-2600(20)30076-X.
2. Tse GM, To KF, Chan PK, Lo AW, Ng KC, Wu A, Lee N, Wong HC, Mak SM, Chan KF, Hui DS, Sung JJ, Ng HK. 2004. Pulmonary pathological features in coronavirus associated severe acute respiratory syndrome (SARS). J Clin Pathol 57:260–265. https://doi.org/10.1136/jcp.2003.013276.
3. Smits SL, de Lang A, van den Brand JMA, Leijten LM, van IJcken WF, Eijkemans MJC, van Amerongen G, Kuiken T, Andeweg AC, Osterhaus ADME, Haagmans BL. 2010. Exacerbated innate host response to SARS-CoV in aged non-human primates. PLoS Pathog 6:e1000756. https://doi.org/10.1371/journal.ppat.1000756.
4. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, Wu X, Xu J, Tu S, Zhang Y, Chen H, Cao B. 2020. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet 395:1054–1062. https://doi.org/10.1016/S0140-6736(20)30566-3.
5. World Health Organization. 2020. Report of the WHO-China Joint Mission on Coronavirus Disease 2019 (COVID-19). World Health Organization, Geneva, Switzerland. https://www.who.int/docs/default-source/coronaviruse/who-china-joint-mission-on-covid-19-final-report.pdf.
6. Gorse GJ, Patel GB, Vitale JN, O'Connor TZ. 2010. Prevalence of antibodies to four human coronaviruses is lower in nasal secretions than in serum. Clin Vaccine Immunol 17:1875–1880. https://doi.org/10.1128/CVI.00278-10.
7. Prill MM, Iwane MK, Edwards KM, Williams JV, Weinberg GA, Staat MA, Willby MJ, Talbot HK, Hall CB, Szilagyi PG, Griffin MR, Curns AT, Erdman DD, New Vaccine Surveillance Network. 2012. Human coronavirus in young children hospitalized for acute respiratory illness and asymptomatic controls. Pediatr Infect Dis J 31:235–240. https://doi.org/10.1097/INF.0b013e31823e07fe.
8. Wu K, Li W, Peng G, Li F. 2009. Crystal structure of NL63 respiratory

Downloaded from http://msphere.asm.org/ on April 22, 2020 by guest

coronavirus receptor-binding domain complexed with its human receptor. Proc Natl Acad Sci U S A 106:19970–19974. https://doi.org/10.1073/pnas.0908837106.
9. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. 2004. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J Pathol 203:631–637. https://doi.org/10.1002/path.1570.
10. Tang A, Tong ZD, Wang HL, Dai YX, Li KF, Liu JN, Wu WJ, Yuan C, Yu ML, Li P, Yan JB. 9 March 2020. Detection of novel coronavirus by RT-PCR in stool specimen from asymptomatic child, China. Emerg Infect Dis https://doi.org/10.3201/eid2606.200301.
11. Wan Y, Shang J, Sun S, Tai W, Chen J, Geng Q, He L, Chen Y, Wu J, Shi Z, Zhou Y, Du L, Li F. 2019. Molecular mechanism for antibody-dependent enhancement of coronavirus entry. J Virol 94:e02015-19. https://doi.org/10.1128/JVI.02015-19.
12. Walls AC, Xiong X, Park YJ, Tortorici MA, Snijder J, Quispe J, Cameroni E, Gopal R, Dai M, Lanzavecchia A, Zambon M, Rey FA, Corti D, Veesler D. 2019. Unexpected receptor functional mimicry elucidates activation of coronavirus fusion. Cell 176:1026–1039.e15. https://doi.org/10.1016/j.cell.2018.12.028.
13. Li M, Zhao L, Zhang C, Wang X, Hong W, Sun J, Liu R, Yu L, Wang J, Zhang F, Jin X. 2018. Dengue immune sera enhance Zika virus infection in human peripheral blood monocytes through Fc gamma receptors. PLoS One 13:e0200478. https://doi.org/10.1371/journal.pone.0200478.
14. Anania JC, Chenoweth AM, Wines BD, Hogarth PM. 2019. The human Fc! RII (CD32) family of leukocyte FcR in health and disease. Front Immunol 10:464. https://doi.org/10.3389/fimmu.2019.00464.
15. Liu D, Mamorska-Dyga A. 2017. Syk inhibitors in clinical development for hematological malignancies. J Hematol Oncol 10:145. https://doi.org/10.1186/s13045-017-0512-1.
16. Choy EH. 2019. Clinical significance of Janus kinase inhibitor selectivity. Rheumatology (Oxford) 58:1122. https://doi.org/10.1093/rheumatology/kez002.
17. Nicholls JM, Butany J, Poon LL, Chan KH, Beh SL, Poutanen S, Peiris JS, Wong M. 2006. Time course and cellular localization of SARS-CoV nucleoprotein and RNA in lungs from fatal cases of SARS. PLoS Med 3:e27. https://doi.org/10.1371/journal.pmed.0030027.
18. Chen J, Subbarao K. 2007. The immunobiology of SARS. Annu Rev Immunol 25:443–472. https://doi.org/10.1146/annurev.immunol.25.022106.141706.
19. Arabi YM, Balkhy HH, Hayden FG, Bouchama A, Luke T, Baillie JK, Al-Omari A, Hajeer AH, Senga M, Denison MR, Nguyen-Van-Tam JS, Shindo N, Bermingham A, Chappell JD, Van Kerkhove MD, Fowler RA. 2017. Middle East respiratory syndrome. N Engl J Med 376:584–594. https://doi.org/10.1056/NEJMsr1408795.
20. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ. 2020. Covid-19: consider cytokine storm syndrome and immunosuppression. Lancet 395:1033–1034. https://doi.org/10.1016/S0140-6736(20)30628-0.
21. Liu Y, Yan LM, Wan L, Xiang TX, Le A, Liu JM, Peiris M, Poon LLM, Zhang W. 19 March 2020. Viral dynamics in mild and severe cases of COVID-19. Lancet Infect Dis https://doi.org/10.1016/S1473-3099(20)30232-2.
22. Schlenz H, Intemann T, Wolters M, González-Gil EM, Nappo A, Fraterman A, Veidebaum T, Molnar D, Tornaritis M, Sioen I, Mårild S, Iacoviello L, Ahrens W, IDEFICS Consortium. 2014. C-reactive protein reference percentiles among pre-adolescent children in Europe based on the IDEFICS study population. Int J Obes (Lond) 38(Suppl 2):S26–S31. https://doi.org/10.1038/ijo.2014.132.
23. Bharadwaj D, Stein MP, Volzer M, Mold C, Du Clos TW. 1999. The major receptor for C-reactive protein on leukocytes is fcgamma receptor II. J Exp Med 190:585–590. https://doi.org/10.1084/jem.190.4.585.
24. Devaraj S, Chen X, Adams-Huet B, Jialal I. 2013. Increased expression of Fc-! receptors on monocytes in patients with nascent metabolic syndrome. J Clin Endocrinol Metab 98:E1510–E1515. https://doi.org/10.1210/jc.2013-2112.
25. Bhoori S, Rossi RE, Citterio D, Mazzaferro V. 9 April 2020. COVID-19 in long-term liver transplant patients: preliminary experience from an Italian transplant centre in Lombardy. Lancet Gastroenterol Hepatol https://doi.org/10.1016/S2468-1253(20)30116-3.
26. Batteux F, Palmer P, Daëron M, Weill B, Lebon P. 1999. FCgammaRII (CD32)-dependent induction of interferon-alpha by serum from patients with lupus erythematosus. Eur Cytokine Netw 10:509–514.

Downloaded from http://msphere.asm.org/ on April 22, 2020 by guest

PERSPECTIVE
Host-Microbe Biology

COVID-19: an Immunopathological View

Kamran Kadkhoda[a]

[a]Immunopathology Laboratory, Robert J. Tomsich Pathology & Laboratory Medicine Institute, Cleveland Clinic, Cleveland, Ohio, USA

ABSTRACT Since its emergence in December 2019, it took only a couple of months for an outbreak of the novel coronavirus disease 2019 (COVID-19) to be declared a pandemic by the World Health Organization (WHO). This along with the highly infectious nature of the disease and the associated mortality call for particular attention to the underlying (immuno)pathomechanism(s). The latter will inform case management and vaccine design. Unravelling these mechanisms can assist basic scientists, laboratory medicine practitioners, clinicians, public health practitioners, funding agencies, and health care policymakers in responding to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic.

KEYWORDS COVID-19, SARS-CoV-2, immunopathology

Xu et al. (1) reported on a 50-year-old man with no known underlying conditions who presented with pneumonia. Despite the presence of lymphopenia and absence of neutropenia, all inflammatory cells observed on the lung biopsy specimen were mononuclear cells. Peripheral blood lymphocytes showed high levels of activation markers (HLA-DR and CD38) by flow cytometry. These cells were strongly perforin- and/or granulysin-positive $CD8^{ffi}$ T cells or were inflammatory Th17 cells. This had led to significant damage to the lung tissues as demonstrated by diffuse alveolar damage, indicating acute respiratory distress syndrome (ARDS). This is consistent with high-level surface expression of angiotensin-converting enzyme 2 (ACE2), the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) receptor, on pneumocytes (2). Unfortunately, the patient passed away, but it is important to note that methylprednisolone was started on day 8 after the onset of symptoms, while the catastrophic ARDS cascade was already under way. Interestingly, inhalational alpha interferon (IFN-ffi) was also instituted along with methylprednisolone. Since IFN-ffi can boost antiviral immune responses, it might have further played a role in the tissue damage in this case. On a related note, previous studies with macaques using SARS-CoV had shown that older macaques had stronger innate immune responses (including those related to IFN-ffi) compared with younger macaques. In contrast, expression of IFN-ffi, as an anti-inflammatory cytokine, was reduced in older macaques (3). It has recently been shown that interleukin-6 (IL-6) was also expressed significantly higher in patients who succumbed to coronavirus disease 2019 (COVID-19) than in survivors (4). Observations like this case made by a number of front-line clinicians since the inception of the COVID-19 pandemic have led to pointing fingers at immunopathology as the potential main culprit.

There have been several studies including the large epidemiological joint report by WHO and China (5) stating that the case fatality rate (CFR) is exceedingly low in pediatric patients especially in the very young (CFR of 21.9% for individuals over 80 years of age versus 0% for individuals under 8 years of age). This is a striking finding that further suggests an immunopathological component to this observation. Another observation is the seroprevalence of community-acquired coronaviruses among adults is very high (90 to 100%) (6), but this is not necessarily the case in pediatric patients (7). Additionally, there are antigenic commonalities among coronaviruses (CoVs); for in-

Citation Kadkhoda K. 2020. COVID-19: an immunopathological view. mSphere 5:e00344-20. https://doi.org/10.1128/mSphere.00344-20

Editor Helene F. Rosenberg, National Institute of Allergy and Infectious Diseases

Copyright © 2020 Kadkhoda. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Address correspondence to kadkhok@ccf.org.

Published 22 April 2020

Downloaded from http://msphere.asm.org/ on April 22, 2020 by guest

stance, similar to SARS-CoV-2, CoV-NL63 also uses ACE2 as the receptor (8). As individuals age, the chance of exposure to common community-acquired CoVs (229E, OC43, NL63, and HKU1) increases. As a consequence, with such background anamnesis, once individuals are exposed to novel (zoonotic) CoVs such as SARS-CoV-2, the fast and furious immune response does the damage (the original antigenic sin [OAS]). According to OAS, the initial antigen imprints an immune response so that subsequent exposure to related antigen(s) preferentially chooses the already-existing memory cells. The resultant response, although brisk and apparently strong (a high-titer IgG response would be faster than that of IgM), may be unduly insufficient and inappropriate to the point that it might be initially nonprotective. In the context of COVID-19, since ACE2 is highly expressed in the gastrointestinal (GI) tract (9), shedding the virus in the stool is prolonged (10); however, diarrhea is uncommon likely because virus-specific effector memory T cells typically home to the mucosal surfaces they had previously encountered with an infection with a common CoV, i.e., upper and lower respiratory tract. As a result, despite ARDS in the lungs, almost no significant intestinal damage occurs, if at all. To further confound matters, lungs also express high levels of CD32a (Fc! RIIa, typically on alveolar macrophages), whereas GI tissues express almost no CD32a protein (The Human Protein Atlas [https://www.proteinatlas.org/] [accessed March 2020]). It has recently been shown that SARS-CoV and the Middle East respiratory syndrome (MERS)-CoV take advantage of non- or subneutralizing antibodies and enter cells via surface CD32a receptors (Trojan horse mechanism) (11, 12). Therefore, another even more biologically plausible pathomechanism is the so-called antibody-dependent enhancement (ADE). ADE has also been shown in infection with dengue viruses, Zika virus, Ebola virus, and human immunodeficiency virus. ADE is not only triggered by neutralizing antibodies but also by nonneutralizing antibodies. Additionally, antibody affinity plays an important role in ADE (11, 13). The most important pathomechanism in COVID-19, therefore, could be ADE in which CD32a plays the central role. CD32a is expressed on the surfaces of monocytes and macrophages among other cells such as alveolar macrophages, and once aggregated by several IgG molecules, it transduces its signal through the associated immunoreceptor tyrosine-based activation motif (ITAM). This results in the release of proinflammatory cytokines including IFN-! , tumor necrosis factor alpha, interleukin-1 (IL-1), and IL-6 (14). This IL-6 overproduction is the justification for using IL-6 receptor antagonists such as tocilizumab in COVID-19 clinical trials. It should be noted, however, that activation of ITAM occurs through phosphorylation by certain members of Src family of tyrosine kinases such as Fyn and Syk. Interestingly, agents such as cerdulatinib have dual inhibitory function against Syk as well as against Janus kinase family members (such as JAK1/2) (15), the latter also play key roles in IL-6 receptor signaling (16).

To complete the picture, SARS-CoV nucleoprotein and RNA had previously been found within alveolar macrophages of nonsurvivors (17). These cells also express dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN; CD209 and also CD209L). These surface molecules have been reported to be coreceptors for SARS-CoV entry (18). In this way, alveolar macrophages can function as a local niche for the virus and thereby pass it on to other cells.

Findings similar to the aforementioned epidemiological finding have been made for MERS-CoV in which disease severity increases with age and severe disease has been uncommon among pediatric patients (19). Furthermore, along the lines of ADE, it has been known for decades that immunization of cats with spike protein of feline CoV leads to more severe future infections with feline CoV in cats (11). Therefore, ADE is perhaps more effective when the time intervals between CoV infections are just long enough so antibody titers drop to at least subneutralizing levels. This may explain why association of older age with severe COVID-19 is not always the rule. Infection of alveolar macrophages through ADE may explain their excessive activation and generation of local hyperinflammatory environment and the resultant systemic cytokine storm that may even fulfill criteria for secondary hemophagocytic lymphohistiocytosis and macrophage activation syndrome. The pivotal involvement of macrophages is further highlighted by the fact that very high levels of ferritin are seen in severe

Downloaded from http://msphere.asm.org/ on April 22, 2020 by guest

COVID-19 cases (20). Additionally, the fact that severe COVID-19 is associated with much higher viral load compared with mild cases further suggests that ADE could play an important role in severe cases (21). Another explanation for age-associated severity of COVID-19 may be attributed to C-reactive protein (CRP). It is known that CRP levels increase with age and that serum CRP levels in adults are higher than in children, with the highest median level observed in the elderly (22). Furthermore, it has been demonstrated that CD32a is the major biological receptor for CRP (23).

Furthermore, the course of COVID-19 is in general more severe in those with underlying conditions such as hypertension, poorly controlled diabetes mellitus, and cardiovascular disease among others (4). This may be attributed to the known increased expression of CD32a on monocytes and macrophages in these patients (24). However, it is a common assumption that immunocompromised patients are at increased risk of developing severe COVID-19. To challenge this assumption, a report from Italy (25) showed that 3 out of 111 liver transplant recipients who passed away due to COVID-19 had received their transplants 10 years prior to death. Interestingly, these patients were stable with very low trough concentrations of calcineurin inhibitors in plasma. They, however, were all male, older than 65 years, overweight, diabetic, and on treatment for hypertension. In contrast, in the same study, 3 out of 40 individuals who had recently received liver transplants and were on intense immunosuppression drugs (without the other risk factors mentioned for the former group) and who tested positive for SARS-CoV-2 experienced an uneventful course of COVID-19. The CFR in the former group was 3% in contrast to the overall CFR of ca. 10% in Italy. Abatement of IFN-ffi signaling by calcineurin inhibitors may explain these observations. IFN-ffi can also be largely produced as a result of CD32a activation (26). Similar observations in other immunocompromised patient populations with larger sample sizes will be paramount to further understand the role of immunopathology in COVID-19.

All in all, further corroborative research is urgently needed to find out why advanced age is a major risk factor for COVID-19 and whether immunopathomechanisms can potentially be harnessed early enough to prevent irreversible consequences. This may be ground-breaking and would not only change the current individual patient management but also potentially inform prudent vaccine design and recommendations as was the case with dengue virus vaccination. The implications of ADE will be immense if IgG serology is used to determine immune status for health care workers, as a positive result does not necessarily mean one is immune to COVID-19 (even with a test specificity of 100%). More importantly, ADE may cause harm if plasma from clinically resolved patients is used for treatment. A very careful balance between risks and benefits established through large multicenter randomized controlled trials will provide answers to all these questions.

ACKNOWLEDGMENT

I sincerely thank Stanley Plotkin for reviewing the article.

REFERENCES

1. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, Song J, Xia P, Dong J, Zhao J, Wang FS. 2020. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. Lancet Respir Med 8:420–422. https://doi.org/10.1016/S2213-2600(20)30076-X.
2. Tse GM, To KF, Chan PK, Lo AW, Ng KC, Wu A, Lee N, Wong HC, Mak SM, Chan KF, Hui DS, Sung JJ, Ng HK. 2004. Pulmonary pathological features in coronavirus associated severe acute respiratory syndrome (SARS). J Clin Pathol 57:260–265. https://doi.org/10.1136/jcp.2003.013276.
3. Smits SL, de Lang A, van den Brand JMA, Leijten LM, van IJcken WF, Eijkemans MJC, van Amerongen G, Kuiken T, Andeweg AC, Osterhaus ADME, Haagmans BL. 2010. Exacerbated innate host response to SARS-CoV in aged non-human primates. PLoS Pathog 6:e1000756. https://doi.org/10.1371/journal.ppat.1000756.
4. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, Wu X, Xu J, Tu S, Zhang Y, Chen H, Cao B. 2020. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet 395:1054–1062. https://doi.org/10.1016/S0140-6736(20)30566-3.
5. World Health Organization. 2020. Report of the WHO-China Joint Mission on Coronavirus Disease 2019 (COVID-19). World Health Organization, Geneva, Switzerland. https://www.who.int/docs/default-source/coronaviruse/who-china-joint-mission-on-covid-19-final-report.pdf.
6. Gorse GJ, Patel GB, Vitale JN, O'Connor TZ. 2010. Prevalence of antibodies to four human coronaviruses is lower in nasal secretions than in serum. Clin Vaccine Immunol 17:1875–1880. https://doi.org/10.1128/CVI.00278-10.
7. Prill MM, Iwane MK, Edwards KM, Williams JV, Weinberg GA, Staat MA, Willby MJ, Talbot HK, Hall CB, Szilagyi PG, Griffin MR, Curns AT, Erdman DD, New Vaccine Surveillance Network. 2012. Human coronavirus in young children hospitalized for acute respiratory illness and asymptomatic controls. Pediatr Infect Dis J 31:235–240. https://doi.org/10.1097/INF.0b013e31823e07fe.
8. Wu K, Li W, Peng G, Li F. 2009. Crystal structure of NL63 respiratory

Downloaded from http://msphere.asm.org/ on April 22, 2020 by guest

coronavirus receptor-binding domain complexed with its human receptor. Proc Natl Acad Sci U S A 106:19970–19974. https://doi.org/10.1073/pnas.0908837106.
9. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. 2004. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J Pathol 203:631–637. https://doi.org/10.1002/path.1570.
10. Tang A, Tong ZD, Wang HL, Dai YX, Li KF, Liu JN, Wu WJ, Yuan C, Yu ML, Li P, Yan JB. 9 March 2020. Detection of novel coronavirus by RT-PCR in stool specimen from asymptomatic child, China. Emerg Infect Dis https://doi.org/10.3201/eid2606.200301.
11. Wan Y, Shang J, Sun S, Tai W, Chen J, Geng Q, He L, Chen Y, Wu J, Shi Z, Zhou Y, Du L, Li F. 2019. Molecular mechanism for antibody-dependent enhancement of coronavirus entry. J Virol 94:e02015-19. https://doi.org/10.1128/JVI.02015-19.
12. Walls AC, Xiong X, Park YJ, Tortorici MA, Snijder J, Quispe J, Cameroni E, Gopal R, Dai M, Lanzavecchia A, Zambon M, Rey FA, Corti D, Veesler D. 2019. Unexpected receptor functional mimicry elucidates activation of coronavirus fusion. Cell 176:1026–1039.e15. https://doi.org/10.1016/j.cell.2018.12.028.
13. Li M, Zhao L, Zhang C, Wang X, Hong W, Sun J, Liu R, Yu L, Wang J, Zhang F, Jin X. 2018. Dengue immune sera enhance Zika virus infection in human peripheral blood monocytes through Fc gamma receptors. PLoS One 13:e0200478. https://doi.org/10.1371/journal.pone.0200478.
14. Anania JC, Chenoweth AM, Wines BD, Hogarth PM. 2019. The human Fc! RII (CD32) family of leukocyte FcR in health and disease. Front Immunol 10:464. https://doi.org/10.3389/fimmu.2019.00464.
15. Liu D, Mamorska-Dyga A. 2017. Syk inhibitors in clinical development for hematological malignancies. J Hematol Oncol 10:145. https://doi.org/10.1186/s13045-017-0512-1.
16. Choy EH. 2019. Clinical significance of Janus kinase inhibitor selectivity. Rheumatology (Oxford) 58:1122. https://doi.org/10.1093/rheumatology/kez002.
17. Nicholls JM, Butany J, Poon LL, Chan KH, Beh SL, Poutanen S, Peiris JS, Wong M. 2006. Time course and cellular localization of SARS-CoV nucleoprotein and RNA in lungs from fatal cases of SARS. PLoS Med 3:e27. https://doi.org/10.1371/journal.pmed.0030027.
18. Chen J, Subbarao K. 2007. The immunobiology of SARS. Annu Rev Immunol 25:443–472. https://doi.org/10.1146/annurev.immunol.25.022106.141706.
19. Arabi YM, Balkhy HH, Hayden FG, Bouchama A, Luke T, Baillie JK, Al-Omari A, Hajeer AH, Senga M, Denison MR, Nguyen-Van-Tam JS, Shindo N, Bermingham A, Chappell JD, Van Kerkhove MD, Fowler RA. 2017. Middle East respiratory syndrome. N Engl J Med 376:584–594. https://doi.org/10.1056/NEJMsr1408795.
20. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ. 2020. Covid-19: consider cytokine storm syndrome and immunosuppression. Lancet 395:1033–1034. https://doi.org/10.1016/S0140-6736(20)30628-0.
21. Liu Y, Yan LM, Wan L, Xiang TX, Le A, Liu JM, Peiris M, Poon LLM, Zhang W. 19 March 2020. Viral dynamics in mild and severe cases of COVID-19. Lancet Infect Dis https://doi.org/10.1016/S1473-3099(20)30232-2.
22. Schlenz H, Intemann T, Wolters M, González-Gil EM, Nappo A, Fraterman A, Veidebaum T, Molnar D, Tornaritis M, Sioen I, Mårild S, Iacoviello L, Ahrens W, IDEFICS Consortium. 2014. C-reactive protein reference percentiles among pre-adolescent children in Europe based on the IDEFICS study population. Int J Obes (Lond) 38(Suppl 2):S26–S31. https://doi.org/10.1038/ijo.2014.132.
23. Bharadwaj D, Stein MP, Volzer M, Mold C, Du Clos TW. 1999. The major receptor for C-reactive protein on leukocytes is fcgamma receptor II. J Exp Med 190:585–590. https://doi.org/10.1084/jem.190.4.585.
24. Devaraj S, Chen X, Adams-Huet B, Jialal I. 2013. Increased expression of Fc-! receptors on monocytes in patients with nascent metabolic syndrome. J Clin Endocrinol Metab 98:E1510–E1515. https://doi.org/10.1210/jc.2013-2112.
25. Bhoori S, Rossi RE, Citterio D, Mazzaferro V. 9 April 2020. COVID-19 in long-term liver transplant patients: preliminary experience from an Italian transplant centre in Lombardy. Lancet Gastroenterol Hepatol https://doi.org/10.1016/S2468-1253(20)30116-3.
26. Batteux F, Palmer P, Daëron M, Weill B, Lebon P. 1999. FCgammaRII (CD32)-dependent induction of interferon-alpha by serum from patients with lupus erythematosus. Eur Cytokine Netw 10:509–514.

Downloaded from http://msphere.asm.org/ on April 22, 2020 by guest

JCI insight

Anti–spike IgG causes severe acute lung injury by skewing macrophage responses during acute SARS-CoV infection

Li Liu, … , Kwok-Yung Yuen, Zhiwei Chen

JCI Insight. 2019;4(4):e123158. https://doi.org/10.1172/jci.insight.123158 .

Research Article | Infectious disease | Pulmonology

Newly emerging viruses, such as severe acute respiratory syndrome coronavirus (SARS-CoV), Middle Eastern respiratory syndrome CoVs (MERS-CoV), and H7N9, cause fatal acute lung injury (ALI) by driving hypercytokinemia and aggressive inflammation through mechanisms that remain elusive. In SARS-CoV/macaque models, we determined that anti–spike IgG (S-IgG), in productively infected lungs, causes severe ALI by skewing inflammation-resolving response. Alveolar macrophages underwent functional polarization in acutely infected macaques, demonstrating simultaneously both proinflammatory and wound-healing characteristics. The presence of S-IgG prior to viral clearance, however, abrogated wound-healing responses and promoted MCP1 and IL-8 production and proinflammatory monocyte/macrophage recruitment and accumulation. Critically, patients who eventually died of SARS (hereafter referred to as deceased patients) displayed similarly accumulated pulmonary proinflammatory, absence of wound-healing macrophages, and faster neutralizing antibody responses. Their sera enhanced SARS-CoV–induced MCP1 and IL-8 production by human monocyte–derived wound-healing macrophages, whereas blockade of FcγR reduced such effects. Our findings reveal a mechanism responsible for virus-mediated ALI, define a pathological consequence of viral specific antibody response, and provide a potential target for treatment of SARS-CoV or other virus-mediated lung injury.

Find the latest version:

http://jci.me/123158/pdf

JCI insight

RESEARCH ARTICLE

Anti–spike IgG causes severe acute lung injury by skewing macrophage responses during acute SARS-CoV infection

Li Liu,[1,2] Qiang Wei,[3] Qingqing Lin,[1] Jun Fang,[1] Haibo Wang,[1] Hauyee Kwok,[1] Hangying Tang,[1] Kenji Nishiura,[1] Jie Peng,[1] Zhiwu Tan,[1] Tongjin Wu,[1] Ka-Wai Cheung,[1] Kwok-Hung Chan,[1] Xavier Alvarez,[4] Chuan Qin,[3] Andrew Lackner,[4] Stanley Perlman,[5,6] Kwok-Yung Yuen,[1] and Zhiwei Chen[1,2]

[1]AIDS Institute and Department of Microbiology, State Key Laboratory of Emerging Infectious Disease, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China. [2]HKU-AIDS Institute Shenzhen Research Laboratory and AIDS Clinical Research Laboratory, Shenzhen Key Laboratory of Infection and Immunity, Shenzhen Key Clinical Department of Emerging Infectious Diseases, Shenzhen Third People's Hospital, Shenzhen, China. [3]Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College (PUMC), Beijing, China. [4]Division of Comparative Pathology, Tulane National Primate Research Center, Covington, Louisiana, USA. [5]Department of Microbiology and Immunology, University of Iowa, Iowa City, Iowa, USA. [6]State Key Laboratory of Respiratory Disease, National Clinical Research Center for Respiratory Disease, Guangzhou Institute of Respiratory Health, the First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China.

Newly emerging viruses, such as severe acute respiratory syndrome coronavirus (SARS-CoV), Middle Eastern respiratory syndrome CoVs (MERS-CoV), and H7N9, cause fatal acute lung injury (ALI) by driving hypercytokinemia and aggressive inflammation through mechanisms that remain elusive. In SARS-CoV/macaque models, we determined that anti–spike IgG (S-IgG), in productively infected lungs, causes severe ALI by skewing inflammation-resolving response. Alveolar macrophages underwent functional polarization in acutely infected macaques, demonstrating simultaneously both proinflammatory and wound-healing characteristics. The presence of S-IgG prior to viral clearance, however, abrogated wound-healing responses and promoted MCP1 and IL-8 production and proinflammatory monocyte/macrophage recruitment and accumulation. Critically, patients who eventually died of SARS (hereafter referred to as deceased patients) displayed similarly accumulated pulmonary proinflammatory, absence of wound-healing macrophages, and faster neutralizing antibody responses. Their sera enhanced SARS-CoV–induced MCP1 and IL-8 production by human monocyte–derived wound-healing macrophages, whereas blockade of Fcγ reduced such effects. Our findings reveal a mechanism responsible for virus-mediated ALI, define a pathological consequence of viral specific antibody response, and provide a potential target for treatment of SARS-CoV or other virus-mediated lung injury.

Authorship note: AL is decease

Conflict of interest: This work w
financially supported by grants fro
the US NIH RO1HL080211 and HH
TRS T11-706/18-N to ZC, the TNPRC
(base grant RR00164), RO1060699 to
SP, HMRF 16150662, and the University
Development Fund/Li Ka Shing Faculty
of Medicine Matching Fund of the
University of Hong Kong to its AIDS
Institute and Hong Kong RGV.

License: Copyright 2019, American
Society for Clinical Investigation.

Submitted: July 23, 2018
Accepted: January 11, 2019
Published: February 21, 2019

Reference information:
JCI Insight. 2019;4(4):e123158.
https://doi.org/10.1172/jci.
insight.123158.

Introduction

Severe acute respiratory syndrome coronavirus (SARS-CoV) causes fatal human respiratory disease (1–3). Patients with SARS (hereafter referred to as SARS patients) displayed the characteristics of acute lung injury (ALI), including diffuse alveolar damage (DAD), epithelial necrosis, and fibrin and hyaline deposition (4, 5). Most patients who die of SARS develop acute respiratory distress syndrome (ARDS), the most severe form of ALI (6, 7). Recent outbreaks of severe acute respiratory infections of emerging viruses, including Middle Eastern respiratory syndrome CoVs (MERS-CoV), highly pathogenic avian influenza viruses (e.g., H5N1 and H7N9), highlight the need to characterize the mechanisms responsible for virus-mediated ALI or ARDS.

Fundamental to ARDS is the acute onset of lung inflammation, which is intimately tied to monocyte/macrophage polarization and function (7, 8). Lung macrophages are highly plastic and heterogeneous cells that are resident in the lung interstitium and alveoli or recruited upon inflammatory stimuli. Inflammatory monocytes and resident tissue macrophages play critical roles in initiating and maintaining inflammation

during the acute stage of ARDS, as well as in the resolution phages of inflammation and recovery from ARDS. At steady state, resident macrophages are normally quiescent to prevent damaging the alveoli and are critically involved in normal tissue homeostasis. After tissue injury or during infection, resident macrophages become activated. Circulating monocytes can be efficiently recruited to the site of injury. Inflammatory monocytes/macrophages (IMMs) and resident macrophages undergo marked phenotypic and functional changes, and they can be classified into proinflammatory (M1 or classically activated) and inflammatory-resolving (M2, alternatively activated, wound-healing, or antiinflammatory) macrophages, with a continuum of macrophage polarization existing beyond these discrete categories (9). During acute infection, monocytes/macrophages often display a phenotype of classically activated macrophages. These cells mediate host defenses against viruses and also promote lung injury by producing nitric oxide (NO); ROS; IL-1, IL-6, and IL-8; and TNF. Simultaneously, some macrophages may become alternatively activated, exerting antiinflammatory function and regulating wound healing by producing matrix metalloproteinases (MMPs), growth factors, and antiinflammatory cytokines, particularly TGF-β. When the pathogen or inflammatory stimulus is eliminated, proinflammatory macrophages diminish. The predominant macrophage population assumes a wound-healing phenotype. At the final recovery stage, macrophages show a regulatory/suppressive phenotype, secreting increased levels of IL-10, which facilitates the resolution of wound healing and restores homeostasis. When the wound-healing response is well organized and controlled, the inflammatory response resolves quickly, and normal tissue architecture is restored. Disturbances in wound-healing response can lead to uncontrolled production of inflammatory mediators, contributing to a state of persistent injury (9–11). In patients who eventually died of SARS (hereafter referred to as deceased patients) and animal models, extensive lung damage is associated with high initial viral loads, increased IMM accumulation in the lungs, and elevated serum proinflammatory cytokine (IL-1, IL-6, IL-8, CXCL-10, and MCP1) levels (4, 5, 12, 13). While much is known about the terminal phase of SARS, little is known about the early immune events during the acute phase of infection. Studies defining macrophage heterogeneity and function during acute infection and ALI using nonhuman primate and patient specimens are limited, and the factors driving the hypercytokinemia and aggressive inflammation remain elusive.

During the SARS outbreak in Hong Kong, most patients (70%–80%) presented with abnormal chest radiographs, with approximately one-quarter of these individuals progressing to ALI. After 12 days, 80% of these SARS patients developed ARDS, coincident with IgG seroconversion (6). In a detailed analysis of antibody responses against SARS-CoV spike (S) glycoprotein, we reported that the anti-S neutralizing antibody (NAb) response developed significantly faster in deceased patients compared with recovered patients after the onset of clinical symptoms (14). It took an average of 20 days for the recovered patients to reach their peak of NAb activities, as opposed to only 14.7 days for the deceased patients. Moreover, the actual NAb titer is significantly higher in deceased patients compared with that in the recovered patients during the same time period (14). These findings suggest a role of anti-S antibodies in SARS-CoV–mediated ALI during acute infection. Consistently, preexisting serum antibodies against influenza antigens were found to associate with worse clinical severity and poor outcomes in patients during the 2009 influenza pandemic (15, 16). Moreover, multiple vaccine platforms and viral infection appeared to induce SARS-CoV–specific immune memory that enhanced lung inflammation following homologous challenge in mice and African green monkeys (17–19). The mechanism responsible for the immunopathologic reaction remains elusive. Recent studies suggested that T cells play a crucial role in protection of mice against lethal SARS-CoV infection (20–22). Enhanced pulmonary immunopathology in vaccinated and challenged animals reflects an inadequate Th1 response (22, 23). The role of virus-specific antibody responses in SARS-CoV–induced lung injury has yet to be clearly defined. Therefore, we used vaccination and anti–S-IgG passive immunization strategies to evaluate the effects of anti-S antibodies on SARS-CoV–induced ALI in Chinese rhesus monkeys (*Macaca mulatta*). Our results now show that, in productively infected lungs, anti–S–IgG causes severe ALI by skewing inflammatory resolving responses during acute infection.

Results

Vaccine-induced S-specific immunity resulted in severe ALI in SARS-CoV–infected Chinese macaques. We initially compared the pathological changes in the lungs of rhesus macaques vaccinated with a modified vaccinia Ankara (MVA) virus encoding full-length SARS-CoV S glycoprotein (ADS-MVA) or control at 7 and 35 days after pathogenic SARS-CoV$_{PUMC}$ (1×10^5 tissue culture infectious dose of 50% [$TCID_{50}$]) challenge (24). Three healthy macaques were included as controls. Sixteen macaques were i.m. immunized twice: 8 animals with

ADS-MVA and another 8 animals with a control MVA (ADC-MVA) (Figure 1A) (24). Vaccination with ADS-MVA induced high levels of anti–SARS-CoV NAbs in all 8 macaques, with sera IC_{50} values ranging from 10,232- to 28,703-fold dilutions (Figure 1B). None of the macaques developed fever during or after vaccination (normal body temperature 37.8°C–38.1°C). The IC_{50} values of these sera were maintained, with an over-2,000–fold reciprocal serum dilution at 4 weeks after the secondary immunization, a time when all of the animals were i.n. challenged (Figure 1B). After viral challenge, all macaques developed fever between 1 and 5 days after inoculation (dpi) ranging from 38.6°C–39.2°C, and sera from control vaccinated macaques showed increased neutralizing activity after 14 dpi, indicating establishment of infection (Figure 1B). SARS-CoV RNA was readily detected on oral swabs from all control macaques examined at multiple time points using real-time reverse transcription PCR (real-time RT-PCR) but only in 3 of the 8 ADS-MVA–vaccinated macaques with lower NAb titers at 2 dpi (SL11, SE13, and SL14) (Figure 1C), suggesting reduced productive viral infection in the immunized macaques through S-specific immunity.

However, histological examination revealed acute DAD with various degrees of severity in 6 ADS-MVA–vaccinated macaques at 7 and 35 dpi, whereas most control macaques in the ADC-MVA group showed minor to moderate inflammation (Figure 1D). To better characterize the pathological changes, we adopted a 6-grade scoring system to describe the severity of the lung damage from least severe to most severe: 0, –; 1, +; 2, ++; 3, +++; 4, ++++; and 5, +++++ (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.123158DS1). In the control vaccinated group, 5 macaques showed minor inflammation with slight septa broadening and inflammatory cells infiltration that we scored as + (CL23, CE25, CE20, CE21, and CL19; Figure 1D, Supplemental Figure 1B, and Supplemental Figure 2). Two macaques showed moderate inflammation with more interstitial mononuclear inflammatory infiltration and were scored as ++ (CL26, CE24; Supplemental Figure 1C and Supplemental Figure 2). Only 1 macaque showed typical symptoms of acute DAD with extensive exudation and cell infiltration at 35 dpi (CL22, scored as ++++; Supplemental Figure 2). In the ADS-MVA–vaccinated group, 4 macaques showed typical symptom of acute DAD with extensive exudation and many-cell infiltration in alveolar cavities (SL11, SE12, SL16, and SL18, scored as ++++; Supplemental Figure 1E and Supplemental Figure 2). One macaque showed severe acute DAD with exudation, hyaline membrane formation along the alveoli, pneumocyte desquamation, and damaged alveolus filled with hemorrhage and inflammatory cells (SL15, scored as +++++; Figure 1D). One macaque showed early symptom of acute DAD (SL14, scored as +++; Supplemental Figure 1D), and the remaining 2 macaques showed moderate inflammation (SE13 and SE17, scored as ++; Supplemental Figure 2). The comparison of lung histopathological scores of 2 groups showed significantly enhanced lung injury in the ADS-MVA–vaccinated group at both 7 and 35 dpi compared with the control ADC-MVA group, suggesting that S-specific— but not MVA-specific — immunity promotes ALI during SARS-CoV infection (Figure 1E). Moreover, there is a moderate correlation between lung pathological scores and sera NAb titers at 0 dpi (Figure 1F), suggesting a role of S-specific antibody in enhancing SARS-CoV–mediated lung injury.

Anti–S-IgG induced severe lung injury during acute SARS-CoV infection. In our previous study, i.n. inoculation of Chinese macaques with $SARS\text{-}CoV_{PUMC}$ (1×10^5 $TCID_{50}$) led to lower respiratory tract infection in all animals within 2 days (25). However, most of them rapidly cleared infection in the lungs, and all of the animals ($n = 8$) exhibited mild lung lesions before 3 dpi (25). Severe lung injury in SARS-CoV–infected Chinese macaques has not been detected until 7 dpi (26, 27). To determine the effect of anti-S antibody on the extent of SARS-CoV–mediated lung injury, we adoptively transferred 5 mg (low dose) and 200 mg (high dose) purified anti–S-IgG from ADS-MVA–vaccinated but unchallenged macaques into 2 groups of unvaccinated macaques ($n = 6$/group) via i.v. injection, and then i.n. challenged recipients with $SARS\text{-}CoV_{PUMC}$ (1×10^5 $TCID_{50}$) (Figure 2A). As controls, another 2 macaques were administered 200 mg of control IgG (C-IgG) derived from ADC-MVA–vaccinated macaques. We sacrificed half of the macaques in each group at 2 dpi to avoid potential disruption by virus infection–induced antibodies against nucleocapsid protein (N) and other viral proteins — and prior to when induction of ALI was typically observed. The remaining half of the macaques in each group was sacrificed at 21 dpi to evaluate long-term impact (Figure 2A). At day 2, sera from macaques that received low- or high-dose S-IgG showed neutralizing activity, with IC_{50} values ranging from 10–260 and 1,000–10,000, corresponding to the dose of sera transfer; macaques that received C-IgG failed to show neutralizing activity (Figure 2B and Supplemental Table 1). While NAb titers in the sera of macaques in the high-dose group declined steadily between 2 and 21 dpi, macaques in the low-dose and control groups displayed increased NAb titers over time (Figure 2B), suggesting active viral replication.

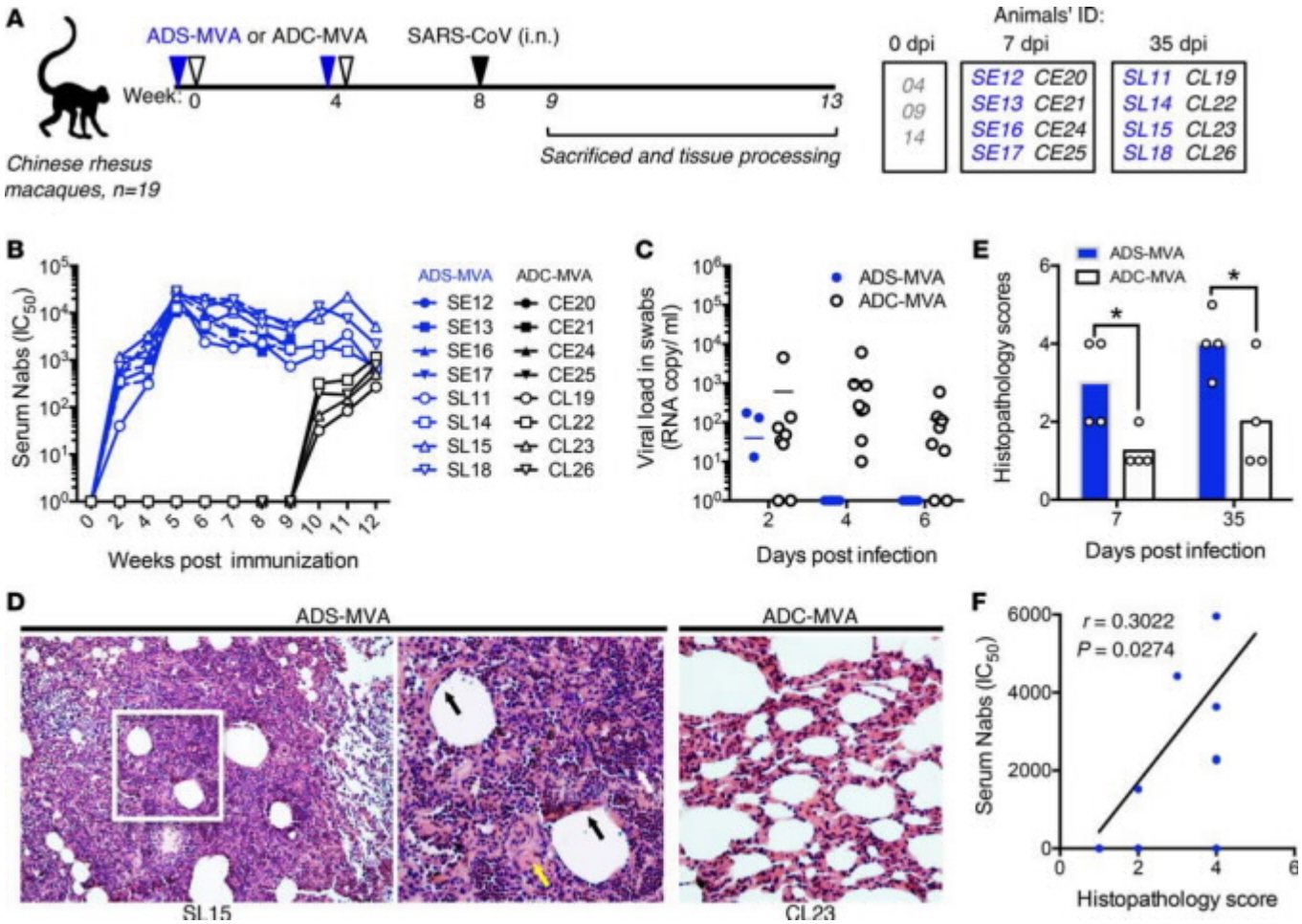


Figure 1. ADS-MVA–induced S-specific immune response enhanced pulmonary pathology in SARS-CoV–infected Chinese rhesus macaques. (**A**) imental design used to investigate the influence of S-specific immunity on SARS-CoV–induced lung injury. Two groups of Chinese rhesus macaques (*n* = 8/group) were subjected to i.m. injections of ADS-MVA or control vaccine ADC-MVA at weeks 0 and 4, followed by i.n. challenge with live pathogenic SARS-CoV_{PUMC} (1×10^5 $TCID_{50}$) at 4 weeks after the second vaccination. Four animals each were sacrificed at 1 and 5 weeks after inoculation. Three healthy macaques were included as controls. (**B**) Serum neutralizing activity. Sera collected from macaques were tested for a capacity to neutralize SARS-CoV pseudotype virus. (**C**) Detection of viral RNA in oral swabs. SARS-CoV RNA was detected by nested RT-PCR in the swabs at the indicated time points relative to infection. (**D**) Pathology changes of the lung tissue. Sections were stained with H&E. **D** shows symptom of acute DAD exhibited in 6 of 8 ADS-MVA–vaccinated macaques with extensive exudation (yellow arrow), hyaline membranes lining the alveolar walls (black arrows), and massive cell infiltration in alveolar cavities (white arrow). Left image shows a low magnification overview (100×). Middle image shows higher magnification of the boxed area in left image (200×). Right image shows minor inflammation observed in 7 macaques received ADC-MVA (*n* = 8) with slight alveolar septa broadening and sparse monocyte infiltration (original magnification, 100×). (**E**) Histopathological scores of the ADS-MVA group, including lung samples collected at both 7 and 35 dpi, were compared against the ADC-MVA control group. See Supplemental Figure 1 for the scoring index based on severity of lung histopathology. Data represent mean ± SEM values. Statistical analysis was undertaken using 2-tailed unpaired Student's *t* test. $^*P < 0.05$, *n* = 4. (**F**) Correlation of lung histopathological scores of all macaques with sera NAb titers at 0 dpi. Solid lines denote the relationship between histopathology scores and serum neutralizing activity. Statistical analysis was performed using Spearman's rank correlation test.

Indeed, SARS-CoV RNA was detected using real-time RT-PCR on oral swabs, and/or virus was recovered from lung tissue in both control animals and 5 of 6 animals (83%) in the low-dose group. Only 2 of 6 animals (33%) in the high-dose group were viral RNA^+ (Figure 2C and Supplemental Table 1), suggesting reduced viral production by high-dose S-IgG.

Consistent with the findings in the control ADC-MVA–vaccinated group, histopathological examination of the lungs indicated minor and moderate inflammation at day 2 and 21 in C-IgG recipients, respectively. In contrast, all S-IgG recipients exhibited symptoms of acute DAD with various degrees of exudation, hyaline membrane formation, and hemorrhage and inflammatory cells within alveolus at both day 2 and 21 (Figure 2, D and E, and Supplemental Table 2). Of note, despite the presence of high titer sera NAbs during the chronic stage of infection (Figure 1B and Figure 2B), only 1 of 5 C-IgG and control

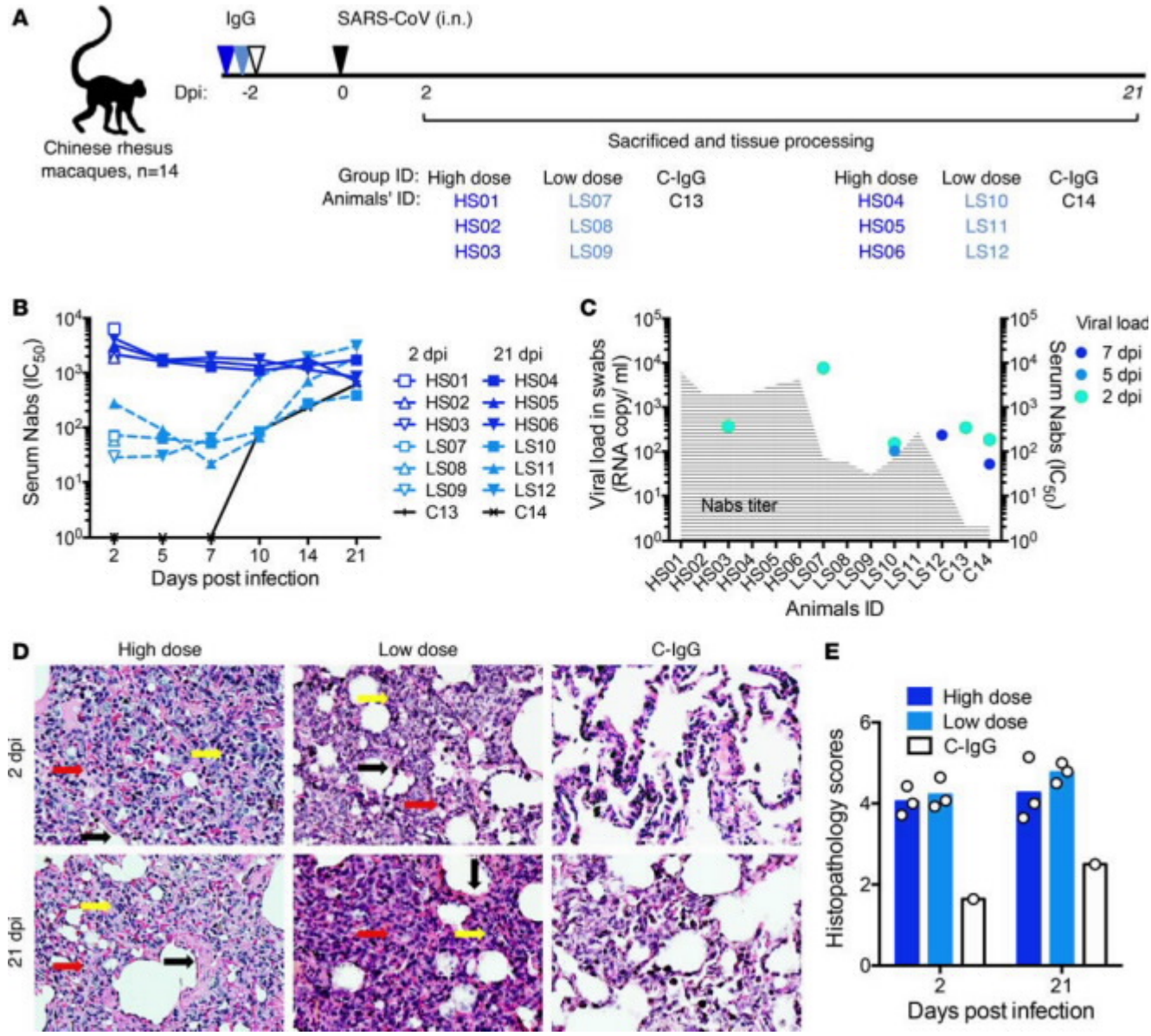


Figure 2. Anti-spike antibodies induced ALI in SARS-CoV–infected Chinese rhesus macaques. (**A**) Experimental design used to investigate the influence of S-IgG on SARS-CoV–induced lung injury. Two groups of Chinese rhesus macaques (*n* = 6/group) were subjected to i.v. injection of high-dose (200 mg) or low-dose (5 mg) purified S-IgG from ADS-MVA–vaccinated but unchallenged macaques. As controls, another 2 macaques were administered 200 mg of C-IgG derived from ADC-MVA–vaccinated macaques. After 2 days, 3 groups of macaques were challenged i.n. with SARS-CoV (10^6 $TCID_{50}$). Half of the animals from each group were sacrificed at 2 and 21 dpi. Sera (**B**) from macaques at the indicated time points were tested for the capacity to neutralize SARS-CoV pseudotype virus. (**C**) Detection of viral RNA in oral swabs. SARS-CoV RNA was detected by nested RT-PCR in the swabs from one of the high-dose S-IgG–treated macaques (*n* = 6), 3 low-dose S-IgG–treated macaques (*n* = 6), and 2 C-IgG–treated macaques (*n* = 2) at the indicated time points. Left *y* axis shows the viral RNA copy number per milliliter swab. Right *y* axis shows the serum NAb titers of each macaque at 2 dpi, which are highlighted by shaded area. (**D**) Pathology changes of the lung tissue (200×). Sections from macaques were stained with H&E. Images show symptom of acute DAD exhibited in macaques received high-dose and low-dose S-IgG (*n* = 2) with extensive exudation (red arrows), hyaline membranes (black arrows), and massive cell infiltration (yellow arrows) at 2 and 21 dpi. Right panel shows minor and moderate inflammation in the macaques received C-IgG with slight alveolar septa broadening and sparse monocyte infiltration at 2 and 21 dpi. (**E**) Histological scores of the high-dose and low-dose S-IgG groups, including lung samples collected at both 2 and 21 dpi, were compared against the C-IgG group. See Supplemental Figure 1 for the scoring index based on severity of lung histopathology.

ADC-MVA vaccine recipients showed acute DAD (Figure 1E and Figure 2E), indicating a time-restricted role for S-IgG, which mostly causes severe ALI in acutely infected macaques. Therefore, we conclude that, despite viral suppression, the presence of S-IgG at the acute stage of SARS-CoV infection caused severe ALI that persists until late stages.

S-IgG failed to prevent SARS-CoV lower respiratory tract infection and amplified IMM infiltration and accumulation in the lungs. To determine the potential cause of S-IgG–enhanced ALI, we measured viral infection and monocytes/macrophages infiltration in the lungs and serum cytokine profile at 2 dpi. We previously found that pulmonary infection of Chinese macaques could be rapidly controlled. Viral RNA$^+$ cells were detectable in the lungs of only 2 of 4 animals at 2 dpi, although all of them were viral nucleoprotein-positive (NP$^+$) in the lungs and the hilar lymph nodes (LNs), where the lymphatics of the lungs drain (25). Therefore, we measured viral RNA$^+$ cells by in situ hybridization (ISH), as well as NP signals in the lungs and the hilar LNs by IHC staining to evaluate the actual pulmonary infection of each macaque. Consistent with the results by viral isolation (Supplemental Table 1), viral RNA was detected in pneumocytes of 1 macaque in the high-dose (HS02, 33%), 2 macaques in the low-dose (LS01, LS02; 66%), and the C-IgG–treated macaque (Figure 3A and Supplemental Table 3), suggesting reduced productive infection by S-IgG. However, an NP antibody identified positive signals in the lungs and hilar LNs of all S-IgG and C-IgG recipients (7 of 7 macaques) but not healthy controls (Figure 3B and Supplemental Table 3), suggesting that S-IgG failed to prevent SARS-CoV entry, and SARS-CoV established lower respiratory tract infection in all of the challenged animals.

Consistent with previous findings in SARS-CoV–inoculated naive Chinese macaques (n = 4) (25), double-immunostaining with the NP antibody and antibodies specific to the stages of macrophage differentiation and inflammation (MAC387, CD68, CD163) revealed significant infiltration of IMMs in the lungs of C-IgG recipients, consisting of newly infiltrated (MAC387$^+$) and inflammatory (CD163$^+$) monocytes/macrophages (Figure 3C and Supplemental Figure 3). The CD163$^+$ cells greatly exceeded the CD68$^+$ population and typically gathered around NP$^+$ cells to form cell clusters (Figure 3C), showing an association between viral infection and IMM infiltration in these macaques.

Compared with C-IgG recipients, more robust signals for MAC387 and CD163 were identified in the lungs of S-IgG recipients (Figure 3C and Supplemental Figure 3), suggesting enhanced IMM infiltration by S-IgG. MAC387$^+$ and CD163$^+$ monocytes/macrophages not only gathered around NP$^+$ cells, but also formed clusters in tissues with little NP signal (Figure 3C), suggesting that infection-induced recruitment from circulation continued after viral clearance and accumulated at the inflamed site. Moreover, more-concentrated CD68$^+$ signals were found, indicating altered macrophages functional response by S-IgG treatment (Figure 3C). Consistently, elevated serum levels of IL-8 were observed in macaques treated with high-dose S-IgG at 2 dpi (Supplemental Figure 3), and the IL-8 levels are strongly correlated with anti–SARS-CoV NAb titers (Supplemental Figure 3). IL-8 is a macrophage-derived inflammatory cytokine important in initiating ALI. These results, therefore, suggest that S-IgG likely altered macrophage functional response in the lungs during acute infection, resulted in increase in IL-8 production, and enhanced ALI and IMM infiltration and accumulation.

Alveolar monocytes/macrophages assumed wound-healing function as early as 2 dpi in macaques not treated with S-IgG. Macrophage activation is dynamic and plastic. Different monocyte/macrophage activation statues have different specialized and critically timed roles, taking part in either the initiation and maintenance or resolution of inflammation (9). A well-organized wound-healing response leads to timely resolution of inflammation, whereas disturbance in wound-healing response results in persistent inflammation and injury. To understand the S-IgG–caused excessive inflammation, we further defined macrophage heterogeneity and activation statues in the lung lesions of C-IgG (n = 1) and S-IgG recipients (n = 3) at 2 dpi. Three healthy macaques and 4 naive Chinese macaques challenged with the same dose of SARS-CoV$_{PUMC}$ and scarified at 2 dpi from a previous study were included as controls (25).

By combining 5 macrophages markers (MAC387, CD68, CD163, HAM56, and CD206) (28–30), 2 subpopulations of resident macrophages were shown in the lungs of Chinese rhesus macaques at steady state as previously described (29). They are interstitial macrophages (IMs; MAC387$^+$HAM56$^-$CD206$^-$) (Figure 4A) and resident alveolar macrophages (AMs; CD68$^+$ HAM56$^-$) expressing low levels of CD163 and CD206, a marker specific to the macrophage wound-healing response, corresponding to their intrinsic antiinflammatory function (Figure 4B) (29). In lung lesions of both C-IgG–treated and naive macaque groups challenged with SARS-CoV$_{PUMC}$, IMMs were further characterized and divided into 2 subpopulations. One subpopulation was the recently recruited MAC387$^+$HAM56$^-$CD68$^-$ monocytes (Figure 4C) (29), which is like IM but is larger in size and primarily coexpressed CD163 (Supplemental Figure 4). The other subpopulation was single CD163$^+$ inflammatory macrophages that had differentiated from newly recruited MAC387$^+$ monocytes/macrophages (Figure 4D and Supplemental Figure 4) (29). Importantly, resident

AMs (CD68$^+$) differentiated into HAM56$^+$ cells, with a slight increase in numbers and increased size, with stronger CD163 and — more importantly — CD206 staining, suggesting that they are alternatively activated (Figure 4D). Strikingly, elevated levels of TGF-β expression were detected in all MAC387$^+$ — and most enlarged CD163$^+$ — as well as CD68$^+$ macrophages in the lungs of both C-IgG–treated and unvaccinated macaques compared with healthy controls (Figure 4, G–I), indicating that many IMMs and activated resident AMs have assumed a wound-healing function in infected lungs within 2 days of infection.

S-IgG treatment skewed wound-healing macrophage response in the lungs during acute SARS-CoV infection. Compared with the macaques not treated with S-IgG, the number of IMMs (MAC387$^+$ and CD163$^+$HAM56$^-$) significantly increased in S-IgG recipients (Figure 4, E and F). Moreover, many recently recruited MAC387$^+$HAM56$^-$ monocytes/macrophages did not coexpress CD163 (Supplemental Figure 4), representing the newest recruited blood monocytes, as a result of enhanced monocytes infiltration by S-IgG. Importantly, the majority of CD163$^+$HAM56$^-$ IMMs coexpressed CD68 (Supplemental Figure 4), suggesting a change of function of this subpopulation by S-IgG. Moreover, resident macrophages (CD68$^+$HAM56$^+$) in the lungs of S-IgG recipients no longer express CD206, which — being surrounded by many CD163$^+$ IMMs (Figure 4F) — suggests a loss of wound-healing function and a role in recruiting blood monocytes. Consistently, S-IgG–treated macaques displayed a loss of TGF-β and high expression of IL-6 in all macrophage subpopulations (Figure 4, G, H, and J). IL-6 favors macrophage accumulation at the site of injury and is important for the development of persistent inflammation and ALI (31, 32). Therefore, we conclude that, while AMs underwent phenotypic and functional changes in acutely infected lungs, many monocytes/macrophages assumed a wound-healing function for inflammation resolution, but the presence of S-IgG skewed the wound-healing response, leading to uncontrolled inflammation and tissue injury.

Onset of an antibody response prior to viral clearance is associated with abrogated wound-healing responses and increased IMM lung infiltration in unvaccinated Chinese rhesus macaques. To understand the mechanism underlying the time-restricted role of S-IgG in promoting ALI, we conducted a temporal analysis of the productive viral infection, antibody response, and macrophage functional changes in unvaccinated Chinese macaques during the first week of infection. Naive macaques were challenged i.n. with 1×10^6 $TCID_{50}$ $SARS\text{-}CoV_{PUMC}$ and sacrificed at 2, 3, and 7 dpi (4 macaques/group) (Figure 5A) (25). Successful lower respiratory tract infection was confirmed by detection of viral NP using IHC staining in the lungs of all macaques (25). Viral RNA$^+$ alveolar pneumocytes were primarily found at 2 dpi (2 of 4 macaques), and infrequently at 3 dpi (0 of 4 macaques; Figure 5B and Supplemental Table 4) (25). At 7 dpi, viral RNA was detectable in the lungs of 2 macaques, AD0516 and AD0518, suggesting productive infection there (Figure 5, B and C). Only few scattered NP$^+$ cells were found in AD0517 (Figure 5D). Neither NP nor viral RNA was found in the lungs or swabs of AD0515, suggesting clearance of productive viral pulmonary infection in this macaque (Figure 5D and Supplemental Table 4). Serum NAb was not observed at 2 and 3 dpi until 7 dpi in 2 of 4 macaques, AD0515 and AD0516 (Supplemental Table 4 and Figure 5E).

In agreement with the findings in the C-IgG group, temporal analysis of macrophage heterogeneity revealed significantly increased number of IMMs (CD163$^+$CD206$^-$) in viral RNA$^+$ lungs at 2 dpi (Figure 5F, right image). The IMMs diminished at 3 dpi (4 of 4 macaques) (Figure 5G) and became nearly completely absent at 7 dpi in macaques without productive pulmonary viral infection (2 of 4 macaques, AD0515 and AD0517) (Figure 5H), indicating that inflammatory response resolved with 7 days following the control of productive infection. Critically, wound-healing macrophages (CD163$^+$CD206$^+$ and CD163$^+$TGF-β$^+$) were observed in the lungs of all macaques at 2 dpi, despite productive pulmonary viral infection in macaques AD0506 and AD0508 (Figure 5F). In contrast, macaque AD0516, with serum NAbs and productive virus infection in the lungs, displayed a significantly enhanced proinflammatory macrophage response and reduced inflammatory-resolving macrophages (Figure 5I), which was not observed in macaques with productive viral infection but undetectable NAb responses (AD0518) or in macaques with detectable NAbs but undetectable viral replication (AD0515) (Figure 5D and Supplemental Table 4). Therefore, persistent viral replication and concomitant NAb detection is associated with increased IMM lung infiltration and reduced wound-healing macrophage numbers.

S-IgG modifies functional response of alternatively but not classically activated macrophages to SARS-CoV infection. To determine whether S-IgG skews wound-healing response through modifying wound-healing macrophage functional response during SARS-CoV infection, we treated in vitro–polarized alternatively activated human monocyte–derived macrophages (MDM) for 20 hours with SARS-CoV pseudovirus and heat-inactivated sera of high-dose S-IgG–treated macaques (S-IgG sera) collected at 2 dpi. We

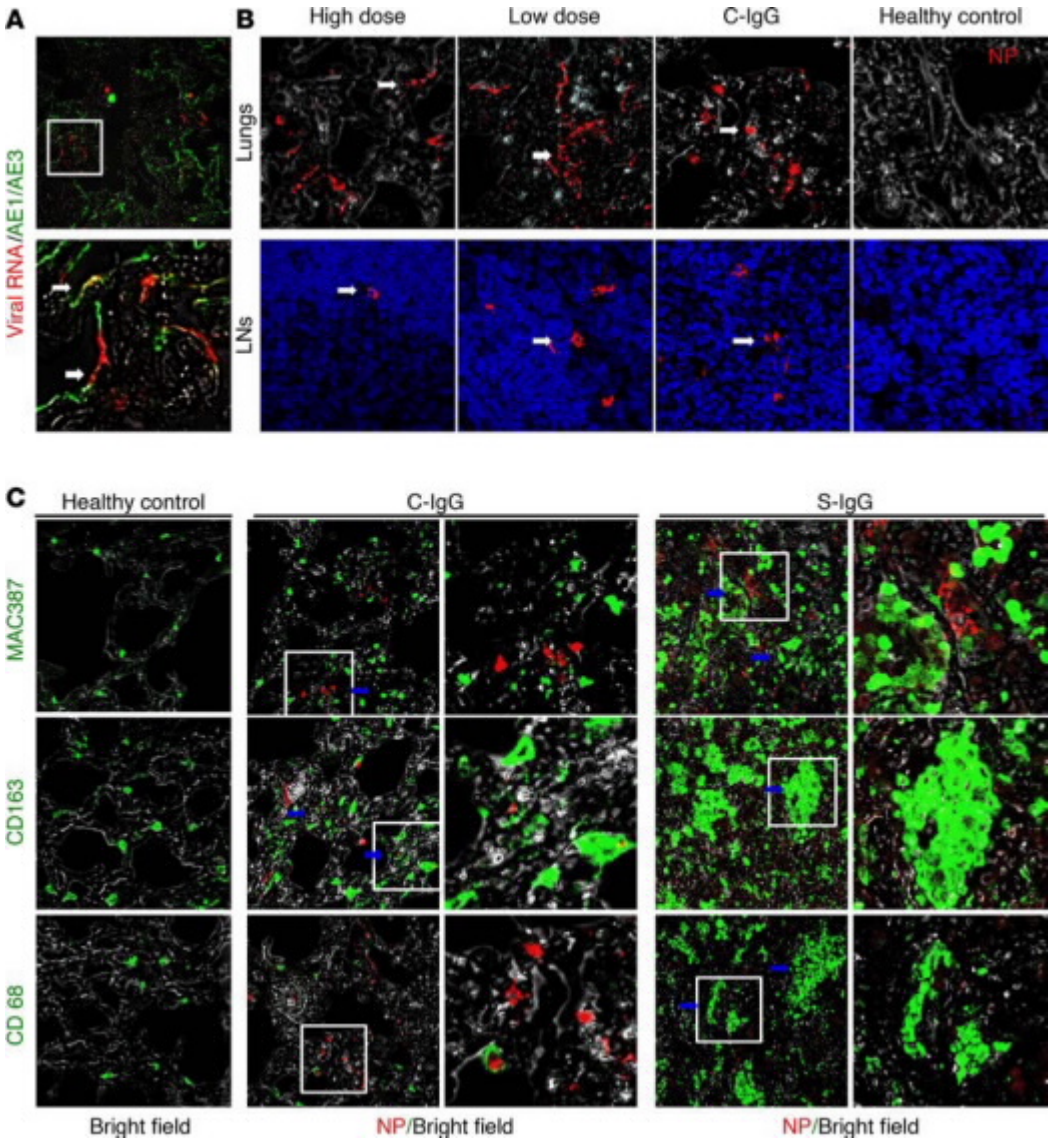


Figure 3. SARS-CoV infection and monocytes/macrophages infiltration in the lungs of C-IgG– and S-IgG–treated macaques at 2 dpi. (**A**) Represen images of SARS-CoV RNA (TRITC) and AE1/AE3 (FITC) cells in the lungs of infected macaques (white arrows). The upper photo shows a low magnification overview (200×); the bottom photo shows the boxed area in the upper photo. (**B**) Representative images of viral protein (NP) immunostaining of the lungs and hilar lymph nodes (LN) of 7 infected and 3 uninfected animals (TRITC, white arrows) (original magnification, 200×). (**C**) Representative images of viral NP and monocytes/macrophages. These sections showed significantly increased IMMs in the lungs of the S-IgG group compared with the C-IgG group (MAC387, CD163, or CD68, blue arrows). Tissue samples are double-immunostained for the SARS-CoV NP (TRITC) and markers for macrophages, including MAC387 (FITC), CD163 (FITC), and CD68 (FITC). Within the panel of representative images for the C-IgG and S-IgG groups, the left panel shows low magnification overview; the right panel shows the boxed area in the left panel (original magnification, 200×).

then measured the concentrations of 13 inflammatory cytokines in cell culture supernatants, including IL-1β, IFN-α, IFN-γ, TNF-α, MCP1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33. Sera from C-IgG recipients or healthy macaques were included as controls. In vitro–polarized classically activated MDM was also studied and compared with alternatively activated MDM in parallel. Consistent with previous report, unstimulated classically activated MDM produced high levels of IL-8 and IL-6; alternatively activated MDM only produced minimal levels of those cytokines (Figure 6A). Addition of SARS-CoV pseudovirus induced IL-8 production in alternatively activated MDM to levels comparable with those found in unstimulated, classically activated MDM, as well as in low-level MCP1 and IL-6 production (Figure 6B). By contrast, SARS-CoV pseudovirus caused less than a 1-fold increase in IL-8 secretion, but not other cytokine secretion, by classically activated MDM (Figure 6C). Strikingly, while none of the sera samples induced cytokine production when they were administered into MDMs alone,

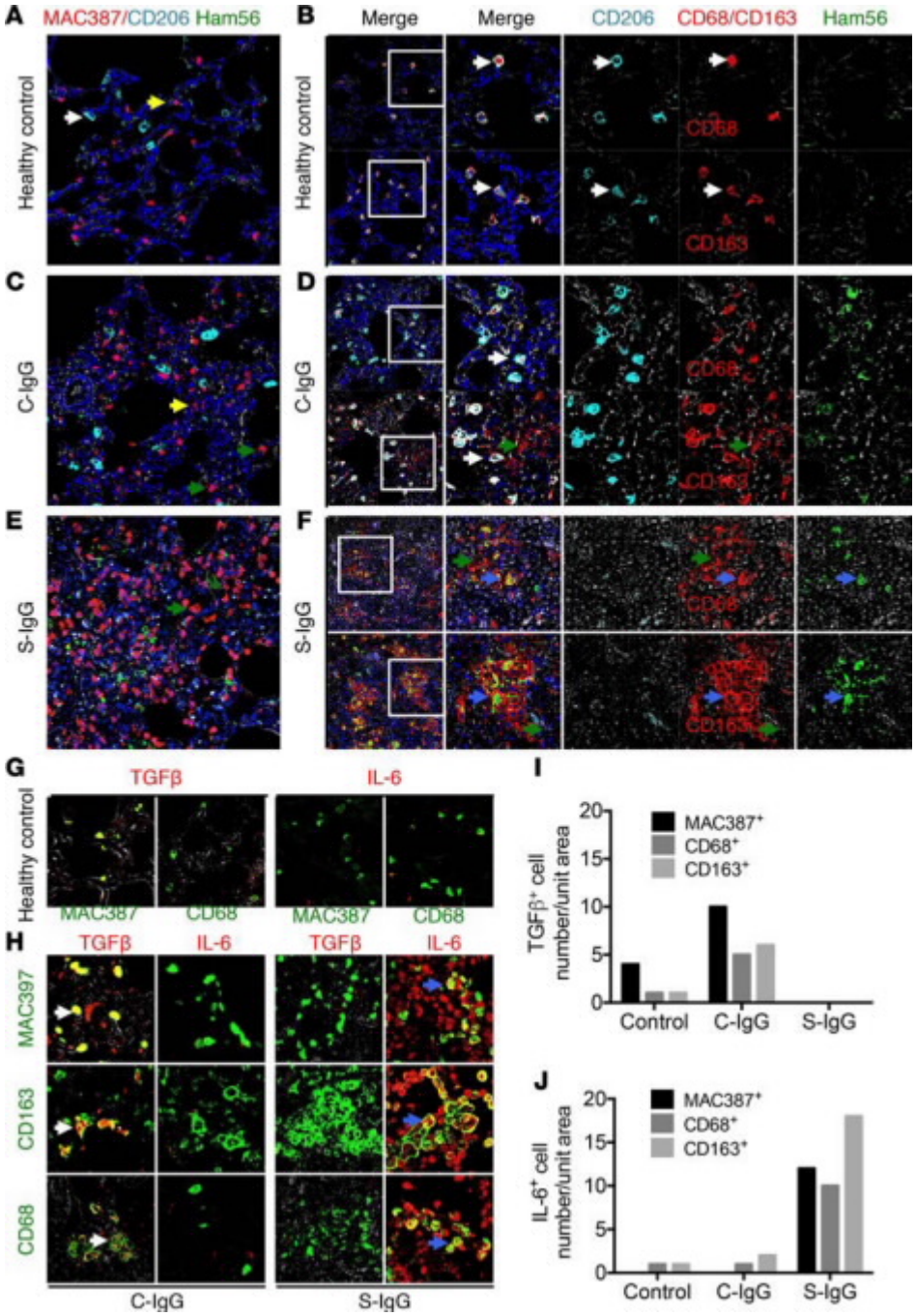


Figure 4. Comparison of monocyte/macrophage phenotype and function in the lungs of S-IgG– and C-IgG–treated macaques. (**A**–**F**) Phenotype of monocytes/macrophages subpopulations in the lungs of healthy, C-IgG–, and S-IgG–treated macaques (original magnification, 200×). These sections were triple-immunostained with antibodies for CD206 (cyan), HAM56 (FITC), and MAC387 (TRITC) (**C**, **E**, and **A**); CD206 (cyan), HAM56 (FITC), and CD68 (TRITC) (upper panel, **D**, **B**, and **F**); or CD163 (TRITC) (lower panel, **D**, **B**, and **F**). **A** and **B** show 2 subpopulations at steady states, including interstitial macrophages (IM) (MAC387$^+$HAM56$^-$CD206$^+$) (yellow arrow), and resident alveolar macrophages (AM) (CD68$^+$CD163$^+$CD206$^+$HAM56$^-$) (white arrows). **C** and **D** show the presence of alternatively activated resident AM and accumulated IMMs in the C-IgG group. Alternatively activated AMs are CD206$^+$CD68$^+$CD163$^+$HAM56$^-$ (**D**, white arrows). IM are MAC387$^+$HAM56$^-$CD206$^+$ (**C**, yellow arrow). IMMs include newly infiltrating monocytes/macrophages (MAC387$^+$) (**C**, green arrow); and inflammatory macrophages (CD68$^+$CD206$^-$HAM56$^-$) (**D**, green arrow). **E** and **F** show significantly increased number of IMMs and decreased number of alternatively activated macrophages in the S-IgG group. IMMs include newly infiltrating monocytes/macrophages (MAC387$^+$) (**E**, green arrow) and inflammatory macrophages that are CD68$^+$HAM56$^-$ (green arrow, **F**, upper panel) and CD163$^+$CD206$^+$HAM56$^-$ (green arrow, **F**, lower panel). Resident AMs are no longer expressing CD206$^+$ (CD68$^+$CD163$^+$CD206$^-$) (blue arrow, **F**, upper panel). (**G**–**J**) TGF-β and IL-6 expression in macrophages in the lungs. These sections were double-immunostained with antibodies for TGF-β (TRITC) and MAC387, CD163, or CD68 (FITC). (**G**) shows low expression levels of TGF-β and IL-6 in IMs (MAC387$^+$) and AMs (CD68$^+$) in healthy macaques. **H** shows increased TGF-β expression in the C-IgG group (white arrows) but increased IL-6 in the S-IgG group (blue arrows). (**I** and **J**) show the numbers of TGF-β$^+$ and IL-6$^+$ monocytes/macrophages in 3 groups in a 200× field.

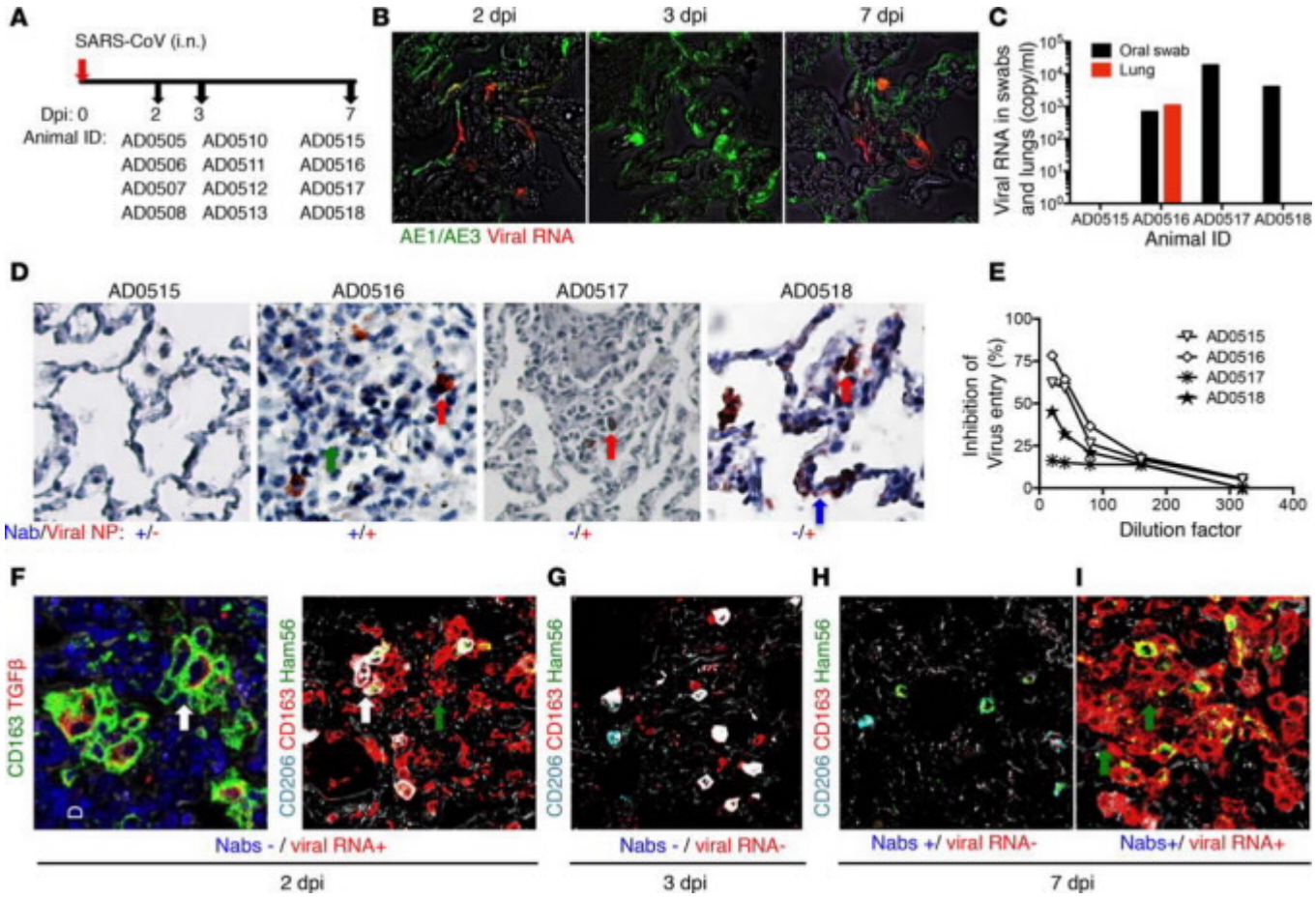


Figure 5. Dynamics of the viral replication, antibody response, and macrophage activation during acute SARS-CoV infection. (**A**) Experimental scheme. A total of 12 Chinese rhesus macaques were challenged i.n. with SARS-CoV ($\times 10^{6}$ $TCID_{50}$). Four animals each were sacrificed at 2, 3, and 7 dpi. (**B**) SARS-CoV RNA detection by ISH in the lungs at 2, 3, and 7 dpi (original magnification, 200 ×). Viral RNA in type I pneumocytes (FITC) was found at 2 dpi ($n = 2$) and 7 dpi ($n = 1$). (**C**) Viral RNA in the swabs and lung homogenates on 7 dpi. SARS-CoV RNA was detected using nested RT-PCR in swabs from 3 macaques (AD0516, AD0517, and AD0518) and lung homogenates from macaque AD0516 and AD0518. (**D**) Alveolar antigen and inflammatory infiltrates in the lungs at 7 dpi (200×). These sections were stained for SARS-CoV NP (red) and nucleus (blue) by IHC, showing NP signal in macrophage-like cells in AD0516 and AD0517 (red arrows), and type I pneumocytes in AD0518 (blue arrows), but substantial inflammatory infiltrates were only observed in AD0516 (green arrows). (**E**) Serum neutralizing activity. Serum neutralizing activity was detected in macaques AD0515 and AD0516 at 7 dpi. (**F**–**I**) Decreased wound-healing response in macaques with productive pulmonary viral infection and serum neutralizing activity (200×). These sections were double-immunostained for TGF-β (TRITC) and CD163 (FITC) or triple stained for CD206 (cyan), HAM56 (FITC), and CD163 (TRITC). The figure shows that wound-healing response took place within 2 dpi, with alternatively activated monocytes/macrophages (TGF-β, CD206, white arrows) and IMMs (CD163, CD206, green arrows) coexisting in the lungs (**F**). After viral clearance, IMMs diminish at 3 dpi (**G**), and the homeostasis was restored at 7 dpi (AD0515) (**H**). When pulmonary viral infection persists, IMM (CD163, CD206, green arrows) infiltration and accumulation is enhanced, and wound-healing macrophages (CD206) are reduced in macaques that have faster NAb response (AD0516) (**I**).

administration of S-IgG sera caused a dose-dependent increase in the production of all 3 inflammatory cytokines in SARS-CoV–stimulated alternatively activated MDM, resulted in a greater than 10-fold increase in IL-6, an 8-fold increase in MCP1, and a 5-fold increase in IL-8 (Figure 6, D and E). This enhancement, however, was not observed when S-IgG sera were replaced with sera from C-IgG recipients or healthy macaques (Figure 6, F, G, and H), suggesting that S-IgG, but not other sera components, enhanced SARS-CoV–stimulated cytokine production by alternatively activated MDM. Administration of S-IgG sera had no effect on cytokine production by SARS-CoV–treated classically activated MDM (Figure 6, I, J, and K), indicating that S-IgG do not modulate virus-mediated classically activated macrophage functional response. MCP1 and IL-8 are key cytokines that regulate monocytes/macrophages migration and promote neutrophils infiltration, while IL-6 promotes persistent inflammation and injury. These results suggest that, in response to SARS-CoV infection, wound-healing macrophages produce IL-8 and low amounts of MCP1 to recruit neutrophils and blood monocytes, whereas the presence

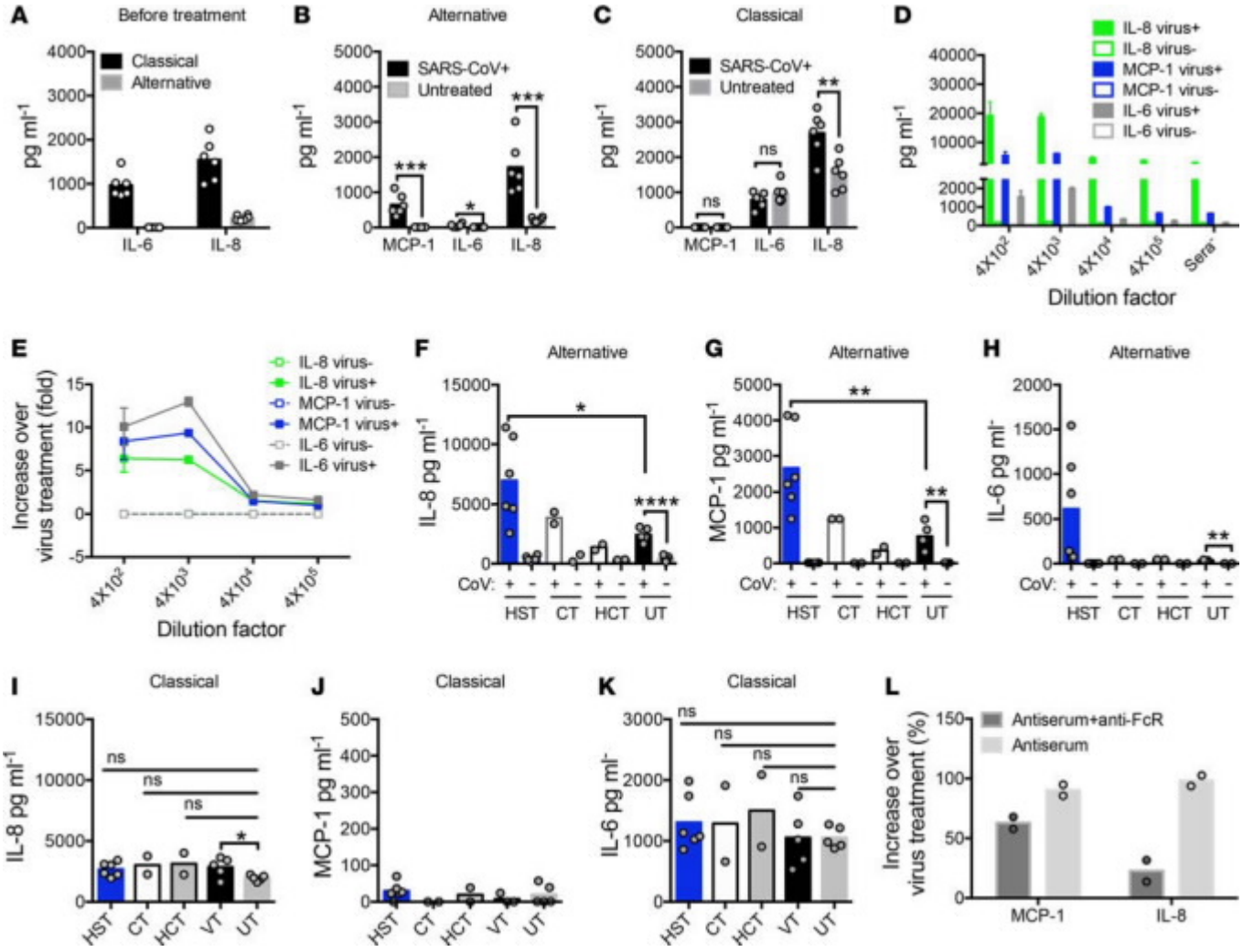


Figure 6. S-IgG significantly amplified proinflammatory cytokine production by SARS-CoV–treated alternatively activated macrophages. In vit polarized alternatively activated macrophages were either left unstimulated or were incubated with SARS-CoV pseudovirus alone or cocultured with SARS-CoV pseudovirus and diluted sera from the high-dose S-IgG group (n = 6) or C-IgG group (n = 2) collected at 2 dpi or from healthy macaques (n = 2) for 20 hours. Secreted cytokine and chemokine levels were measured in the culture supernatant and are shown in data represented as a column graph (**A–D** and **F–K**) and fold or percentage over supernatants from macrophages treated with virus alone and (**E**). **A** shows the levels of IL-8 and IL-6 in the supernatant of classically activated and alternatively activated MDM before treatment and **B** and **C** show that SARS-CoV treatment induced MCP1, IL-8, and very low levels of IL-6 production by alternatively activated MDM, as well as enhanced IL-8 production by classically activated MDM. **D** and **E** show that sera from S-IgG recipients caused dose-dependent increase in cytokine production from SARS-CoV–treated alternatively activated MDM. **F–H** show that virus treated alternatively activated MDM (VT), but not sera treated or untreated cells (UT), secreted proinflammatory cytokines IL-8 (**F**), MCP1 (**G**), and very low levels of IL-6 (**H**). Addition of sera (1:4,000 dilutions) from high-dose S-IgG (HST), but not C-IgG treated macaques (CT) or healthy controls (HCT), significantly amplified IL-8 (**F**), MCP1 (**G**), and IL-6 (**H**) production. **I–K** show that addition of sera (1:4,000 diluted) from high-dose S-IgG–treated (HST), C-IgG treated macaques (CT), or healthy controls (HCT) had no effect on MCP-1 (**J**), and IL-6 (**K**) production by SARS-CoV–treated classically activated MDM. **L** shows that blockade of FcγR significantly reduced antiserum-enhanced IL-8 secretion and partially reduced MCP1 secretion. Data represent mean values or mean values ± SEM of at least 3 independent experiments. Two-tailed unpaired Student's t test was used for statistical analysis. *P ≤ 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

of S-IgG can result in 5- to 10-fold increases in IL-8, MCP1, and IL-6 production by wound-healing macrophages, leading to significantly enhanced and persistent monocytes infiltration and tissue injury. Critically, in the presence of Fcγ receptor (FcγR) blocking antibody, IL-8 was significantly reduced when cells were treated with SARS-CoV pseudovirus and sera from high-dose S-IgG–treated macaques (diluted 1:4,000), indicating that anti–SARS-CoV antibody modifies wound-healing response partially through FcγRs (Figure 6L). Therefore, we conclude that S-IgG directly modifies functional response of alternatively activated macrophages during acute infection, thus skewing wound-healing response, leading to hypercytokinemia, aggressive inflammation and severe lung injury.

Deceased SARS patients displayed higher levels of serum NAbs, accumulated proinflammatory macrophage infiltration, and absence of wound-healing macrophages in the lungs. To examine the role of S-IgG in promoting ALI in a clinical setting, we investigated antibody responses during the early stage of infection in 2 groups of deceased patients or individuals recovered from SARS and characterized the heterogeneity of monocytes/macrophages in the lungs of 3 deceased SARS patients. H&E staining of lung specimens derived from 3 deceased SARS patients revealed severe ALI (Figure 7A). Consistent with the macaque findings, IHC analysis revealed significant infiltration of $CD163^+$ monocytes/macrophages into the lungs of patients, with little TGF-β expression and loss of the wound-healing marker $CD206^+$, indicating predominantly proinflammatory macrophage activation and reduced inflammatory-resolving response (Figure 7B). Moreover, significantly higher levels of anti-S NAbs were detected in the sera of deceased patients (n = 6) during acute infection compared with recovered patients (n = 8) (Figure 7C). These results, therefore, are consistent with findings in experimental macaques.

Antisera from deceased SARS patients skewed wound-healing response during SARS-CoV infection partially through the FcγR. To examine whether human anti–SARS-CoV antibody modifies wound-healing response during SARS-CoV infection, we treated in vitro–polarized alternatively activated MDM with SARS-CoV pseudovirus for 20 hours in the presence of sera collected from SARS patients during acute infection. Macrophages treated with SARS-CoV pseudovirus or sera alone were included as controls. We then examined the protein levels of cytokines/chemokines in the cell culture supernatant. Consistent with findings in macaques, administration of sera from a deceased SARS patient (D1; Supplemental Table 5) into SARS-CoV–treated alternatively activated MDM caused a dose-dependent increase in the production of cytokines, leading to a 2- to 3-fold increase in MCP1 and IL-8 after 20 hours (Figure 8, A and B). This enhancement, however, was not observed in cells treated with sera alone (Figure 8, A–D). Administration of sera from recovered patients (n = 8; Supplemental Table 5) had no effect on cytokine production except 1 sample (Figure 8, C and D). These differences may be explained, at least in part, by their lower NAb titers, although it is also possible that sera from recovered patients contain different antibody populations. Consistently, the only serum sample — R2, which enhanced chemokine production — has a relatively higher NAb titer among the samples tested (Supplemental Table 5). Moreover, statistical analysis shows that IL-8 production strongly correlates with NAb titer of sera from deceased patients (Figure 8E). Furthermore, IL-8 production was significantly reduced when cells were treated with SARS-CoV pseudovirus and sera from deceased SARS patient (diluted 1:4,000) in the presence of FcγR blocking antibody, indicating that anti–SARS-CoV antibody modifies wound-healing response in humans partially through FcγRs (Figure 8F).

Discussion

Here, we present evidence of a detrimental role of anti–S-IgG in ALI during SARS-CoV infection. Respiratory CoVs infection poses a unique challenge to the immune system: not only must the virus be rapidly eliminated, but lung inflammation must also be controlled to prevent acute respiratory failure (33). In the present study, we show that, despite markedly reducing virus titers, anti–S-IgG caused lung injury during the early stages of infection by abrogating a wound-healing macrophage response and TGF-β production, while promoting proinflammatory cytokine IL-8 and MCP1 production and inflammatory macrophage accumulation. To our knowledge, our data demonstrate a previously unrecognized mechanism underlying virus-mediated ALI and suggest that modulation of the anti-S antibody response or blockage of Fcγ receptors during acute infection might be needed for effective treatment for respiratory CoV infection.

Many studies have demonstrated the role of NAbs induced by the S glycoproteins in protecting viral replication in susceptible hosts, including mice, ferrets, hamsters, and macaques (24, 34, 35). However, the effect of S-specific immunity in protecting against pulmonary immunopathology has been controversial. In some cases, vaccination-induced immunity assists the viral clearance and protects mice or ferrets against lethal challenge (36–39), whereas in many other situations, multiple vaccine platforms appear to induce increased eosinophilic proinflammatory pulmonary response upon challenge (18, 23, 40). These differences may be explained, in part, by the characteristics of the vaccine being studied, the infectious dose, and the viral strain employed, as well as by the type or quality of immune response elicited. Recent studies suggest that $CD8^+$ T cell response plays a crucial role in viral clearance and protection of mice against eosinophilic immunopathology and lethal SARS-CoV infection, whereas immunopathology predominantly reflects an inadequate vaccine-induced Th1 response (20–23).

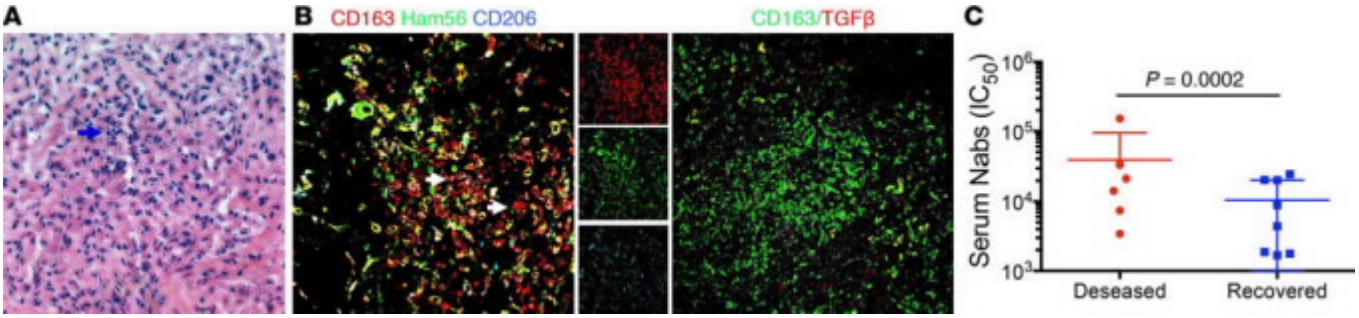


Figure 7. NAb response and phenotype analysis of accumulating monocytes/macrophages in the lungs in deceased SARS patients. (**A**) Pathological changes of the lung tissue. Sections from deceased SARS patients were stained with H&E. A representative of 3 patients (original magnification, 100×), showing symptom of acute DAD with massive cell infiltration in alveolar cavities (blue arrow). (**B**) Massive accumulation of IMMs and absence of wound-healing macrophage response in the lungs of deceased patients. These sections were triple-immunostained with antibodies for CD206 (cyan), HAM56 (FITC), and CD163 (TRITC) or double-immunostained with TGF-β (FITC) and CD163 (FITC) (original magnification, 200×). The right panel in the left image shows single colors from the left image (200×). These representative images show accumulation of IMMs (CD163+HAM56+, white arrows) and the absence of wound-healing response, indicated by loss of the signal for CD206 (cyan, B1) and TGF-β (B2) in the lungs. (**C**) Serum-neutralizing activity against SARS-CoV. Sera collected from deceased ($n = 6$), or recovered (blue, $n = 8$) SARS patients at the early stage of infection were tested for the capacity to neutralize SARS-CoV pseudotype virus. Two-tailed unpaired Student's *t* test was used for statistical analysis.

In fact, a protective effect against pulmonary immunopathology mediated by full length S protein–based vaccine in SARS-CoV–infected nonhuman primate models has not been described, to our knowledge. Although several vaccine candidates protected macaques against viral replication in the lungs, pulmonary immunopathology was not evaluated in these studies (24, 41, 42). By contrast, it was shown that SARS-CoV–specific immune memory induced by prior infection enhanced lung inflammation following homologous challenge in African green monkeys (17). Similarly, augmentation of disease by vaccination has also been described in studies of atypical measles and dengue hemorrhagic fever, as well as several respiratory diseases, including respiratory syncytial virus (RSV) and pandemic influenza (16, 43). In the study of RSV vaccine conducted in 1966 and 1967, 80% of RSV vaccinees needed hospitalization, whereas only 5% of RSV-infected children in the control vaccine group required admission, although the underlying mechanisms remain incompletely understood. Using Chinese rhesus macaques, we show that an ADS-MVA–based vaccine protected macaques against viral replication but significantly enhanced acute DAD at both 7 and 35 dpi compared with the control ADC-MVA group, suggesting that S-specific but not MVA-specific immunity promotes ALI (Figure 1). Until now, it remains unknown if SARS vaccine candidates with a focus on the receptor-binding domain (RBD) of S protein can avoid the induction of ALI in nonhuman primates. These vaccines have been shown to induce highly potent NAb responses and elicit long-term protective immunity in immunized small animals (38).

SARS-CoV infection of Chinese macaques is often characterized by the rapid control of viral replication with mild lung lesions in most macaques (25). The mechanisms underlying why Chinese macaques do not often develop ALI, as observed in most SARS patients, are largely unknown. Our data suggest that these effects, in part, reflect the rapid control of viral replication in the lungs, which peaked between 24 and 48 hours after inoculation (hpi) and diminished within 7 days, thus creating an interval between lung inflammation and high titer antibody production in most macaques. Indeed, although SARS-CoV infection resulted in significant infiltration of macrophages in the lungs, the number of macrophages was rapidly reduced following virus clearance at 3 dpi (Figure 5). At 7 dpi, inflammation appeared to be mostly resolved (Figure 5). By contrast, control of virus replication is less efficient in SARS patients, and the peak in viral load coincides with the first appearance of an antibody response, approximately 10 days after the onset of symptoms (6).

The low rate of ALI and interval between lung inflammation resolution and antibody production, however, makes Chinese macaques an ideal animal model for the present study. Using vaccination and passive immunization, we conditioned anti-S antibody titers in macaques during the early stage of infection when ALI was not typically observed. We found that prior administration of anti–S-IgG led to massive accumulation of monocytes/macrophages in the lungs within 2 dpi. This result is consistent with previous reports, in which mice given the SARS vaccine exhibited an immunopathologic lung reaction after the subsequent challenge with SARS-CoV (18, 19). Moreover, in SARS patients, clinical course and outcome are more favorable in children younger than 12 years of age compared with adolescents and adults (44), indicating that prior alternative CoV infection might

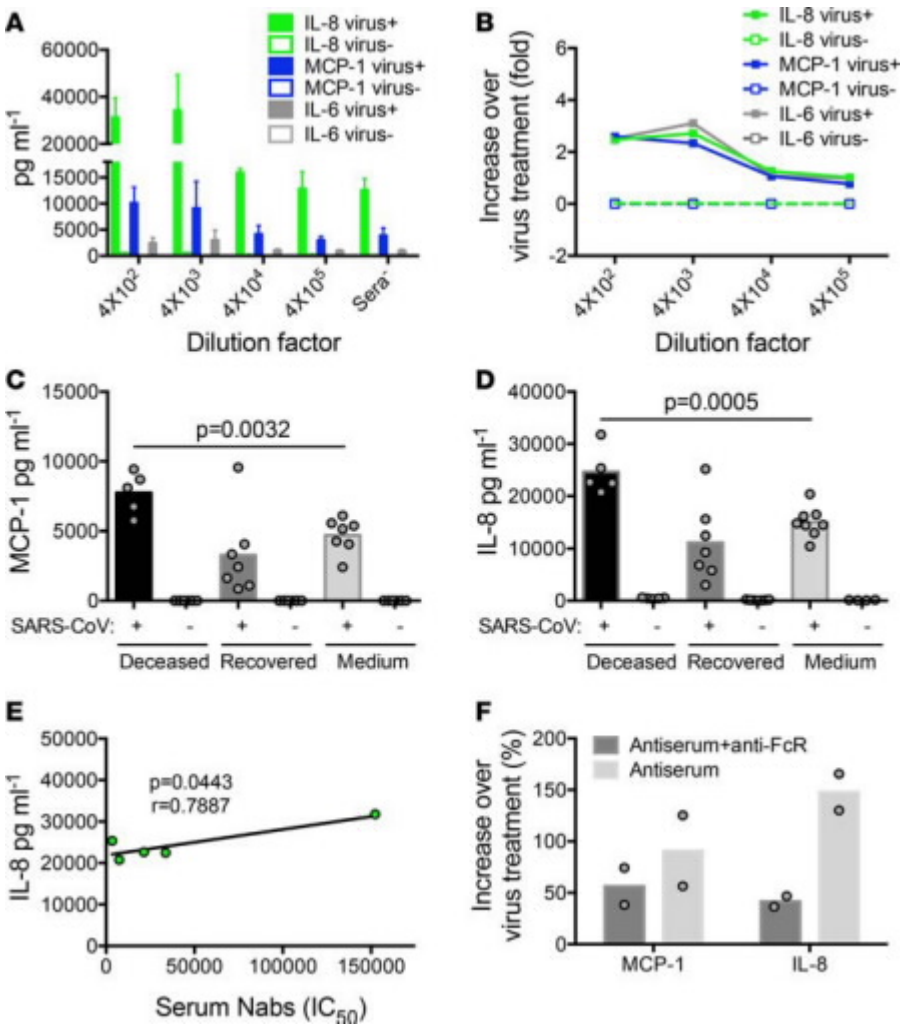


Figure 8. Sera from deceased SARS patients skewed wound-healing macrophage response partially through FcγR. In vitro–polarized alternatively activated MDM were either left unstimulated or were incubated with SARS-CoV pseudovirus alone or cocultured with SARS-CoV pseudovirus and 1–400,000 serials diluted or 1/4,000 diluted sera from deceased (n = 5) or recovered SARS patients (n = 7) for 20 hours. Cells treated with sera from patients alone served as controls. Secreted cytokine and chemokine levels were measured in the culture supernatant and are shown in data represented as a column graph (**A**, **C**, and **D**) and fold or percentage over supernatants from macrophages treated with virus alone (**B** and **F**). **A** and **B** show that addition of sera from deceased patient (D1) dose-dependently enhanced production of IL-8, IL-6, and MCP-1 by SARS-CoV–treated alternatively activated MDM. **C** and **D** show significantly enhanced IL-8 and MCP-1 production by alternatively activated macrophages treated with sera from deceased SARS patients and virus compared with cells treated with virus alone. Sera treatment alone did not induce IL-8 or MCP-1 production. (**E**) Correlation of IL-8 production with NAb titers of sera of deceased patients. Solid lines denote the relationship between histopathology scores and serum neutralizing activity. (**F**) Blockade of human FcγR significantly reduced antiserum-enhanced IL-8 secretion and partially reduced MCP-1 secretion. In vitro–polarized alternatively activated human monocyte–derived macrophages were cocultured with SARS-CoV pseudovirus and 1–4,000 diluted sera from deceased patient (D1) with or without FcγR blocking antibody for 20 hours. Secreted cytokine and chemokine levels were measured in the culture supernatant and are shown in data represented as increase over supernatants from macrophages treated with virus alone. Data represent mean values or mean values ± SEM of at least 3 independent experiments. Two-tailed unpaired Student's t test and Spearman's rank correlation test were used for statistical analysis.

play a role in driving enhanced pulmonary inflammation. A recent study reported that pathogenic immune complexes promoted lung injury during the 2009 H1N1 pandemic (16). It remains unknown whether immune complexes may also play a role in driving ALI during SARS-CoV infection.

Detailed analyses of macrophage heterogeneity at different stages of infection and in lungs with severe injury or mild lesion revealed distinct roles for proinflammatory and wound-healing macrophages in SARS disease progression. We observed that the early transition of macrophage responses from proinflammatory

to wound healing with increased TGF-β expression is associated with inflammation resolution and mild lung injury. By contrast, abrogated wound healing by S-IgG resulted in decreased TGF-β production and prolonged classical macrophage activation, and it promoted severe lung injury. Our findings are primarily based on imaging of formalin-fixed tissues of infected macaques, which may have technical limitations. A detailed flow cytometric analysis would be helpful to further characterize macrophage subpopulations in infected lungs. Unfortunately, we were not allowed to obtain any live cells, including macrophages, for analysis by flow cytometry outside the BSL-3 laboratory in China, which is in line with "WHO biosafety guidelines for handling of SARS specimens" (https://www.who.int/csr/sars/biosafety2003_04_25/en/). Having said this, TGF-β is mainly derived from wound-healing macrophages but not from classically activated macrophages. Therefore, by staining both TGF-β and CD206 (another biomarker of wound-healing macrophages), we believe that the finding of reduction of this type of macrophages in the S-IgG–treated lungs is convincing.

Patients who received convalescent plasma from recovered SARS patients had shorter hospital stays and lower mortality rates without evident adverse effects (45–47). These results might reflect a different quality of antibodies in recovered patients from deceased patients during early infection. Indeed, we previously observed that deceased patients had significantly higher NAbs with a very low level of anti-N antibodies in serum during the early stage of infection (14). In contrast, recovered patients had more rapid anti-N antibody and slower NAb development (14). Consistently, serum from deceased patients — but not SARS survivors during early stages of infection — enhanced IL-6, IL-8, and MCP1 production by wound-healing macrophages (Figure 8). We previously identified epitopes in S protein that elicited NAbs and antibodies that enhanced SARS-CoV infection (48, 49). Further studies that will attempt to identify antibodies and epitopes that mediate prevention or enhancement of ALI might be needed for future vaccine design and immunotherapy.

Lastly, blockade of FcγRs reduced proinflammatory cytokine production, suggesting a potential role of FcγRs for the postulated reprogramming of alternatively activated macrophage FcγRs are functionally divided into activating and inhibitory receptors. Activating FcγRs, such as FcγRI, FcγRIIA, and FcγRIII, triggers production of proinflammatory chemokines and cytokines through an immunoreceptor tyrosine–based activation motif (ITAM) in their intracytoplasmic domain and SRC family kinases and spleen tyrosine kinase (SYK), whereas the inhibitory FcγR, FcγRIIB, has an immunoreceptor tyrosine–based inhibition motif (ITIM) in its intracytoplasmic domain and counteracts the signals that are mediated by activating FcγRs. Based on literature and our experiments, human monocyte–derived wound-healing macrophages express higher levels of CD16 (FcγRIII) and CD32A (FcγRIIA) (50). Therefore, it is likely that S-IgG promoted proinflammatory cytokine production through FcγRI and/or FcγRIIA. S-IgG did not affect classically activated macrophage function in vitro. This difference can be possibly explained by the high baseline level of proinflammatory cytokines before infection and the downregulated expression of FcγRs on classically activated MDM by SARS-CoV treatment.

Methods

Immunization of animals. We vaccinated Chinese rhesus macaques as previously described (51). Briefly, 16 Chinese rhesus monkeys were immunized twice on days 0 and 28. Eight of these animals received ADS-MVA, whereas the other 8 animals received ADC-MVA. ADS-MVA and ADC-MVA vectors encoding SARS-CoV S protein or HCV E1E2 were constructed and produced as previously described (51). The dose for both immunizations was 5×10^8 $TCID_{50}$. The vaccine strain SARS-CoV HKU39849 (AY278491) shares 100% sequence homology of the S gene with the challenge strain $SARS\text{-}CoV_{PUMC}$ (AY350750).

Animals, tissues, and SARS-CoV infection. SARS-CoV–infected animals and control animals were all adult female Chinese rhesus macaques. A total of 42 Chinese rhesus macaques were challenged i.n. with a live pathogenic SARS-CoV (PUM, $TCID_{50}$ = 50) as previously described (25). An additional 3 uninfected macaques were inoculated with PBS as negative controls. Animals were subjected to daily measurement of anal temperature, routine blood assays, and chest radiography. Animals were euthanized at 2, 3, 7, 21, and 35 days after inoculation (Figure 1, Figure 2, and Figure 6). All animals were humanely euthanized with an i.v. overdose of pentobarbital and were immediately necropsied. Lung tissues (left and right cranial, middle and caudal lobes, and right accessory lobe) were collected and fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 5 μm.

Passive transfer of IgG. SARS-CoV S protein–immune serum was generated by vaccination with ADS-MVA vectors. IgG was purified from pooled sera by protein G affinity chromatography to >95% purity. IgG was depyrogenated, concentrated to 20–30 mg protein ml^{-1}, and administered i.v. Recipients received 200 mg or 5 mg of purified IgG per kg body weight at 48 hours before the viral challenge.

Neutralization. A neutralization assay was established to determine the humoral immune responses generated by the vaccine as previously described (51). The neutralizing activity of heat-inactivated animal sera was determined using a pseudotype viral entry assay. The pseudotype virus was generated through cotransfection of 293T cells with 2 plasmids, pcDNA-Sopt9 and pNL4-3Luc_Env_Vpr_, carrying the optimized S gene and a human immunodeficiency virus type 1 backbone, respectively. The serial diluted serum samples were incubated with equal amounts of pseudotype virus at 37°C for 1 hour. The serum-virus mixtures were subsequently added into preseeded HEK 293T-ACE2 cells. After 56 hours, infected cells were lysed to measure luciferase activity.

Nested RT-PCR and isolation of SARS-CoV. Pharyngeal swab samples were collected from infected monkeys on the first day after inoculation and subsequently tested for SARS-CoV using nested RT-PCR as previously described (25). Briefly, RNA was isolated using TRIZOL (Invitrogen). RT-PCR was performed in a 50 μl reaction volume with outer primer pair (5′-GCTGCATTGGTTTGTTATATCGTTA-TGC-3′) and (5′-ATACAGAATACAT-AGATTGCTGTTATCC-3′), inner primer pair (5′-TCACTTGCTTC-CGTTGAGGTAGC-CAGCGTGGT-GGTTCATACAA-3′) and (5′-GGTTTCGGATGTTACAGC-GTCTCCCGG-CAGAAAGCTGTAAGCT-3′). All PCR products were verified by nucleotide sequencing. SARS-CoV was isolated as previously described. Briefly, pharyngeal swab samples from the infected macaques were inoculated onto Vero-E6 cells and cultured in DMEM (Thermo Fisher Scientific). Both cytopathic effect (CPE) and immunofluorescence assay (IFA) (incubation with 1:10 dilution of SARS patient serum) were used to determine the infection status of the animals.

Double and triple immunofluorescence staining on paraffin sections. The samples were deparaffinized and rehydrated. After blocking with normal goat serum for 30 minutes at room temperature, a rabbit anti–SARS-CoV nucleocapsid antibody (ss-006-0100, ENZYME) was applied at 4°C overnight, followed by an Alexa Fluor 448 conjugated goat anti–rabbit IgG antibody for 1 hour at room temperature. Additional immunofluorescence staining was subsequently performed by overnight incubation with primary antibodies, followed by the detection of signals using appropriate fluorescent secondary antibodies (MilliporeSigma) for 1 hour at room temperature.

Primary antibodies for immunofluresence staining. The following primary antibodies were used in this study: SARS-CoV NP (ss-006-0100, ENZYME); MAC387 (clone MAC387, AbD, Serotec); CD68 (clone KPI, DAKO; ab125047, Abcam); CD163 (clone EDHu1, AbD and ab183476, Abcam); HAM56 (clone HAM56, DAKO); CD206 (HPA004114, MilliporeSigma); TGF-β (sc-146, Santa Cruz Biotechnology Inc.); IL-6 (sc-1265, Santa Cruz Biotechnology Inc.); AE1/AE3 (clone AE1/AE3, DAKO).

ISH studies. ISH was developed for the detection of SARS-CoV. DNA fragments of N and S proteins from the SARS-CoV strain from Hong Kong (AY278491) were used as templates to generate RNA probes. The probes were labeled with digoxigenin (GENEWIZ Inc.). After deparaffinization and rehydration, the sections were treated with microwave heating in an 800 W domestic microwave oven (NN-ST556M, Panasonic) at medium power for 10 minutes in 0.01 M sodium citrate buffer. Hybridization was performed at high stringency with 150 ng/ml of denatured probe at 45°C for 16 hours in 50 μl of hybridization mix (50% formamide, 10% dextran sulfate in 2× saline sodium citrate [SSC]) in a moist chamber. Excess probe was removed by washing twice in 2× SSC for 20 minutes at 45°C, followed by 2 washes in 1× SSC and 0.1× SSC for 20 minutes at 45°C. Immunological detection was conducted using the DIG Nuclear Acid Detection Kit (Roche Diagnostics) according to the manufacturer's instructions. Briefly, after incubation with 1% blocking reagent for 30 minutes, the sections were incubated with sheep anti-digoxigenin alkaline phosphatase conjugate (11093274910, Roche Diagnostics) diluted at 1: 500 for 2 hours at room temperature. After 3 separate washes with washing buffer (0.1 M maleic acid, 0.15 M NaCl [pH 7.5], 0.3% Tween 20), the signal was developed using 200 μl of a HNPP/Fast Red TR mix (1758888, Roche Diagnostics) in the dark for 45 minutes. The sections were counterstained with DAPI. Tissue sections incubated with sense RNA probe were used as a negative control.

Confocal microscopy. Confocal microscopy was performed using a Carl Zeiss LSM 700 confocal microscope equipped with 4 lasers (Zeiss). ZEN (Zeiss) was used to assign colors to the 4 channels collected: Alexa Fluor 568 and HNPP/Fast Red, which fluoresces when exposed to a 568-nm wavelength laser and appears red; Alexa Fluor 488 (Molecular Probes), which appears green; Alexa Fluor 647 (Molecular Probes), which appears cyan; and the differential interference contrast (DIC) image, which is in gray scale. The 4 channels were simultaneously collected. To differentiate between individual cells, Hoechst 33258 (nuclear marker; Molecular Probes) was used at 1 μg/ml and incubated for 5 minutes, followed by rinsing with water. The colocalization of antigens was demonstrated by the addition of colors as indicated in the figure legend.

Monocyte isolation, differentiation, and polarization of macrophages. Monocytes were purified from peripheral blood mononuclear cells obtained from blood buffy coats (provided by Red Cross, Hong Kong, China) or monkey peripheral blood, as we previously described (52). Monkey peripheral blood was obtained in heparinized vacutainer collection tubes from healthy female rhesus monkeys. To generate M1 macrophages, monocytes were differentiated in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with GM-CSF (400 IU/ml), 2 mM glutamine, 10% decomplemented FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific) for 4 days, followed by exposure to fresh medium supplemented with 5% FCS and containing GM-CSF and LPS (100 ng/ml) + IFN-γ (20 ng/ml) for an additional 72 hours. To generate M2 macrophages, monocytes were differentiated in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with M-CSF (50 ng/ml), 2 mM glutamine, 10% decomplemented FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin for 4 days, followed by exposure to fresh medium supplemented with 5% FCS and containing M-CSF (50 ng/ml) + IL-4 (20 ng/ml) for an additional 72 hours.

Quantification of cytokine levels. A panel of 13 cytokines, including IL-1β, IFN-α, IFN-γ, TNF-α, MCP1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33 in monkey sera and in culture medium were quantified using multiplexing laser bead technology (BioLegend). To assess the cytokine production of macrophages, in vitro–polarized cells were washed 3 times with PBS and were subsequently activated with SARS-CoV pseudotype virus in RPMI 1640 medium (Thermo Fisher Scientific) with or without administration of highly diluted and heat-inactivated (56°C) sera from macaques or SARS patients. FcγR-specific mouse monoclonal antibody (5 μg, clone 3G8, anti-hCD16, 16016682; clone FLI8.26, anti-hCD32, 16032981; and clone 10.1, anti-hCD64, 16064981 [BD Pharmingen]) was used for blockade of human FcγR. The supernatant of these cultures was recovered after 20 hours of stimulation, and the cytokine levels were measured and analyzed.

Statistics. All statistical analyses were performed using the 2-tailed Student's *t* test. *P* values less than 0.05 were considered statistically significant. Data are presented as the mean values ± SEM of at least 3 independent experiments unless otherwise indicated.

Study approval. The experiment was conducted in a Biosafety Level 3 animal facility. Handling and experimental procedures, including injections, blood collection, virus challenge, and sacrificing were approved and performed according to the animal welfare committee on the Use of Live Animals in Teaching and Research of Institute of Laboratory Animal Science, Beijing, China. All study subjects gave informed consent, and procedures were reviewed and approved by the University of Hong Kong Research Ethics Committee.

Author contributions

ZC, LL, and QW designed the study. LL and QW analyzed the data. LL, ZC, KYY, SP, and AL contributed to drafting the manuscript. ZC is the principal investigator, with overall responsibility for the design of the study. QW and CQ coordinated the autopsy organization and collection of autopsy materials of Chinese macaques. QW was involved in the development and performance of the RT-PCR assay. LL, HT, TW, and KWC performed the neutralization assay. LL, XA, and AL were involved in the development of the H&E, SARS-CoV IHC, and ISH assays. HW performed the ISH assay and the IHC staining of NP and calculation of the mean florescence intensity in the lungs. LL, HK, and KN performed the H&E and double- or triple-IHC staining. JF and HT performed culture of monocyte-derived macrophages. QL, HT, JP, and ZT performed analysis of cytokine production in cell culture supernatant and monkey sera. KHC and KYY coordinated the collection of sera and tissue sample from SARS patients. All authors were involved in the correlative interpretation of the pathological and molecular data.

Acknowledgments

This work was financially supported by grants from the U.S. NIH RO1HL080211 and Hong Kong Research Grants Council TRS T11-706/18-N to ZC, the TNPRC (base grant RR00164), RO1060699 to SP, HMRF 16150662, and the University Development Fund/Li Ka Shing Faculty of Medicine Matching Fund of the University of Hong Kong to its AIDS Institute and Hong Kong RGV. The authors would like to thank D.D. Ho and K. Liu for support and scientific advice, and Wenjie Yu for assistance of S-IgG and C-IgG purification.

Address correspondence to: Zhiwei Chen or Li Liu, AIDS Institute and Department of Microbiology, State Key Laboratory of Emerging Infectious Disease, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong S.A.R., China. Phone: 852.28199831; Email: zchenai@hku.hk (ZC). Phone: 852.39179094; Email: liuli71@hku.hk (LL).

1. Drosten C, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med*. 2003;348(20):1967–1976.
2. Ksiazek TG, et al. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med*. 2003;348(20):1953–1966.
3. Hwang DM, Chamberlain DW, Poutanen SM, Low DE, Asa SL, Butany J. Pulmonary pathology of severe acute respiratory syndrome in Toronto. *Mod Pathol*. 2005;18(1):1–10.
4. Peiris JS, Guan Y. Confronting SARS: a view from Hong Kong. *Philos Trans R Soc Lond, B, Biol Sci*. 2004;359(1447):1075–1079.
5. Nicholls J, Dong XP, Jiang G, Peiris M. SARS: clinical virology and pathogenesis. *Respirology*. 2003;8 Suppl:S6–S8.
6. Peiris JS, et al. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. *Lancet*. 2003;361(9371):1767–1772.
7. Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med*. 2000;342(18):1334–1349.
8. Herold S, Mayer K, Lohmeyer J. Acute lung injury: how macrophages orchestrate resolution of inflammation and tissue repair. *Front Immunol*. 2011;2:65.
9. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol*. 2011;11(11):723–737.
10. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8(12):958–969.
11. Wynn TA, Vannella KM. Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity*. 2016;44(3):450–462.
12. Perlman S, Dandekar AA. Immunopathogenesis of coronavirus infections: implications for SARS. *Nat Rev Immunol*. 2005;5(12):917–927.
13. Channappanavar R, et al. Dysregulated Type I Interferon and Inflammatory Monocyte-Macrophage Responses Cause Lethal Pneumonia in SARS-CoV-Infected Mice. *Cell Host Microbe*. 2016;19(2):181–193.
14. Zhang L, et al. Antibody responses against SARS coronavirus are correlated with disease outcome of infected individuals. *J Med Virol*. 2006;78(1):1–8.
15. To KK, et al. High titer and avidity of nonneutralizing antibodies against influenza vaccine antigen are associated with severe influenza. *Clin Vaccine Immunol*. 2012;19(7):1012–1018.
16. Monsalvo AC, et al. Severe pandemic 2009 H1N1 influenza disease due to pathogenic immune complexes. *Nat Med*. 2011;17(2):195–199.
17. Clay C, et al. Primary severe acute respiratory syndrome coronavirus infection limits replication but not lung inflammation upon homologous rechallenge. *J Virol*. 2012;86(8):4234–4244.
18. Bolles M, et al. A double-inactivated severe acute respiratory syndrome coronavirus vaccine provides incomplete protection in mice and induces increased eosinophilic proinflammatory pulmonary response upon challenge. *J Virol*. 2011;85(23):12201–12215.
19. Tseng CT, et al. Immunization with SARS coronavirus vaccines leads to pulmonary immunopathology on challenge with the SARS virus. *PLoS ONE*. 2012;7(4):e35421.
20. Zhao J, et al. Airway Memory CD4(+) T Cells Mediate Protective Immunity against Emerging Respiratory Coronaviruses. *Immunity*. 2016;44(6):1379–1391.
21. Channappanavar R, Fett C, Zhao J, Meyerholz DK, Perlman S. Virus-specific memory CD8 T cells provide substantial protection from lethal severe acute respiratory syndrome coronavirus infection. *J Virol*. 2014;88(19):11034–11044.
22. Zhao J, Zhao J, Perlman S. T cell responses are required for protection from clinical disease and for virus clearance in severe acute respiratory syndrome coronavirus-infected mice. *J Virol*. 2010;84(18):9318–9325.
23. Honda-Okubo Y, Barnard D, Ong CH, Peng BH, Tseng CT, Petrovsky N. Severe acute respiratory syndrome-associated coronavirus vaccines formulated with delta inulin adjuvants provide enhanced protection while ameliorating lung eosinophilic immunopathology. *J Virol*. 2015;89(6):2995–3007.
24. Chen Z, et al. Recombinant modified vaccinia virus Ankara expressing the spike glycoprotein of severe acute respiratory syndrome coronavirus induces protective neutralizing antibodies primarily targeting the receptor binding region. *J Virol*. 2005;79(5):2678–2688.
25. Liu L, et al. Spatiotemporal interplay of severe acute respiratory syndrome coronavirus and respiratory mucosal cells drives viral dissemination in rhesus macaques. *Mucosal Immunol*. 2016;9(4):1089–1101.
26. Li BJ, et al. Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nat Med*. 2005;11(9):944–951.
27. Qin C, et al. An animal model of SARS produced by infection of Macaca mulatta with SARS coronavirus. *J Pathol*. 2005;206(3):251–259.
28. Soulas C, et al. Recently infiltrating MAC387(+) monocytes/macrophages a third macrophage population involved in SIV and HIV encephalitic lesion formation. *Am J Pathol*. 2011;178(5):2121–2135.
29. Cai Y, Sugimoto C, Arainga M, Alvarez X, Didier ES, Kuroda MJ. In vivo characterization of alveolar and interstitial lung macrophages in rhesus macaques: implications for understanding lung disease in humans. *J Immunol*. 2014;192(6):2821–2829.
30. Murray PJ, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*. 2014;41(1):14–20.
31. Imai Y, et al. Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell*. 2008;133(2):235–249.
32. Atreya R, et al. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med*. 2000;6(5):583–588.
33. Braciale TJ, Sun J, Kim TS. Regulating the adaptive immune response to respiratory virus infection. *Nat Rev Immunol*. 2012;12(4):295–305.

34. Subbarao K, et al. Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome coronavirus in the respiratory tract of mice. *J Virol*. 2004;78(7):3572–3577.
35. Yang ZY, et al. A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. *Nature*. 2004;428(6982):561–564.
36. Du L, et al. Priming with rAA V encoding RBD of SARS-CoV S protein and boosting with RBD-specific peptides for T cell epitopes elevated humoral and cellular immune responses against SARS-CoV infection. *Vaccine*. 2008;26(13):1644–1651.
37. Du L, et al. Intranasal vaccination of recombinant adeno-associated virus encoding receptor-binding domain of severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein induces strong mucosal immune responses and provides long-term protection against SARS-CoV infection. *J Immunol*. 2008;180(2):948–956.
38. Du L, et al. Receptor-binding domain of SARS-CoV spike protein induces long-term protective immunity in an animal model. *Vaccine*. 2007;25(15):2832–2838.
39. Fett C, DeDiego ML, Regla-Nava JA, Enjuanes L, Perlman S. Complete protection against severe acute respiratory syndrome coronavirus-mediated lethal respiratory disease in aged mice by immunization with a mouse-adapted virus lacking E protein. *J Virol*. 2013;87(12):6551–6559.
40. Iwata-Yoshikawa N, et al. Effects of Toll-like receptor stimulation on eosinophilic infiltration in lungs of BALB/c mice immunized with UV-inactivated severe acute respiratory syndrome-related coronavirus vaccine. *J Virol*. 2014;88(15):8597–8614.
41. Qin E, et al. Immunogenicity and protective efficacy in monkeys of purified inactivated Vero-cell SARS vaccine. *Vaccine*. 2006;24(7):1028–1034.
42. Zhou J, et al. Immunogenicity, safety, and protective efficacy of an inactivated SARS-associated coronavirus vaccine in rhesus monkeys. *Vaccine*. 2005;23(24):3202–3209.
43. Openshaw PJ, Tregoning JS. Immune responses and disease enhancement during respiratory syncytial virus infection. *Clin Microbiol Rev*. 2005;18(3):541–555.
44. Stockman LJ, et al. Severe acute respiratory syndrome in children. *Pediatr Infect Dis J*. 2007;26(1):68–74.
45. Mair-Jenkins J, et al. The effectiveness of convalescent plasma and hyperimmune immunoglobulin for the treatment of severe acute respiratory infections of viral etiology: a systematic review and exploratory meta-analysis. *J Infect Dis*. 2015;211(1):80–90.
46. Cheng Y, et al. Use of convalescent plasma therapy in SARS patients in Hong Kong. *Eur J Clin Microbiol Infect Dis*. 2005;24(1):44–46.
47. Soo YO, et al. Retrospective comparison of convalescent plasma with continuing high-dose methylprednisolone treatment in SARS patients. *Clin Microbiol Infect*. 2004;10(7):676–678.
48. Wang Q, et al. Immunodominant SARS Coronavirus Epitopes in Humans Elicited both Enhancing and Neutralizing Effects on Infection in Non-human Primates. *ACS Infect Dis*. 2016;2(5):361–376.
49. Yi CE, Ba L, Zhang L, Ho DD, Chen Z. Single amino acid substitutions in the severe acute respiratory syndrome coronavirus spike glycoprotein determine viral entry and immunogenicity of a major neutralizing domain. *J Virol*. 2005;79(18):11638–11646.
50. Pahl JH, et al. Macrophages inhibit human osteosarcoma cell growth after activation with the bacterial cell wall derivative liposomal muramyl tripeptide in combination with interferon-γ. *J Exp Clin Cancer Res*. 2014;33:27.
51. Kieran J, et al. The relative efficacy of boceprevir and telaprevir in the treatment of hepatitis C virus genotype 1. *Clin Infect Dis*. 2013;56(2):228–235.
52. Cheng L, et al. Monoclonal antibodies specific to human Δ42PD1: A novel immunoregulator potentially involved in HIV-1 and tumor pathogenesis. *MAbs*. 2015;7(3):620–629.

nature medicine

A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence

Vineet D Menachery[1], Boyd L Yount Jr[1], Kari Debbink[1,2], Sudhakar Agnihothram[3], Lisa E Gralinski[1], Jessica A Plante[1], Rachel L Graham[1], Trevor Scobey[1], Xing-Yi Ge[4], Eric F Donaldson[1], Scott H Randell[5,6], Antonio Lanzavecchia[7], Wayne A Marasco[8,9], Zhengli-Li Shi[4] & Ralph S Baric[1,2]

The emergence of severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome (MERS)-CoV underscores the threat of cross-species transmission events leading to outbreaks in humans. Here we examine the disease potential of a SARS-like virus, SHC014-CoV, which is currently circulating in Chinese horseshoe bat populations[1]. Using the SARS-CoV reverse genetics system[2], we generated and characterized a chimeric virus expressing the spike of bat coronavirus SHC014 in a mouse-adapted SARS-CoV backbone. The results indicate that group 2b viruses encoding the SHC014 spike in a wild-type backbone can efficiently use multiple orthologs of the SARS receptor human angiotensin converting enzyme II (ACE2), replicate efficiently in primary human airway cells and achieve *in vitro* titers equivalent to epidemic strains of SARS-CoV. Additionally, *in vivo* experiments demonstrate replication of the chimeric virus in mouse lung with notable pathogenesis. Evaluation of available SARS-based immune-therapeutic and prophylactic modalities revealed poor efficacy; both monoclonal antibody and vaccine approaches failed to neutralize and protect from infection with CoVs using the novel spike protein. On the basis of these findings, we synthetically re-derived an infectious full-length SHC014 recombinant virus and demonstrate robust viral replication both *in vitro* and *in vivo*. Our work suggests a potential risk of SARS-CoV re-emergence from viruses currently circulating in bat populations.

The emergence of SARS-CoV heralded a new era in the cross-species transmission of severe respiratory illness with globalization leading to rapid spread around the world and massive economic impact[3,4]. Since then, several strains—including influenza A strains H5N1, H1N1 and H7N9 and MERS-CoV—have emerged from animal populations, causing considerable disease, mortality and economic hardship in the afflicted regions[5]. Although public health measures were able to stop the SARS-CoV outbreak[4], recent metagenomics studies have identified sequences of closely related SARS-like viruses circulating in Chinese bat populations that may pose a future threat[1,6–11]. However, sequence data alone provides minimal insights to identify and prepare for future prepandemic viruses. Therefore, to examine the emergence potential (that is, the potential to infect humans) of circulating bat CoVs, we built a chimeric virus encoding a novel, zoonotic CoV spike protein—from the RsSHC014-CoV sequence that was isolated from Chinese horseshoe bats[1]—in the context of the SARS-CoV mouse-adapted backbone. The hybrid virus allowed us to evaluate the ability of the novel spike protein to cause disease independently of other necessary adaptive mutations in its natural backbone. Using this approach, we characterized CoV infection mediated by the SHC014 spike protein in primary human airway cells and *in vivo*, and tested the efficacy of available immune therapeutics against SHC014-CoV. Together, the strategy translates metagenomics data to help predict and prepare for future emergent viruses.

The sequences of SHC014 and the related RsWIV1-CoV show that these CoVs are the closest relatives to the epidemic SARS-CoV strains (**Fig. 1a**,**b**); however, there are important differences in the 14 residues that bind human ACE2, the receptor for SARS-CoV, including the five that are critical for host range: Y442, L472, N479, T487 and Y491 (ref. 7). In WIV1, three of these residues vary from the epidemic SARS-CoV Urbani strain, but they were not expected to alter binding to ACE2 (**Supplementary Fig. 1a** and **Supplementary Table 1**). This fact is confirmed by both pseudotyping experiments that measured the ability of lentiviruses encoding WIV1 spike proteins to enter cells expressing human ACE2 (**Supplementary Fig. 1**) and by *in vitro* replication assays of WIV1-CoV (ref. 1). In contrast, 7 of 14 ACE2-interaction residues in SHC014 are different from those in SARS-CoV, including all five residues critical for host range (**Supplementary Fig. 1c** and **Supplementary Table 1**). These changes, coupled with

[1]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA. [2]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA. [3]National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas, USA. [4]Key Laboratory of Special Pathogens and Biosafety, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China. [5]Department of Cell Biology and Physiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA. [6]Cystic Fibrosis Center, Marsico Lung Institute, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA. [7]Institute for Research in Biomedicine, Bellinzona Institute of Microbiology, Zurich, Switzerland. [8]Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts, USA. [9]Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA. Correspondence should be addressed to R.S.B. (rbaric@email.unc.edu) or V.D.M. (vineet@email.unc.edu).

Received 12 June; accepted 8 October; published online 9 November 2015; corrected online 20 November 2015 (details online); doi:10.1038/nm.3985

© 2015 Nature America, Inc. All rights reserved.

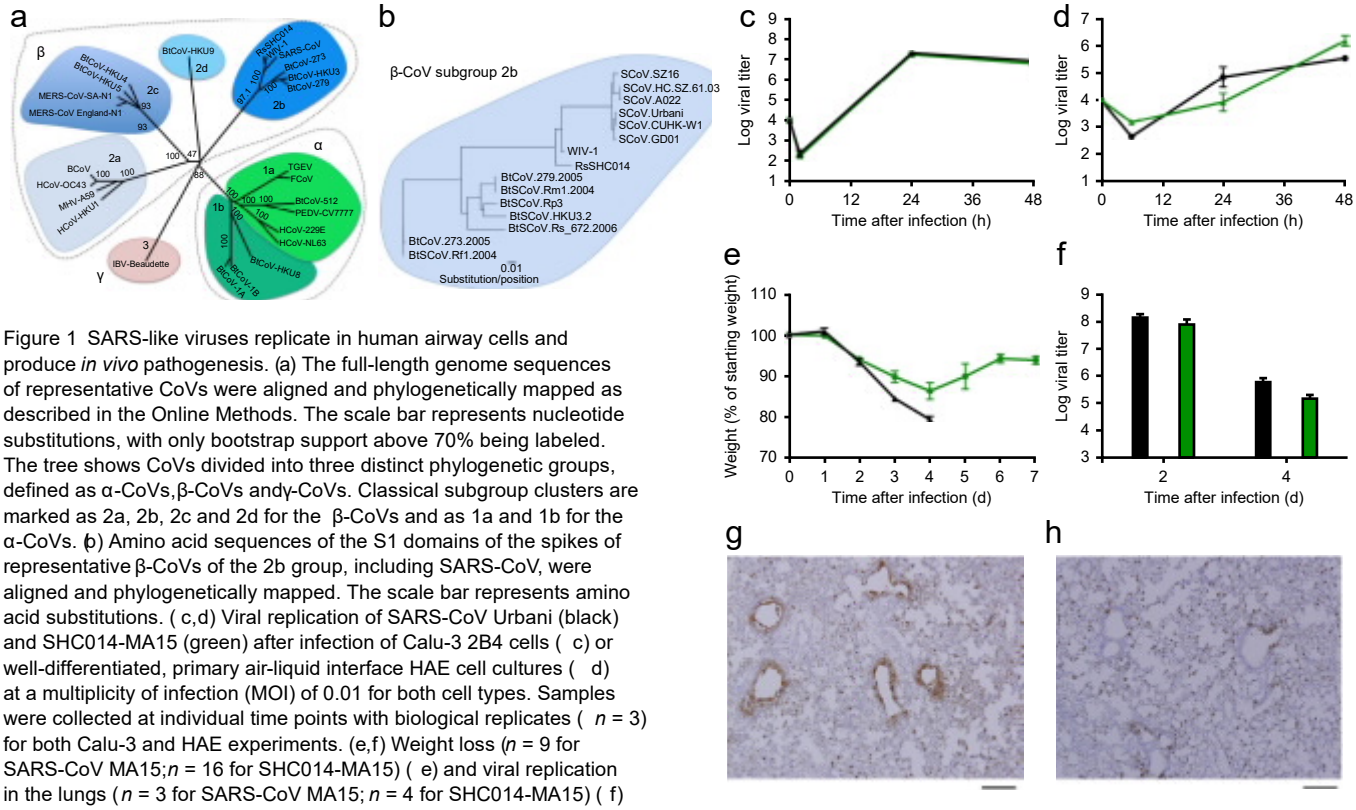


Figure 1 SARS-like viruses replicate in human airway cells and produce *in vivo* pathogenesis. (a) The full-length genome sequences of representative CoVs were aligned and phylogenetically mapped as described in the Online Methods. The scale bar represents nucleotide substitutions, with only bootstrap support above 70% being labeled. The tree shows CoVs divided into three distinct phylogenetic groups, defined as α-CoVs, β-CoVs and γ-CoVs. Classical subgroup clusters are marked as 2a, 2b, 2c and 2d for the β-CoVs and as 1a and 1b for the α-CoVs. (b) Amino acid sequences of the S1 domains of the spikes of representative β-CoVs of the 2b group, including SARS-CoV, were aligned and phylogenetically mapped. The scale bar represents amino acid substitutions. (c,d) Viral replication of SARS-CoV Urbani (black) and SHC014-MA15 (green) after infection of Calu-3 2B4 cells (c) or well-differentiated, primary air-liquid interface HAE cell cultures (d) at a multiplicity of infection (MOI) of 0.01 for both cell types. Samples were collected at individual time points with biological replicates ($n = 3$) for both Calu-3 and HAE experiments. (e,f) Weight loss ($n = 9$ for SARS-CoV MA15; $n = 16$ for SHC014-MA15) (e) and viral replication in the lungs ($n = 3$ for SARS-CoV MA15; $n = 4$ for SHC014-MA15) (f) of 10-week-old BALB/c mice infected with 1×10^4 p.f.u. of mouse-adapted SARS-CoV MA15 (black) or SHC014-MA15 (green) via the intranasal (i.n.) route. (g,h) Representative images of lung sections stained for SARS-CoV N antigen from mice infected with SARS-CoV MA15 ($n = 3$ mice) (g) or SHC014-MA15 ($n = 4$ mice) (h) are shown. For each graph, the center value represents the group mean, and the error bars define the s.e.m. Scale bars, 1 mm.

the failure of pseudotyped lentiviruses expressing the SHC014 spike to enter cells (**Supplementary Fig. 1d**), suggested that the SHC014 spike is unable to bind human ACE2. However, similar changes in related SARS-CoV strains had been reported to allow ACE2-binding[7,8], suggesting that additional functional testing was required for verification. Therefore, we synthesized the SHC014 spike in the context of the replication-competent, mouse-adapted SARS-CoV backbone (we hereafter refer to the chimeric CoV as SHC014-MA15) (**Fig. 1g**) to maximize the opportunity for pathogenesis and vaccine studies in mice (**Supplementary Fig. 2a**). Despite predictions from both structure-based modeling and pseudotyping experiments, SHC014-MA15 was viable and replicated to high titers in Vero cells (**Supplementary Fig. 2b**). Similarly to SARS, SHC014-MA15 also required a functional ACE2 molecule for entry and could use human, civet and bat ACE2 orthologs (**Supplementary Fig. 2c**). To test the ability of the SHC014 spike to mediate infection of the human airway, we examined the sensitivity of the human epithelial airway cell line Calu-3 2B4 (ref. 9) to infection and found robust SHC014-MA15 replication, comparable to that of SARS-CoV Urbani (**Fig. 1c**). To extend these findings, primary human airway epithelial (HAE) cultures were infected and showed robust replication of both viruses (**Fig. 1d**). Together, the data confirm the ability of viruses with the SHC014 spike to infect human airway cells and underscore the potential threat of cross-species transmission of SHC014-CoV.

To evaluate the role of the SHC014 spike in mediating infection *in vivo*, we infected 10-week-old BALB/c mice with 10^4 plaque-forming units (p.f.u.) of either SARS-MA15 or SHC014-MA15 (**Fig. 1e–h**). Animals infected with SARS-MA15 experienced rapid weight loss and lethality by 4 d post infection (d.p.i.); in contrast, SHC014-MA15 infection produced substantial weight loss (10%) but no lethality in mice (**Fig. 1e**). Examination of viral replication revealed nearly equivalent viral titers from the lungs of mice infected with SARS-MA15 or SHC014-MA15 (**Fig. 1f**). Whereas lungs from the SARS-MA15–infected mice showed robust staining in both the terminal bronchioles and the lung parenchyma 2 d.p.i. (**Fig. 1g**), those of SHC014-MA15–infected mice showed reduced airway antigen staining (**Fig. 1h**); in contrast, no deficit in antigen staining was observed in the parenchyma or in the overall histology scoring, suggesting differential infection of lung tissue for SHC014-MA15 (**Supplementary Table 2**). We next analyzed infection in more susceptible, aged (12-month-old) animals. SARS-MA15–infected animals rapidly lost weight and succumbed to infection (**Supplementary Fig. 3a**). SHC014-MA15 infection induced robust and sustained weight loss, but had minimal lethality. Trends in the histology and antigen staining patterns that we observed in young mice were conserved in the older animals (**Supplementary Table 3**). We excluded the possibility that SHC014-MA15 was mediating infection through an alternative receptor on the basis of experiments using *Ace2*$^{-/-}$ mice, which did not show weight loss or antigen staining after SHC014-MA15 infection (**Supplementary Fig. 4a** and **Supplementary Table 2**). Together, the data indicate that viruses with the SHC014 spike are capable of inducing weight loss in mice in the context of a virulent CoV backbone.

Given the preclinical efficacy of Ebola monoclonal antibody therapies, such as ZMApp[10], we next sought to determine the efficacy of SARS-CoV monoclonal antibodies against infection with

© 2015 Nature America, Inc. All rights reserved.

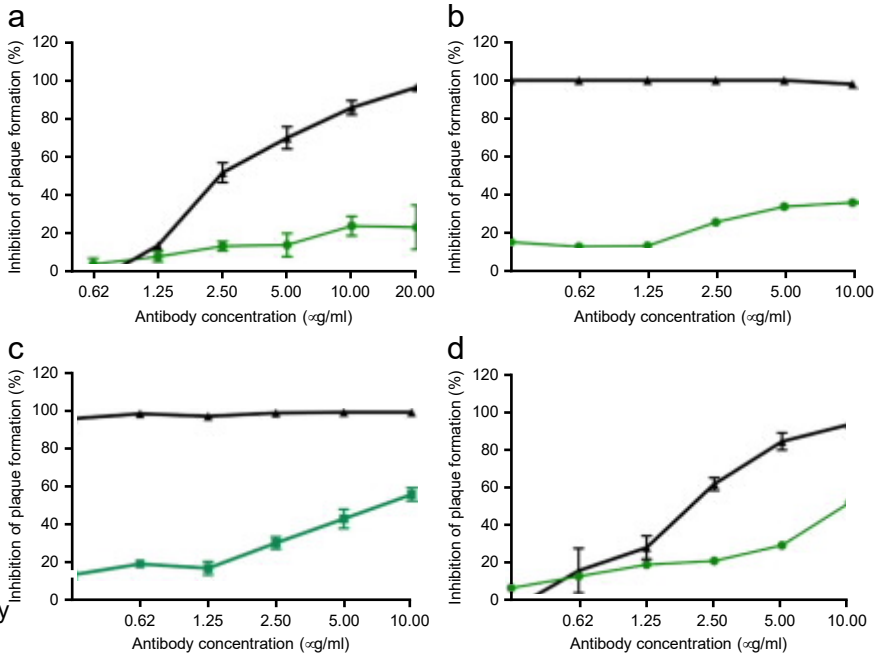


Figure 2 SARS-CoV monoclonal antibodies have marginal efficacy against SARS-like CoVs. (a–d) Neutralization assays evaluating efficacy (measured as reduction in the number of plaques) of a panel of monoclonal antibodies, which were all originally generated against epidemic SARS-CoV, against infection of Vero cells with SARS-CoV Urbani (black) or SHC014-MA15 (green). The antibodies tested were fm6 (n = 3 for Urbani; n = 5 for SHC014-MA15)[11,12] (a), 230.15 (n = 3 for Urbani; n = 2 for SHC014-MA15) (b), 227.15 (n = 3 for Urbani; n = 5 for SHC014-MA15) (c) and 109.8 (n = 3 for Urbani; n = 2 for SHC014-MA15)[13] (d). Each data point represents the group mean and error bars define the s.e.m. Note that the error bars in SARS-CoV Urbani–infected Vero cells in b,c are overlapped by the symbols and are not visible.

SHC014-MA15. Four broadly neutralizing human monoclonal antibodies targeting SARS-CoV spike protein had been previously reported and are probable reagents for immunotherapy[11–13]. We examined the effect of these antibodies on viral replication (expressed as percentage inhibition of viral replication) and found that whereas wild-type SARS-CoV Urbani was strongly neutralized by all four antibodies at relatively low antibody concentrations (**Fig. 2a–d**), neutralization varied for SHC014-MA15. Fm6, an antibody generated by phage display and escape mutants[11,12], achieved only background levels of inhibition of SHC014-MA15 replication (**Fig. 2a**). Similarly, antibodies 230.15 and 227.14, which were derived from memory B cells of SARS-CoV-infected patients[13], also failed to block SHC014-MA15 replication (**Fig. 2b,c**). For all three antibodies, differences between the SARS and SHC014 spike amino acid sequences corresponded to direct or adjacent residue changes found in SARS-CoV escape mutants (fm6 N479R; 230.15 L443V; 227.14 K390Q/E), which probably explains the absence of the antibodies' neutralizing activity against SHC014. Finally, monoclonal antibody 109.8 was able to achieve 50% neutralization of SHC014-MA15, but only at high concentrations (>10 μg/ml) (**Fig. 2d**). Together, the results demonstrate that broadly neutralizing antibodies against SARS-CoV may only have marginal efficacy against emergent SARS-like CoV strains such as SHC014.

To evaluate the efficacy of existing vaccines against infection with SHC014-MA15, we vaccinated aged mice with double-inactivated whole SARS-CoV (DIV). Previous work showed that DIV could neutralize and protect young mice from challenge with a homologous virus[14]; however, the vaccine failed to protect aged animals in which augmented immune pathology was also observed, indicating the possibility of the animals being harmed because of the vaccination[15]. Here we found that DIV did not provide protection from challenge with SHC014-MA15 with regards to weight loss or viral titers (**Supplementary Fig. 5a,b**). Consistent with a previous report with other heterologous group 2b CoVs[15], serum from DIV-vaccinated, aged mice also failed to neutralize SHC014-MA15 (**Supplementary Fig. 5c**). Notably, DIV vaccination resulted in robust immune pathology (**Supplementary Table 4**) and eosinophilia (**Supplementary Fig. 5d–f**). Together, these results confirm that the DIV vaccine would not be protective against infection with SHC014 and could possibly augment disease in the aged vaccinated group.

In contrast to vaccination of mice with DIV, the use of SHC014-MA15 as a live, attenuated vaccine showed potential cross-protection against challenge with SARS-CoV, but the results have important caveats. We infected young mice with 10^4 p.f.u. of SHC014-MA15 and observed them for 28 d. We then challenged the mice with SARS-MA15 at day 29 (**Supplementary Fig. 6a**). The prior infection of the mice with the high dose of SHC014-MA15 conferred protection against challenge with a lethal dose of SARS-MA15, although there was only a minimal SARS-CoV neutralization response from the sera elicited 28 d after SHC014-MA15 infection (**Supplementary Fig. 6b**, 1:200). In the absence of a secondary antigen boost, 28 d.p.i. represents the expected peak of antibody titers and implies that there will be diminished protection against SARS-CoV over time[16,17]. Similar results showing protection against challenge with a lethal dose of SARS-CoV were observed in aged BALB/c mice with respect to weight loss and viral replication (**Supplementary Fig. 6c**). However, the SHC014-MA15 infection dose of 10^4 p.f.u. induced >10% weight loss and lethality in some aged animals (**Fig. 1** and **Supplementary Fig. 3**). We found that vaccination with a lower dose of SHC014-MA15 (10^2 p.f.u.), did not induce weight loss, but it also failed to protect aged animals from a SARS-MA15 lethal dose challenge (**Supplementary Fig. 6e,f**). Together, the data suggest that SHC014-MA15 challenge may confer cross-protection against SARS-CoV through conserved epitopes, but the required dose induces pathogenesis and precludes use as an attenuated vaccine.

Having established that the SHC014 spike has the ability to mediate infection of human cells and cause disease in mice, we next synthesized a full-length SHC014-CoV infectious clone based on the approach used for SARS-CoV (**Fig. 3a**)[2]. Replication in Vero cells revealed no deficit for SHC014-CoV relative to that for SARS-CoV (**Fig. 3b**); however, SHC014-CoV was significantly ($P < 0.01$) attenuated in primary HAE cultures at both 24 and 48 h after infection (**Fig. 3c**). *In vivo* infection of mice demonstrated no significant weight loss but showed reduced viral replication in lungs of full-length SHC014-CoV infection, as compared to SARS-CoV Urbani (**Fig. 3d,e**). Together, the results establish the viability of full-length

© 2015 Nature America, Inc. All rights reserved.

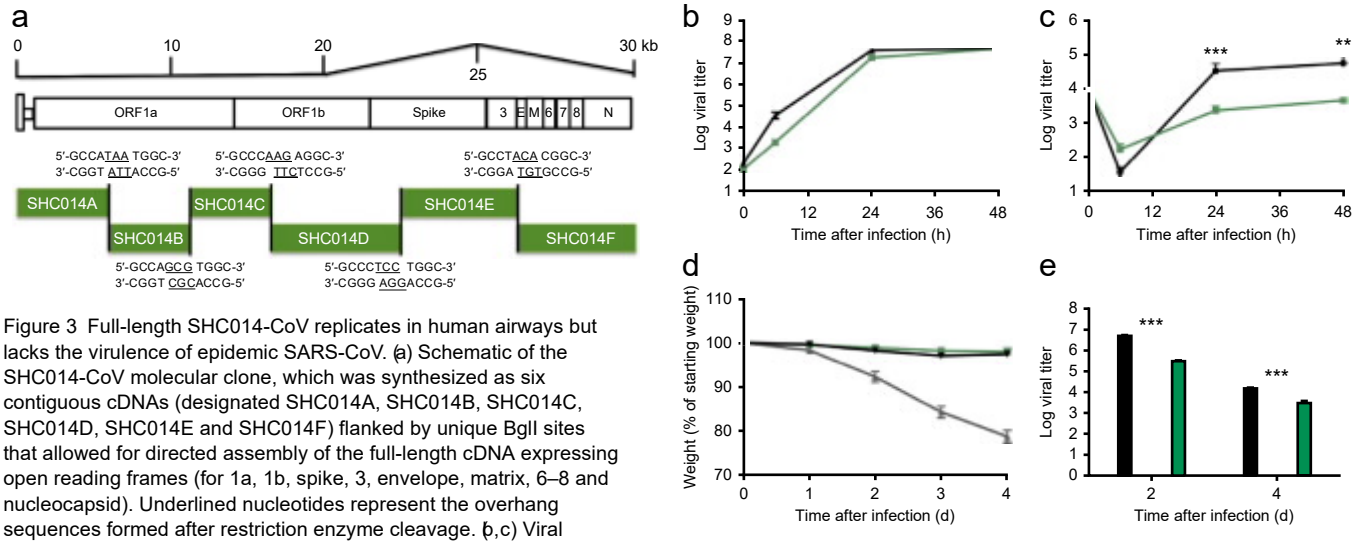


Figure 3 Full-length SHC014-CoV replicates in human airways but lacks the virulence of epidemic SARS-CoV. (a) Schematic of the SHC014-CoV molecular clone, which was synthesized as six contiguous cDNAs (designated SHC014A, SHC014B, SHC014C, SHC014D, SHC014E and SHC014F) flanked by unique BglI sites that allowed for directed assembly of the full-length cDNA expressing open reading frames (for 1a, 1b, spike, 3, envelope, matrix, 6–8 and nucleocapsid). Underlined nucleotides represent the overhang sequences formed after restriction enzyme cleavage. (b,c) Viral replication of SARS-CoV Urbani (black) or SHC014-CoV (green) after infection of Vero cells (b) or well-differentiated, primary air-liquid interface HAE cell cultures (c) at an MOI of 0.01. Samples were collected at individual time points with biological replicates ($n = 3$) for each group. Data represent one experiment for both Vero and HAE cells. (d,e) Weight loss ($n = 3$ for SARS-CoV MA15, $n = 7$ for SHC014-CoV; $n = 6$ for SARS-Urbani) (d) and viral replication in the lungs ($n = 3$ for SARS-Urbani and SHC014-CoV) (e) of 10-week-old BALB/c mice infected with 1×10^5 p.f.u. of SARS-CoV MA15 (gray), SHC014-CoV (green) or SARS-CoV Urbani (black) via the i.n. route. Each data point represents the group mean, and error bars define the s.e.m. ** $P < 0.01$ and *** $P < 0.001$ using two-tailed Student's *t*-test of individual time points.

SHC014-CoV, but suggest that further adaptation is required for its replication to be equivalent to that of epidemic SARS-CoV in human respiratory cells and in mice.

During the SARS-CoV epidemic, links were quickly established between palm civets and the CoV strains that were detected in humans[4]. Building on this finding, the common emergence paradigm argues that epidemic SARS-CoV originated as a bat virus, jumped to civets and incorporated changes within the receptor-binding domain (RBD) to improve binding to civet Ace2 (ref. 8). Subsequent exposure to people in live-animal markets permitted human infection with the civet strain, which, in turn, adapted to become the epidemic strain (**Fig. 4a**). However, phylogenetic analysis suggests that early human SARS strains appear more closely related to bat strains than to civet strains[18]. Therefore, a second paradigm argues that direct bat-human transmission initiated SARS-CoV emergence and that palm civets served as a secondary host and reservoir for continued infection (**Fig. 4b**)[19]. For both paradigms, spike adaptation in a secondary host is seen as a necessity, with most mutations expected to occur within the RBD, thereby facilitating improved infection. Both theories imply that pools of bat CoVs are limited and that host-range mutations are

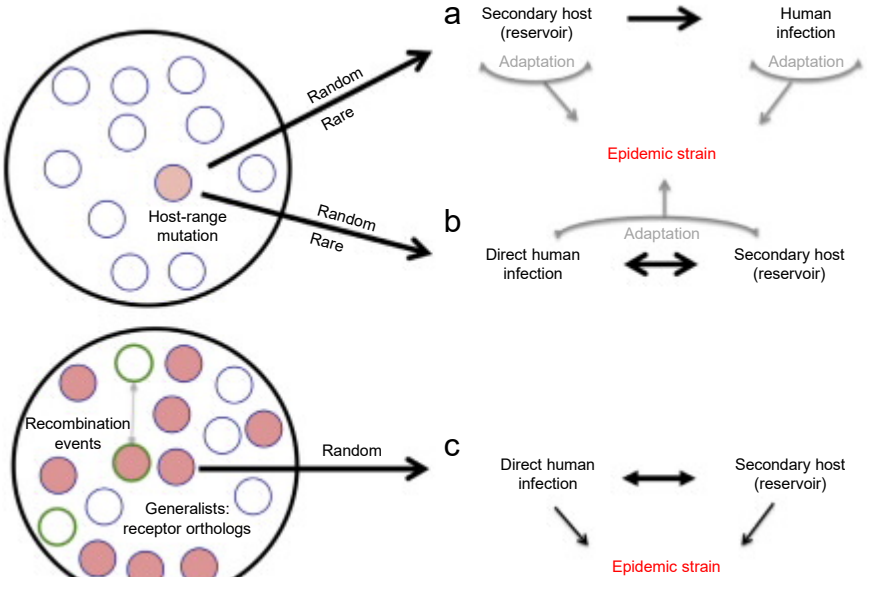


Figure 4 Emergence paradigms for coronaviruses. Coronavirus strains are maintained in quasi-species pools circulating in bat populations. (a,b) Traditional SARS-CoV emergence theories posit that host-range mutants (red circle) represent random and rare occurrences that permit infection of alternative hosts. The secondary-host paradigm (a) argues that a nonhuman host is infected by a bat progenitor virus and, through adaptation, facilitates transmission to humans; subsequent replication in humans leads to the epidemic viral strain. The direct paradigm (b) suggests that transmission occurs between bats and humans without the requirement of an intermediate host; selection then occurs in the human population with closely related viruses replicating in a secondary host, permitting continued viral persistence and adaptation in both. (c) The data from chimeric SARS-like viruses argue that the quasi-species pools maintain multiple viruses capable of infecting human cells without the need for mutations (red circles). Although adaptations in secondary or human hosts may be required for epidemic emergence, if SHC014 spike–containing viruses recombined with virulent CoV backbones (circles with green outlines), then epidemic disease may be the result in humans. Existing data support elements of all three paradigms.

© 2015 Nature America, Inc. All rights reserved.

both random and rare, reducing the likelihood of future emergence events in humans.

Although our study does not invalidate the other emergence routes, it does argue for a third paradigm in which circulating bat CoV pools maintain 'poised' spike proteins that are capable of infecting humans without mutation or adaptation (**Fig. 4c**). This hypothesis is illustrated by the ability of a chimeric virus containing the SHC014 spike in a SARS-CoV backbone to cause robust infection in both human airway cultures and in mice without RBD adaptation. Coupled with the observation of previously identified pathogenic CoV backbones[3,20], our results suggest that the starting materials required for SARS-like emergent strains are currently circulating in animal reservoirs. Notably, although full-length SHC014-CoV probably requires additional backbone adaption to mediate human disease, the documented high-frequency recombination events in CoV families underscores the possibility of future emergence and the need for further preparation.

To date, genomics screens of animal populations have primarily been used to identify novel viruses in outbreak settings[21]. The approach here extends these data sets to examine questions of viral emergence and therapeutic efficacy. We consider viruses with the SHC014 spike a potential threat owing to their ability to replicate in primary human airway cultures, the best available model for human disease. In addition, the observed pathogenesis in mice indicates a capacity for SHC014-containing viruses to cause disease in mammalian models, without RBD adaptation. Notably, differential tropism in the lung as compared to that with SARS-MA15 and attenuation of full-length SHC014-CoV in HAE cultures relative to SARS-CoV Urbani suggest that factors beyond ACE2 binding—including spike processivity, receptor bio-availability or antagonism of the host immune responses—may contribute to emergence. However, further testing in nonhuman primates is required to translate these findings into pathogenic potential in humans. Importantly, the failure of available therapeutics defines a critical need for further study and for the development of treatments. With this knowledge, surveillance programs, diagnostic reagents and effective treatments can be produced that are protective against the emergence of group 2b–specific CoVs, such as SHC014, and these can be applied to other CoV branches that maintain similarly heterogeneous pools.

In addition to offering preparation against future emerging viruses, this approach must be considered in the context of the US government–mandated pause on gain-of-function (GOF) studies[22]. On the basis of previous models of emergence (**Fig. 4a,b**), the creation of chimeric viruses such as SHC014-MA15 was not expected to increase pathogenicity. Although SHC014-MA15 is attenuated relative to its parental mouse-adapted SARS-CoV, similar studies examining the pathogenicity of CoVs with the wild-type Urbani spike within the MA15 backbone showed no weight loss in mice and reduced viral replication[23]. Thus, relative to the Urbani spike–MA15 CoV, SHC014-MA15 shows a gain in pathogenesis (**Fig. 1**). On the basis of these findings, scientific review panels may deem similar studies building chimeric viruses based on circulating strains too risky to pursue, as increased pathogenicity in mammalian models cannot be excluded. Coupled with restrictions on mouse-adapted strains and the development of monoclonal antibodies using escape mutants, research into CoV emergence and therapeutic efficacy may be severely limited moving forward. Together, these data and restrictions represent a crossroads of GOF research concerns; the potential to prepare for and mitigate future outbreaks must be weighed against the risk of creating more dangerous pathogens. In developing policies moving forward, it is important to consider the value of the data generated by these studies and whether these types of chimeric virus studies warrant further investigation versus the inherent risks involved.

Overall, our approach has used metagenomics data to identify a potential threat posed by the circulating bat SARS-like CoV SHC014. Because of the ability of chimeric SHC014 viruses to replicate in human airway cultures, cause pathogenesis *in vivo* and escape current therapeutics, there is a need for both surveillance and improved therapeutics against circulating SARS-like viruses. Our approach also unlocks the use of metagenomics data to predict viral emergence and to apply this knowledge in preparing to treat future emerging virus infections.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

Research in this manuscript was supported by grants from the National Institute of Allergy & Infectious Disease and the National Institute of Aging of the US National Institutes of Health (NIH) under awards U19AI109761 (R.S.B.), U19AI107810 (R.S.B.), AI085524 (W.A.M.), F32AI102561 (V.D.M.) and K99AG049092 (V.D.M.), and by the National Natural Science Foundation of China awards 81290341 (Z.-L.S.) and 31470260 (X.-Y.G.), and by USAID-EPT-PREDICT funding from EcoHealth Alliance (Z.-L.S.). Human airway epithelial cultures were supported by the National Institute of Diabetes and Digestive and Kidney Disease of the NIH under award NIH DK065988 (S.H.R.). We also thank M.T. Ferris (Dept. of Genetics, University of North Carolina) for the reviewing of statistical approaches and C.T. Tseng (Dept. of Microbiology and Immunology, University of Texas Medical Branch) for providing Calu-3 cells. Experiments with the full-length and chimeric SHC014 recombinant viruses were initiated and performed before the GOF research funding pause and have since been reviewed and approved for continued study by the NIH. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

AUTHOR CONTRIBUTIONS

V.D.M. designed, coordinated and performed experiments, completed analysis and wrote the manuscript. B.L.Y. designed the infectious clone and recovered chimeric viruses; S.A. completed neutralization assays; L.E.G. helped perform mouse experiments; T.S. and J.A.P. completed mouse experiments and plaque assays; X.-Y.G. performed pseudotyping experiments; K.D. generated structural figures and predictions; E.F.D. generated phylogenetic analysis; R.L.G. completed RNA analysis; S.H.R. provided primary HAE cultures; A.L. and W.A.M. provided critical monoclonal antibody reagents; and Z.-L.S. provided SHC014 spike sequences and plasmids. R.S.B. designed experiments and wrote manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Ge, X.Y. *et al.* Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. *Nature* 503, 535–538 (2013).
2. Yount, B. *et al.* Reverse genetics with a full-length infectious cDNA of severe acute respiratory syndrome coronavirus *Proc. Natl. Acad. Sci. USA* 100, 12995–13000 (2003).
3. Becker, M.M. *et al.* Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice. *Proc. Natl. Acad. Sci. USA* 105, 19944–19949 (2008).
4. Peiris, J.S., Guan, Y. & Yuen, K.Y. Severe acute respiratory syndrome *Nat. Med.* 10, S88–S97 (2004).
5. Al-Tawfiq, J.A. *et al.* Surveillance for emerging respiratory viruses. *Lancet Infect. Dis.* 14, 992–1000 (2014).
6. He, B. *et al.* Identification of diverse alphacoronaviruses and genomic characterization of a novel severe acute respiratory syndrome–like coronavirus from bats in China. *J. Virol.* 88, 7070–7082 (2014).
7. Li, F. Receptor recognition and cross-species infections of SARS coronavirus. *Antiviral Res.* 100, 246–254 (2013).

© 2015 Nature America, Inc. All rights reserved.

8. Sheahan, T. *et al.* Mechanisms of zoonotic severe acute respiratory syndrome coronavirus host range expansion in human airway epithelium. *J. Virol.* 82, 2274–2285 (2008).
9. Yoshikawa, T. *et al.* Dynamic innate immune responses of human bronchial epithelial cells to severe acute respiratory syndrome–associated coronavirus infection. *PLoS ONE* 5, e8729 (2010).
10. Qiu, X. *et al.* Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. *Nature* 514, 47–53 (2014).
11. Sui, J. *et al.* Broadening of neutralization activity to directly block a dominant antibody-driven SARS-coronavirus evolution pathway. *PLoS Pathog.* 4, e1000197 (2008).
12. Sui, J. *et al.* Effects of human anti–spike protein receptor binding domain antibodies on severe acute respiratory syndrome coronavirus neutralization escape and fitness. *J. Virol.* 88, 13769–13780 (2014).
13. Rockx, B. *et al.* Escape from human monoclonal antibody neutralization affects *in vitro* and *in vivo* fitness of severe acute respiratory syndrome coronavirus. *J. Infect. Dis.* 201, 946–955 (2010).
14. Spruth, M. *et al.* A double-inactivated whole-virus candidate SARS coronavirus vaccine stimulates neutralizing and protective antibody responses. *Vaccine* 24, 652–661 (2006).
15. Bolles, M. *et al.* A double-inactivated severe acute respiratory syndrome coronavirus vaccine provides incomplete protection in mice and induces increased eosinophilic proinflammatory pulmonary response upon challenge. *J. Virol.* 85, 12201–12215 (2011).
16. Siegrist, C.-A. in *Vaccines* 6th edn. (eds. Plotkin, S.A., Orenstein, W.A. & Offit, P.A.) 14–32 (W.B. Saunders, 2013).
17. Deming, D. *et al.* Vaccine efficacy in senescent mice challenged with recombinant SARS-CoV bearing epidemic and zoonotic spike variants. *PLoS Med.* 3, e525 (2006).
18. Graham, R.L., Donaldson, E.F. & Baric, R.S. A decade after SARS: strategies for controlling emerging coronaviruses. *Nat. Rev. Microbiol.* 11, 836–848 (2013).
19. Graham, R.L. & Baric, R.S. Recombination, reservoirs and the modular spike: mechanisms of coronavirus cross-species transmission. *J. Virol.* 84, 3134–3146 (2010).
20. Agnihothram, S. *et al.* A mouse model for betacoronavirus subgroup 2c using a bat coronavirus strain HKU5 variant. *MBio* 5, e00047-14 (2014).
21. Relman, D.A. Metagenomics, infectious disease diagnostics and outbreak investigations: sequence first, ask questions later? *J. Am. Med. Assoc.* 309, 1531–1532 (2013).
22. Kaiser, J. Moratorium on risky virology studies leaves work at 14 institutions in limbo. *ScienceInsider* http://news.sciencemag.org/biology/2014/11/moratorium-risky-virology-studies-leaves-work-14-institutions-limbo (2014).
23. Frieman, M. *et al.* Molecular determinants of severe acute respiratory syndrome coronavirus pathogenesis and virulence in young and aged mouse models of human disease. *J. Virol.* 86, 884–897 (2012).

© 2015 Nature America, Inc. All rights reserved.

ONLINE METHODS

Viruses, cells and *in vitro* infection and plaque assays. Wild-type SARS-CoV (Urbani), mouse-adapted SARS-CoV (MA15) and chimeric SARS-like CoVs were cultured on Vero E6 cells (obtained from United States Army Medical Research Institute of Infectious Diseases), grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, CA) and 5% fetal clone serum (FCS) (Hyclone, South Logan, UT) along with antibiotic/antimycotic (Gibco, Carlsbad, CA). DBT cells (Baric laboratory, source unknown) expressing *ACE2* orthologs have been previously described for both human and civet; *Ace2* sequence was based on that from *Rhinolophus leschenaulti* and DBT cells expressing bat *Ace2* were established as described previously[8]. Pseudotyping experiments were similar to those using an HIV-based pseudovirus, prepared as previously described[10], and examined on HeLa cells (Wuhan Institute of Virology) that expressed *ACE2* orthologs. HeLa cells were grown in minimal essential medium (MEM) (Gibco, CA) supplemented with 10% FCS (Gibco, CA) as previously described[34]. Growth curves in Vero E6, DBT, Calu-3 2B4 and primary human airway epithelial cells were performed as previously described[8,25]. None of the working cell line stocks were authenticated or tested for mycoplasma recently, although the original seed stocks used to create the working stocks are free from contamination. Human lungs for HAE cultures were procured under University of North Carolina at Chapel Hill Institutional Review Board–approved protocols. HAE cultures represent highly differentiated human airway epithelium containing ciliated and non-ciliated epithelial cells as well as goblet cells. The cultures are also grown on an air-liquid interface for several weeks before use, as previously described[26]. Briefly, cells were washed with PBS and inoculated with virus or mock-diluted in PBS for 40 min at 37 °C. After inoculation, cells were washed three times and fresh medium was added to signify time 0. Three or more biological replicates were harvested at each described time point. No blinding was used in any sample collections nor were samples randomized. All virus cultivation was performed in a biosafety level (BSL) 3 laboratory with redundant fans in the biosafety cabinets, as described previously by our group[7]. All personnel wore powered air purifying respirators (Breathe Easy, 3M) with Tyvek suits, aprons and booties and were double-gloved.

Sequence clustering and structural modeling. The full-length genomic sequences and the amino acid sequences of the S1 domains of the spike of representative CoVs were downloaded from Genbank or Pathosystems Resource Integration Center (PATRIC), aligned with ClustalX and phylogenetically compared by using maximum likelihood estimation using 100 bootstraps or by using the PhyML[1] (https://code.google.com/p/phyml/) package, respectively. The tree was generated using maximum likelihood with the PhyML package. The scale bar represents nucleotide substitutions. Only nodes with bootstrap support above 70% are labeled. The tree shows that CoVs are divided into three distinct phylogenetic groups defined as α-CoVs, β-CoVs and γ-CoVs. Classical subgroup clusters are marked as 2a, 2b, 2c and 2d for β-CoVs, and 1a and 1b for the α-CoVs. Structural models were generated using Modeller (Max Planck Institute Bioinformatics Toolkit) to generate homology models for SHC014 and Rs3367 of the SARS RBD in complex with ACE2 based on crystal structure 2AJF (Protein Data Bank). Homology models were visualized and manipulated in MacPyMol (version 1.3).

Construction of SARS-like chimeric viruses. Both wild-type and chimeric viruses were derived from either SARS-CoV Urbani or the corresponding mouse-adapted (SARS-CoV MA15) infectious clone (ic) as previously described[37]. Plasmids containing spike sequences for SHC014 were extracted by restriction digest and ligated into the E and F plasmid of the MA15 infectious clone. The clone was designed and purchased from Bio Basic as six contiguous cDNAs using published sequences flanked by unique class IIS restriction endonuclease sites (BglI). Thereafter, plasmids containing wild-type, chimeric SARS-CoV and SHC014-CoV genome fragments were amplified, excised, ligated and purified. *In vitro* transcription reactions were then preformed to synthesize full-length genomic RNA, which was transfected into Vero E6 cells as previously described[2]. The medium from transfected cells was harvested and served as seed stocks for subsequent experiments. Chimeric and full-length viruses were confirmed by sequence analysis before use in these studies. Synthetic construction of chimeric mutant and full-length SHC014-CoV was approved by the University of North Carolina Institutional Biosafety Committee and the Dual Use Research of Concern committee.

Ethics statement. This study was carried out in accordance with the recommendations for the care and use of animals by the Office of Laboratory Animal Welfare (OLAW), NIH. The Institutional Animal Care and Use Committee (IACUC) of The University of North Carolina at Chapel Hill (UNC, Permit Number A-3410-01) approved the animal study protocol (IACUC #13-033) used in these studies.

Mice and *in vivo* infection. Female, 10-week-old and 12-month-old BALB/cAnNHsD mice were ordered from Harlan Laboratories. Mouse infections were done as previously described[29]. Briefly, animals were brought into a BSL3 laboratory and allowed to acclimate for 1 week before infection. For infection and live-attenuated virus vaccination, mice were anesthetized with a mixture of ketamine and xylazine and infected intranasally, when challenged, with 50 µl of phosphate-buffered saline (PBS) or diluted virus with three or four mice per time point, per infection group per dose as described in the figure legends. For individual mice, notations for infection including failure to inhale the entire dose, bubbling of inoculum from the nose, or infection through the mouth may have led to exclusion of mouse data at the discretion of the researcher; post-infection, no other pre-established exclusion or inclusion criteria are defined. No blinding was used in any animal experiments, and animals were not randomized. For vaccination, young and aged mice were vaccinated by footpad injection with a 20-µl volume of either 0.2 µg of double-inactivated SARS-CoV vaccine with alum or mock PBS; mice were then boosted with the same regimen 22 d later and challenged 21 d thereafter. For all groups, as per protocol, animals were monitored daily for clinical signs of disease (hunching, ruffled fur and reduced activity) for the duration of the experiment. Weight loss was monitored daily for the first 7 d, after which weight monitoring continued until the animals recovered to their initial starting weight or displayed weight gain continuously for 3 d. All mice that lost greater than 20% of their starting body weight were group-fed and further monitored multiple times per day as long as they were under the 20% cutoff. Mice that lost greater than 30% of their starting body weight were immediately sacrificed as per protocol. Any mouse deemed to be moribund or unlikely to recover was also humanely sacrificed at the discretion of the researcher. Euthanasia was performed using an isoflurane overdose and death was confirmed by cervical dislocation. All mouse studies were performed at the University of North Carolina (Animal Welfare Assurance #A3410-01) using protocols approved by the UNC Institutional Animal Care and Use Committee (IACUC).

Histological analysis. The left lung was removed and submerged in 10% buffered formalin (Fisher) without inflation for 1 week. Tissues were embedded in paraffin and 5-µm sections were prepared by the UNC Lineberger Comprehensive Cancer Center histopathology core facility. To determine the extent of antigen staining, sections were stained for viral antigen using a commercially available polyclonal SARS-CoV anti-nucleocapsid antibody (Imgenex) and scored in a blinded manner by for staining of the airway and parenchyma as previously described[29]. Images were captured using an Olympus BX41 microscope with an Olympus DP71 camera.

Virus neutralization assays. Plaque reduction neutralization titer assays were performed with previously characterized antibodies against SARS-CoV, as previously described[11–13]. Briefly, neutralizing antibodies or serum was serially diluted twofold and incubated with 100 p.f.u. of the different infectious clone SARS-CoV strains for 1 h at 37 °C. The virus and antibodies were then added to a 6-well plate with 5×10^5 Vero E6 cells/well with multiple replicates ($n \geq 2$). After a 1-h incubation at 37 °C, cells were overlaid with 3 ml of 0.8% agarose in medium. Plates were incubated for 2 d at 37 °C, stained with neutral red for 3 h and plaques were counted. The percentage of plaque reduction was calculated as (1 − (no. of plaques with antibody/no. of plaques without antibody)) × 100.

© 2015 Nature America, Inc. All rights reserved.

Statistical analysis. All experiments were conducted contrasting two experimental groups (either two viruses, or vaccinated and unvaccinated cohorts). Therefore, significant differences in viral titer and histology scoring were determined by a two-tailed Students *t* test at individual time points. Data was normally distributed in each group being compared and had similar variance.

Biosafety and biosecurity. Reported studies were initiated after the University of North Carolina Institutional Biosafety Committee approved the experimental protocol (Project Title: Generating infectious clones of bat SARS-like CoVs; Lab Safety Plan ID: 20145741; Schedule G ID: 12279). These studies were initiated before the US Government Deliberative Process Research Funding Pause on Selected Gain-of-Function Research Involving Influenza, MERS and SARS Viruses (http://www.phe.gov/s3/dualuse/Documents/gain-of-function.pdf). This paper has been reviewed by the funding agency, the NIH. Continuation of these studies was requested, and this has been approved by the NIH.

SARS-CoV is a select agent. All work for these studies was performed with approved standard operating procedures (SOPs) and safety conditions for SARS-CoV, MERs-CoV and other related CoVs. Our institutional CoV BSL3 facilities have been designed to conform to the safety requirements that are recommended in the Biosafety in Microbiological and Biomedical Laboratories (BMBL), the US Department of Health and Human Services, the Public Health Service, the Centers for Disease Control (CDC) and the NIH. Laboratory safety plans were submitted to, and the facility has been approved for use by, the UNC Department of Environmental Health and Safety (EHS) and the CDC. Electronic card access is required for entry into the facility. All workers have been trained by EHS to safely use powered air purifying respirators (PAPRs), and appropriate work habits in a BSL3 facility and active medical surveillance plans are in place. Our CoV BSL3 facilities contain redundant fans, emergency power to fans and biological safety cabinets and freezers, and our facilities can accommodate SealSafe mouse racks. Materials classified as BSL3 agents consist of SARS-CoV, bat CoV precursor strains, MERS-CoV and mutants derived from these pathogens. Within the BSL3 facilities, experimentation with infectious virus is performed in a certified Class II Biosafety Cabinet (BSC). All members of the staff wear scrubs, Tyvek suits and aprons, PAPRs and shoe covers, and their hands are double-gloved. BSL3 users are subject to a medical surveillance plan monitored by the University Employee Occupational Health Clinic (UEOHC), which includes initially physical, annual influenza vaccination and mandatory reporting of any symptoms associated with CoV infection during periods when working in the BSL3. All BSL3 users are trained in exposure management and reporting protocols, are prepared to self-quarantine and have been trained for safe delivery to a local infectious disease management department in an emergency situation. All potential exposure events are reported and investigated by EHS and UEOHC, with reports filed to both the CDC and the NIH.

24. Li, W. *et al.* Difference in receptor usage between severe acute respiratory syndrome (SARS) coronavirus and SARS-like coronavirus of bat origin. *J. Virol.* 82, 1899–1907 (2008).
25. Sims, A.C. *et al.* Release of severe acute respiratory syndrome coronavirus nuclear import block enhances host transcription in human lung cells. *J. Virol.* 87, 3885–3902 (2013).
26. Fulcher, M.L., Gabriel, S., Burns, K.A., Yankaskas, J.R. & Randell, S.H. Well-differentiated human airway epithelial cell cultures. *Methods Mol. Med.* 107, 183–206 (2005).
27. Roberts, A. *et al.* A mouse-adapted SARS-coronavirus causes disease and mortality in BALB/c mice. *PLoS Pathog.* 3, e5.

© 2015 Nature America, Inc. All rights reserved.

Corrigendum: A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence

Vineet D Menachery, Boyd L Yount Jr, Kari Debbink, Sudhakar Agnihothram, Lisa E Gralinski, Jessica A Plante, Rachel L Graham, Trevor Scobey, Xing-Yi Ge, Eric F Donaldson, Scott H Randell, Antonio Lanzavecchia, Wayne A Marasco, Zhengli-Li Shi & Ralph S Baric

***Nat. Med.*; doi:10.1038/nm.3985 Corrected 20 November 2015**

In the version of this article initially published online, the authors omitted to acknowledge a funding source, USAID-EPT-PREDICT funding from EcoHealth Alliance, to Z.-L.S. The error has been corrected for the print, PDF and HTML versions of this article.

© 2015 Nature America, Inc. All rights reserved.

Calibr
at Scripps Research

ReFRAME Workflow

To improve efficiency and limit excess compound usage, ReFRAME screening follows a strict workflow. A well-defined protocol helps to reduce or eliminate time-consuming and sometimes confusing email exchanges. To ensure a successful screen, please make sure you understand the process before engaging in a ReFRAME screen.

You should have received this document as a Zip Package. To keep the hyperlinks working (example Assay Description), unzip the package to one location and do not move again.

Before Screening Begins

1	MTA or Collaboration Agreement Signed (provided by Kelli Kuhen, Director of Global Health)
2	Final *Assay Description* form for ReFRAMEDB.org delivered to *reframescreens@scripps.edu*
3	Final *Plate Layout* form delivered to *reframescreens@scripps.edu*
4	By default, partner agrees to a standard primary screen (singlicate) and two dose response sets for initial follow-up on. Dose sets are provided as replicate plates. Plates are prepared as 10-point 1:3 serial dilutions, starting at 10 µM unless otherwise discussed. Exceptions must be approved by the ReFRAME committee. For these the partner must complete the *Quantity Exception* form. More information on standard usage can be found *here*.
5	Optional: Partner sends Calibr controls dissolved in DMSO. Active concentration adjusted to match volume of the screening compounds whenever possible.

Screening Round 1

1	Calibr sends partner pre-spotted plates and compound keys (as blinded RFM IDs) for primary screen.
2	Partner performs screen and select hits (up to 1% of the library)
3	Partner sends hitlist as RFM IDs using the *Primary Screening* form.
4	Calibr sends partner two sets of pre-spotted dose response plates.
5	Collaborator sends Calibr dose response data via *Dose Response Data* form
6	Calibr sends collaborator structure and identities of confirmed hits

Additional Counter Screens (On ReFRAME committee approval)

1	In some cases, additional material for counter screen assays can be provided by Calibr. Requests for additional material or other resources will be reviewed by the ReFRAME committee. Partners are asked to complete *Screening Round 1* before additional requests for compounds.

Post Screening Begins

1	Dose response data uploaded to ReFRAMEdb.org by Calibr 1 year after partner receives structure identities. Partner can contact Calibr to request a delay under some circumstances.
2	Public success is the best way for our partners to support the ReFRAME project. Please notify Calibr of any publications, grants or studies that resulted from the ReFRAME screen using the *Milestone* document.

Calibr
at Scripps Research

How to access the ReFRAME Library

ReFRAME partnerships are determined on a case-by-case basis to avoid redundant screens, minimize compound consumption and ensure proposed assays have the requisite quality. Successful partner screens will meet the following requirements:

- Relevant to Global Health
- Execute a Material Transfer Agreement that ensures global access to data generated by the screen (12-month delay before deposition of data onto a public database maintained by Calibr (reframedb.org)
- Differentiated assay from prior or on-going screens (e.g. Pathogen species tested, clinical isolate vs. lab-adapted strain, lifecycle stage, assay readout (proliferation vs viability, etc.)
- Miniaturized and optimized to suit limited compound supply
    - Assay volume less than 50 µL (384- or 1536-well format)
    - Assay amenable to a single point screen and <1% hit rate follow-up

If approved, the typical screening partner receives ~0.5 nanomoles of each compound during primary screening (i.e., **50 nL @ 10 mM**) and up to 4 nanomoles for “hits” during the entire campaign (includes primary screen, dose response reconfirmation and counter screen).

For more information contact *reframescreens@scripps.edu*

Calibr
at Scripps Research

Calibr ReFrame Assay Form

*A screening library of 12,000 molecules assembled by combining three databases (Clarivate Integrity, GVK Excelra GoStar and Citeline Pharmaprojects) to facilitate drug repurposing. **Information provided in this form will be posted on the public reframe portal website. Example of a completed form is at the end of page. Please visit https://reframedb.org/#/assays to see more examples.*

Screening Workflow: After MTA is signed, collaborator will provide plates and control. Primary screen of compounds will be pre-spotted and sent on assay ready plates. Collaborator will determine hits up to 1%. Hits will be pre-spotted in dose response in two sets. Structures and identities of the hits from dose response will be revealed. Any deviations from typical workflow require approval from the ReFrame Committee.

General Information

Assay ID	**Provided by Calibr**
Contact Name	Click or tap here to enter text.
Contact Phone	Click or tap here to enter text.
Contact Email(s)	Click or tap here to enter text.
Mailing Address	Click or tap here to enter text.
Assay Title	Click or tap here to enter text.
Assay Title Short (less than 50 characters)	Click or tap here to enter text.
Author In format "Name, Titles (Location)"	Click or tap here to enter text.
Summary Brief sentence or two about the type of screen and the target. If secondary assay start with "Secondary assay for <assay title short>"	*Click or tap here to enter text.*

Compound Transfer Description

Assay Volume (uL of volume at time of compound exposure)

Ex: 50uL

Primary Screening Volume Transfer from 10mM Source Plates.

Ex: 50nL for 50uL assay volume at 10uM.

Plate Type. Format: Vendor Catalog number, (description)

Ex: 384-well Corning 8793 (black solid bottom)

Dose Response Volume (High point and dilution). Dose response delivered as two singlicate dose sets.

Calibr
at Scripps Research

Ex: Start at 10uM (final), 1:3 dilution, 8pt.

☐ By checking this box, I understand the primary screen of compounds will be pre-spotted and sent on assay ready plates. I, the collaborator will determine hits up to 1%. Then, hits will be pre-spotted in dose response in duplicate.

Assay Description

Description (Word count between 100-200**. The background biology needed to understand the screen. Should be detailed and specific to the assay. Keep general discussion of a disease to a minimum. Examples: the details and significance of the particular larval stage being studied in the assay; the mechanism of action of the enzyme being targeted by a biochemical screen; the benefits of your screen over other formats, details on cell type and modification if any; how your assay works to detect viability, activity or other factors etc.)**

Click or tap here to enter text.

Protocol/Workflow (A light-weight materials and methods section, but be as detailed as possible in sentence form. Please no bullet form.)

Click or tap here to enter text.

Read out and Normalization (Clarify exactly what is being measured and how. Include information on normalization routines)

Click or tap here to enter text.

Indication (Indicate disease that is targeting)

Click or tap here to enter text.

Assay Type (Type of assay: cell based, biochemical, phenotypic…)

Click or tap here to enter text.

Calibr
at Scripps Research

AC50 Units (If another metric is used, please specify)

Click or tap here to enter text.

Detection Method (Describe the way the assay is read)

Click or tap here to enter text.

Detection Reagent (Provide the reagents used for detection)

Click or tap here to enter text.

Components (Any species that are involved in the assay)

Click or tap here to enter text.

Neutral Control (Position on plate, description, and amount transferred in nL)

Click or tap here to enter text.

Stimulator or Inhibitor Control (Position on plate, description, source concentration provided by collaborator, final concentration in uM, and volume transferred in nL)

Click or tap here to enter text.

DMSO Sensitivity (Highest percentage of DMSO that assay is tolerable at)

Click or tap here to enter text.

Pre-screening Notes

Previous experiments showed a 10 fold decrease with positive control. Assay underwent several round of optimization to achieve a Z' score of 0.52. After pilot screen and consideration of control, hits will be selected when compounds reduce signal 7 standard deviations below mean. A 0.7% hit-rate was observed in the pilot with a 68% confirmation rate.)

Assay Development Statistics (Z', fold change, CV's of strongest control)

Pilot (triplicate plates) had Z' > 0.5 (10 fold change)

Calibr
at Scripps Research

Calibr Notes (leave blank)

Click or tap here to enter text.

Calibr
at Scripps Research

Pre-spotted plate description

Description of pre-potted plates for single assay and corresponding counter screens. If this description is for a secondary or counter screen, please indicate the primary screen and leave the rest blank.

For primary screen 50 nL compound from 10 mM source in singlicate (layout: described in 1_AssayRegistration.PlateLayout.xlsx)

ReFRAME Process

1	MTA Approval and partner completes *Assay Registration Description* Form
2	Partner completes *Plate Layout Form* and finalizes all details (i.e. plate type, volumes, controls, etc.). Typical concentration of compound transferred goes up to 10uM. Typical volume of compound transferred ranges from 2.5nL to 100nL from a 10mM source.
3	Partner sends Calibr plate and controls. Controls are given at 10mM in DMSO.
4	Calibr sends partner pre-spotted plates and compound keys (as blinded RFM IDs) for primary screen. Plates will be in singlicate in the format defined in *Plate Layout Form.*
5	Partner performs screen and select hits (up to 1% of the library)
6	Partner sends hitlist as RFM IDs
7	Calibr sends partner two sets of pre-spotted dose response plates. Sets are provided as replicate plates. Plates are prepared as 8-point 1:3 serial dilutions, starting at 20 uM unless otherwise discussed.
8	Collaborator sends Calibr dose response data via *Dose Response Data* form
9	Calibr sends collaborator structure and identities of confirmed hits
10	Final review of Assay Registration Description and publication date
11	In some cases, additional material for secondary assays can be provided by Calibr. Requests for additional material or other resources will be reviewed by the ReFRAME committee. Request are considered after steps 1 through 10 are complete.

Calibr
at Scripps Research

Example Calibr ReFrame Assay Form

*A screening library of 12,000 molecules assembled by combining three databases (Clarivate Integrity, GVK Excelra GoStar and Citeline Pharmaprojects) to facilitate drug repurposing. **Information provided in this form will be posted on the public reframe portal website. Example of a filled-out form is on the end of page.*

General Information

Assay ID	**Provided by Calibr**
Contact Name	Dr. Jane Doe, PhD
Contact Phone	(123-456-7890)
Contact Email(s)	JaneDoe@scripps.edu
Mailing Address	11119 N Torrey Pines Rd, La Jolla, CA 92037
Assay Title	Crypto-C. hominis high-content imaging proliferation
Assay Title Short (less than 50 characters)	Crypto-C. hominis proliferation
Author In format "Name, Titles (Location)"	Jane Doe, PhD (California Institute for Biomedical Research (Calibr), La Jolla, CA)
Summary Brief sentence or two about the type of screen and the target. If secondary assay start with "Secondary assay for <assay title short>"	Secondary assay for Crypto-C. hominis proliferation. Phenotypic high-content imaging-based high-throughput screen against the protozoan pathogen C. parvum to find new small molecule inhibitors of Cryptosporidium, which causes diarrheal disease in humans.

Compound Transfer Description

Assay Volume (uL of volume at time of compound exposure)

50uL

Primary Screening Volume Transfer from 10mM Source Plates.

50nL for 50uL assay volume at 10uM

Plate Type. Format: Vendor Catalog number, (description)

384-well Corning 8793 (black solid bottom)

Dose Response Volume (High point and dilution). Dose response delivered as two singlicate dose sets.

Start at 10uM (final), 1:3 dilution, 8pt.

☒ By checking this box, I understand the primary screen of compounds will be pre-spotted and sent on assay ready plates. I, the collaborator will determine hits up to 1%. Then, hits will be pre-spotted in dose response in duplicate.

Calibr
at Scripps Research

Assay Description

Description (Word count between 100-200**. The background biology needed to understand the screen. Should be detailed and specific to the assay. Keep general discussion of a disease to a minimum. Examples: the details and significance of the particular larval stage being studied in the assay; the mechanism of action of the enzyme being targeted by a biochemical screen; the benefits of your screen over other formats, details on cell type and modification if any; how your assay works to detect viability, activity or other factors etc.)**

This is a secondary assay for hits active against C. parvum IOWA (Sterling and Bunch Grass Farm strains). Cryptosporidium parvum and the related species Cryptosporidium hominis cause diarrheal disease in humans. The current standard of care is ineffective in patients most at risk of cryptosporidiosis. This is a confirmatory secondary high-content imaging-based high-throughput screen of the hits from the full deck against the protozoan pathogen Cryptosporidium parvum. The assay measures inhibition of parasite proliferation within the human ileocecal colorectal adenocarcinoma cell line HCT-8 (a commonly used cell line for studying Cryptosporidium spp. in vitro. HCI detection of this cell-based assay allows for concomitant measurement of Cryptosporidium parasite inhibition and host cell cytotoxicity.

Protocol/Workflow (A light-weight materials and methods section, but be as detailed as possible)

This specific ID is for C. hominis (TU502), which has been routinely passaged through neonatal gnotobiotic piglets at Tufts University by the Tzipori lab. For the assay, HCT-8 cells are seeded into 1536-well clear-bottom plates and incubated at 37C. 24 hours later, compounds are transferred (1.88 uM for primary screening and 25 uM top concentration for dose-response), and then excysted Cryptosporidium sporozoites and oocysts are dispensed onto cells. 48 hours later, cells are fixed with 4% paraformaldehyde. Cells are then washed, permeabilized with 0.25% Triton X-100 in PBS, blocked with SuperBlock (Thermo), and finally stained with a 1:10 dilution of Superblock with 1ug/mL FITC-tagged Vicia Villosa lectin and 3uM DAPI nuclear stain. Cells are then washed with PBS-T to remove residual stain and plates are sealed with aluminum adhesive seals. Plates are imaged (10x objective) on the CX5 Cellomics imager using the SpotDetector algorithm. Compounds that inhibit Cryptosporidium proliferation by >= 70% and do not inhibit HCT-8 growth by more than 40% are picked for dose response testing.

Read out and Normalization (Clarify exactly what is being measured and how. Include information on normalization routines)

High-content imaging; normalized to neutral control minus inhibitor.

Indication (Indicate disease that is targeting)

Cryptosporidiosis

Assay Type (Type of assay: cell based, biochemical, phenotypic…)

cell based, High-Content Imaging

AC50 Units (If another metric is used, please specify)

AC50 in Molar

Detection Method (Describe the way the assay is read)

High-Content Imaging; Nitazoxanide and floxuridine are used as positive controls, and DMSO is the neutral control. Spot

Counts are normalized by Neutral Controls Minus Inhibitors.

Detection Reagent (Provide the reagents used for detection)

FITC 485/521

Components (Any species that are involved in the assay)

C. hominis (TU502); HCT-8 cells; Detection reagent

Neutral Control (Position on plate, description, and amount transferred in nL)

100nL of DMSO in column 22

Stimulator or Inhibitor Control (Position on plate, description, source concentration provided by collaborator, final concentration in uM, and volume transferred in nL)

100nL of DMSO (worms added by collaborator) in column 23

DMSO Sensitivity (Highest percentage of DMSO that assay is tolerable at)

1%

Pre-screening Notes

Previous experiments showed a 10 fold decrease with positive control. Assay underwent several round of optimization to achieve a Z' score of 0.52. After pilot screen and consideration of control, hits will be selected when compounds reduce signal 7 standard deviations below mean. A 0.7% hit-rate was observed in the pilot with a 68% confirmation rate.

Assay Development Statistics (Z', fold change, CV's of strongest control)

Pilot (triplicate plates) had Z' > 0.5 (10 fold change)

California Institute for Biomedical Research

11119 N Torrey Pines Rd, La Jolla, CA 92037
(858)242-1130
echen@calibr.org

Summary of Process

Step	Description
1	MTA Approval/Pre-screening meeting
2	Assay Registration Form and Plate Layout completed
3	Collaborator sends plates and controls to be used
4	Calibr sends collaborator prespotted plates and plate ID for primary screening (one assay, singlicate, single concentration)
5	Collaborator performs screen and selects hit (<= 1% of library)
6	Collaborator sends hitlist as RFM IDs
7	Calibr sends collaborator 2 sets of dose response prespotted plates starting at 20uM high
8	Collaborator sends Calibr data from dose response experiment
9	Calibr sends collaborator identities of confirmed hits
10	Discuss for further follow up compounds

Materials Needed from Collaborator

Completet MTA and Assay Registration Form
Plates
Control
Hitlist from Primary Screen
Data from dose response experiment (used for reframe portal)

Materials Provided to Collaborator

Plates with Compounds
File with Plate layout
Stucture and smiles of compounds confirmed in dose response

Assay ready plates will be spotted exactly as defined by the information below. Please verify all calculations and concentrations are correct and match values defined in the assay description form.

PRIMARY SCREEN

	type/description	catalog number	notes	Assay Volume µL
Plate	µCLEAR®, BLACK, TC, STERILE	781092		50

PRIMARY SCREEN

Number	type	volume (nL)	source	final concentration in assay (µM)
0	empty/blank	0		na
1	compound library	50	10 mM library compounds	10
2	stimulator control	10	10 mM <cmpd name> provided by researcher for positive control	
3	DMSO/neutral	50		

One set per assay

	Screen Description
Set 1	Screen name

Transfer 1 (e.g. compounds)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
C	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
D	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
E	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
F	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
G	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
H	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
I	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
J	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
K	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
L	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
M	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
N	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
O	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
P	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

DOSE RESPONSE SCREEN

Number	type	volume (nL)	source	concentration/notes
0	empty/blank	0		na
1	compound library	50	10 mM library compounds	10 µM
2	compound library	50	library or intermediate plate	1:3 dilution
3	compound library	50	library or intermediate plate	1:3 dilution
4	compound library	50	library or intermediate plate	1:3 dilution
5	compound library	50	library or intermediate plate	1:3 dilution
6	compound library	50	library or intermediate plate	1:3 dilution
7	compound library	50	library or intermediate plate	1:3 dilution
8	compound library	50	library or intermediate plate	1:3 dilution
9	stimulator control	10	10 mM <cmpd name> provided by researcher for positive control	
10	DMSO/neutral	50		

Two sets per dose reponse follow-up

	Screen Description
Set 1	Screen name
Set 2	Counterscreen name

Transfer 1 (e.g. compounds)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	0	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	9	10	0
C	0	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	0
D	0	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	9	10	0
E	0	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	0
F	0	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	9	10	0
G	0	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	0
H	0	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	9	10	0
I	0	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	0
J	0	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	9	10	0
K	0	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	0
L	0	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	9	10	0
M	0	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	0
N	0	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	9	10	0
O	0	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	0
P	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Notes: See Notes and Rules sheet

	type/description	catalog number	notes	Assay Volume
Plate				

Number	type	volume (nL)	source	final concentration in assay
0	empty/blank	0		na
1	DMSO/neutral	100,200	OTF Plate	
2	compound library	10	10 mM or 2mM library compounds	
3	stimulator control	10	OTF Plate	

Transfer 1 (e.g. compounds)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
A	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
B	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
C	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
D	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
E	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
F	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
G	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
H	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
I	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
J	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
K	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
L	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
M	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
N	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
O	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
P	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
Q	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
R	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
S	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
T	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
U	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
V	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
W	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
X	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
Y	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
Z	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
AA	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
AB	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
AC	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
AD	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
AE	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
AF	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0

Number	type	volume (nL)	source	final concentration in assay
0	empty/blank	0		na
1	DMSO/neutral	400	DMSO plate	

Transfer 2 (e.g. backfill)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
E	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
F	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
G	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
I	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
J	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
N	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
P	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Q	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
U	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
V	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
W	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
X	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Y	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Z	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AB	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AC	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AD	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AF	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Notes: See Notes and Rules sheet

	type/description	catalog number	notes	Assay Volume
Plate				

Number	type	volume (nL)	source	final concentration in assay
0	empty/blank	0		na
1	DMSO/neutral	100	OTF Plate	
2	compound library	100	10 mM or 2mM library compounds	
3	stimulator control	10	OTF Plate	

Transfer 1 (e.g. compounds)

	1	2	3	4	5	6	7	8	9	10	11	12
A	2	2	2	2	2	2	2	2	2	2	2	1
B	2	2	2	2	2	2	2	2	2	2	2	1
C	2	2	2	2	2	2	2	2	2	2	2	1
D	2	2	2	2	2	2	2	2	2	2	2	1
E	2	2	2	2	2	2	2	2	2	2	2	3
F	2	2	2	2	2	2	2	2	2	2	2	3
G	2	2	2	2	2	2	2	2	2	2	2	3
H	2	2	2	2	2	2	2	2	2	2	2	3

Number	type	volume (nL)	source	final concentration in assay
0	empty/blank	0		na
1	DMSO/neutral	400	Combi	

Transfer 2 (e.g. backfill)

	1	2	3	4	5	6	7	8	9	10	11	12
A	2	2	2	2	2	2	2	2	2	2	2	2
B	2	2	2	2	2	2	2	2	2	2	2	2
C	2	2	2	2	2	2	2	2	2	2	2	2
D	2	2	2	2	2	2	2	2	2	2	2	2
E	2	2	2	2	2	2	2	2	2	2	2	2
F	2	2	2	2	2	2	2	2	2	2	2	2
G	2	2	2	2	2	2	2	2	2	2	2	2
H	2	2	2	2	2	2	2	2	2	2	2	2

Calibr
at Scripps Research

Why “Pre-spotted” Plates?

Calibr has worked with dozens of screening partners to provide the ReFRAME collection for screening and it is intended to fuel hundreds of assays. Because the collection is finite, we provide the collection in nanomole quantities to prevent premature depletion of the library. The typical screening partner receives ~0.5 nanomoles of each compound during primary screening (i.e., **50 nL @ 10 mM**) and up to 4 nanomoles for “hits” during the campaign (includes primary screen, dose response experiments and counter screens). To achieve this, Calibr uses acoustic dispensing to “pre-spot” assay plates or intermediate plates for our partners. In addition to conserving the library, this format limits our partners’ need for automation and information management—all tracking, dosing and compound management are performed at Calibr. After the campaign, we work with our partners to provide larger quantities for the validated hits. However, until then the library is provided in two formats:

Format 1, Pre-spotted Assay plates: Calibr “dry spots” compound directly onto assay plates and ships to partner for assay. Optimal workflow when the assay can accommodate addition of compound at the start of the assay.

Format 2, Pre-spotted Intermediate plates: Calibr “dry spots” compound onto intermediate plates and ships to partner. The partner then resuspends compounds in assay buffer for transfer to the assay plate at the appropriate time. This workflow is necessary when the partner needs to delay addition of the compound to the assay well (e.g. some adherent cell-based assays).

Exceptions can be made. However, exceptions are rare and only made when the assay is considered very high priority with no other options. We typically will not accommodate assay well volumes greater than 50 µL and we expect our partners to conserve compound to the best of their ability. To date, a large majority of ReFRAME screens have been in 384- or 1536-well format.

Summary of Standard Workflow

1	MTA Approval and partner completes *Assay Registration Description* Form
2	Partner completes *Plate Layout Form* and finalizes all details (i.e. plate type, volumes, controls, etc.). Typical concentration of compound transferred goes up to 10uM. Typical volume of compound transferred ranges from 2.5nL to 100nL from a 10mM source.
3	Partner sends Calibr plate and controls. Controls are given at 10mM in DMSO.
4	Calibr sends partner pre-spotted plates and compound keys (as blinded RFM IDs) for primary screen. Plates will be in singlicate in the format defined in *Plate Layout Form.*
5	Partner performs screen and select hits (up to 1% of the library)
6	Partner sends hitlist as RFM IDs
7	Calibr sends partner two sets of pre-spotted dose response plates. Sets are provided as replicate plates. Plates are prepared as 8-point 1:3 serial dilutions, starting at 10 µM unless otherwise discussed.
8	Collaborator sends Calibr dose response data via *Dose Response Data* form
9	Calibr sends collaborator structure and identities of confirmed hits

10	Final review of Assay Registration Description and publication date
11	In some cases, additional material for secondary assays can be provided by Calibr. Requests for additional material or other resources will be reviewed by the ReFRAME committee. Request are considered after steps 1 through 10 are complete.

Primary Screening Stats (average per plate)	example
Z' Factor	0.5
Fold Change	5.5
CV's of DMSO	7.5%
CV's of compound	10%
CV's of control (strongest control)	15%
hit selection method (fold change, Z score, etc.)	z-score
hit selection cut-off	z-score > 5

ReFRAMe IDs of Hits from Primary Screen (Only up to 1%)

RFM-xxx-xxx-x
RFM-xxx-xxx-x
RFM-xxx-xxx-x

Instructions: See examples in grey text. Hover over column headers for more information. Please include one data point of each determined hit from the dose response experiment. Highlighting not necessary, this is to point out and explain exceptions. Please delete examples upon submission.

RFM ID	ac50 (M)	Precision	Normalization Routine	Efficacy	r-squared	Datamode	Note	Examples
RFM-000-077-1	*0.000002*	*EQUAL*	*Neutral Controls Minus Inhibitors*	*-111.97322*	*0.9997*	*DECREASING*		*typical data example 1*
RFM-000-077-2	*5.00E-05*	*EQUAL*	*Neutral Controls Minus Inhibitors*	*-111.97322*	*0.910*	*INCREASING*		*typical data example 2*
RFM-000-077-3	*N/A*	*LESS THAN*	*Neutral Controls Minus Inhibitors*	*N/A*	*N/A*	*SUPER ACTIVE*	*super activity due to toxicity-- no further follow up*	
RFM-000-077-2	*5.00E-06*	*EQUAL*	*Neutral Controls Minus Inhibitors*	*NULL?*	*0.910*	*INCREASING*	*data unable to be characterized via control due to xxxxxx*	
RFM-000-077-2	*5.00E-06*	*EQUAL*	*None*	*-111.97322*	*0.910*	*INCREASING*	*data not normalized due to xxxxxxxxx*	
RFM-000-077-2	*2.00E-05*	*GREATER THAN*	*Neutral Controls Minus Inhibitors*	*-100*	*NULL?*	*INCREASING*	*r-squared vavlue cound not be calculated because of xxxxxxx*	

RFM ID	RFM ID: List RFM compound identifier of every hit. Plate-well to RFM ID keys were provided by Calibr during screening (i.e. RFM-000-077-1)
ac50 (M)	ac50 (M): Half Maximal Activity Concentration AC50, IC50, EC50 or CC50 depending on assay type. Must be a number or blank. Do not use greater than or less than signs. Indicate this in precision column.
Precision	Precision: Describe the precision of the experimental concentration using EQUAL, LESS THAN, or GREATER THAN. The result is nearly always EQUAL. -EQUAL if ac50 was found in the concentration range tested -LESS THAN for super active compounds and ac50 was below the curve -GREATER THAN if ac50 is higher than indicated by curve
Normalization Routine	Normalization Routine: Description of how data was normalized (normalized to compounds, normalized to neutral controls minus inhibitors, Robust Z-score, etc.)
Efficacy	Efficacy: Measurement of efficacy (example: compound identical to control inhibitor would be -100 if data normalized by Neutral Controls Minus Inhibitors). Must be a number or blank. If no value for effiacy, type NULL.
r-squared	r-squared: Must be a number or blank. R squared value of curve
Datamode	Datamode: Direction of the curve: DECREASING, INCREASING, SUPER ACTIVE (see notes in example)
Note	Note: Provide explaination for every exception here and any additional comments that you may want to include.

Calibr
at Scripps Research

ReFRAME Library Milestone Check-In

For the purpose of tracking ReFRAME metrics, please indicate milestone achieved with any ReFRAME hits of interest in your screen follow-up:

Current as of (Day/Month/Year)	***In vivo* activity demonstrated in relevant animal model of disease**	**Publication (add citation, or journal submission)**	**IND-enabling studies initiated for repurposed indication**	**Grant proposals funded or in the process of submission for funding approval**	**Licensing opportunity, either executed or exploring**
Yes (# of compounds)					
No					
Comments (if possible, please indicate compound name)					

Calibr
at Scripps Research

Calibr ReFrame Portal Form

*A screening library of 12,000 molecules assembled by combining three databases (Clarivate Integrity, GVK Excelra GoStar and Citeline Pharmaprojects) to facilitate drug repurposing. **Information provided in this form will be posted on the public reframe portal website. Example of a filled out form is on the end of page.*

Summary of Screening Workflow

Step 1	MTA is signed and approved
Step 2	Assay Description Form and Plate Layout is filled out
Step 3	Collaborator sends plate and control to be used
Step 4	Calibr sends pre-spotted plates for primary screening at no more than 10uM (one assay, one concentration, single copy)
Step 5	Collaborator performs screen and select hits up to ~1%
Step 6	Calibr receives hitlist as RFM ID and sends 2 sets of pre-spotted 8 point dose response plates (starting at 20uM highest concentration with 1 to 3 dilution)
Step 7	Collaborator sends dose response hits data (ac50, r squared, efficacy, normalization)
Step 8	Calibr sends collaborator identities and structure of confirmed hits
Step 9	Discuss providing larger quantities of final hits
Deviations from Screening Workflow must be approved by Director of Global Health Program	Approval Signature:_____ Approval Date:_____

General Information

Assay ID	**Provided by Calibr**
Assay Title	
Assay Title Short (less than 50 characters)	
Author in format "Name, Titles (Location)"	
Summary (Brief sentence or two about the type of screen and the target)	

© 2018 Bill & Melinda Gates Foundation

Calibr
at Scripps Research

Assay Description

Description (Word count between 100-200**. The background biology needed to understand the screen. Should be detailed and specific to the assay. Keep general discussion of a disease to a minimum. Examples: the details and significance of the particular larval stage being studied in the assay; the mechanism of action of the enzyme being targeted by a biochemical screen; the benefits of your screen over other formats, details on cell type and modification if any; how your assay works to detect viability, activity or other factors etc.)**

Protocol/Workflow (A light-weight materials and methods section, but be as detailed as possible)

Detection/measurement (Clarify exactly what is being measure and how. Include information on normalization routines)

Indication (Indicate disease that is targeting)

Assay Type (Type of assay: cell based, biochemical, phenotypic…)

Calibr
at Scripps Research

Detection Method (Describe the way the assay is read)

Detection Reagent (Provide the reagents used for detection)

Components (Any species that are involved in the assay)

Drug Concentration (Final concentration of compounds and volume dispensed from 10mM or 2mM source)

Plate Type (Provide catalog number and description)

Calibr
at Scripps Research

Example Calibr ReFrame Portal Form

*A screening library of 12,000 molecules assembled by combining three databases (Clarivate Integrity, GVK Excelra GoStar and Citeline Pharmaprojects) to facilitate drug repurposing. **Information provided in this form will be posted on the public reframe portal website. Example of a filled out form is on the end of page.*

General Information

Assay ID	**Provided by Calibr**
Assay Title	**Helminth: B. malayi microfilariae (6 Day)**
Assay Title Short (less than 50 characters)	**Helminth: B. malayi microfilariae (6 Day)**
Author in format "Name, Titles (Location)"	**Jane Doe, PhD (University of California, San Francisco)**
Summary (Brief sentence or two about the type of screen and the target)	**Brugia pahangi worm motility assay**

Assay Description

Description (Word count between 100-200**. The background biology needed to understand the screen. Should be detailed and specific to the assay. Keep general discussion of a disease to a minimum. Examples: the details and significance of the particular larval stage being studied in the assay; the mechanism of action of the enzyme being targeted by a biochemical screen; the benefits of your screen over other formats, details on cell type and modification if any; how your assay works to detect viability, activity or other factors etc.)**

Adult female Brugia pahangi worms are used in the primary screens to identify a macrofilaricidal candidate. Adult female Brugia are initially screened in quadruplicate in 24 well plates at a single concentration from 1-10μM. Worms treated with 1% DMSO only are used as controls. Worms are kept in a 37°C, 5% CO2 incubator for the duration of the assay. Worm motility (phenotypic screening) is assessed on Day 0 (the day worms are treated with compound), Day 1, Day 2, Day 3, and Day 6 using an imaging system called The Worminator. Mean movement units (worms moving are assessed as pixels displaced per second) generated from The Worminator on Day 3 and Day 6 are converted into % inhibitions of worm motility compared to controls treated with 1% DMSO only. A compound hit is defined as one that inhibits the motility of the adult female worm ≥ 75% by Day 6 of the assay. If a compound inhibits motility of the worms consistently in at least two assays at single concentrations, an IC50 is performed using a 6-point serial dilution starting at 30μM. IC50s are calculated using GraphPad Prism.

Protocol/Workflow (A light-weight materials and methods section, but be as detailed as possible)

Adult female Brugia pahangi were shipped from Univ. of Missouri-Columbia and assayed using methods described by Marcellino et al. (2012). Individual females are placed in each well of a 24-well plate with media (RPMI-1640 with 25 mM HEPES, 2.0 g/L NaHCO3, 5% heat inactivated FBS, and 1X Antibiotic/Antimycotic solution). Worms are transferred to new 24-well plates upon arrival with 500 μL of media per well. Initial ReFrame compound screening is conducted at 3 μM per compound, and 1% DMSO is used as a negative control. All drugs are dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) stock solutions are stored at -20°C. Four worms were used as replicates for each concentration and worm plates are kept in a 37°C, 5% CO2 incubator for the duration of the assay except during motility readings. To determine the effect of a compound on worm motility, individual worm movement are counted as the number of pixels displaced per second by

each worm in each well using the Worminator. Each plate of worms is video recorded for approximately 60 seconds, and mean movement units (MMUs) are determined for individual worms. Percent inhibition of motility is calculated by dividing the MMUs of the treated worms by the control average MMUs, subtracting the value from 1.0, flooring the values to zero and multiplying by 100%. Videos are recorded each day, including the first day of the assay (Day 0). Compounds that inhibit worm motility can be tested in IC50 assays. Prism is used to calculate IC50 values using a non-linear regression curve fit.

Detection/measurement (Clarify exactly what is being measure and how. Include information on normalization routines)

Individual worm movements are counted as number of pixels displaced per second by each worm in each well using the Worminator. Each plate of worms is video recorded for approximately 60 seconds, and the mean movement units (MMUs) are determined for individual worms. Percent inhibition of motility is calculated by dividing the MMUs of the treated worms by the control average MMUs, subtracting the value from 1.0, flooring the values to zero and multiplying by 100%. Videos are recorded each day, including the first day of the assay (Day 0). Compounds that inhibit worm motility can be tested in IC50 assays. Prism is used to calculate IC50 values using a non-linear regression curve fit.

Indication (Indicate disease that is targeting)

Lymphatic Filariasis

Assay Type (Type of assay: cell based, biochemical, phenotypic…)

Cell based, Phenotypic

Detection Method (Describe the way the assay is read)

Video motion capture

Detection Reagent (Provide the reagents used for detection)

None

Components (Any species that are involved in the assay)

Adult female Brugia pahangi worms

Drug Concentration (Final concentration of compounds and volume dispensed from 10mM or 2mM source)

15 nL cmpd (2 mM) into 10 µL assay buffer. 3 µM final concentration

Plate Type (Provide catalog number and description)

1536 well black clear bottom plate from Greiner 789091-F

Calibr
at Scripps Research

Calibr ReFrame: Request Format Exception

Because the collection is finite and intended for dozens of partners, each partner is expected to minimize library consumption. A majority of ReFRAME screens are performed in singlicate and miniaturized to a 384 or 1536-well format. However, we recognize this is not always possible (i.e. larger parasites). Please justify your need for an exception. This will be reviewed by the ReFRAME committee and must be approved by Calibr's Director of Global Health.

thepharmaletter
Up to date news for the Pharmaceutical and Biotechnology industries

In vitro promise of Aplidin for COVID- 19 sends PharmaMar upwards

13-03-2020

A bolstering of hopes that Spanish drugmaker PharmaMar (MSE: PHM) might have an answer for the COVID-19 coronavirus sent the comp share price spiralling upwards on Friday.

PharmaMar was 38% higher later on Friday, having earlier in the day shown gains of more than 50%, as markets digested an update on a d that the company sees as a potential treatment for the virus.

The firm reported that *in vitro* studies results of Aplidin (plitidepsin) on the human coronavirus HCoV-229E, which has a multiplication and propagation mechanism very similar to Covid-19, have been positive with a potency of the nanomolar order.

These studies have been carried out at the National Biotechnology Centre of the Spanish National Research Council. They confirm the hypothesis that the therapeutic target of Aplidin (plitidepsin), which is EF1A, is key to the multiplication and spread of the virus.

PharmaMar has said that it will contact regulatory authorities to analyze the possibilities of studies on patients infected with Covid-19.

Aplidin (plitidepsin) is a compound that is currently approved in Australia for the treatment of multiple myeloma, having been rejected by the European Medicines Agency (EMA) in 2017. That decision is being appealed in the European Court of Justice. And, in January 2019, Pharn

This website uses cookies to improve your experience. Continue if you are OK with this.
Please read our privacy policy for more details.

Anti-virals Aplidin Biotechnology Coronavirus Drug Trial Focus On Oncology PharmaMar Research Spain

This website uses cookies to improve your experience. Continue if you are OK with this.

*

Please read our privacy policy for more details.

Preclinical therapy information request form:
BOLD: Concept categories (Individual data request questions are below)
ITALICS: Actual input expected from the request.

Therapy Sponsor

Name of contact
Therapy owner affiliation
Therapy sponsor affiliation (if not the same as owner)

Summary paragraph of compound and presumed target of action (virus/host). *(250 words or less)*

Summary of any existing data in humans (stage, initial indication, safety issues) *(500 words or less)*

For antivirals

1) Biochemical Assay
    a. Specificity Index (other protein targets in class; *PanLabs or equivalent*) *(what is the metric?)*
2) Cell types tested: EC50, CC50
    a. Primary Screen cell type (*EC50, CC50)*
    b. Secondary screen cell type *(EC50, CC50)*
3) Cell type endpoints tested:
    a. Reduction in viral yield assay *(how many fold, at what concentration)*
4) Graph of dose-dependency of (1) and (2)
5) Data in small animal infection models for viral targets. *(what is the metric?)*

For immunomodulatory agents.

2) Biochemical Assay
    a. Specificity Index (other protein targets in class; PanLabs or equivalent) *(what is the metric?)*
3) Cellular assays
    a. Specificity index *(what is the metric?)*
    b. Cytotoxicity determination *(what is the metric?)*
4) Small animal model data (Transgenic animals useful but not always predictive)
    a. Animal models tested (target based); more than 2 are preferred.
    b. Specificity of model to target pathway *(what is the metric?)*
    c. Animal models tested (infection based?: [preferred model like hamster?])
    d. Expected off-target activities of immunomodulator *(what is the metric?)*

PK/PD data

1) Rodent
    a. the fold achievable free-drug over the IC50s in the secondary in vitro assay
2) Non-rodent (or NHP PK or Human PK if available)
    a. the fold achievable free-drug over the IC50s in the secondary in vitro assay
    b. Plasma Protein Binding *(what is the metric?)*
3) Acute Toxicity (*what is the metric?)*
4) Gene Toxicity (*what is the metric?)*
5) Expected Drug-drug interactions (*what is the metric?)*
6) If pro-drug: conversion in animals vs. human (*what is the metric?)*
7) Human PK prediction (*what is the metric?)*

Lung tissue penetration in model studies.

1) Route *(oral/IV/pulmonary)*
2) Partition function (*percent lung exposure compared to whole body*)
3) Solubility (*what is the metric?* And is this a PK item?)

Clinical data (if any)

1) Previous indication *(disease(s))*
    a. *Stage of development*
    b.
2) Human Efficacy data in previous indication
3) Human PK/PD data
    a. the fold achievable free-drug over the IC50s in the secondary in vitro assay
    b. Plasma Protein Binding *(what is the metric?)*
4) Human Safety data
5) Availability (*what is the metric?)*

Calibr
at Scripps Research

Calibr ReFrame Assay Form

*A screening library of 12,000 molecules assembled by combining three databases (Clarivate Integrity, GVK Excelra GoStar and Citeline Pharmaprojects) to facilitate drug repurposing. **Information provided in this form will be posted on the public reframe portal website. Example of a completed form is at the end of page. Please visit https://reframedb.org/#/assays to see more examples.*

Screening Workflow: After MTA is signed, collaborator will provide plates and control. Primary screen of compounds will be pre-spotted and sent on assay ready plates. Collaborator will determine hits up to 1%. Hits will be pre-spotted in dose response in two sets. Structures and identities of the hits from dose response will be revealed. Any deviations from typical workflow require approval from the ReFrame Committee.

General Information

Assay ID	**Provided by Calibr**
Contact Name	Click or tap here to enter text.
Contact Phone	Click or tap here to enter text.
Contact Email(s)	Click or tap here to enter text.
Mailing Address	Click or tap here to enter text.
Assay Title	Click or tap here to enter text.
Assay Title Short (less than 50 characters)	Click or tap here to enter text.
Author In format "Name, Titles (Location)"	Click or tap here to enter text.
Summary Brief sentence or two about the type of screen and the target. If secondary assay start with "Secondary assay for <assay title short>"	*Click or tap here to enter text.*

Compound Transfer Description

Assay Volume (uL of volume at time of compound exposure)

Ex: 50uL

Primary Screening Volume Transfer from 10mM Source Plates. (For cell-based assays, consider toxicity. A higher concentration (i.e. 10 µM) can lead to false negatives in many assays.)

Ex: 50nL for 50uL assay volume at 10uM.

Plate Type. Format: Vendor Catalog number, (description)

Ex: 384-well Corning 8793 (black solid bottom)

Calibr
at Scripps Research

Dose Response Volume (High point and dilution). Dose response delivered as two singlicate dose sets.

Ex: Start at 10uM (final), 1:3 dilution, 8pt.

☐ By checking this box, I understand the primary screen of compounds will be pre-spotted and sent on assay ready plates. I, the collaborator, will determine hits up to 1%. Then, hits will be pre-spotted in dose response in duplicate.

Assay Description

Description (Word count between 100-200**. The background biology needed to understand the screen. Should be detailed and specific to the assay. Keep general discussion of a disease to a minimum. Examples: the details and significance of the particular larval stage being studied in the assay; the mechanism of action of the enzyme being targeted by a biochemical screen; the benefits of your screen over other formats, details on cell type and modification if any; how your assay works to detect viability, activity or other factors etc.)**

Click or tap here to enter text.

Protocol/Workflow (A light-weight materials and methods section, but be as detailed as possible in sentence form. Please no bullet form.)

Click or tap here to enter text.

Read out and Normalization (Clarify exactly what is being measured and how. Include information on normalization routines)

Click or tap here to enter text.

Indication (Indicate disease that is targeting)

Click or tap here to enter text.

Assay Type (Type of assay: cell based, biochemical, phenotypic…)

Calibr
at Scripps Research

Click or tap here to enter text.

AC50 Units (If another metric is used, please specify)

Click or tap here to enter text.

Detection Method (Describe the way the assay is read)

Click or tap here to enter text.

Detection Reagent (Provide the reagents used for detection)

Click or tap here to enter text.

Components (Any species that are involved in the assay)

Click or tap here to enter text.

Neutral Control (Position on plate, description, and amount transferred in nL)

Click or tap here to enter text.

Stimulator or Inhibitor Control (Position on plate, description, source concentration provided by collaborator, final concentration in uM, and volume transferred in nL)

Click or tap here to enter text.

DMSO Sensitivity (Highest percentage of DMSO that assay is tolerable at)

Click or tap here to enter text.

Pre-screening Notes

Previous experiments showed a 10 fold decrease with positive control. Assay underwent several round of optimization to achieve a Z' score of 0.52. After pilot screen and consideration of control, hits will be selected when compounds reduce signal 7 standard deviations below mean. A 0.7% hit-rate was observed in the pilot with a 68% confirmation rate.)

Assay Development Statistics (Z', fold change, CV's of strongest control)

Pilot (triplicate plates) had Z' > 0.5 (10 fold change)

Calibr
at Scripps Research

Calibr Notes (leave blank)

Click or tap here to enter text.

Calibr
at Scripps Research

Pre-spotted plate description

Description of pre-potted plates for single assay and corresponding counter screens. If this description is for a secondary or counter screen, please indicate the primary screen and leave the rest blank.

For primary screen 50 nL compound from 10 mM source in singlicate (layout: described in 1_AssayRegistration.PlateLayout.xlsx)

ReFRAME Process

1	MTA Approval and partner completes *Assay Registration Description* Form
2	Partner completes *Plate Layout Form* and finalizes all details (i.e. plate type, volumes, controls, etc.). Typical concentration of compound transferred goes up to 10uM. Typical volume of compound transferred ranges from 2.5nL to 100nL from a 10mM source.
3	Partner sends Calibr plate and controls. Controls are given at 10mM in DMSO.
4	Calibr sends partner pre-spotted plates and compound keys (as blinded RFM IDs) for primary screen. Plates will be in singlicate in the format defined in *Plate Layout Form.*
5	Partner performs screen and select hits (up to 1% of the library)
6	Partner sends hitlist as RFM IDs
7	Calibr sends partner two sets of pre-spotted dose response plates. Sets are provided as replicate plates. Plates are prepared as 8-point 1:3 serial dilutions, starting at 20 uM unless otherwise discussed.
8	Collaborator sends Calibr dose response data via *Dose Response Data* form
9	Calibr sends collaborator structure and identities of confirmed hits
10	Final review of Assay Registration Description and publication date
11	In some cases, additional material for secondary assays can be provided by Calibr. Requests for additional material or other resources will be reviewed by the ReFRAME committee. Request are considered after steps 1 through 10 are complete.

Calibr
at Scripps Research

Example Calibr ReFrame Assay Form

*A screening library of 12,000 molecules assembled by combining three databases (Clarivate Integrity, GVK Excelra GoStar and Citeline Pharmaprojects) to facilitate drug repurposing. **Information provided in this form will be posted on the public reframe portal website. Example of a filled-out form is on the end of page.*

General Information

Assay ID	**Provided by Calibr**
Contact Name	Dr. Jane Doe, PhD
Contact Phone	(123-456-7890)
Contact Email(s)	JaneDoe@scripps.edu
Mailing Address	11119 N Torrey Pines Rd, La Jolla, CA 92037
Assay Title	Crypto-C. hominis high-content imaging proliferation
Assay Title Short (less than 50 characters)	Crypto-C. hominis proliferation
Author In format "Name, Titles (Location)"	Jane Doe, PhD (California Institute for Biomedical Research (Calibr), La Jolla, CA)
Summary Brief sentence or two about the type of screen and the target. If secondary assay start with "Secondary assay for <assay title short>"	Secondary assay for Crypto-C. hominis proliferation. Phenotypic high-content imaging-based high-throughput screen against the protozoan pathogen C. parvum to find new small molecule inhibitors of Cryptosporidium, which causes diarrheal disease in humans.

Compound Transfer Description

Assay Volume (uL of volume at time of compound exposure)

50uL

Primary Screening Volume Transfer from 10mM Source Plates.

50nL for 50uL assay volume at 10uM

Plate Type. Format: Vendor Catalog number, (description)

384-well Corning 8793 (black solid bottom)

Dose Response Volume (High point and dilution). Dose response delivered as two singlicate dose sets.

Start at 10uM (final), 1:3 dilution, 8pt.

☒ By checking this box, I understand the primary screen of compounds will be pre-spotted and sent on assay ready plates. I, the collaborator will determine hits up to 1%. Then, hits will be pre-spotted in dose response in duplicate.

Assay Description

Description (Word count between 100-200**. The background biology needed to understand the screen. Should be detailed and specific to the assay. Keep general discussion of a disease to a minimum. Examples: the details and significance of the particular larval stage being studied in the assay; the mechanism of action of the enzyme being targeted by a biochemical screen; the benefits of your screen over other formats, details on cell type and modification if any; how your assay works to detect viability, activity or other factors etc.)**

This is a secondary assay for hits active against C. parvum IOWA (Sterling and Bunch Grass Farm strains). Cryptosporidium parvum and the related species Cryptosporidium hominis cause diarrheal disease in humans. The current standard of care is ineffective in patients most at risk of cryptosporidiosis. This is a confirmatory secondary high-content imaging-based high-throughput screen of the hits from the full deck against the protozoan pathogen Cryptosporidium parvum. The assay measures inhibition of parasite proliferation within the human ileocecal colorectal adenocarcinoma cell line HCT-8 (a commonly used cell line for studying Cryptosporidium spp. in vitro. HCI detection of this cell-based assay allows for concomitant measurement of Cryptosporidium parasite inhibition and host cell cytotoxicity.

Protocol/Workflow (A light-weight materials and methods section, but be as detailed as possible)

This specific ID is for C. hominis (TU502), which has been routinely passaged through neonatal gnotobiotic piglets at Tufts University by the Tzipori lab. For the assay, HCT-8 cells are seeded into 1536-well clear-bottom plates and incubated at 37C. 24 hours later, compounds are transferred (1.88 uM for primary screening and 25 uM top concentration for dose-response), and then excysted Cryptosporidium sporozoites and oocysts are dispensed onto cells. 48 hours later, cells are fixed with 4% paraformaldehyde. Cells are then washed, permeabilized with 0.25% Triton X-100 in PBS, blocked with SuperBlock (Thermo), and finally stained with a 1:10 dilution of Superblock with 1ug/mL FITC-tagged Vicia Villosa lectin and 3uM DAPI nuclear stain. Cells are then washed with PBS-T to remove residual stain and plates are sealed with aluminum adhesive seals. Plates are imaged (10x objective) on the CX5 Cellomics imager using the SpotDetector algorithm. Compounds that inhibit Cryptosporidium proliferation by >= 70% and do not inhibit HCT-8 growth by more than 40% are picked for dose response testing.

Read out and Normalization (Clarify exactly what is being measured and how. Include information on normalization routines)

High-content imaging; normalized to neutral control minus inhibitor.

Indication (Indicate disease that is targeting)

Cryptosporidiosis

Assay Type (Type of assay: cell based, biochemical, phenotypic…)

cell based, High-Content Imaging

AC50 Units (If another metric is used, please specify)

AC50 in Molar

Detection Method (Describe the way the assay is read)

High-Content Imaging; Nitazoxanide and floxuridine are used as positive controls, and DMSO is the neutral control. Spot

Counts are normalized by Neutral Controls Minus Inhibitors.

Detection Reagent (Provide the reagents used for detection)

FITC 485/521

Components (Any species that are involved in the assay)

C. hominis (TU502); HCT-8 cells; Detection reagent

Neutral Control (Position on plate, description, and amount transferred in nL)

100nL of DMSO in column 22

Stimulator or Inhibitor Control (Position on plate, description, source concentration provided by collaborator, final concentration in uM, and volume transferred in nL)

100nL of DMSO (worms added by collaborator) in column 23

DMSO Sensitivity (Highest percentage of DMSO that assay is tolerable at)

1%

Pre-screening Notes

Previous experiments showed a 10 fold decrease with positive control. Assay underwent several round of optimization to achieve a Z' score of 0.52. After pilot screen and consideration of control, hits will be selected when compounds reduce signal 7 standard deviations below mean. A 0.7% hit-rate was observed in the pilot with a 68% confirmation rate.

Assay Development Statistics (Z', fold change, CV's of strongest control)

Pilot (triplicate plates) had Z' > 0.5 (10 fold change)

California Institute for Biomedical Research

11119 N Torrey Pines Rd, La Jolla, CA 92037
(858)242-1130
echen@calibr.org

Summary of Process

Step	Description
1	MTA Approval/Pre-screening meeting
2	Assay Registration Form and Plate Layout completed
3	Collaborator sends plates and controls to be used
4	Calibr sends collaborator prespotted plates and plate ID for primary screening (one assay, singlicate, single concentration)
5	Collaborator performs screen and selects hit (<= 1% of library)
6	Collaborator sends hitlist as RFM IDs
7	Calibr sends collaborator 2 sets of dose response prespotted plates starting at 20uM high
8	Collaborator sends Calibr data from dose response experiment
9	Calibr sends collaborator identities of confirmed hits
10	Discuss for further follow up compounds

Materials Needed from Collaborator

Completet MTA and Assay Registration Form
Plates
Control
Hitlist from Primary Screen
Data from dose response experiment (used for reframe portal)

Materials Provided to Collaborator

Plates with Compounds
File with Plate layout
Stucture and smiles of compounds confirmed in dose response

Assay ready plates will be spotted exactly as defined by the information below. Please verify all calculations and concentrations are correct and match values defined in the assay description form.

PRIMARY SCREEN

	type/description	catalog number	notes	Assay Volume µL
Plate	µCLEAR®, BLACK, TC, STERILE	781092		50

PRIMARY SCREEN

Number	type	volume (nL)	source	final concentration in assay (µM)
0	empty/blank	0		na
1	compound library	50	10 mM library compounds	10
2	stimulator control	10	10 mM <cmpd name> provided by researcher for positive control	
3	DMSO/neutral	50		

One set per assay

	Screen Description
Set 1	Screen name

Transfer 1 (e.g. compounds)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
C	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
D	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
E	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
F	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
G	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
H	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
I	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
J	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
K	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
L	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
M	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
N	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
O	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	0
P	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

DOSE RESPONSE SCREEN

Number	type	volume (nL)	source	concentration/notes
0	empty/blank	0		na
1	compound library	50	10 mM library compounds	10 µM
2	compound library	50	library or intermediate plate	1:3 dilution
3	compound library	50	library or intermediate plate	1:3 dilution
4	compound library	50	library or intermediate plate	1:3 dilution
5	compound library	50	library or intermediate plate	1:3 dilution
6	compound library	50	library or intermediate plate	1:3 dilution
7	compound library	50	library or intermediate plate	1:3 dilution
8	compound library	50	library or intermediate plate	1:3 dilution
9	stimulator control	10	10 mM <cmpd name> provided by researcher for positive control	
10	DMSO/neutral	50		

Two sets per dose reponse follow-up

	Screen Description
Set 1	Screen name
Set 2	Counterscreen name

Transfer 1 (e.g. compounds)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	0	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	9	10	0
C	0	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	0
D	0	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	9	10	0
E	0	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	0
F	0	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	9	10	0
G	0	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	0
H	0	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	9	10	0
I	0	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	0
J	0	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	9	10	0
K	0	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	0
L	0	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	9	10	0
M	0	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	0
N	0	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	9	10	0
O	0	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9	10	0
P	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Notes: See Notes and Rules sheet

	type/description	catalog number	notes	Assay Volume
Plate				

Number	type	volume (nL)	source	final concentration in assay
0	empty/blank	0		na
1	DMSO/neutral	100,200	OTF Plate	
2	compound library	10	10 mM or 2mM library compounds	
3	stimulator control	10	OTF Plate	

Transfer 1 (e.g. compounds)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
A	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
B	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
C	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
D	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
E	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
F	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
G	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
H	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
I	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
J	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
K	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
L	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
M	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
N	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
O	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
P	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
Q	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
R	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
S	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
T	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
U	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
V	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
W	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
X	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
Y	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
Z	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
AA	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
AB	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
AC	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
AD	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
AE	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0
AF	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	1	0	0

Number	type	volume (nL)	source	final concentration in assay
0	empty/blank	0		na
1	DMSO/neutral	400	DMSO plate	

Transfer 2 (e.g. backfill)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
E	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
F	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
G	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
I	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
J	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
N	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
P	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Q	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
R	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
U	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
V	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
W	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
X	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Y	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Z	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AB	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AC	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AD	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AF	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Notes: See Notes and Rules sheet

	type/description	catalog number	notes	Assay Volume
Plate				

Number	type	volume (nL)	source	final concentration in assay
0	empty/blank	0		na
1	DMSO/neutral	100	OTF Plate	
2	compound library	100	10 mM or 2mM library compounds	
3	stimulator control	10	OTF Plate	

Transfer 1 (e.g. compounds)

	1	2	3	4	5	6	7	8	9	10	11	12
A	2	2	2	2	2	2	2	2	2	2	2	1
B	2	2	2	2	2	2	2	2	2	2	2	1
C	2	2	2	2	2	2	2	2	2	2	2	1
D	2	2	2	2	2	2	2	2	2	2	2	1
E	2	2	2	2	2	2	2	2	2	2	2	3
F	2	2	2	2	2	2	2	2	2	2	2	3
G	2	2	2	2	2	2	2	2	2	2	2	3
H	2	2	2	2	2	2	2	2	2	2	2	3

Number	type	volume (nL)	source	final concentration in assay
0	empty/blank	0		na
1	DMSO/neutral	400	Combi	

Transfer 2 (e.g. backfill)

	1	2	3	4	5	6	7	8	9	10	11	12
A	2	2	2	2	2	2	2	2	2	2	2	2
B	2	2	2	2	2	2	2	2	2	2	2	2
C	2	2	2	2	2	2	2	2	2	2	2	2
D	2	2	2	2	2	2	2	2	2	2	2	2
E	2	2	2	2	2	2	2	2	2	2	2	2
F	2	2	2	2	2	2	2	2	2	2	2	2
G	2	2	2	2	2	2	2	2	2	2	2	2
H	2	2	2	2	2	2	2	2	2	2	2	2

Calibr
at Scripps Research

Why “Pre-spotted” Plates?

Calibr has worked with dozens of screening partners to provide the ReFRAME collection for screening and it is intended to fuel hundreds of assays. Because the collection is finite, we provide the collection in nanomole quantities to prevent premature depletion of the library. The typical screening partner receives ~0.5 nanomoles of each compound during primary screening (i.e., **50 nL @ 10 mM**) and up to 4 nanomoles for “hits” during the campaign (includes primary screen, dose response experiments and counter screens). To achieve this, Calibr uses acoustic dispensing to “pre-spot” assay plates or intermediate plates for our partners. In addition to conserving the library, this format limits our partners’ need for automation and information management—all tracking, dosing and compound management are performed at Calibr. After the campaign, we work with our partners to provide larger quantities for the validated hits. However, until then the library is provided in two formats:

Format 1, Pre-spotted Assay plates: Calibr “dry spots” compound directly onto assay plates and ships to partner for assay. Optimal workflow when the assay can accommodate addition of compound at the start of the assay.

Format 2, Pre-spotted Intermediate plates: Calibr “dry spots” compound onto intermediate plates and ships to partner. The partner then resuspends compounds in assay buffer for transfer to the assay plate at the appropriate time. This workflow is necessary when the partner needs to delay addition of the compound to the assay well (e.g. some adherent cell-based assays).

Exceptions can be made. However, exceptions are rare and only made when the assay is considered very high priority with no other options. We typically will not accommodate assay well volumes greater than 50 µL and we expect our partners to conserve compound to the best of their ability. To date, a large majority of ReFRAME screens have been in 384- or 1536-well format.

Summary of Standard Workflow

1	MTA Approval and partner completes *Assay Registration Description* Form
2	Partner completes *Plate Layout Form* and finalizes all details (i.e. plate type, volumes, controls, etc.). Typical concentration of compound transferred goes up to 10uM. Typical volume of compound transferred ranges from 2.5nL to 100nL from a 10mM source.
3	Partner sends Calibr plate and controls. Controls are given at 10mM in DMSO.
4	Calibr sends partner pre-spotted plates and compound keys (as blinded RFM IDs) for primary screen. Plates will be in singlicate in the format defined in *Plate Layout Form.*
5	Partner performs screen and select hits (up to 1% of the library)
6	Partner sends hitlist as RFM IDs
7	Calibr sends partner two sets of pre-spotted dose response plates. Sets are provided as replicate plates. Plates are prepared as 8-point 1:3 serial dilutions, starting at 10 µM unless otherwise discussed.
8	Collaborator sends Calibr dose response data via *Dose Response Data* form
9	Calibr sends collaborator structure and identities of confirmed hits

10	Final review of Assay Registration Description and publication date
11	In some cases, additional material for secondary assays can be provided by Calibr. Requests for additional material or other resources will be reviewed by the ReFRAME committee. Request are considered after steps 1 through 10 are complete.

Primary Screening Stats (average per plate)	example
Z' Factor	0.5
Fold Change	5.5
CV's of DMSO	7.5%
CV's of compound	10%
CV's of control (strongest control)	15%
hit selection method (fold change, Z score, etc.)	z-score
hit selection cut-off	z-score > 5

ReFRAMe IDs of Hits from Primary Screen (Only up to 1%)

RFM-xxx-xxx-x
RFM-xxx-xxx-x
RFM-xxx-xxx-x

Instructions: See examples in grey text. Hover over column headers for more information. Please include one data point of each determined hit from the dose response experiment. Highlighting not necessary, this is to point out and explain exceptions. Please delete examples upon submission.

RFM ID	ac50 (M)	Precision	Normalization Routine	Efficacy	r-squared	Datamode	Note	Examples
RFM-000-077-1	*0.000002*	*EQUAL*	*Neutral Controls Minus Inhibitors*	*-111.97322*	*0.9997*	*DECREASING*		*typical data example 1*
RFM-000-077-2	*5.00E-05*	*EQUAL*	*Neutral Controls Minus Inhibitors*	*-111.97322*	*0.910*	*INCREASING*		*typical data example 2*
RFM-000-077-3	*N/A*	*LESS THAN*	*Neutral Controls Minus Inhibitors*	*N/A*	*N/A*	*SUPER ACTIVE*	*super activity due to toxicity-- no further follow up*	
RFM-000-077-2	*5.00E-06*	*EQUAL*	*Neutral Controls Minus Inhibitors*	*NULL?*	*0.910*	*INCREASING*	*data unable to be characterized via control due to xxxxxx*	
RFM-000-077-2	*5.00E-06*	*EQUAL*	*None*	*-111.97322*	*0.910*	*INCREASING*	*data not normalized due to xxxxxxxxx*	
RFM-000-077-2	*2.00E-05*	*GREATER THAN*	*Neutral Controls Minus Inhibitors*	*-100*	*NULL?*	*INCREASING*	*r-squared vavlue cound not be calculated because of xxxxxxx*	

RFM ID	RFM ID: List RFM compound identifier of every hit. Plate-well to RFM ID keys were provided by Calibr during screening (i.e. RFM-000-077-1)
ac50 (M)	ac50 (M): Half Maximal Activity Concentration AC50, IC50, EC50 or CC50 depending on assay type. Must be a number or blank. Do not use greater than or less than signs. Indicate this in precision column.
Precision	Precision: Describe the precision of the experimental concentration using EQUAL, LESS THAN, or GREATER THAN. The result is nearly always EQUAL. -EQUAL if ac50 was found in the concentration range tested -LESS THAN for super active compounds and ac50 was below the curve -GREATER THAN if ac50 is higher than indicated by curve
Normalization Routine	Normalization Routine: Description of how data was normalized (normalized to compounds, normalized to neutral controls minus inhibitors, Robust Z-score, etc.)
Efficacy	Efficacy: Measurement of efficacy (example: compound identical to control inhibitor would be -100 if data normalized by Neutral Controls Minus Inhibitors). Must be a number or blank. If no value for effiacy, type NULL.
r-squared	r-squared: Must be a number or blank. R squared value of curve
Datamode	Datamode: Direction of the curve: DECREASING, INCREASING, SUPER ACTIVE (see notes in example)
Note	Note: Provide explaination for every exception here and any additional comments that you may want to include.

Calibr
at Scripps Research

ReFRAME Library Milestone Check-In

For the purpose of tracking ReFRAME metrics, please indicate milestone achieved with any ReFRAME hits of interest in your screen follow-up:

Current as of (Day/Month/Year)	***In vivo* activity demonstrated in relevant animal model of disease**	**Publication (add citation, or journal submission)**	**IND-enabling studies initiated for repurposed indication**	**Grant proposals funded or in the process of submission for funding approval**	**Licensing opportunity, either executed or exploring**
Yes (# of compounds)					
No					
Comments (if possible, please indicate compound name)					

Calibr
at Scripps Research

Calibr ReFrame Portal Form

*A screening library of 12,000 molecules assembled by combining three databases (Clarivate Integrity, GVK Excelra GoStar and Citeline Pharmaprojects) to facilitate drug repurposing. **Information provided in this form will be posted on the public reframe portal website. Example of a filled out form is on the end of page.*

Summary of Screening Workflow

Step 1	MTA is signed and approved
Step 2	Assay Description Form and Plate Layout is filled out
Step 3	Collaborator sends plate and control to be used
Step 4	Calibr sends pre-spotted plates for primary screening at no more than 10uM (one assay, one concentration, single copy)
Step 5	Collaborator performs screen and select hits up to ~1%
Step 6	Calibr receives hitlist as RFM ID and sends 2 sets of pre-spotted 8 point dose response plates (starting at 20uM highest concentration with 1 to 3 dilution)
Step 7	Collaborator sends dose response hits data (ac50, r squared, efficacy, normalization)
Step 8	Calibr sends collaborator identities and structure of confirmed hits
Step 9	Discuss providing larger quantities of final hits
Deviations from Screening Workflow must be approved by Director of Global Health Program	Approval Signature:_____ Approval Date:_____

General Information

Assay ID	**Provided by Calibr**
Assay Title	
Assay Title Short (less than 50 characters)	
Author in format "Name, Titles (Location)"	
Summary (Brief sentence or two about the type of screen and the target)	

© 2018 Bill & Melinda Gates Foundation

Calibr
at Scripps Research

Assay Description

Description (Word count between 100-200**. The background biology needed to understand the screen. Should be detailed and specific to the assay. Keep general discussion of a disease to a minimum. Examples: the details and significance of the particular larval stage being studied in the assay; the mechanism of action of the enzyme being targeted by a biochemical screen; the benefits of your screen over other formats, details on cell type and modification if any; how your assay works to detect viability, activity or other factors etc.)**

Protocol/Workflow (A light-weight materials and methods section, but be as detailed as possible)

Detection/measurement (Clarify exactly what is being measure and how. Include information on normalization routines)

Indication (Indicate disease that is targeting)

Assay Type (Type of assay: cell based, biochemical, phenotypic…)

Calibr
at Scripps Research

Detection Method (Describe the way the assay is read)

Detection Reagent (Provide the reagents used for detection)

Components (Any species that are involved in the assay)

Drug Concentration (Final concentration of compounds and volume dispensed from 10mM or 2mM source)

Plate Type (Provide catalog number and description)

Calibr
at Scripps Research

Example Calibr ReFrame Portal Form

*A screening library of 12,000 molecules assembled by combining three databases (Clarivate Integrity, GVK Excelra GoStar and Citeline Pharmaprojects) to facilitate drug repurposing. **Information provided in this form will be posted on the public reframe portal website. Example of a filled out form is on the end of page.*

General Information

Assay ID	**Provided by Calibr**
Assay Title	**Helminth: B. malayi microfilariae (6 Day)**
Assay Title Short (less than 50 characters)	**Helminth: B. malayi microfilariae (6 Day)**
Author in format "Name, Titles (Location)"	**Jane Doe, PhD (University of California, San Francisco)**
Summary (Brief sentence or two about the type of screen and the target)	**Brugia pahangi worm motility assay**

Assay Description

Description (Word count between 100-200**. The background biology needed to understand the screen. Should be detailed and specific to the assay. Keep general discussion of a disease to a minimum. Examples: the details and significance of the particular larval stage being studied in the assay; the mechanism of action of the enzyme being targeted by a biochemical screen; the benefits of your screen over other formats, details on cell type and modification if any; how your assay works to detect viability, activity or other factors etc.)**

Adult female Brugia pahangi worms are used in the primary screens to identify a macrofilaricidal candidate. Adult female Brugia are initially screened in quadruplicate in 24 well plates at a single concentration from 1-10μM. Worms treated with 1% DMSO only are used as controls. Worms are kept in a 37°C, 5% CO2 incubator for the duration of the assay. Worm motility (phenotypic screening) is assessed on Day 0 (the day worms are treated with compound), Day 1, Day 2, Day 3, and Day 6 using an imaging system called The Worminator. Mean movement units (worms moving are assessed as pixels displaced per second) generated from The Worminator on Day 3 and Day 6 are converted into % inhibitions of worm motility compared to controls treated with 1% DMSO only. A compound hit is defined as one that inhibits the motility of the adult female worm ≥ 75% by Day 6 of the assay. If a compound inhibits motility of the worms consistently in at least two assays at single concentrations, an IC50 is performed using a 6-point serial dilution starting at 30μM. IC50s are calculated using GraphPad Prism.

Protocol/Workflow (A light-weight materials and methods section, but be as detailed as possible)

Adult female Brugia pahangi were shipped from Univ. of Missouri-Columbia and assayed using methods described by Marcellino et al. (2012). Individual females are placed in each well of a 24-well plate with media (RPMI-1640 with 25 mM HEPES, 2.0 g/L NaHCO3, 5% heat inactivated FBS, and 1X Antibiotic/Antimycotic solution). Worms are transferred to new 24-well plates upon arrival with 500 μL of media per well. Initial ReFrame compound screening is conducted at 3 μM per compound, and 1% DMSO is used as a negative control. All drugs are dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) stock solutions are stored at -20°C. Four worms were used as replicates for each concentration and worm plates are kept in a 37°C, 5% CO2 incubator for the duration of the assay except during motility readings. To determine the effect of a compound on worm motility, individual worm movement are counted as the number of pixels displaced per second by

each worm in each well using the Worminator. Each plate of worms is video recorded for approximately 60 seconds, and mean movement units (MMUs) are determined for individual worms. Percent inhibition of motility is calculated by dividing the MMUs of the treated worms by the control average MMUs, subtracting the value from 1.0, flooring the values to zero and multiplying by 100%. Videos are recorded each day, including the first day of the assay (Day 0). Compounds that inhibit worm motility can be tested in IC50 assays. Prism is used to calculate IC50 values using a non-linear regression curve fit.

Detection/measurement (Clarify exactly what is being measure and how. Include information on normalization routines)

Individual worm movements are counted as number of pixels displaced per second by each worm in each well using the Worminator. Each plate of worms is video recorded for approximately 60 seconds, and the mean movement units (MMUs) are determined for individual worms. Percent inhibition of motility is calculated by dividing the MMUs of the treated worms by the control average MMUs, subtracting the value from 1.0, flooring the values to zero and multiplying by 100%. Videos are recorded each day, including the first day of the assay (Day 0). Compounds that inhibit worm motility can be tested in IC50 assays. Prism is used to calculate IC50 values using a non-linear regression curve fit.

Indication (Indicate disease that is targeting)

Lymphatic Filariasis

Assay Type (Type of assay: cell based, biochemical, phenotypic…)

Cell based, Phenotypic

Detection Method (Describe the way the assay is read)

Video motion capture

Detection Reagent (Provide the reagents used for detection)

None

Components (Any species that are involved in the assay)

Adult female Brugia pahangi worms

Drug Concentration (Final concentration of compounds and volume dispensed from 10mM or 2mM source)

15 nL cmpd (2 mM) into 10 µL assay buffer. 3 µM final concentration

Plate Type (Provide catalog number and description)

1536 well black clear bottom plate from Greiner 789091-F

Calibr
at Scripps Research

Calibr ReFrame: Request Format Exception

Because the collection is finite and intended for dozens of partners, each partner is expected to minimize library consumption. A majority of ReFRAME screens are performed in singlicate and miniaturized to a 384 or 1536-well format. However, we recognize this is not always possible (i.e. larger parasites). Please justify your need for an exception. This will be reviewed by the ReFRAME committee and must be approved by Calibr's Director of Global Health.

Routine Venous Thromboembolism Prophylaxis May Be Inadequate in the Hypercoagulable State of Severe Coronavirus Disease 2019

Thomas K. Maatman, MD[1]; Farid Jalali, MD[2]; Cyrus Feizpour, MD[1]; Anthony Douglas II, BS[3]; Sean P. McGuire, MD[1]; Gabriel Kinnaman, BS[3]; Jennifer L. Hartwell, MD[1]; Benjamin T. Maatman, MD[4]; Rolf P. Kreutz, MD[4]; Rajat Kapoor, MD[5]; Omar Rahman, MD[5]; Nicholas J. Zyromski, MD[1]; Ashley D. Meagher, MD, MPH

Objectives: The aim of this study was to determine the frequency of venous thromboembolism in critically ill coronavirus disease 2019 patients and associate a degree of inflammatory marker elevation to venous thromboembolism development.

Design: An observational study that identified patients with severe coronavirus disease 2019 between March 12, 2020, and March 31, 2020. Data reported are those available through May 6, 2020.

Setting: A multicenter study including three Indianapolis area academic hospitals.

Patients: Two-hundred forty consecutive patients with confirmed severe acute respiratory syndrome coronavirus 2 infection were admitted to one of three hospitals. One-hundred nine critically ill coronavirus disease 2019 patients admitted to the ICU were included in the analysis.

Interventions: All patients received routine subcutaneous chemical venous thromboembolism prophylaxis.

Measurements and Main Results: The primary outcome of this study was to determine the frequency of venous thromboembolism and the degree of inflammatory and coagulation marker elevation associated with venous thromboembolism development. Descriptive statistics outlined the frequency of venous thromboembolism at any time during severe coronavirus disease 2019. Clinical course and laboratory metrics were compared between patients that developed venous thromboembolism and patients that did not develop venous thromboembolism. Hypercoagulable thromboelastography was defined as two or more hypercoagulable parameters.

Main Results: One-hundred nine patients developed severe coronavirus disease 2019 requiring ICU care. The mean (± sd) age was 61 ± 16 years and 57% were male. Seventy-five patients (69%) were discharged home, 7 patients (6%) remain in the hospital, and 27 patients (25%) died. Venous thromboembolism was diagnosed in 31 patients (28%) 8 ± 7 days after hospital admission, including two patients diagnosed with venous thromboembolism at presentation to the hospital. Elevated admission $_d$-dimer and peak $_d$-dimer were associated with venous thromboembolism development ($p < 0.05$). $_d$-dimer greater than 2,600 ng/mL predicted venous thromboembolism with an area under the receiver operating characteristic curve of 0.760 (95% CI, 0.661–0.858; $p < 0.0001$), sensitivity of 89.7%, and specificity of 59.5%. Twelve patients (11%) had thromboelastography performed and 58% of these patients had a hypercoagulable study. The calculated coagulation index was hypercoagulable in 50% of patients with thromboelastography.

Conclusions: These data show that coronavirus disease 2019 results in a hypercoagulable state. Routine chemical venous thromboembolism prophylaxis may be inadequate in preventing venous thromboembolism in severe coronavirus disease 2019. (*Crit Care Med* 2020; XX:00–00)

Key Words: deep vein thrombosis; low-molecular-weight heparin; novel coronavirus; pulmonary embolism; venous thromboembolism

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in a pandemic passing three million worldwide cases of coronavirus disease 2019 (COVID-19) with an associated case-fatality rate of 6.9% (1). The first death from COVID-19 in the state of Indiana was reported on March 16; 65 deaths and 2,565 cases were confirmed by March 31 (2). Severe COVID-19 is

[1]Department of Surgery, Indiana University School of Medicine, Indianapolis, IN.

[2]Department of Gastroenterology, Saddleback Medical Group, Laguna Hills, CA.

[3]Indiana University School of Medicine, Indianapolis, IN.

[4]Krannert Institute of Cardiology, Department of Internal Medicine, Indiana University School of Medicine, Indianapolis, IN.

[5]Department of Pulmonary and Critical Care Medicine, Indiana University School of Medicine, Indianapolis, IN.

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

DOI: 10.1097/CCM.0000000000004466

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

Downloaded from https://journals.lww.com/ccmjournal by A1V36RY9v17Hyzsxk/VM6HA7rlTGhx+v6vG6mv+q3Yq0hk3sgJeFTuBFrqTCW3yGJhnBpRs/o0dEhZTOktnRYkAN3zR0G9NFek6LvfC2XmPK5L4K6bRDr5o4GpBBVP on 05/29/2020

characterized by acute respiratory failure often culminating in the need for mechanical ventilation (3–6). Studies have associated increased acute phase reactants and inflammatory markers with severe COVID-19 (3–5,7–10). Abnormal coagulation parameters have additionally been associated with severe or fatal COVID-19 (11–13). These reports describe a biochemical profile suspicious for a prothrombotic and hypercoagulable state characterized by significant elevations in fibrinogen and a rapid unexpected ascent of serum d-dimer. This degree of profound systemic inflammation has a well-known association to a hypercoagulable state (14). These findings, and anecdotal clinical experience, have generated the hypothesis that a prothrombotic state may play a dominant pathophysiologic role in severe or fatal COVID-19. Supporting this thesis is a study from China describing decreased mortality in critically ill patients receiving prophylactic anticoagulation with low-molecular-weight heparin (15).

Profound systemic inflammation, critical illness, immobility, and organ failure are documented risk factors for a prothrombotic and hypercoagulable state (16–18). Recent autopsy reports in patients with COVID-19 identified cardiac and pulmonary microvascular thrombosis (19), supporting clinical suspicion for this pathophysiologic mechanism in COVID-19 shared among physicians on preprint platforms and social media. The frequency of venous thromboembolism (VTE) in critically ill patients with COVID-19, including deep vein thrombosis (DVT) and pulmonary embolism (PE), is becoming better understood, ranging from 25% to 27% in recent publications (12,13). Thromboelastography is a dynamic evaluation of the viscoelastic properties of whole blood throughout the clot formation and lysis process, allowing for quantitative measurement of clot formation kinetics (20, 21). In various clinical circumstances including critical illness, a hypercoagulable thromboelastography profile has been associated with increased thrombotic risk; however, thromboelastography has not been explored in patients with severe COVID-19.

The aim of this study was to determine the frequency of VTE in critically ill COVID-19 patients and associate a degree of inflammatory marker elevation to VTE development.

MATERIALS AND METHODS

Study Population

All patients with laboratory-confirmed SARS-CoV-2 infection admitted to three Indianapolis area hospitals between March 12 and March 31 were evaluated in this study. Patients less than 18 years old, imprisoned patients, pregnant patients, and patients who elected for comfort care on arrival were excluded from analysis. All data available through May 6, 2020, were included.

Two-hundred forty adult patients were admitted; 122 patients had mild/moderate COVID-19 and 118 patients had severe/fatal COVID-19. Definitions of COVID-19 severity are defined in subsequent text. Nine patients with severe COVID-19 elected for comfort care only and were excluded from the analysis, resulting in 109 patients meeting inclusion criteria (**Fig. 1**). Twelve patients with thromboelastography were included in a subgroup analysis. The Indiana University (IU) Institutional Review Board (IRB) approved the conduct of this study (IRB Study Number 2004134287) and deemed it exempt. Informed consent was waived, and anonymized data were analyzed.

The severity of COVID-19 is defined in subsequent text. All patients admitted with COVID-19 receive VTE chemoprophylaxis including either 5,000 U subcutaneous heparin every 8 hours, 40 mg enoxaparin daily, or 30 mg enoxaparin bid. Mild COVID-19 patients received best supportive treatment. Moderate and severe COVID-19 patients, in addition to best supportive care, were treated with hydroxychloroquine 400 mg orally every 12 hours for two doses, then 400 mg orally daily for four doses and azithromycin 500 mg IV daily for three doses. Acute respiratory distress syndrome (ARDS) was defined according to the Berlin criteria (22). In patients with low pulmonary compliance, similar to traditional ARDS, standard ARDS Network strategy was used (23). Although standardized treatment protocols were applied generally, clinical judgment in individual cases dictated treatment strategies.

Study Definitions

Coexisting medical conditions and medications were recorded according to physician documentation at the time of hospital admission. Individual patient's clinical examination findings, laboratory results, diagnostic imaging, treatment, and outcomes were retrospectively reviewed.

Testing for COVID-19 was obtained in accordance with the Centers for Disease Control and Prevention (CDC) guidelines and clinical specimens were tested using the CDC's assay (24). Testing occurred at the Indiana State Department of Health Laboratory from March 1 to March 19. All testing on and after March 20 was performed at the IU Health Pathology Laboratory.

Per institutional guidelines, mild COVID-19 was defined as Spo_2 greater than 94% on room air with or without radiographic evidence of pneumonia. Moderate COVID-19 was defined as a measured peripheral oxygen saturation (Spo_2) less than or equal to 94% on room air with or without radiographic evidence of pneumonia. Severe COVID-19 was defined as Spo_2 less than or equal to 94% with respiratory rate greater than or equal to 30 breaths per minute and Pao_2/Fio_2 ratio less than or equal to 300 mm Hg. Any patient requiring mechanical ventilation was considered to have severe COVID-19.

Extremity DVT was diagnosed on four-extremity duplex ultrasound performed for clinical suspicion for venous thrombosis and defined as a loss of compressibility of the vein and abnormal or absent blood flow. PE was diagnosed on contrast-enhanced cross-sectional imaging and defined as a filling defect within the lumen of the pulmonary artery or its branches. All patients developing VTE at any time period during their illness were included in this analysis.

Kaolin activated thromboelastography was performed with the TEG5000 hemostasis analyzer (Haemonetics, Braintree, MA) and used heparinase coated cups. Each

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

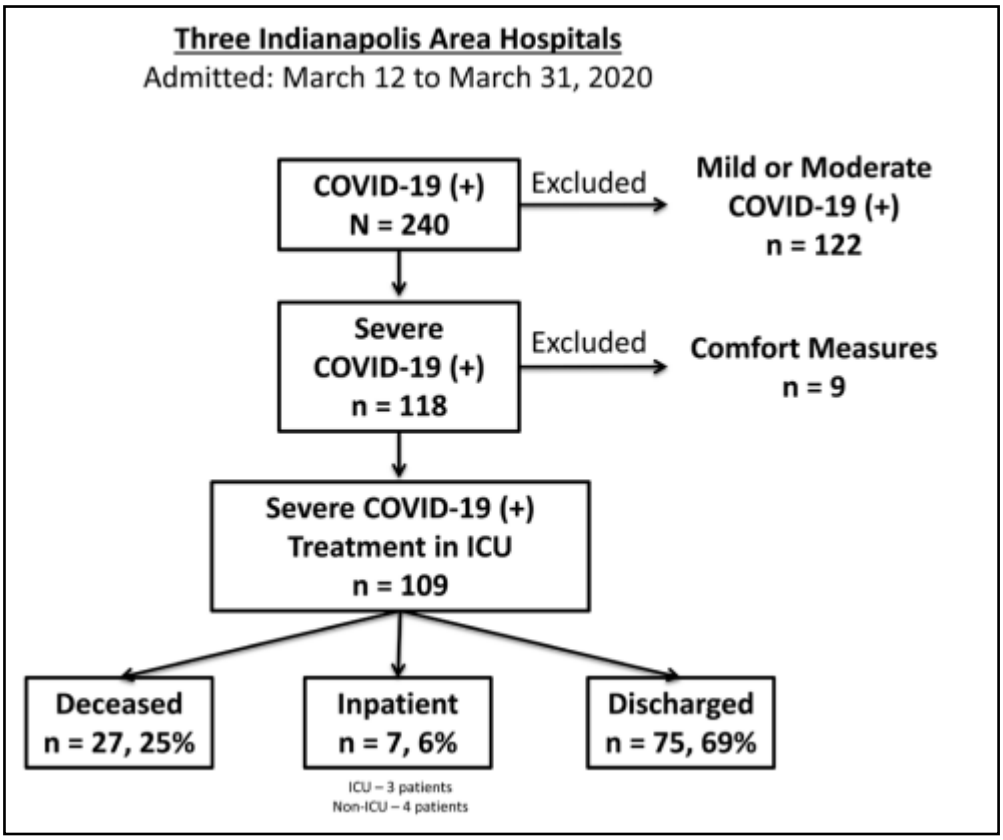


Figure 1. Flow chart of study patients. COVID-19 = coronavirus disease 2019.

thromboelastography assay included reaction time (R), kinetics (K), alpha angle (α), maximum amplitude (MA), and fibrinolytic activity 30 minutes after MA (LY30). A hypercoagulable thromboelastography was defined as two or more thromboelastography parameters beyond one sd of the age- and gender-matched controls (25). Of the individual thromboelastography parameters, a decreased R-time and K-time and an increased alpha angle and MA were considered hypercoagulable. A coagulation index (CI) was calculated for each thromboelastography and considered hypercoagulable if elevated beyond the laboratory reference range.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics Version 25.0 with deidentified data (IBM, Armonk, NY). Categorical variables are summarized as number with percentages. Variables with greater than 50% of patients missing data were not reported or included in the analysis. Continuous variables are reported as mean values with sd or median values with interquartile range (IQR). No imputation was made for missing data. As appropriate, the chi-square test and Mann-Whitney *U* tests were performed to evaluate the bivariate relationship between variables of interest and COVID-19 outcomes. Receiver operator characteristic curves were plotted and Youden Index was used to determine optimal laboratory cutoff points. The discriminatory ability of these cutoff points to predict VTE was assessed by calculating the area under the curve (AUC), sensitivity, and specificity. *p* values of less than 0.05 were accepted as statistically significant.

RESULTS

Demographic and Clinical Characteristics

One-hundred nine patients with confirmed SARS-CoV-2 infection were treated for severe COVID-19 requiring intensive care. Demographic and clinical characteristics are shown in **Table 1**. Fifty-five patients (50%) were black, the mean age was 61 ± 16 years, and 57% of patients were male. The mean body mass index was 34.8 ± 11.8 kg/m^2. Sixty-one patients (56%) were admitted directly to the ICU. The mean duration of symptoms prior to ICU admission was 8 ± 5 days. Respiratory failure requiring mechanical ventilation developed in 103 patients (94%), cardiovascular failure requiring vasopressors developed in 70 patients (64%), and renal failure requiring renal replacement therapy (RRT) developed in 16 patients (15%).

As of May 6, 7 patients (6%) continue to receive inpatient treatment (non-ICU, *n* = 4; ICU, *n* = 3), 75 patients (69%) had been discharged from the hospital, and 27 patients (22%) died (Fig. 1). Patients discharged from the ICU had a median duration of 13 days (IQR, 9–17 d) of intensive care. Of the patients discharged from the hospital, the median hospital length of stay was 20 days (IQR, 16–27 d). Death occurred a median of 11 days (IQR, 8–16 d) after hospital admission.

The median time to routine chemical VTE prophylaxis was hospital day 0 (IQR, 0–1 d) and included subcutaneous heparin every 8 hours (*n* = 61, 56%), enoxaparin daily (*n* = 26, 24%), or enoxaparin every 12 hours (*n* = 14, 13%). Seven patients (6%) were treated with full anticoagulation immediately upon admission for existing medical comorbidities (*n* = 4, 4%) or VTE diagnosed at presentation (*n* = 2, 2%). One patient was transferred from an outside hospital in which the timing of VTE prophylaxis initiation was unable to be determined.

Venous Thromboembolism

Of the 109 patients with severe COVID-19, 31 patients (28%) developed VTE. Two patients were diagnosed with VTE upon presentation to the hospital; the remaining 29 patients developed VTE while on routine chemical VTE chemoprophylaxis (*n* = 26) or on full anticoagulation for medical comorbidities (*n* = 3). Isolated DVT was diagnosed in 26 patients (84%) and isolated PE was diagnosed in one patient (3%); four patients

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

TABLE 1. Clinical Characteristics of Patients at Baseline and Laboratory Values at Presentation

Baseline Variables	Cohort (*n* = 109)	No VTE (*n* = 78)	VTE (*n* = 31)
Characteristic			
Mean age (range), yr	61 ± 16 (18–95)	62 ± 15 (18–83)	60 ± 17 (31–87)
Sex,*n* (%)			
Male	62 (57)	42 (54)	20 (65)
Female	47 (43)	36 (46)	11 (35)
Race,*n* (%)			
Black	55 (50)	39 (50)	16 (52)
White	44 (40)	33 (42)	11 (35)
Other	10 (9)	6 (8)	4 (13)
Mean body mass index, kg/m^2	34.8 ± 11.8	34.8 ± 11.5	34.7 ± 12.7
Comorbidity			
Hypertension	74 (68)	57 (73)	17 (55)
Hyperlipidemia	59 (54)	46 (59)	13 (42)
Diabetesmellitus	43 (39)	33 (42)	10 (32)
Current smoker (within 30 d)	33 (30)	**28 (36)**	**5 (16)**
Chronic obstructive pulmonary disorder	18 (16)	**17 (22)**	**1 (3)**
Congestive heart failure	17 (15)	14 (18)	3 (10)
Chronic kidney disease	16 (15)	13 (17)	3 (10)
Asthma	16 (15)	13 (17)	3 (10)
Mean duration of symptoms before admission, d	7 ± 5	7 ± 5	7 ± 3
Mean duration of symptoms before ICU admission, d	8 ± 5	9 ± 5	8 ± 4
Laboratory data	*n*, median (IQR)	*n*, median (IQR)	*n*, median (IQR)
WBC count (per mm^3)	109, 7,200 (5,100–9,800)	78, 7,000 (4,875–9,200)	31, 9,000 (5,150–11,450)
Hemoglobin (g/dL)	109, 13.3 (12–14.5)	78, 13.4 (11.9–14.3)	31, 13.0 (12.4–14.8)
Platelet count (per mm^3)	**109, 207,000 (152,000–255,000)**	**78, 189,000 (141,000–252,000**	**31, 224,000 (191,000–261,000)**
Neutrophil-to-lymphocyte ratio	99, 6.5 (4.2–12.2)	71, 6.2 (3.8–11.8)	28, 7.7 (5.6–12.6)
Creatinine (mg/dL)	109, 1.2 (0.9–1.9)	78, 1.30 (0.87–2.13)	31, 1.10 (0.89–1.42)
Alanine aminotransferase (U/L)	103, 24 (18–44)	76, 23.5 (18–38.5)	27, 31 (19.5–47.5)
Aspartate aminotransferase (U/L)	**103, 39 (29.5–63)**	**76, 38 (27–58.5)**	**27, 51 (37–72.5)**
Hemoglobin A1c (%)	78, 6.4 (5.9–7.2)	57, 6.4 (5.9–7.2)	21, 6.1 (5.8–6.9)
Lactate dehydrogenase (U/L)	**91, 437 (348.5–573)**	**66, 426 (332–581)**	**25, 525 (431–568)**
Fibrinogen (mg/dL)	57, 535 (435–651)	36, 535 (425–681)	21, 528 (435–632)
d-dimer (ng/mL)	**84, 506 (321–973)**	**59, 464 (282–755)**	**25, 900 (432–3,570)**
Troponin (ng/mL)	**81, 0.04 (0.03–0.10)**	**61, 0.03 (0.03–0.06)**	**20, 0.10 (0.05–0.22)**
Ferritin (ng/mL)	76, 579 (339–1,057)	52, 579 (339–1,050)	24, 638 (345–1,190)
C-reactive protein (mg/dL)	91, 14.6 (10.1–22.7)	66, 14.8 (8.0–22.8)	25, 14.5 (12.5–21.3)
Procalcitonin (ng/mL)	81, 0.23 (0.12–0.92)	58, 0.20 (0.14–0.58)	23, 0.26 (0.11–1.78)
Interleukin-6 (pg/mL)	55, 14 (7.5–40.5)	39, 14 (7–40)	16, 14 (11–48)

IQR = interquartile range, VTE = venous thromboembolism.
Unless otherwise stated, means values are reported ± sd and median values are reported with IQR.
Bold values represent statistical significance with $p < 0.05$.

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

had both DVT and PE (13%). Five DVT were associated with central venous catheters (CVCs) in the internal jugular vein (n = 3) and femoral vein (n = 2); however, in four of these patients, additional DVT were detected in locations remote from the CVC. The number of DVT identified per patient was 3 ± 2. Of the 30 patients with DVT, the location of the DVT was most commonly the lower extremities (n = 18, 60%); however, upper extremity DVT and both upper and lower extremity DVT were diagnosed in six each (20%). The mean time from hospital admission to VTE diagnosis was 8 ± 7 days. Initial treatment for VTE included unfractionated heparin IV infusion (n = 13, 42%), weight-based enoxaparin subcutaneously bid (n = 13, 42%), oral apixaban (n = 2, 6%), and unfractionated heparin IV infusion with alteplase infusion (n = 2, 6%). In one patient, anticoagulation was contraindicated due to recent cerebrovascular accident.

On univariate analysis, the development of VTE was significantly associated with higher serum platelet count, aspartate aminotransferase, lactate dehydrogenase, d-dimer, and troponin concentrations at admission (Table 1). The frequency of VTE was no different in patients without or with respiratory failure requiring mechanical ventilation (33% vs 28%; p = 0.785), cardiovascular failure requiring vasopressors (26% vs 30%; p = 0.629), or renal failure requiring RRT (31% vs 12.5%; p = 0.126). When comparing the peak serum concentration of laboratory values in patients prior to VTE diagnosis to patients that did not develop VTE, increased d-dimer concentration and decreased platelet counts were observed (**Table 2**).

Admission d-dimer concentration, peak d-dimer concentration, and peak platelet count were plotted on a receiver-operating characteristic curve; the AUC, ideal laboratory cutoff points to detect VTE, and associated sensitivity and specificity are shown in **Figure 2**. A serum d-dimer concentration greater than 2,600 ng/mL (normal range, 0–292 ng/mL) was the test with the best discriminatory ability to detect VTE with an AUC of 0.760 (p < 0.0001) and a sensitivity and specificity of 89.7% and 59.5%, respectively. Patients developing VTE had no difference in mortality (8/31, 26%) when compared with patients that did not develop VTE (19/78, 24%; p = 0.875).

Thromboelastography

Twelve patients (11%) had thromboelastography analyzed during their hospital stay and the mean value of each thromboelastography parameter is shown in **Figure 3**. Thromboelastography was performed a median of 3.5 days (IQR, 1–6 d) after hospital admission and was performed for significantly elevated d-dimer (n = 8, 67%) or as a baseline prior to extracorporeal membrane oxygenation initiation (n = 3, 25%). In one patient (8%), thromboelastography was ordered due to persistent clotting of the RRT filter. Hypercoagulable thromboelastography (≥ 2 hypercoagulable thromboelastography parameters) were observed in 58% of patients and at least one thromboelastography parameter was hypercoagulable in 83% of patients. The most common hypercoagulable feature of thromboelastography was a decreased R-time in 67% of patients. The calculated CI was hypercoagulable in 50% of patients. The individual patterns of patients with hypercoagulable and nonhypercoagulable thromboelastography are shown in Figure 3. The mean d-dimer in patients with a hypercoagulable thromboelastography was 2,240 ± 599 ng/mL. Three patients (43%) with a hypercoagulable thromboelastography developed VTE; however, one patient with a normal thromboelastography developed VTE.

DISCUSSION

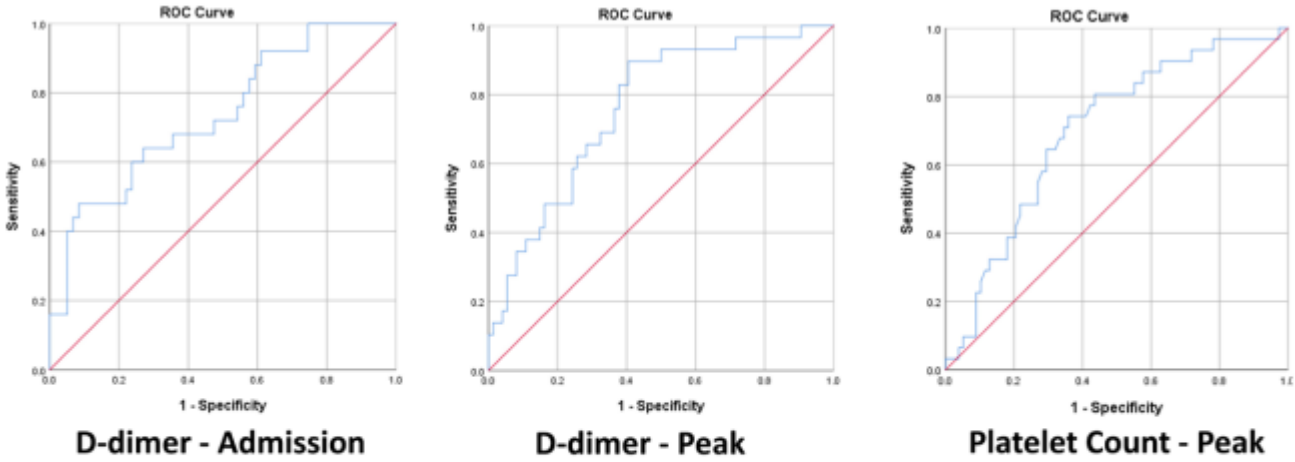
In this series of 109 critically ill COVID-19 patients among three Indianapolis area hospitals, we report an extremely high frequency of VTE despite the prompt initiation of routine chemical VTE prophylaxis. Risk factors associated with VTE development included an elevated d-dimer at hospital presentation and an elevated d-dimer or decreased platelet count during intensive care. Thromboelastography documented hypercoagulability in the majority of patients in which it was

TABLE 2. Peak Inflammatory and Coagulation Markers Prior to Venous Thromboembolism Diagnosis

Laboratory Value	No VTE (n = 80), n, Median (IQR)	VTE (n = 29), n, Median (IQR)	p
Platelet count (per mm^3)	**78, 383,000 (291,000–472,000)**	**31, 294,000 (223,000–340,000)**	**0.001**
Neutrophil-to-lymphocyte ratio	74, 12.2 (8.6–23.7)	29, 16.0 (9.3–21.0)	0.695
Lactate dehydrogenase (U/L)	75, 541 (401–708)	26, 534 (495–721)	0.870
Fibrinogen (mg/dL)	46, 680 (521–872)	23, 651 (465–771)	0.243
d-dimer (ng/mL)	**74, 1,934 (695–3,573)**	**29, 4,046 (3,071–13,324)**	**0.00004**
Ferritin (ng/mL)	70, 910 (447–1,775)	26, 1,022 (459–1,643)	0.882
C-reactive protein (mg/dL)	73, 24.3 (17.3–32.3)	27, 26.4 (13.6–33.4)	0.877
Procalcitonin (ng/mL)	63, 0.34 (0.20–1.93)	25, 0.66 (0.15–3.98)	0.554

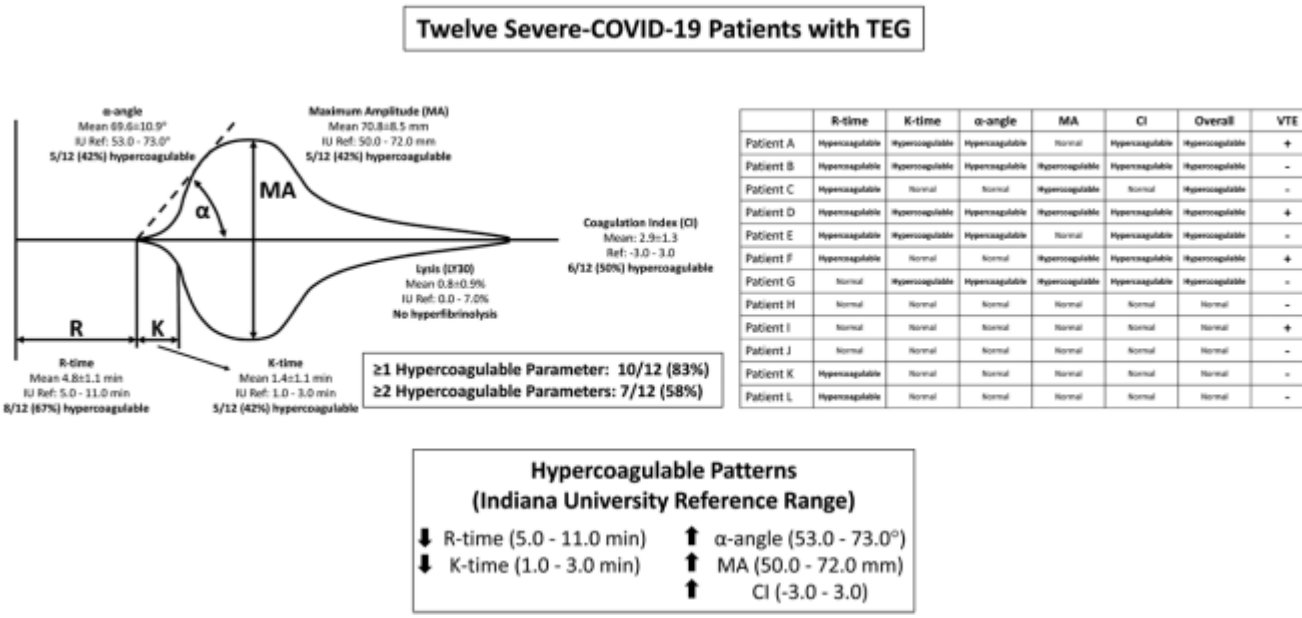
IQR = interquartile range, VTE = venous thromboembolism.
Unless otherwise stated, median values are reported with IQR.
Bold values represent statistical significance with p < 0.05.

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.



Receiver Operator Characteristic Curve	Area Under the Curve (95% Confidence Interval)	*P*	Sensitivity	Specificity
Admission Laboratory Value				
D-Dimer > 728 ng/mL	0.735 (0.617-0.852)	0.001	64.0%	72.9%
Peak Laboratory Value				
D-Dimer > 2600 ng/mL	0.760 (0.661-0.858)	<0.0001	89.7%	59.5%
Platelet Count < 360000 per mm^3	0.700 (0.595-0.805)	0.001	80.6%	55.1%

Figure 2. Receiver operator characteristic (ROC) curve, area under the curve, sensitivity, and specificity of d-dimer and platelet count to detect venous thromboembolism.



	R-time	K-time	α-angle	MA	CI	Overall	VTE
Patient A	Hypercoagulable	Hypercoagulable	Hypercoagulable	Normal	Hypercoagulable	Hypercoagulable	+
Patient B	Hypercoagulable	Hypercoagulable	Hypercoagulable	Hypercoagulable	Hypercoagulable	Hypercoagulable	-
Patient C	Hypercoagulable	Normal	Normal	Hypercoagulable	Normal	Hypercoagulable	-
Patient D	Hypercoagulable	Hypercoagulable	Hypercoagulable	Hypercoagulable	Hypercoagulable	Hypercoagulable	+
Patient E	Hypercoagulable	Hypercoagulable	Hypercoagulable	Normal	Hypercoagulable	Hypercoagulable	-
Patient F	Hypercoagulable	Normal	Normal	Hypercoagulable	Hypercoagulable	Hypercoagulable	+
Patient G	Normal	Hypercoagulable	Hypercoagulable	Hypercoagulable	Hypercoagulable	Hypercoagulable	-
Patient H	Normal	Normal	Normal	Normal	Normal	Normal	-
Patient I	Normal	Normal	Normal	Normal	Normal	Normal	+
Patient J	Normal	Normal	Normal	Normal	Normal	Normal	-
Patient K	Hypercoagulable	Normal	Normal	Normal	Normal	Normal	-
Patient L	Hypercoagulable	Normal	Normal	Normal	Normal	Normal	-

Figure 3. Thromboelastography in 12 patients with severe coronavirus disease 2019 (COVID-19). Values are reported as mean ± SD. CI = coagulation index, IU = Indiana University, K = kinetics, LY30 = fibrinolytic activity 30 minutes after maximum amplitude, MA = maximum amplitude, R = reaction time, TEG = thromboelastography, VTE = venous thromboembolism, α = alpha angle.

performed. Mortality was not significantly different in patients developing VTE when compared with patients who did not develop VTE.

We report a frequency of VTE of 28% in critically ill patients with COVID-19 and identified an elevated d-dimer at presentation and an elevated d-dimer or thrombocytopenia

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

during intensive care as risk factors for VTE development. About 5–10% of critically ill patients develop VTE; thus, the risk for VTE in critically ill patients with COVID-19 is about 2.5–5 times higher than the general ICU population (26, 27). The frequency of VTE identified in the current study is similar to that reported in two recent studies from China (25%) and the Netherlands (27%) (12, 13) and similar to that reported in critically ill patients with severe acute respiratory syndrome (24%) during the outbreak in the early 2000s (28). COVID-19 is associated with a profound systemic inflammatory response, hypercoagulable serum coagulation profile, organ failure, and immobility; thus, it is no surprise that the frequency of VTE is significant and higher than expected despite prompt initiation of routine chemical VTE prophylaxis. This study likely underestimates the true frequency of VTE in this population, as diagnostic evaluation for VTE was deferred to prevent untoward risk to healthcare workers and inpatients.

The mechanism by which critically ill patients with COVID-19 develop VTE at a higher frequency than the general ICU population remains unclear, specifically whether a hypercoagulable state is induced by the degree of viremia and the virus itself, or if this is simply in response to the profound systemic inflammation and critical illness, which are known risk factors for VTE development (14, 16, 26, 27). The association between elevated d-dimer and VTE development is likely representative of both the degree of systemic inflammation in critically ill COVID-19 patients and inappropriate activation of the coagulation cascade; however, the degree to which each of these components contribute to VTE development was not possible to determine in this study. The frequency of VTE in severe COVID-19, its risk factors, and outcomes warrant ongoing worldwide investigation, as COVID-19 will likely continue to contribute to worldwide morbidity and mortality for some years. Given the documented hypercoagulability and frequency of VTE in this population, consideration should be given to an escalated dose of chemical VTE prophylaxis using either weight-based or anti-factor-Xa guided dosing in COVID-19 patients with d-dimer greater than 728 ng/mL at presentation. In addition to this increased dosing of chemical VTE prophylaxis, critically ill patients with a d-dimer greater than 2,600 ng/mL (or > 10 times the upper limit of laboratory normal) should be evaluated with four-extremity duplex ultrasound; empiric anticoagulation in the setting of elevated d-dimer and presumptive diagnosis of VTE should be made on a case-by-case basis. Early detection of VTE and prevention of PE in patients with severe COVID-19 is paramount given the mounting clinical and autopsy evidence of lung microvascular and macrovascular thrombosis (12, 13, 19, 29), which plausibly may be contributing to the progressive and fatal hypoxemic respiratory failure in these patients without prompt initiation of adequate anticoagulation.

In this series of 12 patients with severe COVID-19 undergoing thromboelastography analysis, 58% met objective criteria for hypercoagulability and 83% of patients had at least one hypercoagulable thromboelastography parameter. The most common hypercoagulable parameter was the reaction (R) time in 67% of patients. This suggests that SARS-CoV-2 infection and the associated systemic inflammation, in the most severe cases, results in activation of the coagulation cascade. Three of seven patients with a hypercoagulable thromboelastography went on to develop VTE; thus, larger volume, prospective studies may be warranted to evaluate this association further and define the utility of thromboelastography in COVID-19. Given the degree of inflammatory and coagulation marker elevation in severe COVID-19, serial thromboelastography analysis may provide useful insights as to which patients are at highest risk for microvascular and macrovascular thrombosis, potentially offering a window for improved and effective microvascular and macrovascular thrombosis prophylaxis. Our study corroborates a recently published study that reported hypercoagulable thromboelastography in COVID-19 patients (30). Thromboelastography represents an objective metric (thromboelastography) to describe the hypercoagulability encountered in COVID-19 and provides a more complete picture of the coagulation cascade compared with traditional laboratory values. Large, prospective studies evaluating thromboelastography could provide detailed insight into the abnormal coagulation in COVID-19. Further, the potential role of platelet activation and aggregation in VTE development can be evaluated with thromboelastography, providing an additional target for improved VTE prophylaxis with antiplatelet therapy. The current study and the study published by Panigada et al (30) were unable to evaluate platelet function in COVID-19, as thromboelastography with platelet mapping was not routinely performed. A multi-institutional observational study evaluating the utility of serial thromboelastography in COVID-19 is currently underway. Future investigation may evaluate for an association between thromboelastography parameters and anti-factor Xa guided VTE prophylaxis, as thromboelastography may not be available at all institutions treating COVID-19 patients.

Given the gravity and scope of the COVID-19 pandemic, rapid dissemination of significant clinical observations is paramount to improving patient outcomes; however, several limitations of this study deserve mention. Although this is a relatively large series it is an observational study and the inherent bias introduced by a retrospective evaluation of any patient population is a limitation. The retrospective nature of this study precludes the ability to establish any causal relationship between d-dimer concentration or platelet count and VTE development. Critically ill COVID-19 patients often had multiple CVCs simultaneously and/or at different time points throughout their treatment and the location of CVC as a risk factor for VTE development was unable to be evaluated. Measurement of prothrombin time was not included in the institutional protocol at the time of this study and not routinely performed, thereby preventing the ability of this study to evaluate disseminated intravascular coagulation. Only critically ill COVID-19 patients were included in this study; therefore, this study lacked a comparator group of ICU patients without COVID-19 in which to compare VTE frequency. The current study did not evaluate the degree of viremia as a risk factor for

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

VTE and this should be considered in future studies. The small number of patients with thromboelastography analysis may not accurately reflect the trends of the entire cohort and more detailed analysis is warranted. Given the number of patients with thromboelastography data, we were unable to associate thromboelastography parameters to VTE. The number of comparisons was intentionally kept to a minimum to avoid the introduction of type II error. Finally, Patients with sudden clinical deterioration and death were not evaluated postmortem for the development of VTE.

CONCLUSIONS

These data show that SARS-CoV-2 infection results in systemic hypercoagulability resulting in VTE. Although current data on outcomes in patients receiving therapeutic anticoagulation in COVID-19 are lacking, it is apparent that routine chemical VTE prophylaxis may be inadequate in preventing thrombotic complications in severe COVID-19.

All authors involved in acquisition, analysis, and interpretation of data. All authors involved in conception, drafting, and revision of work. All authors involved in final approval of work.

Dr. Kreutz's institution received funding from Idorsia, and he received funding from Haemonetics. The remaining authors have disclosed that they do not have any potential conflicts of interest.

For information regarding this article, E-mail: tkmaatma@iu.edu

---

REFERENCES

1. World Health Organization: Coronavirus Disease (COVID-2019) Situation Report 107. Geneva, Switzerland, World Health Organization, 2020. Available at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports. Accessed May 6, 2020
2. Indiana State Department of Health: 2019 Novel Coronavirus (COVID-19). 2020. State of Indiana. Available at: coronavirus.in.gov. Accessed April 11, 2020
3. Chen T, Wu D, Chen H, et al: Clinical characteristics of 113 deceased patients with coronavirus disease 2019: Retrospective study. *BMJ* 2020; 368:m1091
4. Zhang JJ, Dong X, Cao YY, et al: Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. *Allergy* 2020 Feb 19. [online ahead of print]
5. Zhou F, Yu T, Du R, et al: Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: A retrospective cohort study. *Lancet* 2020; 395:1054–1062
6. Bhatraju PK, Ghassemieh BJ, Nichols M, et al: Covid-19 in critically ill patients in the Seattle region - case series. *N Engl J Med* 2020; 382:2012–2022
7. Chen G, Wu D, Guo W, et al: Clinical and immunologic features in severe and moderate coronavirus disease 2019. *J Clin Invest* 2020; 130:2620–2629
8. Wan S, Xiang Y, Fang W, et al: Clinical features and treatment of COVID-19 patients in northeast Chongqing. *J Med Virol* 2020 Mar 21. [online ahead of print]
9. Wu C, Chen X, Cai Y, et al: Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. *JAMA Intern Med* 2020 Mar 13. [online ahead of print]
10. Zhang G, Zhang J, Wang B, et al: Analysis of clinical characteristics and laboratory findings of 95 cases of 2019 novel coronavirus pneumonia in Wuhan, China: A retrospective analysis. *Respir Res* 2020; 21:74
11. Tang N, Li D, Wang X, et al: Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. *J Thromb Haemost* 2020; 18:844–847
12. Cui S, Chen S, Li X, et al: Prevalence of venous thromboembolism in patients with severe novel coronavirus pneumonia. *J Thromb Haemost* 2020 Apr 9. [online ahead of print]
13. Klok FA, Kruip MJHA, van der Meer NJM, et al: Frequency of thrombotic complications in critically ill ICU patients with COVID-19. *Thromb Res* 2020 Apr 10. [online ahead of print]
14. Rodriguez AL, Wojcik BM, Wrobleski SK, et al: Statins, inflammation and deep vein thrombosis: A systematic review. *J Thromb Thrombolysis* 2012; 33:371–382
15. Tang N, Bai H, Chen X, et al: Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. *J Thromb Haemost* 2020; 18:1094–1099
16. Di Nisio M, van Es N, Büller HR: Deep vein thrombosis and pulmonary embolism. *Lancet* 2016; 388:3060–3073
17. Gando S: Microvascular thrombosis and multiple organ dysfunction syndrome. *Crit Care Med* 2010; 38:S35–S42
18. Roch AM, Maatman TK, Carr RA, et al: Venous thromboembolism in necrotizing pancreatitis: An underappreciated risk. *J Gastrointest Surg* 2019; 23:2430–2438
19. Fox SE, Akmatbekov A, Harbert JL, et al: Pulmonary and cardiac pathology in covid-19: The first autopsy series from New Orleans. *medRxiv* 2020.04.06.20050575
20. Ganter MT, Hofer CK: Coagulation monitoring: Current techniques and clinical use of viscoelastic point-of-care coagulation devices. *Anesth Analg* 2008; 106:1366–1375
21. Othman M, Kaur H: Thromboelastography (TEG). *In*: Hemostasis and Thrombosis: Methods and Protocols. Favaloro EJ, Lippi G (Eds). New York, NY, Springer, 2017, pp 533–543
22. Ranieri VM, Rubenfeld GD, Thompson BT, et al; The ARDS Definition Task Force*: Acute respiratory distress syndrome: The Berlin definition. *JAMA* 2012; 307:2526–2533
23. Brower RG, Matthay MA, Morris A, et al: The Acute Respiratory Distress Syndrome Network*: Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. *N Engl J Med* 2000; 342:1301–1308
24. Centers for Disease Control and Prevention: Coronavirus Disease 2019 (COVID-19). 2020. Available at: https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-detection-instructions.html. Accessed March 4, 2020
25. Toukh M, Siemens DR, Black A, et al: Thromboelastography identifies hypercoagulablilty and predicts thromboembolic complications in patients with prostate cancer. *Thromb Res* 2014; 133:88–95
26. Cook D, Crowther M, Meade M, et al: Deep venous thrombosis in medical-surgical critically ill patients: Prevalence, frequency, and risk factors. *Crit Care Med* 2005; 33:1565–1571
27. Cook D, Meade M, Guyatt G, et al: Dalteparin versus unfractionated heparin in critically ill patients. *N Engl J Med* 2011; 364:1305–1314
28. Lew TW, Kwek TK, Tai D, et al: Acute respiratory distress syndrome in critically ill patients with severe acute respiratory syndrome. *JAMA* 2003; 290:374–380
29. Barton LM, Duval EJ, Stroberg E, et al: COVID-19 autopsies, Oklahoma, USA. *Am J Clin Pathol* 2020; 153:725–733
30. Panigada M, Bottino N, Tagliabue P, et al: Hypercoagulability of COVID-19 patients in intensive care unit. A report of thromboelastography findings and other parameters of hemostasis. *J Thromb Haemost* 2020 Apr 17. [online ahead of print]

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

Coagulopathy of Coronavirus Disease 2019

Toshiaki Iba, MD[1]; Jerrold H. Levy, MD[2]; Marcel Levi, MD[3]; Jean Marie Connors, MD[4]; Jecko Thachil, MD[5]

Downloaded from https://journals.lww.com/ccmjournal by A1V36RY5v17t+yzsxkVM6HA7rlTGhx+v6vG6mv+q3Yq0hk3sgJeRTu8Fog1CW3yGJhnBpPdv0x6ErC7OktRrYkAN3zR0G9NFeklLvfC2XrnPK5L4K6bRDr5o4GpBEVP on 05/29/2020

Objectives: Recent studies have reported a high prevalence of thrombotic events in coronavirus disease 2019. However, the significance of thromboembolic complications has not been widely appreciated. The purpose of this review is to provide current knowledge of this serious problem.

Design: Narrative review.

Data Sources: Online search of published medical literature through PubMed using the term "COVID-19," "SARS," "acute respiratory distress syndrome," "coronavirus," "coagulopathy," "thrombus," and "anticoagulants."

Study Selection and Data Extraction: Articles were chosen for inclusion based on their relevance to coagulopathy and thrombosis in coronavirus disease 2019, and anticoagulant therapy. Reference lists were reviewed to identify additional relevant articles.

Data Synthesis: Coronavirus disease 2019 is associated with a strikingly high prevalence of coagulopathy and venous thromboembolism that may contribute to respiratory deterioration. Monitoring coagulation variables is important, as abnormal coagulation tests are related to adverse outcomes and may necessitate adjuvant antithrombotic interventions. In the initial phase of the infection, d-dimer and fibrinogen levels are increased, while activated partial prothrombin time, prothrombin time, and platelet counts are often relatively normal. Increased d-dimer levels three times the upper limit of normal may trigger screening for venous thromboembolism. In all hospitalized patients, thromboprophylaxis using low-molecular-weight heparin is currently recommended. The etiology of the procoagulant responses is complex and thought to be a result of specific interactions between host defense mechanisms and the coagulation system. Although the coagulopathy is reminiscent of disseminated intravascular coagulation and thrombotic microangiopathy, it has features that are markedly distinct from these entities.

Conclusions: Severe acute respiratory syndrome coronavirus 2/coronavirus disease 2019 frequently induces hypercoagulability with both microangiopathy and local thrombus formation, and a systemic coagulation defect that leads to large vessel thrombosis and major thromboembolic complications, including pulmonary embolism in critically ill hospitalized patients. d-dimers and fibrinogen levels should be monitored, and all hospitalized patients should undergo thromboembolism prophylaxis with an increase in therapeutic anticoagulation in certain clinical situations. (*Crit Care Med* 2020; XX:00–00)

Key Words: coagulopathy; coronavirus; coronavirus disease 2019; disseminated intravascular coagulation; hypercoagulability; thromboembolism

Increasing communications worldwide have reported that hospitalized, critically ill coronavirus disease 2019 (COVID-19) patients are frequently developing laboratory abnormalities compatible with hypercoagulability and clinically a high prevalence of thromboembolic events (1). In addition to deep vein thrombosis (DVT) and pulmonary embolism (PE), thrombosis in extracorporeal circuits and arterial thrombosis have been reported (2). Patients with COVID-19 often present with dyspnea, hypoxemia, and hemodynamic instability with acute respiratory distress syndrome (ARDS), and in such clinical condition, venous thromboembolism (VTE) may be overlooked (3, 4). Standard imaging in critically ill patients utilizing contrast-enhanced CT may not always be feasible, and additionally, concerns exist with disease transmission to health-care staff.

Ranucci et al (5) recently reported comprehensive coagulation analyses including d-dimers, fibrinogen levels, and viscoelastic testing in the COVID-19 patients with ARDS, and reported the procoagulant profile on ICU admission with median d-dimer levels of 5.5 mg/L (10 times the upper limit of normal), fibrinogen levels of 7.8 g/L, and increased clot strength by thromboelastometry. Panigada et al (6) performed a similar analysis and also noted increased fibrinogen levels, enhanced platelet activation, and increased viscoelastic variables. Beyond

[1]Department of Emergency and Disaster Medicine, Juntendo University Graduate School of Medicine, Tokyo, Japan.

[2]Department of Anesthesiology, Critical Care, and Surgery, Duke University School of Medicine, Durham, NC.

[3]Department of Medicine and Cardio-metabolic Programme-NIHR UCLH/UCL BRC, University College London Hospitals NHS Foundation Trust, London, United Kingdom.

[4]Hematology Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA.

[5]Department of Haematology, Manchester Royal Infirmary, Manchester, United Kingdom.

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

DOI: 10.1097/CCM.0000000000004458

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

VTE, the relevance of microthrombus formation to organ dysfunction and acro-ischemic change has also been suggested (7–9). Although the number of postmortem pathologic reports are limited, Luo et al (10) described vascular wall thickening, stenosis of the vascular lumen, and microthrombus formation accompanying the findings of ARDS. Similar pathologic findings are found in small vessels of other organs (8–11). Magro et al (12) reported the deposition of C5b-9 (membrane attack complex), C4d, and mannose-binding lectin-associated serine protease-2 in the lung capillaries and the skin microvasculature. Notably, the deposition was co-localized with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike glycoprotein. A recent report also noted mononuclear and polymorphonuclear leukocyte infiltration and pulmonary microcirculation along with apoptosis as induced by caspase-3 staining (13).

In critically ill COVID-19 patients, there appear to be at least two separate pathologic coagulation pathologic processes that are important in producing clinical manifestations. In the microcirculation of the lung and potentially other organs, there is local direct vascular and endothelial injury producing microvascular clot formation and angiopathy (13, 14). Postmortem biopsy of the lung revealed mononuclear and polymorphonuclear infiltration along with apoptosis of endothelial and mononuclear cells (13). In the systemic circulation, due to hypercoagulability with hyperfibrinogenemia, there is also the potential for large vessel thrombosis and major thromboembolic sequelae including PE that is reported in 20–30% of ICU patients (15–17) (**Fig. 1**). Because of the important role of coagulation activation in critically ill COVID-19 patients, this review summarizes the current knowledge about the coagulopathy and role of anticoagulation in these patients.

PREVALENCE OF COAGULOPATHY AND DISSEMINATED INTRAVASCULAR COAGULATION

Coagulopathy is a common feature of SARS-CoV-2 infection, and an increase in d-dimer is the most common finding. One of the larger initial studies found abnormally elevated d-dimer levels in 260 of 560 cases (46.4%) with a prevalence of 43% in nonsevere patients compared with 60% in critically ill ICU patients (18). In another series, elevated d-dimers were associated with a poor prognosis (19). More recently, Zhang et al (20) examined 343 cases and showed that d-dimer levels of over 2.0 mg/L could predict mortality with a sensitivity of 92.3% and a specificity of 83.3%. Tang et al (21) reported increased d-dimers and fibrin degradation products along with mildly to moderately increased prothrombin times (PTs) and activated partial thromboplastin times (aPTT) in COVID-19. Of note is they reported that 71.4% of nonsurvivors fulfilled the criteria of sepsis-induced coagulopathy, while 0.6% of the survivors met the criteria.

Platelet counts in COVID-19 patients are variable depending on the reported studies. A meta-analysis reported significantly lower platelet counts in critically ill COVID-19 patients with weighted mean difference of -31×10^9/L (95% CI, -35 to -29×10^9/L), and thrombocytopenia defined as below the lower limit of the reference range was associated with more than five-fold higher risk of severe disease, which may reflects secondary infections (22). However, as noted above, thrombocytopenia is not a significant finding initially in COVID-19 (23). Huang et al (24) reported platelet counts of less than 100×10^9/L in only 8% of ICU and 4% in non-ICU patients initially at admission. Yin et al (25) compared the platelet count between COVID-19–associated ARDS patients and non-COVID-19 ARDS patients and reported minor clinical differences in platelet counts (215 ± 100 vs $188 \pm 98 \times 10^9$/L). The lack of thrombocytopenia reflects that this is not a consumptive coagulopathy typical of disseminated intravascular coagulation (DIC). The potential for increases in platelet counts in COVID-19 patients is suspected to be caused by increased proinflammatory cytokines such as interleukin (IL)-1β and IL-6 produced by the macrophages and monocytes in the lung (26), and activated platelets may contribute to the lung injury (27). Interestingly, only 6.4% of COVID-19 patients who died met DIC criteria of the International Society on Thrombosis and Haemostasis (ISTH) (28). More recent data suggests the COVID-19

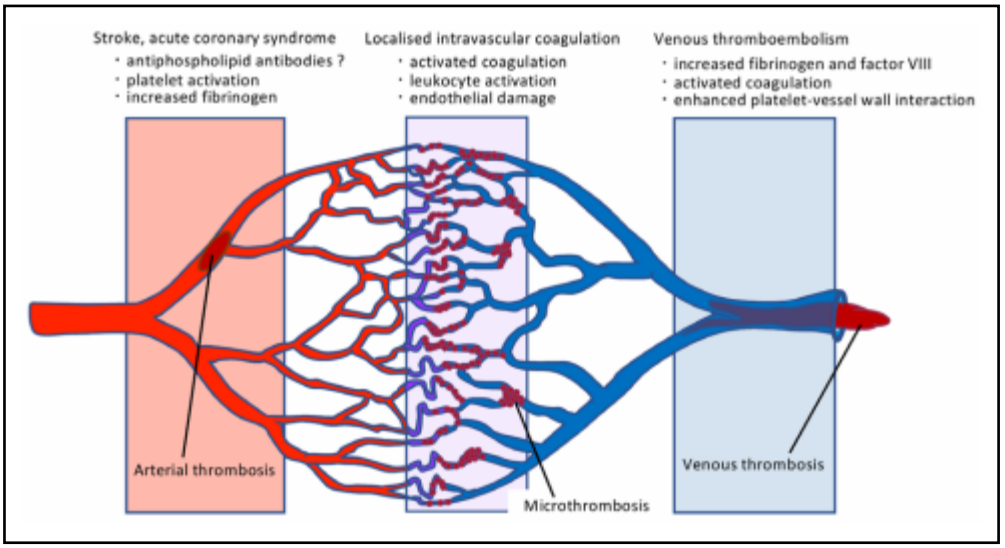


Figure 1. Various types of thrombus formation in coronavirus disease 2019 (COVID-19). Venous thrombosis and pulmonary thromboembolism are common complications in COVID-19. Increased fibrinogen and factor VIII, activated coagulation, direct viral endothelial infection and endothelial injury, and increased platelet-vessel wall interaction activation play roles in the development of thrombotic complications. In addition, there may be pulmonary microvascular coagulation in COVID-19. The prevalence of arterial thrombosis is also high and involvement of antiphospholipid antibodies has been suggested.

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

coagulopathy is different from common bacterial infection-induced DIC with relatively minimal changes in platelet counts, antithrombin levels, PT, and aPTT (21, 29).

The association with increased microvascular thrombosis, increased levels of lactate dehydrogenase (LDH) and ferritin, and mild increase in PT and aPTT are reminiscent of thrombotic microangiopathy (30). Although thrombocytopenia and hemolytic anemia are uncommon in COVID-19, clinical presentations of increased d-dimers, vascular endothelial injury, and multiple organ damage are also common features of atypical hemolytic uremic syndrome (12).

CHANGES IN COAGULATION AND FIBRINOLYSIS

The coagulation cascade is activated in viral infections as a host defense to limit the spread of the pathogens (31). Initially, an adaptive hemostasis response occurs that is associated with a systemic inflammatory response. As a result of increased inflammatory activity, fibrinogen is significantly increased, and thrombin generation occurs (32). The enhanced cytokine production during virus infection also stimulates additional procoagulant reactions, with increased tissue factor expression, a major initiator of the activation in coagulation. However, other factors such as phosphatidylserine on the cellular membrane, neutrophil extracellular traps, and damage-associated molecular patterns (DAMPs) may also be involved in the procoagulant profile in COVID-19 (33). In some cases, the presence of antiphospholipid antibodies that can induce arterial thrombosis is reported (2), and the relevance to stroke and acute coronary disease should be examined in future studies.

There is an association between bronchoalveolar coagulation/fibrinolysis and the pathogenesis of ARDS that enhances intrapulmonary deposition of fibrin (34). In cases of bacterial infection, measurement of coagulation and fibrinolysis factors in bronchoalveolar lavage fluid (BALF) have demonstrated enhanced intrapulmonary thrombin generation, insufficiently balanced physiologic anticoagulation, and suppressed fibrinolysis mediating the pathogenesis of ARDS (35). In COVID-19, procoagulant activity is increased through tissue factor pathway, and plasmin activity suppressed by the reduced urokinase-type plasminogen activator and increased plasminogen activator inhibitor-1 (36, 37). By contrast, Ji et al (38) reported activated plasmin and increased fibrinolytic activity resulting in d-dimer elevation in COVID-19 and reported that the preexisting increased plasmin activity recognized in hypertension, diabetes, and cardiovascular disease enhances the virulence and infectivity of the SARS-CoV-2 virus by cleaving its spike proteins. The pathologic findings of ARDS in COVID-19 suggest the inflammation and diffuse alveolar damage with exudates that mimic sepsis-induced ARDS are lymphocyte predominant (39).

Gattinoni et al (40) hypothesized that there are two distinct types of lung damage in ARDS. Type-L is characterized by the low elastance and high compliance, which is rarely seen in ARDS, while type-H shows high elastance and low compliance that is the typical style of ARDS. They explain the primary cause of hypoxemia in type-L is perfusion defects presumably caused by vasoconstriction and high shunt fraction. In contrast, the high elastance in type-H is thought to be induced by lung edema. We hypothesize that the intravascular coagulation and clot formation, in addition to the vasoconstriction, contribute to the disturbance of perfusion. The findings as mentioned above suggest that the procoagulant changes are present in both intra-alveolar and intravascular spaces (**Fig. 2**). Indeed, Dolhnikoff et al (41) reported the fibrinous thrombi in pulmonary arteriole in areas of both damaged and preserved lung parenchyma. The endothelial damage in the pulmonary capillary is also accelerated by the vascular endothelial damage. SARS-CoV-2 infects endothelial cells through an angiotensin-converting enzyme 2 receptor (42); the rapid viral replication causes massive endothelial cell apoptosis and triggers the loss of anticoagulant function of the vascular lumen. In addition to the derangement of coagulation/fibrinolysis and platelet function, endothelial dysfunction contributes to the procoagulant change in COVID-19 (**Fig. 3**).

INFLAMMATORY THROMBUS, MICROCIRCULATORY INJURY, AND ORGAN DYSFUNCTION

Various proinflammatory cytokines are known to be elevated in COVID-19, and a "cytokine storm" is estimated to be relevant in the progression and modification of the disease. Tumor necrosis factor-α, IL-1β, IL-6, interferon-γ, and granulocyte-colony stimulating factor are the representative cytokines that mediate inflammation and coagulation. Elevation in the circulating blood cytokine levels is also increased in the lung. Xiong et al (43) revealed increased levels of chemokines such as monocyte chemotactic protein 1 (MCP-1), interferon-inducible protein-10, macrophage inflammatory protein-1α in the BALF obtained from COVID-19 patients. This cytokine storm leads to the systemic intravascular coagulation, multiple organ dysfunction syndrome (MODS), and fatal outcome (9). Indeed, Cao et al (44) integrated data obtained from more than 46,000 COVID-19 cases, and reported that the prevalence of ARDS was 28.8%, that of MODS was 8.5%, and the fatality rate was 6.8%. Therefore, the regulation of the overproduced cytokine is a focus of treatments targeting suppression of IL-1 family and IL-6 currently in trials (26). The multiple inflammatory mediators also can produce microcirculatory injury and thrombus formation. In the postmortem evaluation of COVID-19 pulmonary tissues, the arterial vessels demonstrated neutrophilic and mononuclear cellular infiltration, and apoptosis of endothelial cells and mononuclear cells based on caspase 3 immunostaining (13).

Mehta et al (45) reported that severe COVID-19 resembles hemophagocytic lymphohistiocytosis (HLH) most frequently triggered by viral infection, a clinical scenario with uncontrolled cytokine production and typically presentation of fever, splenomegaly, cytopenia of two or more lineages, increased ferritin, low fibrinogen level, and MODS, including ARDS (46, 47). Chest radiographic findings in HLH include bilateral

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

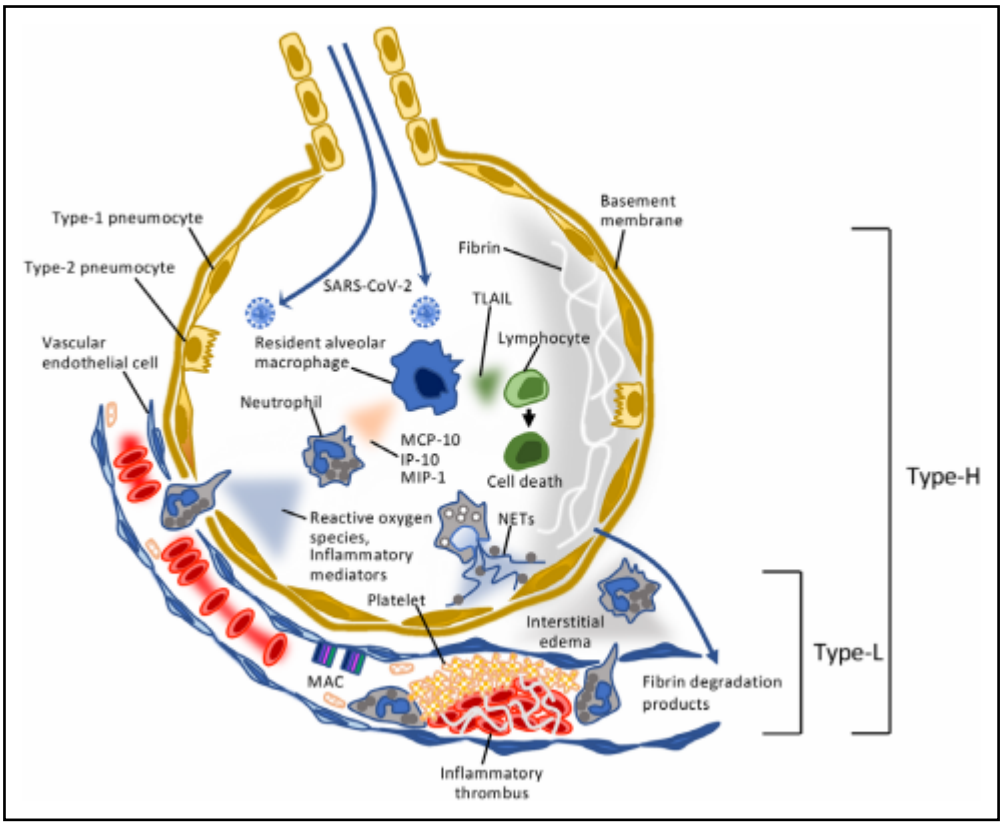


Figure 2. Lung injury in coronavirus disease 2019 (COVID-19). COVID-19 infection causes acute lung injury induced by activation of residential macrophages, lymphocyte apoptosis, and neutrophils. The macrophages produce cytokines and chemokines including monocyte chemotactic protein 1 (MCP-1), interferon-inducible protein (IP)-10, macrophage inflammatory protein (MIP)-1, and releases these mediators into the alveolar space. Increased tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) stimulates the lymphocyte apoptosis. COVID-19 also induces vascular endothelial damage through activating the complement system that leads to increased permeability and inflammatory thrombus formation. The fibrinolytic system is activated releasing fibrin degradation fragments (d-dimers) in the circulation. When the changes in the blood vessel are dominant and the damage in the alveolar space is relatively mild, that situation is considered as type-L, and when the damage advances to the alveolar space, it turns to type-H. MAC = membrane attack complex, NETs = neutrophil extracellular traps, SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2.

ground-glass opacities and consolidation that are similar to COVID-19. In laboratory testing, hyperferritinemia and high LDH levels are common, but low fibrinogen levels and cytopenias of more than two cell lineages by hemophagocytosis are not reported in COVID-19. Taken together, it may be reasonable to think that the pathophysiology of COVID-19 overlaps with low-grade HLH, and the differences and similarities of HLH and COVID-19 should be examined in future studies.

VENOUS THROMBOEMBOLISM AND PULMONARY EMBOLISM

Immobilization, inflammation, activated coagulation, and suppressed fibrinolysis increase the risk of VTE and PE. Increasing reports have indicated an increased risk of VTE and PE in COVID-19 (1, 48). Cui et al (49) examined thrombosis in non-symptomatic lower limbs by ultrasonography in COVID-19 pneumonia patients treated in ICU and reported that the prevalence was 25% (20/81). In another study in SARS-CoV-1 infected patients, it was reported that 20.5% of patients had DVT, and 11.4% had PE (50). Klok et al (15) studied 184 ICU patients and confirmed VTE in 27% and arterial thrombotic events in 3.7% patients. It should be kept in mind that the prevalence of VTE and PE is underestimated since the access to contrast-enhanced CT may be limited in critically ill patients for practical reasons. d-dimer levels can also not accurately differentiate between the presence of thrombosis and high levels due to the critical illness state. Therefore, all critically ill patients should receive VTE prophylaxis, preferably using low-molecular-weight (LMW) heparin regardless of their d-dimer level. In case of sudden deterioration, increased oxygenation requirements, right heart failure, and/or shock, a very high suspicion for PE should be maintained (51).

COAGULATION DISORDERS OF SARS-COV-2 INFECTION IN PREGNANCY

The effect of SARS-CoV-2 infection on pregnancy is not clear and, Liu et al (52) reported pregnancy and childbirth did not aggravate the course of COVID-19 pneumonia in the small case study (15 cases). However, Dashraath et al (53) suggested that 2% of pregnant women will require mechanical ventilation, 9% will have fetal growth restriction, and 43% will deliver preterm. In addition, since there are changes in coagulation/fibrinolysis that occur naturally during pregnancy, with hyperfibrinogenemia, there are considerable concerns for the risk of pregnant patients for thrombotic complications and coagulopathy. ISTH released an interim guideline for the management of COVID-19–associated coagulopathy and DIC in pregnant women (54), and this guidance recommends admission of any patients with markedly raised d-dimer, prolonged PT, platelet county less than 100×10^9/L, or fibrinogen less than 2 g/L, even in absence of other concerns, with the consideration for the use of prophylactic LMW heparin in all patients who require hospital admission in the absence of contraindications.

COAGULATION DISORDER IN CANCER PATIENTS

Patients with cancer are more susceptible to SARS-CoV-2 infection due to immunosuppression, poor nutrition,

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

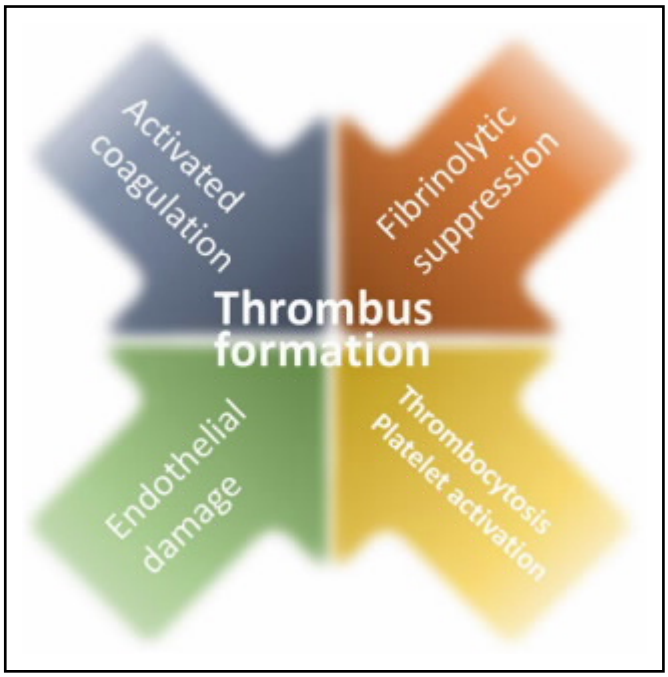


Figure 3. Thrombus formation in coronavirus disease 2019 (COVID-19). There are four major factors that accelerate thrombosis formation. First, severe acute respiratory syndrome coronavirus 2 infection-induced cytokine storm activates coagulation. Proinflammatory cytokines such as interleukin (IL)-1β and IL-6 stimulate the expression of tissue factor on immune cells and initiates extrinsic coagulation cascade activation. Second, the fibrinolytic system is suppressed by the decreased activity of urokinase-type plasminogen activator and increased release of plasminogen activator inhibitor-1. Third, platelets are activated by various proinflammatory cytokines and the damaged endothelium readily bind platelets. Fourth, endothelial damage induced by inflammation further accelerates the thrombotic reaction.

comorbidities, and immobilization (55). COVID-19 affected cancer patients require specific attention as they are prone to thromboembolic complications, and thromboprophylaxis is mandatory. However, evidence on the clinical course of COVID-19 in cancer patients is scarce so far, and more studies are warranted.

TREATMENT WITH ANTICOAGULANTS

Heparin for VTE Prevention

ISTH interim guidance recommends the use of prophylactic LMW heparin in severe COVID-19 patients (51). The effect of heparin, mainly LMW heparin, is reported by Tang et al (56) showing a reduced mortality in cases with coagulopathy and treated with heparin compared with the patients who did have coagulopathy and were not treated with heparin (40.0% vs 64.2%, respectively, $p = 0.029$). In the same study, increasing levels of d-dimer were related to increasing mortality in non-heparin treated patients. Heparin exhibits anti-inflammatory effects by neutralizing DAMPs to protect the endothelial cells by reducing the toxicity of histones on endothelial tight junctions, and decrease lung edema and vascular leakage (57, 58). Regarding the type and dose of heparins, we summarize the current recommendation in **Table 1**. Caution is required for the application of the treatment dose of heparins. The overall effectiveness is still under debate (59).

TABLE 1. Dosing Recommendations for Unfractionated and Low-Molecular-Weight Heparins

Drug	Prophylaxis	Treatment
Dalteparin (subcutaneously)	5,000 IU once daily	200 U/kg daily
Enoxaparin (subcutaneously)	40 mg once daily	1 mg/kg bid
Nadroparin (subcutaneously)	2,850 IU once daily	171 IU/kg once daily
Tinzaparin (subcutaneously)	4,500 IU once daily	175 IU/kg once daily
Unfractionated heparin	5,000 units twice/three times a day (subcutaneously)	IV titrated to target laboratory values

IU = international units.

Anticoagulant Therapies for Inflammatory Thrombus Prevention

In addition to VTE prevention, anticoagulant therapy may also have anti-inflammatory effects. Glas et al (34) proposed the administration of anticoagulants such as antithrombin and activated protein C for the treatment of classical ARDS. Others have suggested therapies to reverse pulmonary microthrombi in ARDS with tissue plasminogen activator; however, supporting evidence in humans is currently unavailable (60, 61).

Antiplatelet Therapies

Although platelets may be involved in the local and systemic thrombotic response in COVID-19 coagulopathy, adding a platelet inhibitor to unfractionated heparin or LMW heparin at therapeutic doses would increase the potential for risk for bleeding. This is a known phenomenon in acute coronary syndromes where anticoagulant therapy along with antiplatelet therapy may decrease arterial thrombosis, but it is associated with increased bleeding risk that also increases adverse events and most P2Y12 inhibitors have long half-lives without the availability of any reversal agent (62). Further, platelet function testing is cumbersome, and research studies on platelet activation biomarkers are still premature. The microvascular thrombosis, DVT, and pulmonary artery thrombosis appear to be due to abnormally elevated coagulation factor levels and the absence of the usual protective effects of the vascular endothelium. The role of platelet activation in this process is less well defined and not clearly implicated.

SUMMARY

SARS-CoV-2/COVID-19 frequently induces hypercoagulability with inflammation driving increased levels of procoagulant clotting factors and disruption of the normal

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

homeostasis of vascular endothelial cells resulting in microangiopathy, local thrombus formation, and a systemic coagulation defect leading to large vessel thrombosis and major thromboembolic complications including PE in critically ill hospitalized patients. In patients with infection-induced coagulopathies, a critical component of management is treating the underlying disease. In COVID-19, because we currently do not have a standard antiviral therapy, we believe some of the unique microvascular and macrovascular hypercoagulability clinician are observing represent thromboinflammatory responses to the continuing infection. As a result, sequential monitoring of coagulation tests every 2–3 days is recommended. Surveillance for development of VTE is important with heightened suspicion in patients with sudden decompensation not attributable to other factors. All hospitalized patients should receive VTE prophylaxis; higher than conventional doses of LMW heparin are currently being investigated in clinical trials, although many centers have adopted escalated or intermediate doses for VTE prophylaxis. Whether anticoagulation alone is sufficient to prevent these thrombotic events, especially those driven by endothelial dysfunction, is unknown. Additional strategies and studies to address all factors that result in microvascular and macrovascular thrombosis are needed.

Dr. Levy received funding from research, data safety, or advisory committees for CSL Behring, Instrumentation Labs, Janssen, Merck, and Octapharma. Dr. Connors' institution received funding from CSL Behring; received funding from Abbott (consulting), Bristol-Myers Squibb (consulting), and Portola (scientific advisory board). The remaining authors have disclosed that they do not have any potential conflicts of interest.

For information regarding this article, E-mail: toshiiba@juntendo.ac.jp

REFERENCES

1. Danzi GB, Loffi M, Galeazzi G, et al: Acute pulmonary embolism and COVID-19 pneumonia: A random association? *Eur Heart J* 2020; 41:1858
2. Zhang Y, Xiao M, Zhang S, et al: Coagulopathy and antiphospholipid antibodies in patients with Covid-19. *N Engl J Med* 2020; 382:e38
3. Wu C, Chen X, Cai Y, et al: Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. *JAMA Intern Med* 2020 Mar 13. [online ahead of print]
4. Rodriguez-Morales AJ, Cardona-Ospina JA, Gutiérrez-Ocampo E, et al: Clinical, laboratory and imaging features of COVID-19: A systematic review and meta-analysis. *Travel Med Infect Dis* 2020; 34:101623
5. Ranucci M, Ballotta A, Di Dedda U, et al: The procoagulant pattern of patients with Covid-19 acute respiratory distress syndrome. *J Thromb Haemost* 2020 Apr 17. [online ahead of print]
6. Panigada M, Bottino N, Tagliabue P, et al: Hypercoagulability of COVID-19 patients in intensive care unit. A report of thromboelastography findings and other parameters of hemostasis. *J Thromb Haemost* 2020 Apr 17. [online ahead of print]
7. Zhang Y, Cao W, Xiao M, et al: Clinical and coagulation characteristics of 7 patients with critical COVID-2019 pneumonia and acro-ischemia. *Zhonghua Xue Ye Xue Za Zhi* 2020; 41:E006
8. Li T, Lu H, Zhang W: Clinical observation and management of COVID-19 patients. *Emerg Microbes Infect* 2020; 9:687–690
9. Lin L, Lu L, Cao W, et al: Hypothesis for potential pathogenesis of SARS-CoV-2 infection-a review of immune changes in patients with viral pneumonia. *Emerg Microbes Infect* 2020; 9:727–732
10. Luo W, Yu H, Gou J, et al: Clinical pathology of critical patient with novel coronavirus pneumonia (COVID-19). *Preprints* 2020:2020020407
11. Zhou M, Zhang X, Qu J: Coronavirus disease 2019 (COVID-19): A clinical update. *Front Med* 2020; 14:126–135
12. Magro C, Mulvey JJ, Berlin D, et al: Complement associated microvascular injury and thrombosis in the pathogenesis of severe COVID-19 infection: A report of five cases. *Transl Res* 2020 Apr 15. [online ahead of print]
13. Varga Z, Flammer AJ, Steiger P, et al: Endothelial cell infection and endotheliitis in COVID-19. *Lancet* 2020; 395:1417–1418
14. Levi M, Scully M: How I treat disseminated intravascular coagulation. *Blood* 2018; 131:845–854
15. Klok FA, Kruip MJHA, van der Meer NJM, et al: Incidence of thrombotic complications in critically ill ICU patients with COVID-19. *Thromb Res* 2020 Apr 10. [online ahead of print]
16. Poissy J, Goutay J, Caplan M, et al; Lille ICU Haemostasis COVID-19 Group: Pulmonary embolism in COVID-19 patients: Awareness of an increased prevalence. *Circulation* 2020 Apr 24. [online ahead of print]
17. Leonard-Lorant I, Delabranche X, et al: Acute pulmonary embolism in COVID-19 patients on CT angiography and relationship to D-dimer levels. *Radiology* 2020 Apr 23. [online ahead of print]
18. Guan WJ, Ni ZY, Hu Y, et al: China medical treatment expert group for covid-19. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med* 2020; 382:1708–1720
19. Zhou F, Yu T, Du R, et al: Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: A retrospective cohort study. *Lancet* 2020; 395:1054–1062
20. Zhang L, Yan X, Fan Q, et al: D-dimer levels on admission to predict in-hospital mortality in patients with Covid-19. *J Thromb Haemost* 2020 Apr 19. [online ahead of print]
21. Tang N, Li D, Wang X, et al: Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. *J Thromb Haemost* 2020; 18:844–847
22. Lippi G, Plebani M, Michael Henry B: Thrombocytopenia is associated with severe coronavirus disease 2019 (COVID-19) infections: A meta-analysis. *Clin Chim Acta* 2020; 506:145–148
23. Yang X, Yu Y, Xu J, et al: Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: A single-centered, retrospective, observational study. *Lancet Respir Med* 2020; 8:475–481
24. Huang C, Wang Y, Li X, et al: Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; 395:497–506
25. Yin S, Huang M, Li D, et al: Difference of coagulation features between severe pneumonia induced by SARS-CoV2 and non-SARS CoV2. *J Thromb Thrombolysis* 2020 Apr 3. [online ahead of print]
26. Conti P, Ronconi G, Caraffa A, et al: Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by coronavirus-19 (COVI-19 or SARS-CoV-2): Anti-inflammatory strategies. *J Biol Regul Homeost Agents* 2020; 34:1
27. Amgalan A, Othman M: Exploring possible mechanisms for COVID-19 induced thrombocytopenia: Unanswered questions. *J Thromb Haemost* 2020 Apr 11. [online ahead of print]
28. Deng Y, Liu W, Liu K, et al: Clinical characteristics of fatal and recovered cases of coronavirus disease 2019 (COVID-19) in Wuhan, China: A retrospective study. *Chin Med J* 2020 Mar 20. [online ahead of print]
29. Han H, Yang L, Liu R, et al: Prominent changes in blood coagulation of patients with SARS-CoV-2 infection. *Clin Chem Lab Med* 2020 Mar 16. [online ahead of print]
30. Campbell CM, Kahwash R: Will complement inhibition be the new target in treating COVID-19 related systemic thrombosis? *Circulation* 2020 Apr 9. [online ahead of print]
31. Antoniak S, Mackman N: Multiple roles of the coagulation protease cascade during virus infection. *Blood* 2014; 123:2605–2613
32. Delabranche X, Helms J, Meziani F: Immunohaemostasis: A new view on haemostasis during sepsis. *Ann Intensive Care* 2017; 7:117
33. Barnes BJ, Adrover JM, Baxter-Stoltzfus A, et al: Targeting potential drivers of COVID-19: Neutrophil extracellular traps. *J Exp Med* 2020; 217:e20200652

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

34. Glas GJ, Van Der Sluijs KF, Schultz MJ, et al: Bronchoalveolar hemostasis in lung injury and acute respiratory distress syndrome. *J Thromb Haemost* 2013; 11:17–25
35. Günther A, Mosavi P, Heinemann S, et al: Alveolar fibrin formation caused by enhanced procoagulant and depressed fibrinolytic capacities in severe pneumonia. Comparison with the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2000; 161:454–462
36. Idell S: Coagulation, fibrinolysis, and fibrin deposition in acute lung injury. *Crit Care Med* 2003; 31:S213–S220
37. Gralinski LE, Bankhead A 3rd, Jeng S, et al: Mechanisms of severe acute respiratory syndrome coronavirus-induced acute lung injury. *mBio* 2013; 4:e00271–e00313
38. Ji HL, Zhao R, Matalon S, et al: Elevated plasmin(ogen) as a common risk factor for COVID-19 susceptibility. *Physiol Rev* 2020; 100:1065–1075
39. Hanley B, Lucas SB, Youd E, et al: Autopsy in suspected COVID-19 cases. *J Clin Pathol* 2020; 73:239–242
40. Gattinoni L, Chiumello D, Caironi P, et al: COVID-19 pneumonia: Different respiratory treatments for different phenotypes? *Intensive Care Med* 2020; 14:1–4
41. Dolhnikoff M, Duarte-Neto AN, de Almeida Monteiro RA, et al: Pathological evidence of pulmonary thrombotic phenomena in severe COVID-19. *J Thromb Haemost* 2020 Apr 15. [online ahead of print]
42. Zhang H, Penninger JM, Li Y, et al: Angiotensin-converting enzyme 2 (ACE2) as a SARS-CoV-2 receptor: Molecular mechanisms and potential therapeutic target. *Intensive Care Med* 2020; 46:586–590
43. Xiong Y, Liu Y, Cao L, et al: Transcriptomic characteristics of bronchoalveolar lavage fluid and peripheral blood mononuclear cells in COVID-19 patients. *Emerg Microbes Infect* 2020; 9:761–770
44. Cao Y, Liu X, Xiong L, et al: Imaging and clinical features of patients with 2019 novel coronavirus SARS-CoV-2: A systematic review and meta-analysis. *J Med Virol* 2020 Apr 3. [online ahead of print]
45. Mehta P, McAuley DF, Brown M, et al; HLH Across Speciality Collaboration, UK: COVID-19: Consider cytokine storm syndromes and immunosuppression. *Lancet* 2020; 395:1033–1034
46. Valade S, Mariotte E, Azoulay E: Coagulation disorders in hemophagocytic lymphohistiocytosis/macrophage activation syndrome. *Crit Care Clin* 2020; 36:415–426
47. Seguin A, Galicier L, Boutboul D, et al: Pulmonary involvement in patients with hemophagocytic lymphohistiocytosis. *Chest* 2016; 149:1294–1301
48. Xie Y, Wang X, Yang P: COVID-19 complicated by acute pulmonary embolism. *Radiology: Cardiothoracic Imaging* 2020; 2:e200067
49. Cui S, Chen S, Li X, et al: Prevalence of venous thromboembolism in patients with severe novel coronavirus pneumonia. *J Thromb Haemost* 2020 Apr 9. [online ahead of print]
50. Chong PY, Chui P, Ling AE, et al: Analysis of deaths during the severe acute respiratory syndrome (SARS) epidemic in Singapore: Challenges in determining a SARS diagnosis. *Arch Pathol Lab Med* 2004; 128:195–204
51. Thachil J, Wada H, Gando S, et al: ISTH interim guidance on recognition and management of coagulopathy in COVID-19. *J Thromb Haemost* 2020; 18:1023–1026
52. Liu D, Li L, Wu X, et al: Pregnancy and perinatal outcomes of women with coronavirus disease (COVID-19) pneumonia: A preliminary analysis. *AJR Am J Roentgenol* 2020 Mar 18. [online ahead of print]
53. Dashraath P, Jing Lin Jeslyn W, Mei Xian Karen L, et al: Coronavirus disease 2019 (COVID-19) pandemic and pregnancy. *Am J Obstet Gynecol* 2020 Mar 23. [online ahead of print]
54. Abdul-Kadir R, Kobayashi T, Iba T, et al: ISTH interim guidance in recognition and management of coagulopathy or DIC in COVID-19-specific issues for consideration in pregnant women. *J Thromb Haemost* 2020; 18:1023–1026
55. Liang W, Guan W, Chen R, et al: Cancer patients in SARS-CoV-2 infection: A nationwide analysis in China. *Lancet Oncol* 2020; 21:335–337
56. Tang N, Bai H, Chen X, et al: Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. *J Thromb Haemost* 2020; 18:1094–1099
57. Thachil J: The versatile heparin in COVID-19. *J Thromb Haemost* 2020; 18:1020–1022
58. Liu Y, Mu S, Li X, et al: Unfractionated heparin alleviates sepsis-induced acute lung injury by protecting tight junctions. *J Surg Res* 2019; 238:175–185
59. Connors JM, Levy JH: COVID-19 and its implications for thrombosis and anticoagulation. *Blood* 2020 Apr 27. [online ahead of print]
60. Hardaway RM, Harke H, Tyroch AH, et al: Treatment of severe acute respiratory distress syndrome: A final report on a phase I study. *Am Surg* 2001; 67:377–382
61. Moore HB, Barrett CD, Moore EE, et al: Is there a role for tissue plasminogen activator (tPA) as a novel treatment for refractory COVID-19 associated acute respiratory distress syndrome (ARDS). *J Trauma Acute Care Surg* 2020 Mar 20. [online ahead of print]
62. Hobl EL, Derhaschnig U, Firbas C, et al: Reversal strategy in antagonizing the P2Y12 -inhibitor ticagrelor. *Eur J Clin Invest* 2013; 43:1258–1261

Copyright © 2020 by the Society of Critical Care Medicine and Wolters Kluwer Health, Inc. All Rights Reserved.

EDITORIAL

A Randomized Trial of Convalescent Plasma for COVID-19—Potentially Hopeful Signals

Arturo Casadevall, MD, PhD; Michael J. Joyner, MD; Liise-Anne Pirofski, MD

Convalescent plasma for the treatment of infectious diseases has been used since the early 20th century and was associated with reduced mortality during the 1918 influenza,[1] 2003 SARS,[2] and 2009 influenza H1N1[3] pandemics. However, most published studies of these diseases were case series and retrospective comparisons of treated and nontreated individuals. Consistent with this, several uncontrolled case series of convalescent plasma use in patients with coronavirus disease (2019) COVID-19 have suggested a possible benefit.[4-6] Given encouraging historical precedents and the absence of proven SARS-CoV-2 (severe acute respiratory disease coronavirus 2) antiviral therapies, convalescent plasma therapy has been proposed as a treatment option for COVID-19.[7] The availability of clinical information generated from randomized clinical trials is therefore of substantial importance given that the world remains in the grip of the COVID-19 epidemic and convalescent plasma is currently in use in many countries, including the US.

Related article

In their article in *JAMA*, Li et al[8] present findings from the first randomized clinical trial of convalescent plasma therapy for patients with COVID-19 conducted in China. In contrast to most other reports of convalescent plasma use in past epidemics, this study is noteworthy in that it used a randomized trial design and well-characterized plasma units with a high titer of antibody to SARS-CoV-2. It was an important accomplishment to conduct a carefully controlled trial during a pandemic with an entirely new highly contagious disease that stressed health systems in an unprecedented way.

However, the authors report that because the COVID-19 outbreak in China was being contained while the trial was ongoing and new cases were unavailable for enrollment, the trial was terminated before it reached its targeted original sample size of 200 patients; only 103 were enrolled (for whom randomization was stratified by disease severity). Consequently, the study was underpowered and many comparisons between the convalescent plasma group and the control group were not statistically significant.

In the primary analysis, based on 52 patients who were randomized to receive convalescent plasma in addition to standard treatment and 51 patients who were randomized to receive standard treatment alone (control), the primary outcome of time to clinical improvement within 28 days (defined as being discharged alive or having a reduction of 2 points on a 6-point disease severity scale) was 2.15 days shorter (95% CI, −5.28 to 0.99 days) in the intervention group compared with the control group, and clinical improvement at 28 days occurred in 27 patients (51.9%) in the intervention group vs 22 patients (43.1%) in the control group (difference, 8.8%; 95% CI, −10.4% to 28%; hazard ratio, 1.40 [95% CI, 0.79-2.49]; $P = .26$).

In analyses stratified by disease severity, among patients with severe disease (23 in the convalescent plasma group and 22 in the control group), time to clinical improvement within 28 days was 4.94 days shorter (95% CI, −9.33 to −0.54 days) in the intervention group compared with the control group, and clinical improvement at 28 days occurred in 21 patients (91.3%) in the intervention group vs 15 patients (68.2%) in the control group (hazard ratio, 2.15 [95% CI, 1.07-4.32]; $P = .03$). Among the subgroup of patients with life-threatening disease (29 in the convalescent plasma group and 29 in the control group), there were no significant differences in the primary outcome or rates of clinical improvement at 28 days: 6 patients (20.7%) in the convalescent plasma group vs 7 patients (24.1%) in the control group (HR, 0.88 [95% CI, 0.30-2.63]; $P = .83$) (P for interaction = .17).

In the entire study population, the findings for several of the secondary end points appeared to signal a more favorable outcome for patients who received convalescent plasma, although there were no statistically significant differences between the convalescent plasma group vs the control group in any of the major secondary outcomes, including 28-day mortality (15.7% vs 24.0%, respectively; $P = .30$) or rate of discharge at 28 days (51% vs 36%; $P = .13$).

Convalescent plasma use in the study by Li et al[8] was associated with some clinical improvement in severely ill patients, but not in critically ill patients. Greater efficacy in less ill individuals is expected because antibody therapies generally work best when administered earlier in disease.[9] Historically, antibody therapy was effective in reducing the mortality of pneumococcal pneumonia when instituted in the first 3 days of symptom onset.[10] Consequently, it is not surprising that patients with COVID-19 who had tachypnea and hypoxia might benefit more from convalescent plasma than those who required mechanical ventilation. However, any indication of possible benefit in the severely ill group is noteworthy because these individuals had advanced disease, which is not considered optimal for antibody therapy. Lack of efficacy among patients who were receiving mechanical ventilation, some with multiorgan failure, highlights that the pathologic process in these individuals is likely irreversible.

The convalescent plasma used in the study by Li et al had high titers of IgG to SARS-CoV-2, which correlated with neutralizing activity. While neutralizing activity is considered to be the main determinant of convalescent plasma efficacy,

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

other antibody functions may also mediate protection. Correlates of antibody efficacy should be investigated in future studies. As reported in case series from Wuhan,[4,5] plasma-treated patients had large reductions in their serum viral loads and most were virus negative 3 days after infusion. This observation establishes that convalescent plasma has antiviral activity, which is important because it indicates that antibody administration mediates a clear biological effect. The precedent of antiviral drug use against HIV and hepatitis C shows that reductions in viral load translate into clinical improvement, and earlier therapy is more effective than later therapy when organ damage is already present. In this regard, antibody-mediated viral elimination removes damaging antigens, which may translate into reduced tissue damage and inflammation. Hence, the antiviral effect of COVID-19 convalescent plasma suggests that its use earlier in the course of disease could have potentially important therapeutic activity, especially in less severely ill individuals.

Significant concerns have been raised about the use of convalescent plasma in COVID-19.[11] These include transfusion-related lung injury and transfusion-related circulatory overload. In addition, there have been theoretical concerns that the administration of antibodies might aggravate disease through antibody-mediated enhancement of proinflammatory effects.[11] The study by Li et al[8] reported only 2 adverse events among the 52 individuals who received convalescent plasma, each of whom responded to corticosteroid administration. The occurrence of one episode within 2 hours of plasma administration characterized by chills and rash suggests a transfusion reaction. However, the second episode occurred within 6 hours and its association with plasma infusion is less certain. Overall, the paucity of adverse effects is reassuring and reduces concerns about adverse effects from antibody administration.

Although the observed differences in mortality rates and hospital discharge rates between the convalescent plasma group and the control group did not reach statistical significance, these data provide valuable information for the magnitude of effects that may be expected in convalescent plasma studies. For example, the observed overall mortality difference of 24% vs 15.7% provides actionable information for the design of future trials to help ensure they are adequately powered. This difference in mortality is smaller than mortality reductions associated with convalescent plasma reported in prior studies involving 1918 influenza,[1] SARS,[2] and 2009 influenza H1N1,[3] which ranged from 50% to 70%. Hence, assuming the results of the study by Li et al[8] are generalizable, the findings may be helpful in estimating effect sizes for future studies of convalescent plasma use in hospitalized patients with COVID-19.

In the study by Li et al, the median age of the patients with severe disease was 70 years, and the median time between symptom onset and randomization was 30 days. Promising results with convalescent plasma treatment in patients with SARS[2] and influenza H1N1[3] were obtained among younger patients, and in the case of SARS, earlier in the disease. The importance of a possible treatment benefit in older persons, in whom mortality from COVID-19 is markedly higher than in younger persons,[12] cannot be overstated. In addition, the apparent improvement in the clinical status of the subgroup of less severely ill patients a month after the onset of symptoms suggests that the beneficial effects of antibodies in COVID-19 may be measurable as an improvement in inflammatory markers and viral elimination before clinical improvement is observed. The prolonged course of COVID-19 in patients who recover also should be considered in the design of future studies.

However, the study by Li et al has several important limitations, which are acknowledged by the investigators. The early termination of the trial most likely resulted in an underpowered study, thereby precluding any definitive conclusions about the role and potential efficacy of convalescent plasma for patients with COVID-19. In addition, the open-label design, the possibility of an element of subjectivity for the primary outcome, lack of a protocolized approach to standard therapy, and variability among study centers also must be considered when interpreting the study findings. Despite these limitations, by virtue of its randomized design, this study takes prior case studies[5,6] one step further by helping to separate the effects of convalescent plasma from concurrently administered agents, such as corticosteroids and antiviral agents.[13]

The signal of potential benefit of convalescent plasma in the subgroup of patients with severe COVID-19 disease (ie, those without life-threatening COVID-19 disease) is similar to findings from a recent preliminary report of a clinical trial of remdesivir for COVID-19.[14] Like remdesivir, convalescent plasma administration was associated with clinical improvement without a statistically significant effect on mortality, with the important caveat that remdesivir was evaluated in a larger study (n = 1063 randomized patients), whereas the study by Li et al[8] was terminated prematurely and underpowered. For both studies, the importance of clinical improvement as a primary end point became apparent as the trials progressed.[14]

The availability of both convalescent plasma and remdesivir means that physicians now have at least 2 therapeutic options for COVID-19, which raises the question of combination therapy. Despite only a few studies of the efficacy of combination therapy with antiviral drugs and specific antibodies, there is evidence that these agents may work well in combination.[9] Given that the mechanisms of action of antiviral drugs and neutralizing antibodies are distinct, they could be synergistic. Future trials should consider the efficacy of combination antiviral and antibody therapies.

In summary, the first randomized clinical trial of convalescent plasma in COVID-19, reported by Li et al in *JAMA*, showed no statistically significant benefit in clinical improvement at 28 days or mortality among all randomized patients, but does provide an important signal of possible benefit in the subgroup of severely ill patients and suggests that high titer antibody against SARS-CoV-2 may have antiviral efficacy. These results, while preliminary and subject to important study limitations, should stimulate more clinical trials to establish the optimal conditions for antibody therapies against COVID-19 and suggest that future studies

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

should focus on determining efficacy in less severely ill patients. If the efficacy of convalescent plasma is established by future studies, the ratio of donor to patients is favorable because individuals who recover from COVID-19 can donate 2 or 3 units of plasma, which could be used to treat more than 1 person with COVID-19 disease. Therapeutic success against such a complex and challenging disease as COVID-19 is likely to require more than 1 modality, and the results from Li et al [8] provide optimism for the future of antibody therapy in this disease.

ARTICLE INFORMATION

Author Affiliations: Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland (Casadevall); Department of Anesthesiology and Perioperative Medicine, Mayo Clinic, Rochester, Minnesota (Joyner); Montefiore Medical Center, Division of Infectious Diseases, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York (Pirofski).

Corresponding Author: Arturo Casadevall, MD, PhD, Johns Hopkins Bloomberg School of Public Health, 605 N Wolfe St, Baltimore, MD 21205 (acasade1@jhu.edu).

Published Online: June 3, 2020. doi:10.1001/jama.2020.10218

Conflict of Interest Disclosures: Dr Casadevall serves on the scientific advisory board of SAB Therapeutics, which is working to develop cow-derived human antibodies for the therapy of COVID-19. No other disclosures were reported.

REFERENCES

1. Luke TC, Kilbane EM, Jackson JL, Hoffman SL. Meta-analysis: convalescent blood products for Spanish influenza pneumonia: a future H5N1 treatment? *Ann Intern Med*. 2006;145(8):599-609. doi:10.7326/0003-4819-145-8-200610170-00139

2. Cheng Y, Wong R, Soo YO, et al. Use of convalescent plasma therapy in SARS patients in Hong Kong. *Eur J Clin Microbiol Infect Dis*. 2005;24(1):44-46. doi:10.1007/s10096-004-1271-9

3. Hung IF, To KK, Lee CK, et al. Convalescent plasma treatment reduced mortality in patients with severe pandemic influenza A (H1N1) 2009 virus infection. *Clin Infect Dis*. 2011;52(4):447-456. doi:10.1093/cid/ciq106

4. Duan K, Liu B, Li C, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc Natl Acad Sci U S A*. 2020;117(17):9490-9496. doi:10.1073/pnas.2004168117

5. Shen C, Wang Z, Zhao F, et al. Treatment of 5 critically ill patients with COVID-19 with convalescent plasma. *JAMA*. 2020;323(16):1582-1589. doi:10.1001/jama.2020.4783

6. Zhang B, Liu S, Tan T, et al. Treatment with convalescent plasma for critically ill patients with severe acute respiratory syndrome coronavirus 2 infection. *Chest*. Published online May 18, 2020. doi:10.1016/j.chest.2020.03.039

7. Casadevall A, Pirofski LA. The convalescent sera option for containing COVID-19. *J Clin Invest*. 2020;130(4):1545-1548. doi:10.1172/JCI138003

8. Li L, Zhang W, Hu Y, et al. Effect of convalescent plasma therapy on time to clinical improvement in patients with severe and life-threatening COVID-19: a randomized clinical trial. *JAMA*. Published online June 3, 2020. doi:10.1001/jama.2020.10044

9. Casadevall A, Scharff MD. Return to the past: the case for antibody-based therapies in infectious diseases. *Clin Infect Dis*. 1995;21(1):150-161. doi:10.1093/clinids/21.1.150

10. Casadevall A, Scharff MD. Serum therapy revisited: animal models of infection and development of passive antibody therapy. *Antimicrob Agents Chemother*. 1994;38(8):1695-1702. doi:10.1128/AAC.38.8.1695

11. Dzik S. COVID-19 convalescent plasma: now is the time for better science. *Transfus Med Rev*. 2020;S0887-7963(20)30026-2.

12. Zhou F, Yu T, Du R, et al. Clinical course and risk factors for mortality of adult in patients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*. 2020;395(10229):1054-1062. doi:10.1016/S0140-6736(20)30566-3

13. Roback JD, Guarner J. Convalescent plasma to treat COVID-19: possibilities and challenges. *JAMA*. 2020;323(16):1561-1562. doi:10.1001/jama.2020.4940

14. Beigel JH, Tomashek KM, Dodd LE, et al; ACTT-1 Study Group Members. Remdesivir for the treatment of Covid-19—preliminary report. *N Engl J Med*. Published online May 22, 2020. doi:10.1056/NEJMoa2007764

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

JAMA | Original Investigation

Effect of Convalescent Plasma Therapy on Time to Clinical Improvement in Patients With Severe and Life-threatening COVID-19 A Randomized Clinical Trial

Ling Li, MD, PhD; Wei Zhang, MD; Yu Hu, MD, PhD; Xunliang Tong, MD, PhD; Shangen Zheng, MD; Juntao Yang, PhD; Yujie Kong, MD; Lili Ren, PhD; Qing Wei, MD; Heng Mei, MD, PhD; Caiying Hu, MD; Cuihua Tao, MD; Ru Yang, MD; Jue Wang, MD; Yongpei Yu, PhD; Yong Guo, PhD; Xiaoxiong Wu, MD; Zhihua Xu, MD; Li Zeng, MD; Nian Xiong, MD; Lifeng Chen, MD; Juan Wang, MD; Ning Man, MD; Yu Liu, PhD; Haixia Xu, MD; E. Deng, MS; Xuejun Zhang, MS; Chenyue Li, MD; Conghui Wang, PhD; Shisheng Su, PhD; Linqi Zhang, PhD; Jianwei Wang, PhD; Yanyun Wu, MD, PhD; Zhong Liu, MD, PhD

IMPORTANCE Convalescent plasma is a potential therapeutic option for patients with coronavirus disease 2019 (COVID-19), but further data from randomized clinical trials are needed.

OBJECTIVE To evaluate the efficacy and adverse effects of convalescent plasma therapy for patients with COVID-19.

DESIGN, SETTING, AND PARTICIPANTS Open-label, multicenter, randomized clinical trial performed in 7 medical centers in Wuhan, China, from February 14, 2020, to April 1, 2020, with final follow-up April 28, 2020. The trial included 103 participants with laboratory-confirmed COVID-19 that was severe (respiratory distress and/or hypoxemia) or life-threatening (shock, organ failure, or requiring mechanical ventilation). The trial was terminated early after 103 of a planned 200 patients were enrolled.

INTERVENTION Convalescent plasma in addition to standard treatment (n = 52) vs standard treatment alone (control) (n = 51), stratified by disease severity.

MAIN OUTCOMES AND MEASURES Primary outcome was time to clinical improvement within 28 days, defined as patient discharged alive or reduction of 2 points on a 6-point disease severity scale (ranging from 1 [discharge] to 6 [death]). Secondary outcomes included 28-day mortality, time to discharge, and the rate of viral polymerase chain reaction (PCR) results turned from positive at baseline to negative at up to 72 hours.

RESULTS Of 103 patients who were randomized (median age, 70 years; 60 [58.3%] male), 101 (98.1%) completed the trial. Clinical improvement occurred within 28 days in 51.9% (27/52) of the convalescent plasma group vs 43.1% (22/51) in the control group (difference, 8.8% [95% CI, −10.4% to 28.0%]; hazard ratio [HR], 1.40 [95% CI, 0.79-2.49]; P = .26). Among those with severe disease, the primary outcome occurred in 91.3% (21/23) of the convalescent plasma group vs 68.2% (15/22) of the control group (HR, 2.15 [95% CI, 1.07-4.32]; P = .03); among those with life-threatening disease the primary outcome occurred in 20.7% (6/29) of the convalescent plasma group vs 24.1% (7/29) of the control group (HR, 0.88 [95% CI, 0.30-2.63]; P = .83) (P for interaction = .17). There was no significant difference in 28-day mortality (15.7% vs 24.0%; OR, 0.65 [95% CI, 0.29-1.46]; P = .30) or time from randomization to discharge (51.0% vs 36.0% discharged by day 28; HR, 1.61 [95% CI, 0.88-2.93]; P = .12). Convalescent plasma treatment was associated with a negative conversion rate of viral PCR at 72 hours in 87.2% of the convalescent plasma group vs 37.5% of the control group (OR, 11.39 [95% CI, 3.91-33.18]; P < .001). Two patients in the convalescent plasma group experienced adverse events within hours after transfusion that improved with supportive care.

CONCLUSION AND RELEVANCE Among patients with severe or life-threatening COVID-19, convalescent plasma therapy added to standard treatment, compared with standard treatment alone, did not result in a statistically significant improvement in time to clinical improvement within 28 days. Interpretation is limited by early termination of the trial, which may have been underpowered to detect a clinically important difference.

TRIAL REGISTRATION Chinese Clinical Trial Registry: ChiCTR2000029757

JAMA doi:10.1001/jama.2020.10044
Published online June 3, 2020.

Editorial
Supplemental content

Author Affiliations: Author affiliations are listed at the end of this article.

Corresponding Authors: Zhong Liu, MD, PhD, Institute of Blood Transfusion, Chinese Academy of Medical Sciences and Peking Union Medical College, 26 Huacai Rd, Longtan Industry Zone, Chenghua District, 610052, Chengdu, Sichuan, China (liuz@ibt.pumc.edu.cn); Yanyun Wu, MD, PhD, University of Miami, Department of Pathology, 1611 NW 12th Ave, Miami, FL 33136 (yxw1366@med.miami.edu).

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

Since December 2019, coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), has spread rapidly around the world, with high rates of transmission and substantial mortality. COVID-19 symptoms can range from mild, self-limited respiratory disease to severe progressive pneumonia, multiple organ failure, and even death. [1,2] To date, there is no effective treatment or vaccine to contain the disease.

Convalescent plasma therapy has been used to treat patients with infections using plasma collected from recovered patients. [3] This approach has been evaluated in the treatment of SARS,[4,5] Middle East respiratory syndrome (MERS), [6] and Ebola, [7] but not well studied and with no definitive results. Recently, case series from China reported improved outcomes after COVID-19 convalescent plasma transfusion. [8,9] The US Food and Drug Administration (FDA) recently approved the emergency use of convalescent plasma for patients with severe or life-threatening COVID-19. [10] Although the use of convalescent plasma shows promise, the evidence supporting its use in the treatment of COVID-19 remains limited, and thus its use remains investigational.

In addition, due to the limited understanding of the mechanism and precise therapeutic components of convalescent plasma, there is no standardization or evidence-based rationale for donor selection, quality control of convalescent plasma, or recipient transfusion indications for convalescent plasma. This may help to explain the varied therapeutic effects of convalescent plasma seen in a variety of infectious diseases. To address these issues, the World Health Organization issued a guideline on the use of convalescent plasma in a pandemic, advocating for standardization in donor selection and convalescent plasma quality control to maximize therapeutic potency. [11]

The objective of this randomized clinical trial was to evaluate the efficacy and adverse effects of convalescent plasma added to standard treatment, compared with standard treatment alone, for patients with severe or life-threatening COVID-19 using a standardized approach in donor selection and convalescent plasma quality control.

Key Points

Question What is the effect of convalescent plasma therapy added to standard treatment, compared with standard treatment alone, on clinical outcomes in patients with severe or life-threatening coronavirus disease 2019 (COVID-19)?

Finding In this randomized clinical trial that included 103 patients and was terminated early, the hazard ratio for time to clinical improvement within 28 days in the convalescent plasma group vs the standard treatment group was 1.40 and was not statistically significant.

Meaning Among patients with severe or life-threatening COVID-19, convalescent plasma therapy added to standard treatment did not significantly improve the time to clinical improvement within 28 days, although the trial was terminated early and may have been underpowered to detect a clinically important difference.

Methods

This study was a collaborative effort by the Institute of Blood Transfusion of the Chinese Academy of Medical Sciences; Union Hospital of Tongji Medical College of Huazhong University of Science and Technology; the Guanggu District Maternal and Child Health Hospital of Hubei Province; Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology; General Hospital of the Central Theater Command of the People's Liberation Army; Wuhan Red Cross Hospital; Wuhan Asia Heart Hospital; and Wuhan Pulmonary Hospital.

This study was approved by the ethics committee of the Institute of Blood Transfusion of the Chinese Academy of Medical Sciences and the ethics committees of the participating hospitals. Written informed consent was obtained from all study participants or their legal representatives. The trial protocol and statistical analysis plan are available in Supplement 1 and Supplement 2 .

Participants

Patients were recruited from 7 medical centers. The study recruitment was from February 14, 2020, to April 1, 2020. Follow-up was completed on April 28, 2020.

Inclusion Criteria

Inclusion criteria were the following: (1) signed informed consent; (2) aged at least 18 years; (3) COVID-19 diagnosis based on polymerase chain reaction (PCR) testing; (4) positive PCR result within 72 hours prior to randomization; (5) pneumonia confirmed by chest imaging; (6) clinical symptoms meeting the definitions of severe or life-threatening COVID-19; (7) acceptance of random group assignment; (8) hospital admission; (9) willingness to participate in all necessary research studies and be able to complete the study follow-up; and (10) no participation in other clinical trials, such as antiviral trials, during the study period.

Severe COVID-19 was defined as respiratory distress (≥30 breaths/min; in resting state, oxygen saturation of 93% or less on room air; or arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FIO_2) of 300 or less. Life-threatening COVID-19 was defined as respiratory failure requiring mechanical ventilation; shock; or other organ failure (apart from lung) requiring intensive care unit (ICU) monitoring.

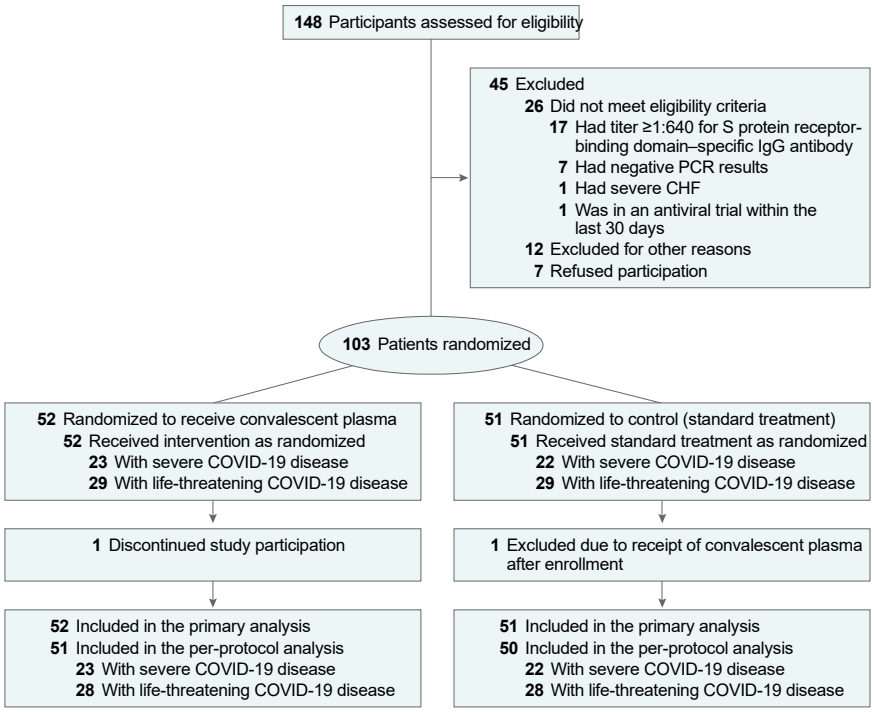
Exclusion Criteria

Exclusion criteria were the following: (1) pregnancy or lactation; (2) immunoglobulin allergy; (3) IgA deficiency; (4) preexisting comorbidity that could increase the risk of thrombosis; (5) life expectancy less than 24 hours; (6) disseminated intravascular coagulation; (7) severe septic shock; (8) PaO_2/FIO2 of less than 100; (9) severe congestive heart failure; (10) detection of high titer of S protein–RBD-specific (receptor binding domain) IgG antibody (≥1:640); (11) other contraindications as determined by the patient's physicians; and (12) participation in any antiviral clinical trials for COVID-19 within 30 days prior to enrollment.

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

Figure 1. Patient Enrollment and Treatment Assignment



PCR indicates polymerase chain reaction; CHF, congestive heart failure; COVID-19, coronavirus disease 2019.

Randomization

Potential study participants were screened for eligibility 72 hours prior to study randomization. Patients were randomly assigned via computer-generated random numbering (1:1) to receive standard treatment coupled with convalescent plasma transfusion or standard treatment alone (control group) (Figure 1). The randomization was stratified based on the severity of COVID-19 (severe or life-threatening) and a randomization schedule was generated using block randomization with block size of 4 for each type of COVID-19 by SAS software. Patients and clinicians were not masked to treatment assignment.

Procurement of Convalescent Plasma

In brief, patients with a laboratory-confirmed COVID-19 diagnosis, who had fully recovered and been discharged from the hospital for more than 2 weeks, were recruited. Convalescent plasma–specific donor screening and selection were based on the following criteria: age of 18 through 55 years, suitable for blood donation, initially diagnosed with COVID-19 but with 2 negative PCR test results from nasopharyngeal swabs (at least 24 hours apart) prior to hospital discharge, discharged for more than 2 weeks from the hospital, and no persisting COVID-19 symptoms. Convalescent plasma collection was performed based on routine plasma collection procedures via plasmapheresis. The plasma products were prepared as fresh-f rozen plasma. COVID-19 convalescent plasma was collected and processed at the Wuhan Blood Center.

S-RBD–specific IgG antibody titer was measured for convalescent plasma products and reported as the following: less than 1:160, 1:160, 1:320, 1:640, 1: 1280, or greater than 1:1280. There was a positive correlation between the SARS-CoV-2 viral neutralization titer and the S-RBD–specific IgG titer (r = 0.622, P = .03). A serum neutralization titer of 1:80 is approximately equivalent to a titer of 1:1280 for S-RBD–specific IgG. To ensure the therapeutic potency of the convalescent plasma, only the plasma units with an S-RBD–specific IgG titer of at least 1:640 were used for this study.

Additional details regarding plasma preparation standards can be found in the eMethods in Supplement 3 , and the preparation requirements of convalescent plasma used were similar to the recently updated FDA recommendations. [10]

Convalescent Plasma Transfusion

The transfusion dose of COVID-19 convalescent plasma was approximately 4 to 13 mL/kg of recipient body weight. ABO type of the convalescent plasma transfused was compatible with the patient's ABO type. In addition, the convalescent plasma was crossmatched with the patient's red blood cells to ensure compatibility. Convalescent plasma transfusion was administered at approximately 10 mL for the first 15 minutes, which was then increased to approximately 100 mL per hour with close monitoring. Adjustments in the infusion rates were allowed based on the patient's risk for volume overload and tolerance, at the discretion of the treating physicians. No premedication was given before convalescent plasma transfusion.

Standard Treatment

Standard treatment consisted of symptomatic control and supportive care for COVID-19, mostly based on the evolving Chinese national COVID-19 treatment guidelines and hospital

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

practice.[12] Possible treatments included antiviral medications, antibacterial medications, steroids, human immunoglobulin, Chinese herbal medicines, and other medications.

OutcomeMeasures

The primary end point was time to clinical improvement within a 28-day period. Clinical improvement was defined as patient discharge or a reduction of 2 points on a 6-point disease severity scale.[13] The scale was defined as follows: 6 points, death; 5 points, hospitalization plus extracorporeal membrane oxygenation (ECMO) or invasive mechanical ventilation; 4 points, hospitalization plus noninvasive ventilation or high-flow supplemental oxygen; 3 points, hospitalization plus supplemental oxygen (not high-flow or noninvasive ventilation); 2 points, hospitalization with no supplemental oxygen; 1 point, hospital discharge.

Patient discharge criteria included body temperature returned to normal for longer than 3 days, respiratory symptoms significantly improved without the need for oxygen support, and 2 consecutive negative PCR test results from nasopharyngeal swabs at least 24 hours apart.

Secondary clinical outcomes were as follows: (1) 28-day mortality, including analysis of time from randomization to death; (2) duration of hospitalization, including analyses of time from randomization to discharge, time from admission to discharge, and 28-day discharge rates; and (3) conversion of nasopharyngeal swab viral PCR results from positive at baseline to negative at follow-up assessed at 24, 48, and 72 hours. Once nasopharyngeal swab viral PCR testing yielded negative results 2 times consecutively, no further testing was performed.

A post hoc analysis was added to compare rates of improvement at days 7, 14, and 28.

This clinical trial was an open-label, randomized clinical study. To avoid assessment bias, the evaluation of clinical outcomes was performed by an investigator who was blind to the study group allocation.

StatisticalAnalysis

The original sample size was determined to be 100 for each group, which would provide 80% power, with a 2-sided significance level of $\alpha = .05$, to detect an 8-day change for the convalescent plasma group in time to clinical improvement, assuming that this would be 20 days in the control group and 60% of the patients would reach clinical improvement.

Unless otherwise stated, analyses were performed based on the full analysis set, which is defined as the set of all randomized patients who received at least one treatment specified in the trial. Statistical analysis was performed on randomly assigned treatment groups. Continuous variables were summarized by presenting the median and interquartile range (IQR) for the total number of patients who contributed values. Categorical variables were summarized by presenting the frequency and proportion of patients in each category. Time-to-event data were analyzed using the Kaplan-Meier method, and the median time to event and corresponding 95% CI were calculated. For the cases in which more than 50% of patients were censored and therefore the median time to event was indeterminate, the restricted mean survival time would be used for post hoc analysis.

For the primary end point of time to clinical improvement, death, withdrawal, and crossover between groups before day 28 were considered to be right-censored at day 28, and otherwise would be considered to be right-censored at the last observation date. Hazard ratios (HRs) with 95% CIs were calculated using Cox proportional hazards models. Three Cox proportional hazards models were fitted in this study. We referred to the model that included only the treatment group as the unadjusted model. The model that included disease severity (severe or life-threatening) and treatment group is referred to as model 1, and the model that further considered the interaction between disease severity and treatment group is referred to as model 2. Study sites were considered as a random effect in these models. Proportionality hazard assumption was assessed for treatment group and disease severity by extending the Cox models to include the corresponding time-dependent covariates.[14] If the coefficient of the time-dependent covariate was statistically significant, the proportionality hazard assumption would be considered to be violated.

A per-protocol analysis was performed for the primary end point as a sensitivity analysis. The per-protocol set was defined as the set of all randomized patients who received at least one treatment specified in the trial and who had no significant protocol violations that affected the efficacy evaluation.

Treatment effects for secondary end points were assessed using odds ratios and HRs with 95% CIs for the discrete variables and time-to-event data, respectively. For the analyses of time from randomization to discharge, time from randomization to death, and length of stay, the definition of censoring was consistent with the primary end point. Missing data for secondary outcomes and adverse events were not imputed. Only observed values were used for data analysis and presentation.

Subgroup analyses of efficacy were performed according to disease severity. Interactions between treatment group and disease severity group were tested using model 2.

Statistical analyses were performed with SAS software, version 9.4. Statistical significance was defined using a 2-sided significance level of $\alpha = .05$. Because of the potential for type I error due to multiple comparisons, findings for analyses of secondary end points should be interpreted as exploratory.

EarlyStudyTermination

Due to the containment of the COVID-19 epidemic in Wuhan, China, the numbers of patients with COVID-19 decreased in late March 2020. No new cases were reported in Wuhan for 7 consecutive days after March 24 (data from the National Health Commission of the People's Republic of China).[15] The last patient enrolled in this study was on March 27, 2020, and for the next 3 days, we were not able to recruit more patients and did not have any recruitment targets. After discussion with the expert committee of the Institute of Blood Transfusion, the study was terminated by the sponsor (Chinese Academy of Medical Sciences) and the leading primary investigator on April 1, 2020, with a total of 103 patients enrolled.

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

Table 1. Baseline Demographics and Clinical Characteristics of All Patients With COVID-19[a]

	Convalescent plasma group (n = 52)	Control group (n = 51)
Demographic and clinical characteristics		
Age, median (IQR), y	70 (62-80)	69 (63-76)
Sex, No. (%)		
Male	27 (51.9)	33 (64.7)
Female	25 (48.1)	18 (35.3)
Allergy history, No. (%)[b]	6 (11.5)	5 (9.8)
Coexisting diseases, No. (%)[c]		
Hypertension	29 (55.8)	27 (52.9)
Cardiovascular disease	14 (26.9)	12 (23.5)
Cerebrovascular disease	11 (21.2)	7 (13.7)
Diabetes	9 (17.3)	12 (23.5)
Liver disease	5 (9.6)	5 (9.8)
Cancer	3 (5.8)	0
Kidney disease	2 (3.9)	4 (7.8)
Laboratory values[d]		
Body temperature, median (IQR), °C	36.5 (36.2-36.7) [n = 52]	36.4 (36.2-36.8) [n = 50]
≥37.3 °C, No. (%)	4/52 (7.7)	7/50 (14.0)
Respiratory rate >24/min, No. (%)	11/52 (21.2)	7/49 (14.3)
Heart rate >100/min, No. (%)	13/52 (25.0)	8/50 (16.0)
Systolic blood pressure >140 mm Hg, No. (%)	10/52 (19.2)	15/50 (30.0)
White blood cell count, median (IQR), cells/μL	7590 (6300-11 460)	7160 (6130-11 200)
White blood cell count categories, No. (%)		
<4000/μL	5 (9.6)	4 (7.8)
4000-10 000/μL	31 (59.6)	29 (56.9)
>10 000/μL	16 (30.8)	18 (35.3)
Neutrophil count, median (IQR), cells/μL	7030 (4890-10 350)	5800 (4420-10 150)
Neutrophil count categories, No. (%)		
<1800/μL	0	2 (3.9)
1800-6300/μL	23 (44.2)	25 (49.0)
>6300/μL	29 (55.8)	24 (47.1)
Lymphocyte count, median (IQR), cells/μL	830 (570-1420)	800 (500-1370)
Lymphocyte count categories, No. (%)		
<1000/μL	32 (61.5)	32 (62.8)
≥1000/μL	20 (38.5)	19 (37.3)
Platelet count, median (IQR), $\times 10^3$/μL	164.5 (99.0-248.0)	214.0 (138.0-274.0)
Platelet count categories, No. (%)		
<100 × 10^3/μL	13 (25.0)	7 (13.7)
≥100 × 10^3/μL	39 (75.0)	44 (86.3)
CRP, median (IQR), mg/L	20.40 (5.13-65.60) [n = 49]	8.87 (1.73-40.32) [n = 48]
>5 mg/L, No. (%)	37/49 (75.5)	29/48 (60.4)
IL-6, median (IQR), pg/mL	16.62 (5.76-73.68) [n = 44]	21.67 (5.10-64.00) [n = 35]
>7 pg/mL, No. (%)	32/44 (72.7)	25/35 (71.4)
Prothrombin time, median (IQR), s	13.50 (12.00-15.20) [n = 51]	13.30 (12.35-14.15) [n = 48]
APTT, median (IQR), s	33.10 (28.30-41.10) [n = 49]	33.85 (29.95-42.95) [n = 48]
Thrombin time, median (IQR), s	16.45 (14.60-18.90) [n = 46]	16.10 (15.10-18.55) [n = 48]
Fibrinogen, median (IQR), mg/dL	3.86 (2.93-4.71) [n = 50]	4.00 (3.29-5.12) [n = 48]
D-dimer, median (IQR), μg/mL	1.88 (0.91-4.78) [n = 47]	2.23 (0.79-5.21) [n = 46]
>0.2 μg/mL, No. (%)	45/47 (95.7)	43/46 (93.5)

(continued)

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

Table 1. Baseline Demographics and Clinical Characteristics of All Patients With COVID-19 (continued)

	Convalescent plasma group (n = 52)	Control group (n = 51)
ALT, median (IQR), U/L	35.05 (22.25-55.90) [n = 52]	28.50 (18.95-59.50) [n = 48]
ALT categories, No. (%)		
≤50 U/L	36/52 (69.2)	33/48 (68.8)
>50 U/L	16/52 (30.8)	15/48 (31.3)
AST, median (IQR), U/L	28.50 (20.95-42.00) [n = 52]	24.50 (19.10-33.50) [n = 48]
AST categories, No. (%)		
≤40 U/L	35/52 (67.3)	40/48 (83.3)
>40 U/L	17/52 (32.7)	8/48 (16.7)
Urea nitrogen, median (IQR), mg/dL	20.36 (14.34-28.07) [n = 50]	20.08 (16.22-32.97) [n = 49]
Urea nitrogen categories, No. (%)		
<5.0 mg/dL	0/50	0/49
5.0-19.9 mg/dL	23/50 (46.0)	24/49 (49.0)
>19.9 mg/dL	27/50 (54.0)	25/49 (51.0)
Serum creatinine, median (IQR), mg/dL	0.75 (0.60-0.89) [n = 50]	0.83 (0.62-1.04) [n = 49]
Serum creatinine categories, No. (%)		
≤1.5 mg/dL	46/50 (92.0)	47/49 (95.9)
>1.5 mg/dL	4/50 (8.0)	2/49 (4.1)

Abbreviations: ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; IQR, interquartile range.

SI conversion factors: To convert D-dimer to nmol/L, multiply values by 5.476; to convert urea nitrogen to mmol/L, multiply values by 0.357; to convert creatinine to μmol/L, multiply values by 88.4.

[a] The values shown were based on total number of patients who contributed values.

[b] History of allergy to certain allergens, including food, medicine, etc.

[c] Details of coexisting diseases were collected from medical records.

[d] The vital signs and laboratory values are the last available values within 72 hours prior to randomization. The laboratory values selected were associated with the clinical status and factors that may affect convalescent plasma therapy. The values used for categorization of laboratory values are local divisions of low, normal, and high values.

There was no interim or preliminary data review prior to making this decision.

Results

Study Population

A total of 103 patients were enrolled in this randomized clinical trial. They were assigned to either the convalescent plasma group or the control group in a 1:1 ratio and were categorized as follows: 23 patients in the convalescent plasma group and 22 patients in the control group had severe COVID-19, and 29 patients in the convalescent plasma group and 29 patients in the control group had life-threatening COVID-19. Of these, 1 patient with life-threatening disease in the convalescent plasma treatment group withdrew from the study and 1 patient with life-threatening disease in the control group received convalescent plasma transfusion after randomization (protocol violation). Thus, 103 patients were included in the full analysis set and 101 patients were included in the per-protocol set (Figure 1).

The median age was 70 years (IQR, 62-78 years) among all patients, 71 years (IQR, 66-82 years) for patients with severe COVID-19, and 69 years (IQR, 61-76 years) for patients with life-threatening COVID-19. Of the patients included in the study, 60 (58.3%) were men, and the percentages of men with severe and life-threatening COVID-19 were 53.3% and 62.1%, respectively. A total of 89.2% of the patients had a normal body temperature at the time of participation, and the median body temperature was 36.5 °C (IQR, 36.2-36.7 °C) (Table 1 and eTable 1 and eTable 2 in Supplement 3).

The median interval between the onset of symptoms and randomization was 30 days (IQR, 20-39 days) overall, 33 days (IQR, 22-43 days) for patients with severe disease, and 26 days (IQR, 20-36 days) for patients with life-threatening disease. There were 5 patients with severe disease and 3 patients with life-threatening disease who had an interval between the onset of symptoms and randomization that was fewer than 14 days (Table 2).

Overall and within disease severity strata, the convalescent plasma and control groups were similar in terms of demographic characteristics, baseline laboratory results, and distribution on the 6-point disease severity scale at baseline, with the exception of systolic blood pressure in the patients with severe COVID-19 and sex in the patients with life-threatening COVID-19 (eTable 1 and eTable 2 in Supplement 3). Additional details regarding patients' clinical status at the time of randomization and additional medications received are provided in Table 2 and eTable 3, eTable 4, and eTable 7 in Supplement 3 . For patients in the convalescent plasma group, median plasma infusion volume was 200 mL (IQR, 200-300 mL), and 96% received a single dose of plasma infusion.

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

Primary Clinical Outcome

For all patients combined, there was no significant difference in the primary outcome of time to clinical improvement within 28 days: 51.9% (27/52) in the convalescent plasma group vs 43.1% (22/51) in the control group (difference, 8.8% [95% CI, −10.4% to 28.0%]; HR, 1.40 [95% CI, 0.79-2.49]; $P = .26$). Results for the per-protocol population were not materially different (eTable 5 in Supplement 3). Among those with severe disease, the primary outcome occurred in 91.3% (21/23) vs 68.2% (15/22) (HR, 2.15 [95% CI, 1.07-4.32]; $P = .03$). Among those with life-threatening disease, the primary outcome occurred in 20.7% (6/29) vs 24.1% (7/29) (HR, 0.88 [95% CI, 0.30-2.63]; $P = .83$) (P for interaction = .17) (Table 3 , Figure 2 , and eTable 6 in Supplement 3). For all proportional hazards models, the proportionality assumptions were met.

Secondary Clinical Outcomes

There was no significant difference in the secondary outcome of 28-day mortality (15.7% in the convalescent plasma group vs 24.0% in the control group; OR, 0.65 [95% CI, 0.29-1.46]; $P = .30$). There was also no significant difference in the time from randomization to death between the convalescent group and the control group (HR, 0.74 [95% CI, 0.30-1.82]; $P = .52$) (Table 3). Among patients with severe disease, no patients died in the convalescent plasma group, while 2 patients (9.1%) died in the control group. Among patients with life-threatening disease, 8 patients (28.6%) died in the convalescent plasma group, while 10 patients (35.7%) died in the control group.

There was also no significant difference in the secondary outcome of time from randomization to discharge (51.0% in the convalescent plasma group vs 36.0% in the control group were discharged by day 28; HR, 1.61 [95% CI, 0.88-2.93]; $P = .12$). The 28-day discharge rate of the convalescent plasma group reached 51%, among which the discharge rate of those with severe disease in the convalescent plasma group reached 91.3%.

At 24, 48, and 72 hours, the rates of negative SARS-CoV-2 viral PCR in the convalescent plasma group were all significantly higher than that of the control group (44.7% vs 15.0%, $P = .003$ at 24 hours; 68.1% vs 32.5%, $P = .001$ at 48 hours; 87.2% vs 37.5%, $P < .001$ at 72 hours) (Table 3 and eFigure 1 in Supplement 3). Among patients with severe disease, the rate of negative viral PCR at 72 hours was significantly higher in the convalescent plasma group compared with the control group, but there was no significant difference at 24 hours and 48 hours. Among patients with life-threatening disease, there was a statistically significant difference in favor of the convalescent plasma group at 24, 48, and 72 hours.

Post hoc Analysis

The clinical improvement rates overall and by disease severity subgroups at various time points during the study are presented in Table 3.

Adverse Events

Two participants reported transfusion-related adverse events following convalescent plasma transfusion. One patient in the severe COVID-19 group developed chills and rashes within 2 hours of transfusion but recovered fully after treatment with dexamethasone and promethazine. This was determined to be a definite nonsevere allergic transfusion reaction and also a probable nonsevere febrile nonhemolytic transfusion reaction. The other patient, who was in the life-threatening COVID-19 group, presented with shortness of breath, cyanosis, and severe dyspnea within 6 hours of transfusion. The patient was given dexamethasone, aminophylline, and other supportive care immediately and gradually improved after 2 hours. This was determined to be possible severe transfusion-associated dyspnea.

Table 2. Patients' Clinical Status at Randomization and Medications Received[a]

All patients	Convalescent plasma group (n = 52)	Control group (n = 51)
Time between symptom onset and randomization, median (IQR), d	27 (22-39) [n = 49]	30 (19-38) [n = 48]
≤14 d, No. (%)	3/49 (6.1)	5/48 (10.4)
>14 d, No. (%)	46/49 (93.9)	43/48 (89.6)
Interval between symptom onset and admission, median (IQR), d	12 (5-20) [n = 49]	10 (6-16) [n = 48]
6-Point scale at study day 1, No. (%)		
2- Hospitalization, no supplemental oxygen	1/51 (2.0)	1/50 (2.0)
3- Hospitalization, requiring supplemental oxygen (not high-flow or noninvasive ventilation)	15/51 (29.4)	15/50 (30.0)
4- Hospitalization, requiring noninvasive ventilation and/or high-flow supplemental oxygen	21/51 (41.2)	23/50 (46.0)
5- Hospitalization, requiring extracorporeal membrane oxygenation and/or invasive mechanical ventilation	14/51 (27.5)	11/50 (22.0)
Medications used after randomization		
Antiviral	41/46 (89.1)	44/49 (89.8)
Antibacterial	38/46 (82.6)	39/49 (79.6)
Chinese herbal medicine	26/46 (56.5)	30/49 (61.2)
Steroids	21/46 (45.7)	16/49 (32.7)
Antifungal	15/46 (32.6)	13/49 (26.5)
Human immunoglobulin	13/46 (28.3)	11/49 (22.5)
Interferon	12/46 (26.1)	7/49 (14.3)

[a] The values shown were based on total number of patients who contributed values. Details of medications used were provided in eTable 7 in Supplement 3.

Discussion

In this randomized clinical trial of patients with severe or life-threatening COVID-19, there was no significant difference in the time to clinical improvement between patients who received convalescent plasma transfusion therapy combined with standard treatment vs those who received standard treatment alone. There was also no significant difference in secondary outcomes of 28-day mortality or time from randomization to discharge. Convalescent plasma treatment was

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

Table 3. Primary and Secondary Clinical Outcomes at Day 28[a]

	Convalescent plasma group (n = 52)	Control group (n = 51)	Absolute difference (95% CI)[b]	Effect estimate (95% CI)	*P* value[c]
All patients					
Primary clinical outcome					
Time to clinical improvement, median (IQR), d[d]	28.00 (13.00-Indeterminate)	Indeterminate (18.00-Indeterminate)	−2.15 (−5.28 to 0.99)	HR, 1.40 (0.79-2.49)	.26
Clinical improvement rate, No./total (%)[e]					
At day 7	5/52 (9.6)	5/51 (9.8)	−0.2% (−11.6% to 11.2%)	OR, 0.98 (0.30-3.19)	.97
At day 14	17/52 (32.7)	9/51 (17.6)	15.0% (−1.4% to 31.5%)	OR, 1.85 (0.91-3.77)	.08
At day 28	27/52 (51.9)	22/51 (43.1)	8.8% (−10.4% to 28.0%)	OR, 1.20 (0.80-1.81)	.37
Secondary clinical outcomes					
Discharge rate at 28 d, No./total (%)	26/51 (51.0)	18/50 (36.0)	15.0% (−4.1% to 34.1%)	OR, 1.42 (0.90-2.24)	.13
Time from randomization to discharge, median (IQR), d[d]	28.00 (13.00-Indeterminate)	Indeterminate (19.00-Indeterminate)	−2.43 (−5.56 to 0.69)	HR, 1.61 (0.88-2.93)	.12
Time from hospitalization to discharge, median (IQR), d[d]	41.00 (31.00-Indeterminate)	53.00 (35.00-Indeterminate)	−11.95 (−26.33 to 2.43)	HR, 1.68 (0.92-3.08)	.09
Mortality at 28 d, No./total (%)	8/51 (15.7)	12/50 (24.0)	−8.3% (−23.8% to 7.2%)	OR, 0.65 (0.29-1.46)	.30
Time from randomization to death, median (IQR), d[d]	Indeterminate	Indeterminate (26.00-Indeterminate)	0.52 (−2.10 to 3.14)	HR, 0.74 (0.30-1.82)	.52
Viral nucleic acid negative rate, No./total (%)					
At 24 h	21/47 (44.7)	6/40 (15.0)	29.7% (11.7% to 47.7%)	OR, 4.58 (1.62-12.96)	.003
At 48 h	32/47 (68.1)	13/40 (32.5)	35.6% (15.9% to 55.3%)	OR, 4.43 (1.80-10.92)	.001
At 72 h	41/47 (87.2)	15/40 (37.5)	49.7% (32.0% to 67.5%)	OR, 11.39 (3.91-33.18)	<.001
Patients with severe disease					
Primary clinical outcome					
Time to clinical improvement, median (IQR), d[d]	13.00 (9.00-21.00)	19.00 (15.00-Indeterminate)	−4.94 (−9.33 to −0.54)	HR, 2.15 (1.07-4.32)	.03
Clinical improvement rate, No./total (%)[e]					
At day 7	3/23 (13.0)	4/22 (18.2)	−5.1% (−26.3% to 16.1%)	OR, 0.72 (0.18-2.85)	.70
At day 14	14/23 (60.9)	6/22 (27.3)	33.6% (6.3% to 60.9%)	OR, 2.23 (1.05-4.76)	.02
At day 28	21/23 (91.3)	15/22 (68.2)	23.1% (−3.9% to 50.2%)	OR, 1.34 (0.98-1.83)	.07
Secondary clinical outcomes					
Discharge rate at 28 d, No./total (%)	21/23 (91.3)	15/22 (68.2)	23.1% (−3.9% to 50.2%)	OR, 1.34 (0.98-1.83)	.07
Time from randomization to discharge, median (IQR), d[d]	13.00 (10.00-16.00)	19.00 (11.00-Indeterminate)	−4.09 (−8.44 to 0.27)	HR, 1.97 (1.00-3.88)	.05
Time from hospitalization to discharge, median (IQR), d	32.00 (26.00-40.00)	41.00 (30.00-53.00)	−9.38 (−23.63 to 4.88)	HR, 1.74 (0.89-3.41)	.11
Mortality at 28 d, No./total (%)	0/23	2/22 (9.1)	−9.1% (−25.6% to 7.4%)		.49
Time from randomization to death, median (IQR), d[d]	Indeterminate	Indeterminate (26.00-Indeterminate)	1.42 (−0.88 to 3.71)	HR, 0.00	>.99
Viral nucleic acid negative rate, No./total (%)					
At 24 h	7/21 (33.3)	2/17 (11.8)	21.6% (−9.1% to 52.2%)	OR, 3.75 (0.66-21.20)	.15
At 48 h	13/21 (61.9)	6/17 (35.3)	26.6% (−4.2% to 57.4%)	OR, 2.98 (0.79-11.25)	.10
At 72 h	19/21 (90.5)	7/17 (41.2)	49.3% (22.7% to 75.9%)	OR, 13.57 (2.36-77.95)	<.001
Patients with life-threatening disease					
Primary clinical outcome					
Time to clinical improvement, median (IQR), d[d]	Indeterminate	Indeterminate	0.23 (−3.11 to 3.57)	HR, 0.88 (0.30-2.63)	.83
Clinical improvement rate, No./total (%)[e]					
At day 7	2/29 (6.9)	1/29 (3.4)	3.4% (−11.4% to 18.3%)	OR, 2.00 (0.19-20.86)	>.99
At day 14	3/29 (10.3)	3/29 (10.3)	0.0% (−19.1% to 19.1%)	OR, 1.00 (0.22-4.55)	>.99
At day 28	6/29 (20.7)	7/29 (24.1)	−3.4% (−24.9% to 18.0%)	OR, 0.86 (0.33-2.24)	.75

(continued)

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

Table 3. Primary and Secondary Clinical Outcomes at Day 28[a] (continued)

	Convalescent plasma group (n = 52)	Control group (n = 51)	Absolute difference (95% CI)[b]	Effect estimate (95% CI)	*P* value[c]
Secondary clinical outcomes					
Discharge rate at 28 d, No./total (%)	5/28 (17.9)	3/28 (10.7)	7.1% (−14.7% to 28.9%)	OR, 1.67 (0.44-6.32)	.71
Time from randomization to discharge, median (IQR), d[d]	Indeterminate	Indeterminate	−0.80 (−3.74 to 2.14)	HR, 1.77 (0.42-7.40)	.44
Time from hospitalization to discharge, median (IQR), d[d]	Indeterminate (46.00-Indeterminate)	Indeterminate	−4.61 (−15.07 to 5.85)	HR, 1.90 (0.45-8.04)	.38
Mortality at 28 d, No./total (%)	8/28 (28.6)	10/28 (35.7)	−7.1% (−31.5% to 17.2%)	OR, 0.80 (0.37-1.72)	.57
Time from randomization to death, median (IQR), d[d]	Indeterminate (22.00-Indeterminate)	Indeterminate (15.00-Indeterminate)	−0.04 (−3.86 to 3.77)	HR, 0.86 (0.34-2.17)	.74
Viral nucleic acid negative rate, No./total (%)					
At 24 h	14/26 (53.8)	4/23 (17.4)	36.5% (11.8% to 61.1%)	OR, 5.54 (1.47-20.86)	.01
At 48 h	19/26 (73.1)	7/23 (30.4)	42.6% (17.3% to 68.0%)	OR, 6.20 (1.79-21.46)	.003
At 72 h	22/26 (84.6)	8/23 (34.8)	49.8% (25.9% to 73.7%)	OR, 10.31 (2.63-40.50)	<.001

Abbreviations: HR, hazard ratio; IQR, interquartile range; OR, odds ratio.

[a] The values shown were based on total number of patients who contributed values. The primary clinical outcome was analyzed by primary analysis set. Times to outcomes in the secondary clinical outcomes were analyzed by primary analysis set. Indeterminate events could not be calculated due to the low percentage of outcome events.

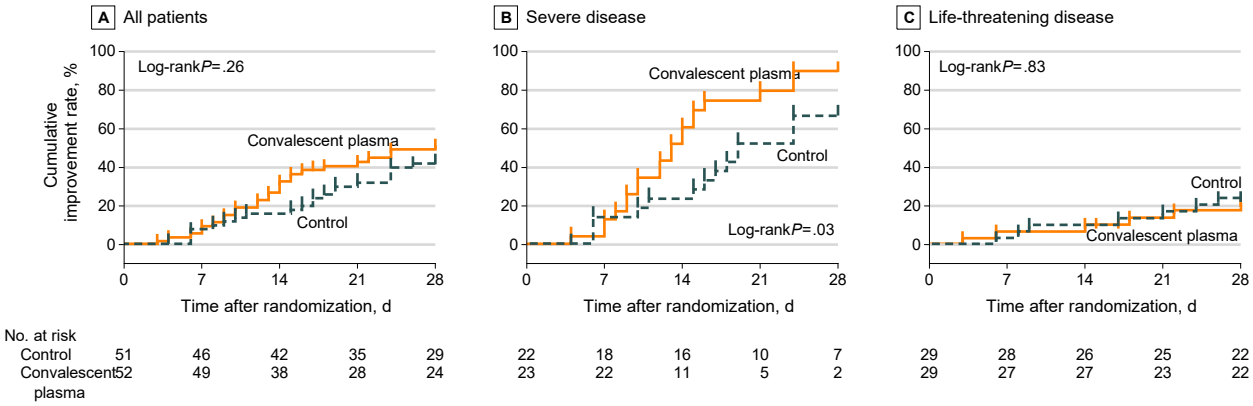
[b] The absolute differences of time to clinical improvement, times to discharge, and times from randomization to death were calculated based on restricted mean survival time.

[c] *P* value was calculated by Cox regression, χ^2 test, or Fisher exact test.

[d] Medians and quartiles of time to clinical improvement, times to discharge, and times from randomization to death could not be determined because too few patients had reached improvement or discharge by the end of the study. By the end of the study, for all, severe, and life-threatening patients, respectively, 27 (51.9%), 21 (91.3%), and 6 (20.7%) were clinically improved in the convalescent plasma group; 22 (43.1%), 15 (68.2%), and 7 (24.1%) were clinically improved in the control group; 26 (51.0%), 21 (91.3%), and 5 (17.9%) were discharged in the convalescent plasma group; 18 (36.0%), 15 (68.2%), and 3 (10.7%) were discharged in the control group; 8 (15.7%), 0, and 8 (28.6%) died in the convalescent plasma group; and 12 (24.0%), 2 (9.1%), and 10 (35.7%) died in the control group.

[e] These analyses were developed post hoc to better illustrate disease progression.

Figure 2. Time to Clinical Improvement in Patients With COVID-19



The cumulative improvement rate is the percentage of patients who experienced a 2-point improvement or were discharged alive from the hospital. Ticks on the curves indicate censored events. All patients who did not reach clinical improvement were observed for the full 28-day period or until death. COVID-19 indicates coronavirus disease 2019.

The median (IQR) follow-up times for the convalescent plasma group and control group, respectively, were 15 (10-28) days and 24 (13-28) days overall; 13 (10-16) and 18.5 (11-26) days among those with severe COVID-19; and 28 (12-28) and 26 (15-28) days among those with life-threatening COVID-19.

associated with higher rates of negative SARS-CoV-2 viral PCR results from nasopharyngeal swabs at 24, 48, and 72 hours, demonstrating that the convalescent plasma treatment was associated with antiviral activity in patients with COVID-19.

Plasma transfusions can result in transfusion-related adverse events including febrile and allergic transfusion reactions, transfusion-associated dyspnea, hypotensive transfusion reaction, to hemolytic transfusion reactions, septic transfusion reaction, transfusion-related acute lung injury, and transfusion-associated circulatory overload. In this study, most patients tolerated convalescent plasma transfusions well. There were 2 cases of reported transfusion-associated adverse events. One case was determined to be a definite nonsevere allergic transfusion reaction and also a probable nonsevere febrile

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

nonhemolytic transfusion reaction, and the other case was determined to be possible severe transfusion-associated dyspnea. The rate is somewhat higher than the general rate of reactions associated with plasma transfusion, possibly due to the small sample size and active surveillance. [16]

In the subgroup analysis by disease severity, there was a signal of possible clinical benefit for convalescent plasma among patients with severe COVID-19. However, bec ause the test for interaction by disease severity was not statistically significant, the findings for the severe and life-threatening subgroups should not be interpreted as different. Given the early study termination, it is possible that the study was underpowered to detect a statistically significant interaction, and a larger trial of convalescent plasma focusing on patients with severe COVID-19 may be warranted.

Patients with COVID-19 who were recruited in this study were heterogeneous with regard to the duration and severity of the illness. The possible antiviral activity with convalescent plasma treatment in patients aged 60 to 80 years and after 14 days of disease onset is notable. To our knowledge, no other antiviral treatments have demonstrated therapeutic effects in this age group or this late in the course of the disease. However, the con valescent plasma treatment was given at least 14 days after the onset of symptoms in most cases, and it is not known whether earlier convalescent plasma treatment could have resulted in better clinical outcomes. Further studies are needed to optimize patient selection and timing of convalescent plasma treatment.

While the use of convalescent plasma has been investigated and used many times in the past, [3,17] its use has not been well studied. It is notable that for most studies of the use of convalescent plasma, [4,5,18] there was lack of standardization and procedure control with regard to the donor selection process and the nature or level of antibodies in convalescent plasma units. This may explain the varied therapeutic effects seen across a variety of diseases or even across patients with the same disease. The guidance on convalescent plasma issued by the World Health Organization [11] highlighted the importance of standardized processes and laboratory testing–based quality control of convalescent plasma units, the selection of clinical indications, as well as program deployment to recruit adequate numbers of donors and maintain sufficient inventory.

One of the potential strengths of this study was the controlled process for donor selection and convalescent plasma quality control. A predefined process was used to enroll donors for convalescent plasma collection. Only units with a high titer of S-RBD–specific IgG antibody were used for convalescent plasma treatment in this study.

Limitations

This study has several limitations. First, the sample size was small and the study was terminated early. It is possible that the study was underpowered to detect a clinically important benefit of convalescent plasma therapy. Second, the median time between the onset of symptoms and randomization was 30 days, and it is unclear whether earlier treatment would have resulted in greater benefit. Third, this was an open-label study and the primary outcome was based to some degree on physicians' clinical management decisions. Fourth, the use of standard therapy was allowed in both groups and was not protocolized, which could have potentially influenced outcomes. Fifth, the relatively short 28-day time frame of the study follow-up may have precluded the observation of clinical improvement in patients with severe diseases, especially life-threatening COVID-19, as they may take longer time to respond and recover. Sixth, plasma was not used for the control group, which would have been a more ideal control group, making blinded design possible. Seventh, the study findings should be interpreted cautiously given that practices may vary from country to country, and hospital to hospital, such as the types of standard treatment, supportive care, and thresholds for intubation and hospital admission. The outcomes of this study may be related to a combination of many factors, such as the quality of the convalescent plasma products in terms of potency, the selection of the patients (severe and life-threatening COVID-19), and the timing of convalescent plasma transfusion.

Conclusion

Among patients with severe or life-threatening COVID-19, convalescent plasma therapy added to standard treatment, compared with standard treatment alone, did not significantly improve the time to clinical improvement within 28 days. Interpretation is limited by early termination of the trial, which may have been underpowered to detect a clinically important difference.

ARTICLE INFORMATION

Accepted for Publication: May 23, 2020.

Published Online: June 3, 2020. doi:10.1001/jama.2020.10044

Author Affiliations: Institute of Blood Transfusion, Chinese Academy of Medical Sciences and Peking Union Medical College, Chengdu, China (L. Li, Kong, J. Wang, Y. Liu, H. Xu, Deng, X. Zhang, C. Li, Z. Liu); Key Laboratory of Transfusion Adverse Reactions, CAMS, Chengdu, China (L. Li, Kong, J. Wang, H. Xu, C. Li, Z. Liu); Guanggu District Maternal and Child Health Hospital of Hubei Province, Wuhan, China. (W. Zhang, Zeng); Department of Respiratory and Critical Care Medicine, First Affiliated Hospital, the Second Military Medical University, Shanghai, China (W. Zhang); Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (Y. Hu, Mei); Department of Pulmonary and Critical Care Medicine, Beijing Hospital, National Respiratory quality control center, National Center of Gerontology, Beijing, China (Tong); Department of Transfusion, General Hospital of Central Theater Command of PLA, Wuhan, China (Zheng); State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union, Beijing, China (J. Yang); NHC Key Laboratory of Systems Biology of Pathogens and Christophe Mérieux Laboratory, Institute of Pathogen Biology, Chinese Academy of Medical Sciences (CAMS) & Peking Union Medical College, Beijing, China (Ren, C. Wang, J. Wang); Key Laboratory of Respiratory Disease Pathogenomics, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China (Ren, J. Wang); Department of Blood Transfusion, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (Wei, J. Wang); Wuhan Red Cross Hospital, Wuhan, China (C. Hu, Z. Xu, Xiong); Department of Blood Transfusion, Wuhan Asia Heart Hospital, Wuhan, China (Tao); Department of Blood Transfusion, Wuhan Asia General Hospital, Wuhan, China (Tao); Wuhan Blood Center, Wuhan, China

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

(R. Yang); Peking University Clinical Research Institute, Peking University Health Science Center, Beijing, China (Yu); Department of Biomedical Engineering, School of Medicine, Tsinghua University, Beijing, China (Guo, Su); Department of Emergency, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (X. Wu); Mianyang Central Hospital, Mianyang, China (Z. Xu); Department of Organ Transplantation, First Affiliated Hospital, the Second Military Medical University, Shanghai, China (Zeng); Department of Neurology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (Xiong); Wuhan Pulmonary Hospital, Wuhan, China (Chen); Department of Respiratory Medicine, Wuhan Asia General Hospital, Wuhan, China (Man); Center for Global Health and Infectious Diseases, Comprehensive AIDS Research Center, and Beijing Advanced Innovation Center for Structural Biology, School of Medicine, Tsinghua University, Beijing, China (L. Zhang); Department of Pathology, University of Miami, Miami, Florida (Y. Wu).

Author Contributions: Dr Z. Liu had full access to all of the data in this study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Drs L. Li, W. Zhang, and Y. Hu are co–first authors. Drs L. Li, W. Zhang, Y. Hu, X. Tong, S. Zheng, J. Yang, Y. Kong, L. Ren, Q. Wei, H. Mei, C. Hu, C. Tao, R. Yang, J. Wang, and Y. Yu contributed equally to the study.
Concept and design: L. Li, W. Zhang, X. Tong, J. Yang, N. Xiong, L. Zhang, J.W. Wang, Y. Wu, Z. Liu.
Acquisition, analysis, or interpretation of data: W. Zhang, S. Zheng, Y. Kong, L. Ren, Q. Wei, H. Mei, C. Hu, C. Tao, R. Yang, Jue Wang, Y. Yu, Y. Guo, X. Wu, Z. Xu, N. Xiong, L. Chen, Juan Wang, N. Man, Y. Liu, H. Xu, E. Deng, X. Zhang, C. Li, C. Wang, S. Su, Y. Hu, Y. Wu, Z. Liu.
Drafting of the manuscript: L. Li, W. Zhang, Y. Kong, L. Ren, H. Xu, E. Deng, C. Wang, Y. Wu, Z. Liu.
Critical revision of the manuscript for important intellectual content: W. Zhang, X. Tong, S. Zheng, J. Yang, Q. Wei, H. Mei, C. Hu, C. Tao, R. Yang, Jue Wang, Y. Yu, Y. Guo, X. Wu, Z. Xu, L. Zeng, N. Xiong, L. Chen, Juan Wang, N. Man, Y. Liu, X. Zhang, C. Li, S. Su, L. Zhang, Juan Wang, Y. Hu, Y. Wu, Z. Liu.
Statistical analysis: J. Yang, Y. Kong, Y. Yu.
Obtained funding: L. Li, Z. Liu.
Administrative, technical, or material support: L. Li, W. Zhang, X. Tong, L. Ren, H. Mei, Jue Wang, Y. Guo, N. Xiong, Y. Liu, H. Xu, E. Deng, C. Wang, S. Su, J.W. Wang, Z. Liu.
Supervision: W. Zhang, J. Yang, H. Mei, Y. Hu.

Conflict of Interest Disclosures: Dr Liu reports holding a pending patent on COVID-19 testing. Dr Wu reports consulting for Verax Medical and Grifols, receiving royalties from UptoDate and AABB, and being a volunteer visiting professor and receiving travel support for giving medical education from the Chinese Institute of Blood Transfusion. No other disclosures were reported.

Funding/Support: This work was supported by the Chinese Academy of Medical Sciences Innovation Fund for Medical Sciences (CIFMS) grants 2020-I2M-CoV19-006, 2016-I2M-3-024 (Dr Z. Liu), and 2017-I2M-1-009 (Dr L. Li) and the Nonprofit Central Research Institute Fund of Chinese Academy of Medical Sciences grant 2018PT32016 (Dr Z. Liu).

Role of the Funder/Sponsor: The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Data Sharing Statement See Supplement 4

Additional Contributions: We would like to thank the following individuals for their guidance, expertise, and assistance with the study design: Peter W. Marks, MD, PhD, Center for Biologics Evaluation and Research, FDA; Anne Eder, MD, PhD, Center for Biologics Evaluation and Research, FDA; Nicole Verdun, MD, Center for Biologics Evaluation and Research, FDA; Tim Uyeki, MD, MPH, MPP, National Center for Immunization and Respiratory Diseases, CDC; Scott Koepsell, MD, PhD, University of Nebraska Medical Center; Annie Winkler, MD, Instrumentation Laboratory; Xuan Qin, PhD, Seattle Children's Hospital and University of Washington. None received compensation related to this study.

REFERENCES

1. Wang D, Hu B, Hu C, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA* 2020;323(11):1061-1069. doi:10.1001/jama.2020.1585

2. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72 314 cases from the Chinese Center for Disease Control and Prevention. *JAMA* 2020;323(13):1239-1242. doi:10.1001/jama.2020.2648

3. Garraud O, Heshmati F, Pozzetto B, et al. Plasma therapy against infectious pathogens, as of yesterday, today and tomorrow. *Transfus Clin Biol*. 2016;23(1):39-44. doi:10.1016/j.tracli.2015.12.003

4. Cheng Y, Wong R, Soo YO, et al. Use of convalescent plasma therapy in SARS patients in Hong Kong. *Eur J Clin Microbiol Infect Dis*. 2005;24(1):44-46. doi:10.1007/s10096-004-1271-9

5. Yeh KM, Chiueh TS, Siu LK, et al. Experience of using convalescent plasma for severe acute respiratory syndrome among healthcare workers in a Taiwan hospital. *J Antimicrob Chemother*. 2005;56(5):919-922. doi:10.1093/jac/dki346

6. Arabi YM, Hajeer AH, Luke T, et al. Feasibility of using convalescent plasma immunotherapy for MERS-CoV infection, Saudi Arabia. *Emerg Infect Dis*. 2016;22(9):1554-1561. doi:10.3201/eid2209.151164

7. Kraft CS, Hewlett AL, Koepsell S, et al; Nebraska Biocontainment Unit and the Emory Serious Communicable Diseases Unit. The use of TKM-100802 and convalescent plasma in 2 patients with Ebola virus disease in the United States. *Clin Infect Dis*. 2015;61(4):496-502. doi:10.1093/cid/civ334

8. Shen C, Wang Z, Zhao F, et al. Treatment of 5 critically ill patients with COVID-19 with convalescent plasma. *JAMA* 2020;323(16):1582-1589. doi:10.1001/jama.2020.4783

9. Duan K, Liu B, Li C, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc Natl Acad Sci U S A* 2020;117(17):9490-9496. doi: 10.1073/pnas.2004168117

10. Recommendations for Investigational COVID-19 Convalescent Plasma. US FDA. Published May 1, 2020. Accessed May 26, 2020. https://www.fda.gov/vaccines-blood-biologics/investigational-new-drug-ind-or-device-exemption-ide-process-cber/recommendations-investigational-covid-19-convalescent-plasma

11 Position Paper on Use of Convalescent Plasma, Serum or Immune Globulin Concentrates as an Element in Response to an Emerging Virus. In: Network WBR, ed. 2017. Accessed April 18, 2020. https://www.who.int/bloodproducts/brn/en/

12 National Health Commission of the People's Republic of China. Covid-19 treatment plan (trial version 6). Accessed April 20, 2020. http://www.nhc.gov.cn/yzygj/s7653p/202002/8334a8326dd94d329df351d7da8aefc2/files/b218cfeb1bc54639af227f922bf6b817

13 Wang Y, Zhang D, Du G, et al. Remdesivir in adults with severe COVID-19: a randomised, double-blind, placebo-controlled, multicentre trial. *Lancet* 2020;395(10236):1569-1578. doi:10.1016/S0140-6736(20)31022-9

14 Kleinbaum DG. Evaluating the proportional hazards assumption. In: *Survival Analysis: Statistics in the Health Sciences*. Springer; 1996:183-184.

15 Daily update on covid-19. National Health Commission of the People's Republic of China. Accessed May 24, 2020. http://www.nhc.gov.cn/xcs/yqtb/202003/f01fc26a8a7b48debe194bd1277fdba3.shtml

16 Annual SHOT (Serious Hazards of Transfusion) Report 2018. Published July 2019. Accessed May 25, 2020. https://www.shotuk.org/

17 Beigel JH, Aga E, Elie-Turenne MC, et al; IRC005 Study Team. Anti-influenza immune plasma for the treatment of patients with severe influenza A: a randomised, double-blind, phase 3 trial. *Lancet Respir Med*. 2019;7(11):941-950. doi:10.1016/S2213-2600(19)30199-7

18 Soo YO, Cheng Y, Wong R, et al. Retrospective comparison of convalescent plasma with continuing high-dose methylprednisolone treatment in SARS patients. *Clin Microbiol Infect*. 2004;10(7):676-678. doi:10.1111/j.1469-0691.2004.00956.x

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

EDITORIAL

A Randomized Trial of Convalescent Plasma for COVID-19—Potentially Hopeful Signals

Arturo Casadevall, MD, PhD; Michael J. Joyner, MD; Liise-Anne Pirofski, MD

Convalescent plasma for the treatment of infectious diseases has been used since the early 20th century and was associated with reduced mortality during the 1918 influenza,[1] 2003 SARS,[2] and 2009 influenza H1N1[3] pandemics. However, most published studies of these diseases were case series and retrospective comparisons of treated and nontreated individuals. Consistent with this, several uncontrolled case series of convalescent plasma use in patients with coronavirus disease (2019) COVID-19 have suggested a possible benefit.[4-6] Given encouraging historical precedents and the absence of proven SARS-CoV-2 (severe acute respiratory disease coronavirus 2) antiviral therapies, convalescent plasma therapy has been proposed as a treatment option for COVID-19.[7] The availability of clinical information generated from randomized clinical trials is therefore of substantial importance given that the world remains in the grip of the COVID-19 epidemic and convalescent plasma is currently in use in many countries, including the US.

Related article

In their article in *JAMA*, Li et al[8] present findings from the first randomized clinical trial of convalescent plasma therapy for patients with COVID-19 conducted in China. In contrast to most other reports of convalescent plasma use in past epidemics, this study is noteworthy in that it used a randomized trial design and well-characterized plasma units with a high titer of antibody to SARS-CoV-2. It was an important accomplishment to conduct a carefully controlled trial during a pandemic with an entirely new highly contagious disease that stressed health systems in an unprecedented way.

However, the authors report that because the COVID-19 outbreak in China was being contained while the trial was ongoing and new cases were unavailable for enrollment, the trial was terminated before it reached its targeted original sample size of 200 patients; only 103 were enrolled (for whom randomization was stratified by disease severity). Consequently, the study was underpowered and many comparisons between the convalescent plasma group and the control group were not statistically significant.

In the primary analysis, based on 52 patients who were randomized to receive convalescent plasma in addition to standard treatment and 51 patients who were randomized to receive standard treatment alone (control), the primary outcome of time to clinical improvement within 28 days (defined as being discharged alive or having a reduction of 2 points on a 6-point disease severity scale) was 2.15 days shorter (95% CI, −5.28 to 0.99 days) in the intervention group compared with the control group, and clinical improvement at 28 days occurred in 27 patients (51.9%) in the intervention group vs 22 patients (43.1%) in the control group (difference, 8.8%; 95% CI, −10.4% to 28%; hazard ratio, 1.40 [95% CI, 0.79-2.49]; $P = .26$).

In analyses stratified by disease severity, among patients with severe disease (23 in the convalescent plasma group and 22 in the control group), time to clinical improvement within 28 days was 4.94 days shorter (95% CI, −9.33 to −0.54 days) in the intervention group compared with the control group, and clinical improvement at 28 days occurred in 21 patients (91.3%) in the intervention group vs 15 patients (68.2%) in the control group (hazard ratio, 2.15 [95% CI, 1.07-4.32]; $P = .03$). Among the subgroup of patients with life-threatening disease (29 in the convalescent plasma group and 29 in the control group), there were no significant differences in the primary outcome or rates of clinical improvement at 28 days: 6 patients (20.7%) in the convalescent plasma group vs 7 patients (24.1%) in the control group (HR, 0.88 [95% CI, 0.30-2.63]; $P = .83$) (P for interaction = .17).

In the entire study population, the findings for several of the secondary end points appeared to signal a more favorable outcome for patients who received convalescent plasma, although there were no statistically significant differences between the convalescent plasma group vs the control group in any of the major secondary outcomes, including 28-day mortality (15.7% vs 24.0%, respectively; $P = .30$) or rate of discharge at 28 days (51% vs 36%; $P = .13$).

Convalescent plasma use in the study by Li et al[8] was associated with some clinical improvement in severely ill patients, but not in critically ill patients. Greater efficacy in less ill individuals is expected because antibody therapies generally work best when administered earlier in disease.[9] Historically, antibody therapy was effective in reducing the mortality of pneumococcal pneumonia when instituted in the first 3 days of symptom onset.[10] Consequently, it is not surprising that patients with COVID-19 who had tachypnea and hypoxia might benefit more from convalescent plasma than those who required mechanical ventilation. However, any indication of possible benefit in the severely ill group is noteworthy because these individuals had advanced disease, which is not considered optimal for antibody therapy. Lack of efficacy among patients who were receiving mechanical ventilation, some with multiorgan failure, highlights that the pathologic process in these individuals is likely irreversible.

The convalescent plasma used in the study by Li et al had high titers of IgG to SARS-CoV-2, which correlated with neutralizing activity. While neutralizing activity is considered to be the main determinant of convalescent plasma efficacy,

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

other antibody functions may also mediate protection. Correlates of antibody efficacy should be investigated in future studies. As reported in case series from Wuhan,[4,5] plasma-treated patients had large reductions in their serum viral loads and most were virus negative 3 days after infusion. This observation establishes that convalescent plasma has antiviral activity, which is important because it indicates that antibody administration mediates a clear biological effect. The precedent of antiviral drug use against HIV and hepatitis C shows that reductions in viral load translate into clinical improvement, and earlier therapy is more effective than later therapy when organ damage is already present. In this regard, antibody-mediated viral elimination removes damaging antigens, which may translate into reduced tissue damage and inflammation. Hence, the antiviral effect of COVID-19 convalescent plasma suggests that its use earlier in the course of disease could have potentially important therapeutic activity, especially in less severely ill individuals.

Significant concerns have been raised about the use of convalescent plasma in COVID-19.[11] These include transfusion-related lung injury and transfusion-related circulatory overload. In addition, there have been theoretical concerns that the administration of antibodies might aggravate disease through antibody-mediated enhancement of proinflammatory effects.[11] The study by Li et al[8] reported only 2 adverse events among the 52 individuals who received convalescent plasma, each of whom responded to corticosteroid administration. The occurrence of one episode within 2 hours of plasma administration characterized by chills and rash suggests a transfusion reaction. However, the second episode occurred within 6 hours and its association with plasma infusion is less certain. Overall, the paucity of adverse effects is reassuring and reduces concerns about adverse effects from antibody administration.

Although the observed differences in mortality rates and hospital discharge rates between the convalescent plasma group and the control group did not reach statistical significance, these data provide valuable information for the magnitude of effects that may be expected in convalescent plasma studies. For example, the observed overall mortality difference of 24% vs 15.7% provides actionable information for the design of future trials to help ensure they are adequately powered. This difference in mortality is smaller than mortality reductions associated with convalescent plasma reported in prior studies involving 1918 influenza,[1] SARS,[2] and 2009 influenza H1N1,[3] which ranged from 50% to 70%. Hence, assuming the results of the study by Li et al[8] are generalizable, the findings may be helpful in estimating effect sizes for future studies of convalescent plasma use in hospitalized patients with COVID-19.

In the study by Li et al, the median age of the patients with severe disease was 70 years, and the median time between symptom onset and randomization was 30 days. Promising results with convalescent plasma treatment in patients with SARS[2] and influenza H1N1[3] were obtained among younger patients, and in the case of SARS, earlier in the disease. The importance of a possible treatment benefit in older persons, in whom mortality from COVID-19 is markedly higher than in younger persons,[12] cannot be overstated. In addition, the apparent improvement in the clinical status of the subgroup of less severely ill patients a month after the onset of symptoms suggests that the beneficial effects of antibodies in COVID-19 may be measurable as an improvement in inflammatory markers and viral elimination before clinical improvement is observed. The prolonged course of COVID-19 in patients who recover also should be considered in the design of future studies.

However, the study by Li et al has several important limitations, which are acknowledged by the investigators. The early termination of the trial most likely resulted in an underpowered study, thereby precluding any definitive conclusions about the role and potential efficacy of convalescent plasma for patients with COVID-19. In addition, the open-label design, the possibility of an element of subjectivity for the primary outcome, lack of a protocolized approach to standard therapy, and variability among study centers also must be considered when interpreting the study findings. Despite these limitations, by virtue of its randomized design, this study takes prior case studies[5,6] one step further by helping to separate the effects of convalescent plasma from concurrently administered agents, such as corticosteroids and antiviral agents.[13]

The signal of potential benefit of convalescent plasma in the subgroup of patients with severe COVID-19 disease (ie, those without life-threatening COVID-19 disease) is similar to findings from a recent preliminary report of a clinical trial of remdesivir for COVID-19.[14] Like remdesivir, convalescent plasma administration was associated with clinical improvement without a statistically significant effect on mortality, with the important caveat that remdesivir was evaluated in a larger study (n = 1063 randomized patients), whereas the study by Li et al[8] was terminated prematurely and underpowered. For both studies, the importance of clinical improvement as a primary end point became apparent as the trials progressed.[14]

The availability of both convalescent plasma and remdesivir means that physicians now have at least 2 therapeutic options for COVID-19, which raises the question of combination therapy. Despite only a few studies of the efficacy of combination therapy with antiviral drugs and specific antibodies, there is evidence that these agents may work well in combination.[9] Given that the mechanisms of action of antiviral drugs and neutralizing antibodies are distinct, they could be synergistic. Future trials should consider the efficacy of combination antiviral and antibody therapies.

In summary, the first randomized clinical trial of convalescent plasma in COVID-19, reported by Li et al in *JAMA*, showed no statistically significant benefit in clinical improvement at 28 days or mortality among all randomized patients, but does provide an important signal of possible benefit in the subgroup of severely ill patients and suggests that high titer antibody against SARS-CoV-2 may have antiviral efficacy. These results, while preliminary and subject to important study limitations, should stimulate more clinical trials to establish the optimal conditions for antibody therapies against COVID-19 and suggest that future studies

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

should focus on determining efficacy in less severely ill patients. If the efficacy of convalescent plasma is established by future studies, the ratio of donor to patients is favorable because individuals who recover from COVID-19 can donate 2 or 3 units of plasma, which could be used to treat more than 1 person with COVID-19 disease. Therapeutic success against such a complex and challenging disease as COVID-19 is likely to require more than 1 modality, and the results from Li et al [8] provide optimism for the future of antibody therapy in this disease.

ARTICLE INFORMATION

Author Affiliations: Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland (Casadevall); Department of Anesthesiology and Perioperative Medicine, Mayo Clinic, Rochester, Minnesota (Joyner); Montefiore Medical Center, Division of Infectious Diseases, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York (Pirofski).

Corresponding Author: Arturo Casadevall, MD, PhD, Johns Hopkins Bloomberg School of Public Health, 605 N Wolfe St, Baltimore, MD 21205 (acasade1@jhu.edu).

Published Online: June 3, 2020. doi:10.1001/jama.2020.10218

Conflict of Interest Disclosures: Dr Casadevall serves on the scientific advisory board of SAB Therapeutics, which is working to develop cow-derived human antibodies for the therapy of COVID-19. No other disclosures were reported.

REFERENCES

1. Luke TC, Kilbane EM, Jackson JL, Hoffman SL. Meta-analysis: convalescent blood products for Spanish influenza pneumonia: a future H5N1 treatment? *Ann Intern Med*. 2006;145(8):599-609. doi:10.7326/0003-4819-145-8-200610170-00139

2. Cheng Y, Wong R, Soo YO, et al. Use of convalescent plasma therapy in SARS patients in Hong Kong. *Eur J Clin Microbiol Infect Dis*. 2005;24(1):44-46. doi:10.1007/s10096-004-1271-9

3. Hung IF, To KK, Lee CK, et al. Convalescent plasma treatment reduced mortality in patients with severe pandemic influenza A (H1N1) 2009 virus infection. *Clin Infect Dis*. 2011;52(4):447-456. doi:10.1093/cid/ciq106

4. Duan K, Liu B, Li C, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc Natl Acad Sci U S A*. 2020;117(17):9490-9496. doi:10.1073/pnas.2004168117

5. Shen C, Wang Z, Zhao F, et al. Treatment of 5 critically ill patients with COVID-19 with convalescent plasma. *JAMA*. 2020;323(16):1582-1589. doi:10.1001/jama.2020.4783

6. Zhang B, Liu S, Tan T, et al. Treatment with convalescent plasma for critically ill patients with severe acute respiratory syndrome coronavirus 2 infection. *Chest*. Published online May 18, 2020. doi:10.1016/j.chest.2020.03.039

7. Casadevall A, Pirofski LA. The convalescent sera option for containing COVID-19. *J Clin Invest*. 2020;130(4):1545-1548. doi:10.1172/JCI138003

8. Li L, Zhang W, Hu Y, et al. Effect of convalescent plasma therapy on time to clinical improvement in patients with severe and life-threatening COVID-19: a randomized clinical trial. *JAMA*. Published online June 3, 2020. doi:10.1001/jama.2020.10044

9. Casadevall A, Scharff MD. Return to the past: the case for antibody-based therapies in infectious diseases. *Clin Infect Dis*. 1995;21(1):150-161. doi:10.1093/clinids/21.1.150

10. Casadevall A, Scharff MD. Serum therapy revisited: animal models of infection and development of passive antibody therapy. *Antimicrob Agents Chemother*. 1994;38(8):1695-1702. doi:10.1128/AAC.38.8.1695

11. Dzik S. COVID-19 convalescent plasma: now is the time for better science. *Transfus Med Rev*. 2020;S0887-7963(20)30026-2.

12. Zhou F, Yu T, Du R, et al. Clinical course and risk factors for mortality of adult in patients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*. 2020;395(10229):1054-1062. doi:10.1016/S0140-6736(20)30566-3

13. Roback JD, Guarner J. Convalescent plasma to treat COVID-19: possibilities and challenges. *JAMA*. 2020;323(16):1561-1562. doi:10.1001/jama.2020.4940

14. Beigel JH, Tomashek KM, Dodd LE, et al; ACTT-1 Study Group Members. Remdesivir for the treatment of Covid-19—preliminary report. *N Engl J Med*. Published online May 22, 2020. doi:10.1056/NEJMoa2007764

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

JAMA | Original Investigation

Effect of Convalescent Plasma Therapy on Time to Clinical Improvement in Patients With Severe and Life-threatening COVID-19 A Randomized Clinical Trial

Ling Li, MD, PhD; Wei Zhang, MD; Yu Hu, MD, PhD; Xunliang Tong, MD, PhD; Shangen Zheng, MD; Juntao Yang, PhD; Yujie Kong, MD; Lili Ren, PhD; Qing Wei, MD; Heng Mei, MD, PhD; Caiying Hu, MD; Cuihua Tao, MD; Ru Yang, MD; Jue Wang, MD; Yongpei Yu, PhD; Yong Guo, PhD; Xiaoxiong Wu, MD; Zhihua Xu, MD; Li Zeng, MD; Nian Xiong, MD; Lifeng Chen, MD; Juan Wang, MD; Ning Man, MD; Yu Liu, PhD; Haixia Xu, MD; E. Deng, MS; Xuejun Zhang, MS; Chenyue Li, MD; Conghui Wang, PhD; Shisheng Su, PhD; Linqi Zhang, PhD; Jianwei Wang, PhD; Yanyun Wu, MD, PhD; Zhong Liu, MD, PhD

IMPORTANCE Convalescent plasma is a potential therapeutic option for patients with coronavirus disease 2019 (COVID-19), but further data from randomized clinical trials are needed.

OBJECTIVE To evaluate the efficacy and adverse effects of convalescent plasma therapy for patients with COVID-19.

DESIGN, SETTING, AND PARTICIPANTS Open-label, multicenter, randomized clinical trial performed in 7 medical centers in Wuhan, China, from February 14, 2020, to April 1, 2020, with final follow-up April 28, 2020. The trial included 103 participants with laboratory-confirmed COVID-19 that was severe (respiratory distress and/or hypoxemia) or life-threatening (shock, organ failure, or requiring mechanical ventilation). The trial was terminated early after 103 of a planned 200 patients were enrolled.

INTERVENTION Convalescent plasma in addition to standard treatment (n = 52) vs standard treatment alone (control) (n = 51), stratified by disease severity.

MAIN OUTCOMES AND MEASURES Primary outcome was time to clinical improvement within 28 days, defined as patient discharged alive or reduction of 2 points on a 6-point disease severity scale (ranging from 1 [discharge] to 6 [death]). Secondary outcomes included 28-day mortality, time to discharge, and the rate of viral polymerase chain reaction (PCR) results turned from positive at baseline to negative at up to 72 hours.

RESULTS Of 103 patients who were randomized (median age, 70 years; 60 [58.3%] male), 101 (98.1%) completed the trial. Clinical improvement occurred within 28 days in 51.9% (27/52) of the convalescent plasma group vs 43.1% (22/51) in the control group (difference, 8.8% [95% CI, −10.4% to 28.0%]; hazard ratio [HR], 1.40 [95% CI, 0.79-2.49]; P = .26). Among those with severe disease, the primary outcome occurred in 91.3% (21/23) of the convalescent plasma group vs 68.2% (15/22) of the control group (HR, 2.15 [95% CI, 1.07-4.32]; P = .03); among those with life-threatening disease the primary outcome occurred in 20.7% (6/29) of the convalescent plasma group vs 24.1% (7/29) of the control group (HR, 0.88 [95% CI, 0.30-2.63]; P = .83) (P for interaction = .17). There was no significant difference in 28-day mortality (15.7% vs 24.0%; OR, 0.65 [95% CI, 0.29-1.46]; P = .30) or time from randomization to discharge (51.0% vs 36.0% discharged by day 28; HR, 1.61 [95% CI, 0.88-2.93]; P = .12). Convalescent plasma treatment was associated with a negative conversion rate of viral PCR at 72 hours in 87.2% of the convalescent plasma group vs 37.5% of the control group (OR, 11.39 [95% CI, 3.91-33.18]; P < .001). Two patients in the convalescent plasma group experienced adverse events within hours after transfusion that improved with supportive care.

CONCLUSION AND RELEVANCE Among patients with severe or life-threatening COVID-19, convalescent plasma therapy added to standard treatment, compared with standard treatment alone, did not result in a statistically significant improvement in time to clinical improvement within 28 days. Interpretation is limited by early termination of the trial, which may have been underpowered to detect a clinically important difference.

TRIAL REGISTRATION Chinese Clinical Trial Registry: ChiCTR2000029757

JAMA doi:10.1001/jama.2020.10044
Published online June 3, 2020.

Editorial
Supplemental content

Author Affiliations: Author affiliations are listed at the end of this article.

Corresponding Authors: Zhong Liu, MD, PhD, Institute of Blood Transfusion, Chinese Academy of Medical Sciences and Peking Union Medical College, 26 Huacai Rd, Longtan Industry Zone, Chenghua District, 610052, Chengdu, Sichuan, China (liuz@ibt.pumc.edu.cn); Yanyun Wu, MD, PhD, University of Miami, Department of Pathology, 1611 NW 12th Ave, Miami, FL 33136 (yxw1366@med.miami.edu).

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

Since December 2019, coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), has spread rapidly around the world, with high rates of transmission and substantial mortality. COVID-19 symptoms can range from mild, self-limited respiratory disease to severe progressive pneumonia, multiple organ failure, and even death. [1,2] To date, there is no effective treatment or vaccine to contain the disease.

Convalescent plasma therapy has been used to treat patients with infections using plasma collected from recovered patients. [3] This approach has been evaluated in the treatment of SARS,[4,5] Middle East respiratory syndrome (MERS), [6] and Ebola, [7] but not well studied and with no definitive results. Recently, case series from China reported improved outcomes after COVID-19 convalescent plasma transfusion. [8,9] The US Food and Drug Administration (FDA) recently approved the emergency use of convalescent plasma for patients with severe or life-threatening COVID-19. [10] Although the use of convalescent plasma shows promise, the evidence supporting its use in the treatment of COVID-19 remains limited, and thus its use remains investigational.

In addition, due to the limited understanding of the mechanism and precise therapeutic components of convalescent plasma, there is no standardization or evidence-based rationale for donor selection, quality control of convalescent plasma, or recipient transfusion indications for convalescent plasma. This may help to explain the varied therapeutic effects of convalescent plasma seen in a variety of infectious diseases. To address these issues, the World Health Organization issued a guideline on the use of convalescent plasma in a pandemic, advocating for standardization in donor selection and convalescent plasma quality control to maximize therapeutic potency. [11]

The objective of this randomized clinical trial was to evaluate the efficacy and adverse effects of convalescent plasma added to standard treatment, compared with standard treatment alone, for patients with severe or life-threatening COVID-19 using a standardized approach in donor selection and convalescent plasma quality control.

Key Points

Question What is the effect of convalescent plasma therapy added to standard treatment, compared with standard treatment alone, on clinical outcomes in patients with severe or life-threatening coronavirus disease 2019 (COVID-19)?

Finding In this randomized clinical trial that included 103 patients and was terminated early, the hazard ratio for time to clinical improvement within 28 days in the convalescent plasma group vs the standard treatment group was 1.40 and was not statistically significant.

Meaning Among patients with severe or life-threatening COVID-19, convalescent plasma therapy added to standard treatment did not significantly improve the time to clinical improvement within 28 days, although the trial was terminated early and may have been underpowered to detect a clinically important difference.

Methods

This study was a collaborative effort by the Institute of Blood Transfusion of the Chinese Academy of Medical Sciences; Union Hospital of Tongji Medical College of Huazhong University of Science and Technology; the Guanggu District Maternal and Child Health Hospital of Hubei Province; Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology; General Hospital of the Central Theater Command of the People's Liberation Army; Wuhan Red Cross Hospital; Wuhan Asia Heart Hospital; and Wuhan Pulmonary Hospital.

This study was approved by the ethics committee of the Institute of Blood Transfusion of the Chinese Academy of Medical Sciences and the ethics committees of the participating hospitals. Written informed consent was obtained from all study participants or their legal representatives. The trial protocol and statistical analysis plan are available in Supplement 1 and Supplement 2 .

Participants

Patients were recruited from 7 medical centers. The study recruitment was from February 14, 2020, to April 1, 2020. Follow-up was completed on April 28, 2020.

Inclusion Criteria

Inclusion criteria were the following: (1) signed informed consent; (2) aged at least 18 years; (3) COVID-19 diagnosis based on polymerase chain reaction (PCR) testing; (4) positive PCR result within 72 hours prior to randomization; (5) pneumonia confirmed by chest imaging; (6) clinical symptoms meeting the definitions of severe or life-threatening COVID-19; (7) acceptance of random group assignment; (8) hospital admission; (9) willingness to participate in all necessary research studies and be able to complete the study follow-up; and (10) no participation in other clinical trials, such as antiviral trials, during the study period.

Severe COVID-19 was defined as respiratory distress (≥30 breaths/min; in resting state, oxygen saturation of 93% or less on room air; or arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FIO_2) of 300 or less. Life-threatening COVID-19 was defined as respiratory failure requiring mechanical ventilation; shock; or other organ failure (apart from lung) requiring intensive care unit (ICU) monitoring.

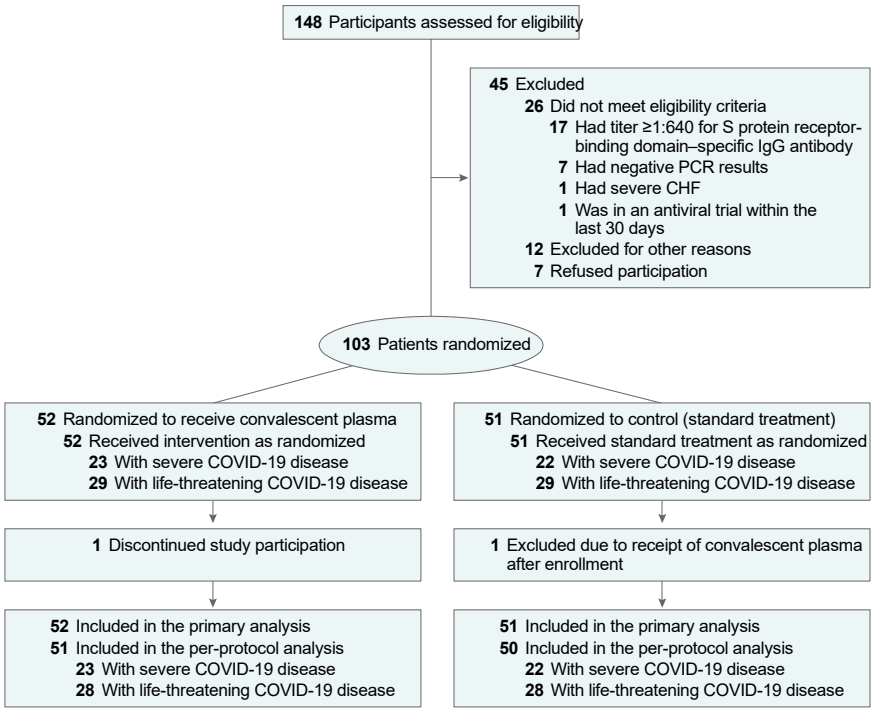
Exclusion Criteria

Exclusion criteria were the following: (1) pregnancy or lactation; (2) immunoglobulin allergy; (3) IgA deficiency; (4) preexisting comorbidity that could increase the risk of thrombosis; (5) life expectancy less than 24 hours; (6) disseminated intravascular coagulation; (7) severe septic shock; (8) PaO_2/FIO2 of less than 100; (9) severe congestive heart failure; (10) detection of high titer of S protein–RBD-specific (receptor binding domain) IgG antibody (≥1:640); (11) other contraindications as determined by the patient's physicians; and (12) participation in any antiviral clinical trials for COVID-19 within 30 days prior to enrollment.

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

Figure 1. Patient Enrollment and Treatment Assignment



PCR indicates polymerase chain reaction; CHF, congestive heart failure; COVID-19, coronavirus disease 2019.

Randomization

Potential study participants were screened for eligibility 72 hours prior to study randomization. Patients were randomly assigned via computer-generated random numbering (1:1) to receive standard treatment coupled with convalescent plasma transfusion or standard treatment alone (control group) (Figure 1). The randomization was stratified based on the severity of COVID-19 (severe or life-threatening) and a randomization schedule was generated using block randomization with block size of 4 for each type of COVID-19 by SAS software. Patients and clinicians were not masked to treatment assignment.

Procurement of Convalescent Plasma

In brief, patients with a laboratory-confirmed COVID-19 diagnosis, who had fully recovered and been discharged from the hospital for more than 2 weeks, were recruited. Convalescent plasma–specific donor screening and selection were based on the following criteria: age of 18 through 55 years, suitable for blood donation, initially diagnosed with COVID-19 but with 2 negative PCR test results from nasopharyngeal swabs (at least 24 hours apart) prior to hospital discharge, discharged for more than 2 weeks from the hospital, and no persisting COVID-19 symptoms. Convalescent plasma collection was performed based on routine plasma collection procedures via plasmapheresis. The plasma products were prepared as fresh-f rozen plasma. COVID-19 convalescent plasma was collected and processed at the Wuhan Blood Center.

S-RBD–specific IgG antibody titer was measured for convalescent plasma products and reported as the following: less than 1:160, 1:160, 1:320, 1:640, 1: 1280, or greater than 1:1280. There was a positive correlation between the SARS-CoV-2 viral neutralization titer and the S-RBD–specific IgG titer (r = 0.622, P = .03). A serum neutralization titer of 1:80 is approximately equivalent to a titer of 1:1280 for S-RBD–specific IgG. To ensure the therapeutic potency of the convalescent plasma, only the plasma units with an S-RBD–specific IgG titer of at least 1:640 were used for this study.

Additional details regarding plasma preparation standards can be found in the eMethods in Supplement 3 , and the preparation requirements of convalescent plasma used were similar to the recently updated FDA recommendations. [10]

Convalescent Plasma Transfusion

The transfusion dose of COVID-19 convalescent plasma was approximately 4 to 13 mL/kg of recipient body weight. ABO type of the convalescent plasma transfused was compatible with the patient's ABO type. In addition, the convalescent plasma was crossmatched with the patient's red blood cells to ensure compatibility. Convalescent plasma transfusion was administered at approximately 10 mL for the first 15 minutes, which was then increased to approximately 100 mL per hour with close monitoring. Adjustments in the infusion rates were allowed based on the patient's risk for volume overload and tolerance, at the discretion of the treating physicians. No premedication was given before convalescent plasma transfusion.

Standard Treatment

Standard treatment consisted of symptomatic control and supportive care for COVID-19, mostly based on the evolving Chinese national COVID-19 treatment guidelines and hospital

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

practice.[12] Possible treatments included antiviral medications, antibacterial medications, steroids, human immunoglobulin, Chinese herbal medicines, and other medications.

OutcomeMeasures

The primary end point was time to clinical improvement within a 28-day period. Clinical improvement was defined as patient discharge or a reduction of 2 points on a 6-point disease severity scale.[13] The scale was defined as follows: 6 points, death; 5 points, hospitalization plus extracorporeal membrane oxygenation (ECMO) or invasive mechanical ventilation; 4 points, hospitalization plus noninvasive ventilation or high-flow supplemental oxygen; 3 points, hospitalization plus supplemental oxygen (not high-flow or noninvasive ventilation); 2 points, hospitalization with no supplemental oxygen; 1 point, hospital discharge.

Patient discharge criteria included body temperature returned to normal for longer than 3 days, respiratory symptoms significantly improved without the need for oxygen support, and 2 consecutive negative PCR test results from nasopharyngeal swabs at least 24 hours apart.

Secondary clinical outcomes were as follows: (1) 28-day mortality, including analysis of time from randomization to death; (2) duration of hospitalization, including analyses of time from randomization to discharge, time from admission to discharge, and 28-day discharge rates; and (3) conversion of nasopharyngeal swab viral PCR results from positive at baseline to negative at follow-up assessed at 24, 48, and 72 hours. Once nasopharyngeal swab viral PCR testing yielded negative results 2 times consecutively, no further testing was performed.

A post hoc analysis was added to compare rates of improvement at days 7, 14, and 28.

This clinical trial was an open-label, randomized clinical study. To avoid assessment bias, the evaluation of clinical outcomes was performed by an investigator who was blind to the study group allocation.

StatisticalAnalysis

The original sample size was determined to be 100 for each group, which would provide 80% power, with a 2-sided significance level of $\alpha = .05$, to detect an 8-day change for the convalescent plasma group in time to clinical improvement, assuming that this would be 20 days in the control group and 60% of the patients would reach clinical improvement.

Unless otherwise stated, analyses were performed based on the full analysis set, which is defined as the set of all randomized patients who received at least one treatment specified in the trial. Statistical analysis was performed on randomly assigned treatment groups. Continuous variables were summarized by presenting the median and interquartile range (IQR) for the total number of patients who contributed values. Categorical variables were summarized by presenting the frequency and proportion of patients in each category. Time-to-event data were analyzed using the Kaplan-Meier method, and the median time to event and corresponding 95% CI were calculated. For the cases in which more than 50% of patients were censored and therefore the median time to event was indeterminate, the restricted mean survival time would be used for post hoc analysis.

For the primary end point of time to clinical improvement, death, withdrawal, and crossover between groups before day 28 were considered to be right-censored at day 28, and otherwise would be considered to be right-censored at the last observation date. Hazard ratios (HRs) with 95% CIs were calculated using Cox proportional hazards models. Three Cox proportional hazards models were fitted in this study. We referred to the model that included only the treatment group as the unadjusted model. The model that included disease severity (severe or life-threatening) and treatment group is referred to as model 1, and the model that further considered the interaction between disease severity and treatment group is referred to as model 2. Study sites were considered as a random effect in these models. Proportionality hazard assumption was assessed for treatment group and disease severity by extending the Cox models to include the corresponding time-dependent covariates.[14] If the coefficient of the time-dependent covariate was statistically significant, the proportionality hazard assumption would be considered to be violated.

A per-protocol analysis was performed for the primary end point as a sensitivity analysis. The per-protocol set was defined as the set of all randomized patients who received at least one treatment specified in the trial and who had no significant protocol violations that affected the efficacy evaluation.

Treatment effects for secondary end points were assessed using odds ratios and HRs with 95% CIs for the discrete variables and time-to-event data, respectively. For the analyses of time from randomization to discharge, time from randomization to death, and length of stay, the definition of censoring was consistent with the primary end point. Missing data for secondary outcomes and adverse events were not imputed. Only observed values were used for data analysis and presentation.

Subgroup analyses of efficacy were performed according to disease severity. Interactions between treatment group and disease severity group were tested using model 2.

Statistical analyses were performed with SAS software, version 9.4. Statistical significance was defined using a 2-sided significance level of $\alpha = .05$. Because of the potential for type I error due to multiple comparisons, findings for analyses of secondary end points should be interpreted as exploratory.

EarlyStudyTermination

Due to the containment of the COVID-19 epidemic in Wuhan, China, the numbers of patients with COVID-19 decreased in late March 2020. No new cases were reported in Wuhan for 7 consecutive days after March 24 (data from the National Health Commission of the People's Republic of China).[15] The last patient enrolled in this study was on March 27, 2020, and for the next 3 days, we were not able to recruit more patients and did not have any recruitment targets. After discussion with the expert committee of the Institute of Blood Transfusion, the study was terminated by the sponsor (Chinese Academy of Medical Sciences) and the leading primary investigator on April 1, 2020, with a total of 103 patients enrolled.

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

Table 1. Baseline Demographics and Clinical Characteristics of All Patients With COVID-19[a]

	Convalescent plasma group (n = 52)	Control group (n = 51)
Demographic and clinical characteristics		
Age, median (IQR), y	70 (62-80)	69 (63-76)
Sex, No. (%)		
Male	27 (51.9)	33 (64.7)
Female	25 (48.1)	18 (35.3)
Allergy history, No. (%)[b]	6 (11.5)	5 (9.8)
Coexisting diseases, No. (%)[c]		
Hypertension	29 (55.8)	27 (52.9)
Cardiovascular disease	14 (26.9)	12 (23.5)
Cerebrovascular disease	11 (21.2)	7 (13.7)
Diabetes	9 (17.3)	12 (23.5)
Liver disease	5 (9.6)	5 (9.8)
Cancer	3 (5.8)	0
Kidney disease	2 (3.9)	4 (7.8)
Laboratory values[d]		
Body temperature, median (IQR), °C	36.5 (36.2-36.7) [n = 52]	36.4 (36.2-36.8) [n = 50]
≥37.3 °C, No. (%)	4/52 (7.7)	7/50 (14.0)
Respiratory rate >24/min, No. (%)	11/52 (21.2)	7/49 (14.3)
Heart rate >100/min, No. (%)	13/52 (25.0)	8/50 (16.0)
Systolic blood pressure >140 mm Hg, No. (%)	10/52 (19.2)	15/50 (30.0)
White blood cell count, median (IQR), cells/μL	7590 (6300-11 460)	7160 (6130-11 200)
White blood cell count categories, No. (%)		
<4000/μL	5 (9.6)	4 (7.8)
4000-10 000/μL	31 (59.6)	29 (56.9)
>10 000/μL	16 (30.8)	18 (35.3)
Neutrophil count, median (IQR), cells/μL	7030 (4890-10 350)	5800 (4420-10 150)
Neutrophil count categories, No. (%)		
<1800/μL	0	2 (3.9)
1800-6300/μL	23 (44.2)	25 (49.0)
>6300/μL	29 (55.8)	24 (47.1)
Lymphocyte count, median (IQR), cells/μL	830 (570-1420)	800 (500-1370)
Lymphocyte count categories, No. (%)		
<1000/μL	32 (61.5)	32 (62.8)
≥1000/μL	20 (38.5)	19 (37.3)
Platelet count, median (IQR), $\times 10^3$/μL	164.5 (99.0-248.0)	214.0 (138.0-274.0)
Platelet count categories, No. (%)		
<100 × 10^3/μL	13 (25.0)	7 (13.7)
≥100 × 10^3/μL	39 (75.0)	44 (86.3)
CRP, median (IQR), mg/L	20.40 (5.13-65.60) [n = 49]	8.87 (1.73-40.32) [n = 48]
>5 mg/L, No. (%)	37/49 (75.5)	29/48 (60.4)
IL-6, median (IQR), pg/mL	16.62 (5.76-73.68) [n = 44]	21.67 (5.10-64.00) [n = 35]
>7 pg/mL, No. (%)	32/44 (72.7)	25/35 (71.4)
Prothrombin time, median (IQR), s	13.50 (12.00-15.20) [n = 51]	13.30 (12.35-14.15) [n = 48]
APTT, median (IQR), s	33.10 (28.30-41.10) [n = 49]	33.85 (29.95-42.95) [n = 48]
Thrombin time, median (IQR), s	16.45 (14.60-18.90) [n = 46]	16.10 (15.10-18.55) [n = 48]
Fibrinogen, median (IQR), mg/dL	3.86 (2.93-4.71) [n = 50]	4.00 (3.29-5.12) [n = 48]
D-dimer, median (IQR), μg/mL	1.88 (0.91-4.78) [n = 47]	2.23 (0.79-5.21) [n = 46]
>0.2 μg/mL, No. (%)	45/47 (95.7)	43/46 (93.5)

(continued)

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

Table 1. Baseline Demographics and Clinical Characteristics of All Patients With COVID-19 (continued)

	Convalescent plasma group (n = 52)	Control group (n = 51)
ALT, median (IQR), U/L	35.05 (22.25-55.90) [n = 52]	28.50 (18.95-59.50) [n = 48]
ALT categories, No. (%)		
≤50 U/L	36/52 (69.2)	33/48 (68.8)
>50 U/L	16/52 (30.8)	15/48 (31.3)
AST, median (IQR), U/L	28.50 (20.95-42.00) [n = 52]	24.50 (19.10-33.50) [n = 48]
AST categories, No. (%)		
≤40 U/L	35/52 (67.3)	40/48 (83.3)
>40 U/L	17/52 (32.7)	8/48 (16.7)
Urea nitrogen, median (IQR), mg/dL	20.36 (14.34-28.07) [n = 50]	20.08 (16.22-32.97) [n = 49]
Urea nitrogen categories, No. (%)		
<5.0 mg/dL	0/50	0/49
5.0-19.9 mg/dL	23/50 (46.0)	24/49 (49.0)
>19.9 mg/dL	27/50 (54.0)	25/49 (51.0)
Serum creatinine, median (IQR), mg/dL	0.75 (0.60-0.89) [n = 50]	0.83 (0.62-1.04) [n = 49]
Serum creatinine categories, No. (%)		
≤1.5 mg/dL	46/50 (92.0)	47/49 (95.9)
>1.5 mg/dL	4/50 (8.0)	2/49 (4.1)

Abbreviations: ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; IQR, interquartile range.

SI conversion factors: To convert D-dimer to nmol/L, multiply values by 5.476; to convert urea nitrogen to mmol/L, multiply values by 0.357; to convert creatinine to μmol/L, multiply values by 88.4.

[a] The values shown were based on total number of patients who contributed values.

[b] History of allergy to certain allergens, including food, medicine, etc.

[c] Details of coexisting diseases were collected from medical records.

[d] The vital signs and laboratory values are the last available values within 72 hours prior to randomization. The laboratory values selected were associated with the clinical status and factors that may affect convalescent plasma therapy. The values used for categorization of laboratory values are local divisions of low, normal, and high values.

There was no interim or preliminary data review prior to making this decision.

Results

Study Population

A total of 103 patients were enrolled in this randomized clinical trial. They were assigned to either the convalescent plasma group or the control group in a 1:1 ratio and were categorized as follows: 23 patients in the convalescent plasma group and 22 patients in the control group had severe COVID-19, and 29 patients in the convalescent plasma group and 29 patients in the control group had life-threatening COVID-19. Of these, 1 patient with life-threatening disease in the convalescent plasma treatment group withdrew from the study and 1 patient with life-threatening disease in the control group received convalescent plasma transfusion after randomization (protocol violation). Thus, 103 patients were included in the full analysis set and 101 patients were included in the per-protocol set (Figure 1).

The median age was 70 years (IQR, 62-78 years) among all patients, 71 years (IQR, 66-82 years) for patients with severe COVID-19, and 69 years (IQR, 61-76 years) for patients with life-threatening COVID-19. Of the patients included in the study, 60 (58.3%) were men, and the percentages of men with severe and life-threatening COVID-19 were 53.3% and 62.1%, respectively. A total of 89.2% of the patients had a normal body temperature at the time of participation, and the median body temperature was 36.5 °C (IQR, 36.2-36.7 °C) (Table 1 and eTable 1 and eTable 2 in Supplement 3).

The median interval between the onset of symptoms and randomization was 30 days (IQR, 20-39 days) overall, 33 days (IQR, 22-43 days) for patients with severe disease, and 26 days (IQR, 20-36 days) for patients with life-threatening disease. There were 5 patients with severe disease and 3 patients with life-threatening disease who had an interval between the onset of symptoms and randomization that was fewer than 14 days (Table 2).

Overall and within disease severity strata, the convalescent plasma and control groups were similar in terms of demographic characteristics, baseline laboratory results, and distribution on the 6-point disease severity scale at baseline, with the exception of systolic blood pressure in the patients with severe COVID-19 and sex in the patients with life-threatening COVID-19 (eTable 1 and eTable 2 in Supplement 3). Additional details regarding patients' clinical status at the time of randomization and additional medications received are provided in Table 2 and eTable 3, eTable 4, and eTable 7 in Supplement 3 . For patients in the convalescent plasma group, median plasma infusion volume was 200 mL (IQR, 200-300 mL), and 96% received a single dose of plasma infusion.

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

Primary Clinical Outcome

For all patients combined, there was no significant difference in the primary outcome of time to clinical improvement within 28 days: 51.9% (27/52) in the convalescent plasma group vs 43.1% (22/51) in the control group (difference, 8.8% [95% CI, −10.4% to 28.0%]; HR, 1.40 [95% CI, 0.79-2.49]; $P = .26$). Results for the per-protocol population were not materially different (eTable 5 in Supplement 3). Among those with severe disease, the primary outcome occurred in 91.3% (21/23) vs 68.2% (15/22) (HR, 2.15 [95% CI, 1.07-4.32]; $P = .03$). Among those with life-threatening disease, the primary outcome occurred in 20.7% (6/29) vs 24.1% (7/29) (HR, 0.88 [95% CI, 0.30-2.63]; $P = .83$) (P for interaction = .17) (Table 3 , Figure 2 , and eTable 6 in Supplement 3). For all proportional hazards models, the proportionality assumptions were met.

Secondary Clinical Outcomes

There was no significant difference in the secondary outcome of 28-day mortality (15.7% in the convalescent plasma group vs 24.0% in the control group; OR, 0.65 [95% CI, 0.29-1.46]; $P = .30$). There was also no significant difference in the time from randomization to death between the convalescent group and the control group (HR, 0.74 [95% CI, 0.30-1.82]; $P = .52$) (Table 3). Among patients with severe disease, no patients died in the convalescent plasma group, while 2 patients (9.1%) died in the control group. Among patients with life-threatening disease, 8 patients (28.6%) died in the convalescent plasma group, while 10 patients (35.7%) died in the control group.

There was also no significant difference in the secondary outcome of time from randomization to discharge (51.0% in the convalescent plasma group vs 36.0% in the control group were discharged by day 28; HR, 1.61 [95% CI, 0.88-2.93]; $P = .12$). The 28-day discharge rate of the convalescent plasma group reached 51%, among which the discharge rate of those with severe disease in the convalescent plasma group reached 91.3%.

At 24, 48, and 72 hours, the rates of negative SARS-CoV-2 viral PCR in the convalescent plasma group were all significantly higher than that of the control group (44.7% vs 15.0%, $P = .003$ at 24 hours; 68.1% vs 32.5%, $P = .001$ at 48 hours; 87.2% vs 37.5%, $P < .001$ at 72 hours) (Table 3 and eFigure 1 in Supplement 3). Among patients with severe disease, the rate of negative viral PCR at 72 hours was significantly higher in the convalescent plasma group compared with the control group, but there was no significant difference at 24 hours and 48 hours. Among patients with life-threatening disease, there was a statistically significant difference in favor of the convalescent plasma group at 24, 48, and 72 hours.

Post hoc Analysis

The clinical improvement rates overall and by disease severity subgroups at various time points during the study are presented in Table 3.

Adverse Events

Two participants reported transfusion-related adverse events following convalescent plasma transfusion. One patient in the severe COVID-19 group developed chills and rashes within 2 hours of transfusion but recovered fully after treatment with dexamethasone and promethazine. This was determined to be a definite nonsevere allergic transfusion reaction and also a probable nonsevere febrile nonhemolytic transfusion reaction. The other patient, who was in the life-threatening COVID-19 group, presented with shortness of breath, cyanosis, and severe dyspnea within 6 hours of transfusion. The patient was given dexamethasone, aminophylline, and other supportive care immediately and gradually improved after 2 hours. This was determined to be possible severe transfusion-associated dyspnea.

Table 2. Patients' Clinical Status at Randomization and Medications Received[a]

All patients	Convalescent plasma group (n = 52)	Control group (n = 51)
Time between symptom onset and randomization, median (IQR), d	27 (22-39) [n = 49]	30 (19-38) [n = 48]
≤14 d, No. (%)	3/49 (6.1)	5/48 (10.4)
>14 d, No. (%)	46/49 (93.9)	43/48 (89.6)
Interval between symptom onset and admission, median (IQR), d	12 (5-20) [n = 49]	10 (6-16) [n = 48]
6-Point scale at study day 1, No. (%)		
2- Hospitalization, no supplemental oxygen	1/51 (2.0)	1/50 (2.0)
3- Hospitalization, requiring supplemental oxygen (not high-flow or noninvasive ventilation)	15/51 (29.4)	15/50 (30.0)
4- Hospitalization, requiring noninvasive ventilation and/or high-flow supplemental oxygen	21/51 (41.2)	23/50 (46.0)
5- Hospitalization, requiring extracorporeal membrane oxygenation and/or invasive mechanical ventilation	14/51 (27.5)	11/50 (22.0)
Medications used after randomization		
Antiviral	41/46 (89.1)	44/49 (89.8)
Antibacterial	38/46 (82.6)	39/49 (79.6)
Chinese herbal medicine	26/46 (56.5)	30/49 (61.2)
Steroids	21/46 (45.7)	16/49 (32.7)
Antifungal	15/46 (32.6)	13/49 (26.5)
Human immunoglobulin	13/46 (28.3)	11/49 (22.5)
Interferon	12/46 (26.1)	7/49 (14.3)

[a] The values shown were based on total number of patients who contributed values. Details of medications used were provided in eTable 7 in Supplement 3.

Discussion

In this randomized clinical trial of patients with severe or life-threatening COVID-19, there was no significant difference in the time to clinical improvement between patients who received convalescent plasma transfusion therapy combined with standard treatment vs those who received standard treatment alone. There was also no significant difference in secondary outcomes of 28-day mortality or time from randomization to discharge. Convalescent plasma treatment was

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

Table 3. Primary and Secondary Clinical Outcomes at Day 28[a]

	Convalescent plasma group (n = 52)	Control group (n = 51)	Absolute difference (95% CI)[b]	Effect estimate (95% CI)	*P* value[c]
All patients					
Primary clinical outcome					
Time to clinical improvement, median (IQR), d[d]	28.00 (13.00-Indeterminate)	Indeterminate (18.00-Indeterminate)	−2.15 (−5.28 to 0.99)	HR, 1.40 (0.79-2.49)	.26
Clinical improvement rate, No./total (%)[e]					
At day 7	5/52 (9.6)	5/51 (9.8)	−0.2% (−11.6% to 11.2%)	OR, 0.98 (0.30-3.19)	.97
At day 14	17/52 (32.7)	9/51 (17.6)	15.0% (−1.4% to 31.5%)	OR, 1.85 (0.91-3.77)	.08
At day 28	27/52 (51.9)	22/51 (43.1)	8.8% (−10.4% to 28.0%)	OR, 1.20 (0.80-1.81)	.37
Secondary clinical outcomes					
Discharge rate at 28 d, No./total (%)	26/51 (51.0)	18/50 (36.0)	15.0% (−4.1% to 34.1%)	OR, 1.42 (0.90-2.24)	.13
Time from randomization to discharge, median (IQR), d[d]	28.00 (13.00-Indeterminate)	Indeterminate (19.00-Indeterminate)	−2.43 (−5.56 to 0.69)	HR, 1.61 (0.88-2.93)	.12
Time from hospitalization to discharge, median (IQR), d[d]	41.00 (31.00-Indeterminate)	53.00 (35.00-Indeterminate)	−11.95 (−26.33 to 2.43)	HR, 1.68 (0.92-3.08)	.09
Mortality at 28 d, No./total (%)	8/51 (15.7)	12/50 (24.0)	−8.3% (−23.8% to 7.2%)	OR, 0.65 (0.29-1.46)	.30
Time from randomization to death, median (IQR), d[d]	Indeterminate	Indeterminate (26.00-Indeterminate)	0.52 (−2.10 to 3.14)	HR, 0.74 (0.30-1.82)	.52
Viral nucleic acid negative rate, No./total (%)					
At 24 h	21/47 (44.7)	6/40 (15.0)	29.7% (11.7% to 47.7%)	OR, 4.58 (1.62-12.96)	.003
At 48 h	32/47 (68.1)	13/40 (32.5)	35.6% (15.9% to 55.3%)	OR, 4.43 (1.80-10.92)	.001
At 72 h	41/47 (87.2)	15/40 (37.5)	49.7% (32.0% to 67.5%)	OR, 11.39 (3.91-33.18)	<.001
Patients with severe disease					
Primary clinical outcome					
Time to clinical improvement, median (IQR), d[d]	13.00 (9.00-21.00)	19.00 (15.00-Indeterminate)	−4.94 (−9.33 to −0.54)	HR, 2.15 (1.07-4.32)	.03
Clinical improvement rate, No./total (%)[e]					
At day 7	3/23 (13.0)	4/22 (18.2)	−5.1% (−26.3% to 16.1%)	OR, 0.72 (0.18-2.85)	.70
At day 14	14/23 (60.9)	6/22 (27.3)	33.6% (6.3% to 60.9%)	OR, 2.23 (1.05-4.76)	.02
At day 28	21/23 (91.3)	15/22 (68.2)	23.1% (−3.9% to 50.2%)	OR, 1.34 (0.98-1.83)	.07
Secondary clinical outcomes					
Discharge rate at 28 d, No./total (%)	21/23 (91.3)	15/22 (68.2)	23.1% (−3.9% to 50.2%)	OR, 1.34 (0.98-1.83)	.07
Time from randomization to discharge, median (IQR), d[d]	13.00 (10.00-16.00)	19.00 (11.00-Indeterminate)	−4.09 (−8.44 to 0.27)	HR, 1.97 (1.00-3.88)	.05
Time from hospitalization to discharge, median (IQR), d	32.00 (26.00-40.00)	41.00 (30.00-53.00)	−9.38 (−23.63 to 4.88)	HR, 1.74 (0.89-3.41)	.11
Mortality at 28 d, No./total (%)	0/23	2/22 (9.1)	−9.1% (−25.6% to 7.4%)		.49
Time from randomization to death, median (IQR), d[d]	Indeterminate	Indeterminate (26.00-Indeterminate)	1.42 (−0.88 to 3.71)	HR, 0.00	>.99
Viral nucleic acid negative rate, No./total (%)					
At 24 h	7/21 (33.3)	2/17 (11.8)	21.6% (−9.1% to 52.2%)	OR, 3.75 (0.66-21.20)	.15
At 48 h	13/21 (61.9)	6/17 (35.3)	26.6% (−4.2% to 57.4%)	OR, 2.98 (0.79-11.25)	.10
At 72 h	19/21 (90.5)	7/17 (41.2)	49.3% (22.7% to 75.9%)	OR, 13.57 (2.36-77.95)	<.001
Patients with life-threatening disease					
Primary clinical outcome					
Time to clinical improvement, median (IQR), d[d]	Indeterminate	Indeterminate	0.23 (−3.11 to 3.57)	HR, 0.88 (0.30-2.63)	.83
Clinical improvement rate, No./total (%)[e]					
At day 7	2/29 (6.9)	1/29 (3.4)	3.4% (−11.4% to 18.3%)	OR, 2.00 (0.19-20.86)	>.99
At day 14	3/29 (10.3)	3/29 (10.3)	0.0% (−19.1% to 19.1%)	OR, 1.00 (0.22-4.55)	>.99
At day 28	6/29 (20.7)	7/29 (24.1)	−3.4% (−24.9% to 18.0%)	OR, 0.86 (0.33-2.24)	.75

(continued)

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

Table 3. Primary and Secondary Clinical Outcomes at Day 28[a] (continued)

	Convalescent plasma group (n = 52)	Control group (n = 51)	Absolute difference (95% CI)[b]	Effect estimate (95% CI)	*P* value[c]
Secondary clinical outcomes					
Discharge rate at 28 d, No./total (%)	5/28 (17.9)	3/28 (10.7)	7.1% (−14.7% to 28.9%)	OR, 1.67 (0.44-6.32)	.71
Time from randomization to discharge, median (IQR), d[d]	Indeterminate	Indeterminate	−0.80 (−3.74 to 2.14)	HR, 1.77 (0.42-7.40)	.44
Time from hospitalization to discharge, median (IQR), d[d]	Indeterminate (46.00-Indeterminate)	Indeterminate	−4.61 (−15.07 to 5.85)	HR, 1.90 (0.45-8.04)	.38
Mortality at 28 d, No./total (%)	8/28 (28.6)	10/28 (35.7)	−7.1% (−31.5% to 17.2%)	OR, 0.80 (0.37-1.72)	.57
Time from randomization to death, median (IQR), d[d]	Indeterminate (22.00-Indeterminate)	Indeterminate (15.00-Indeterminate)	−0.04 (−3.86 to 3.77)	HR, 0.86 (0.34-2.17)	.74
Viral nucleic acid negative rate, No./total (%)					
At 24 h	14/26 (53.8)	4/23 (17.4)	36.5% (11.8% to 61.1%)	OR, 5.54 (1.47-20.86)	.01
At 48 h	19/26 (73.1)	7/23 (30.4)	42.6% (17.3% to 68.0%)	OR, 6.20 (1.79-21.46)	.003
At 72 h	22/26 (84.6)	8/23 (34.8)	49.8% (25.9% to 73.7%)	OR, 10.31 (2.63-40.50)	<.001

Abbreviations: HR, hazard ratio; IQR, interquartile range; OR, odds ratio.

[a] The values shown were based on total number of patients who contributed values. The primary clinical outcome was analyzed by primary analysis set. Times to outcomes in the secondary clinical outcomes were analyzed by primary analysis set. Indeterminate events could not be calculated due to the low percentage of outcome events.

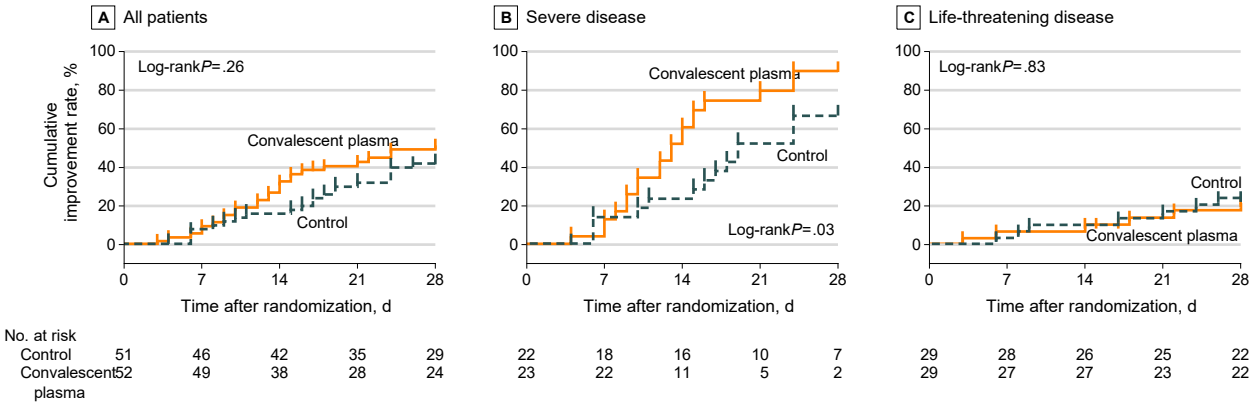
[b] The absolute differences of time to clinical improvement, times to discharge, and times from randomization to death were calculated based on restricted mean survival time.

[c] *P* value was calculated by Cox regression, χ^2 test, or Fisher exact test.

[d] Medians and quartiles of time to clinical improvement, times to discharge, and times from randomization to death could not be determined because too few patients had reached improvement or discharge by the end of the study. By the end of the study, for all, severe, and life-threatening patients, respectively, 27 (51.9%), 21 (91.3%), and 6 (20.7%) were clinically improved in the convalescent plasma group; 22 (43.1%), 15 (68.2%), and 7 (24.1%) were clinically improved in the control group; 26 (51.0%), 21 (91.3%), and 5 (17.9%) were discharged in the convalescent plasma group; 18 (36.0%), 15 (68.2%), and 3 (10.7%) were discharged in the control group; 8 (15.7%), 0, and 8 (28.6%) died in the convalescent plasma group; and 12 (24.0%), 2 (9.1%), and 10 (35.7%) died in the control group.

[e] These analyses were developed post hoc to better illustrate disease progression.

Figure 2. Time to Clinical Improvement in Patients With COVID-19



The cumulative improvement rate is the percentage of patients who experienced a 2-point improvement or were discharged alive from the hospital. Ticks on the curves indicate censored events. All patients who did not reach clinical improvement were observed for the full 28-day period or until death. COVID-19 indicates coronavirus disease 2019.

The median (IQR) follow-up times for the convalescent plasma group and control group, respectively, were 15 (10-28) days and 24 (13-28) days overall; 13 (10-16) and 18.5 (11-26) days among those with severe COVID-19; and 28 (12-28) and 26 (15-28) days among those with life-threatening COVID-19.

associated with higher rates of negative SARS-CoV-2 viral PCR results from nasopharyngeal swabs at 24, 48, and 72 hours, demonstrating that the convalescent plasma treatment was associated with antiviral activity in patients with COVID-19.

Plasma transfusions can result in transfusion-related adverse events including febrile and allergic transfusion reactions, transfusion-associated dyspnea, hypotensive transfusion reaction, to hemolytic transfusion reactions, septic transfusion reaction, transfusion-related acute lung injury, and transfusion-associated circulatory overload. In this study, most patients tolerated convalescent plasma transfusions well. There were 2 cases of reported transfusion-associated adverse events. One case was determined to be a definite nonsevere allergic transfusion reaction and also a probable nonsevere febrile

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

nonhemolytic transfusion reaction, and the other case was determined to be possible severe transfusion-associated dyspnea. The rate is somewhat higher than the general rate of reactions associated with plasma transfusion, possibly due to the small sample size and active surveillance. [16]

In the subgroup analysis by disease severity, there was a signal of possible clinical benefit for convalescent plasma among patients with severe COVID-19. However, bec ause the test for interaction by disease severity was not statistically significant, the findings for the severe and life-threatening subgroups should not be interpreted as different. Given the early study termination, it is possible that the study was underpowered to detect a statistically significant interaction, and a larger trial of convalescent plasma focusing on patients with severe COVID-19 may be warranted.

Patients with COVID-19 who were recruited in this study were heterogeneous with regard to the duration and severity of the illness. The possible antiviral activity with convalescent plasma treatment in patients aged 60 to 80 years and after 14 days of disease onset is notable. To our knowledge, no other antiviral treatments have demonstrated therapeutic effects in this age group or this late in the course of the disease. However, the con valescent plasma treatment was given at least 14 days after the onset of symptoms in most cases, and it is not known whether earlier convalescent plasma treatment could have resulted in better clinical outcomes. Further studies are needed to optimize patient selection and timing of convalescent plasma treatment.

While the use of convalescent plasma has been investigated and used many times in the past, [3,17] its use has not been well studied. It is notable that for most studies of the use of convalescent plasma, [4,5,18] there was lack of standardization and procedure control with regard to the donor selection process and the nature or level of antibodies in convalescent plasma units. This may explain the varied therapeutic effects seen across a variety of diseases or even across patients with the same disease. The guidance on convalescent plasma issued by the World Health Organization [11] highlighted the importance of standardized processes and laboratory testing–based quality control of convalescent plasma units, the selection of clinical indications, as well as program deployment to recruit adequate numbers of donors and maintain sufficient inventory.

One of the potential strengths of this study was the controlled process for donor selection and convalescent plasma quality control. A predefined process was used to enroll donors for convalescent plasma collection. Only units with a high titer of S-RBD–specific IgG antibody were used for convalescent plasma treatment in this study.

Limitations

This study has several limitations. First, the sample size was small and the study was terminated early. It is possible that the study was underpowered to detect a clinically important benefit of convalescent plasma therapy. Second, the median time between the onset of symptoms and randomization was 30 days, and it is unclear whether earlier treatment would have resulted in greater benefit. Third, this was an open-label study and the primary outcome was based to some degree on physicians' clinical management decisions. Fourth, the use of standard therapy was allowed in both groups and was not protocolized, which could have potentially influenced outcomes. Fifth, the relatively short 28-day time frame of the study follow-up may have precluded the observation of clinical improvement in patients with severe diseases, especially life-threatening COVID-19, as they may take longer time to respond and recover. Sixth, plasma was not used for the control group, which would have been a more ideal control group, making blinded design possible. Seventh, the study findings should be interpreted cautiously given that practices may vary from country to country, and hospital to hospital, such as the types of standard treatment, supportive care, and thresholds for intubation and hospital admission. The outcomes of this study may be related to a combination of many factors, such as the quality of the convalescent plasma products in terms of potency, the selection of the patients (severe and life-threatening COVID-19), and the timing of convalescent plasma transfusion.

Conclusion

Among patients with severe or life-threatening COVID-19, convalescent plasma therapy added to standard treatment, compared with standard treatment alone, did not significantly improve the time to clinical improvement within 28 days. Interpretation is limited by early termination of the trial, which may have been underpowered to detect a clinically important difference.

ARTICLE INFORMATION

Accepted for Publication: May 23, 2020.

Published Online: June 3, 2020. doi:10.1001/jama.2020.10044

Author Affiliations: Institute of Blood Transfusion, Chinese Academy of Medical Sciences and Peking Union Medical College, Chengdu, China (L. Li, Kong, J. Wang, Y. Liu, H. Xu, Deng, X. Zhang, C. Li, Z. Liu); Key Laboratory of Transfusion Adverse Reactions, CAMS, Chengdu, China (L. Li, Kong, J. Wang, H. Xu, C. Li, Z. Liu); Guanggu District Maternal and Child Health Hospital of Hubei Province, Wuhan, China. (W. Zhang, Zeng); Department of Respiratory and Critical Care Medicine, First Affiliated Hospital, the Second Military Medical University, Shanghai, China (W. Zhang); Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (Y. Hu, Mei); Department of Pulmonary and Critical Care Medicine, Beijing Hospital, National Respiratory quality control center, National Center of Gerontology, Beijing, China (Tong); Department of Transfusion, General Hospital of Central Theater Command of PLA, Wuhan, China (Zheng); State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union, Beijing, China (J. Yang); NHC Key Laboratory of Systems Biology of Pathogens and Christophe Mérieux Laboratory, Institute of Pathogen Biology, Chinese Academy of Medical Sciences (CAMS) & Peking Union Medical College, Beijing, China (Ren, C. Wang, J. Wang); Key Laboratory of Respiratory Disease Pathogenomics, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China (Ren, J. Wang); Department of Blood Transfusion, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (Wei, J. Wang); Wuhan Red Cross Hospital, Wuhan, China (C. Hu, Z. Xu, Xiong); Department of Blood Transfusion, Wuhan Asia Heart Hospital, Wuhan, China (Tao); Department of Blood Transfusion, Wuhan Asia General Hospital, Wuhan, China (Tao); Wuhan Blood Center, Wuhan, China

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

(R. Yang); Peking University Clinical Research Institute, Peking University Health Science Center, Beijing, China (Yu); Department of Biomedical Engineering, School of Medicine, Tsinghua University, Beijing, China (Guo, Su); Department of Emergency, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (X. Wu); Mianyang Central Hospital, Mianyang, China (Z. Xu); Department of Organ Transplantation, First Affiliated Hospital, the Second Military Medical University, Shanghai, China (Zeng); Department of Neurology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (Xiong); Wuhan Pulmonary Hospital, Wuhan, China (Chen); Department of Respiratory Medicine, Wuhan Asia General Hospital, Wuhan, China (Man); Center for Global Health and Infectious Diseases, Comprehensive AIDS Research Center, and Beijing Advanced Innovation Center for Structural Biology, School of Medicine, Tsinghua University, Beijing, China (L. Zhang); Department of Pathology, University of Miami, Miami, Florida (Y. Wu).

Author Contributions: Dr Z. Liu had full access to all of the data in this study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Drs L. Li, W. Zhang, and Y. Hu are co–first authors. Drs L. Li, W. Zhang, Y. Hu, X. Tong, S. Zheng, J. Yang, Y. Kong, L. Ren, Q. Wei, H. Mei, C. Hu, C. Tao, R. Yang, J. Wang, and Y. Yu contributed equally to the study.
Concept and design: L. Li, W. Zhang, X. Tong, J. Yang, N. Xiong, L. Zhang, J.W. Wang, Y. Wu, Z. Liu.
Acquisition, analysis, or interpretation of data: W. Zhang, S. Zheng, Y. Kong, L. Ren, Q. Wei, H. Mei, C. Hu, C. Tao, R. Yang, Jue Wang, Y. Yu, Y. Guo, X. Wu, Z. Xu, N. Xiong, L. Chen, Juan Wang, N. Man, Y. Liu, H. Xu, E. Deng, X. Zhang, C. Li, C. Wang, S. Su, Y. Hu, Y. Wu, Z. Liu.
Drafting of the manuscript: L. Li, W. Zhang, Y. Kong, L. Ren, H. Xu, E. Deng, C. Wang, Y. Wu, Z. Liu.
Critical revision of the manuscript for important intellectual content: W. Zhang, X. Tong, S. Zheng, J. Yang, Q. Wei, H. Mei, C. Hu, C. Tao, R. Yang, Jue Wang, Y. Yu, Y. Guo, X. Wu, Z. Xu, L. Zeng, N. Xiong, L. Chen, Juan Wang, N. Man, Y. Liu, X. Zhang, C. Li, S. Su, L. Zhang, Juan Wang, Y. Hu, Y. Wu, Z. Liu.
Statistical analysis: J. Yang, Y. Kong, Y. Yu.
Obtained funding: L. Li, Z. Liu.
Administrative, technical, or material support: L. Li, W. Zhang, X. Tong, L. Ren, H. Mei, Jue Wang, Y. Guo, N. Xiong, Y. Liu, H. Xu, E. Deng, C. Wang, S. Su, J.W. Wang, Z. Liu.
Supervision: W. Zhang, J. Yang, H. Mei, Y. Hu.

Conflict of Interest Disclosures: Dr Liu reports holding a pending patent on COVID-19 testing. Dr Wu reports consulting for Verax Medical and Grifols, receiving royalties from UptoDate and AABB, and being a volunteer visiting professor and receiving travel support for giving medical education from the Chinese Institute of Blood Transfusion. No other disclosures were reported.

Funding/Support: This work was supported by the Chinese Academy of Medical Sciences Innovation Fund for Medical Sciences (CIFMS) grants 2020-I2M-CoV19-006, 2016-I2M-3-024 (Dr Z. Liu), and 2017-I2M-1-009 (Dr L. Li) and the Nonprofit Central Research Institute Fund of Chinese Academy of Medical Sciences grant 2018PT32016 (Dr Z. Liu).

Role of the Funder/Sponsor: The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Data Sharing Statement See Supplement 4

Additional Contributions: We would like to thank the following individuals for their guidance, expertise, and assistance with the study design: Peter W. Marks, MD, PhD, Center for Biologics Evaluation and Research, FDA; Anne Eder, MD, PhD, Center for Biologics Evaluation and Research, FDA; Nicole Verdun, MD, Center for Biologics Evaluation and Research, FDA; Tim Uyeki, MD, MPH, MPP, National Center for Immunization and Respiratory Diseases, CDC; Scott Koepsell, MD, PhD, University of Nebraska Medical Center; Annie Winkler, MD, Instrumentation Laboratory; Xuan Qin, PhD, Seattle Children's Hospital and University of Washington. None received compensation related to this study.

REFERENCES

1. Wang D, Hu B, Hu C, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA* 2020;323(11):1061-1069. doi:10.1001/jama.2020.1585

2. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72 314 cases from the Chinese Center for Disease Control and Prevention. *JAMA* 2020;323(13):1239-1242. doi:10.1001/jama.2020.2648

3. Garraud O, Heshmati F, Pozzetto B, et al. Plasma therapy against infectious pathogens, as of yesterday, today and tomorrow. *Transfus Clin Biol*. 2016;23(1):39-44. doi:10.1016/j.tracli.2015.12.003

4. Cheng Y, Wong R, Soo YO, et al. Use of convalescent plasma therapy in SARS patients in Hong Kong. *Eur J Clin Microbiol Infect Dis*. 2005;24(1):44-46. doi:10.1007/s10096-004-1271-9

5. Yeh KM, Chiueh TS, Siu LK, et al. Experience of using convalescent plasma for severe acute respiratory syndrome among healthcare workers in a Taiwan hospital. *J Antimicrob Chemother*. 2005;56(5):919-922. doi:10.1093/jac/dki346

6. Arabi YM, Hajeer AH, Luke T, et al. Feasibility of using convalescent plasma immunotherapy for MERS-CoV infection, Saudi Arabia. *Emerg Infect Dis*. 2016;22(9):1554-1561. doi:10.3201/eid2209.151164

7. Kraft CS, Hewlett AL, Koepsell S, et al; Nebraska Biocontainment Unit and the Emory Serious Communicable Diseases Unit. The use of TKM-100802 and convalescent plasma in 2 patients with Ebola virus disease in the United States. *Clin Infect Dis*. 2015;61(4):496-502. doi:10.1093/cid/civ334

8. Shen C, Wang Z, Zhao F, et al. Treatment of 5 critically ill patients with COVID-19 with convalescent plasma. *JAMA* 2020;323(16):1582-1589. doi:10.1001/jama.2020.4783

9. Duan K, Liu B, Li C, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc Natl Acad Sci U S A* 2020;117(17):9490-9496. doi: 10.1073/pnas.2004168117

10. Recommendations for Investigational COVID-19 Convalescent Plasma. US FDA. Published May 1, 2020. Accessed May 26, 2020. https://www.fda.gov/vaccines-blood-biologics/investigational-new-drug-ind-or-device-exemption-ide-process-cber/recommendations-investigational-covid-19-convalescent-plasma

11 Position Paper on Use of Convalescent Plasma, Serum or Immune Globulin Concentrates as an Element in Response to an Emerging Virus. In: Network WBR, ed. 2017. Accessed April 18, 2020. https://www.who.int/bloodproducts/brn/en/

12 National Health Commission of the People's Republic of China. Covid-19 treatment plan (trial version 6). Accessed April 20, 2020. http://www.nhc.gov.cn/yzygj/s7653p/202002/8334a8326dd94d329df351d7da8aefc2/files/b218cfeb1bc54639af227f922bf6b817

13 Wang Y, Zhang D, Du G, et al. Remdesivir in adults with severe COVID-19: a randomised, double-blind, placebo-controlled, multicentre trial. *Lancet* 2020;395(10236):1569-1578. doi:10.1016/S0140-6736(20)31022-9

14 Kleinbaum DG. Evaluating the proportional hazards assumption. In: *Survival Analysis: Statistics in the Health Sciences*. Springer; 1996:183-184.

15 Daily update on covid-19. National Health Commission of the People's Republic of China. Accessed May 24, 2020. http://www.nhc.gov.cn/xcs/yqtb/202003/f01fc26a8a7b48debe194bd1277fdba3.shtml

16 Annual SHOT (Serious Hazards of Transfusion) Report 2018. Published July 2019. Accessed May 25, 2020. https://www.shotuk.org/

17 Beigel JH, Aga E, Elie-Turenne MC, et al; IRC005 Study Team. Anti-influenza immune plasma for the treatment of patients with severe influenza A: a randomised, double-blind, phase 3 trial. *Lancet Respir Med*. 2019;7(11):941-950. doi:10.1016/S2213-2600(19)30199-7

18 Soo YO, Cheng Y, Wong R, et al. Retrospective comparison of convalescent plasma with continuing high-dose methylprednisolone treatment in SARS patients. *Clin Microbiol Infect*. 2004;10(7):676-678. doi:10.1111/j.1469-0691.2004.00956.x

© 2020 American Medical Association. All rights reserved.

Downloaded From: https://jamanetwork.com/ by a Mayo Clinic Library User on 06/03/2020

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Board on Health Sciences Policy

HOST GENOMICS RESEARCH AND DATA COLLECTION EFFORTS RELATED TO COVID-19

Joint Meeting of the
Roundtable on Genomics and Precision Health
and the
Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats

Virtual Meeting July 1, 2020
Zoom: https://nasem.zoom.us/j/95540901323

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Board on Health Sciences Policy

Host Genomics Research and Data Collection Efforts Related to COVID-19

July 1, 2020

Table of Contents

Agenda 3

Agenda 4

ROUNDTABLE AND STANDING COMMITTEE INFORMATION 7

Roundtable on Genomics and Precision Health 8

Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats 10

MEETING INFORMATION 13

Speaker and Chair Biographies 14

BACKGROUND MATERIALS 17

Online links to background materials 18

Rapid Expert Consultations on the COVID-19 Pandemic 20

A blood-based host gene expression assay for early detection of viral infection 21

AGENDA

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Board on Health Sciences Policy

Host Genomics Research and Data Collection Efforts Related to COVID-19

Joint Meeting
of the
Roundtable on Genomics and Precision Health
and the
Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats

A VIRTUAL MEETING
July 1, 2020
1:00 PM – 2:45 PM ET
Zoom Link: https://nasem.zoom.us/j/95540901323

Aims for the meeting:

- To understand the scientific challenges that the field of genomics is facing
  - What has been the effect of the pandemic on genomics research and data collection and sharing? What is the role of the genomics community during the pandemic? What research is underway? What research still needs to be done?
  - What gaps and challenges in the field is this crisis highlighting? What unique contributions of the field are not being leveraged effectively?
- To explore innovative approaches to overcoming those challenges
  - What new approaches are you using to solve a problem? Could these be applied to the field of genomics? Are there lessons from genomics that are being used in light of the pandemic? What are the opportunities for connecting efforts and data sets (e.g. across institutions, genotype and phenotype data)?
- To discuss opportunities for the Genomics Roundtable and for the Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats to facilitate progress
  - Are there opportunities for the Roundtable and Standing Committee to act that align with strategic priorities?
  - Are the Roundtable and Standing Committee uniquely positioned to address specific challenges?

AGENDA:

1:00 p.m. ET Welcoming Remarks

GEOFFREY GINSBURG, *Roundtable Co-Chair*
Director, Duke Center for Applied Genomics & Precision Medicine
Professor, Medicine, Pathology, and Biomedical Engineering
Duke University Medical Center

MICHELLE PENNY, *Roundtable Co-Chair*
Vice President and Head of Genomics
Goldfinch Bio

HARVEY FINEBERG, *Standing Committee Chair*
President
Gordon and Betty Moore Foundation

1:15 p.m. **An Introduction to the Host Genetics of COVID-19**

MAXIMILIAN MUENKE
CEO
American College of Medical Genetics and Genomics (ACMG)

Understanding Host Genetics and Genomics Related to COVID-19 and Opportunities for Forming Data Sharing COVID-19 Communities

1:25 p.m. SHARON TERRY
Founder and CEO
Genetic Alliance

1:35 p.m. JOYCE TUNG
Vice President, Research
23andMe

1:45 p.m. GEOFFREY GINSBURG, *Roundtable Co-Chair*
Director, Duke Center for Applied Genomics & Precision Medicine
Professor, Medicine, Pathology, and Biomedical Engineering
Duke University Medical Center

1:55 p.m. **Discussion with Roundtable and Standing Committee Members**

2:30 p.m. **Next Steps**

Questions:

- What challenges or gaps were exposed today?
- Is there an area that should be incorporated into the Roundtable's strategic planning or the Standing Committee's work?

GEOFFREY GINSBURG, *Roundtable Co-Chair*
Director, Duke Center for Applied Genomics & Precision Medicine
Professor, Medicine, Pathology, and Biomedical Engineering
Duke University Medical Center

MICHELLE PENNY, *Roundtable Co-Chair*
Vice President and Head of Genomics
Goldfinch Bio

HARVEY FINEBERG, *Standing Committee Chair*
President
Gordon and Betty Moore Foundation

2:45 p.m. Adjourn

ROUNDTABLE AND STANDING COMMITTEE INFORMATION

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Roundtable on GENOMICS and PRECISION HEALTH

The sequencing of the human genome is rapidly opening new doors to research and progress in biology, medicine, and health care. At the same time, these developments have produced a diversity of new issues to be addressed.

The National Academies of Sciences, Engineering, and Medicine has convened a Roundtable on Genomics and Precision Health (previously the Roundtable on Translating Genomic-Based Research for Health) that brings together leaders from academia, industry, government, foundations and associations, and representatives of patient and consumer interests who have a mutual concern and interest in addressing the issues surrounding the translation of genome-based research for use in maintaining and improving health. The mission of the Roundtable is to advance the field of genomics and improve the translation of research findings to health care, education, and policy. The Roundtable will discuss the translation process, identify challenges at various points in the process, and discuss approaches to address those challenges.

The field of genomics and its translation involves many disciplines, and takes place within different economic, social, and cultural contexts, necessitating a need for increased communication and understanding across these fields. As a convening mechanism for interested parties from diverse perspectives to meet and discuss complex issues of mutual concern in a neutral setting, the Roundtable: fosters dialogue across sectors and institutions; illuminates issues, but does not necessarily resolve them; and fosters collaboration among stakeholders.

To achieve its objectives, the Roundtable conducts structured discussions, workshops, and symposia. Workshop summaries will be published and collaborative efforts among members are encouraged (e.g., journal articles). Specific issues and agenda topics are determined by the Roundtable membership, and span a broad range of issues relevant to the translation process.

Issues may include the integration and coordination of genomic information into health care and public health including encompassing standards for genetic screening and testing, improving information technology for use in clinical decision making, ensuring access while protecting privacy, and using genomic information to reduce health disparities. The patient and family perspective on the use of genomic information for translation includes social and behavioral issues for target populations. There are evolving requirements for the health professional community, and the need to be able to understand and responsibly apply genomics to medicine and public health.

Of increasing importance is the need to identify the economic implications of using genome-based research for health. Such issues include incentives, cost-effectiveness, and sustainability.

Issues related to the developing science base are also important in the translation process. Such issues could include studies of gene-environment interactions, as well as the implications of genomics for complex disorders such as addiction, mental illness, and chronic diseases.

Roundtable sponsors include federal agencies, pharmaceutical companies, medical and scientific associations, foundations, and patient/public representatives. For more information about the Roundtable on Genomics and Precision Health, please visit our website at nationalacademies.org/GenomicsRT or contact Sarah Beachy at 202-334-2217, or by e-mail at sbeachy@nas.edu.

The National Academy of Sciences, National Academy of Engineering, and National Academy of Medicine work together as the National Academies of Sciences, Engineering, and Medicine ("the Academies") to provide independent, objective analysis and advice to the nation and conduct other activities to solve complex problems and inform public policy decisions. The Academies also encourage education and research, recognize outstanding contributions to knowledge, and increase public understanding in matters of science, engineering, and medicine.

Roundtable on Genomics and Precision Health Membership

Geoffrey Ginsburg, M.D., Ph.D. (Co-Chair) Duke University
Michelle Penny, Ph.D. (Co-Chair) Goldfinch Bio

Naomi Aronson, Ph.D., BlueCross/BlueShield Association

Aris Baras, M.D., M.B.A., Regeneron Pharmaceuticals

Karina Bienfait, Ph.D., Merck and Co., Inc.

Vence Bonham, Jr., J.D., National Human Genome Research Institute

Robert B. Darnell, M.D. Ph.D., The Rockefeller University / NY Genome Center

Stephanie Devaney, Ph.D., All of Us Research Program, NIH

Katherine Donigan, Ph.D., U.S. Food and Drug Administration

W. Gregory Feero, M.D., Ph.D., Journal of the American Medical Association

Jessica M. Gill, Ph.D., R.N., FAAN, National Institute of Nursing Research

Jennifer Goldsack, MChem, M.A., M.B.A., Digital Medicine Society (DiMe)

Marc Grodman, M.D., Genosity

Richard Hodes, M.D., National Institute on Aging

Praduman Jain, M.S., Vibrent Health

Sally John, Ph.D., Biogen

Sekar Kathiresan, M.D., Massachusetts General Hospital

Muin Khoury, M.D., Ph.D., Centers for Disease Control and Prevention

David Ledbetter, Ph.D., Geisinger

Charles Lee, Ph.D., FACMG, The Jackson Laboratory for Genomic Medicine

Thomas Lehner, Ph.D., M.P.H., National Institute of Mental Health

Debra Leonard, M.D., Ph.D., College of American Pathologists

Patrick Loerch, Ph.D., Johnson & Johnson

James Lu, M.D., Ph.D., Helix

Sean McConnell, Ph.D., American Medical Association

Mona Miller, M.P.P., American Society of Human Genetics

Jennifer Moser, Ph.D., U.S. Department of Veterans Affairs

Maximilian Muenke, M.D., FACMG, American College of Medical Genetics and Genomics

Anna Pettersson, Ph.D., Pfizer Inc.

Victoria M. Pratt, Ph.D., FACMG, Association for Molecular Pathology

Kunal Sanghavi, M.B.B.S., M.S., C.G.C., The Jackson Laboratory for Genomic Medicine

Nadeem Sarwar, Ph.D., Eisai Inc.

Sheri Schully, Ph.D., Formerly NIH Office of Disease Prevention, now All of Us

Joan A. Scott, M.S., C.G.C., Health Resources and Services Administration

Sam Shekar, M.D., M.P.H., American College of Preventive Medicine

Nonniekaye Shelburne, C.R.N.P., M.S., A.O.C.N., National Cancer Institute

Nikoletta Sidiropoulos, M.D., University of Vermont Health Network Medical Group

Katherine Johansen Taber, Ph.D., Myriad Women's Health

Ryan Taft, Ph.D., Illumina

Jacquelyn Taylor, Ph.D., New York University

Sharon Terry, M.A., Genetic Alliance

Joyce Tung, Ph.D., 23andMe, Inc.

Jameson Voss, M.D., Air Force Medical Support Agency

Catherine A. Wicklund, M.S., C.G.C., National Society of Genetic Counselors

Huntington F. Willard, Ph.D., Genome Medical

Janet K. Williams, Ph.D., R.N., FAAN, American Academy of Nursing

Sarah Wordsworth, Ph.D., University of Oxford

Alicia Zhou, Ph.D., Color Genomics

Project Staff
Sarah H. Beachy, Ph.D., *Roundtable Director*
Siobhan Addie, Ph.D., *Program Officer*
Meredith Hackmann, *Associate Program Officer*
Kelly Choi, *Senior Program Assistant*

NAM Greenwall Fellow in Bioethics
Rachel Fabi, Ph.D., *Upstate Medical University*

500 Fifth Street, NW, Washington, DC 20001
Phone 202.334.2217 E-mail sbeachy@nas.edu nationalacademies.org/GenomicsRT

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Health and Medicine Division

Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats

In response to a request from the Office of Science and Technology Policy (OSTP) and the Office of the Assistant Secretary for Preparedness and Response (ASPR), the National Academies of Sciences, Engineering, and Medicine will convene a standing committee of experts to help inform the federal government on critical science and policy issues related to emerging infectious diseases and other 21st century health threats. The standing committee will include members with expertise in emerging infectious diseases, public health, public health preparedness and response, biological sciences, clinical care and crisis standards of care, risk communication, epidemiology, and regulatory issues, as well as veterinary science, One Health, ethics, and community engagement. The standing committee will provide a venue for the exchange of ideas among federal government agencies, the private sector, and the academic community, as well as other relevant stakeholders.

The standing committee will:

- Stand ready to respond on short notice to requests from the federal government to assess and consider the science and policy implications of an emerging infectious disease or significant public health threat;
- Provide a venue to enable science and policy discussions relevant to the federal government on emerging issues, research, and activities through in-depth knowledge of the sponsor's programs, goals, and objectives;
- Identify opportunities to integrate science into national preparedness and response decision making;
- Explore lessons learned and best practices from previous preparedness and response efforts, and identify opportunities to disseminate that information to a variety of stakeholders;
- Serve as a focal point for national policy discussions by experts and other leaders in the field;
- Consider, identify, and discuss strategies for addressing misinformation; and
- Respond to the federal government's needs for continuing dialog related to strategic planning and program development to address emerging infectious diseases, biosecurity, and public health and medical preparedness.

At the request of the sponsors, the standing committee will be involved in the planning, development, and oversight of related ad hoc activities undertaken by separately appointed committees operating under its auspices.

The standing committee will serve as a focal point for the discussion of scientific, technical, and policy issues relevant to emerging infectious diseases and public health preparedness and response that warrant detailed examination. Topics for discussion with the standing committee may include:

- Technical assistance and/or assessment of response to emerging infectious diseases;
- Availability of and access to information, samples, and other materials to determine the origin and evolution of emerging infectious diseases;
- International coordination and engagement;
- Technical assessment of ecological and evolutionary drivers of disease emergence;
- Approaches to proactive public messaging and strategies to address misinformation;
- Other science and policy issues relevant to emerging infectious diseases and 21st century health threats.

The committee will carry out its charge at its in-person and virtual meetings by gathering evidence from experts, deliberating, and, when necessary, by preparing short reports.

COMMITTEE SPONSORS

White House Office of Science and Technology Policy and HHS Office of Assistant Secretary for Preparedness and Response

COMMITTEE ROSTER

Harvey Fineberg, M.D., Ph.D. ***(Chair)***
President
Gordon and Betty Moore Foundation

Kristian Andersen, Ph.D.
Associate Professor and Director of Infectious Disease Genomics, Scripps Research Translational Institute
The Scripps Research Institute

Ralph Steven Baric, Ph.D.
William R. Kenan, Jr. Distinguished Professor
The University of North Carolina at Chapel Hill

Mary Bassett, M.D., M.P.H.
Director of the François-Xavier Bagnoud Center for Health and Human Rights
Harvard School of Public Health

Trevor Bedford, Ph.D.
Associate Faculty Member, Vaccine and Infectious Disease Division, Public Health Sciences Division, and Human Biology Division
Fred Hutchinson Cancer Research Center

Georges Benjamin, M.D.
Executive Director
American Public Health Association

Donald Berwick, M.D., M.P.P., F.R.C.P, KBE
Lecturer of Health Care Policy
Harvard Medical School

Richard Besser, M.D.
President and CEO
Robert Wood Johnson Foundation

R. Alta Charo, J.D.
Warren P. Knowles Professor of Law and Bioethics
University of Wisconsin at Madison

Peter Daszak, Ph.D.
President and CEO
EcoHealth Alliance

Jeffrey S. Duchin, M.D.
Health Officer and Chief, Communicable Disease Epidemiology & Immunization Section and Professor in Medicine

Ellen Embrey
President/CEO
Stratitia, Inc.

Baruch Fischhoff, Ph.D.
Howard Heinz University Professor
Department of Engineering and Public Policy
Carnegie Mellon University

Diane Griffin, M.D., Ph.D.
Professor, Department of Molecular Microbiology and Immunology
Johns Hopkins Bloomberg School of Public Health

Robert Groves, Ph.D., M.A.
Executive Vice President and Provost
Gerard J. Campbell, S.J. Professor
Math and Statistics Department and Sociology Department
Georgetown University

Margaret Hamburg, M.D.
Foreign Secretary
National Academy of Medicine

Dan Hanfling, M.D.
Vice President, Technical Staff
In-Q-Tel

John Hick, M.D.
Associate Medical Director for EMS
Medical Director of Emergency Medicine
Hennepin County Medical Center

Kent E. Kester, M.D.
Vice President and Head, Translational Science and Biomarkers
Sanofi Pasteur

Patricia King, J.D.
Professor Emerita
Georgetown University Law Center

Jonna Mazet, D.V.M., M.P.V.M., Ph.D.
Executive Director, One Health Institute
UC Davis School of Veterinary Medicine

Phyllis Meadows, Ph.D., M.S.N., R.N.
Senior Fellow, Health
The Kresge Foundation

Tara O'Toole, M.D., M.P.H.
Executive Vice President
In-Q-Tel

Alexandra Phelan, S.J.D., LL.M., LL.B.
Assistant Professor
Center for Global Health Science and Security
Georgetown University

David Relman, M.D.
Thomas C. and Joan M. Merigan Professor of Medicine, and of Microbiology & Immunology; Chief of Infectious Diseases
Stanford University; VA Palo Alto Health Care System

Mark Smolinski, M.D., M.P.H.
President
Ending Pandemics

David Walt, Ph.D.
Hansjörg Wyss Professor of Biologically Inspired Engineering
Harvard Medical School

CONTACT INFORMATION

Andrew Pope, Ph.D.
Senior Director, Board on Health Sciences Policy
202-334-1739
apope@nas.edu

Lisa Brown, M.P.H.
Study Director, Board on Health Sciences Policy
202-334-2487
lbrown@nas.edu

ADDITIONAL INFORMATION

For additional information, please visit https://www.nationalacademies.org/our-work/standing-committee-on-emerging-infectious-diseases-and-21st-century-health-threats

MEETING INFORMATION

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Board on Health Sciences Policy

Host Genomics Research and Data Collection Efforts Related to COVID-19

Joint Meeting
of the
Roundtable on Genomics and Precision Health
and the
Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats

SPEAKER AND CHAIR BIOGRAPHIES

Harvey V. Fineberg, M.D., Ph.D. ***(Standing Committee Chair)***
Dr. Fineberg is the president of the Gordon and Betty Moore Foundation, which fosters path-breaking scientific discovery, environmental conservation, improvements in patient care and preservation of the special character of the San Francisco Bay Area. He previously held the Presidential Chair for 2014-2015 as visiting professor at the University of California, San Francisco. Prior to that, he served as president of the Institute of Medicine from 2002 to 2014 and as provost of Harvard University from 1997 to 2001, following 13 years as dean of the Harvard School of Public Health. He has devoted most of his academic career to the fields of health policy and medical decision-making. His past research has focused on the process of policy development and implementation, assessment of medical technology, evaluation and use of vaccines, and dissemination of medical innovations. Fineberg chairs the board of the Carnegie Endowment for International Peace and serves on the boards of the William and Flora Hewlett Foundation and the China Medical Board. He helped found and served as president of the Society for Medical Decision Making and also served as consultant to the World Health Organization. Fineberg is co-author of the books Clinical Decision Analysis, Innovators in Physician Education and The Epidemic That Never Was, an analysis of the controversial federal immunization program against swine flu in 1976. He has co-edited several books on such diverse topics as AIDS prevention, vaccine safety, understanding risk in society and global health. He has also authored numerous articles published in professional journals. Fineberg is the recipient of several honorary degrees—the Frank A. Calderone Prize in Public Health, the Henry G. Friesen International Prize in Health Research and the Harvard Medal, awarded by the alumni association of the university from which he earned his bachelor's and doctoral degrees.

Geoffrey Ginsburg, M.D., Ph.D. (Roundtable *Co-Chair*)
Dr. Ginsburg is the founding director for the Center for Applied Genomics & Precision Medicine at the Duke University Medical Center and for MEDx, a partnership between the Schools of Medicine and Engineering to spark and translate innovation. His research addresses the challenges for translating genomic and digital information into medical practice and the integration of precision medicine into healthcare. In 2017 he received Duke's Translational Research Mentorship Award and is a finalist in the NIH/BARDA Antimicrobial Resistance Prize.

He is a member of the Advisory Council to the Director of NIH and is co-chair of the National Academies Roundtable on Genomics and Precision Health and is founder and president of the Global Genomic Medicine

Collaborative, a not for profit organization aimed creating international partnerships to advance the implementation of precision medicine. He has recently served as a member of the Board of External Experts for the NHLBI, the advisory council for the National Center for Accelerating Translational Science, the chair of the review for Genome Canada's Large Scale Applied Research Competition in Genomics and Precision Medicine, and the World Economic Forum's Global Agenda Council on the Future of the Health Sector. He is a founder of Predigen Inc and MeTree&You Inc. He was previously Vice President of Molecular Medicine at Millennium Pharmaceuticals, Inc and a faculty member at Harvard Medical School.

Maximilian Muenke, M.D., FACMG
Dr. Muenke joined the American College of Medical Genomics (ACMG) as CEO in 2019. Recognized as a highly acclaimed physician-scientist and dedicated clinical and research mentor, Dr. Muenke has more than three decades of experience to the ACMG, including 10 years on the faculty of the University of Pennsylvania School of Medicine. Since 2000, Dr. Muenke has served as Senior Investigator, Head of the Human Development Section, and Chief of the Medical Genetics Branch at the National Human Genome Research Institute (NHGRI) of the National Institutes of Health (NIH). The focus of his laboratory's research was on the delineation and identification of the underlying causes of craniofacial and related anomalies in humans including holoprosencephaly as well as Muenke syndrome, a relatively common craniosynostosis syndrome. Dr. Muenke is passionate about training the next generation of leaders in the field of genetics and genomic medicine. He has directed medical genetics training since 1994, first at the University of Pennsylvania and then at the NIH, where he was the Director of the NIH Medical Genetics and Genomic Medicine Residency and Fellowship Training Programs.

Michelle Penny, Ph.D. ***(Roundtable Co-Chair)***
Dr. Penny is a Vice President and Head of Genomics at Golfich Bio. Prior to that, she was the Head of Translational Genome Sciences at Biogen. She received her Ph.D. in Genetics from the University of Birmingham, UK in 1993. After a postdoctoral fellowship in the Virology Division at the National Institute of Medical Research, Mill Hill, London, she joined Imperial College London, Department of Medical and Community Genetics where she was a postdoctoral research scientist until taking a lectureship in Human Molecular Genetics in 1998. Her academic research career focused on the study of complex polygenic diseases particularly autoimmune disease and susceptibility to infection. In 2002, Dr. Penny joined the Clinical Pharmacogenomics group at Pfizer in Sandwich, UK, and moved to New London, CT in 2006 to take on the role of Oncology Molecular Medicine Lead until 2009 when she moved to Indianapolis to lead the Pharmacogenomics work at Eli Lilly and Company.

Sharon Terry, M.A.
Sharon Terry is President and CEO of Genetic Alliance, a large network engaging individuals, families and communities to transform health. Genetic Alliance works to provide programs, products and tools for ordinary people to take charge of their health.

As 'just a Mom' with a master's degree in Theology, she cofounded PXE International, a research advocacy organization for the genetic condition pseudoxanthoma elasticum (PXE), in response to the diagnosis of PXE in her two children in 1994. With her husband, she co-discovered the ABCC6 gene, patented it to ensure ethical stewardship in 2000, and assigned their rights to the foundation. She subsequently developed a diagnostic test and conducts clinical trials. She is the author of 140 peer-reviewed papers, of which 30 are clinical PXE studies.

In her focus at the forefront of consumer participation in genetics research, services and policy, she serves in a leadership role on many of the major international and national organizations, including the Precision Medicine Initiative Cohort Advisory Panel, Accelerating Medicines Partnership, Institute of Medicine (IOM) Science and Policy Board, the IOM Roundtable on Translating Genomic-Based Research for Health, the PubMed Central National Advisory Committee, the PhenX scientific advisory board, the Global Alliance for Genomics and Health, the International Rare Disease Research Consortium Executive Committee and as Founding President of EspeRare Foundation of Geneva, Switzerland. Terry is co-founder of the Genetic Alliance Registry and Biobank. She is on the editorial boards of several journals. She led the coalition that was instrumental in the passage of the Genetic Information Nondiscrimination Act. She received an honorary doctorate from Iona College for her

community engagement work in 2006; the Research!America Distinguished Organization Advocacy Award in 2009; and the Clinical Research Forum and Foundation's Annual Award for Leadership in Public Advocacy in 2011. She was named one of FDA's "30 Heroes for the Thirtieth Anniversary of the Orphan Drug Act" in 2013. She is co-inventor of the Platform for Engaging Everyone Responsibly (PEER). PEER received $500,000 from the Robert Wood Johnson Foundation in 2014. PEER undergirds the Community Engaged Network for All (CENA), a PCORnet member funded in 2013 for Phase I and in 2015 for Phase II. She is Co-PI of the PCORnet Coordinating Center and Chair of the PCORnet Engagment Committee.

Joyce Tung, Ph.D.
Joyce joined 23andMe in 2007 and manages the 23andMe research team, which is responsible for consumer health and ancestry research and development, academic and industry collaborations, computational analyses for therapeutics, and new research methods and tools development. While a postdoctoral fellow at Stanford University, Joyce studied the genetics of mouse and human pigmentation. She graduated from Stanford with honors and distinction with a B.S. in Biological Sciences and a minor in computer science, and earned her Ph.D. in Genetics from the University of California, San Francisco where she was a National Science Foundation graduate research fellow.

BACKGROUND MATERIALS

Links to Background Materials

Materials from 23andMe

- The information page for the external recruitment portion of our COVID-19 study
- Blog post on initial launch of our study
- Blog post on expansion of our study to enroll severe cases
- Blog post on preliminary findings

Genomewide Association Study of Severe Covid-19 with Respiratory Failure

BACKGROUND

There is considerable variation in disease behavior among patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes coronavirus disease 2019 (Covid-19). Genomewide association analysis may allow for the identification of potential genetic factors involved in the development of Covid-19.

A Host Transcriptional Signature for Presymptomatic Detection of Infection in Humans Exposed to Influenza H1N1 or H3N2

BACKGROUND

There is great potential for host-based gene expression analysis to impact the early diagnosis of infectious diseases. In particular, the influenza pandemic of 2009 highlighted the challenges and limitations of traditional pathogen-based testing for suspected upper respiratory viral infection.

A crowdsourced analysis to identify ab initio molecular signatures predictive of susceptibility to viral infection

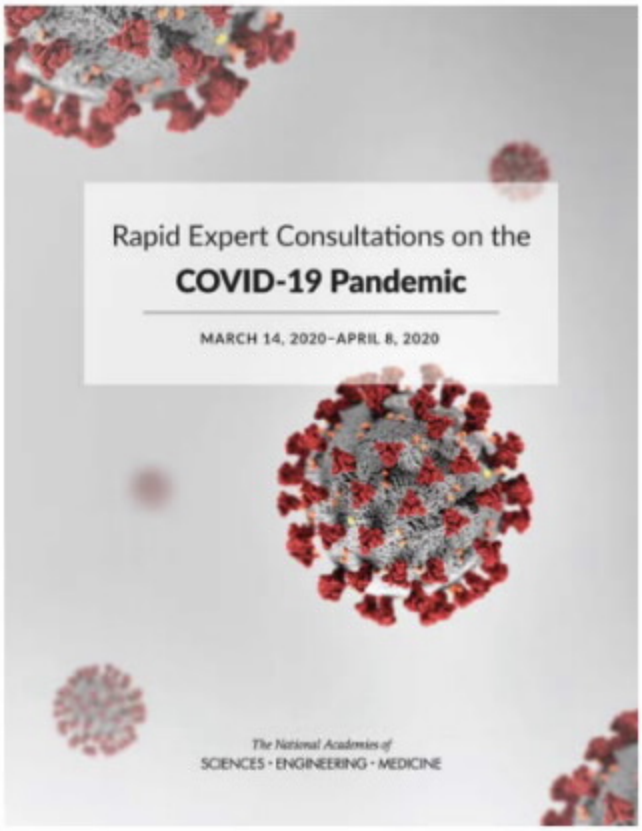
BACKGROUND

The response to respiratory viruses varies substantially between individuals, and there are currently no known molecular predictors from the early stages of infection. Here we conduct a community-based analysis to determine whether pre- or early post-exposure molecular factors could predict physiologic responses to viral exposure.

A Host-Based RT-PCR Gene Expression Signature to Identify Acute Respiratory Viral Infection

Improved ways to diagnose acute respiratory viral infections could decrease inappropriate antibacterial use and serve as a vital triage mechanism in the event of a potential viral pandemic. Measurement of the host response to infection is an alternative to pathogen-based

diagnostic testing and may improve diagnostic accuracy. We have developed a host-based assay with a reverse transcription polymerase chain reaction (RT-PCR) TaqMan low-density array (TLDA) platform for classifying respiratory viral infection.



Rapid Expert Consultations on the COVID-19 Pandemic

In response to a request from the Office of Science and Technology Policy and the Office of the Assistant Secretary for Preparedness and Response, the National Academies of Sciences, Engineering, and Medicine convened a standing committee of experts to help inform the federal government on critical science and policy issues related to emerging infectious diseases and other 21st century health threats. This set of Rapid Expert Consultations are the first of their kind and represent the best evidence available to the Committee at the time each publication was released.

This paper is in press at The Lancet Infectious Diseases

A blood-based host gene expression assay for early detection of viral infection

Micah T. McClain MD, PhD[1-3], Florica J. Constantine, MS[1], Bradly P. Nicholson, PhD[4], Marshall Nichols, MS[1], Thomas W. Burke, PhD[1], Ricardo Henao, PhD[1], Daphne C. Jones, MD[2], Lori L. Hudson, PhD[1], L. Brett Caram, MD[3], Timothy Veldman, PhD[1], Anna Mazur, MS[1], Lawrence P. Park, PhD[2,3], Sunil Suchindran, PhD[1], Ephraim L. Tsalik, MD, PhD[1-3], Geoffrey S. Ginsburg, MD[1], and Christopher W. Woods, MD, MPH[1-3]

Affiliations:

[1]Center for Applied Genomics and Precision Medicine, Duke University, Durham, NC, [2]Durham VA Medical Center, Durham, NC, [3]Division of Infectious Diseases, Duke University Medical Center, Durham, NC, [4]Institute for Medical Research, Durham, NC

Corresponding Author:

Micah T. McClain, MD, PhD

Division of Infectious Diseases

Center for Applied Genomics and Precision Medicine

Duke University Medical Center

DUMC Box 102359

Durham, NC 27710

e-mail: micah.mcclain@duke.edu

Office: 919-613-5636

Research in context

Evidence before this study

An increasing body of research has demonstrated that host gene expression patterns in peripheral blood offer a high degree of accuracy for diagnosis of acute infections at the time of clinical presentation.

Added value of this study

For the first time we demonstrate in a real-world setting that a blood-based gene expression test can accurately predict impending viral infection even before typical symptoms are present.

Implications of all the available evidence

These data show that biomarkers of viral infection are present and detectable even before clinical disease develops, opening the door to novel approaches to early treatment and outbreak management.

Abstract

Background

Early and accurate identification of persons with viral infections can be of critical importance for clinical management and public health interventions. Measuring host responses through gene signature-based biomarkers offer promise for improved diagnosis of respiratory viral infections, but little is known how early in disease such measures may be effective.

Methods

We performed a modified index-cluster study in 1465 university dormitory residents over five years. Each year, we monitored for symptomatic Index Cases (IC) and identified clusters of their Close Contacts (CC) who would be at presumed increased risk of developing symptomatic respiratory tract infection (RTI) while under observation. We then monitored each CC for 5 days for development of RTI and measured transcriptomic responses at each timepoint via a blood-based 36-gene RT-PCR assay.

Findings

Of 264 ICs with RTI symptoms, 150 (56%) had a confirmed viral etiology detected. Of their 555 CCs, 106 (19%) developed symptomatic RTI with a proven viral etiology during the observation window. The transcriptomic assay accurately predicts infection at the time of peak symptoms despite heterogeneity of hosts and nine different viral etiologies (auROC 0·96). Furthermore, these genomic biomarkers are capable of identifying early disease one, two and three days prior to peak illness when minimal to no symptoms are present (auROC 0·87, 0·86, and 0·77, respectively), and when nearly two-thirds of subjects (62%) are not yet producing detectable viral shedding.

Interpretation

Transcriptional biomarkers accurately predict and diagnose infection across diverse viral etiologies and stages of disease, and may prove useful for guiding administration of early effective therapy, quarantine decisions, and other clinical and public health interventions in the setting of endemic and pandemic infectious disease.

Funding: US Defense Advanced Research Projects Agency

Introduction

Acute viral infections are among the most common causes of acute physician visits in the United States[1]. In a given year, influenza alone affects 5-20% of the population in the US, causes over 400,000 hospitalizations, and as many as 56,000 deaths.[2,3] Outbreaks of viral infection continue to plague modern society, including heavy resource-requiring outbreaks of measles in the developed world, global pandemics like the novel H1N1 outbreak of 2009, or the deadly epidemics of SARS and MERS-CoV.[4-6] However, molecular tools that can contribute to outbreak investigation or directing early clinical management are often lacking. Traditional pathogen-focused diagnostic modalities for viral infection (culture, serology, antigen detection, PCR) offer considerable utility but can also be slow, costly, limited in terms of breadth of pathogen detection, require an *a priori* knowledge of the pathogen to be tested, may not detect emerging strains (as with the 2009 influenza pandemic[7]), or are incapable of distinguishing between active infection and colonization, all of which can minimize the impact of their use. Therefore, there is increasing interest in host-derived biomarkers to aid in the determination of the presence and/or type of infection in at-risk hosts - from single-analyte biomarkers like procalcitonin to composite, multiplex biomarker panels such as those included in a growing number of gene expression-based studies.[8-21] However, the published works on diagnostics for naturally-acquired infection focus on identifying symptomatic subjects at the time of clinical presentation for medical care[19], which is often late in the time course of many viral diseases. Identification of infectious etiologies in earlier, even pre-symptomatic phases of illness, offers the ability to optimize and deliver timely and thus more effective therapy, refine prophylaxis decisions, and guide public health interventions such as isolation and quarantine.[13,22] However, identifying subjects who have been exposed to infectious virus but are not yet exhibiting symptoms of clinical illness is logistically challenging, resulting in a dearth of literature to address these problems.

We have previously shown that during experimental influenza infection of healthy volunteers the host genomic response is robust and detectable well before typical symptoms become apparent.[16,22,23] Other

investigators have confirmed that transcriptomic markers can be indicative of early viral infection in experimental human challenge models as well as animal models of disease.[24-27] However, the mechanism of challenge infection is contrived rather than natural, typically consists of highly egg-adapted viral strains, and the severity of clinical illness does not fully replicate that seen in naturally-acquired infection, all of which can limit the broad applicability of findings from this approach.[16,22,28-31] To date there has been no significant description of the host response to infection during the pre-symptomatic phase of naturally-occurring disease. Here we have utilized a unique index-cluster study design with a focus on serial sampling of 'real world', healthy close contacts of infected individuals to enrich for cases of naturally-occurring infection during these early, post-exposure, but pre-symptomatic time points. This unique design has permitted real-world validation of a transcriptomic signature in peripheral blood that is capable of accurately identifying exposed but apparently healthy individuals who will go on to develop symptomatic viral infection.

Methods

Surveillance Cohort

Over five academic years, we enrolled 1465 Duke University undergraduate students (age 18-24, most of whom live in dormitories on campus) in a modified index-cluster study design (Figure 1). At the time of enrollment, we collected baseline biospecimens and standardized questionnaires to ascertain demographic, behavioral, and other medical characteristics. After enrollment, participating students completed a daily, web-based survey to describe the presence and severity of eight symptoms of RTI (scored 0-4) on a daily basis (see eMethods for details). We monitored the surveys daily for a 6-point increase in a subject's cumulative daily symptom score which would trigger a sample and data collection event for a potential Index Case. Suspected ICs were then assessed for confirmation of illness and biospecimen collection. Using these sentinel cases of RTI suggestive of viral etiology we then identified and monitored a prospective observational cohort of asymptomatic but potentially exposed Close Contacts (people living in the same dormitory or identified by the IC as a Close Contact), who were then monitored and sampled for up to 5 days (the Observation Window).

Sample and data collection

Confirmed IC's provided blood and nasal swab samples at the time of illness identification. Viral etiology was assessed using a commercial multiplex PCR assay (QIAGEN ResPlex II v2·0, Luminex RVP, or Biofire FilmArray RP). Healthy CCs living in proximity to an IC provided blood and respiratory samples for up to five consecutive days and were monitored for symptomatic conversion and viral shedding.

Real-time PCR

Peripheral blood was collected in PAXgene™ Blood RNA tubes (PreAnalytiX), and total RNA extracted using the PAXgene™ Blood miRNA Kit (QIAGEN). cDNA synthesis was performed using SuperScript VILO™ Master Mix (Invitrogen) according to manufacturer's instructions. Real-time PCR was performed using custom TLDA 384-well microfluidic cards with TaqMan Gene Expression Master Mix, and run on a ViiA7 Real-Time PCR System (Applied Biosystems).

Statistical Analysis

For RT-PCR data the Cq values were transformed into normalized relative quantities using reference genes - those with the most stable combined coefficient of variation across all samples - FPGS and TRAP1 were used for the first batch, and DECR1 and TRAP1 were used for the second batch, and data was normalized as previously described.[8,9] We utilized a regularized (2-norm) logistic regression model to discriminate cases from controls (see eMethods for complete details). The data was split into training and testing sets 25 times using repeated random subsampling and each of the 25 models was trained using all timepoints. We evaluated the model's performance separately at each individual timepoint. All model analyses were implemented in Python and Tensorflow.[32]

Results

Brief Description of Study

Healthy first year students at Duke University (n=1465) were enrolled into a surveillance cohort at the beginning of each academic year. After enrollment, we electronically monitored self-reported daily symptoms throughout the duration of the academic year via an online portal. When one or more subjects indicated a change in respiratory symptoms, the sample collection protocol was initiated for individuals (Index Cases, ICs) that met the clinical case definition for acute, community-acquired RTI (see Methods), as well as for a subset of other currently healthy study participants who were in close proximity to the Index Case (Close Contacts, CCs). CCs were monitored for symptoms and serially sampled for serum, whole blood PAXgene RNA, and respiratory samples for 5 days from identification of their Index Case, regardless of symptomatology (Figure 1). This design permitted enrichment for, and collection of, samples from CCs during the timeframe between exposure (which occurred at an unknown time) and subsequent development of symptomatic disease (which occurred under observation).

Prevalence, Transmission, and Clinical Outcomes of Viral Respiratory Tract Infection

We identified 264 clinical ICs, of whom 150 had a confirmed respiratory viral etiology by multiplex PCR testing of NP samples, and 555 CCs, 19% (106) of whom developed symptomatic RTI with concomitant confirmatory viral PCR. Only 57% of the CCs were infected with the same virus as their associated IC. Nine different viral pathogens were detected, and there was marked variation in viral etiologies detected from year to year (Table 1). Infection resulted in many common symptoms of upper respiratory tract infection with clinical variation amongst viral etiologies (Figure 2). For example, symptoms were almost twice as severe in individuals with influenza infection compared to all other viruses (mean symptom score of 20 for influenza vs 13 for all others ($p=0.005$)), although all subjects in the study remained well enough to be managed in the outpatient setting.

Since the exact time of exposure/transmission during naturally-acquired infection is unknown, for analysis CC days were aligned to the time of maximal illness (day of highest symptom score) for each subject (Time "T") in order to attempt to place subjects on broadly similar time scales in terms of the host response to infection. The average total symptom score for all CCs at Time T was 14·8, although this varied by viral etiology (Figures 1,2). For each individual CC, the day before their day of maximal symptoms was termed T-minus 1 ("T-1"), 48 hours prior to symptom max was "T-2" and 72 hours prior to symptom max was time "T-3". At times prior to maximal illness the average total symptom score for CCs was 7·0 (T-1), 4·1 (T-2), and 1·7 (T-3). In our studies a daily symptom score of 6 represents mild clinical illness and was the threshold for defining a 'symptomatic' day. Thus, at days T-2 and earlier, most subjects exhibited subclinical or absent illness (65% asymptomatic at Time T-3, 32% at T-2).

An RT-PCR signature identifies symptomatic subjects with naturally-acquired viral RTI

We previously developed a host response-derived transcriptomic signature of symptomatic viral infection using microarray data[30]. However, microarray and bulk RNA sequencing platforms are impractical for rapid clinical testing. We therefore migrated our prior 'pan-viral' signature to an RT-PCR platform with potential for more direct translation to point-of-care testing [8,9]. Using sparsity-imposing techniques (see eMethods), we selected 36 pre-designed TaqMan probes representing genes comprising the acute respiratory viral signature (and normalization controls) to be used on a TaqMan Low Density Array (TLDA) platform[8]. We then utilized a regularized (2-norm) logistic regression model to determine coefficients for each gene in the signature[33], such that the output of the model is a probability that a given subject will go on to have a symptomatic, viral PCR-positive event during the observation window. The performance of the RT-PCR signature for classification of symptomatic individuals was evaluated using a repeated random subsampling validation strategy on relevant participants across all study years. Overall, the RT-PCR-based host gene expression assay distinguished symptomatic, virus-infected subjects from asymptomatic, viral PCR-negative controls with 92% accuracy (Figure 3). However, accuracy varied depending on viral etiology, ranging from 99% accuracy in influenza infection (where symptoms are

typically more severe) to 92% with Coronavirus and 81% accuracy with metapneumovirus infections (eFigure 1). Interestingly, analysis of individual gene components of the model demonstrates that many individual genes performed similar to the aggregate model across all iterations (eFigure 2-3). In fact, many individual gene targets alone had mean area under Receiver Operating Characteristic (auROC) of >0·90 at the time of maximal illness with the top performing genes at time T being IFIT3 (0·97), HERC5 (0·97), and RSAD2 (0·96)

The gene expression signature identifies naturally-acquired viral RTI during the pre-symptomatic phase.

In order to evaluate performance of the gene signature early in the course of natural infection, we selected CCs with positive microbiology and at least three days of sampling prior to the development of maximal symptoms. As noted previously, the RT-PCR gene signature correctly identified symptomatic subjects with 92% accuracy on the day of maximal symptoms. This decreased to 88% on day T-1 (24 hours before maximal symptoms), and 83% accuracy on day T-2 (48 hours before maximal symptoms) (Figure 3), although performance varies from virus to virus. Only one case of influenza infection had available pre-symptomatic samples at all times, and the signature correctly classified that individual at each timepoint (T-1, T-2, T-3, and T-4). For a fixed FPR of 0·2 (specificity 0·8), the model achieves greater sensitivity than either symptoms alone or viral PCR at all timepoints other than time T and for ICs (where symptom-based diagnosis by definition has a 100% sensitivity, eFigures 4-6). Critically, 62% of subjects who would develop proven viral RTI lacked detectable viral shedding at time T-3, and 44% were still negative for virus by routine clinical testing at day T-2, times where the gene signature outperforms the clinical standards of care - symptom evaluation and viral PCR.

Discussion

The unique design of this community study has allowed us to identify individuals (CCs) at increased risk for developing viral RTI while under observation. This approach has enabled us to demonstrate for the first time that host responses to naturally-acquired respiratory viral infection, as measured by gene expression in peripheral blood, are present well before the time of maximal illness and can be used to predict those at highest risk for developing active infection.

This study demonstrates that conserved genomic responses can be used to correctly classify individuals with a broad array of naturally-acquired respiratory viral infections well before the time when they would present for clinical evaluation, and even before detectable viral shedding begins for the majority of subjects (62%). The promise of this approach is manifested by the discriminative ability of the genomic signature from two and three days prior to maximal illness, when many individuals (65% at Time T-3, 32% at T-2) have no symptoms at all. Even the mild symptoms some subjects do have at early times are clinically vague and would be typical of seasonal allergies, mild COPD flares, or even symptoms due to sequelae of chronic smoking.[34] The potential utility of genomic analysis to classify viral infection among asymptomatic individuals or those with common, nonspecific upper respiratory symptoms is profound. That this holds true across 9 different respiratory viruses, each with its own incubation times and variable clinical progression and duration, also speaks to the potential power of this approach. Additionally, it is important to note that the signature utilized represents conserved underlying antiviral response pathways that are active, and thus discriminatory, across a wide range of illness and not just at early times. This is vital as any real-world use of such a biomarker assay would often be agnostic to where in the course of infection an individual exists at the moment of testing.

While this transcriptomic signature accurately classifies a wide spectrum of viral RTIs, it performs best in a subset of viruses including influenza and coronavirus, where it performs with a high degree of accuracy even during early and pre-symptomatic phases of disease. Influenza is one of the most important viral etiologies to identify early given its importance in pandemics where existing diagnostics may perform

poorly (especially for emerging strains as in 2009[35]), where questions of triage and quarantine are paramount, and where effective therapeutic options are readily available and most effective when given early in disease. Similarly, a number of novel highly virulent coronaviruses, including SARS-CoV and MERS-CoV, have recently arisen and driven global outbreaks, highlighting another area of need for such diagnostics.[5,36].It seems likely that our ability to accurately detect exposure to these types of emerging infections will only become more critical over time. Availability of tools that can aid in assessment of those with potential exposure, especially those that offer diagnostic utility prior to the time of detectable viral shedding, would be a valuable aid to existing epidemiologic and molecular approaches in outbreak settings.

A number of other studies have described interferon response-based transcriptomic signatures of acute respiratory infections which vary by target population, pathogens, modelling approaches, and numbers of genes required to discriminate disease states. [9,10,13-18,30] Some of this work has also been validated, combined, or expanded upon by secondary *ex vivo* analysis of publicly available data. [11,12,37] While the focused signature described here clearly represents a limited subset of all discriminating genes, some observations regarding simplified, IFN-driven signatures can be made. Our data confirm that that some genes from these prior works (such as IFI44L and IFI27[10,38]) both perform well as classifiers distinguishing between viral illness and the healthy state (median auROC = 0·92 and 0·87), being strongly upregulated in cases of viral infection. The data further suggest that a number of other single targets also perform this task well including RSAD2 (0·96), IFIT3 (0·97), and IFI44 (0·93). Based on our experience and the published literature, it seems that relatively simple classification tasks - where the biological responses are highly conserved, unique in character, and of great magnitude - may be reasonably addressed with pauci-analyte biomarker panels.[10,12,39-41] However, as complexity increases by adding more powerful interhost variability (such as immunosuppression), discriminating clinically similar groups with more varied underlying pathophysiology, or discriminating multiple clinical mimics at once,

more intricate biomarker panels will likely be required to maintain high fidelity in real-world settings.[9,14,42]

Our study has several limitations. This project was designed to validate the predictive power of a small set of genes which already had proven discriminatory capability during maximal illness, and thus was not designed to discover whether additional, untested gene sets may offer improved discriminatory performance at early times. Future work with RNASeq on whole blood, single cell sequencing, or other methods will be needed to more completely define early genomic changes in the naturally-infected host. Additionally, our patient cohort was comprised of otherwise young, healthy individuals with community-acquired viral RTI of mild to moderate severity. Therefore, application to patient populations from other age groups, those with immunologic comorbidities, or with more severe viral infections will need to be evaluated. Finally, we did not identify any confirmed infections due to bacterial or other pathogens. As a result, while these transcriptomic markers show great promise for differentiating early infection and pre-symptomatic viral exposure from noninfectious states (which it may mimic at early times), this study did not directly validate the ability of these markers to differentiate viral exposure from early forms of other types of infection[9,10,14,43]. As we move towards bringing transcriptomic biomarkers to the bedside, consideration of broad applicability and performance across a wide array of clinical settings will continue to be paramount.

We have for the first time defined the pre-symptomatic and temporal dynamics of transcriptomic biomarkers characterizing the host response to naturally-acquired viral infections in humans. Clinic-ready platforms capable of operationalizing PCR-based signatures of the size tested herein already exist, some of which are CLIA-approved with point-of-care functionality, offering a proximal pathway to clinical application of these findings. Thus, analysis of the evolution of gene expression-based biomarkers over time shows promise for driving development of diagnostics for early detection of viral exposure and infection which may prove useful for guiding administration of early effective therapy, quarantine

decisions, and other clinical and public health interventions in the setting of endemic and pandemic infectious disease.

References

1. Hong CY, Lin RT, Tan ES, et al. Acute respiratory symptoms in adults in general practice. *Fam Pract* 2004; **21**(3): 317-23.
2. Shrestha SS, Swerdlow DL, Borse RH, et al. Estimating the burden of 2009 pandemic influenza A (H1N1) in the United States (April 2009-April 2010). *Clin Infect Dis* 2011; **52 Suppl 1**: S75-82.
3. Rebecca Garten PLB, MPH1; Anwar Isa Abd Elal1; Noreen Alabi, MPH1; John Barnes, PhD1; Matthew Biggerstaff, ScD1; Lynnette Brammer, MPH1; Alicia P. Budd, MPH1; Erin Burns, MA1; Charisse N. Cummings, MPH1; Todd Davis, PhD1; Shikha Garg, MD1; Larisa Gubareva, PhD1; Yunho Jang, PhD1; Krista Kniss, MPH1; Natalie Kramer1; Stephen Lindstrom, PhD1; Desiree Mustaquim, MPH1; Alissa O'Halloran, MSPH1; Wendy Sessions, MPH1; Calli Taylor, MPH1; Xiyan Xu, MD1; Vivien G. Dugan, PhD1; Alicia M. Fry, MD1; David E. Wentworth, PhD1; Jacqueline Katz, PhD1; Daniel Jernigan, MD. Update: Influenza Activity in the United States During the 2017–18 Season and Composition of the 2018–19 Influenza Vaccine. *MMWR Morb Mortal Wkly Rep* 2018; (67): 634-42.
4. de Wit E, van Doremalen N, Falzarano D, Munster VJ. SARS and MERS: recent insights into emerging coronaviruses. *Nature reviews Microbiology* 2016; **14**(8): 523-34.
5. Arabi YM, Balkhy HH, Hayden FG, et al. Middle East Respiratory Syndrome. *New England Journal of Medicine* 2017; **376**(6): 584-94.
6. Angelo KM, Gastanaduy PA, Walker AT, et al. Spread of Measles in Europe and Implications for US Travelers. *Pediatrics* 2019; **144**(1).
7. Emergence of a Novel Swine-Origin Influenza A (H1N1) Virus in Humans. *New England Journal of Medicine* 2009; **360**(25): 2605-15.
8. Zaas AK, Burke T, Chen M, et al. A host-based RT-PCR gene expression signature to identify acute respiratory viral infection. *Sci Transl Med* 2013; **5**(203): 203ra126.
9. Tsalik EL, Henao R, Nichols M, et al. Host gene expression classifiers diagnose acute respiratory illness etiology. *Sci Transl Med* 2016; **8**(322): 322ra11.
10. Herberg JA, Kaforou M, Wright VJ, et al. Diagnostic Test Accuracy of a 2-Transcript Host RNA Signature for Discriminating Bacterial vs Viral Infection in Febrile Children. *JAMA* 2016; **316**(8): 835-45.
11. Andres-Terre M, McGuire HM, Pouliot Y, et al. Integrated, Multi-cohort Analysis Identifies Conserved Transcriptional Signatures across Multiple Respiratory Viruses. *Immunity* 2015; **43**(6): 1199-211.
12. Sweeney TE, Braviak L, Tato CM, Khatri P. Genome-wide expression for diagnosis of pulmonary tuberculosis: a multicohort analysis. *Lancet Respir Med* 2016; **4**(3): 213-24.

13. Liu X, Speranza E, Munoz-Fontela C, et al. Transcriptomic signatures differentiate survival from fatal outcomes in humans infected with Ebola virus. *Genome Biol* 2017; **18**(1): 4.
14. Suarez NM, Bunsow E, Falsey AR, Walsh EE, Mejias A, Ramilo O. Superiority of transcriptional profiling over procalcitonin for distinguishing bacterial from viral lower respiratory tract infections in hospitalized adults. *J Infect Dis* 2015; **212**(2): 213-22.
15. Kaforou M, Wright VJ, Oni T, et al. Detection of tuberculosis in HIV-infected and -uninfected African adults using whole blood RNA expression signatures: a case-control study. *PLoS Med* 2013; **10**(10): e1001538.
16. Woods CW, McClain MT, Chen M, et al. A Host Transcriptional Signature for Presymptomatic Detection of Infection in Humans Exposed to Influenza H1N1 or H3N2. *PLoS One* 2013; **8**(1): e52198.
17. Mejias A, Dimo B, Suarez NM, et al. Whole blood gene expression profiles to assess pathogenesis and disease severity in infants with respiratory syncytial virus infection. *PLoS Med* 2013; **10**(11): e1001549.
18. Hu X, Yu J, Crosby SD, Storch GA. Gene expression profiles in febrile children with defined viral and bacterial infection. *Proc Natl Acad Sci U S A* 2013; **110**(31): 12792-7.
19. Zhai Y, Franco LM, Atmar RL, et al. Host Transcriptional Response to Influenza and Other Acute Respiratory Viral Infections--A Prospective Cohort Study. *PLoS pathogens* 2015; **11**(6): e1004869.
20. Roe J, Venturini C, Gupta RK, et al. Blood transcriptomic stratification of short-term risk in contacts of tuberculosis. *Clin Infect Dis* 2019.
21. Afroz S, Giddaluru J, Abbas MM, Khan N. Transcriptome meta-analysis reveals a dysregulation in extra cellular matrix and cell junction associated gene signatures during Dengue virus infection. *Scientific reports* 2016; **6**: 33752.
22. McClain MT, Nicholson BP, Park LP, et al. A Genomic Signature of Influenza Infection Shows Potential for Presymptomatic Detection, Guiding Early Therapy, and Monitoring Clinical Responses. *Open Forum Infect Dis* 2016; **3**(1): ofw007.
23. Huang Y, Zaas AK, Rao A, et al. Temporal Dynamics of Host Molecular Responses Differentiate Symptomatic and Asymptomatic Influenza A Infection. *PLoS Genet* 2011; **7**(8): e1002234.
24. Shurtleff AC, Whitehouse CA, Ward MD, Cazares LH, Bavari S. Pre-symptomatic diagnosis and treatment of filovirus diseases. *Frontiers in microbiology* 2015; **6**(108).
25. Caballero IS, Yen JY, Hensley LE, Honko AN, Goff AJ, Connor JH. Lassa and Marburg viruses elicit distinct host transcriptional responses early after infection. *BMC Genomics* 2014; **15**(1): 960.
26. Duy J, Koehler JW, Honko AN, et al. Circulating microRNA profiles of Ebola virus infection. *Scientific reports* 2016; **6**: 24496.
27. Wang K, Langevin S, O'Hern CS, et al. Anomaly Detection in Host Signaling Pathways for the Early Prognosis of Acute Infection. *PLoS One* 2016; **11**(8): e0160919.
28. Memoli MJ, Czajkowski L, Reed S, et al. Validation of the wild-type influenza A human challenge model H1N1pdMIST: an A(H1N1)pdm09 dose-finding investigational new drug study. *Clin Infect Dis* 2015; **60**(5): 693-702.
29. Han A, Poon JL, Powers JH, 3rd, Leidy NK, Yu R, Memoli MJ. Using the Influenza Patient-reported Outcome (FLU-PRO) diary to evaluate symptoms of influenza viral infection in a healthy human challenge model. *BMC infectious diseases* 2018; **18**(1): 353.

30. Zaas AK, Chen M, Varkey J, et al. Gene expression signatures diagnose influenza and other symptomatic respiratory viral infections in humans. *Cell Host Microbe* 2009; **6**(3): 207-17.
31. Sobel Leonard A, McClain MT, Smith GJ, et al. Deep Sequencing of Influenza A Virus from a Human Challenge Study Reveals a Selective Bottleneck and Only Limited Intrahost Genetic Diversification. *Journal of virology* 2016; **90**(24): 11247-58.
32. Friedman J, Hastie T, Tibshirani R. Regularization Paths for Generalized Linear Models via Coordinate Descent. *Journal of statistical software* 2010; **33**(1): 1-22.
33. Goeman JJ. L1 penalized estimation in the Cox proportional hazards model. *Biometrical journal Biometrische Zeitschrift* 2010; **52**(1): 70-84.
34. Stanton N, Hood K, Kelly MJ, et al. Are smokers with acute cough in primary care prescribed antibiotics more often, and to what benefit? An observational study in 13 European countries. *European Respiratory Journal* 2010; **35**(4): 761-7.
35. LeBlanc JJ, Li Y, Bastien N, Forward KR, Davidson RJ, Hatchette TF. Switching gears for an influenza pandemic: validation of a duplex reverse transcriptase PCR assay for simultaneous detection and confirmatory identification of pandemic (H1N1) 2009 influenza virus. *Journal of clinical microbiology* 2009; **47**(12): 3805-13.
36. Christian MD, Poutanen SM, Low DE, Muller MP, Loutfy MR. Severe Acute Respiratory Syndrome. *Clinical Infectious Diseases* 2004; **38**(10): 1420-7.
37. Bongen E, Vallania F, Utz PJ, Khatri P. KLRD1-expressing natural killer cells predict influenza susceptibility. *Genome medicine* 2018; **10**(1): 45.
38. Tang BM, McLean AS, Dawes IW, Huang SJ, Lin RC. Gene-expression profiling of peripheral blood mononuclear cells in sepsis. *Crit Care Med* 2009; **37**(3): 882-8.
39. Warsinske HC, Rao AM, Moreira FMF, et al. Assessment of Validity of a Blood-Based 3-Gene Signature Score for Progression and Diagnosis of Tuberculosis, Disease Severity, and Treatment Response. *JAMA network open* 2018; **1**(6): e183779.
40. Tang BM, Shojaei M, Parnell GP, et al. A novel immune biomarker IFI27 discriminates between influenza and bacteria in patients with suspected respiratory infection. *The European respiratory journal* 2017; **49**(6).
41. Herberg JA, Kaforou M, Wright VJ, et al. Diagnostic Test Accuracy of a 2-Transcript Host RNA Signature for Discriminating Bacterial vs Viral Infection in Febrile Children. *Jama* 2016; **316**(8): 835-45.
42. Maslove DM, Wong HR. Gene expression profiling in sepsis: timing, tissue, and translational considerations. *Trends Mol Med* 2014; **20**(4): 204-13.
43. Ramilo O, Allman W, Chung W, et al. Gene expression patterns in blood leukocytes discriminate patients with acute infections. *Blood* 2007; **109**(5): 2066-77.

Acknowledgements

Special thanks to Debra Freeman, Sara Hoffman, Olga M. Better, Mert Aydin, Stephanie Dobos, Kyle R. Breitschwert, Ashlee M. Valente for their tireless work enrolling subjects, processing samples and data, and engaging with the undergraduate campus.

Funding

Funding for this study was provided by the US Defense Advanced Research Projects Agency (DARPA), project # N66001-07-C-2024 and W911NF1410052. The statements and conclusions herein are those of the authors and do not reflect the position or policy of the United States Government.

Author contributions

MTM, ELT, GSG and CWW drafted the manuscript, which was critically revised by all remaining authors. MTM, BPN, TB, TV, GSG, and CWW helped conceive and implement the study. All authors helped acquire, analyze, or interpret data. Statistical analyses were performed by FJC, RH, SS, MN, and LP.

Figures

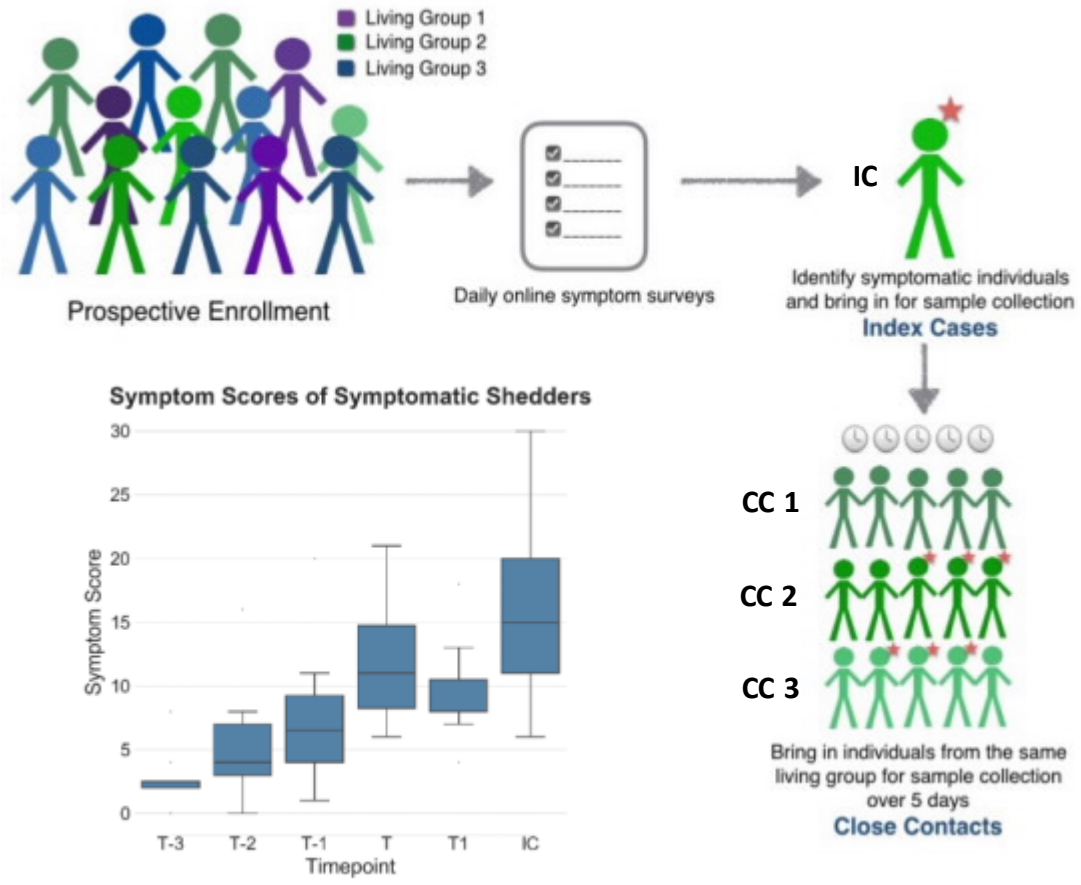


Figure 1: Study design. Students were enrolled at the beginning of the school year. Index Cases were identified when daily symptom surveys indicated potential respiratory illness. This flagged serial sampling of their Close Contacts, some of whom would also develop RTI during the 5-day observation window (red stars). This unique model permits blood sampling of some Close Contacts during early and presymptomatic phases of illness. Mean symptom scores of Close Contacts who developed PCR-proven RTI under observation are presented.

A

	Cough	Fever	Headache	Malaise	Nasal Congestion	Nasal Discharge	Sneezing	Sore Throat	Total Symptoms
Influenza*	3.2	1.9	2.4	3.0	2.5	2.4	1.8	2.7	20.0
RSV	2.1	0.7	1.6	2.9	2.6	2.6	1.5	2.6	16.6
Human Metapneumovirus	2.6	0.8	1.5	2.5	2.4	2.5	1.9	2.2	16.3
Rhinovirus	2.2	0.5	1.1	2.1	2.3	2.3	1.5	2.1	14.0
Coxsackie/Echovirus	2.1	0.5	1.1	2.2	2.4	2.4	1.6	1.8	14.0
Coronavirus	1.8	0.7	1.0	2.1	2.3	2.5	1.6	1.8	13.8
Parainfluenzavirus	2.3	0.5	1.2	2.3	1.8	1.7	1.3	2.3	13.4
Adenovirus	2.2	1.2	1.4	2.2	1.6	1.6	0.2	2.2	12.6
Bocavirus	1.0	0.0	0.0	1.0	2.0	2.0	1.0	1.0	8.0

B

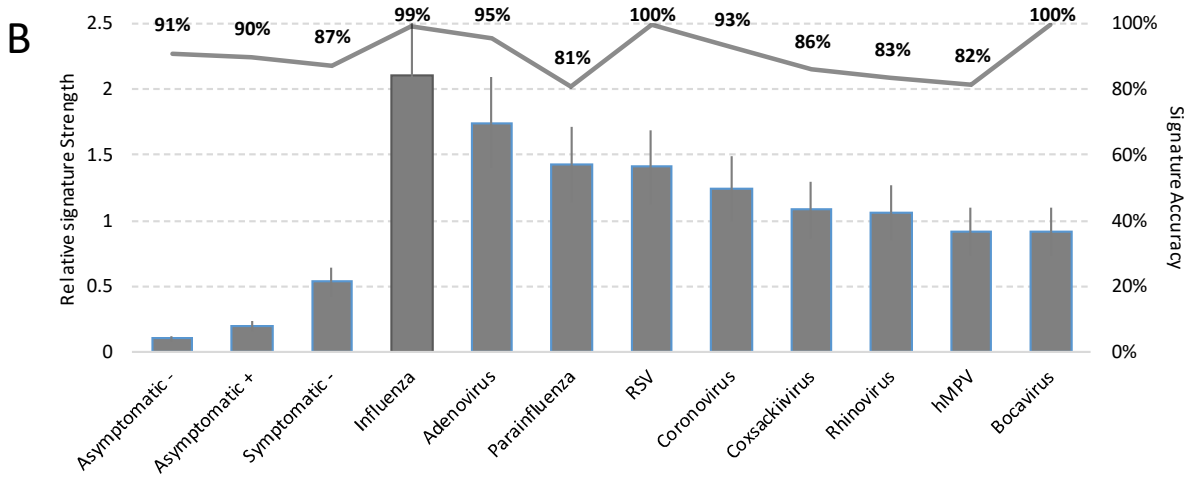


Figure 2. Clinical and genomic variation amongst viruses. For each type of viral infection, mean scores for each symptom measured at the time of maximal illness are presented (Panel A). The relative quantitative transcriptomic signature strength (bars) and accuracy (black line) at the time of maximal illness for each virus, as well as for Asymptomatic nonshedders (-), Asymptomatic shedders (+), and Symptomatic nonshedders (-) are presented in Panel B.

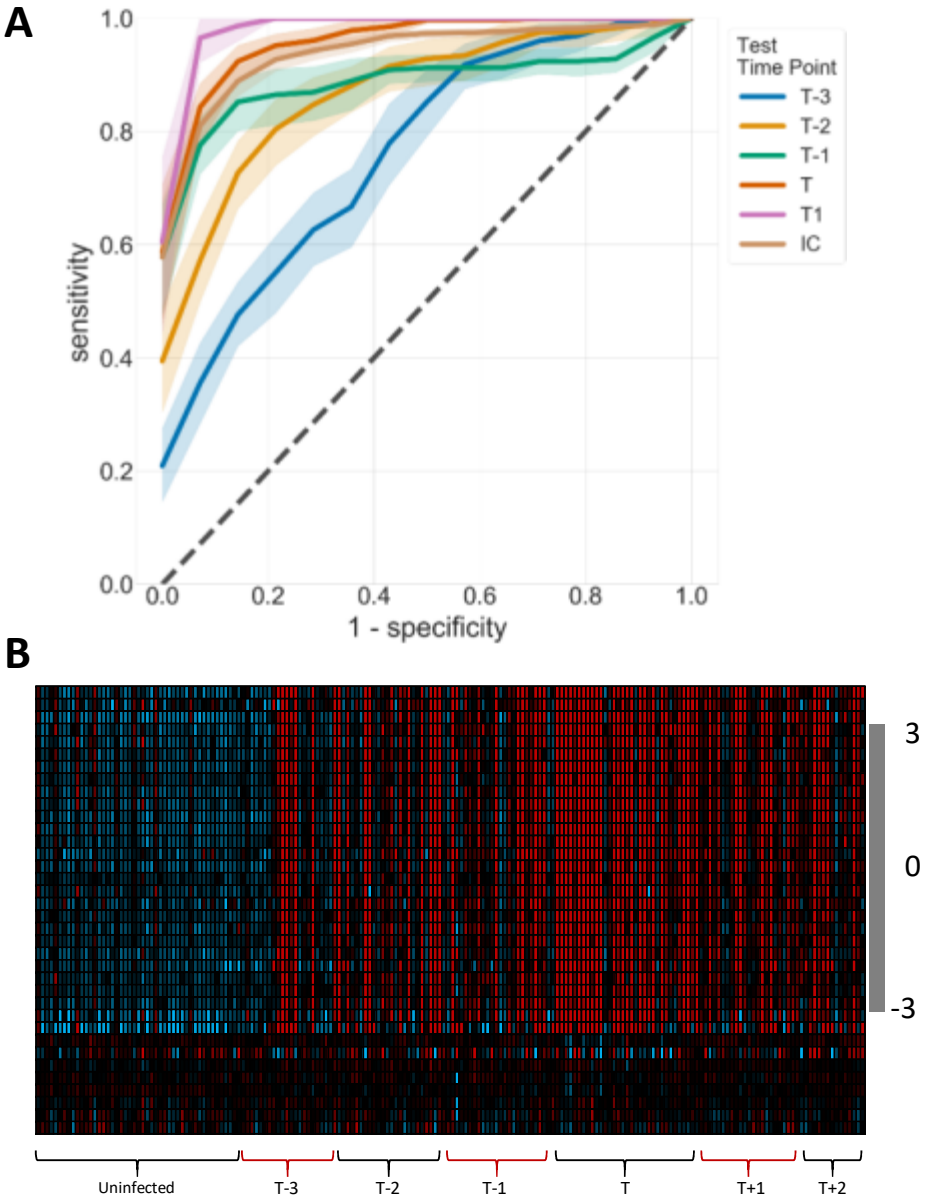


Figure 3: Signature performance across time. An RT-PCR-based 36-gene signature accurately discriminates subjects with naturally-acquired respiratory viral infection at both symptomatic and presymptomatic stages of infection (A). Heatmap of z-scores for each gene at each timepoint in subjects with proven symptomatic viral infection (Panel B), where time 'T' is the day of maximal illness.

Table 1. Viral etiologies of RTI in the Student Cohort

	2009-2010	2010-2011	2011-2012	2013-2014[a]	2014-2015	Total
Adenovirus	1	1	0	1	2	**5**
Bocavirus	0	0	0	0	2	**2**
Coronavirus	11	23	8	3	9	**54**
Coxsackie/Echovirus[b]	21	38	12	0	0	**71**
Human Metapneumovirus	2	1	3	4	1	**11**
Influenza	9	7	1	1	1	**19**
Parainfluenzavirus	3	10	1	0	7	**21**
RSV	3	1	3	0	3	**10**
Rhinovirus	20	23	29	3	37	**112**
Total	**70**	**104**	**57**	**12**	**62**	**305**

[a]truncated season, Spring enrollment only
[b]not present in 2013-2015 testing

ECONOMIST
Laboratory mice

An animal model of covid-19 is now available

Another is on its way

Science & technology Jun 11th 2020 edition

Jun 11th 2020

Mice are, as it were, the guinea pigs of science. And these days they are often genetically engineered guinea pigs, to boot. The emergence of covid-19, for example, has created demand for laboratory animals that have human versions of a protein called the ace2 receptor. This molecule is the hook that sars-cov-2, the virus which causes covid-19, uses to attach itself to a cell before entering and turning that cell into a virus factory. The murine version of ace2 is, however, the wrong shape for the virus to link up with. That means unmodified mice cannot catch the infection. Hence the need for genetic engineering.

The first version of such a mouse has recently become available courtesy of the Jackson Laboratory, a not-for-profit biomedical research institution in Maine that specialises in breeding laboratory mice. By luck, the team that produced it had a head start. The original sars virus (now known as sars-cov-1), which came close to causing a pandemic in 2003, also uses ace2 as its point of entry. As a consequence Stanley Perlman and Paul McCray of the University of Iowa, who were researching sars, created a mouse with human ace2 receptors in 2007.

That project was eventually put on ice. But not before Dr Perlman had given some semen from his modified mice to the Jackson Lab. On hearing about the virus in early January researchers there, led by Cat Lutz, quickly thawed out this stored semen and started work. Five months later they have created enough of Dr Perlman’s mice to start making them widely available.

The Perlman mice are, however, not quite perfect for the job. Though their genome has had human ace2 added, the mouse variety has not been

subtracted. This means they make both versions of the protein. Also, the human version of the gene is in the wrong place in the cell nucleus. The ace2 gene's proper home is the x chromosome, one of the sex chromosomes. In Perlman mice it is elsewhere. That could change its activity.

Help may be on its way, though, from Wang Youchun at the National Institutes for Food and Drug Control in Beijing. As they describe in this week's *Cell Host & Microbe*, Dr Wang and his colleagues have excised the murine version of the ace2 gene completely from their own mice and substituted the human version in exactly the same place. They did this using crispr/Cas9 gene-editing technology, a technique unavailable to Dr Perlman back in 2007.

Spot-on

Dr Wang's initial tests of the new mouse suggest it generates ace2 receptor molecules in all of the parts of the body where researchers think the virus makes its initial attacks in human beings. They show, too, that sars-cov-2 replicates well in the animals' lungs and tracheas, and also in their digestive systems—another part of the human body that seems susceptible to sars-cov-2 infection. In the case of the digestive systems, however, the dose of virus needed to establish a gut infection is ten times that needed to start a respiratory-tract infection. Dr Wang suspects that this might be important. If alimentary-canal infection requires an equivalently large initial dose of viruses in people, as well, then that might make it easier to stop it happening in human intestines, by the practice of good food hygiene.

One further result of Dr Wang's investigation was the discovery that the virus also replicates in the brains of his mice. This suggests it may be making use of human nervous tissue for replication in a way that is not currently being detected, but is suspected by some researchers because one symptom of some people who have the illness is loss of the sense of smell.

Curiously, despite their propensity to infection by sars-cov-2, Dr Wang's mice, unlike Dr Perlman's, do not show obvious symptoms when infected. Given the apparently high rate of symptomless infection in human beings, that, too, may be a pertinent observation

Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

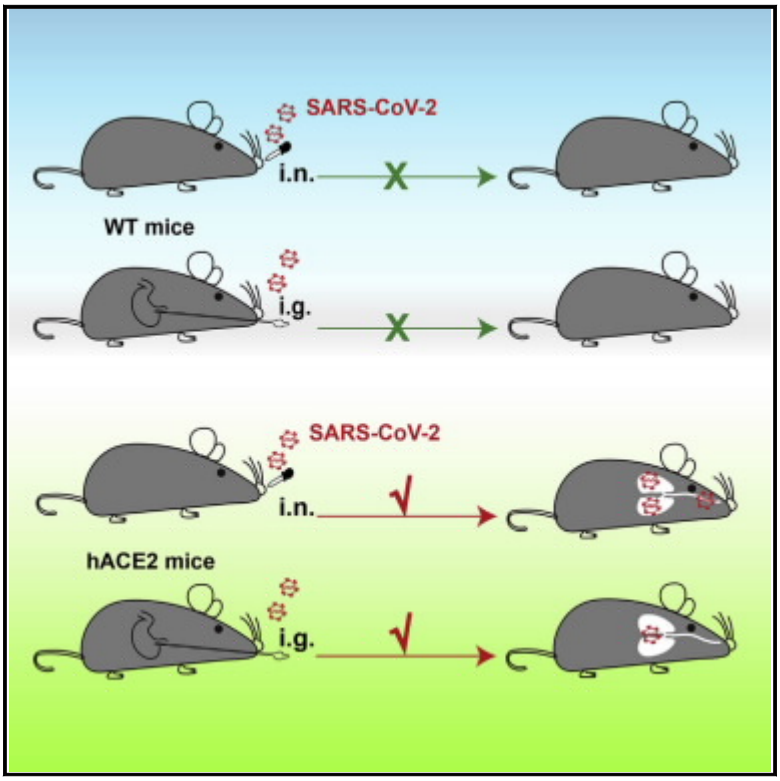
Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Resource

Cell Host & Microbe

A Mouse Model of SARS-CoV-2 Infection and Pathogenesis

Graphical Abstract



Authors

Shi-Hui Sun, Qi Chen, Hong-Jing Gu, ..., Yu-Sen Zhou, Cheng-Feng Qin, You-Chun Wang

Correspondence

fancf@nifdc.org.cn (C.-F.F.), yszhou@bmi.ac.cn (Y.-S.Z.), qincf@bmi.ac.cn (C.-F.Q.), wangyc@nifdc.org.cn (Y.-C.W.)

In Brief

The COVID-19 pandemic has brought an urgent need for small animal models. Here, Sun et al. established an ACE2 humanized mouse by CRISPR/Cas9 knockin technology. These hACE2 mice are susceptible to SARS-CoV-2 infection upon intranasal inoculation, and the resulting pulmonary infection and pathological changes resemble those observed in COVID-19 patients.

Highlights

- Human ACE2 knockin mice were generated by using CRISPR/Cas9 technology
- SARS-CoV-2 leads to robust replication in lung, trachea, and brain
- SARS-CoV-2 causes interstitial pneumonia and elevated cytokine in aged hACE2 mice
- High dose of SARS-CoV-2 can establish infection via intragastric route in hACE2 mice

Sun et al., 2020, Cell Host & Microbe 28, 1–10
July 8, 2020 [a] 2020 Elsevier Inc.
https://doi.org/10.1016/j.chom.2020.05.020

CellPress

Pleasecite this article in press as: Sun et al., A Mouse Model of SARS-CoV-2 Infection and Pathogenesis,Cell Host & Microbe (2020),https://doi.org/10.1016/j.chom.2020.05.020

Cell Host & Microbe

CellPress

Resource

A Mouse Model of SARS-CoV-2 Infection and Pathogenesis

Shi-Hui Sun,[1,7] Qi Chen,[1,7] Hong-Jing Gu,[1,7] Guan Yang,[2,7] Yan-Xiao Wang,[2,7] Xing-Yao Huang,[1,7] Su-Su Liu,[3] Na-Na Zhang,[1] Xiao-Feng Li,[1] Rui Xiong,[3] Yan Guo,[1] Yong-Qiang Deng,[1] Wei-Jin Huang,[4] Quan Liu,[3] Quan-Ming Liu,[3] Yue-Lei Shen,[5] Yong Zhou,[6] Xiao Yang,[2] Tong-Yan Zhao,[1] Chang-Fa Fan,[3,*] Yu-Sen Zhou,[1,*] Cheng-Feng Qin,[1,*] and You-Chun Wang[4,8,*]

[1]State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences (AMMS), Beijing 100071, China

[2]State Key Laboratory of Proteomics, Beijing Proteome Research Center, National Center for Protein Science（Beijing）, Beijing Institute of Lifeomics, Beijing 102206, China

[3]Division of Animal Model Research, Institute for Laboratory Animal Resources, National Institutes for Food and Drug Control, Beijing 102629, China.

[4]Division of HIV/AIDS and Sex-Transmitted Virus Vaccines, Institute for Biological Product Control, National Institutes for Food and Drug Control (NIFDC),Beijing 102629, China

[5]Beijing Biocytogen Co., Ltd., Beijing 101111, China

[6]Chongqing Weisiteng Biotech Transnational Research Institute, Chongqing 400039, China

[7]These authors contributed equally

[8]Lead Contact

*Correspondence: fancf@nifdc.org.cn (C.-F.F.), yszhou@bmi.ac.cn (Y.-S.Z.), qincf@bmi.ac.cn (C.-F.Q.), wangyc@nifdc.org.cn (Y.-C.W.)

https://doi.org/10.1016/j.chom.2020.05.020

SUMMARY

Since December 2019, a novel coronavirus SARS-CoV-2 has emerged and rapidly spread throughout the world, resulting in a global public health emergency. The lack of vaccine and antivirals has brought an urgent need for an animal model. Human angiotensin-converting enzyme II (ACE2)has been identified as a functional receptor for SARS-CoV-2. In this study, we generated a mouse model expressing human ACE2 (hACE2)by using CRISPR/Cas9 knockin technology. In comparison with wild-type C57BL/6 mice, both young and aged hACE2 mice sustained high viral loads in lung, trachea, and brain upon intranasal infection. Although fatalities were not observed, interstitial pneumonia and elevated cytokines were seen in SARS-CoV-2 infected-aged hACE2 mice. Interestingly, intragastric inoculation of SARS-CoV-2 was seen to cause productive infection and lead to pulmonary pathological changes in hACE2 mice. Overall, this animal model described here provides a useful tool for studying SARS-CoV-2 transmission and pathogenesis and evaluating COVID-19 vaccines and therapeutics.

INTRODUCTION

Coronaviruses (CoVs) are a group of enveloped, positive-sensed, single-stranded RNA viruses within the subfamily *Coronavirinae* in the family *Coronaviridae* and the order *Nidovirales*. All coronaviruses can be divided into four genera: *alphacoronaviruses*, *betacoronaviruses*, *gammacoronaviruses*, and *deltacoronaviruses*. During the past decades, two highly transmissible and pathogenic CoVs, Severe Acute Respiratory Syndrome coronavirus (SARS-CoV)and Middle East respiratory syndrome coronavirus (MERS-CoV), have emerged in humans and caused global concerns. SARS-CoV was first recorded in Guandong, China, in December 2002 and rapidly spread to five continents (Peiris et al., 2003). The SARS-CoV outbreak ultimately caused 8,437 infections and 813 deaths worldwide, according to the World Health Organization (WHO). MERS-CoV first emerged in the Arabian Peninsula in 2012 (Zaki et al., 2012) and has caused 1,728 confirmed cases including 624 deaths in Middle Eastern countries, the United Kingdom, and South Korea, according to WHO. Both SARS-CoV and MERS-CoV likely originated from wild animals and are prone to cause lethal pneumonia in humans (Cui et al., 2019). There is currently no vaccine or specific antiviral drugs to prevent or treat their infections and transmission in humans.

In December 2019, a cluster of pneumonia patients with links to Huanan seafood market, and with potential animal contact, was reported in Wuhan, Hubei Province, China (Li et al., 2020; Zhou et al., 2020b; Zhu et al., 2020). Subsequently, the disease rapidly spread throughout the world to become a pandemic. The etiologic agent of the disease was then identified as a novel coronavirus by several independent groups (Wu et al., 2020; Zhou, et al., 2020b) and has now been named as SARS-CoV-2 because of its close relationship to SARS-CoV. The disease was subsequently termed coronavirus disease 19 (COVID-19). The most common clinical manifestations of the patients are fever, cough, shortness of breath, and fatigue, and some patients

Pleasecite this article in press as: Sun et al., A Mouse Model of SARS-CoV-2Infection and Pathogenesis,Cell Host & Microbe (2020),https://doi.org/10.1016/j.chom.2020.05.020

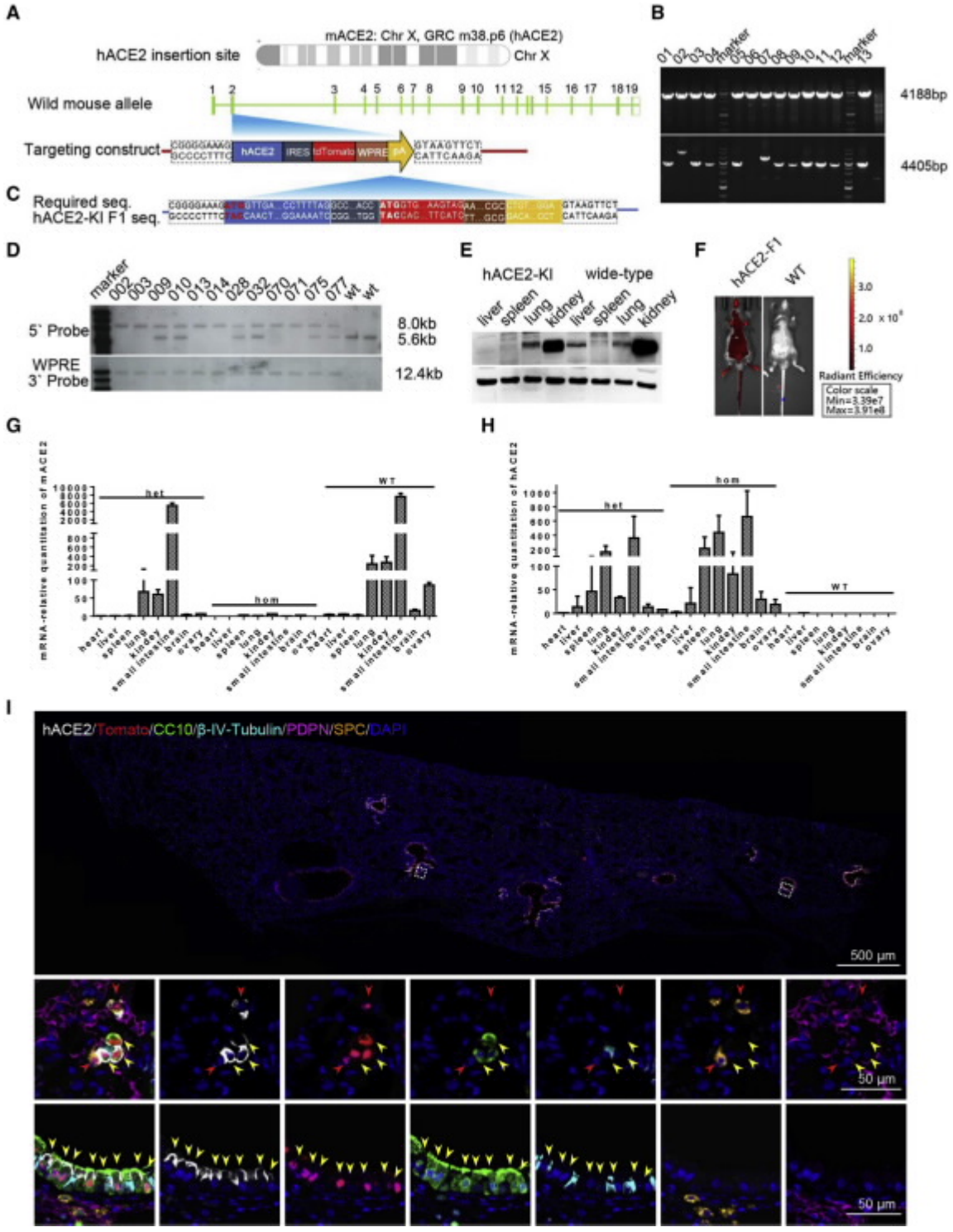


Figure 1. Construction and Characterization of hACE2 Humanized Mouse

(A) The hACE2 gene were inserted into exon2, the first coding exon, of mouse *ACE2* locating in chromosome X GRC m38.p6.

(B) The mice produced both 4,188 bp and 4,405 bp PCR amplicons that were identified as founder. Ten representative positive PCR results were shown.

(C) $F1^{+}$ mice were sequenced to confirm the right insertion. The head and tail bases of each part were aligned with expected one.

(D) Two endonucleases, *NcoI* and *StuI*, were used to digest DNA for identifying the right insertion and no random recombinant. The expected sizes of WT and gene-targeted bands were 5.6 kb and 8.0 kb, respectively. Heterozygous female mice had both bands, whereas homozygous male mice had only one 8.0 kb band. All the mice detected with WPRE probe had only one 12.4 kb band, indicating no random insertion.

(legend continued on next page)

Pleasecite this article in press as: Sun et al., A Mouse Model of SARS-CoV-2 Infection and Pathogenesis,Cell Host & Microbe (2020),https://doi.org/10.1016/j.chom.2020.05.020

CellPress

have radiographic ground-glass lung changes and finally died from acute respiratory distress syndrome (ARDS)(Huang et al., 2020; Wang et al., 2020a).The WHO declared COVID-19 as a public health emergency of international concern on January 30, 2020.

Full genome-sequencing analysis has classified SARS-CoV-2 as a new member of the betacoronavirus genus, which includes SARS-CoV, MERS-CoV, and bat SARS-related coronaviruses (SARSr-CoV). SARS-CoV-2 has 82% nucleotide identity with SARS-CoV and 89% identify with a bat CoV, RaTG13, discovered in a cave of Yunnan, China, in 2013 (Lu et al., 2020; Zhou et al., 2020b). Like other CoVs, the genome RNA of SARS-CoV-2 encodes a non-structural replicase polyprotein and structural proteins, including spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. The S protein of CoVs constitutes the spike on the virion surface and plays a vital role in viral binding, fusion, and entry as well as the induction of neutralizing antibody and T cell responses (Du et al., 2009). More importantly, several pioneering investigations have demonstrated that both SARS-CoV-2 and SARS-CoV utilize the same cellular receptor, human angiotensin-converting enzyme 2 (hACE2) to enter cells (Walls et al., 2020; Li et al., 2003; Zhou et al., 2020b). The crystal structure of the SARS-CoV-2 S protein receptor-binding domain (RBD) binding to hACE2 has been resolved, with an estimated 10- to 20-fold higher affinity toward hACE2 than SARS-CoV has (Lan et al., 2020; Shang et al., 2020). These findings pave the way for potent intervention strategies targeting RBD/hACE2 interactions. Exogenous hACE2 has been demonstrated to inhibit SARS-CoV-2 infection in cell cultures (Monteil et al., 2020; Ou et al., 2020), and several monoclonal antibodies targeting RBDs of SARS-CoV or SARS-CoV-2 are being tested with *in vitro* cultures (Walls et al., 2020). At present, no vaccine or antiviral drugs have been tested effectively in animal models.

Small animal models that reproduce the clinical course and pathology observed in COVID-19 patients are highly needed. In the present study, we generated a stable humanized ACE2 mice by using CRISPR/Cas9 knockin technology, replacing the endogenous mouse ACE2 (mACE2) with the human ACE2. Further characterization demonstrated that these humanized mice are highly susceptible to SARS-CoV-2 infection via intranasal inoculation, and the viral kinetics and pathological outcomes seen in the SARS-CoV-2-infected animals recapitulated the major finding from COVID-19 patients. Additionally, intragastric injection of SARS-CoV-2 could also establish infection and cause lung injury in the humanized ACE2 mice. This unique mouse model described here provides a useful tool for studying SARS-CoV-2 infection and transmission, as well as evaluating SARS-CoV-2 vaccines and therapeutics.

RESULTS

An hACE2 Humanized Mouse Was Established by Using CRISPR/Cas9

Previous studies have demonstrated that transgenic mice expressing human ACE2 became highly susceptible to SARS-CoV infection (McCray et al., 2007; Menachery et al., 2016; Netland et al., 2008; Tseng et al., 2007; Yang et al., 2007). Here, we aimed to establish a humanized ACE2 mouse by using CRISPR/Cas9 knockin technology. As shown in Figure 1A, the full cDNAs of hACE2 were inserted into Exon 2 (Figure 1A), the first coding exon, of the m*Ace2* gene located in GRC m38.p6 sites on chromosome X. This disrupted the m*Ace2* gene and terminated its expression. The *tdTomato* gene was inserted downstream of *hAce2* with an internal ribosome entry site (IRES),allowing co-expression of hACE2 and tdTomato. A woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and poly (A) sequence were added to enhance mRNA stability and translation efficiency. This targeting strategy allowed an intrinsic expression profile of *hACE2* under control of m*Ace2* promoter. Along with subgenomic RNA (sgRNA) and Cas9 mRNA, the targeting construct was injected into 370 zygotes of C57BL/6 mice. Successful insertion in 10 of 46 offspring (21.74%) was confirmed by PCR screen (Figure 1B); then three founders were backcrossed with C57BL/6 mice, and 37 $F1^{+}$ offspring were screened. The targeting results of 12 positive F1 mice were further confirmed by sequencing (Figure 1C) and Southern blotting (Figure 1D). As expected, no animals had random insertion as demonstrated by the WPRE internal probe (Figure 1D). Importantly, western blotting results showed that hACE2 expression was strong in lung and kidney but very weak in spleen and liver, which displayed a similar expression pattern of mACE2 protein in wild-type (WT) animals (Figure 1E). More importantly, the m*Ace2* gene was not detected by PCR in homozygous hACE2 mice and decreased largely in heterozygous mice (Figure 1F), whereas significant hACE2 expression was detected in lung, small intestine, spleen, and kidney from both hACE2 heterozygous and homozygous mice but not in the WT mice (Figure 1G). Profiting from the co-expression design, the rough global expression patterns of hACE2 were examined visually via bioluminescent imaging in the hACE2 mice (Figure 1H). Furthermore, immunofluorescence staining of the lung sections from the homozygous mice showed that hACE2, as well as tdTomato, were predominantly expressed in the $CC10^{+}$ Clara cells along the airway, as well as a small population of surfactant protein C positive (SPC^{+}) alveolar type II cells (Figure 1I). Thus, we established a homozygous mouse model that stably expresses hACE2 under the control of mACE2 promoter, which were termed hACE2-KI/NIFDC mice (abbreviated to hACE2 mice) accordingly.

(E) western blotting results of tissue distribution of hACE2 in humanized mice and WT mice. The primary antibody used here cross-reacted with both mACE2 and hACE2.

(F and G) The expression pattern of m*ACE2* (F) and h*ACE2* (G) in hACE2 heterozygous and homozygous and WT mice (n = 3) detected by real-time qPCR.

(H) Bioluminescence imaging (BLI) of homozygous mice and WT mice.

(I) Immunofluorescence staining analysis for the hAEC2-expressed cell types in lung of hACE2 mice. Paraffin lung sections were stained for hACE2 (white), tdTomato (red) CC10 (green), b-IV-Tubulin (cyan), PDPN (magenta), and SPC (gold). Two white frames were magnified below. Solid red and yellow arrows indicated the $ACE2^{+}$/tdTomato $^{+}$/SPC^{+} cells and $ACE2^{+}$/tdTomato $^{+}$/$CC10^{+}$ cells, respectively.

Pleasecite this article in press as: Sun et al., A Mouse Model of SARS-CoV-2Infection and Pathogenesis,Cell Host & Microbe (2020),https://doi.org/10.1016/j.chom.2020.05.020

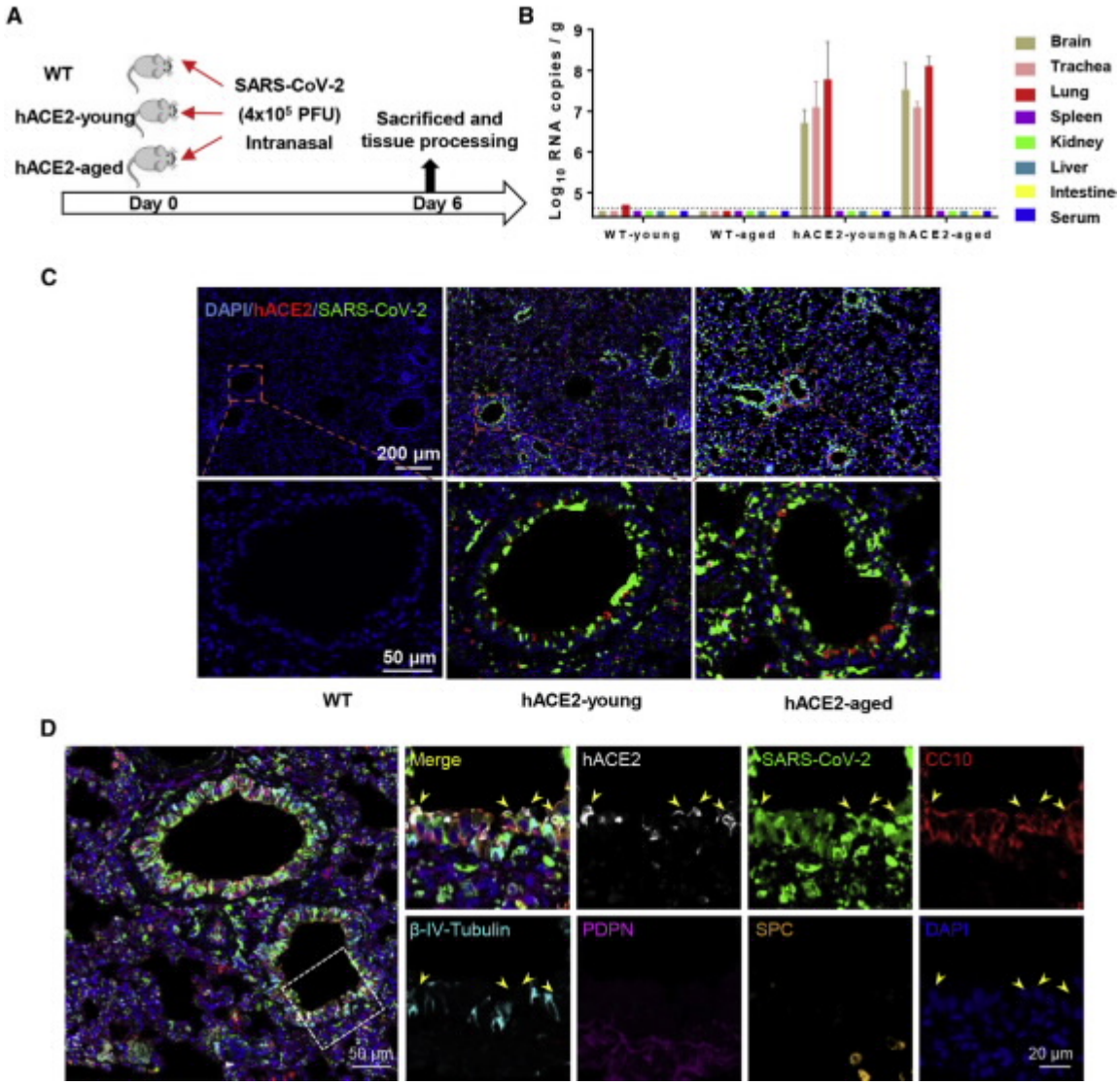


Figure 2. Intranasal Infection of SARS-CoV-2 in hACE2 Mice

(A)WT C57BL/6 mice, young (4.5-week-old), and aged (30-week-old) hACE2mice (n = 3)were intranasally infected with 4 3 10^5 PFU of SARS-CoV-2, and all mice were sacrificed on 6 dpi for tissue collection.

(B) Tissue distribution of SARS-CoV-2 viral RNAs. Each tissue and serum were processed with viral RNA copies analysis by real-time qPCR. Dotted lines denote the detection limit.

(C) Immunofluorescence staining of mouse lung paraffin sections for SARS-CoV-2 S protein (green), ACE2 (red), and DAPI (blue).

(D)Immunofluorescence staining analysis for the infected cells in lung. Cell markers for hACE2 (white), CC10 (red), b-IV-Tubulin (cyan), PDPN (magenta), and SPC (gold). White frame was magnified on the right. Solid yellow arrows indicate the SARS-CoV-2^+/hACE2^+/CC10^+ cells.

The hACE2 Mice Are Susceptible to Intranasal Infection of SARS-CoV-2

To further characterize the susceptibility of hACE2 mice to SARS-CoV-2 infection, groups of young (4.5-week-old) and aged (30-week-old) hACE2 mice were intranasally infected with 4 3 10^5 plaque-forming units (PFU) of SARS-CoV-2, and WT C57BL/6 mice that received the same dose of viral challenge were set as control (Figure 2A). All animals were monitored daily for clinical symptoms and body-weight changes and were sacrificed on day 6 post-infection (dpi). None of the inoculated animals displayed obvious clinical symptoms, and only the aged hACE2 mice lost 10% of their weight at 3 dpi and then recovered (Figure S1). As shown in Figure 2B, robust viral RNA replication was seen in lung, trachea, and brain tissues from both young and aged hACE2 mice, whereas no detectable viral RNA was seen in spleen, kidney, liver, intestine, and serum. Especially, infectious SARS-CoV-2 were successfully rescued from lung samples by using plaque assay. As expected, no viral RNAs were detected in any tested tissues or serum from the WT C57BL/6 mice (Figure 2). Interestingly, we also detected high levels of viral RNAs (2.9 3 10^5 copies/g) in feces of aged hACE2 mice. Further immunostaining of mouse lung section

Pleasecite this article in press as: Sun et al., A Mouse Model of SARS-CoV-2 Infection and Pathogenesis, Cell Host & Microbe (2020), https://doi.org/10.1016/j.chom.2020.05.020

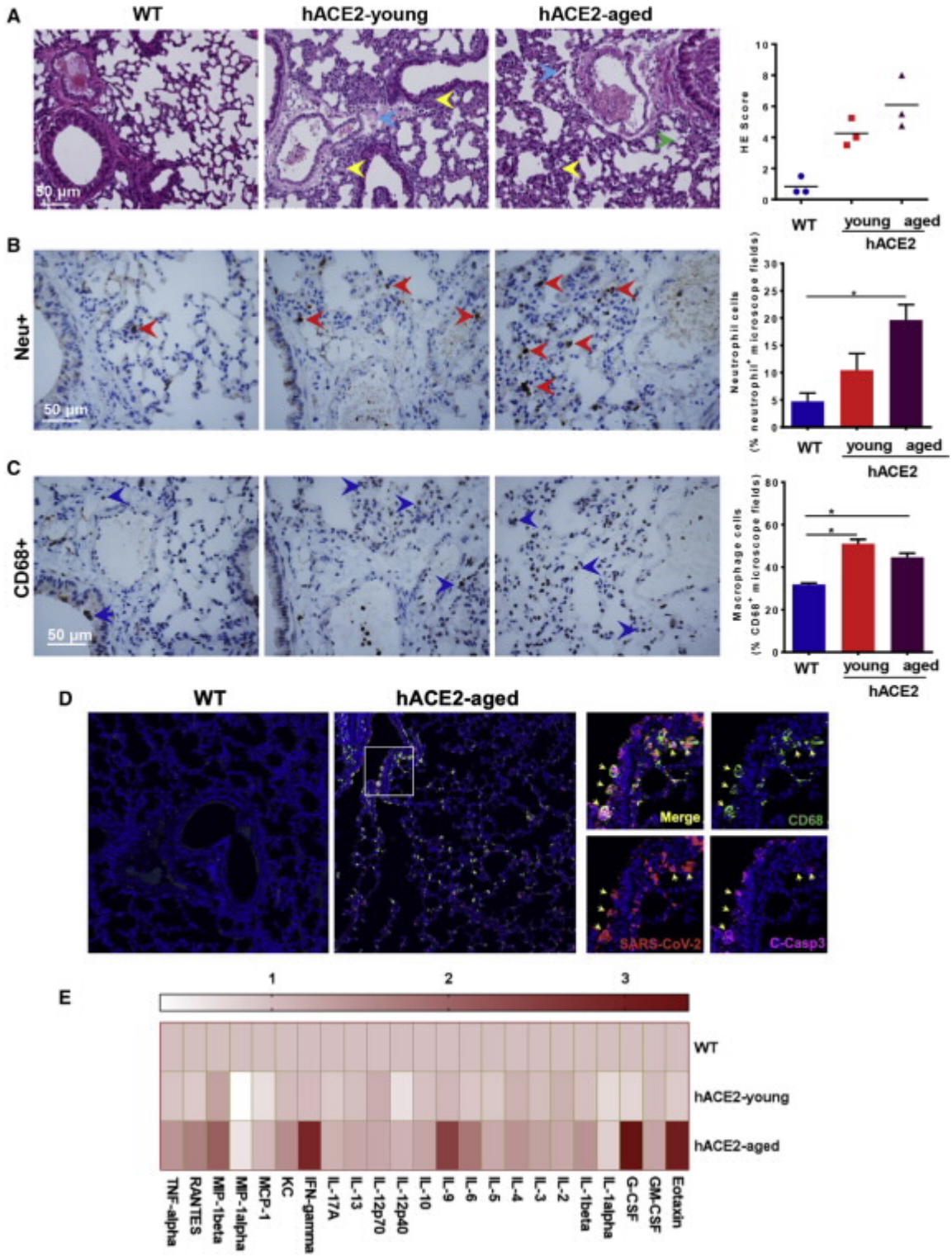


Figure 3. Pathological Changes and Inflammatory Response in SARS-CoV-2-Infected hACE2 Mice

(A) H&E staining analysis showing inflammatory cell infiltration (yellow arrow), alveolar septal thickening, focal hemorrhage (green arrow), and distinctive vascular system injury (blue arrow) in hACE2 mice. Right panel, semiquantitative analysis of the H&E-stained lung sections.

(B and C) IHC staining analysis for neutrophils (Neu$^+$) and macrophages (CD68$^+$) in SARS-CoV-2 infected hACE2 mice. Right panel, semiquantitative analysis of the neutrophil and macrophage counts. Statistical significance was analyzed by unpaired Student's t tests. *$p < 0.05$.

(legend continued on next page)

Pleasecite this article in press as: Sun et al., A Mouse Model of SARS-CoV-2Infection and Pathogenesis,Cell Host & Microbe (2020),https://doi.org/10.1016/j.chom.2020.05.020

showed that robust viral S protein was detected along the airway in both young and aged hACE2 mice, and bare viral protein was detected in the SARS-CoV-2-inoculated WT mice (Figure 2C). To confirm the major target cells of SARS-CoV-2 in our model, we further stained the lung section from the infected hACE2 mice with different cell markers, including Clara cells (marked by Clara cell 10 kDa protein/CC10), ciliated cells (marked by b-IV-tubulin), alveolar type 1 cells (AT1, marked by podoplanin/PDPN), and alveolar type 2 cells (AT2, marked by surfactant protein C/SPC), as well as viral S protein and hACE2. As shown in Figure 2D, SARS-CoV-2 S protein predominantly colocalized with CC10 and hACE2, indicating that $CC10^+$ Clara cells are the major target cells of SARS-CoV-2 along the airway in our model. Additionally, immunostaining of brain sections from the infected hACE2 mice showed that robust viral S protein expression could also be detected in neuron, astrocyte, and microglial cells (Figure S2). Together, these results indicate that the hACE2 mice are highly susceptible to intranasal infection of SARS-CoV-2 in comparison with WT mice, with sustained robust viral RNA replication in lung Clara cells as well as in trachea and brain.

hACE2 Mice Developed Interstitial Pneumonia upon SARS-CoV-2 Infection

To check whether the SARS-CoV-2-infected hACE2 mice produced similar pathological features as those observed in COVID-19 patients, histopathological examination, immunohistochemistry (IHC), and immunofluorescence staining were performed in lung sections from SARS-CoV-2-infected animals. In comparison with SARS-CoV-2-infected WT mice, H&E staining showed that both young and aged hACE2 mice developed interstitial pneumonia characterized with inflammatory cell infiltration, alveolar septal thickening, and distinctive vascular system injury. More lesions of alveolar epithelial cells and focal hemorrhage were observed in the aged mice (Figure 3A). In comparison with WT mice, IHC staining analysis showed SARS-CoV-2 infection induced more neutrophil (Neu^+) and macrophage ($CD68^+$) inf ltration in the aged hACE2 mice (Figures 3B and 3C). Additionally, immunofluorescence co-staining showed SARS-CoV-2 directly infected $CD68^+$ macrophages in lung, resulting in significant apoptosis ($C\text{-}Casp3^+$) in the aged hACE2 mice (Figure 3D). Most importantly, Luminex cytokine analysis showed that SARS-CoV-2 infection led to elevated cytokine production including Eotaxin, G-CSF, IFN-g, IL-9, and MIP-1b in aged hACE2 mice, but it had a weaker response in young mice (Figure 3E; Table S1). Thus, the hACE2 mice not only sustained viral replication but also developed pulmonary pathology upon SARS-CoV-2 infection.

SARS-CoV-2 Established Productive Infection via Intragastric Route in hACE2 Mice

SARS-CoV-2 mainly transmits via droplets and close contact among humans. However, some patients with COVID-19 infection had gastrointestinal symptoms such as diarrhea, abdominal pain, and vomiting (Lin et al., 2020; Wang et al., 2020a), and viral RNAs have been detected in feces of COVID-19 patients (Cai et al., 2020; Xu et al., 2020), highlighting the possibility of fecal-oral transmission of SARS-CoV-2 (Yeo et al., 2020). Here, we sought to investigate whether SARS-CoV-2 could establish productive infection via intragastric route in hACE2 mice. After intragastric administration of SARS-CoV-2, all animals were subjected to daily monitoring and tissue processing on 5 dpi (Figure 4A). Although no clinical signs were seen in the three inoculated hACE2 mice, high levels of viral RNA were detected in trachea (2.9×10^6 copies/g) and lung (3.2×10^6 copies/g) of two animals (Figure 4B), which was comparable to that in animals infected via intranasal route. Importantly, the expression of SARS-CoV-2 S protein was also detected along the lung airway in the infected hACE2 mice (Figure 4C). In addition, interstitial inflammation was observed with alveolar septal thickening in the intagastric SARS-CoV-2-infected hACE2 mice (Figure 4D). Overall, our results demonstrated that intragastric administration of SARS-CoV-2 could also establish productive infection and lead to pulmonary pathological changes in hACE2 mice.

DISCUSSION

In the present study, we generated a stable humanized mouse strain in which hACE2 expression is under the control of IRES in fusion with the tdTomato reporter gene. Further characterization demonstrated that the expression of mACE2 was completely replaced by hACE2 in the homozygous mice with peak hACE2 expression in lung and small intestine (Figure 1). Previously, several hACE2 transgenic mice have been generated with different strategies and used for modeling SARS-CoV infection (McCray et al., 2007; Tseng et al., 2007). Generally, the pulmonary pathology caused by SARS-CoV in transgenic mice correlated with the level of hACE2 expression in lung. Thus, such transgenic mouse has been explored to mimic SARS-CoV-2 infection (Bao et al., 2020). When compared with such transgenic mouse model, our hACE2 mouse model has several advantages. First, the h*Ace2* gene was inserted at GRC m38.p6 sites on chromosome X to replace m*Ace2*, and there is no endogenous mACE2 expression in homozygous hACE2 mice. Because transgene insertion occurs randomly, the integrity of the landing genes would probably be affected. Second, the tissue distribution of hACE2 in our mouse model matches the clinical findings from COVID-19 patients, and high levels of hACE2 expression were detected in lung (Figure 1E). Interestingly, recent analysis based on single-cell sequencing results from human tissues showed that kidney, heart, esophagus, bladder, and ileum also represent tissues of high abundance (Qi et al., 2020; Zou et al., 2020), whereas liver, spleen, small intestine, ovary, and brain are the h*ACE2*-abundant tissues in our model (Figure 1H); this inconsistency between human and mice deserves further investigation. Third, the introduction of tdTomato gene allows real-time monitoring of hACE2 expression. All these ideal physiological features, stable inheritances, and phenotypes of hACE2 mice prompted us to test their application in reproducing SARS-CoV-2 infection.

(D) Immunofluorescence staining analysis for paraffin lung sections with markers for CD68 (green), cleaved-caspase 3 (C-Casp3, magenta), and SARS-CoV-2 S protein (red). White frame was magnified on the right. Solid yellow arrows indicated the $SARS\text{-}CoV\text{-}2^+/CD68^+/C\text{-}Casp3^+$ cells.
(E) Cytokine production in serum of SARS-CoV-2-infected mice.

Pleasecite this article in press as: Sun et al., A Mouse Model of SARS-CoV-2 Infection and Pathogenesis,Cell Host & Microbe (2020),https://doi.org/10.1016/j.chom.2020.05.020

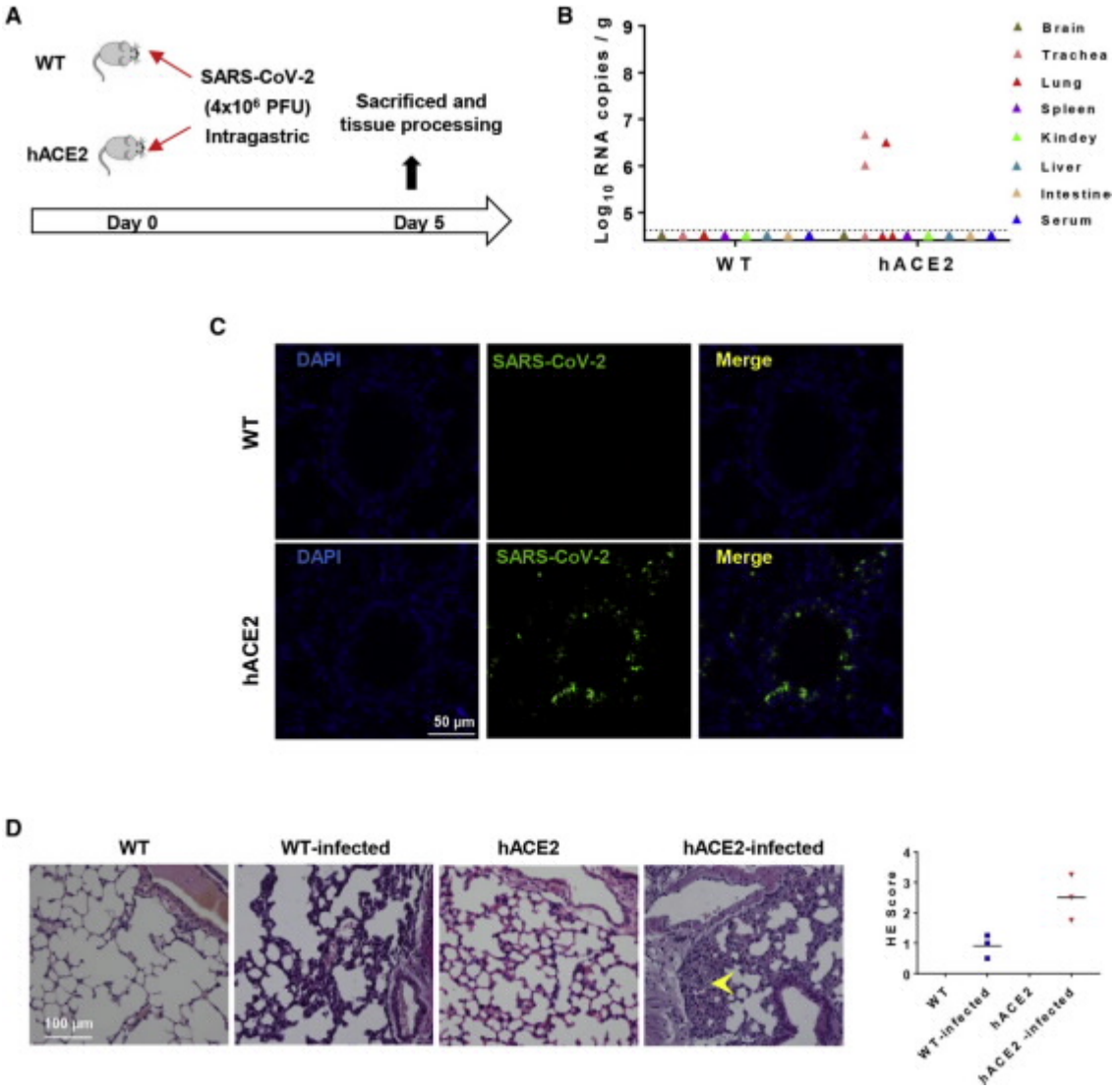


Figure 4. Intragastric Infection with SARS-CoV-2 in hACE2 Mice

(A) WT C57BL/6 mice and hACE2 mice (n = 3) at 4.5 weeks old were infected with 4 3 10^6 PFU of SARS-CoV-2 through intragastric administration, and all mice were sacrificed on 5 dpi for main tissues and serum collection.

(B) Tissue distribution of SARS-CoV-2 viral RNAs. Each tissue and serum were collected and viral RNA copies analysis by real-time qPCR.

(C) Immunofluorescence staining of mouse lung sections for SARS-CoV-2 S protein (green) and DAPI (blue).

(D) Histopathological analysis of mouse lung sections. Alveolar septal thickening was indicated with yellow arrow. Right panel, semiquantitative analysis of the H&E-stained lung sections.

Not surprisingly, our challenge experiment of SARS-CoV-2 clearly demonstrated that these hACE2 mice are highly susceptible to SARS-CoV-2 infection via intranasal inoculation, and 4 3 10^5 PFU of SARS-CoV-2 readily led to robust viral replication in respiratory tracts (lung and trachea) as well as in brain. The peaked viral RNA loads in lung reached 10^8 copies/g, which is much higher than that in hACE2 transgenic mice (Bao et al., 2020). The presence of viral RNAs in brain was somewhat unexpected, because few COVID-19 patients developed neurological systems (Chen et al., 2020). Previous findings from a SARS-CoV-infected transgenic model also confirmed viral tropism to brain (McCray et al., 2007), which is consistent with our findings. The underlying mechanism through which SARS-CoV or SARS-CoV-2 invade the brain and the correlation to disease severity remain to be determined. Further characterization demonstrated that $CC10^+$ Clara cells along the airway are the major target cells of SARS-CoV-2 (Figure 2D) in our model. Specific protease in Clara cells, like tryptase Clara, might contribute to the efficient cleavage of S protein to enhance viral entry into cells. Additionally, $CD68^+$ macrophages in pulmonary alveolus are also infected with SARS-CoV-2, resulting in severe cell apoptosis (Figure 3D). Interestingly, a significant population of cells that do not express hACE2 were also infected with SARS-CoV-2 (Figure 2D). How SARS-CoV-2 invades these cells also deserves further investigation. Overall, our result provides the

Pleasecite this article in press as: Sun et al., A Mouse Model of SARS-CoV-2 Infection and Pathogenesis, Cell Host & Microbe (2020), https://doi.org/10.1016/j.chom.2020.05.020

first line of evidence showing the specific major target cells of SARS-CoV-2 in mouse lung.

Most importantly, all hACE2 mice intranasally challenged with SARS-CoV-2 developed interstitial pneumonia characterized with inflammatory cell infiltration, alveolar septal thickening, and distinctive vascular system injury, which recapitulated the clinical features in most COVID-19 patients. Early epidemiological findings have suggested that advanced age resulted in increased mortality of COVID-19 patients (Chen et al., 2020; Yang et al., 2020; Zhou et al., 2020a), and our results from the hACE2 model reflect this finding well. The aged hACE2 mice (30 weeks old) lost weight upon SARS-CoV-2 infection, whereas young hACE2 mice (4.5 weeks old) as well as WT mice maintained their body weight increase during the observation period (Figure S1). Remarkably, although both young and aged mice sustained similar viral replication in lung, more severe pathological changes as well as increased cytokine response were seen in the aged hACE2 mice (Figure 3). These unique features in aged hACE2 mice resembling clinical findings (Wang et al., 2020b) highlight the potential application in different settings. It should be noted that no clinical symptoms or mortality was observed in our model, and other animal models that can recapitulate severe or lethal disease of COVID-19 should be warranted in the future.

There are several lines of evidence that support potential fecal-oral transmission of SARS-CoV-2. First, gastrointestinal symptoms were seen in many COVID-19 patients (Jin et al., 2020; Lin et al., 2020), and viral shedding in feces have been well reported. Second, ACE2 was found to be highly expressed in gastrointestinal epithelial cells, and *in vitro* studies also indicated SARS-CoV-2 can infect multiple cells from gastrointestinal tracts. Third, experience from SARS and MERS supported that human CoVs maintained their viability in environmental conditions essential for fecal-oral transmission (Chan et al., 2011; van Doremalen et al., 2013). Recent data from SARS-CoV-2 revealed similar stability to SARS-CoV, suggesting similar transmission routes are plausible (van Doremalen et al., 2020). Our results from hACE2 mouse model provide evidence supporting this hypothesis. Upon intranasal infection, viral RNA shading was also confirmed in feces from the hACE2 mice (data not shown). Due to facility limitation, we were not able to collect and quantitate feces samples from each individual animal. Importantly, SARS-CoV-2 given intragastrically caused productive infection in the respiratory tracts of hACE2 mice, as demonstrated by the presence of high level of viral RNAs and active viral protein expression (Figures 4B and 4C). Our preliminary results show, however, that intragastric infection of SARS-CoV is not as efficient as intranasal infection: the challenge dose of intragastric model is 10-fold higher than the intranasal model; however, pulmonary infection was only detected in one of three animals tested (Figure 4B); the viral RNA loads and protein expression were also lower than that in the intranasal model. Finally, whether intragastric infection of SARS-CoV-2 is actually occurring in clinical setting in humans remains to be confirmed. The underlying mechanism through which SARS-CoV-2 survives the gastrointestinal environment and finally invades the respiratory tracts in our mouse model warrants further investigation.

In summary, our results show that hACE2 mice are susceptible to intranasal and intragastric infection of SARS-CoV-2, and robust viral replication was seen in the pulmonary Clara cells and macrophages. Especially, the pathological changes observed in the aged hACE2 mice more closely resemble those observed in COVID-19 patients. Thus, the use of hACE2 mice described in our manuscript provides a small animal model for studying the transmission and pathogenesis of SARS-CoV-2 and for the understanding of unexpected clinical manifestations of SARS-CoV-2 infection in humans. This model will also be valuable for testing vaccines and therapeutics to combat SARS-CoV-2.

STAR+ METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCESTABLE
- RESOURCEAVAILABILITY
  - Lead Contact
  - Materials Availability
  - Data and Code Availability
- EXPERIMENTALMODEL AND SUBJECT DETAILS
  - Experimental Animals
  - Cell Lines
  - SARS-CoV-2 Propagation and Titeration
- METHOD DETAILS
  - Generation of hACE2 Mice
  - RNA Extraction and Real-Time Quantitative PCR
  - Western Blotting
  - Mouse Challenge Experiments
  - Measurement of Viral RNA Loads
  - Histopathological Analysis
  - Multiplex Immunofluorescent Assay
  - Immunohistochemistry
  - Cytokine Assay
- QUANTIFICATIONAND STATISTICALANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.chom.2020.05.020.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Project of China, China (2016YFD0500304, 2020YFA0707801); the National Science and Technology Major Project of China, China (No. 2017ZX103304402, 2018ZX09711003); the National Natural Science Foundation of China, China (82041006, 31871476); and Guangdong Pearl River talent plan, China (2019CX01N111). C.F.Q. was supported by the National Science Fund for Distinguished Young Scholar, China (No. 81925025); the Innovative Research Group (No. 81621005) from the National Natural Science Foundation of China, China; and the Innovation Fund for Medical Sciences (No. 2019-I2M-5-049) from the Chinese Academy of Medical Sciences, China.

AUTHOR CONTRIBUTIONS

C.F.Q., C.F.F., Y.S.Z., and Y.C.W. conceived this project. S.H.S., Q.C., H.J.G., G.Y., Y.X.W., X.Y.H., S.S.L., N.N.Z., X.F.L., Y.G., and Y.Q.D. performed the experiments and analyzed all the results. W.J.H., Q. L., Q.M.L., Y.L.S., Y.Z., X.Y., and T.Y.Z. helped analyze the results. Y.C.W., C.F.Q., Y.S.Z., X.Y., and C.C.F. co-supervised the project. C.F.Q., Q.C., Y.S.Z., Y.C.W., and C.F.F. wrote and revised the manuscript with input from all authors.

Pleasecite this article in press as: Sun et al., A Mouse Model of SARS-CoV-2 Infection and Pathogenesis,Cell Host & Microbe (2020),https://doi.org/10.1016/j.chom.2020.05.020

CellPress

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: April 14, 2020
Revised: May 9, 2020
Accepted: May 22, 2020
Published: May 27, 2020

REFERENCES

Bao, L., Deng, W., Huang, B., Gao, H., Liu, J., Ren, L., Wei, Q., Yu, P., Xu, Y., Qi, F., et al. (2020). The pathogenicity of SARS-CoV-2 in hACE2 transgenic mice. Nature.

Cai, J., Xu, J., Lin, D., Yang, Z., Xu, L., Qu, Z., Zhang, Y., Zhang, H., Jia, R., Liu, P., et al. (2020). A Case Series of children with 2019 novel coronavirus infection: clinical and epidemiological features. Clin. Infect. Dis. ciaa198. https://doi.org/10.1093/cid/ciaa198 .

Chan, K.H., Peiris, J.S., Lam, S.Y., Poon, L.L., Yuen, K.Y., and Seto, W.H. (2011). The Effects of Temperature and Relative Humidity on the Viability of the SARS Coronavirus. Adv. Virol. *2011*. 734690. https://doi.org/10.1155/2011/734690.

Chen, N., Zhou, M., Dong, X., Qu, J., Gong, F., Han, Y., Qiu, Y., Wang, J., Liu, Y., Wei, Y., et al. (2020). Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet *395*, 507–513.

Cui, J., Li, F., and Shi, Z.L. (2019). Origin and evolution of pathogenic coronaviruses. Nat. Rev. Microbiol. *17*, 181–192.

Du, L., He, Y., Zhou, Y., Liu, S., Zheng, B.J., and Jiang, S. (2009). The spike protein of SARS-CoV–a target for vaccine and therapeutic development. Nat. Rev. Microbiol. *7*, 226–236.

Haagmans, B.L., Kuiken, T., Martina, B.E., Fouchier, R.A., Rimmelzwaan, G.F., van Amerongen, G., van Riel, D., de Jong, T., Itamura, S., Chan, K.H., et al. (2004). Pegylated interferon-alpha protects type 1 pneumocytes against SARS coronavirus infection in macaques. Nat. Med. *10*, 290–293.

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., Zhang, L., Fan, G., Xu, J., Gu, X., et al. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet *395*, 497–506.

Jin, X., Lian, J.S., Hu, J.H., Gao, J., Zheng, L., Zhang, Y.M., Hao, S.R., Jia, H.Y., Cai, H., Zhang, X.L., et al. (2020). Epidemiological, clinical and virological characteristics of 74 cases of coronavirus-infected disease 2019 (COVID-19) with gastrointestinal symptoms. Gut *69*, 1002–1009.

Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S., Zhang, Q., Shi, X., Wang, Q., Zhang, L., and Wang, X. (2020). Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. Nature *581*, 215–220.

Li, W., Moore, M.J., Vasilieva, N., Sui, J., Wong, S.K., Berne, M.A., Somasundaran, M., Sullivan, J.L., Luzuriaga, K., Greenough, T.C., et al. (2003). Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature *426*, 450–454.

Li, Q., Guan, X., Wu, P., Wang, X., Zhou, L., Tong, Y., Ren, R., Leung, K.S.M., Lau, E.H.Y., Wong, J.Y., et al. (2020). Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus-Infected Pneumonia. N. Engl. J. Med. *382*, 1199–1207.

Lin, L., Jiang, X., Zhang, Z., Huang, S., Zhang, Z., Fang, Z., Gu, Z., Gao, L., Shi, H., Mai, L., et al. (2020). Gastrointestinal symptoms of 95 cases with SARS-CoV-2 infection. Gut *69*, 997–1001.

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., Wang, W., Song, H., Huang, B., Zhu, N., et al. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. Lancet *395*, 565–574.

McCray, P.B., Jr., Pewe, L., Wohlford-Lenane, C., Hickey, M., Manzel, L., Shi, L., Netland, J., Jia, H.P., Halabi, C., Sigmund, C.D., et al. (2007). Lethal infection of K18-hACE2 mice infected with severe acute respiratory syndrome coronavirus. J. Virol. *81*, 813–821.

Menachery, V.D., Yount, B.L., Jr., Sims, A.C., Debbink, K., Agnihothram, S.S., Gralinski, L.E., Graham, R.L., Scobey, T., Plante, J.A., Royal, S.R., et al. (2016). SARS-like WIV1-CoV poised for human emergence. Proc. Natl. Acad. Sci. USA *113*, 3048–3053.

Monteil, V., Kwon, H., Prado, P., Hagelkrüys, A., Wimmer, R.A., Stahl, M., Leopoldi, A., Garreta, E., Hurtado Del Pozo, C., Prosper, F., et al. (2020). Inhibition of SARS-CoV-2 Infections in Engineered Human Tissues Using Clinical-Grade Soluble Human ACE2. Cell *181*, 905–913.e7.

Netland, J., Meyerholz, D.K., Moore, S., Cassell, M., and Perlman, S. (2008). Severe acute respiratory syndrome coronavirus infection causes neuronal death in the absence of encephalitis in mice transgenic for human ACE2. J. Virol. *82*, 7264–7275.

Ou, X., Liu, Y., Lei, X., Li, P., Mi, D., Ren, L., Guo, L., Guo, R., Chen, T., Hu, J., et al. (2020). Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. Nat. Commun. *11*, 1620.

Peiris, J.S., Lai, S.T., Poon, L.L., Guan, Y., Yam, L.Y., Lim, W., Nicholls, J., Yee, W.K., Yan, W.W., Cheung, M.T., et al.; SARS study group (2003). Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet *361*, 1319–1325.

Qi, F., Qian, S., Zhang, S., and Zhang, Z. (2020). Single cell RNA sequencing of 13 human tissues identify cell types and receptors of human coronaviruses. Biochem. Biophys. Res. Commun. *526*, 135–140.

Shang, J., Ye, G., Shi, K., Wan, Y., Luo, C., Aihara, H., Geng, Q., Auerbach, A., and Li, F. (2020). Structural basis of receptor recognition by SARS-CoV-2. Nature *581*, 221–224.

Tseng, C.T., Huang, C., Newman, P., Wang, N., Narayanan, K., Watts, D.M., Makino, S., Packard, M.M., Zaki, S.R., Chan, T.S., and Peters, C.J. (2007). Severe acute respiratory syndrome coronavirus infection of mice transgenic for the human Angiotensin-converting enzyme 2 virus receptor. J. Virol. *81*, 1162–1173.

van den Brand, J.M., Stittelaar, K.J., van Amerongen, G., Rimmelzwaan, G.F., Simon, J., de Wit, E., Munster, V., Bestebroer, T., Fouchier, R.A., Kuiken, T., and Osterhaus, A.D. (2010). Severity of pneumonia due to new H1N1 influenza virus in ferrets is intermediate between that due to seasonal H1N1 virus and highly pathogenic avian influenza H5N1 virus. J. Infect. Dis. *201*, 993–999.

van Doremalen, N., Bushmaker, T., and Munster, V.J. (2013). Stability of Middle East respiratory syndrome coronavirus (MERS-CoV) under different environmental conditions. Euro Surveill. *18*, 20590.

van Doremalen, N., Bushmaker, T., Morris, D.H., Holbrook, M.G., Gamble, A., Williamson, B.N., Tamin, A., Harcourt, J.L., Thornburg, N.J., Gerber, S.I., et al. (2020). Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1. N. Engl. J. Med. *382*, 1564–1567.

Walls, A.C., Park, Y.-J., Tortorici, M.A., Wall, A., McGuire, A.T., and Veesler, D. (2020). Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. Cell *181*, 281–292.e6.

Wang, D., Hu, B., Hu, C., Zhu, F., Liu, X., Zhang, J., Wang, B., Xiang, H., Cheng, Z., Xiong, Y., et al. (2020a). Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. JAMA. https://doi.org/10.1001/jama.2020.1585 .

Wang, Z., Yang, B., Li, Q., Wen, L., and Zhang, R. (2020b). Clinical Features of 69 Cases with Coronavirus Disease 2019 in Wuhan, China. Clin. Infect. Dis. ciaa272.

Wu, F., Zhao, S., Yu, B., Chen, Y.M., Wang, W., Song, Z.G., Hu, Y., Tao, Z.W., Tian, J.H., Pei, Y.Y., et al. (2020). A new coronavirus associated with human respiratory disease in China. Nature *579*, 265–269.

Xu, Y., Li, X., Zhu, B., Liang, H., Fang, C., Gong, Y., Guo, Q., Sun, X., Zhao, D., Shen, J., et al. (2020). Characteristics of pediatric SARS-CoV-2 infection and potential evidence for persistent fecal viral shedding. Nat. Med. *26*, 502–505.

Yang, X.H., Deng, W., Tong, Z., Liu, Y.X., Zhang, L.F., Zhu, H., Gao, H., Huang, L., Liu, Y.L., Ma, C.M., et al. (2007). Mice transgenic for human angiotensin-converting enzyme 2 provide a model for SARS coronavirus infection. Comp. Med. *57*, 450–459.

Pleasecite this article in press as: Sun et al., A Mouse Model of SARS-CoV-2 Infection and Pathogenesis, Cell Host & Microbe (2020), https://doi.org/10.1016/j.chom.2020.05.020

Yang, X., Yu, Y., Xu, J., Shu, H., Xia, J., Liu, H., Wu, Y., Zhang, L., Yu, Z., Fang, M., et al. (2020). Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Respir. Med. *8*, 475–481.

Yeo, C., Kaushal, S., and Yeo, D. (2020). Enteric involvement of coronaviruses: is faecal-oral transmission of SARS-CoV-2 possible? Lancet Gastroenterol. Hepatol. *5*, 335–337.

Zaki, A.M., van Boheemen, S., Bestebroer, T.M., Osterhaus, A.D., and Fouchier, R.A. (2012). Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N. Engl. J. Med. *367*, 1814–1820.

Zhou, F., Yu, T., Du, R., Fan, G., Liu, Y., Liu, Z., Xiang, J., Wang, Y., Song, B., Gu, X., et al. (2020a). Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet *395*, 1054–1062.

Zhou, P., Yang, X.L., Wang, X.G., Hu, B., Zhang, L., Zhang, W., Si, H.R., Zhu, Y., Li, B., Huang, C.L., et al. (2020b). A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature *579*, 270–273.

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Zhao, X., Huang, B., Shi, W., Lu, R., et al.; China Novel Coronavirus Investigating and Research Team (2020). A Novel Coronavirus from Patients with Pneumonia in China, 2019. N. Engl. J. Med. *382*, 727–733.

Zou, X., Chen, K., Zou, J., Han, P., Hao, J., and Han, Z. (2020). Single-cell RNA-seq data analysis on the receptor ACE2 expression reveals the potential risk of different human organs vulnerable to 2019-nCoV infection. Front. Med. *14*, 185–192.

Please cite this article in press as: Sun et al., A Mouse Model of SARS-CoV-2 Infection and Pathogenesis, Cell Host & Microbe (2020), https://doi.org/10.1016/j.chom.2020.05.020

CellPress

STAR+ METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
ACE2 (immunostaining)	Abcam	Cat#ab108209; RRID: AB_10862654
SARS-CoV Spike	Sinobiological	Cat#40150-T52
b-IV-tubulin	Abcam	Cat#ab179504
CC10	Millipore	Cat#07-623; RRID: AB_310759
Podoplanin	Sinobiological	Cat#50256-R066
SPC	Abcam	Cat#ab211326
CD68	Abcam	Cat#ab125212
Cleaved Caspase-3	CST	Cat#9661; RRID: AB_2341188
Neutrophil Marker	Santa Cruz Biotechnology	Cat#sc-71674; RRID: AB_2167794
GAPDH	Abcam	Cat#ab9485; RRID: AB_307275
horseradish peroxidase (HRP)-conjugated anti-mouse IgG	Abcam	Cat#ab6728; RRID: AB_955440
horseradish peroxidase (HRP)-conjugated anti-rabbit IgG	Abcam	Cat#ab205718; RRID: AB_2819160
ACE2 (Western Blotting)	Huabio	Cat#ET1611-58; RRID: AB_11158910
Virus Strains		
SARS-CoV-2 BetaCoV/Wuhan/AMMS01/2020	This paper	N/A
Chemicals, Peptides, and Recombinant Proteins		
DMEM	GIBCO	Cat#11995-065
Fetal Bovine Serum	PAN Biotech	Cat#P30-3306
Penecillin Streptomycin	GIBCO	Cat#15140-122
HEPES	GIBCO	Cat#15630-080
RNAlater	Invitrogen	Cat#AM7020
retrieval/elution buffer	Abcracker	Cat#ABCFR5L
Streptavidin-peroxidase conjugate	Zhongshan Biotechnology	Cat#SP-9000
low-melting point agarose	Promega	Cat#V2111
Pierce ECL Western Blotting Substrate	Thermo	Cat#32106
pelltobarbitalum natricum	Sigma	Cat#P3761
NcoI	Thermo	Cat#FD0595
StuI	Thermo	Cat#ER0421
Critical Commercial Assays		
RNeasy Mini kit	QIAGEN	Cat#74106
One Step PrimeScript RT-PCR Kit	TaKaRa	Cat#RR064A
Bio-Plex Pro Mouse Cytokine Grp I Panel 23-Plex	BIO-RAD	Cat#M60009RDPD
Reverse transcription (RT)-PCR kit	TaKaRa	Cat#RR096A
NEON 7-color Allround Discovery Kit	Histova Biotechnology	Cat#NEFP750
Experimental Models: Cell Line		
Vero Cells	ATCC	Cat#CCL-81
Experimental Models: Animals		
C57BL/6	Institute for Laboratory Animal Resources, NIFDC, China	N/A
hACE2-KI/NIFDC	This paper	N/A

(*Continued on next page*)

Pleasecite this article in press as: Sun et al., A Mouse Model of SARS-CoV-2 Infection and Pathogenesis, Cell Host & Microbe (2020), https://doi.org/10.1016/j.chom.2020.05.020

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
sgRNA gaaagatgtccagctcctcctgg	This paper	N/A
Primers for genotyping F0 offspring, see Table S2	This paper	N/A
Primers for RT-qPCR quantification of ACE2 expression level, see Table S2	This paper	N/A
Primers and probes for RT-qPCR quantification of SARS-CoV-2 RNA, see Table S2	This paper	N/A
Software and Algorithms		
GraphPad Prism 7.0	Graphpad	https://www.graphpad.com/
Luminex PONENT	Thermo Fisher	https://www.luminexcorp.com/xponent/
Other		
IVIS-Lumina II imaging system	Xenogen	https://health.usf.edu/

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, You-Chun Wang (wangyc@nifdc.org.cn).

Materials Availability

All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability

This study did not generate any unique datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Experimental Animals

Groups of 4.5 weeks and 30 weeks old hACE2 and wild-type C57BL/6 female mice for SARS-CoV-2 infection were maintained in animal biosafety level 3 (ABSL3) containment laboratory of AMMS at 23 C. All animals were allowed free access to water and diet and provided with a 12 h light/dark cycle. All animal experiments were approved by the Experimental Animal Committee of NIFDC (approval number: 2018-B-002) and Laboratory Animal Center, AMMS (approval number: IACUC-DWZX-2020-001).

Cell Lines

Vero Cells for SARS-CoV-2 propagation and titeration were maintained in DMEM medium (GIBCO) supplemented with 2% FBS (PAN Biotech), 1% Penecillin Streptomycin (GIBCO) and 1% HEPES (GIBCO) at 37 C.

SARS-CoV-2 Propagation and Titeration

SARS-CoV-2 strain BetaCoV/wuhan/AMMS01/2020 was originally isolated by CFQ's lab, AMMS, from a patient returning from Wuhan. The virus was amplified on Vero cells and titerated by standard plaque forming assay. Briefly, Vero cells in 12-well plates were infected with a 10-fold serial dilution of viruses. The plates were incubated at 37 C for 1 h and cells were overlaid with 1% low-melting point agarose (Promega) in DMEM containing 2% FBS. After further incubation at 37 C for 2 days, the cells were fixed with 4% formaldehyde and stained with 0.2% crystal violet to visualize the plaques. All experiments involving infectious SARS-CoV-2 were performed in biosafety level 3 (BSL3) containment laboratory in AMMS.

METHOD DETAILS

Generation of hACE2 Mice

To construct the targeting vector, cDNAs encoding hACE2 linked to the red fluorescent protein tdTomato by an IRES sequence, flowed with WPRE and ployA, was inserted into the first coding exon of m*ACE2* by homologous recombination. The expression

Pleasecite this article in press as: Sun et al., A Mouse Model of SARS-CoV-2 Infection and Pathogenesis,Cell Host & Microbe (2020),https://doi.org/10.1016/j.chom.2020.05.020

CellPress

of inserted h*ACE2* and tdTomato gene were driven by the mouse mACE2 promoter. The specific targeting sgRNA (5^0-gaaagatgtc-cagctcctcctgg-3 ^{0}) and Cas9 mRNA were microinjected into C57BL/6 zygotes, which were subsequently implanted into pseudo-pregnant mice. F0 offspring were genotyped using two pairs of primers L-GT-F1, L-GT-R1 and R-GT-F1, R-GT-R1 (Table S2). The expected size of amplicons was 4188 bp and 4405 bp, respectively. Genotype-positive founders were backcrossed with C57BL/6 mice to produce generation F1. DNA samples of F1-positive mice were subjected to Southern blotting to confirm off-targeting (by WPRE 3^0 probe) and correct insertion (by 5^0 probe). Two endonucleases, *NcoI* (Thermo) and *StuI* (Thermo), were used to digest DNA for identifying the right insertion and no random recombinant.

RNA Extraction and Real-Time Quantitative PCR

To confirm the expression of hACE2 and mACE2 in humanized mice, tissues from 4.5 weeks old and 30 weeks old mice were dissected, immediately immersed in RNAlater (Invitrogen) stabilization reagent and stored at 80 C. Total RNA was extracted from individual tissues using TRIzol and quantified using a spectrophotometer. Real-time quantitative PCR (RT-qPCR) was performed using a reverse transcription (RT)-PCR kit (Takara) containing SYBR green dye. Each reaction was performed in triplicate. Relative expression level of hACE2 and mACE2 was determined using primer pairs ehACE2 F1, ehACE2 R1 and emACE2-F1, emACE2-R1 (Table S2). Amplicons were normalized to expression of GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

Western Blotting

Mouse tissues were homogenized in Cell lysis buffer for Western and IP (20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 100 mM EDTA, and 1% Triton X-100) supplemented with Phenylmethanesulfonyl fluoride (PMSF) (Beyotime). The denatured protein lysates were separated using 10% SDS-PAGE gels. After transfer, ACE2 antibody (Huabio, 1:1000) and GAPDH antibody (Abcam, 1:1000) were added, followed by horseradish peroxidase (HRP)-conjugated anti-mouse and rabbit IgG (Abcam, 1:2,000). The Pierce ECL Western Blotting Substrate (Thermo) was used for development. Bioluminescence imaging (BLI) Mice were anesthetized by intraperitoneal injection of pelltobarbitalum natricum (Sigma, 240 mg/kg body weight). tdTomato bioluminescence imaging was performed with IVIS-Lumina II imaging system (Xenogen). The relative intensities were presented as pseudocolored images as photon flux in photon (p/s/cm^2/sr).

Mouse Challenge Experiments

For intranasal infection, wild-type C57BL/6 mice, young (4.5 weeks old) and aged (30 weeks old) hACE2 mice were anesthetized with sodium pentobarbital at a dose of 50 mg/kg though intraperitoneal route, and then intranasally infected with 4 3 10^5 pfu of SARS-CoV-2, respectively. For intragastric infection, all animals were fed with 4 3 10^6 pfu of SARS-CoV-2 (in 200 ml) via intragastric gavage using a ball-tipped 6-gauge feeding needle. Mice were then weighed and monitored daily, and sacrificed on day 5 or 6 post infection for serum collection and tissue processing.

Measurement of Viral RNA Loads

Viral RNA in each sample was extracted with an RNeasy Mini kit (QIAGEN) according to the protocols. The viral RNA quantification was performed by RT-qPCR targeting the S gene of SARS-CoV-2. RT-qPCR was performed using One Step PrimeScript RT-PCR Kit (Takara) with the following primers and probes: CoV-F3, CoV-R3 and CoV-P3 (Table S2).

Histopathological Analysis

Mouse tissues were excised and fixed with 10% neutral buffered formaline, dehydrated and embedded in paraffin. Each embedded tissue was sectioned into 4 mm thickness longitudinal sections. Three tissue sections derived from different parts of each tissue were stained with hematoxylin and eosin (H&E) according to standard procedures for examination by light microscopy. The degree of lung damage under the light microscopy was assessed by the degeneration of alveolar epithelial cells, the expansion of parenchymal wall, edema, hemorrhage, and inflammatory cells infiltration. The semiquantitative assessment were performed as reported previously and described briefly as the following (Haagmans et al., 2004; van den Brand et al., 2010). For the degree of degeneration of alveolar epithelial cells, we scored 0 when no alveolar epithelial cells degeneration was observed, scored 1 when the alveolar epithelial cells degeneration was less than 10%, and scored 2 when the degeneration was 10%–50%. For the degree parenchymal wall expansion, we scored 0 when no parenchymal wall expanded, scored 1 when the area of the expanded parenchymal wall was less than 10% but no focal fusion was observed, and scored 2 when the area was 10%–50% but no focal fusion was observed. For the degree of edema, we scored 0 when no edema was observed, scored 1 when the area of edema was less than 10%, and scored 2 when the area was 10%–50%. For the degree of inflammatory cells infiltration, we scored 0 when no inflammatory cell infiltration, scored 1 when occasional infiltration of single inflammatory cell was visible, and scored 2 when focal infiltration of inflammatory cells was visible. The lesion of lung was observed by an experienced experimental pathologist who focuses on the pathogenesis study of virus infection. The cumulative scores of the severity of the three sections provided the total score per animal, and the average of the three animals was taken as the total score for that group.

Multiplex Immunofluorescent Assay

3 mM paraffin sections were deparaffinised in xylene and rehydrated in a series of graded alcohols. Antigen retrievals were performed in citrate buffer (pH6) with a microwave for 20 min at 95 C followed by a 20 min cool down at room temperature. Multiplex

Pleasecite this article in press as: Sun et al., A Mouse Model of SARS-CoV-2 Infection and Pathogenesis, Cell Host & Microbe (2020), https://doi.org/10.1016/j.chom.2020.05.020

fluorescence labeling was performed using TSA-dendron-fluorophores with NEON 7-color Allround Discovery Kit for FFPE (Histova Biotechnology). Briefly, endogenous peroxidase was quenched in 3% H_2O_2 for 20 min, followed by blocking reagent for 30 min at room temperature. Primary antibody was incubated for 2 h in a humidified chamber at 37 C, followed by detection using the HRP-conjugated secondary antibody and TSA-dendronfluorophores. Afterward, the primary and secondary antibodies were thoroughly eliminated by heating the slides in retrieval/elution buffer (Abcracker) for 10 s at 95 C using microwave. In a serial fashion, each antigen was labeled by distinct fluorophores. Multiplex antibody panels applied in this study are: ACE2 (Abcam, 1:200), SARS-CoV Spike (Sinobiological, 1:2000); b-IV-tubulin (Abcam, 1:1000), CC10 (Millipore, 1:500), Podoplanin (Sinobiological, 1:1000), SPC (Abcam, 1:500), CD68 (Abcam, 1:1000), Cleaved Caspase-3 (C-Casp3,CST, 1:200). After all the antibodies were detected sequentially, the slices were imaged using the confocal laser scanning microscopy platform Zeiss LSM880.

Immunohistochemistry

Paraffin-embedded tissues were sectioned at a thickness of 4 mm for immunohistochemistry staining. Sections were deparaffinized and rehydrated, endogenous peroxidases were inactivated with methanol containing 0.3% hydrogen peroxide for 30 min. Antigen retrieval was performed with citrate buffer (pH6) at 95 C for 30 min. After incubation in blocking solution (5% normal goat serum) for 10 min at room temperature, the slides were incubated with Neutrophil Marker antibody (Santa Cruz Biotechnology, 1:200) and rabbit anti- CD68 polyclonal antibody (Abcam, 1:800) overnight at 4 C. After three washes, the sections were incubated with biotinylated anti-IgG at 37 C for 1 h, followed by Streptavidin-peroxidase conjugate (Zhongshan Biotechnology). Immunoreactivity was detected using 3, 3^0diaminobenzidine and the sections were counterstained with hematoxylin for observation by microscopy. To measure the macrophage and neutrophil infiltration semiquantitatively, 10 arbitrarily chosen 40 3 (neutrophil) or 100 3 (macrophage) objective fields of the lung parenchyma in each lung section were examined by light microscopy for the presence of neutrophils or macrophages. This assessment was performed in a blinded fashion. The cumulative scores for each animal were expressed as the number of positive fields per 100 fields (%).

Cytokine Assay

A total of 25 mL serum from each mouse was adopt for cytokine analysis with Bio-Plex Pro Mouse Cytokine Grp I Panel 23-Plex (BIO-RAD) according to the manufacturer's instructions. The data were collected on Luminex 200 and analyzed by Luminex PONENT (Thermo Fisher).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were carried out using Prism software (GraphPad Prism 7.0). Data of semiquantitative analysis of the H&E-stained lung sections are presented as mean, and the statistical significance between the WT and hACE2 mice was assessed by Mann Whitney test of Student's t tests. The other data are presented as mean ± sem, and the statistical significance of IHC staining analysis between the WT and hACE2 mice was assessed by unpaired Student's t tests. Before the statistical significance was assessed, Shapiro-Wilk normality test was used to determine whether the data met normal distribution. Statistical details of experiments and animal replication numbers (n) are stated in the relevant figure legends and method details. A p value less than 0.05 is considered statistically significant.

Safety Update: COVID-19 Convalescent Plasma in 20,000 Hospitalized Patients

Michael J. Joyner[1#*], MD, Katelyn A. Bruno[2#], PhD, Stephen A. Klassen[1#], PhD, Katie L. Kunze[3#], PhD, Patrick W. Johnson[4#], BS, Elizabeth R. Lesser[4#], MS, Chad C. Wiggins[1#], PhD, Jonathon W. Senefeld[1#], PhD, Allan M. Klompas[1#], MB, BCh, BAO, David O. Hodge[4], MS, John R.A. Shepherd[1], MD, Robert F. Rea[5], MD, Emily R. Whelan[2], BS, Andrew J. Clayburn[1], BS, Matthew R. Spiegel[4], BS, Sarah E. Baker[1], PhD, Kathryn F. Larson[1], MD, Juan G. Ripoll[1], MD, Kylie J. Andersen[1], BS, Matthew R. Buras[3], MS, Matthew N.P. Vogt[1], MD, Vitaly Herasevich[1], MD, PhD, Joshua J. Dennis[1], BS, Riley J. Regimbal[1], BS, Philippe R. Bauer[6], MD, PhD, Janis E. Blair[7], MD, Camille M. Van Buskirk[8], MD, Jeffrey L. Winters[8], MD, James R. Stubbs[8], MD, Noud van Helmond[9], MD, Brian P. Butterfield[1], BS, Matthew A. Sexton[1], MD, Juan C. Diaz Soto[1], MD, Nigel S. Paneth[10,11†], MD, MPH, Nicole C. Verdun[12†], MD, Peter Marks[12†], MD, PhD, Arturo Casadevall[13†], MD, PhD, DeLisa Fairweather[2†], PhD, Rickey E. Carter[4†], PhD, R. Scott Wright[5,14†], MD

[#]Denotes co-first authors (MJJ, KAB, SAK, KLK, PWJ, ERL, CCW, JWS, AMK)
[*]Denotes corresponding author (MJJ)
[†]Denotes co-senior authors (NSP, NCV, PM, AC, DF, REC, RSW)

Affiliations:
[1]Department of Anesthesiology and Perioperative Medicine, Mayo Clinic, Rochester, Minnesota
[2]Department of Cardiovascular Medicine, Mayo Clinic, Jacksonville, Florida
[3]Department of Health Sciences Research, Mayo Clinic, Scottsdale, Arizona
[4]Department of Health Sciences Research, Mayo Clinic, Jacksonville, Florida
[5]Department of Cardiovascular Medicine, Mayo Clinic, Rochester, Minnesota
[6]Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, Mayo Clinic, Rochester, Minnesota
[7]Department of Internal Medicine, Division of Infectious Diseases, Mayo Clinic, Phoenix, Arizona
[8]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota
[9]Department of Anesthesiology, Cooper Medical School of Rowan University, Cooper University Health Care, Camden, New Jersey
[10]Department of Epidemiology and Biostatistics, College of Human Medicine, Michigan State University, East Lansing, Michigan
[11]Department of Pediatrics and Human Development, College of Human Medicine, Michigan State University, East Lansing, Michigan
[12]Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, Maryland
[13]Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland
[14]Director Human Research Protection Program, Mayo Clinic, Rochester, Minnesota

Journal Pre-Proof

 © 2020 Mayo Foundation for Medical Education and Research.

Abstract

Objective: To provide an update on key safety metrics after transfusion of convalescent plasma in hospitalized COVID-19 patients, having previously demonstrated safety in 5,000 hospitalized patients.

Patients and Methods: From April 3 to June 2, 2020, the US FDA Expanded Access Program for COVID-19 convalescent plasma transfused a convenience sample of 20,000 hospitalized patients with COVID-19 convalescent plasma.

Results: The incidence of all serious adverse events was low; these included transfusion reactions (*n*=89; <1%), thromboembolic or thrombotic events (*n*=87; <1%), and cardiac events (*n*=680, ~3%). Notably, the vast majority of the thromboembolic or thrombotic events (*n*=55) and cardiac events (*n*=562) were judged to be unrelated to the plasma transfusion *per se*. The seven-day mortality rate was 8.6% (8.2%, 9.0%), and was higher among more critically-ill patients relative to less ill counterparts, including patients admitted to the intensive care unit vs. not admitted (10.5% vs. 6.0%), mechanically ventilated vs. not ventilated (12.1% vs. 6.2%), and with septic shock or multiple organ dysfunction/failure vs. those without dysfunction/failure (14.0% vs. 7.6%).

Conclusion: These updated data provide robust evidence that transfusion of convalescent plasma is safe in hospitalized patients with COVID-19, and support the notion that earlier administration of plasma within the clinical course of COVID-19 is more likely to reduce mortality.

Journal Pre-Proof

© 2020 Mayo Foundation for Medical Education and Research.

Introduction

Coronavirus disease 2019 (COVID-19) continues to be a world-wide pandemic, and the number of deaths attributed to COVID-19 in the US at the time of this writing (~110,000) exceed that of any other nation in the world[1]. The overall case fatality rate for diagnosed COVID-19 ranges from about 4% to greater than 50%[2-6], with higher mortality rates observed in more critically ill patients. In response to the COVID-19 outbreak in the US and reportedly high case-fatality rates, the US Food and Drug Administration (FDA) in collaboration with the Mayo Clinic and national blood banking community developed a national Expanded Access Program (EAP) to collect and distribute COVID-19 convalescent plasma. Historical precedent indicates that human convalescent plasma is a viable option for mitigation and treatment of COVID-19[7, 8]. The premise of human convalescent plasma therapy is that plasma of recently-infected and currently-recovered COVID-19 patients contains anti-viral antibodies and other bioactive elements that can be used to treat patients with COVID-19. Convalescent plasma has a strong historical record of some efficacy during acute infectious pandemics[7, 9]. As recently summarized[7], convalescent plasma represents a promising treatment strategy with strong historical precedence, biological plausibility, and limited barriers for rapid development and deployment of this investigational therapy.

Recently, our investigation of key safety indicators in 5,000 patients transfused with COVID-19 convalescent plasma demonstrated an incidence of transfusion-related serious adverse events (SAE) of less than 1% and a mortality rate of 14.9%[10]. These early indicators suggest that transfusion of convalescent plasma is safe in hospitalized adults with COVID-19. Because an additional 15,000 hospitalized patients have been transfused with convalescent plasma under the purview of the EAP, a subsequent safety update is warranted. These new data may provide novel insights into the incidence of emerging adverse events associated with COVID-19, including thromboembolic[11, 12] and cardiac events[13]. Further, these data may provide better understanding of the clinical features contributing to the seven-day mortality rate among transfused patients with COVID-19.

Thus, we analyzed key safety metrics following transfusion of convalescent plasma in 20,000 hospitalized adults with severe or life-threatening COVID-19. These data represent a deeper and larger analysis relative to our initial report of 5,000 transfused patients under the EAP. We hypothesized that both the seven-day mortality rate and the number of serious adverse events related to the transfusion of convalescent plasma would continue to be low. Additionally, we hypothesized that higher mortality rates would be observed in more critically-ill patients.

Methods

As described previously[10], the program is a FDA-initiated, national, multicenter, open-label EAP in hospitalized adults that had (or were judged to have high risk of progression to) severe or life-threatening COVID-19. The US COVID-19 Convalescent Plasma EAP was conducted as a pragmatic treatment study, empowering local acute care facilities to use the emerging best evidence for care while allowing for administration of convalescent plasma. It was conducted within a modified clinical trial framework: All participants were allowed access to convalescent plasma per the discretion of the treating physician/ principal investigator given the nature of the

Journal Pre-Proof

 © 2020 Mayo Foundation for Medical Education and Research.

pandemic and the lack of any effective therapies at the time of design. It was the intent *a priori* to create a control comparator group to determine potential efficacy using patients hospitalized with COVID-19 infections during the same time period. This decision was made after collaboration with the US FDA. This report is only on safety of the convalescent plasma for the initial 20,000 subjects. A future publication will discuss potential efficacy.

Between the date of initial Institutional Review Board (IRB) approval of the EAP (April 1st 2020) and June 11 2020, more than 20,000 patients were transfused with COVID-19 convalescent plasma, **Figure 1**. The Mayo Clinic IRB served as the central IRB and empaneled an independent Data and Safety Monitoring Board (DSMB) to oversee the safety analyses. Written informed consent was obtained from the participant or a legally-authorized representative prior to enrollment, or with use of emergency consent procedures recommended by the US FDA and approved by the Mayo Clinic IRB. The protocol was modified to allow inclusion of incarcerated participants once it became clear that this group was especially vulnerable and at risk.

Participants. Eligible patients were aged 18 years or older, hospitalized with a laboratory confirmed diagnosis of infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and had (or were judged by a healthcare provider to be at high risk of progression to) severe or life-threatening COVID-19. The clinical symptoms defining severe or life-threatening COVID-19 are outlined in **Table 1**.

Procedures. As described previously[10], ABO-compatible COVID-19 convalescent plasma had no minimum neutralizing-antibody titer level and was donated by recently-recovered, COVID-19 survivors. Approximately 200 – 500 mL of convalescent plasma was administered intravenously according to institutional transfusion guidelines. Web-based, standardized data reporting surveys were completed to assess clinical status of patients at regular time intervals (four-hours and seven-days after convalescent plasma transfusion) using the Research Electronic Data Capture system (REDCap, v.9.1.15 Vanderbilt University, Nashville, TN)[14, 15].

Serious Adverse Event Reporting. Separate REDCap data collection forms were used to report each SAE that occurred within seven days following the convalescent plasma transfusion. Two primary data capture forms were used to report SAEs: a transfusion form and a SAE form. Serious adverse events which occurred during the time window beginning at the onset of the plasma transfusion and including the four-hour time-period following the transfusion were reported on the transfusion form. By definition, the primary SAEs related to transfusion (including transfusion associated circulatory overload [TACO] and transfusion-related acute lung injury [TRALI]) occurred within six hours of the transfusion. All transfusion-related SAEs occurred within four hours of the transfusion and thus, were all reported on the transfusion form. All other SAEs were reported on the SAE form. The attribution scale used by treating physicians for evaluating SAE relatedness to convalescent plasma transfusion included unrelated, possibility related, probably related, or definitely related. All transfusion-related SAEs were independently adjudicated over the course of the study by the IND Sponsor (MJJ) and trained designee (AMK) using the National

Journal Pre-Proof

 © 2020 Mayo Foundation for Medical Education and Research.

Healthcare Safety Network Biovigilance Component Hemovigilance Module Surveillance Protocol as a conceptual framework[16].

Statistics. Data presented in this safety report may undergo additional data quality control measures as the study continues. The cumulative incidence of each of a series of SAEs was summarized using a point estimate and 95% score confidence interval (CI), as outlined in **Table 2**. To assess mortality, time (in days) between transfusion and mortality was examined using the Kaplan Meier product limit estimator. Participants were censored at their last known vital status and all reported mortalities through seven days were used to estimate the survival function. Data were censored at 0.25 days for patients who did not have follow-up beyond the initial report at four hours post transfusion at time of the analysis. For patients who expired within 24 hours, a survival time of 0.5 days was assigned. Precise time of day for key events was not recorded in the data collection system; thus, these imprecise time estimates were used. The point estimate and 95% CI for mortality were estimated at day seven based on the estimated survival function. All analyses and graphics were produced with R version 3.6.2 (Vienna, Austria).

Results

From April 3 to June 11, 2020, a total of 30,117 patients were enrolled in the EAP and a total of 21,987 enrolled patients received a COVID-19 convalescent plasma transfusion (**Figure 1**). Data from the first 5,000 transfused patients have been reported previously[10]. This update reports data from 20,000 patients including the initial 5,000 and subsequent 15,000 transfused patients. By June 2, 2020, a total of 20,000 patients had been transfused with COVID-19 convalescent plasma, thus, 7-day mortality data is presented for all 20,000 patients.

Patient Characteristics. Key patient characteristics are presented in **Table 1**. The patient enrollment indicates a wide age range of hospitalized patients with COVID-19, consistent with prior CDC published data[17]. The study population was diverse, with 20% of patients being African American, nearly 35% Hispanic and 5% Asian. The recruitment of a diverse population has improved over the time course of the study. Nearly 40% of our subjects were women. Most of the patients enrolled were overweight or obese, consistent with early reports of risk[18]. Nearly all of the patients had severe or life-threatening COVID-19, by design of the investigational protocol. Nearly two-thirds had respiratory failure as well as dyspnea as a primary symptom. Most were hypoxic and nearly half had pulmonary infiltrates. At least one-third had severe respiratory compromise. Equal numbers appeared to have multi-organ failure or septic shock, but these were a small percentage of the total population.

Serious Adverse Events. Key serious adverse events (SAE) related to the transfusion of convalescent plasma are reported in **Table 2**. Our report is not a comprehensive summary of all risks associated with hospitalization of COVID-19 but did assume that convalescent plasma potentially could cause life-threatening cardiac events and thrombotic events, so these were collected with an underlying assumption of attribution. Within four hours of completion of the COVID-19 convalescent plasma transfusion, 146 SAEs classified as transfusion reactions were reported (<1% of all

Journal Pre-Proof

 © 2020 Mayo Foundation for Medical Education and Research.

transfusions). Of these SAEs, there were 83 non-mortality events reported, with 37 reports of transfusion-associated circulatory overload (TACO), 20 reports of transfusion-related acute lung injury (TRALI), and 26 reports of severe allergic transfusion reaction. Of the SAEs reported within four hours of plasma transfusion, there were 63 mortalities (0.3% of all transfusions) and 13 of these mortalities were judged as related (possibly, *n*=12; probably, *n*=1; definitely, *n*=0) to the transfusion of COVID-19 convalescent plasma.

Within seven days of completion of the COVID-19 convalescent plasma transfusion, 1,136 other SAEs were reported. Of these SAEs, 87 thromboembolic or thrombotic events were reported, 406 sustained hypotensive events requiring intravenous pressor support were reported, and 643 patients suffered a cardiac event. Notably, the vast majority of the thromboembolic or thrombotic complications (*n*=55) and cardiac events (*n*=569) were judged to be unrelated to the plasma transfusion.

Seven-day Mortality. Seven-day mortality rates stratified by key clinical status indicators are presented in **Table 2**. Over the first seven days after the COVID-19 convalescent plasma transfusion, a total of 1,711 deaths were observed. The overall seven-day mortality rate was 8.56% (95% CI: 8.18%, 8.95%), **Figure 2**. The seven-day mortality rate was higher among the sickest of our critically-ill patients, including patients *admitted* to the intensive care unit (ICU) vs. not admitted to the ICU (10.5% vs. 6.0%), mechanically ventilated vs. not mechanically ventilated (12.1% vs. 6.2%), and those with septic shock or multiple organ dysfunction/failure vs. without septic shock or multiple organ dysfunction/failure (14.0% vs. 7.6%).

Discussion

In this safety update of the US Convalescent Plasma Expanded Access Program of 20,000 hospitalized patients in the US with severe or life-threatening COVID-19, the overall frequency of SAEs classified as attributable or likely secondary to convalescent plasma transfusion continued to be low (<1% of all transfusions) and the seven-day mortality rate in this extremely high risk cohort was 8.6%. Despite the potential risks associated with plasma transfusion in critically-ill patients[16, 19], these data provide continued optimism for the safety of COVID-19 convalescent plasma.

Emerging COVID-19 Clinical Complications. Although thrombotic and thromboembolic events are emerging clinical complications of COVID-19[11, 12, 20], our data demonstrate a low rate (<1%) of these events within the first seven days after COVID-19 convalescent plasma transfusion. Cardiac events represent another novel clinical concern of COVID-19[13], particularly in the context of open-label use of experimental treatments such as hydroxychloroquine [21]. In aggregate, adverse cardiac events occurred in ~3% of patients transfused with COVID-19 convalescent plasma. The vast majority of adverse cardiac events of interest were deemed unrelated to the plasma transfusion (88%) by the treating physicians. Collectively, these data suggest that transfusion of COVID-19 convalescent plasma *per se* does not demonstrably increase the risk of adverse cardiac events. We note that the incidence of TRALI and TACO reported in this study of 0.18% and 0.10%, respectively is significantly lower than those reported in previous studies, which ranged from 5-8% and 1-4%, respectively[22],

Journal Pre-Proof

 © 2020 Mayo Foundation for Medical Education and Research.

despite the fact that many of the COVID-19 patients receiving plasma were critically ill and likely at risk for clinical conditions which mimic TACO.

Limitations. Although this study was not designed to evaluate efficacy of convalescent plasma, we note with optimism that after 20,000 transfused patients the seven-day mortality rate (8.6%) is lower than the mortality rate observed in the first 5,000 transfused patients (12.0%), **Table 2**. While the mortality rate has fallen (**Figure 2**), we note that the clinical characteristics of the transfused patients in the EAP have shifted toward less critically-ill patients and lower proportions of apparent "rescue therapy". No therapy has been introduced into clinical use during this time period which reduces (to our knowledge) mortality in hospitalized patients with COVID-19. We postulate that several potential explanations may explain the observed decline in mortality. The ability and success of the US Healthcare community at managing hospitalized COVID-19 patients is likely improving. Second, the blood banking communities have enhanced the availability of convalescent plasma such that more patients received plasma earlier in their hospital course compared to the initial cohort of 5000 participants. The more expeditious delivery of plasma to patients reflects improved logistics for the collection, deployment and use of convalescent plasma, and a larger COVID-19 recovered population that may be potential plasma donors. In this regard it is remarkable that there was no system in place for convalescent plasma use in March 2020 and yet within months the nation is now able to meet most of the demand, despite complex logistics[23]. Given the historical experience that antibody therapies are most effective when given earlier and that convalescent plasma has reduced mortality in prior epidemics[7], the lower mortality in more recently treated patients would be consistent with greater efficacy from earlier use. Finally, as recovered COVID-19 patients were more quickly recruited for convalescent plasma donation, the plasma may contain higher levels of neutralizing antibodies or other bioactive elements. However, additional data are required to evaluate these potential explanations for the observed trends.

Conclusion

Data from the first 20,000 patients transfused with COVID-19 convalescent plasma demonstrate that use of convalescent plasma is safe and carries no excess risk of complications. Indeed, convalescent plasma may be associated with improvement in survival, however, this report does not establish efficacy. Additionally, our data demonstrate that the US Health care system is improving in its care for those hospitalized for COVID-19 including managing those critically-ill patients with multiple comorbidities included in these analyses. Overall, the mortality observed has fallen with our observations and continued use of convalescent plasma. Given the accelerating deployment of this therapy, these emerging data provide early safety indicators of convalescent plasma for COVID-19 treatment and suggest research should shift focus from safety toward determining the efficacy of convalescent plasma.

Journal Pre-Proof

© 2020 Mayo Foundation for Medical Education and Research.

Acknowledgements: We thank the dedicated members of the US Convalescent Plasma Expanded Access Program team— Machiko Anderson, Supriya Behl, Lori Bergstrom, Brian Butterfield, Grant Dubbels, Adam Eggert, Ree Erickson, Rebekah Frost, Daniel Gaz, Winston Guo, Starr Guzman, Karina Hex, Vidhu Joshi, Megan Knudson, Tessa Kroeninger, Frances Lynch, Tim Miksch, Lisa Muenkel, Ryan Oldenburg, Amy Olofson, Laura Pacheco-Spann, Dr. Kelly Paulson, Dr. Sumedha Penheiter, Melanie Peterson, Katrina Pierce, Nicloas Saikali, Jeffrey Schmoll, Pamela Skaran, Lindsay Stromback, Edward Swaray, Morgan Swope, Kristine Tree, Joe Wick, Janelle Worthington. We thank the members of the Mayo Clinic Institutional Review Board, the Mayo Clinic Office of Human Research Protection, the Mayo Clinic Office of Research Regulatory support and in particular Mark Wentworth, the Executive Dean of Research at Mayo Clinic Dr. Gregory Gores and the CEO of Mayo Clinic Dr. Gianrico Farrugia for their support and assistance, and the independent Data and Safety Monitoring Board for their work and oversight of the Expanded Access Program— Dr. Allan S. Jaffe (chair), Dr. David O. Warner, Dr. William G. Morice II, Dr. Paula J. Santrach, Dr. Robert L. Frye, Dr. Lawrence J Appel, Dr. Taimur Sher. We thank the members of the National COVID-19 Convalescent Plasma Project (http://ccpp19.org) for their intellectual contributions and support. We thank the participating medical centers and medical teams, and blood centers for their rigorous efforts necessary to make this program possible. We also thank the donors for providing COVID-19 convalescent plasma.

Abbreviations and Acronyms: **COVID-19 =** coronavirus disease 2019; **DSMB =** Data and Safety Monitoring Board; **EAP** = Expanded Access Program; **FDA =** Food and Drug Administration; **ICU** = intensive care unit; **IND =** Investigational New Drug; **IRB =** Institutional Review Board; **SAE =** serious adverse event; **SARS-CoV-2** = severe acute respiratory syndrome coronavirus-2; **TACO =** transfusion-associated circulatory overload; **TRALI =** transfusion-related acute lung injury; **US =** United States

Grant Support: This study was supported in part by a US Department of Health and Human Services (HHS), Biomedical Advanced Research and Development Authority (BARDA) contract 75A50120C00096 (to MJJ), National Center for Advancing Translational Sciences (NCATS) grant UL1TR002377, National Heart, Lung, and Blood Institute (NHLBI) grant 5R35HL139854 (to MJJ), National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) 5T32DK07352 (to JWS and CCW), Natural Sciences and Engineering Research Council of Canada (NSERC) PDF-532926-2019 (to SAK), National Institute of Allergy and Infectious Disease (NIAID) grants R21 AI145356 and R21 AI152318 (to DF), R01 AI152078 9 (to AC), National Heart Lung and Blood Institute RO1 HL059842 (to AC), National Institute of Aging (NIA) U54AG044170 (to SEB), Schwab Charitable Fund (Eric E Schmidt, Wendy Schmidt donors), United Health Group, National Basketball Association (NBA), Millennium Pharmaceuticals, Octapharma USA, Inc, and the Mayo Clinic.

Potential Competing Interests: The authors report no competing interests.

Correspondence: Address to Michael J. Joyner, MD. Mayo Clinic, 200 First St SW, Joseph Building 4-184, Rochester, MN 55905.

Journal Pre-Proof

 © 2020 Mayo Foundation for Medical Education and Research.

References

1. Centers for Disease Control and Prevention. Coronavirus disease 2019 (COVID-19): cases in US.
2. Rajgor DD, Lee MH, Archuleta S, Bagdasarian N, Quek SC. The many estimates of the COVID-19 case fatality rate. *Lancet Infect Dis.* 2020.
3. Wang K, Zhang Z, Yu M, Tao Y, Xie M. 15-day mortality and associated risk factors for hospitalized patients with COVID-19 in Wuhan, China: an ambispective observational cohort study. *Intensive Care Med.* 2020.
4. Yang X, Yu Y, Xu J, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. *Lancet Respir Med.* 2020;8:475-481.
5. Richardson S, Hirsch JS, Narasimhan M, et al. Presenting Characteristics, Comorbidities, and Outcomes Among 5700 Patients Hospitalized With COVID-19 in the New York City Area. *JAMA.* 2020.
6. Bhatraju PK, Ghassemieh BJ, Nichols M, et al. Covid-19 in Critically Ill Patients in the Seattle Region - Case Series. *N Engl J Med.* 2020.
7. Casadevall A, Pirofski LA. The convalescent sera option for containing COVID-19. *J Clin Invest.* 2020;130:1545-1548.
8. Zhang JS, Chen JT, Liu YX, et al. A serological survey on neutralizing antibody titer of SARS convalescent sera. *J Med Virol.* 2005;77:141-150.
9. Luke TC, Kilbane EM, Jackson JL, Hoffman SL. Meta-analysis: convalescent blood products for Spanish influenza pneumonia: a future H5N1 treatment? *Ann Intern Med.* 2006;145:599-609.
10. Joyner M.J. et al. Early Safety Indicators of COVID-19 Convalescent Plasma in 5,000 Patients. *Journal of Clinical Investigation.* 2020;pre-print.
11. Wright FL, Vogler TO, Moore EE, et al. Fibrinolysis Shutdown Correlates to Thromboembolic Events in Severe COVID-19 Infection. *J Am Coll Surg.* 2020.
12. Wichmann D, Sperhake JP, Lutgehetmann M, et al. Autopsy Findings and Venous Thromboembolism in Patients With COVID-19. *Ann Intern Med.* 2020.
13. Inciardi RM, Lupi L, Zaccone G, et al. Cardiac Involvement in a Patient With Coronavirus Disease 2019 (COVID-19). *JAMA Cardiol.* 2020.
14. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)--a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform.* 2009;42:377-381.
15. Harris PA, Taylor R, Minor BL, et al. The REDCap consortium: Building an international community of software platform partners. *J Biomed Inform.* 2019;95:103208.
16. U.S. Centers for Disease Control and Prevention. The National Healthcare Safety Network (NHSN) Manual: Biovigilance Component v2.5. Atlanta, GA: Division of Healthcare Quality Promotion, National Center for Emerging and Zoonotic Infectious Diseases.
17. Garg S. Hospitalization rates and characteristics of patients hospitalized with laboratory-confirmed coronavirus disease 2019—COVID-NET, 14 States, March 1–30, 2020. *MMWR. Morbidity and mortality weekly report.* 2020;69.
18. Goyal P, Choi JJ, Pinheiro LC, et al. Clinical Characteristics of Covid-19 in New York City. *N Engl J Med.* 2020;382:2372-2374.
19. Dzik S. COVID-19 Convalescent Plasma: Now Is the Time for Better Science. *Transfus Med Rev.* 2020.
20. Klok FA, Kruip M, van der Meer NJM, et al. Confirmation of the high cumulative incidence of thrombotic complications in critically ill ICU patients with COVID-19: An updated analysis. *Thromb Res.* 2020.

Journal Pre-Proof

© 2020 Mayo Foundation for Medical Education and Research.

21. Giudicessi JR, Noseworthy PA, Friedman PA, Ackerman MJ. Urgent guidance for navigating and circumventing the QTc-prolonging and torsadogenic potential of possible pharmacotherapies for coronavirus disease 19 (COVID-19). *Mayo Clinic Proceedings*: Elsevier; 2020.
22. Roubinian N. TACO and TRALI: biology, risk factors, and prevention strategies. *Hematology Am Soc Hematol Educ Program.* 2018;2018:585-594.
23. Bloch EM, Shoham S, Casadevall A, et al. Deployment of convalescent plasma for the prevention and treatment of COVID-19. *J Clin Invest.* 2020.

Journal Pre-Proof

 © 2020 Mayo Foundation for Medical Education and Research.

Table 1. Patient Characteristics Stratified by Month of COVID-19 Convalescent Plasma Transfusion.

Characteristic	April n (%)	May n (%)	June n (%)	Total n (%)
No.	6,216	13,357	427	20,000
Age				
18 - 39 years	450 (7.2%)	1,044 (7.8%)	35 (8.2%)	1,529 (7.6%)
40 - 59 years	2,060 (33.2%)	4,157 (31.1%)	138 (32.3%)	6,355 (31.8%)
60 - 69 years	1,802 (29.0%)	3,516 (26.3%)	105 (24.6%)	5,423 (27.1%)
70 - 79 years	1,249 (20.1%)	2,776 (20.8%)	89 (20.8%)	4,114 (20.6%)
80 years or older	650 (10.5%)	1,858 (13.9%)	60 (14.1%)	2,568 (12.8%)
Gender				
Women	2,254 (36.3%)	5,360 (40.1%)	163 (38.2%)	7,777 (38.9%)
Men	3,935 (63.3%)	7,953 (59.5%)	264 (61.8%)	12,152 (60.8%)
Intersex or Transgender	21 (0.3%)	33 (0.2%)	0 (0.0%)	54 (0.3%)
Undisclosed	6 (0.1%)	11 (0.1%)	0 (0.0%)	17 (0.1%)
Weight Status[a]				
Underweight	99 (1.3%)	236 (1.8%)	15 (3.5%)	310 (1.7%)
Normal Weight	828 (17.6%)	2,356 (17.8%)	62 (14.6%)	3,246 (17.7%)
Overweight	1,413 (30.1%)	3,695 (28.0%)	120 (28.2%)	5,228 (28.5%)
Obese	2,397 (51.0%)	6,927 (52.4%)	228 (53.6%)	9,552 (52.1%)
Race				
Asian	408 (6.6%)	580 (4.3%)	18 (4.2%)	1,006 (5.0%)
Black	1,127 (18.1%)	2,690 (20.1%)	94 (22.0%)	3,911 (19.6%)
White	2,988 (48.1%)	6,541 (49.0%)	214 (50.1%)	9,743 (48.7%)
Other or Unknown	1,693 (27.2%)	3,546 (26.5%)	101 (23.7%)	5,340 (26.7%)
Ethnicity				
Hispanic or Latino	2,140 (34.4%)	4,602 (34.5%)	162 (37.9%)	6,904 (34.5%)
Not Hispanic or Latino	4,075 (65.6%)	8,755 (65.5%)	265 (62.1%)	13,095 (65.5%)
Clinical Status				
Current severe or life-threatening COVID-19	4,960 (79.8%)	8,990 (67.3%)	270 (63.2%)	14,220 (71.1%)
High risk of severe or life-threatening COVID-19	1,256 (20.2%)	4,367 (32.7%)	157 (36.8%)	5,780 (28.9%)
Intensive Care Unit (ICU) admission	4,027 (64.9%)	7,270 (55.0%)	171 (48.3%)	11,468 (57.9%)
Mechanical Ventilation[b]	2,291 (46.7%)	3,977 (30.1%)	69 (19.5%)	6,337 (34.3%)

Journal Pre-Proof

© 2020 Mayo Foundation for Medical Education and Research.

Clinical symptoms[c]				
Respiratory failure	3,579 (72.2%)	5,969 (66.4%)	167 (61.9%)	9,715 (68.3%)
Dyspnea	3,131 (63.1%)	6,326 (70.4%)	203 (75.2%)	9,660 (67.9%)
Blood oxygen saturation ≤ 93%	3,083 (62.2%)	6,421 (71.4%)	199 (73.7%)	9,703 (68.2%)
Lung infiltrates > 50% within 24 to 48 hours	2,106 (42.5%)	3,875 (43.1%)	94 (34.8%)	6,075 (42.7%)
Respiratory frequency ≥ 30/min	1,928 (38.9%)	3,838 (42.7%)	118 (43.7%)	5,884 (41.4%)
P_aO_2:FiO_2 ratio[d] < 300	1,649 (33.2%)	2,901 (32.3%)	78 (28.9%)	4,628 (32.5%)
Multiple organ dysfunction or failure	938 (18.9%)	1,143 (12.7%)	36 (13.3%)	2,117 (14.9%)
Septic shock	733 (14.8%)	949 (10.6%)	19 (7.0%)	1,701 (12.0%)

[a] These data include a subset of the sample (*n* = 18,336).
[b] These data include a subset of the sample (*n* = 18,484).
[c] These data include a subset of the sample (*n* = 14,220), only those patients that currently have severe or life-threatening COVID-19.
[d] The ratio of partial pressure of arterial oxygen to fraction of inspired oxygen ratio.

Journal Pre-Proof

© 2020 Mayo Foundation for Medical Education and Research.

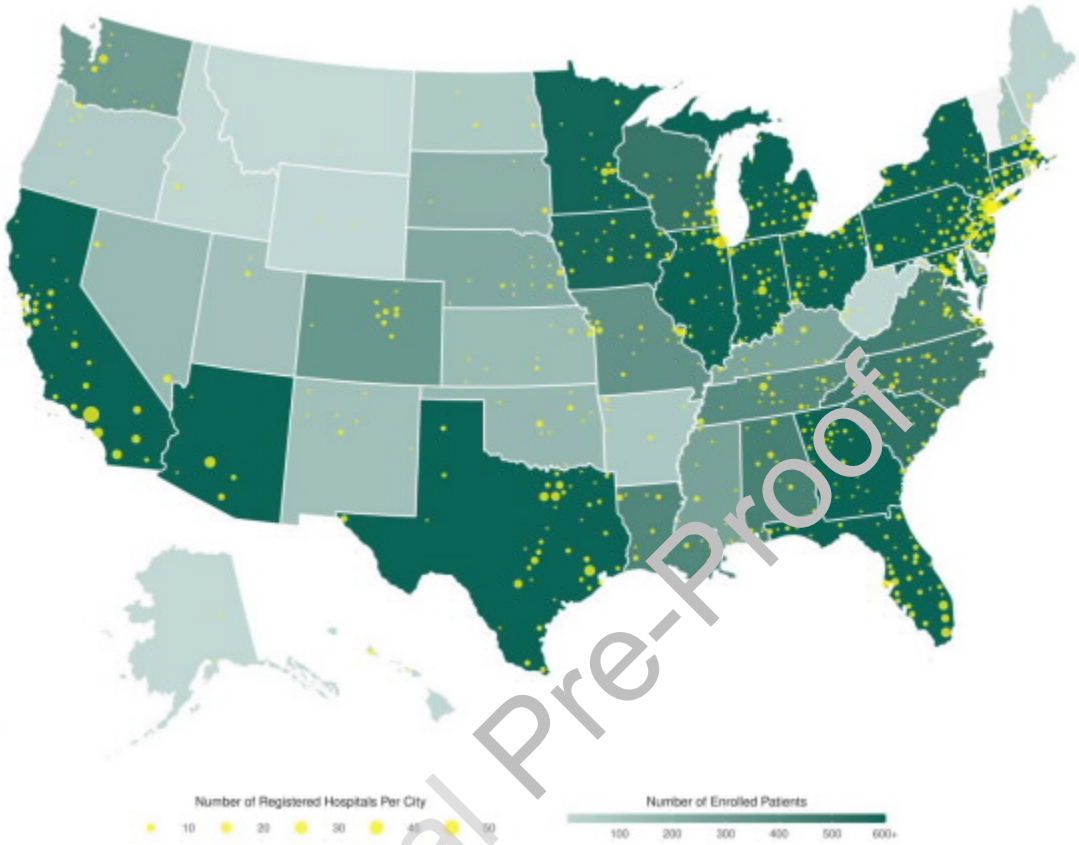
Table 2. SAE Characteristics in Patients Transfused with COVID-19 Convalescent Plasma. (*n* = 20,000)			
Serious Adverse Events (SAE): Transfusion Reactions	**Reported**	**Related**	**Estimate[a] (95% CI)**
Mortality within four hours of transfusion	63	13	0.06% (0.04%, 0.11%)
Transfusion-Associated Circulatory Overload (TACO)	37	37	0.18% (0.13%, 0.25%)
Transfusion-Related Acute Lung Injury (TRALI)	20	20	0.10% (0.06%, 0.15%)
Severe allergic transfusion reaction	26	26	0.13% (0.09%, 0.19%)
Seven-day SAE Reports			
Thrombolic or thromboembolic complication	87	32	0.16% (0.11%, 0.23%)
Sustained Hypotension[b]	406	54	0.27% (0.21%, 0.35%)
Cardiac Events[c]	643	74	0.37% (0.29%, 0.46%)
Seven Day Mortality	**Reported**		**Estimate (95% CI)**
Overall (5,000)[d]	602		12.04% (11.17%, 12.97%)
Overall (20,000)	1,711		8.56% (8.18%, 8.95%)
Clinical Status			
No ICU admission (*n* = 8,323)	501		6.02% (5.53%, 6.55%)
ICU admission (*n* = 11,468)	1,202		10.48% (9.93%, 11.06%)
No Mechanical Ventilation (*n* = 12,147)	749		6.17% (5.75%, 6.61%)
Mechanical Ventilation (*n* = 6,337)	767		12.10% (11.32%, 12.93%)
Clinical Symptoms			
No MOF or Septic Shock (*n* = 17,081)	1,302		7.62% (7.23%, 8.03%)
MOF or Septic Shock (*n* = 2,919)	409		14.01% (12.80%, 15.32%)

Abbreviations: ICU, intensive care unit; MOF, multiple organ failure or dysfunction.

[a] Point estimate of *related* serious adverse event incidence relative to 20,000 transfusions.
[b] Sustained hypotension included events requiring intravenous pressor support.
[c] Cardiac events included ventricular or atrial fibrillation or arrhythmia requiring treatment, and cardiac arrest.
[d] These data were previously published[10] and reported for comparison with current data.

Journal Pre-Proof

© 2020 Mayo Foundation for Medical Education and Research.



Journal Pre-Proof

Figure 1. Participation in the US COVID-19 Convalescent Plasma Expanded Access Program (EAP) including data extracted on June 11, 2020. Choropleth map displaying the number of cumulatively enrolled patients in the EAP within each state, with lower enrollment values displayed in a lighter hue of green and higher enrollment values displayed in a darker hue of green. Registered acute care facilities are represented as filled yellow circles, with larger circles indicating greater number of registered facilities within the metropolitan area of a city. The choropleth map does not display data from US territories, including registered facilities in Puerto Rico, Guam and Northern Mariana Islands.

© 2020 Mayo Foundation for Medical Education and Research.

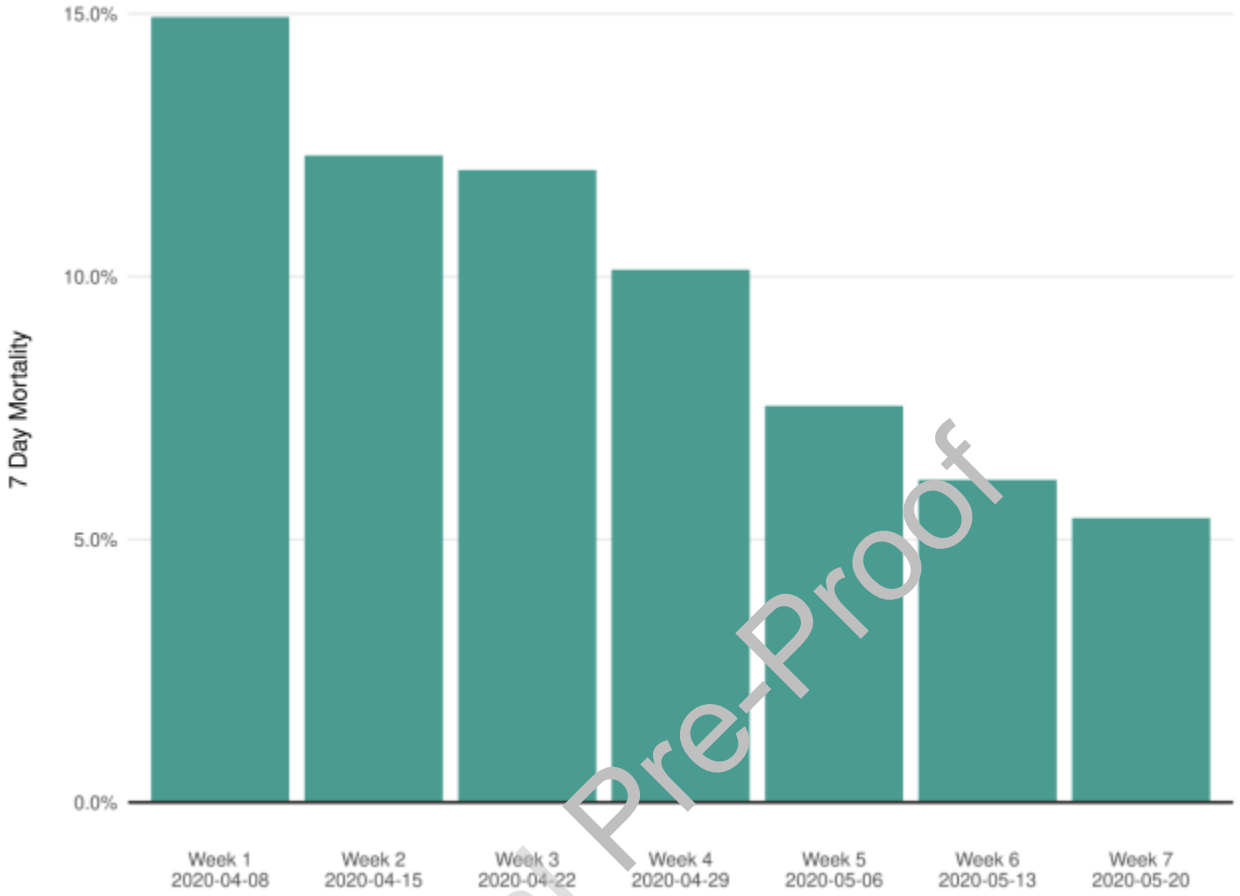


Figure 2. Seven-day Mortality rate in patients transfused with COVID-19 convalescent plasma stratified by week since initiation of the US COVID-19 Convalescent Plasma Expanded Access Program (EAP). Each green bar indicates the seven-day mortality rate stratified by the patients enrolled during a given week since the initiation of the EAP.

Journal Pre-Proof

© 2020 Mayo Foundation for Medical Education and Research.

COVID-19 Convalescent Plasma Project (CCPP19)

"No one cares how much you know until they know how much you care……"

MINUTES

Date: June 26, 2020	**3:00-4:00 PM (CDT)**	**Location: Teleconference**
Facilitators:	Michael Joyner, MD Arturo Casadevall, MD, PhD Nigel Paneth, MD, MPH	Mayo Clinic-Rochester Johns Hopkins Michigan State University
	Safety Update: COVID-19 Convalescent Plasma in 20,000 Hospitalized Patients | Mayo Proceedings: Joyner et al Safety Update Formatted Pr	**Resources:** Website: https://CCPP19.org (barebones for now with protocols and IND available for download) Facebook: https://www.facebook.com/CCPP19/ Twitter (CCPP19): https://twitter.com/CCPP_19 Twitter (Pre-Prints Page): https://twitter.com/SARSCoV192
Note Takers:	• Chad Wiggins, PhD • Stephen Klassen, PhD • Jonathan (Jack) Senefeld, PhD • John-Roger Shepherd, MD	

Introduction — **Arturo Casadevall, M.D., Ph.D.**

- The FDA commissioner testified before congress that convalescent plasma is safe and the signals of efficacy are encouraging
- The second publication from the Mayo Clinic EAP of 20,000 transfused patients indicates that convalescent plasma is safe and there is no evidence of antibody-dependent enhancement (ADE) of COVID-19 (see PDF attached above)

Blood Bank and Transfusion Medicine — **Evan Bloch, M.D.**

- Convalescent plasma is being shared across state lines and amongst blood centers

BARDA Funding — **Ruchika Goel M.D., and Dan Waxman, M.D.**

- BARDA funding is confirmed and will provide an additional $202 million in funding to support ~200,000 additional units of convalescent plasma
- This new BARDA funding will support convalescent plasma that is collected and stockpiled

Clinical Trials — **Shmuel Shoham, M.D.**

- Several positive reports on the regulatory and funding front
  - The FDA has loosened exclusion criteria for randomized-controlled trials, enabling the recruitment of individuals with some co-morbidities
  - Federal funding has increased to allow a larger number of sites to participate in randomized-controlled trials
  - There is nation-wide participation in randomized-controlled trials including sites in Houston, southern California, and Alabama etc. which are currently experiencing COVID-19 spikes

Pediatrics — **Flor Munoz-Rivas, M.D.**

- Convalescent plasma is being used in pediatric cases, although sporadically — most sites have transfused <10 patients
- There are seven sites participating in the Children's National Hospital convalescent plasma study registry
- The main concern in pediatric trials continues to be the lack of funding

Communications — **Nigel Paneth, M.D., M.P.H.**

- There are 20+ sites participating in the Mayo Clinic EAP treatment-control study that will investigate the efficacy of convalescent plasma. For those participating or interested, please contact us if you have any questions about the process including questions about uploading de-identified data from your site to Mayo

	• servers (paneth@msu.edu)
Final Word	**Arturo Casadevall, M.D., Ph.D.**

	• *There is a constellation of information suggesting convalescent plasma has potential to be a promising therapy for COVID-19*
	  ○ New BARDA funding for convalescent plasma collection ($202 million)
	  ○ The Department of Defense has funded sites to investigate the efficacy of convalescent plasma for outpatient treatment
	  ○ New loosened restrictions on exclusion criteria for RCT enrollment
	  ○ The FDA commissioner provided very encouraging support for convalescent plasma in his Congressional testimony

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Board on Health Sciences Policy

HOST GENOMICS RESEARCH AND DATA COLLECTION EFFORTS RELATED TO COVID-19

Joint Meeting of the
Roundtable on Genomics and Precision Health
and the
Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats

Virtual Meeting July 1, 2020
Zoom: https://nasem.zoom.us/j/95540901323

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Board on Health Sciences Policy

Host Genomics Research and Data Collection Efforts Related to COVID-19

July 1, 2020

Table of Contents

Agenda 3

Agenda 4

ROUNDTABLE AND STANDING COMMITTEE INFORMATION 7

Roundtable on Genomics and Precision Health 8

Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats 10

MEETING INFORMATION 13

Speaker and Chair Biographies 14

BACKGROUND MATERIALS 17

Online links to background materials 18

Rapid Expert Consultations on the COVID-19 Pandemic 20

A blood-based host gene expression assay for early detection of viral infection 21

AGENDA

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Board on Health Sciences Policy

Host Genomics Research and Data Collection Efforts Related to COVID-19

Joint Meeting
of the
Roundtable on Genomics and Precision Health
and the
Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats

A VIRTUAL MEETING
July 1, 2020
1:00 PM – 2:45 PM ET
Zoom Link: https://nasem.zoom.us/j/95540901323

Aims for the meeting:

- To understand the scientific challenges that the field of genomics is facing
  - What has been the effect of the pandemic on genomics research and data collection and sharing? What is the role of the genomics community during the pandemic? What research is underway? What research still needs to be done?
  - What gaps and challenges in the field is this crisis highlighting? What unique contributions of the field are not being leveraged effectively?
- To explore innovative approaches to overcoming those challenges
  - What new approaches are you using to solve a problem? Could these be applied to the field of genomics? Are there lessons from genomics that are being used in light of the pandemic? What are the opportunities for connecting efforts and data sets (e.g. across institutions, genotype and phenotype data)?
- To discuss opportunities for the Genomics Roundtable and for the Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats to facilitate progress
  - Are there opportunities for the Roundtable and Standing Committee to act that align with strategic priorities?
  - Are the Roundtable and Standing Committee uniquely positioned to address specific challenges?

AGENDA:

1:00 p.m. ET Welcoming Remarks

GEOFFREY GINSBURG, *Roundtable Co-Chair*
Director, Duke Center for Applied Genomics & Precision Medicine
Professor, Medicine, Pathology, and Biomedical Engineering
Duke University Medical Center

MICHELLE PENNY, *Roundtable Co-Chair*
Vice President and Head of Genomics
Goldfinch Bio

HARVEY FINEBERG, *Standing Committee Chair*
President
Gordon and Betty Moore Foundation

1:15 p.m. **An Introduction to the Host Genetics of COVID-19**

MAXIMILIAN MUENKE
CEO
American College of Medical Genetics and Genomics (ACMG)

Understanding Host Genetics and Genomics Related to COVID-19 and Opportunities for Forming Data Sharing COVID-19 Communities

1:25 p.m. SHARON TERRY
Founder and CEO
Genetic Alliance

1:35 p.m. JOYCE TUNG
Vice President, Research
23andMe

1:45 p.m. GEOFFREY GINSBURG, *Roundtable Co-Chair*
Director, Duke Center for Applied Genomics & Precision Medicine
Professor, Medicine, Pathology, and Biomedical Engineering
Duke University Medical Center

1:55 p.m. **Discussion with Roundtable and Standing Committee Members**

2:30 p.m. **Next Steps**

Questions:

- What challenges or gaps were exposed today?
- Is there an area that should be incorporated into the Roundtable's strategic planning or the Standing Committee's work?

GEOFFREY GINSBURG, *Roundtable Co-Chair*
Director, Duke Center for Applied Genomics & Precision Medicine
Professor, Medicine, Pathology, and Biomedical Engineering
Duke University Medical Center

MICHELLE PENNY, *Roundtable Co-Chair*
Vice President and Head of Genomics
Goldfinch Bio

HARVEY FINEBERG, *Standing Committee Chair*
President
Gordon and Betty Moore Foundation

2:45 p.m. Adjourn

ROUNDTABLE AND STANDING COMMITTEE INFORMATION

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Roundtable on GENOMICS and PRECISION HEALTH

The sequencing of the human genome is rapidly opening new doors to research and progress in biology, medicine, and health care. At the same time, these developments have produced a diversity of new issues to be addressed.

The National Academies of Sciences, Engineering, and Medicine has convened a Roundtable on Genomics and Precision Health (previously the Roundtable on Translating Genomic-Based Research for Health) that brings together leaders from academia, industry, government, foundations and associations, and representatives of patient and consumer interests who have a mutual concern and interest in addressing the issues surrounding the translation of genome-based research for use in maintaining and improving health. The mission of the Roundtable is to advance the field of genomics and improve the translation of research findings to health care, education, and policy. The Roundtable will discuss the translation process, identify challenges at various points in the process, and discuss approaches to address those challenges.

The field of genomics and its translation involves many disciplines, and takes place within different economic, social, and cultural contexts, necessitating a need for increased communication and understanding across these fields. As a convening mechanism for interested parties from diverse perspectives to meet and discuss complex issues of mutual concern in a neutral setting, the Roundtable: fosters dialogue across sectors and institutions; illuminates issues, but does not necessarily resolve them; and fosters collaboration among stakeholders.

To achieve its objectives, the Roundtable conducts structured discussions, workshops, and symposia. Workshop summaries will be published and collaborative efforts among members are encouraged (e.g., journal articles). Specific issues and agenda topics are determined by the Roundtable membership, and span a broad range of issues relevant to the translation process.

Issues may include the integration and coordination of genomic information into health care and public health including encompassing standards for genetic screening and testing, improving information technology for use in clinical decision making, ensuring access while protecting privacy, and using genomic information to reduce health disparities. The patient and family perspective on the use of genomic information for translation includes social and behavioral issues for target populations. There are evolving requirements for the health professional community, and the need to be able to understand and responsibly apply genomics to medicine and public health.

Of increasing importance is the need to identify the economic implications of using genome-based research for health. Such issues include incentives, cost-effectiveness, and sustainability.

Issues related to the developing science base are also important in the translation process. Such issues could include studies of gene-environment interactions, as well as the implications of genomics for complex disorders such as addiction, mental illness, and chronic diseases.

Roundtable sponsors include federal agencies, pharmaceutical companies, medical and scientific associations, foundations, and patient/public representatives. For more information about the Roundtable on Genomics and Precision Health, please visit our website at nationalacademies.org/GenomicsRT or contact Sarah Beachy at 202-334-2217, or by e-mail at sbeachy@nas.edu.

The National Academy of Sciences, National Academy of Engineering, and National Academy of Medicine work together as the National Academies of Sciences, Engineering, and Medicine ("the Academies") to provide independent, objective analysis and advice to the nation and conduct other activities to solve complex problems and inform public policy decisions. The Academies also encourage education and research, recognize outstanding contributions to knowledge, and increase public understanding in matters of science, engineering, and medicine.

Roundtable on Genomics and Precision Health Membership

Geoffrey Ginsburg, M.D., Ph.D. (Co-Chair) Duke University
Michelle Penny, Ph.D. (Co-Chair) Goldfinch Bio

Naomi Aronson, Ph.D., BlueCross/BlueShield Association

Aris Baras, M.D., M.B.A., Regeneron Pharmaceuticals

Karina Bienfait, Ph.D., Merck and Co., Inc.

Vence Bonham, Jr., J.D., National Human Genome Research Institute

Robert B. Darnell, M.D. Ph.D., The Rockefeller University / NY Genome Center

Stephanie Devaney, Ph.D., All of Us Research Program, NIH

Katherine Donigan, Ph.D., U.S. Food and Drug Administration

W. Gregory Feero, M.D., Ph.D., Journal of the American Medical Association

Jessica M. Gill, Ph.D., R.N., FAAN, National Institute of Nursing Research

Jennifer Goldsack, MChem, M.A., M.B.A., Digital Medicine Society (DiMe)

Marc Grodman, M.D., Genosity

Richard Hodes, M.D., National Institute on Aging

Praduman Jain, M.S., Vibrent Health

Sally John, Ph.D., Biogen

Sekar Kathiresan, M.D., Massachusetts General Hospital

Muin Khoury, M.D., Ph.D., Centers for Disease Control and Prevention

David Ledbetter, Ph.D., Geisinger

Charles Lee, Ph.D., FACMG, The Jackson Laboratory for Genomic Medicine

Thomas Lehner, Ph.D., M.P.H., National Institute of Mental Health

Debra Leonard, M.D., Ph.D., College of American Pathologists

Patrick Loerch, Ph.D., Johnson & Johnson

James Lu, M.D., Ph.D., Helix

Sean McConnell, Ph.D., American Medical Association

Mona Miller, M.P.P., American Society of Human Genetics

Jennifer Moser, Ph.D., U.S. Department of Veterans Affairs

Maximilian Muenke, M.D., FACMG, American College of Medical Genetics and Genomics

Anna Pettersson, Ph.D., Pfizer Inc.

Victoria M. Pratt, Ph.D., FACMG, Association for Molecular Pathology

Kunal Sanghavi, M.B.B.S., M.S., C.G.C., The Jackson Laboratory for Genomic Medicine

Nadeem Sarwar, Ph.D., Eisai Inc.

Sheri Schully, Ph.D., Formerly NIH Office of Disease Prevention, now All of Us

Joan A. Scott, M.S., C.G.C., Health Resources and Services Administration

Sam Shekar, M.D., M.P.H., American College of Preventive Medicine

Nonniekaye Shelburne, C.R.N.P., M.S., A.O.C.N., National Cancer Institute

Nikoletta Sidiropoulos, M.D., University of Vermont Health Network Medical Group

Katherine Johansen Taber, Ph.D., Myriad Women's Health

Ryan Taft, Ph.D., Illumina

Jacquelyn Taylor, Ph.D., New York University

Sharon Terry, M.A., Genetic Alliance

Joyce Tung, Ph.D., 23andMe, Inc.

Jameson Voss, M.D., Air Force Medical Support Agency

Catherine A. Wicklund, M.S., C.G.C., National Society of Genetic Counselors

Huntington F. Willard, Ph.D., Genome Medical

Janet K. Williams, Ph.D., R.N., FAAN, American Academy of Nursing

Sarah Wordsworth, Ph.D., University of Oxford

Alicia Zhou, Ph.D., Color Genomics

Project Staff
Sarah H. Beachy, Ph.D., *Roundtable Director*
Siobhan Addie, Ph.D., *Program Officer*
Meredith Hackmann, *Associate Program Officer*
Kelly Choi, *Senior Program Assistant*

NAM Greenwall Fellow in Bioethics
Rachel Fabi, Ph.D., *Upstate Medical University*

500 Fifth Street, NW, Washington, DC 20001
Phone 202.334.2217 E-mail sbeachy@nas.edu nationalacademies.org/GenomicsRT

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Health and Medicine Division

Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats

In response to a request from the Office of Science and Technology Policy (OSTP) and the Office of the Assistant Secretary for Preparedness and Response (ASPR), the National Academies of Sciences, Engineering, and Medicine will convene a standing committee of experts to help inform the federal government on critical science and policy issues related to emerging infectious diseases and other 21st century health threats. The standing committee will include members with expertise in emerging infectious diseases, public health, public health preparedness and response, biological sciences, clinical care and crisis standards of care, risk communication, epidemiology, and regulatory issues, as well as veterinary science, One Health, ethics, and community engagement. The standing committee will provide a venue for the exchange of ideas among federal government agencies, the private sector, and the academic community, as well as other relevant stakeholders.

The standing committee will:

- Stand ready to respond on short notice to requests from the federal government to assess and consider the science and policy implications of an emerging infectious disease or significant public health threat;
- Provide a venue to enable science and policy discussions relevant to the federal government on emerging issues, research, and activities through in-depth knowledge of the sponsor's programs, goals, and objectives;
- Identify opportunities to integrate science into national preparedness and response decision making;
- Explore lessons learned and best practices from previous preparedness and response efforts, and identify opportunities to disseminate that information to a variety of stakeholders;
- Serve as a focal point for national policy discussions by experts and other leaders in the field;
- Consider, identify, and discuss strategies for addressing misinformation; and
- Respond to the federal government's needs for continuing dialog related to strategic planning and program development to address emerging infectious diseases, biosecurity, and public health and medical preparedness.

At the request of the sponsors, the standing committee will be involved in the planning, development, and oversight of related ad hoc activities undertaken by separately appointed committees operating under its auspices.

The standing committee will serve as a focal point for the discussion of scientific, technical, and policy issues relevant to emerging infectious diseases and public health preparedness and response that warrant detailed examination. Topics for discussion with the standing committee may include:

- Technical assistance and/or assessment of response to emerging infectious diseases;
- Availability of and access to information, samples, and other materials to determine the origin and evolution of emerging infectious diseases;
- International coordination and engagement;
- Technical assessment of ecological and evolutionary drivers of disease emergence;
- Approaches to proactive public messaging and strategies to address misinformation;
- Other science and policy issues relevant to emerging infectious diseases and 21st century health threats.

The committee will carry out its charge at its in-person and virtual meetings by gathering evidence from experts, deliberating, and, when necessary, by preparing short reports.

COMMITTEE SPONSORS

White House Office of Science and Technology Policy and HHS Office of Assistant Secretary for Preparedness and Response

COMMITTEE ROSTER

Harvey Fineberg, M.D., Ph.D. ***(Chair)***
President
Gordon and Betty Moore Foundation

Kristian Andersen, Ph.D.
Associate Professor and Director of Infectious Disease Genomics, Scripps Research Translational Institute
The Scripps Research Institute

Ralph Steven Baric, Ph.D.
William R. Kenan, Jr. Distinguished Professor
The University of North Carolina at Chapel Hill

Mary Bassett, M.D., M.P.H.
Director of the François-Xavier Bagnoud Center for Health and Human Rights
Harvard School of Public Health

Trevor Bedford, Ph.D.
Associate Faculty Member, Vaccine and Infectious Disease Division, Public Health Sciences Division, and Human Biology Division
Fred Hutchinson Cancer Research Center

Georges Benjamin, M.D.
Executive Director
American Public Health Association

Donald Berwick, M.D., M.P.P., F.R.C.P, KBE
Lecturer of Health Care Policy
Harvard Medical School

Richard Besser, M.D.
President and CEO
Robert Wood Johnson Foundation

R. Alta Charo, J.D.
Warren P. Knowles Professor of Law and Bioethics
University of Wisconsin at Madison

Peter Daszak, Ph.D.
President and CEO
EcoHealth Alliance

Jeffrey S. Duchin, M.D.
Health Officer and Chief, Communicable Disease Epidemiology & Immunization Section and Professor in Medicine

Ellen Embrey
President/CEO
Stratitia, Inc.

Baruch Fischhoff, Ph.D.
Howard Heinz University Professor
Department of Engineering and Public Policy
Carnegie Mellon University

Diane Griffin, M.D., Ph.D.
Professor, Department of Molecular Microbiology and Immunology
Johns Hopkins Bloomberg School of Public Health

Robert Groves, Ph.D., M.A.
Executive Vice President and Provost
Gerard J. Campbell, S.J. Professor
Math and Statistics Department and Sociology Department
Georgetown University

Margaret Hamburg, M.D.
Foreign Secretary
National Academy of Medicine

Dan Hanfling, M.D.
Vice President, Technical Staff
In-Q-Tel

John Hick, M.D.
Associate Medical Director for EMS
Medical Director of Emergency Medicine
Hennepin County Medical Center

Kent E. Kester, M.D.
Vice President and Head, Translational Science and Biomarkers
Sanofi Pasteur

Patricia King, J.D.
Professor Emerita
Georgetown University Law Center

Jonna Mazet, D.V.M., M.P.V.M., Ph.D.
Executive Director, One Health Institute
UC Davis School of Veterinary Medicine

Phyllis Meadows, Ph.D., M.S.N., R.N.
Senior Fellow, Health
The Kresge Foundation

Tara O'Toole, M.D., M.P.H.
Executive Vice President
In-Q-Tel

Alexandra Phelan, S.J.D., LL.M., LL.B.
Assistant Professor
Center for Global Health Science and Security
Georgetown University

David Relman, M.D.
Thomas C. and Joan M. Merigan Professor of Medicine, and of Microbiology & Immunology; Chief of Infectious Diseases
Stanford University; VA Palo Alto Health Care System

Mark Smolinski, M.D., M.P.H.
President
Ending Pandemics

David Walt, Ph.D.
Hansjörg Wyss Professor of Biologically Inspired Engineering
Harvard Medical School

CONTACT INFORMATION

Andrew Pope, Ph.D.
Senior Director, Board on Health Sciences Policy
202-334-1739
apope@nas.edu

Lisa Brown, M.P.H.
Study Director, Board on Health Sciences Policy
202-334-2487
lbrown@nas.edu

ADDITIONAL INFORMATION

For additional information, please visit https://www.nationalacademies.org/our-work/standing-committee-on-emerging-infectious-diseases-and-21st-century-health-threats

MEETING INFORMATION

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Board on Health Sciences Policy

Host Genomics Research and Data Collection Efforts Related to COVID-19

Joint Meeting
of the
Roundtable on Genomics and Precision Health
and the
Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats

SPEAKER AND CHAIR BIOGRAPHIES

Harvey V. Fineberg, M.D., Ph.D. ***(Standing Committee Chair)***
Dr. Fineberg is the president of the Gordon and Betty Moore Foundation, which fosters path-breaking scientific discovery, environmental conservation, improvements in patient care and preservation of the special character of the San Francisco Bay Area. He previously held the Presidential Chair for 2014-2015 as visiting professor at the University of California, San Francisco. Prior to that, he served as president of the Institute of Medicine from 2002 to 2014 and as provost of Harvard University from 1997 to 2001, following 13 years as dean of the Harvard School of Public Health. He has devoted most of his academic career to the fields of health policy and medical decision-making. His past research has focused on the process of policy development and implementation, assessment of medical technology, evaluation and use of vaccines, and dissemination of medical innovations. Fineberg chairs the board of the Carnegie Endowment for International Peace and serves on the boards of the William and Flora Hewlett Foundation and the China Medical Board. He helped found and served as president of the Society for Medical Decision Making and also served as consultant to the World Health Organization. Fineberg is co-author of the books Clinical Decision Analysis, Innovators in Physician Education and The Epidemic That Never Was, an analysis of the controversial federal immunization program against swine flu in 1976. He has co-edited several books on such diverse topics as AIDS prevention, vaccine safety, understanding risk in society and global health. He has also authored numerous articles published in professional journals. Fineberg is the recipient of several honorary degrees—the Frank A. Calderone Prize in Public Health, the Henry G. Friesen International Prize in Health Research and the Harvard Medal, awarded by the alumni association of the university from which he earned his bachelor's and doctoral degrees.

Geoffrey Ginsburg, M.D., Ph.D. (Roundtable *Co-Chair*)
Dr. Ginsburg is the founding director for the Center for Applied Genomics & Precision Medicine at the Duke University Medical Center and for MEDx, a partnership between the Schools of Medicine and Engineering to spark and translate innovation. His research addresses the challenges for translating genomic and digital information into medical practice and the integration of precision medicine into healthcare. In 2017 he received Duke's Translational Research Mentorship Award and is a finalist in the NIH/BARDA Antimicrobial Resistance Prize.

He is a member of the Advisory Council to the Director of NIH and is co-chair of the National Academies Roundtable on Genomics and Precision Health and is founder and president of the Global Genomic Medicine

Collaborative, a not for profit organization aimed creating international partnerships to advance the implementation of precision medicine. He has recently served as a member of the Board of External Experts for the NHLBI, the advisory council for the National Center for Accelerating Translational Science, the chair of the review for Genome Canada's Large Scale Applied Research Competition in Genomics and Precision Medicine, and the World Economic Forum's Global Agenda Council on the Future of the Health Sector. He is a founder of Predigen Inc and MeTree&You Inc. He was previously Vice President of Molecular Medicine at Millennium Pharmaceuticals, Inc and a faculty member at Harvard Medical School.

Maximilian Muenke, M.D., FACMG
Dr. Muenke joined the American College of Medical Genomics (ACMG) as CEO in 2019. Recognized as a highly acclaimed physician-scientist and dedicated clinical and research mentor, Dr. Muenke has more than three decades of experience to the ACMG, including 10 years on the faculty of the University of Pennsylvania School of Medicine. Since 2000, Dr. Muenke has served as Senior Investigator, Head of the Human Development Section, and Chief of the Medical Genetics Branch at the National Human Genome Research Institute (NHGRI) of the National Institutes of Health (NIH). The focus of his laboratory's research was on the delineation and identification of the underlying causes of craniofacial and related anomalies in humans including holoprosencephaly as well as Muenke syndrome, a relatively common craniosynostosis syndrome. Dr. Muenke is passionate about training the next generation of leaders in the field of genetics and genomic medicine. He has directed medical genetics training since 1994, first at the University of Pennsylvania and then at the NIH, where he was the Director of the NIH Medical Genetics and Genomic Medicine Residency and Fellowship Training Programs.

Michelle Penny, Ph.D. ***(Roundtable Co-Chair)***
Dr. Penny is a Vice President and Head of Genomics at Golfich Bio. Prior to that, she was the Head of Translational Genome Sciences at Biogen. She received her Ph.D. in Genetics from the University of Birmingham, UK in 1993. After a postdoctoral fellowship in the Virology Division at the National Institute of Medical Research, Mill Hill, London, she joined Imperial College London, Department of Medical and Community Genetics where she was a postdoctoral research scientist until taking a lectureship in Human Molecular Genetics in 1998. Her academic research career focused on the study of complex polygenic diseases particularly autoimmune disease and susceptibility to infection. In 2002, Dr. Penny joined the Clinical Pharmacogenomics group at Pfizer in Sandwich, UK, and moved to New London, CT in 2006 to take on the role of Oncology Molecular Medicine Lead until 2009 when she moved to Indianapolis to lead the Pharmacogenomics work at Eli Lilly and Company.

Sharon Terry, M.A.
Sharon Terry is President and CEO of Genetic Alliance, a large network engaging individuals, families and communities to transform health. Genetic Alliance works to provide programs, products and tools for ordinary people to take charge of their health.

As 'just a Mom' with a master's degree in Theology, she cofounded PXE International, a research advocacy organization for the genetic condition pseudoxanthoma elasticum (PXE), in response to the diagnosis of PXE in her two children in 1994. With her husband, she co-discovered the ABCC6 gene, patented it to ensure ethical stewardship in 2000, and assigned their rights to the foundation. She subsequently developed a diagnostic test and conducts clinical trials. She is the author of 140 peer-reviewed papers, of which 30 are clinical PXE studies.

In her focus at the forefront of consumer participation in genetics research, services and policy, she serves in a leadership role on many of the major international and national organizations, including the Precision Medicine Initiative Cohort Advisory Panel, Accelerating Medicines Partnership, Institute of Medicine (IOM) Science and Policy Board, the IOM Roundtable on Translating Genomic-Based Research for Health, the PubMed Central National Advisory Committee, the PhenX scientific advisory board, the Global Alliance for Genomics and Health, the International Rare Disease Research Consortium Executive Committee and as Founding President of EspeRare Foundation of Geneva, Switzerland. Terry is co-founder of the Genetic Alliance Registry and Biobank. She is on the editorial boards of several journals. She led the coalition that was instrumental in the passage of the Genetic Information Nondiscrimination Act. She received an honorary doctorate from Iona College for her

community engagement work in 2006; the Research!America Distinguished Organization Advocacy Award in 2009; and the Clinical Research Forum and Foundation's Annual Award for Leadership in Public Advocacy in 2011. She was named one of FDA's "30 Heroes for the Thirtieth Anniversary of the Orphan Drug Act" in 2013. She is co-inventor of the Platform for Engaging Everyone Responsibly (PEER). PEER received $500,000 from the Robert Wood Johnson Foundation in 2014. PEER undergirds the Community Engaged Network for All (CENA), a PCORnet member funded in 2013 for Phase I and in 2015 for Phase II. She is Co-PI of the PCORnet Coordinating Center and Chair of the PCORnet Engagment Committee.

Joyce Tung, Ph.D.
Joyce joined 23andMe in 2007 and manages the 23andMe research team, which is responsible for consumer health and ancestry research and development, academic and industry collaborations, computational analyses for therapeutics, and new research methods and tools development. While a postdoctoral fellow at Stanford University, Joyce studied the genetics of mouse and human pigmentation. She graduated from Stanford with honors and distinction with a B.S. in Biological Sciences and a minor in computer science, and earned her Ph.D. in Genetics from the University of California, San Francisco where she was a National Science Foundation graduate research fellow.

BACKGROUND MATERIALS

Links to Background Materials

Materials from 23andMe

- The information page for the external recruitment portion of our COVID-19 study
- Blog post on initial launch of our study
- Blog post on expansion of our study to enroll severe cases
- Blog post on preliminary findings

Genomewide Association Study of Severe Covid-19 with Respiratory Failure

BACKGROUND

There is considerable variation in disease behavior among patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes coronavirus disease 2019 (Covid-19). Genomewide association analysis may allow for the identification of potential genetic factors involved in the development of Covid-19.

A Host Transcriptional Signature for Presymptomatic Detection of Infection in Humans Exposed to Influenza H1N1 or H3N2

BACKGROUND

There is great potential for host-based gene expression analysis to impact the early diagnosis of infectious diseases. In particular, the influenza pandemic of 2009 highlighted the challenges and limitations of traditional pathogen-based testing for suspected upper respiratory viral infection.

A crowdsourced analysis to identify ab initio molecular signatures predictive of susceptibility to viral infection

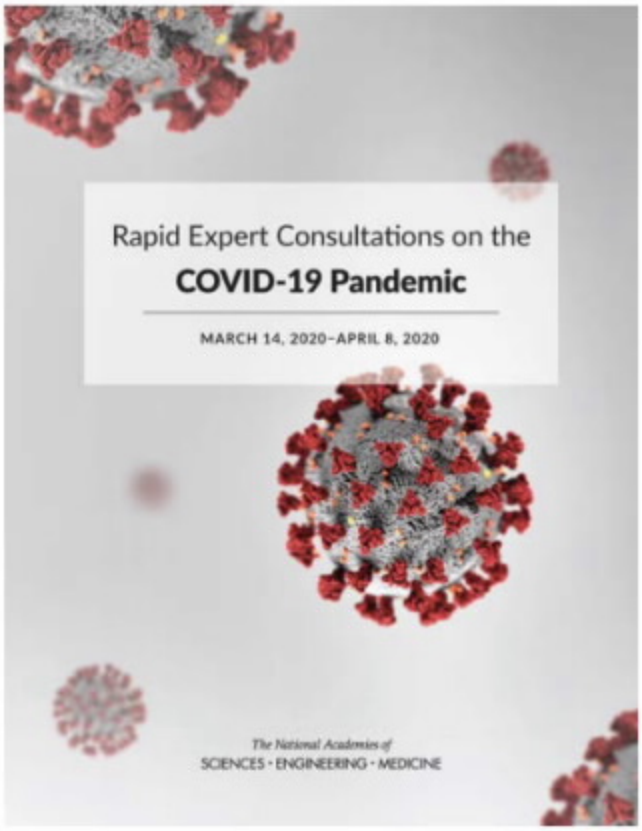
BACKGROUND

The response to respiratory viruses varies substantially between individuals, and there are currently no known molecular predictors from the early stages of infection. Here we conduct a community-based analysis to determine whether pre- or early post-exposure molecular factors could predict physiologic responses to viral exposure.

A Host-Based RT-PCR Gene Expression Signature to Identify Acute Respiratory Viral Infection

Improved ways to diagnose acute respiratory viral infections could decrease inappropriate antibacterial use and serve as a vital triage mechanism in the event of a potential viral pandemic. Measurement of the host response to infection is an alternative to pathogen-based

diagnostic testing and may improve diagnostic accuracy. We have developed a host-based assay with a reverse transcription polymerase chain reaction (RT-PCR) TaqMan low-density array (TLDA) platform for classifying respiratory viral infection.



Rapid Expert Consultations on the COVID-19 Pandemic

In response to a request from the Office of Science and Technology Policy and the Office of the Assistant Secretary for Preparedness and Response, the National Academies of Sciences, Engineering, and Medicine convened a standing committee of experts to help inform the federal government on critical science and policy issues related to emerging infectious diseases and other 21st century health threats. This set of Rapid Expert Consultations are the first of their kind and represent the best evidence available to the Committee at the time each publication was released.

This paper is in press at The Lancet Infectious Diseases

A blood-based host gene expression assay for early detection of viral infection

Micah T. McClain MD, PhD[1-3], Florica J. Constantine, MS[1], Bradly P. Nicholson, PhD[4], Marshall Nichols, MS[1], Thomas W. Burke, PhD[1], Ricardo Henao, PhD[1], Daphne C. Jones, MD[2], Lori L. Hudson, PhD[1], L. Brett Caram, MD[3], Timothy Veldman, PhD[1], Anna Mazur, MS[1], Lawrence P. Park, PhD[2,3], Sunil Suchindran, PhD[1], Ephraim L. Tsalik, MD, PhD[1-3], Geoffrey S. Ginsburg, MD[1], and Christopher W. Woods, MD, MPH[1-3]

Affiliations:

[1]Center for Applied Genomics and Precision Medicine, Duke University, Durham, NC, [2]Durham VA Medical Center, Durham, NC, [3]Division of Infectious Diseases, Duke University Medical Center, Durham, NC, [4]Institute for Medical Research, Durham, NC

Corresponding Author:

Micah T. McClain, MD, PhD

Division of Infectious Diseases

Center for Applied Genomics and Precision Medicine

Duke University Medical Center

DUMC Box 102359

Durham, NC 27710

e-mail: micah.mcclain@duke.edu

Office: 919-613-5636

Research in context

Evidence before this study

An increasing body of research has demonstrated that host gene expression patterns in peripheral blood offer a high degree of accuracy for diagnosis of acute infections at the time of clinical presentation.

Added value of this study

For the first time we demonstrate in a real-world setting that a blood-based gene expression test can accurately predict impending viral infection even before typical symptoms are present.

Implications of all the available evidence

These data show that biomarkers of viral infection are present and detectable even before clinical disease develops, opening the door to novel approaches to early treatment and outbreak management.

Abstract

Background

Early and accurate identification of persons with viral infections can be of critical importance for clinical management and public health interventions. Measuring host responses through gene signature-based biomarkers offer promise for improved diagnosis of respiratory viral infections, but little is known how early in disease such measures may be effective.

Methods

We performed a modified index-cluster study in 1465 university dormitory residents over five years. Each year, we monitored for symptomatic Index Cases (IC) and identified clusters of their Close Contacts (CC) who would be at presumed increased risk of developing symptomatic respiratory tract infection (RTI) while under observation. We then monitored each CC for 5 days for development of RTI and measured transcriptomic responses at each timepoint via a blood-based 36-gene RT-PCR assay.

Findings

Of 264 ICs with RTI symptoms, 150 (56%) had a confirmed viral etiology detected. Of their 555 CCs, 106 (19%) developed symptomatic RTI with a proven viral etiology during the observation window. The transcriptomic assay accurately predicts infection at the time of peak symptoms despite heterogeneity of hosts and nine different viral etiologies (auROC 0·96). Furthermore, these genomic biomarkers are capable of identifying early disease one, two and three days prior to peak illness when minimal to no symptoms are present (auROC 0·87, 0·86, and 0·77, respectively), and when nearly two-thirds of subjects (62%) are not yet producing detectable viral shedding.

Interpretation

Transcriptional biomarkers accurately predict and diagnose infection across diverse viral etiologies and stages of disease, and may prove useful for guiding administration of early effective therapy, quarantine decisions, and other clinical and public health interventions in the setting of endemic and pandemic infectious disease.

Funding: US Defense Advanced Research Projects Agency

Introduction

Acute viral infections are among the most common causes of acute physician visits in the United States[1]. In a given year, influenza alone affects 5-20% of the population in the US, causes over 400,000 hospitalizations, and as many as 56,000 deaths.[2,3] Outbreaks of viral infection continue to plague modern society, including heavy resource-requiring outbreaks of measles in the developed world, global pandemics like the novel H1N1 outbreak of 2009, or the deadly epidemics of SARS and MERS-CoV.[4-6] However, molecular tools that can contribute to outbreak investigation or directing early clinical management are often lacking. Traditional pathogen-focused diagnostic modalities for viral infection (culture, serology, antigen detection, PCR) offer considerable utility but can also be slow, costly, limited in terms of breadth of pathogen detection, require an *a priori* knowledge of the pathogen to be tested, may not detect emerging strains (as with the 2009 influenza pandemic[7]), or are incapable of distinguishing between active infection and colonization, all of which can minimize the impact of their use. Therefore, there is increasing interest in host-derived biomarkers to aid in the determination of the presence and/or type of infection in at-risk hosts - from single-analyte biomarkers like procalcitonin to composite, multiplex biomarker panels such as those included in a growing number of gene expression-based studies.[8-21] However, the published works on diagnostics for naturally-acquired infection focus on identifying symptomatic subjects at the time of clinical presentation for medical care[19], which is often late in the time course of many viral diseases. Identification of infectious etiologies in earlier, even pre-symptomatic phases of illness, offers the ability to optimize and deliver timely and thus more effective therapy, refine prophylaxis decisions, and guide public health interventions such as isolation and quarantine.[13,22] However, identifying subjects who have been exposed to infectious virus but are not yet exhibiting symptoms of clinical illness is logistically challenging, resulting in a dearth of literature to address these problems.

We have previously shown that during experimental influenza infection of healthy volunteers the host genomic response is robust and detectable well before typical symptoms become apparent.[16,22,23] Other

investigators have confirmed that transcriptomic markers can be indicative of early viral infection in experimental human challenge models as well as animal models of disease.[24-27] However, the mechanism of challenge infection is contrived rather than natural, typically consists of highly egg-adapted viral strains, and the severity of clinical illness does not fully replicate that seen in naturally-acquired infection, all of which can limit the broad applicability of findings from this approach.[16,22,28-31] To date there has been no significant description of the host response to infection during the pre-symptomatic phase of naturally-occurring disease. Here we have utilized a unique index-cluster study design with a focus on serial sampling of 'real world', healthy close contacts of infected individuals to enrich for cases of naturally-occurring infection during these early, post-exposure, but pre-symptomatic time points. This unique design has permitted real-world validation of a transcriptomic signature in peripheral blood that is capable of accurately identifying exposed but apparently healthy individuals who will go on to develop symptomatic viral infection.

Methods

Surveillance Cohort

Over five academic years, we enrolled 1465 Duke University undergraduate students (age 18-24, most of whom live in dormitories on campus) in a modified index-cluster study design (Figure 1). At the time of enrollment, we collected baseline biospecimens and standardized questionnaires to ascertain demographic, behavioral, and other medical characteristics. After enrollment, participating students completed a daily, web-based survey to describe the presence and severity of eight symptoms of RTI (scored 0-4) on a daily basis (see eMethods for details). We monitored the surveys daily for a 6-point increase in a subject's cumulative daily symptom score which would trigger a sample and data collection event for a potential Index Case. Suspected ICs were then assessed for confirmation of illness and biospecimen collection. Using these sentinel cases of RTI suggestive of viral etiology we then identified and monitored a prospective observational cohort of asymptomatic but potentially exposed Close Contacts (people living in the same dormitory or identified by the IC as a Close Contact), who were then monitored and sampled for up to 5 days (the Observation Window).

Sample and data collection

Confirmed IC's provided blood and nasal swab samples at the time of illness identification. Viral etiology was assessed using a commercial multiplex PCR assay (QIAGEN ResPlex II v2·0, Luminex RVP, or Biofire FilmArray RP). Healthy CCs living in proximity to an IC provided blood and respiratory samples for up to five consecutive days and were monitored for symptomatic conversion and viral shedding.

Real-time PCR

Peripheral blood was collected in PAXgene™ Blood RNA tubes (PreAnalytiX), and total RNA extracted using the PAXgene™ Blood miRNA Kit (QIAGEN). cDNA synthesis was performed using SuperScript VILO™ Master Mix (Invitrogen) according to manufacturer's instructions. Real-time PCR was performed using custom TLDA 384-well microfluidic cards with TaqMan Gene Expression Master Mix, and run on a ViiA7 Real-Time PCR System (Applied Biosystems).

Statistical Analysis

For RT-PCR data the Cq values were transformed into normalized relative quantities using reference genes - those with the most stable combined coefficient of variation across all samples - FPGS and TRAP1 were used for the first batch, and DECR1 and TRAP1 were used for the second batch, and data was normalized as previously described.[8,9] We utilized a regularized (2-norm) logistic regression model to discriminate cases from controls (see eMethods for complete details). The data was split into training and testing sets 25 times using repeated random subsampling and each of the 25 models was trained using all timepoints. We evaluated the model's performance separately at each individual timepoint. All model analyses were implemented in Python and Tensorflow.[32]

Results

Brief Description of Study

Healthy first year students at Duke University (n=1465) were enrolled into a surveillance cohort at the beginning of each academic year. After enrollment, we electronically monitored self-reported daily symptoms throughout the duration of the academic year via an online portal. When one or more subjects indicated a change in respiratory symptoms, the sample collection protocol was initiated for individuals (Index Cases, ICs) that met the clinical case definition for acute, community-acquired RTI (see Methods), as well as for a subset of other currently healthy study participants who were in close proximity to the Index Case (Close Contacts, CCs). CCs were monitored for symptoms and serially sampled for serum, whole blood PAXgene RNA, and respiratory samples for 5 days from identification of their Index Case, regardless of symptomatology (Figure 1). This design permitted enrichment for, and collection of, samples from CCs during the timeframe between exposure (which occurred at an unknown time) and subsequent development of symptomatic disease (which occurred under observation).

Prevalence, Transmission, and Clinical Outcomes of Viral Respiratory Tract Infection

We identified 264 clinical ICs, of whom 150 had a confirmed respiratory viral etiology by multiplex PCR testing of NP samples, and 555 CCs, 19% (106) of whom developed symptomatic RTI with concomitant confirmatory viral PCR. Only 57% of the CCs were infected with the same virus as their associated IC. Nine different viral pathogens were detected, and there was marked variation in viral etiologies detected from year to year (Table 1). Infection resulted in many common symptoms of upper respiratory tract infection with clinical variation amongst viral etiologies (Figure 2). For example, symptoms were almost twice as severe in individuals with influenza infection compared to all other viruses (mean symptom score of 20 for influenza vs 13 for all others ($p=0.005$)), although all subjects in the study remained well enough to be managed in the outpatient setting.

Since the exact time of exposure/transmission during naturally-acquired infection is unknown, for analysis CC days were aligned to the time of maximal illness (day of highest symptom score) for each subject (Time "T") in order to attempt to place subjects on broadly similar time scales in terms of the host response to infection. The average total symptom score for all CCs at Time T was 14·8, although this varied by viral etiology (Figures 1,2). For each individual CC, the day before their day of maximal symptoms was termed T-minus 1 ("T-1"), 48 hours prior to symptom max was "T-2" and 72 hours prior to symptom max was time "T-3". At times prior to maximal illness the average total symptom score for CCs was 7·0 (T-1), 4·1 (T-2), and 1·7 (T-3). In our studies a daily symptom score of 6 represents mild clinical illness and was the threshold for defining a 'symptomatic' day. Thus, at days T-2 and earlier, most subjects exhibited subclinical or absent illness (65% asymptomatic at Time T-3, 32% at T-2).

An RT-PCR signature identifies symptomatic subjects with naturally-acquired viral RTI

We previously developed a host response-derived transcriptomic signature of symptomatic viral infection using microarray data[30]. However, microarray and bulk RNA sequencing platforms are impractical for rapid clinical testing. We therefore migrated our prior 'pan-viral' signature to an RT-PCR platform with potential for more direct translation to point-of-care testing [8,9]. Using sparsity-imposing techniques (see eMethods), we selected 36 pre-designed TaqMan probes representing genes comprising the acute respiratory viral signature (and normalization controls) to be used on a TaqMan Low Density Array (TLDA) platform[8]. We then utilized a regularized (2-norm) logistic regression model to determine coefficients for each gene in the signature[33], such that the output of the model is a probability that a given subject will go on to have a symptomatic, viral PCR-positive event during the observation window. The performance of the RT-PCR signature for classification of symptomatic individuals was evaluated using a repeated random subsampling validation strategy on relevant participants across all study years. Overall, the RT-PCR-based host gene expression assay distinguished symptomatic, virus-infected subjects from asymptomatic, viral PCR-negative controls with 92% accuracy (Figure 3). However, accuracy varied depending on viral etiology, ranging from 99% accuracy in influenza infection (where symptoms are

typically more severe) to 92% with Coronavirus and 81% accuracy with metapneumovirus infections (eFigure 1). Interestingly, analysis of individual gene components of the model demonstrates that many individual genes performed similar to the aggregate model across all iterations (eFigure 2-3). In fact, many individual gene targets alone had mean area under Receiver Operating Characteristic (auROC) of >0·90 at the time of maximal illness with the top performing genes at time T being IFIT3 (0·97), HERC5 (0·97), and RSAD2 (0·96)

The gene expression signature identifies naturally-acquired viral RTI during the pre-symptomatic phase.

In order to evaluate performance of the gene signature early in the course of natural infection, we selected CCs with positive microbiology and at least three days of sampling prior to the development of maximal symptoms. As noted previously, the RT-PCR gene signature correctly identified symptomatic subjects with 92% accuracy on the day of maximal symptoms. This decreased to 88% on day T-1 (24 hours before maximal symptoms), and 83% accuracy on day T-2 (48 hours before maximal symptoms) (Figure 3), although performance varies from virus to virus. Only one case of influenza infection had available pre-symptomatic samples at all times, and the signature correctly classified that individual at each timepoint (T-1, T-2, T-3, and T-4). For a fixed FPR of 0·2 (specificity 0·8), the model achieves greater sensitivity than either symptoms alone or viral PCR at all timepoints other than time T and for ICs (where symptom-based diagnosis by definition has a 100% sensitivity, eFigures 4-6). Critically, 62% of subjects who would develop proven viral RTI lacked detectable viral shedding at time T-3, and 44% were still negative for virus by routine clinical testing at day T-2, times where the gene signature outperforms the clinical standards of care - symptom evaluation and viral PCR.

Discussion

The unique design of this community study has allowed us to identify individuals (CCs) at increased risk for developing viral RTI while under observation. This approach has enabled us to demonstrate for the first time that host responses to naturally-acquired respiratory viral infection, as measured by gene expression in peripheral blood, are present well before the time of maximal illness and can be used to predict those at highest risk for developing active infection.

This study demonstrates that conserved genomic responses can be used to correctly classify individuals with a broad array of naturally-acquired respiratory viral infections well before the time when they would present for clinical evaluation, and even before detectable viral shedding begins for the majority of subjects (62%). The promise of this approach is manifested by the discriminative ability of the genomic signature from two and three days prior to maximal illness, when many individuals (65% at Time T-3, 32% at T-2) have no symptoms at all. Even the mild symptoms some subjects do have at early times are clinically vague and would be typical of seasonal allergies, mild COPD flares, or even symptoms due to sequelae of chronic smoking.[34] The potential utility of genomic analysis to classify viral infection among asymptomatic individuals or those with common, nonspecific upper respiratory symptoms is profound. That this holds true across 9 different respiratory viruses, each with its own incubation times and variable clinical progression and duration, also speaks to the potential power of this approach. Additionally, it is important to note that the signature utilized represents conserved underlying antiviral response pathways that are active, and thus discriminatory, across a wide range of illness and not just at early times. This is vital as any real-world use of such a biomarker assay would often be agnostic to where in the course of infection an individual exists at the moment of testing.

While this transcriptomic signature accurately classifies a wide spectrum of viral RTIs, it performs best in a subset of viruses including influenza and coronavirus, where it performs with a high degree of accuracy even during early and pre-symptomatic phases of disease. Influenza is one of the most important viral etiologies to identify early given its importance in pandemics where existing diagnostics may perform

poorly (especially for emerging strains as in 2009[35]), where questions of triage and quarantine are paramount, and where effective therapeutic options are readily available and most effective when given early in disease. Similarly, a number of novel highly virulent coronaviruses, including SARS-CoV and MERS-CoV, have recently arisen and driven global outbreaks, highlighting another area of need for such diagnostics.[5,36].It seems likely that our ability to accurately detect exposure to these types of emerging infections will only become more critical over time. Availability of tools that can aid in assessment of those with potential exposure, especially those that offer diagnostic utility prior to the time of detectable viral shedding, would be a valuable aid to existing epidemiologic and molecular approaches in outbreak settings.

A number of other studies have described interferon response-based transcriptomic signatures of acute respiratory infections which vary by target population, pathogens, modelling approaches, and numbers of genes required to discriminate disease states. [9,10,13-18,30] Some of this work has also been validated, combined, or expanded upon by secondary *ex vivo* analysis of publicly available data. [11,12,37] While the focused signature described here clearly represents a limited subset of all discriminating genes, some observations regarding simplified, IFN-driven signatures can be made. Our data confirm that that some genes from these prior works (such as IFI44L and IFI27[10,38]) both perform well as classifiers distinguishing between viral illness and the healthy state (median auROC = 0·92 and 0·87), being strongly upregulated in cases of viral infection. The data further suggest that a number of other single targets also perform this task well including RSAD2 (0·96), IFIT3 (0·97), and IFI44 (0·93). Based on our experience and the published literature, it seems that relatively simple classification tasks - where the biological responses are highly conserved, unique in character, and of great magnitude - may be reasonably addressed with pauci-analyte biomarker panels.[10,12,39-41] However, as complexity increases by adding more powerful interhost variability (such as immunosuppression), discriminating clinically similar groups with more varied underlying pathophysiology, or discriminating multiple clinical mimics at once,

more intricate biomarker panels will likely be required to maintain high fidelity in real-world settings.[9,14,42]

Our study has several limitations. This project was designed to validate the predictive power of a small set of genes which already had proven discriminatory capability during maximal illness, and thus was not designed to discover whether additional, untested gene sets may offer improved discriminatory performance at early times. Future work with RNASeq on whole blood, single cell sequencing, or other methods will be needed to more completely define early genomic changes in the naturally-infected host. Additionally, our patient cohort was comprised of otherwise young, healthy individuals with community-acquired viral RTI of mild to moderate severity. Therefore, application to patient populations from other age groups, those with immunologic comorbidities, or with more severe viral infections will need to be evaluated. Finally, we did not identify any confirmed infections due to bacterial or other pathogens. As a result, while these transcriptomic markers show great promise for differentiating early infection and pre-symptomatic viral exposure from noninfectious states (which it may mimic at early times), this study did not directly validate the ability of these markers to differentiate viral exposure from early forms of other types of infection[9,10,14,43]. As we move towards bringing transcriptomic biomarkers to the bedside, consideration of broad applicability and performance across a wide array of clinical settings will continue to be paramount.

We have for the first time defined the pre-symptomatic and temporal dynamics of transcriptomic biomarkers characterizing the host response to naturally-acquired viral infections in humans. Clinic-ready platforms capable of operationalizing PCR-based signatures of the size tested herein already exist, some of which are CLIA-approved with point-of-care functionality, offering a proximal pathway to clinical application of these findings. Thus, analysis of the evolution of gene expression-based biomarkers over time shows promise for driving development of diagnostics for early detection of viral exposure and infection which may prove useful for guiding administration of early effective therapy, quarantine

decisions, and other clinical and public health interventions in the setting of endemic and pandemic infectious disease.

References

1. Hong CY, Lin RT, Tan ES, et al. Acute respiratory symptoms in adults in general practice. *Fam Pract* 2004; **21**(3): 317-23.
2. Shrestha SS, Swerdlow DL, Borse RH, et al. Estimating the burden of 2009 pandemic influenza A (H1N1) in the United States (April 2009-April 2010). *Clin Infect Dis* 2011; **52 Suppl 1**: S75-82.
3. Rebecca Garten PLB, MPH1; Anwar Isa Abd Elal1; Noreen Alabi, MPH1; John Barnes, PhD1; Matthew Biggerstaff, ScD1; Lynnette Brammer, MPH1; Alicia P. Budd, MPH1; Erin Burns, MA1; Charisse N. Cummings, MPH1; Todd Davis, PhD1; Shikha Garg, MD1; Larisa Gubareva, PhD1; Yunho Jang, PhD1; Krista Kniss, MPH1; Natalie Kramer1; Stephen Lindstrom, PhD1; Desiree Mustaquim, MPH1; Alissa O'Halloran, MSPH1; Wendy Sessions, MPH1; Calli Taylor, MPH1; Xiyan Xu, MD1; Vivien G. Dugan, PhD1; Alicia M. Fry, MD1; David E. Wentworth, PhD1; Jacqueline Katz, PhD1; Daniel Jernigan, MD. Update: Influenza Activity in the United States During the 2017–18 Season and Composition of the 2018–19 Influenza Vaccine. *MMWR Morb Mortal Wkly Rep* 2018; (67): 634-42.
4. de Wit E, van Doremalen N, Falzarano D, Munster VJ. SARS and MERS: recent insights into emerging coronaviruses. *Nature reviews Microbiology* 2016; **14**(8): 523-34.
5. Arabi YM, Balkhy HH, Hayden FG, et al. Middle East Respiratory Syndrome. *New England Journal of Medicine* 2017; **376**(6): 584-94.
6. Angelo KM, Gastanaduy PA, Walker AT, et al. Spread of Measles in Europe and Implications for US Travelers. *Pediatrics* 2019; **144**(1).
7. Emergence of a Novel Swine-Origin Influenza A (H1N1) Virus in Humans. *New England Journal of Medicine* 2009; **360**(25): 2605-15.
8. Zaas AK, Burke T, Chen M, et al. A host-based RT-PCR gene expression signature to identify acute respiratory viral infection. *Sci Transl Med* 2013; **5**(203): 203ra126.
9. Tsalik EL, Henao R, Nichols M, et al. Host gene expression classifiers diagnose acute respiratory illness etiology. *Sci Transl Med* 2016; **8**(322): 322ra11.
10. Herberg JA, Kaforou M, Wright VJ, et al. Diagnostic Test Accuracy of a 2-Transcript Host RNA Signature for Discriminating Bacterial vs Viral Infection in Febrile Children. *JAMA* 2016; **316**(8): 835-45.
11. Andres-Terre M, McGuire HM, Pouliot Y, et al. Integrated, Multi-cohort Analysis Identifies Conserved Transcriptional Signatures across Multiple Respiratory Viruses. *Immunity* 2015; **43**(6): 1199-211.
12. Sweeney TE, Braviak L, Tato CM, Khatri P. Genome-wide expression for diagnosis of pulmonary tuberculosis: a multicohort analysis. *Lancet Respir Med* 2016; **4**(3): 213-24.

13. Liu X, Speranza E, Munoz-Fontela C, et al. Transcriptomic signatures differentiate survival from fatal outcomes in humans infected with Ebola virus. *Genome Biol* 2017; **18**(1): 4.
14. Suarez NM, Bunsow E, Falsey AR, Walsh EE, Mejias A, Ramilo O. Superiority of transcriptional profiling over procalcitonin for distinguishing bacterial from viral lower respiratory tract infections in hospitalized adults. *J Infect Dis* 2015; **212**(2): 213-22.
15. Kaforou M, Wright VJ, Oni T, et al. Detection of tuberculosis in HIV-infected and -uninfected African adults using whole blood RNA expression signatures: a case-control study. *PLoS Med* 2013; **10**(10): e1001538.
16. Woods CW, McClain MT, Chen M, et al. A Host Transcriptional Signature for Presymptomatic Detection of Infection in Humans Exposed to Influenza H1N1 or H3N2. *PLoS One* 2013; **8**(1): e52198.
17. Mejias A, Dimo B, Suarez NM, et al. Whole blood gene expression profiles to assess pathogenesis and disease severity in infants with respiratory syncytial virus infection. *PLoS Med* 2013; **10**(11): e1001549.
18. Hu X, Yu J, Crosby SD, Storch GA. Gene expression profiles in febrile children with defined viral and bacterial infection. *Proc Natl Acad Sci U S A* 2013; **110**(31): 12792-7.
19. Zhai Y, Franco LM, Atmar RL, et al. Host Transcriptional Response to Influenza and Other Acute Respiratory Viral Infections--A Prospective Cohort Study. *PLoS pathogens* 2015; **11**(6): e1004869.
20. Roe J, Venturini C, Gupta RK, et al. Blood transcriptomic stratification of short-term risk in contacts of tuberculosis. *Clin Infect Dis* 2019.
21. Afroz S, Giddaluru J, Abbas MM, Khan N. Transcriptome meta-analysis reveals a dysregulation in extra cellular matrix and cell junction associated gene signatures during Dengue virus infection. *Scientific reports* 2016; **6**: 33752.
22. McClain MT, Nicholson BP, Park LP, et al. A Genomic Signature of Influenza Infection Shows Potential for Presymptomatic Detection, Guiding Early Therapy, and Monitoring Clinical Responses. *Open Forum Infect Dis* 2016; **3**(1): ofw007.
23. Huang Y, Zaas AK, Rao A, et al. Temporal Dynamics of Host Molecular Responses Differentiate Symptomatic and Asymptomatic Influenza A Infection. *PLoS Genet* 2011; **7**(8): e1002234.
24. Shurtleff AC, Whitehouse CA, Ward MD, Cazares LH, Bavari S. Pre-symptomatic diagnosis and treatment of filovirus diseases. *Frontiers in microbiology* 2015; **6**(108).
25. Caballero IS, Yen JY, Hensley LE, Honko AN, Goff AJ, Connor JH. Lassa and Marburg viruses elicit distinct host transcriptional responses early after infection. *BMC Genomics* 2014; **15**(1): 960.
26. Duy J, Koehler JW, Honko AN, et al. Circulating microRNA profiles of Ebola virus infection. *Scientific reports* 2016; **6**: 24496.
27. Wang K, Langevin S, O'Hern CS, et al. Anomaly Detection in Host Signaling Pathways for the Early Prognosis of Acute Infection. *PLoS One* 2016; **11**(8): e0160919.
28. Memoli MJ, Czajkowski L, Reed S, et al. Validation of the wild-type influenza A human challenge model H1N1pdMIST: an A(H1N1)pdm09 dose-finding investigational new drug study. *Clin Infect Dis* 2015; **60**(5): 693-702.
29. Han A, Poon JL, Powers JH, 3rd, Leidy NK, Yu R, Memoli MJ. Using the Influenza Patient-reported Outcome (FLU-PRO) diary to evaluate symptoms of influenza viral infection in a healthy human challenge model. *BMC infectious diseases* 2018; **18**(1): 353.

30. Zaas AK, Chen M, Varkey J, et al. Gene expression signatures diagnose influenza and other symptomatic respiratory viral infections in humans. *Cell Host Microbe* 2009; **6**(3): 207-17.
31. Sobel Leonard A, McClain MT, Smith GJ, et al. Deep Sequencing of Influenza A Virus from a Human Challenge Study Reveals a Selective Bottleneck and Only Limited Intrahost Genetic Diversification. *Journal of virology* 2016; **90**(24): 11247-58.
32. Friedman J, Hastie T, Tibshirani R. Regularization Paths for Generalized Linear Models via Coordinate Descent. *Journal of statistical software* 2010; **33**(1): 1-22.
33. Goeman JJ. L1 penalized estimation in the Cox proportional hazards model. *Biometrical journal Biometrische Zeitschrift* 2010; **52**(1): 70-84.
34. Stanton N, Hood K, Kelly MJ, et al. Are smokers with acute cough in primary care prescribed antibiotics more often, and to what benefit? An observational study in 13 European countries. *European Respiratory Journal* 2010; **35**(4): 761-7.
35. LeBlanc JJ, Li Y, Bastien N, Forward KR, Davidson RJ, Hatchette TF. Switching gears for an influenza pandemic: validation of a duplex reverse transcriptase PCR assay for simultaneous detection and confirmatory identification of pandemic (H1N1) 2009 influenza virus. *Journal of clinical microbiology* 2009; **47**(12): 3805-13.
36. Christian MD, Poutanen SM, Low DE, Muller MP, Loutfy MR. Severe Acute Respiratory Syndrome. *Clinical Infectious Diseases* 2004; **38**(10): 1420-7.
37. Bongen E, Vallania F, Utz PJ, Khatri P. KLRD1-expressing natural killer cells predict influenza susceptibility. *Genome medicine* 2018; **10**(1): 45.
38. Tang BM, McLean AS, Dawes IW, Huang SJ, Lin RC. Gene-expression profiling of peripheral blood mononuclear cells in sepsis. *Crit Care Med* 2009; **37**(3): 882-8.
39. Warsinske HC, Rao AM, Moreira FMF, et al. Assessment of Validity of a Blood-Based 3-Gene Signature Score for Progression and Diagnosis of Tuberculosis, Disease Severity, and Treatment Response. *JAMA network open* 2018; **1**(6): e183779.
40. Tang BM, Shojaei M, Parnell GP, et al. A novel immune biomarker IFI27 discriminates between influenza and bacteria in patients with suspected respiratory infection. *The European respiratory journal* 2017; **49**(6).
41. Herberg JA, Kaforou M, Wright VJ, et al. Diagnostic Test Accuracy of a 2-Transcript Host RNA Signature for Discriminating Bacterial vs Viral Infection in Febrile Children. *Jama* 2016; **316**(8): 835-45.
42. Maslove DM, Wong HR. Gene expression profiling in sepsis: timing, tissue, and translational considerations. *Trends Mol Med* 2014; **20**(4): 204-13.
43. Ramilo O, Allman W, Chung W, et al. Gene expression patterns in blood leukocytes discriminate patients with acute infections. *Blood* 2007; **109**(5): 2066-77.

Acknowledgements

Special thanks to Debra Freeman, Sara Hoffman, Olga M. Better, Mert Aydin, Stephanie Dobos, Kyle R. Breitschwert, Ashlee M. Valente for their tireless work enrolling subjects, processing samples and data, and engaging with the undergraduate campus.

Funding

Funding for this study was provided by the US Defense Advanced Research Projects Agency (DARPA), project # N66001-07-C-2024 and W911NF1410052. The statements and conclusions herein are those of the authors and do not reflect the position or policy of the United States Government.

Author contributions

MTM, ELT, GSG and CWW drafted the manuscript, which was critically revised by all remaining authors. MTM, BPN, TB, TV, GSG, and CWW helped conceive and implement the study. All authors helped acquire, analyze, or interpret data. Statistical analyses were performed by FJC, RH, SS, MN, and LP.

Figures

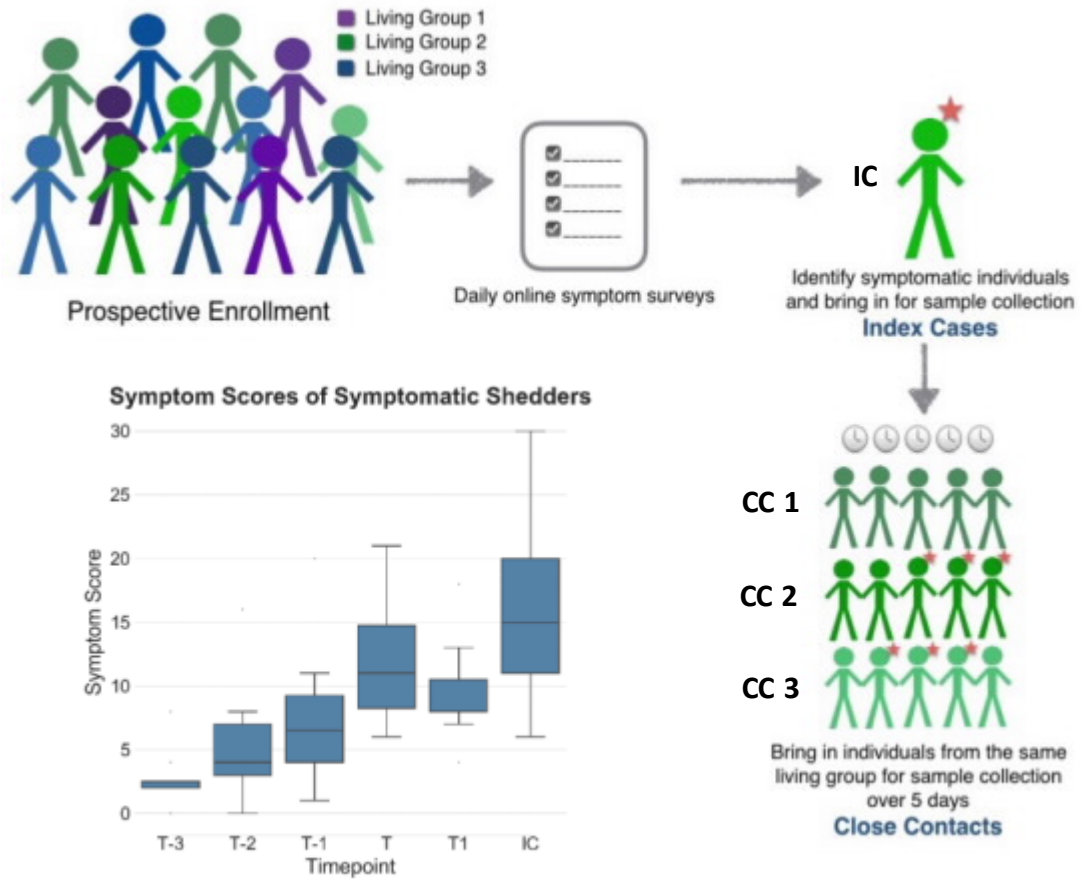


Figure 1: Study design. Students were enrolled at the beginning of the school year. Index Cases were identified when daily symptom surveys indicated potential respiratory illness. This flagged serial sampling of their Close Contacts, some of whom would also develop RTI during the 5-day observation window (red stars). This unique model permits blood sampling of some Close Contacts during early and presymptomatic phases of illness. Mean symptom scores of Close Contacts who developed PCR-proven RTI under observation are presented.

A

	Cough	Fever	Headache	Malaise	Nasal Congestion	Nasal Discharge	Sneezing	Sore Throat	Total Symptoms
Influenza*	3.2	1.9	2.4	3.0	2.5	2.4	1.8	2.7	20.0
RSV	2.1	0.7	1.6	2.9	2.6	2.6	1.5	2.6	16.6
Human Metapneumovirus	2.6	0.8	1.5	2.5	2.4	2.5	1.9	2.2	16.3
Rhinovirus	2.2	0.5	1.1	2.1	2.3	2.3	1.5	2.1	14.0
Coxsackie/Echovirus	2.1	0.5	1.1	2.2	2.4	2.4	1.6	1.8	14.0
Coronavirus	1.8	0.7	1.0	2.1	2.3	2.5	1.6	1.8	13.8
Parainfluenzavirus	2.3	0.5	1.2	2.3	1.8	1.7	1.3	2.3	13.4
Adenovirus	2.2	1.2	1.4	2.2	1.6	1.6	0.2	2.2	12.6
Bocavirus	1.0	0.0	0.0	1.0	2.0	2.0	1.0	1.0	8.0

B

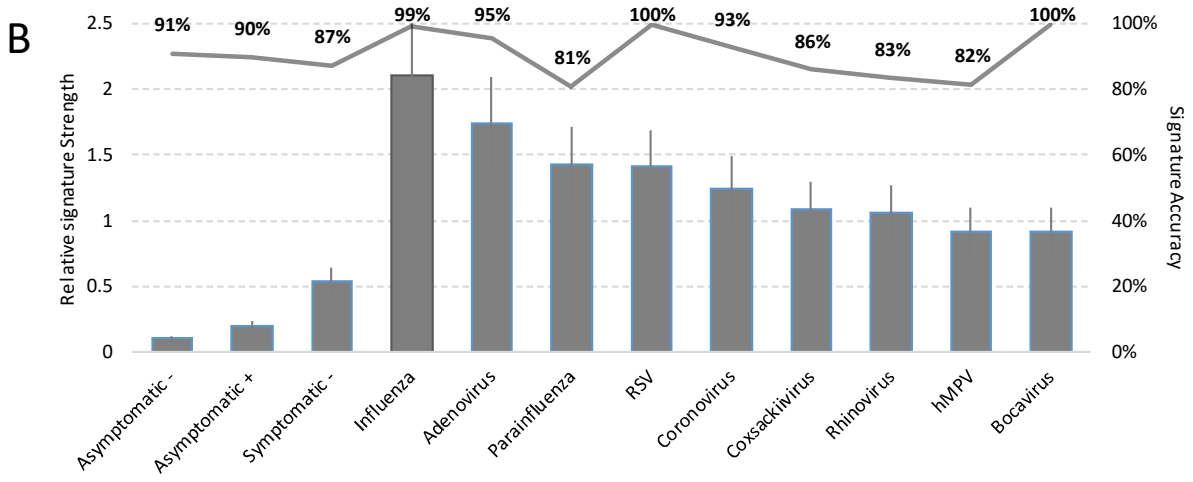


Figure 2. Clinical and genomic variation amongst viruses. For each type of viral infection, mean scores for each symptom measured at the time of maximal illness are presented (Panel A). The relative quantitative transcriptomic signature strength (bars) and accuracy (black line) at the time of maximal illness for each virus, as well as for Asymptomatic nonshedders (-), Asymptomatic shedders (+), and Symptomatic nonshedders (-) are presented in Panel B.

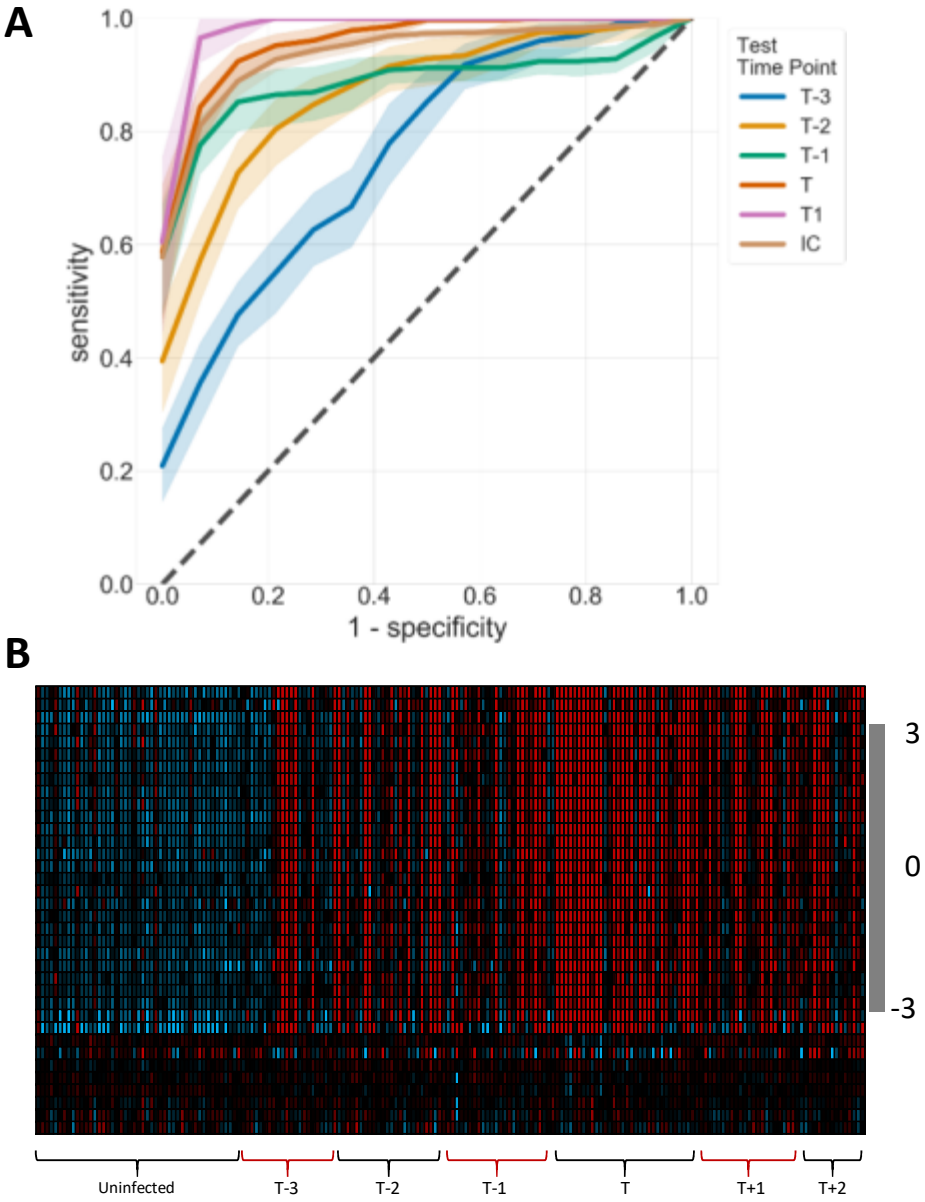


Figure 3: Signature performance across time. An RT-PCR-based 36-gene signature accurately discriminates subjects with naturally-acquired respiratory viral infection at both symptomatic and presymptomatic stages of infection (A). Heatmap of z-scores for each gene at each timepoint in subjects with proven symptomatic viral infection (Panel B), where time 'T' is the day of maximal illness.

Table 1. Viral etiologies of RTI in the Student Cohort

	2009-2010	2010-2011	2011-2012	2013-2014[a]	2014-2015	Total
Adenovirus	1	1	0	1	2	**5**
Bocavirus	0	0	0	0	2	**2**
Coronavirus	11	23	8	3	9	**54**
Coxsackie/Echovirus[b]	21	38	12	0	0	**71**
Human Metapneumovirus	2	1	3	4	1	**11**
Influenza	9	7	1	1	1	**19**
Parainfluenzavirus	3	10	1	0	7	**21**
RSV	3	1	3	0	3	**10**
Rhinovirus	20	23	29	3	37	**112**
Total	**70**	**104**	**57**	**12**	**62**	**305**

[a]truncated season, Spring enrollment only
[b]not present in 2013-2015 testing

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Board on Health Sciences Policy

HOST GENOMICS RESEARCH AND DATA COLLECTION EFFORTS RELATED TO COVID-19

Joint Meeting of the
Roundtable on Genomics and Precision Health
and the
Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats

Virtual Meeting July 1, 2020
Zoom: https://nasem.zoom.us/j/95540901323

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Board on Health Sciences Policy

Host Genomics Research and Data Collection Efforts Related to COVID-19

July 1, 2020

Table of Contents

Agenda 3

Agenda 4

ROUNDTABLE AND STANDING COMMITTEE INFORMATION 7

Roundtable on Genomics and Precision Health 8

Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats 10

MEETING INFORMATION 13

Speaker and Chair Biographies 14

BACKGROUND MATERIALS 17

Online links to background materials 18

Rapid Expert Consultations on the COVID-19 Pandemic 20

A blood-based host gene expression assay for early detection of viral infection 21

AGENDA

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Board on Health Sciences Policy

Host Genomics Research and Data Collection Efforts Related to COVID-19

Joint Meeting
of the
Roundtable on Genomics and Precision Health
and the
Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats

A VIRTUAL MEETING
July 1, 2020
1:00 PM – 2:45 PM ET
Zoom Link: https://nasem.zoom.us/j/95540901323

Aims for the meeting:

- To understand the scientific challenges that the field of genomics is facing
  - What has been the effect of the pandemic on genomics research and data collection and sharing? What is the role of the genomics community during the pandemic? What research is underway? What research still needs to be done?
  - What gaps and challenges in the field is this crisis highlighting? What unique contributions of the field are not being leveraged effectively?
- To explore innovative approaches to overcoming those challenges
  - What new approaches are you using to solve a problem? Could these be applied to the field of genomics? Are there lessons from genomics that are being used in light of the pandemic? What are the opportunities for connecting efforts and data sets (e.g. across institutions, genotype and phenotype data)?
- To discuss opportunities for the Genomics Roundtable and for the Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats to facilitate progress
  - Are there opportunities for the Roundtable and Standing Committee to act that align with strategic priorities?
  - Are the Roundtable and Standing Committee uniquely positioned to address specific challenges?

AGENDA:

1:00 p.m. ET Welcoming Remarks

GEOFFREY GINSBURG, *Roundtable Co-Chair*
Director, Duke Center for Applied Genomics & Precision Medicine
Professor, Medicine, Pathology, and Biomedical Engineering
Duke University Medical Center

MICHELLE PENNY, *Roundtable Co-Chair*
Vice President and Head of Genomics
Goldfinch Bio

HARVEY FINEBERG, *Standing Committee Chair*
President
Gordon and Betty Moore Foundation

1:15 p.m. **An Introduction to the Host Genetics of COVID-19**

MAXIMILIAN MUENKE
CEO
American College of Medical Genetics and Genomics (ACMG)

Understanding Host Genetics and Genomics Related to COVID-19 and Opportunities for Forming Data Sharing COVID-19 Communities

1:25 p.m. SHARON TERRY
Founder and CEO
Genetic Alliance

1:35 p.m. JOYCE TUNG
Vice President, Research
23andMe

1:45 p.m. GEOFFREY GINSBURG, *Roundtable Co-Chair*
Director, Duke Center for Applied Genomics & Precision Medicine
Professor, Medicine, Pathology, and Biomedical Engineering
Duke University Medical Center

1:55 p.m. **Discussion with Roundtable and Standing Committee Members**

2:30 p.m. **Next Steps**

Questions:

- What challenges or gaps were exposed today?
- Is there an area that should be incorporated into the Roundtable's strategic planning or the Standing Committee's work?

GEOFFREY GINSBURG, *Roundtable Co-Chair*
Director, Duke Center for Applied Genomics & Precision Medicine
Professor, Medicine, Pathology, and Biomedical Engineering
Duke University Medical Center

MICHELLE PENNY, *Roundtable Co-Chair*
Vice President and Head of Genomics
Goldfinch Bio

HARVEY FINEBERG, *Standing Committee Chair*
President
Gordon and Betty Moore Foundation

2:45 p.m. Adjourn

ROUNDTABLE AND STANDING COMMITTEE INFORMATION

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Roundtable on GENOMICS and PRECISION HEALTH

The sequencing of the human genome is rapidly opening new doors to research and progress in biology, medicine, and health care. At the same time, these developments have produced a diversity of new issues to be addressed.

The National Academies of Sciences, Engineering, and Medicine has convened a Roundtable on Genomics and Precision Health (previously the Roundtable on Translating Genomic-Based Research for Health) that brings together leaders from academia, industry, government, foundations and associations, and representatives of patient and consumer interests who have a mutual concern and interest in addressing the issues surrounding the translation of genome-based research for use in maintaining and improving health. The mission of the Roundtable is to advance the field of genomics and improve the translation of research findings to health care, education, and policy. The Roundtable will discuss the translation process, identify challenges at various points in the process, and discuss approaches to address those challenges.

The field of genomics and its translation involves many disciplines, and takes place within different economic, social, and cultural contexts, necessitating a need for increased communication and understanding across these fields. As a convening mechanism for interested parties from diverse perspectives to meet and discuss complex issues of mutual concern in a neutral setting, the Roundtable: fosters dialogue across sectors and institutions; illuminates issues, but does not necessarily resolve them; and fosters collaboration among stakeholders.

To achieve its objectives, the Roundtable conducts structured discussions, workshops, and symposia. Workshop summaries will be published and collaborative efforts among members are encouraged (e.g., journal articles). Specific issues and agenda topics are determined by the Roundtable membership, and span a broad range of issues relevant to the translation process.

Issues may include the integration and coordination of genomic information into health care and public health including encompassing standards for genetic screening and testing, improving information technology for use in clinical decision making, ensuring access while protecting privacy, and using genomic information to reduce health disparities. The patient and family perspective on the use of genomic information for translation includes social and behavioral issues for target populations. There are evolving requirements for the health professional community, and the need to be able to understand and responsibly apply genomics to medicine and public health.

Of increasing importance is the need to identify the economic implications of using genome-based research for health. Such issues include incentives, cost-effectiveness, and sustainability.

Issues related to the developing science base are also important in the translation process. Such issues could include studies of gene-environment interactions, as well as the implications of genomics for complex disorders such as addiction, mental illness, and chronic diseases.

Roundtable sponsors include federal agencies, pharmaceutical companies, medical and scientific associations, foundations, and patient/public representatives. For more information about the Roundtable on Genomics and Precision Health, please visit our website at nationalacademies.org/GenomicsRT or contact Sarah Beachy at 202-334-2217, or by e-mail at sbeachy@nas.edu.

The National Academy of Sciences, National Academy of Engineering, and National Academy of Medicine work together as the National Academies of Sciences, Engineering, and Medicine ("the Academies") to provide independent, objective analysis and advice to the nation and conduct other activities to solve complex problems and inform public policy decisions. The Academies also encourage education and research, recognize outstanding contributions to knowledge, and increase public understanding in matters of science, engineering, and medicine.

Roundtable on Genomics and Precision Health Membership

Geoffrey Ginsburg, M.D., Ph.D. (Co-Chair) Duke University
Michelle Penny, Ph.D. (Co-Chair) Goldfinch Bio

Naomi Aronson, Ph.D., BlueCross/BlueShield Association

Aris Baras, M.D., M.B.A., Regeneron Pharmaceuticals

Karina Bienfait, Ph.D., Merck and Co., Inc.

Vence Bonham, Jr., J.D., National Human Genome Research Institute

Robert B. Darnell, M.D. Ph.D., The Rockefeller University / NY Genome Center

Stephanie Devaney, Ph.D., All of Us Research Program, NIH

Katherine Donigan, Ph.D., U.S. Food and Drug Administration

W. Gregory Feero, M.D., Ph.D., Journal of the American Medical Association

Jessica M. Gill, Ph.D., R.N., FAAN, National Institute of Nursing Research

Jennifer Goldsack, MChem, M.A., M.B.A., Digital Medicine Society (DiMe)

Marc Grodman, M.D., Genosity

Richard Hodes, M.D., National Institute on Aging

Praduman Jain, M.S., Vibrent Health

Sally John, Ph.D., Biogen

Sekar Kathiresan, M.D., Massachusetts General Hospital

Muin Khoury, M.D., Ph.D., Centers for Disease Control and Prevention

David Ledbetter, Ph.D., Geisinger

Charles Lee, Ph.D., FACMG, The Jackson Laboratory for Genomic Medicine

Thomas Lehner, Ph.D., M.P.H., National Institute of Mental Health

Debra Leonard, M.D., Ph.D., College of American Pathologists

Patrick Loerch, Ph.D., Johnson & Johnson

James Lu, M.D., Ph.D., Helix

Sean McConnell, Ph.D., American Medical Association

Mona Miller, M.P.P., American Society of Human Genetics

Jennifer Moser, Ph.D., U.S. Department of Veterans Affairs

Maximilian Muenke, M.D., FACMG, American College of Medical Genetics and Genomics

Anna Pettersson, Ph.D., Pfizer Inc.

Victoria M. Pratt, Ph.D., FACMG, Association for Molecular Pathology

Kunal Sanghavi, M.B.B.S., M.S., C.G.C., The Jackson Laboratory for Genomic Medicine

Nadeem Sarwar, Ph.D., Eisai Inc.

Sheri Schully, Ph.D., Formerly NIH Office of Disease Prevention, now All of Us

Joan A. Scott, M.S., C.G.C., Health Resources and Services Administration

Sam Shekar, M.D., M.P.H., American College of Preventive Medicine

Nonniekaye Shelburne, C.R.N.P., M.S., A.O.C.N., National Cancer Institute

Nikoletta Sidiropoulos, M.D., University of Vermont Health Network Medical Group

Katherine Johansen Taber, Ph.D., Myriad Women's Health

Ryan Taft, Ph.D., Illumina

Jacquelyn Taylor, Ph.D., New York University

Sharon Terry, M.A., Genetic Alliance

Joyce Tung, Ph.D., 23andMe, Inc.

Jameson Voss, M.D., Air Force Medical Support Agency

Catherine A. Wicklund, M.S., C.G.C., National Society of Genetic Counselors

Huntington F. Willard, Ph.D., Genome Medical

Janet K. Williams, Ph.D., R.N., FAAN, American Academy of Nursing

Sarah Wordsworth, Ph.D., University of Oxford

Alicia Zhou, Ph.D., Color Genomics

Project Staff
Sarah H. Beachy, Ph.D., *Roundtable Director*
Siobhan Addie, Ph.D., *Program Officer*
Meredith Hackmann, *Associate Program Officer*
Kelly Choi, *Senior Program Assistant*

NAM Greenwall Fellow in Bioethics
Rachel Fabi, Ph.D., *Upstate Medical University*

500 Fifth Street, NW, Washington, DC 20001
Phone 202.334.2217 E-mail sbeachy@nas.edu nationalacademies.org/GenomicsRT

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Health and Medicine Division

Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats

In response to a request from the Office of Science and Technology Policy (OSTP) and the Office of the Assistant Secretary for Preparedness and Response (ASPR), the National Academies of Sciences, Engineering, and Medicine will convene a standing committee of experts to help inform the federal government on critical science and policy issues related to emerging infectious diseases and other 21st century health threats. The standing committee will include members with expertise in emerging infectious diseases, public health, public health preparedness and response, biological sciences, clinical care and crisis standards of care, risk communication, epidemiology, and regulatory issues, as well as veterinary science, One Health, ethics, and community engagement. The standing committee will provide a venue for the exchange of ideas among federal government agencies, the private sector, and the academic community, as well as other relevant stakeholders.

The standing committee will:

- Stand ready to respond on short notice to requests from the federal government to assess and consider the science and policy implications of an emerging infectious disease or significant public health threat;
- Provide a venue to enable science and policy discussions relevant to the federal government on emerging issues, research, and activities through in-depth knowledge of the sponsor's programs, goals, and objectives;
- Identify opportunities to integrate science into national preparedness and response decision making;
- Explore lessons learned and best practices from previous preparedness and response efforts, and identify opportunities to disseminate that information to a variety of stakeholders;
- Serve as a focal point for national policy discussions by experts and other leaders in the field;
- Consider, identify, and discuss strategies for addressing misinformation; and
- Respond to the federal government's needs for continuing dialog related to strategic planning and program development to address emerging infectious diseases, biosecurity, and public health and medical preparedness.

At the request of the sponsors, the standing committee will be involved in the planning, development, and oversight of related ad hoc activities undertaken by separately appointed committees operating under its auspices.

The standing committee will serve as a focal point for the discussion of scientific, technical, and policy issues relevant to emerging infectious diseases and public health preparedness and response that warrant detailed examination. Topics for discussion with the standing committee may include:

- Technical assistance and/or assessment of response to emerging infectious diseases;
- Availability of and access to information, samples, and other materials to determine the origin and evolution of emerging infectious diseases;
- International coordination and engagement;
- Technical assessment of ecological and evolutionary drivers of disease emergence;
- Approaches to proactive public messaging and strategies to address misinformation;
- Other science and policy issues relevant to emerging infectious diseases and 21st century health threats.

The committee will carry out its charge at its in-person and virtual meetings by gathering evidence from experts, deliberating, and, when necessary, by preparing short reports.

COMMITTEE SPONSORS

White House Office of Science and Technology Policy and HHS Office of Assistant Secretary for Preparedness and Response

COMMITTEE ROSTER

Harvey Fineberg, M.D., Ph.D. ***(Chair)***
President
Gordon and Betty Moore Foundation

Kristian Andersen, Ph.D.
Associate Professor and Director of Infectious Disease Genomics, Scripps Research Translational Institute
The Scripps Research Institute

Ralph Steven Baric, Ph.D.
William R. Kenan, Jr. Distinguished Professor
The University of North Carolina at Chapel Hill

Mary Bassett, M.D., M.P.H.
Director of the François-Xavier Bagnoud Center for Health and Human Rights
Harvard School of Public Health

Trevor Bedford, Ph.D.
Associate Faculty Member, Vaccine and Infectious Disease Division, Public Health Sciences Division, and Human Biology Division
Fred Hutchinson Cancer Research Center

Georges Benjamin, M.D.
Executive Director
American Public Health Association

Donald Berwick, M.D., M.P.P., F.R.C.P, KBE
Lecturer of Health Care Policy
Harvard Medical School

Richard Besser, M.D.
President and CEO
Robert Wood Johnson Foundation

R. Alta Charo, J.D.
Warren P. Knowles Professor of Law and Bioethics
University of Wisconsin at Madison

Peter Daszak, Ph.D.
President and CEO
EcoHealth Alliance

Jeffrey S. Duchin, M.D.
Health Officer and Chief, Communicable Disease Epidemiology & Immunization Section and Professor in Medicine

Ellen Embrey
President/CEO
Stratitia, Inc.

Baruch Fischhoff, Ph.D.
Howard Heinz University Professor
Department of Engineering and Public Policy
Carnegie Mellon University

Diane Griffin, M.D., Ph.D.
Professor, Department of Molecular Microbiology and Immunology
Johns Hopkins Bloomberg School of Public Health

Robert Groves, Ph.D., M.A.
Executive Vice President and Provost
Gerard J. Campbell, S.J. Professor
Math and Statistics Department and Sociology Department
Georgetown University

Margaret Hamburg, M.D.
Foreign Secretary
National Academy of Medicine

Dan Hanfling, M.D.
Vice President, Technical Staff
In-Q-Tel

John Hick, M.D.
Associate Medical Director for EMS
Medical Director of Emergency Medicine
Hennepin County Medical Center

Kent E. Kester, M.D.
Vice President and Head, Translational Science and Biomarkers
Sanofi Pasteur

Patricia King, J.D.
Professor Emerita
Georgetown University Law Center

Jonna Mazet, D.V.M., M.P.V.M., Ph.D.
Executive Director, One Health Institute
UC Davis School of Veterinary Medicine

Phyllis Meadows, Ph.D., M.S.N., R.N.
Senior Fellow, Health
The Kresge Foundation

Tara O'Toole, M.D., M.P.H.
Executive Vice President
In-Q-Tel

Alexandra Phelan, S.J.D., LL.M., LL.B.
Assistant Professor
Center for Global Health Science and Security
Georgetown University

David Relman, M.D.
Thomas C. and Joan M. Merigan Professor of Medicine, and of Microbiology & Immunology; Chief of Infectious Diseases
Stanford University; VA Palo Alto Health Care System

Mark Smolinski, M.D., M.P.H.
President
Ending Pandemics

David Walt, Ph.D.
Hansjörg Wyss Professor of Biologically Inspired Engineering
Harvard Medical School

CONTACT INFORMATION

Andrew Pope, Ph.D.
Senior Director, Board on Health Sciences Policy
202-334-1739
apope@nas.edu

Lisa Brown, M.P.H.
Study Director, Board on Health Sciences Policy
202-334-2487
lbrown@nas.edu

ADDITIONAL INFORMATION

For additional information, please visit https://www.nationalacademies.org/our-work/standing-committee-on-emerging-infectious-diseases-and-21st-century-health-threats

MEETING INFORMATION

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

Board on Health Sciences Policy

Host Genomics Research and Data Collection Efforts Related to COVID-19

Joint Meeting
of the
Roundtable on Genomics and Precision Health
and the
Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats

SPEAKER AND CHAIR BIOGRAPHIES

Harvey V. Fineberg, M.D., Ph.D. ***(Standing Committee Chair)***
Dr. Fineberg is the president of the Gordon and Betty Moore Foundation, which fosters path-breaking scientific discovery, environmental conservation, improvements in patient care and preservation of the special character of the San Francisco Bay Area. He previously held the Presidential Chair for 2014-2015 as visiting professor at the University of California, San Francisco. Prior to that, he served as president of the Institute of Medicine from 2002 to 2014 and as provost of Harvard University from 1997 to 2001, following 13 years as dean of the Harvard School of Public Health. He has devoted most of his academic career to the fields of health policy and medical decision-making. His past research has focused on the process of policy development and implementation, assessment of medical technology, evaluation and use of vaccines, and dissemination of medical innovations. Fineberg chairs the board of the Carnegie Endowment for International Peace and serves on the boards of the William and Flora Hewlett Foundation and the China Medical Board. He helped found and served as president of the Society for Medical Decision Making and also served as consultant to the World Health Organization. Fineberg is co-author of the books Clinical Decision Analysis, Innovators in Physician Education and The Epidemic That Never Was, an analysis of the controversial federal immunization program against swine flu in 1976. He has co-edited several books on such diverse topics as AIDS prevention, vaccine safety, understanding risk in society and global health. He has also authored numerous articles published in professional journals. Fineberg is the recipient of several honorary degrees—the Frank A. Calderone Prize in Public Health, the Henry G. Friesen International Prize in Health Research and the Harvard Medal, awarded by the alumni association of the university from which he earned his bachelor's and doctoral degrees.

Geoffrey Ginsburg, M.D., Ph.D. (Roundtable *Co-Chair*)
Dr. Ginsburg is the founding director for the Center for Applied Genomics & Precision Medicine at the Duke University Medical Center and for MEDx, a partnership between the Schools of Medicine and Engineering to spark and translate innovation. His research addresses the challenges for translating genomic and digital information into medical practice and the integration of precision medicine into healthcare. In 2017 he received Duke's Translational Research Mentorship Award and is a finalist in the NIH/BARDA Antimicrobial Resistance Prize.

He is a member of the Advisory Council to the Director of NIH and is co-chair of the National Academies Roundtable on Genomics and Precision Health and is founder and president of the Global Genomic Medicine

Collaborative, a not for profit organization aimed creating international partnerships to advance the implementation of precision medicine. He has recently served as a member of the Board of External Experts for the NHLBI, the advisory council for the National Center for Accelerating Translational Science, the chair of the review for Genome Canada's Large Scale Applied Research Competition in Genomics and Precision Medicine, and the World Economic Forum's Global Agenda Council on the Future of the Health Sector. He is a founder of Predigen Inc and MeTree&You Inc. He was previously Vice President of Molecular Medicine at Millennium Pharmaceuticals, Inc and a faculty member at Harvard Medical School.

Maximilian Muenke, M.D., FACMG
Dr. Muenke joined the American College of Medical Genomics (ACMG) as CEO in 2019. Recognized as a highly acclaimed physician-scientist and dedicated clinical and research mentor, Dr. Muenke has more than three decades of experience to the ACMG, including 10 years on the faculty of the University of Pennsylvania School of Medicine. Since 2000, Dr. Muenke has served as Senior Investigator, Head of the Human Development Section, and Chief of the Medical Genetics Branch at the National Human Genome Research Institute (NHGRI) of the National Institutes of Health (NIH). The focus of his laboratory's research was on the delineation and identification of the underlying causes of craniofacial and related anomalies in humans including holoprosencephaly as well as Muenke syndrome, a relatively common craniosynostosis syndrome. Dr. Muenke is passionate about training the next generation of leaders in the field of genetics and genomic medicine. He has directed medical genetics training since 1994, first at the University of Pennsylvania and then at the NIH, where he was the Director of the NIH Medical Genetics and Genomic Medicine Residency and Fellowship Training Programs.

Michelle Penny, Ph.D. ***(Roundtable Co-Chair)***
Dr. Penny is a Vice President and Head of Genomics at Golfich Bio. Prior to that, she was the Head of Translational Genome Sciences at Biogen. She received her Ph.D. in Genetics from the University of Birmingham, UK in 1993. After a postdoctoral fellowship in the Virology Division at the National Institute of Medical Research, Mill Hill, London, she joined Imperial College London, Department of Medical and Community Genetics where she was a postdoctoral research scientist until taking a lectureship in Human Molecular Genetics in 1998. Her academic research career focused on the study of complex polygenic diseases particularly autoimmune disease and susceptibility to infection. In 2002, Dr. Penny joined the Clinical Pharmacogenomics group at Pfizer in Sandwich, UK, and moved to New London, CT in 2006 to take on the role of Oncology Molecular Medicine Lead until 2009 when she moved to Indianapolis to lead the Pharmacogenomics work at Eli Lilly and Company.

Sharon Terry, M.A.
Sharon Terry is President and CEO of Genetic Alliance, a large network engaging individuals, families and communities to transform health. Genetic Alliance works to provide programs, products and tools for ordinary people to take charge of their health.

As 'just a Mom' with a master's degree in Theology, she cofounded PXE International, a research advocacy organization for the genetic condition pseudoxanthoma elasticum (PXE), in response to the diagnosis of PXE in her two children in 1994. With her husband, she co-discovered the ABCC6 gene, patented it to ensure ethical stewardship in 2000, and assigned their rights to the foundation. She subsequently developed a diagnostic test and conducts clinical trials. She is the author of 140 peer-reviewed papers, of which 30 are clinical PXE studies.

In her focus at the forefront of consumer participation in genetics research, services and policy, she serves in a leadership role on many of the major international and national organizations, including the Precision Medicine Initiative Cohort Advisory Panel, Accelerating Medicines Partnership, Institute of Medicine (IOM) Science and Policy Board, the IOM Roundtable on Translating Genomic-Based Research for Health, the PubMed Central National Advisory Committee, the PhenX scientific advisory board, the Global Alliance for Genomics and Health, the International Rare Disease Research Consortium Executive Committee and as Founding President of EspeRare Foundation of Geneva, Switzerland. Terry is co-founder of the Genetic Alliance Registry and Biobank. She is on the editorial boards of several journals. She led the coalition that was instrumental in the passage of the Genetic Information Nondiscrimination Act. She received an honorary doctorate from Iona College for her

community engagement work in 2006; the Research!America Distinguished Organization Advocacy Award in 2009; and the Clinical Research Forum and Foundation's Annual Award for Leadership in Public Advocacy in 2011. She was named one of FDA's "30 Heroes for the Thirtieth Anniversary of the Orphan Drug Act" in 2013. She is co-inventor of the Platform for Engaging Everyone Responsibly (PEER). PEER received $500,000 from the Robert Wood Johnson Foundation in 2014. PEER undergirds the Community Engaged Network for All (CENA), a PCORnet member funded in 2013 for Phase I and in 2015 for Phase II. She is Co-PI of the PCORnet Coordinating Center and Chair of the PCORnet Engagment Committee.

Joyce Tung, Ph.D.
Joyce joined 23andMe in 2007 and manages the 23andMe research team, which is responsible for consumer health and ancestry research and development, academic and industry collaborations, computational analyses for therapeutics, and new research methods and tools development. While a postdoctoral fellow at Stanford University, Joyce studied the genetics of mouse and human pigmentation. She graduated from Stanford with honors and distinction with a B.S. in Biological Sciences and a minor in computer science, and earned her Ph.D. in Genetics from the University of California, San Francisco where she was a National Science Foundation graduate research fellow.

BACKGROUND MATERIALS

Links to Background Materials

Materials from 23andMe

- The information page for the external recruitment portion of our COVID-19 study
- Blog post on initial launch of our study
- Blog post on expansion of our study to enroll severe cases
- Blog post on preliminary findings

Genomewide Association Study of Severe Covid-19 with Respiratory Failure

BACKGROUND

There is considerable variation in disease behavior among patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes coronavirus disease 2019 (Covid-19). Genomewide association analysis may allow for the identification of potential genetic factors involved in the development of Covid-19.

A Host Transcriptional Signature for Presymptomatic Detection of Infection in Humans Exposed to Influenza H1N1 or H3N2

BACKGROUND

There is great potential for host-based gene expression analysis to impact the early diagnosis of infectious diseases. In particular, the influenza pandemic of 2009 highlighted the challenges and limitations of traditional pathogen-based testing for suspected upper respiratory viral infection.

A crowdsourced analysis to identify ab initio molecular signatures predictive of susceptibility to viral infection

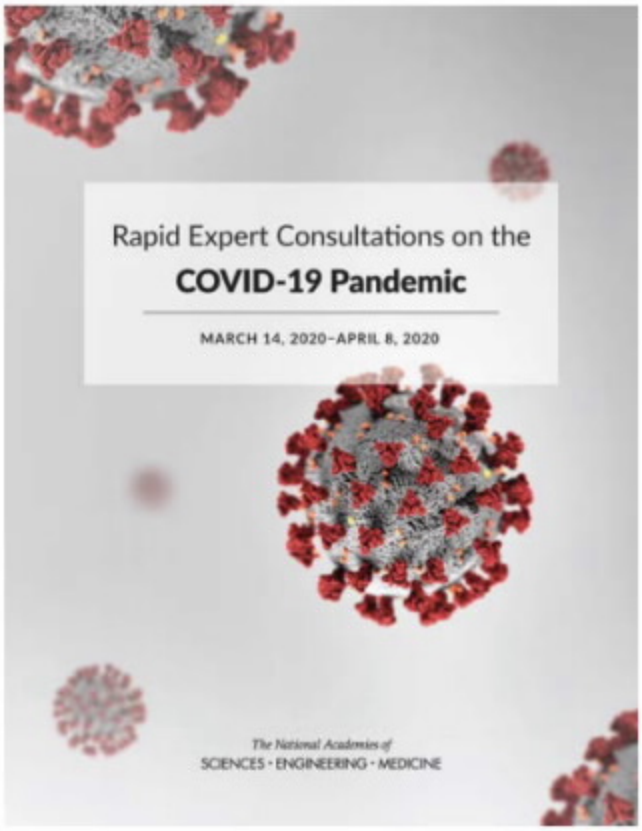
BACKGROUND

The response to respiratory viruses varies substantially between individuals, and there are currently no known molecular predictors from the early stages of infection. Here we conduct a community-based analysis to determine whether pre- or early post-exposure molecular factors could predict physiologic responses to viral exposure.

A Host-Based RT-PCR Gene Expression Signature to Identify Acute Respiratory Viral Infection

Improved ways to diagnose acute respiratory viral infections could decrease inappropriate antibacterial use and serve as a vital triage mechanism in the event of a potential viral pandemic. Measurement of the host response to infection is an alternative to pathogen-based

diagnostic testing and may improve diagnostic accuracy. We have developed a host-based assay with a reverse transcription polymerase chain reaction (RT-PCR) TaqMan low-density array (TLDA) platform for classifying respiratory viral infection.



Rapid Expert Consultations on the COVID-19 Pandemic

In response to a request from the Office of Science and Technology Policy and the Office of the Assistant Secretary for Preparedness and Response, the National Academies of Sciences, Engineering, and Medicine convened a standing committee of experts to help inform the federal government on critical science and policy issues related to emerging infectious diseases and other 21st century health threats. This set of Rapid Expert Consultations are the first of their kind and represent the best evidence available to the Committee at the time each publication was released.

This paper is in press at The Lancet Infectious Diseases

A blood-based host gene expression assay for early detection of viral infection

Micah T. McClain MD, PhD[1-3], Florica J. Constantine, MS[1], Bradly P. Nicholson, PhD[4], Marshall Nichols, MS[1], Thomas W. Burke, PhD[1], Ricardo Henao, PhD[1], Daphne C. Jones, MD[2], Lori L. Hudson, PhD[1], L. Brett Caram, MD[3], Timothy Veldman, PhD[1], Anna Mazur, MS[1], Lawrence P. Park, PhD[2,3], Sunil Suchindran, PhD[1], Ephraim L. Tsalik, MD, PhD[1-3], Geoffrey S. Ginsburg, MD[1], and Christopher W. Woods, MD, MPH[1-3]

Affiliations:

[1]Center for Applied Genomics and Precision Medicine, Duke University, Durham, NC, [2]Durham VA Medical Center, Durham, NC, [3]Division of Infectious Diseases, Duke University Medical Center, Durham, NC, [4]Institute for Medical Research, Durham, NC

Corresponding Author:

Micah T. McClain, MD, PhD

Division of Infectious Diseases

Center for Applied Genomics and Precision Medicine

Duke University Medical Center

DUMC Box 102359

Durham, NC 27710

e-mail: micah.mcclain@duke.edu

Office: 919-613-5636

Research in context

Evidence before this study

An increasing body of research has demonstrated that host gene expression patterns in peripheral blood offer a high degree of accuracy for diagnosis of acute infections at the time of clinical presentation.

Added value of this study

For the first time we demonstrate in a real-world setting that a blood-based gene expression test can accurately predict impending viral infection even before typical symptoms are present.

Implications of all the available evidence

These data show that biomarkers of viral infection are present and detectable even before clinical disease develops, opening the door to novel approaches to early treatment and outbreak management.

Abstract

Background

Early and accurate identification of persons with viral infections can be of critical importance for clinical management and public health interventions. Measuring host responses through gene signature-based biomarkers offer promise for improved diagnosis of respiratory viral infections, but little is known how early in disease such measures may be effective.

Methods

We performed a modified index-cluster study in 1465 university dormitory residents over five years. Each year, we monitored for symptomatic Index Cases (IC) and identified clusters of their Close Contacts (CC) who would be at presumed increased risk of developing symptomatic respiratory tract infection (RTI) while under observation. We then monitored each CC for 5 days for development of RTI and measured transcriptomic responses at each timepoint via a blood-based 36-gene RT-PCR assay.

Findings

Of 264 ICs with RTI symptoms, 150 (56%) had a confirmed viral etiology detected. Of their 555 CCs, 106 (19%) developed symptomatic RTI with a proven viral etiology during the observation window. The transcriptomic assay accurately predicts infection at the time of peak symptoms despite heterogeneity of hosts and nine different viral etiologies (auROC 0·96). Furthermore, these genomic biomarkers are capable of identifying early disease one, two and three days prior to peak illness when minimal to no symptoms are present (auROC 0·87, 0·86, and 0·77, respectively), and when nearly two-thirds of subjects (62%) are not yet producing detectable viral shedding.

Interpretation

Transcriptional biomarkers accurately predict and diagnose infection across diverse viral etiologies and stages of disease, and may prove useful for guiding administration of early effective therapy, quarantine decisions, and other clinical and public health interventions in the setting of endemic and pandemic infectious disease.

Funding: US Defense Advanced Research Projects Agency

Introduction

Acute viral infections are among the most common causes of acute physician visits in the United States[1]. In a given year, influenza alone affects 5-20% of the population in the US, causes over 400,000 hospitalizations, and as many as 56,000 deaths.[2,3] Outbreaks of viral infection continue to plague modern society, including heavy resource-requiring outbreaks of measles in the developed world, global pandemics like the novel H1N1 outbreak of 2009, or the deadly epidemics of SARS and MERS-CoV.[4-6] However, molecular tools that can contribute to outbreak investigation or directing early clinical management are often lacking. Traditional pathogen-focused diagnostic modalities for viral infection (culture, serology, antigen detection, PCR) offer considerable utility but can also be slow, costly, limited in terms of breadth of pathogen detection, require an *a priori* knowledge of the pathogen to be tested, may not detect emerging strains (as with the 2009 influenza pandemic[7]), or are incapable of distinguishing between active infection and colonization, all of which can minimize the impact of their use. Therefore, there is increasing interest in host-derived biomarkers to aid in the determination of the presence and/or type of infection in at-risk hosts - from single-analyte biomarkers like procalcitonin to composite, multiplex biomarker panels such as those included in a growing number of gene expression-based studies.[8-21] However, the published works on diagnostics for naturally-acquired infection focus on identifying symptomatic subjects at the time of clinical presentation for medical care[19], which is often late in the time course of many viral diseases. Identification of infectious etiologies in earlier, even pre-symptomatic phases of illness, offers the ability to optimize and deliver timely and thus more effective therapy, refine prophylaxis decisions, and guide public health interventions such as isolation and quarantine.[13,22] However, identifying subjects who have been exposed to infectious virus but are not yet exhibiting symptoms of clinical illness is logistically challenging, resulting in a dearth of literature to address these problems.

We have previously shown that during experimental influenza infection of healthy volunteers the host genomic response is robust and detectable well before typical symptoms become apparent.[16,22,23] Other

investigators have confirmed that transcriptomic markers can be indicative of early viral infection in experimental human challenge models as well as animal models of disease.[24-27] However, the mechanism of challenge infection is contrived rather than natural, typically consists of highly egg-adapted viral strains, and the severity of clinical illness does not fully replicate that seen in naturally-acquired infection, all of which can limit the broad applicability of findings from this approach.[16,22,28-31] To date there has been no significant description of the host response to infection during the pre-symptomatic phase of naturally-occurring disease. Here we have utilized a unique index-cluster study design with a focus on serial sampling of 'real world', healthy close contacts of infected individuals to enrich for cases of naturally-occurring infection during these early, post-exposure, but pre-symptomatic time points. This unique design has permitted real-world validation of a transcriptomic signature in peripheral blood that is capable of accurately identifying exposed but apparently healthy individuals who will go on to develop symptomatic viral infection.

Methods

Surveillance Cohort

Over five academic years, we enrolled 1465 Duke University undergraduate students (age 18-24, most of whom live in dormitories on campus) in a modified index-cluster study design (Figure 1). At the time of enrollment, we collected baseline biospecimens and standardized questionnaires to ascertain demographic, behavioral, and other medical characteristics. After enrollment, participating students completed a daily, web-based survey to describe the presence and severity of eight symptoms of RTI (scored 0-4) on a daily basis (see eMethods for details). We monitored the surveys daily for a 6-point increase in a subject's cumulative daily symptom score which would trigger a sample and data collection event for a potential Index Case. Suspected ICs were then assessed for confirmation of illness and biospecimen collection. Using these sentinel cases of RTI suggestive of viral etiology we then identified and monitored a prospective observational cohort of asymptomatic but potentially exposed Close Contacts (people living in the same dormitory or identified by the IC as a Close Contact), who were then monitored and sampled for up to 5 days (the Observation Window).

Sample and data collection

Confirmed IC's provided blood and nasal swab samples at the time of illness identification. Viral etiology was assessed using a commercial multiplex PCR assay (QIAGEN ResPlex II v2·0, Luminex RVP, or Biofire FilmArray RP). Healthy CCs living in proximity to an IC provided blood and respiratory samples for up to five consecutive days and were monitored for symptomatic conversion and viral shedding.

Real-time PCR

Peripheral blood was collected in PAXgene™ Blood RNA tubes (PreAnalytiX), and total RNA extracted using the PAXgene™ Blood miRNA Kit (QIAGEN). cDNA synthesis was performed using SuperScript VILO™ Master Mix (Invitrogen) according to manufacturer's instructions. Real-time PCR was performed using custom TLDA 384-well microfluidic cards with TaqMan Gene Expression Master Mix, and run on a ViiA7 Real-Time PCR System (Applied Biosystems).

Statistical Analysis

For RT-PCR data the Cq values were transformed into normalized relative quantities using reference genes - those with the most stable combined coefficient of variation across all samples - FPGS and TRAP1 were used for the first batch, and DECR1 and TRAP1 were used for the second batch, and data was normalized as previously described.[8,9] We utilized a regularized (2-norm) logistic regression model to discriminate cases from controls (see eMethods for complete details). The data was split into training and testing sets 25 times using repeated random subsampling and each of the 25 models was trained using all timepoints. We evaluated the model's performance separately at each individual timepoint. All model analyses were implemented in Python and Tensorflow.[32]

Results

Brief Description of Study

Healthy first year students at Duke University (n=1465) were enrolled into a surveillance cohort at the beginning of each academic year. After enrollment, we electronically monitored self-reported daily symptoms throughout the duration of the academic year via an online portal. When one or more subjects indicated a change in respiratory symptoms, the sample collection protocol was initiated for individuals (Index Cases, ICs) that met the clinical case definition for acute, community-acquired RTI (see Methods), as well as for a subset of other currently healthy study participants who were in close proximity to the Index Case (Close Contacts, CCs). CCs were monitored for symptoms and serially sampled for serum, whole blood PAXgene RNA, and respiratory samples for 5 days from identification of their Index Case, regardless of symptomatology (Figure 1). This design permitted enrichment for, and collection of, samples from CCs during the timeframe between exposure (which occurred at an unknown time) and subsequent development of symptomatic disease (which occurred under observation).

Prevalence, Transmission, and Clinical Outcomes of Viral Respiratory Tract Infection

We identified 264 clinical ICs, of whom 150 had a confirmed respiratory viral etiology by multiplex PCR testing of NP samples, and 555 CCs, 19% (106) of whom developed symptomatic RTI with concomitant confirmatory viral PCR. Only 57% of the CCs were infected with the same virus as their associated IC. Nine different viral pathogens were detected, and there was marked variation in viral etiologies detected from year to year (Table 1). Infection resulted in many common symptoms of upper respiratory tract infection with clinical variation amongst viral etiologies (Figure 2). For example, symptoms were almost twice as severe in individuals with influenza infection compared to all other viruses (mean symptom score of 20 for influenza vs 13 for all others ($p=0.005$)), although all subjects in the study remained well enough to be managed in the outpatient setting.

Since the exact time of exposure/transmission during naturally-acquired infection is unknown, for analysis CC days were aligned to the time of maximal illness (day of highest symptom score) for each subject (Time "T") in order to attempt to place subjects on broadly similar time scales in terms of the host response to infection. The average total symptom score for all CCs at Time T was 14·8, although this varied by viral etiology (Figures 1,2). For each individual CC, the day before their day of maximal symptoms was termed T-minus 1 ("T-1"), 48 hours prior to symptom max was "T-2" and 72 hours prior to symptom max was time "T-3". At times prior to maximal illness the average total symptom score for CCs was 7·0 (T-1), 4·1 (T-2), and 1·7 (T-3). In our studies a daily symptom score of 6 represents mild clinical illness and was the threshold for defining a 'symptomatic' day. Thus, at days T-2 and earlier, most subjects exhibited subclinical or absent illness (65% asymptomatic at Time T-3, 32% at T-2).

An RT-PCR signature identifies symptomatic subjects with naturally-acquired viral RTI

We previously developed a host response-derived transcriptomic signature of symptomatic viral infection using microarray data[30]. However, microarray and bulk RNA sequencing platforms are impractical for rapid clinical testing. We therefore migrated our prior 'pan-viral' signature to an RT-PCR platform with potential for more direct translation to point-of-care testing [8,9]. Using sparsity-imposing techniques (see eMethods), we selected 36 pre-designed TaqMan probes representing genes comprising the acute respiratory viral signature (and normalization controls) to be used on a TaqMan Low Density Array (TLDA) platform[8]. We then utilized a regularized (2-norm) logistic regression model to determine coefficients for each gene in the signature[33], such that the output of the model is a probability that a given subject will go on to have a symptomatic, viral PCR-positive event during the observation window. The performance of the RT-PCR signature for classification of symptomatic individuals was evaluated using a repeated random subsampling validation strategy on relevant participants across all study years. Overall, the RT-PCR-based host gene expression assay distinguished symptomatic, virus-infected subjects from asymptomatic, viral PCR-negative controls with 92% accuracy (Figure 3). However, accuracy varied depending on viral etiology, ranging from 99% accuracy in influenza infection (where symptoms are

typically more severe) to 92% with Coronavirus and 81% accuracy with metapneumovirus infections (eFigure 1). Interestingly, analysis of individual gene components of the model demonstrates that many individual genes performed similar to the aggregate model across all iterations (eFigure 2-3). In fact, many individual gene targets alone had mean area under Receiver Operating Characteristic (auROC) of >0·90 at the time of maximal illness with the top performing genes at time T being IFIT3 (0·97), HERC5 (0·97), and RSAD2 (0·96)

The gene expression signature identifies naturally-acquired viral RTI during the pre-symptomatic phase.

In order to evaluate performance of the gene signature early in the course of natural infection, we selected CCs with positive microbiology and at least three days of sampling prior to the development of maximal symptoms. As noted previously, the RT-PCR gene signature correctly identified symptomatic subjects with 92% accuracy on the day of maximal symptoms. This decreased to 88% on day T-1 (24 hours before maximal symptoms), and 83% accuracy on day T-2 (48 hours before maximal symptoms) (Figure 3), although performance varies from virus to virus. Only one case of influenza infection had available pre-symptomatic samples at all times, and the signature correctly classified that individual at each timepoint (T-1, T-2, T-3, and T-4). For a fixed FPR of 0·2 (specificity 0·8), the model achieves greater sensitivity than either symptoms alone or viral PCR at all timepoints other than time T and for ICs (where symptom-based diagnosis by definition has a 100% sensitivity, eFigures 4-6). Critically, 62% of subjects who would develop proven viral RTI lacked detectable viral shedding at time T-3, and 44% were still negative for virus by routine clinical testing at day T-2, times where the gene signature outperforms the clinical standards of care - symptom evaluation and viral PCR.

Discussion

The unique design of this community study has allowed us to identify individuals (CCs) at increased risk for developing viral RTI while under observation. This approach has enabled us to demonstrate for the first time that host responses to naturally-acquired respiratory viral infection, as measured by gene expression in peripheral blood, are present well before the time of maximal illness and can be used to predict those at highest risk for developing active infection.

This study demonstrates that conserved genomic responses can be used to correctly classify individuals with a broad array of naturally-acquired respiratory viral infections well before the time when they would present for clinical evaluation, and even before detectable viral shedding begins for the majority of subjects (62%). The promise of this approach is manifested by the discriminative ability of the genomic signature from two and three days prior to maximal illness, when many individuals (65% at Time T-3, 32% at T-2) have no symptoms at all. Even the mild symptoms some subjects do have at early times are clinically vague and would be typical of seasonal allergies, mild COPD flares, or even symptoms due to sequelae of chronic smoking.[34] The potential utility of genomic analysis to classify viral infection among asymptomatic individuals or those with common, nonspecific upper respiratory symptoms is profound. That this holds true across 9 different respiratory viruses, each with its own incubation times and variable clinical progression and duration, also speaks to the potential power of this approach. Additionally, it is important to note that the signature utilized represents conserved underlying antiviral response pathways that are active, and thus discriminatory, across a wide range of illness and not just at early times. This is vital as any real-world use of such a biomarker assay would often be agnostic to where in the course of infection an individual exists at the moment of testing.

While this transcriptomic signature accurately classifies a wide spectrum of viral RTIs, it performs best in a subset of viruses including influenza and coronavirus, where it performs with a high degree of accuracy even during early and pre-symptomatic phases of disease. Influenza is one of the most important viral etiologies to identify early given its importance in pandemics where existing diagnostics may perform

poorly (especially for emerging strains as in 2009[35]), where questions of triage and quarantine are paramount, and where effective therapeutic options are readily available and most effective when given early in disease. Similarly, a number of novel highly virulent coronaviruses, including SARS-CoV and MERS-CoV, have recently arisen and driven global outbreaks, highlighting another area of need for such diagnostics.[5,36].It seems likely that our ability to accurately detect exposure to these types of emerging infections will only become more critical over time. Availability of tools that can aid in assessment of those with potential exposure, especially those that offer diagnostic utility prior to the time of detectable viral shedding, would be a valuable aid to existing epidemiologic and molecular approaches in outbreak settings.

A number of other studies have described interferon response-based transcriptomic signatures of acute respiratory infections which vary by target population, pathogens, modelling approaches, and numbers of genes required to discriminate disease states. [9,10,13-18,30] Some of this work has also been validated, combined, or expanded upon by secondary *ex vivo* analysis of publicly available data. [11,12,37] While the focused signature described here clearly represents a limited subset of all discriminating genes, some observations regarding simplified, IFN-driven signatures can be made. Our data confirm that that some genes from these prior works (such as IFI44L and IFI27[10,38]) both perform well as classifiers distinguishing between viral illness and the healthy state (median auROC = 0·92 and 0·87), being strongly upregulated in cases of viral infection. The data further suggest that a number of other single targets also perform this task well including RSAD2 (0·96), IFIT3 (0·97), and IFI44 (0·93). Based on our experience and the published literature, it seems that relatively simple classification tasks - where the biological responses are highly conserved, unique in character, and of great magnitude - may be reasonably addressed with pauci-analyte biomarker panels.[10,12,39-41] However, as complexity increases by adding more powerful interhost variability (such as immunosuppression), discriminating clinically similar groups with more varied underlying pathophysiology, or discriminating multiple clinical mimics at once,

more intricate biomarker panels will likely be required to maintain high fidelity in real-world settings.[9,14,42]

Our study has several limitations. This project was designed to validate the predictive power of a small set of genes which already had proven discriminatory capability during maximal illness, and thus was not designed to discover whether additional, untested gene sets may offer improved discriminatory performance at early times. Future work with RNASeq on whole blood, single cell sequencing, or other methods will be needed to more completely define early genomic changes in the naturally-infected host. Additionally, our patient cohort was comprised of otherwise young, healthy individuals with community-acquired viral RTI of mild to moderate severity. Therefore, application to patient populations from other age groups, those with immunologic comorbidities, or with more severe viral infections will need to be evaluated. Finally, we did not identify any confirmed infections due to bacterial or other pathogens. As a result, while these transcriptomic markers show great promise for differentiating early infection and pre-symptomatic viral exposure from noninfectious states (which it may mimic at early times), this study did not directly validate the ability of these markers to differentiate viral exposure from early forms of other types of infection[9,10,14,43]. As we move towards bringing transcriptomic biomarkers to the bedside, consideration of broad applicability and performance across a wide array of clinical settings will continue to be paramount.

We have for the first time defined the pre-symptomatic and temporal dynamics of transcriptomic biomarkers characterizing the host response to naturally-acquired viral infections in humans. Clinic-ready platforms capable of operationalizing PCR-based signatures of the size tested herein already exist, some of which are CLIA-approved with point-of-care functionality, offering a proximal pathway to clinical application of these findings. Thus, analysis of the evolution of gene expression-based biomarkers over time shows promise for driving development of diagnostics for early detection of viral exposure and infection which may prove useful for guiding administration of early effective therapy, quarantine

decisions, and other clinical and public health interventions in the setting of endemic and pandemic infectious disease.

References

1. Hong CY, Lin RT, Tan ES, et al. Acute respiratory symptoms in adults in general practice. *Fam Pract* 2004; **21**(3): 317-23.
2. Shrestha SS, Swerdlow DL, Borse RH, et al. Estimating the burden of 2009 pandemic influenza A (H1N1) in the United States (April 2009-April 2010). *Clin Infect Dis* 2011; **52 Suppl 1**: S75-82.
3. Rebecca Garten PLB, MPH1; Anwar Isa Abd Elal1; Noreen Alabi, MPH1; John Barnes, PhD1; Matthew Biggerstaff, ScD1; Lynnette Brammer, MPH1; Alicia P. Budd, MPH1; Erin Burns, MA1; Charisse N. Cummings, MPH1; Todd Davis, PhD1; Shikha Garg, MD1; Larisa Gubareva, PhD1; Yunho Jang, PhD1; Krista Kniss, MPH1; Natalie Kramer1; Stephen Lindstrom, PhD1; Desiree Mustaquim, MPH1; Alissa O'Halloran, MSPH1; Wendy Sessions, MPH1; Calli Taylor, MPH1; Xiyan Xu, MD1; Vivien G. Dugan, PhD1; Alicia M. Fry, MD1; David E. Wentworth, PhD1; Jacqueline Katz, PhD1; Daniel Jernigan, MD. Update: Influenza Activity in the United States During the 2017–18 Season and Composition of the 2018–19 Influenza Vaccine. *MMWR Morb Mortal Wkly Rep* 2018; (67): 634-42.
4. de Wit E, van Doremalen N, Falzarano D, Munster VJ. SARS and MERS: recent insights into emerging coronaviruses. *Nature reviews Microbiology* 2016; **14**(8): 523-34.
5. Arabi YM, Balkhy HH, Hayden FG, et al. Middle East Respiratory Syndrome. *New England Journal of Medicine* 2017; **376**(6): 584-94.
6. Angelo KM, Gastanaduy PA, Walker AT, et al. Spread of Measles in Europe and Implications for US Travelers. *Pediatrics* 2019; **144**(1).
7. Emergence of a Novel Swine-Origin Influenza A (H1N1) Virus in Humans. *New England Journal of Medicine* 2009; **360**(25): 2605-15.
8. Zaas AK, Burke T, Chen M, et al. A host-based RT-PCR gene expression signature to identify acute respiratory viral infection. *Sci Transl Med* 2013; **5**(203): 203ra126.
9. Tsalik EL, Henao R, Nichols M, et al. Host gene expression classifiers diagnose acute respiratory illness etiology. *Sci Transl Med* 2016; **8**(322): 322ra11.
10. Herberg JA, Kaforou M, Wright VJ, et al. Diagnostic Test Accuracy of a 2-Transcript Host RNA Signature for Discriminating Bacterial vs Viral Infection in Febrile Children. *JAMA* 2016; **316**(8): 835-45.
11. Andres-Terre M, McGuire HM, Pouliot Y, et al. Integrated, Multi-cohort Analysis Identifies Conserved Transcriptional Signatures across Multiple Respiratory Viruses. *Immunity* 2015; **43**(6): 1199-211.
12. Sweeney TE, Braviak L, Tato CM, Khatri P. Genome-wide expression for diagnosis of pulmonary tuberculosis: a multicohort analysis. *Lancet Respir Med* 2016; **4**(3): 213-24.

13. Liu X, Speranza E, Munoz-Fontela C, et al. Transcriptomic signatures differentiate survival from fatal outcomes in humans infected with Ebola virus. *Genome Biol* 2017; **18**(1): 4.
14. Suarez NM, Bunsow E, Falsey AR, Walsh EE, Mejias A, Ramilo O. Superiority of transcriptional profiling over procalcitonin for distinguishing bacterial from viral lower respiratory tract infections in hospitalized adults. *J Infect Dis* 2015; **212**(2): 213-22.
15. Kaforou M, Wright VJ, Oni T, et al. Detection of tuberculosis in HIV-infected and -uninfected African adults using whole blood RNA expression signatures: a case-control study. *PLoS Med* 2013; **10**(10): e1001538.
16. Woods CW, McClain MT, Chen M, et al. A Host Transcriptional Signature for Presymptomatic Detection of Infection in Humans Exposed to Influenza H1N1 or H3N2. *PLoS One* 2013; **8**(1): e52198.
17. Mejias A, Dimo B, Suarez NM, et al. Whole blood gene expression profiles to assess pathogenesis and disease severity in infants with respiratory syncytial virus infection. *PLoS Med* 2013; **10**(11): e1001549.
18. Hu X, Yu J, Crosby SD, Storch GA. Gene expression profiles in febrile children with defined viral and bacterial infection. *Proc Natl Acad Sci U S A* 2013; **110**(31): 12792-7.
19. Zhai Y, Franco LM, Atmar RL, et al. Host Transcriptional Response to Influenza and Other Acute Respiratory Viral Infections--A Prospective Cohort Study. *PLoS pathogens* 2015; **11**(6): e1004869.
20. Roe J, Venturini C, Gupta RK, et al. Blood transcriptomic stratification of short-term risk in contacts of tuberculosis. *Clin Infect Dis* 2019.
21. Afroz S, Giddaluru J, Abbas MM, Khan N. Transcriptome meta-analysis reveals a dysregulation in extra cellular matrix and cell junction associated gene signatures during Dengue virus infection. *Scientific reports* 2016; **6**: 33752.
22. McClain MT, Nicholson BP, Park LP, et al. A Genomic Signature of Influenza Infection Shows Potential for Presymptomatic Detection, Guiding Early Therapy, and Monitoring Clinical Responses. *Open Forum Infect Dis* 2016; **3**(1): ofw007.
23. Huang Y, Zaas AK, Rao A, et al. Temporal Dynamics of Host Molecular Responses Differentiate Symptomatic and Asymptomatic Influenza A Infection. *PLoS Genet* 2011; **7**(8): e1002234.
24. Shurtleff AC, Whitehouse CA, Ward MD, Cazares LH, Bavari S. Pre-symptomatic diagnosis and treatment of filovirus diseases. *Frontiers in microbiology* 2015; **6**(108).
25. Caballero IS, Yen JY, Hensley LE, Honko AN, Goff AJ, Connor JH. Lassa and Marburg viruses elicit distinct host transcriptional responses early after infection. *BMC Genomics* 2014; **15**(1): 960.
26. Duy J, Koehler JW, Honko AN, et al. Circulating microRNA profiles of Ebola virus infection. *Scientific reports* 2016; **6**: 24496.
27. Wang K, Langevin S, O'Hern CS, et al. Anomaly Detection in Host Signaling Pathways for the Early Prognosis of Acute Infection. *PLoS One* 2016; **11**(8): e0160919.
28. Memoli MJ, Czajkowski L, Reed S, et al. Validation of the wild-type influenza A human challenge model H1N1pdMIST: an A(H1N1)pdm09 dose-finding investigational new drug study. *Clin Infect Dis* 2015; **60**(5): 693-702.
29. Han A, Poon JL, Powers JH, 3rd, Leidy NK, Yu R, Memoli MJ. Using the Influenza Patient-reported Outcome (FLU-PRO) diary to evaluate symptoms of influenza viral infection in a healthy human challenge model. *BMC infectious diseases* 2018; **18**(1): 353.

30. Zaas AK, Chen M, Varkey J, et al. Gene expression signatures diagnose influenza and other symptomatic respiratory viral infections in humans. *Cell Host Microbe* 2009; **6**(3): 207-17.
31. Sobel Leonard A, McClain MT, Smith GJ, et al. Deep Sequencing of Influenza A Virus from a Human Challenge Study Reveals a Selective Bottleneck and Only Limited Intrahost Genetic Diversification. *Journal of virology* 2016; **90**(24): 11247-58.
32. Friedman J, Hastie T, Tibshirani R. Regularization Paths for Generalized Linear Models via Coordinate Descent. *Journal of statistical software* 2010; **33**(1): 1-22.
33. Goeman JJ. L1 penalized estimation in the Cox proportional hazards model. *Biometrical journal Biometrische Zeitschrift* 2010; **52**(1): 70-84.
34. Stanton N, Hood K, Kelly MJ, et al. Are smokers with acute cough in primary care prescribed antibiotics more often, and to what benefit? An observational study in 13 European countries. *European Respiratory Journal* 2010; **35**(4): 761-7.
35. LeBlanc JJ, Li Y, Bastien N, Forward KR, Davidson RJ, Hatchette TF. Switching gears for an influenza pandemic: validation of a duplex reverse transcriptase PCR assay for simultaneous detection and confirmatory identification of pandemic (H1N1) 2009 influenza virus. *Journal of clinical microbiology* 2009; **47**(12): 3805-13.
36. Christian MD, Poutanen SM, Low DE, Muller MP, Loutfy MR. Severe Acute Respiratory Syndrome. *Clinical Infectious Diseases* 2004; **38**(10): 1420-7.
37. Bongen E, Vallania F, Utz PJ, Khatri P. KLRD1-expressing natural killer cells predict influenza susceptibility. *Genome medicine* 2018; **10**(1): 45.
38. Tang BM, McLean AS, Dawes IW, Huang SJ, Lin RC. Gene-expression profiling of peripheral blood mononuclear cells in sepsis. *Crit Care Med* 2009; **37**(3): 882-8.
39. Warsinske HC, Rao AM, Moreira FMF, et al. Assessment of Validity of a Blood-Based 3-Gene Signature Score for Progression and Diagnosis of Tuberculosis, Disease Severity, and Treatment Response. *JAMA network open* 2018; **1**(6): e183779.
40. Tang BM, Shojaei M, Parnell GP, et al. A novel immune biomarker IFI27 discriminates between influenza and bacteria in patients with suspected respiratory infection. *The European respiratory journal* 2017; **49**(6).
41. Herberg JA, Kaforou M, Wright VJ, et al. Diagnostic Test Accuracy of a 2-Transcript Host RNA Signature for Discriminating Bacterial vs Viral Infection in Febrile Children. *Jama* 2016; **316**(8): 835-45.
42. Maslove DM, Wong HR. Gene expression profiling in sepsis: timing, tissue, and translational considerations. *Trends Mol Med* 2014; **20**(4): 204-13.
43. Ramilo O, Allman W, Chung W, et al. Gene expression patterns in blood leukocytes discriminate patients with acute infections. *Blood* 2007; **109**(5): 2066-77.

Acknowledgements

Special thanks to Debra Freeman, Sara Hoffman, Olga M. Better, Mert Aydin, Stephanie Dobos, Kyle R. Breitschwert, Ashlee M. Valente for their tireless work enrolling subjects, processing samples and data, and engaging with the undergraduate campus.

Funding

Funding for this study was provided by the US Defense Advanced Research Projects Agency (DARPA), project # N66001-07-C-2024 and W911NF1410052. The statements and conclusions herein are those of the authors and do not reflect the position or policy of the United States Government.

Author contributions

MTM, ELT, GSG and CWW drafted the manuscript, which was critically revised by all remaining authors. MTM, BPN, TB, TV, GSG, and CWW helped conceive and implement the study. All authors helped acquire, analyze, or interpret data. Statistical analyses were performed by FJC, RH, SS, MN, and LP.

Figures

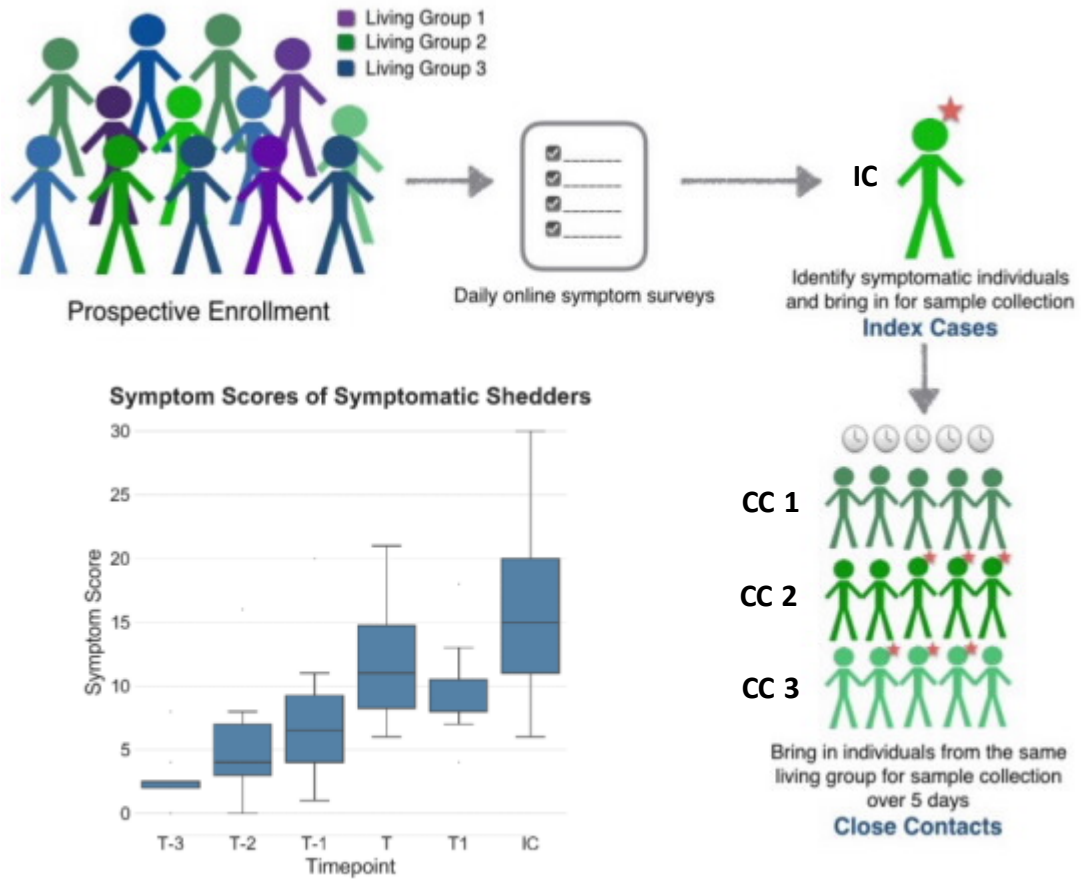


Figure 1: Study design. Students were enrolled at the beginning of the school year. Index Cases were identified when daily symptom surveys indicated potential respiratory illness. This flagged serial sampling of their Close Contacts, some of whom would also develop RTI during the 5-day observation window (red stars). This unique model permits blood sampling of some Close Contacts during early and presymptomatic phases of illness. Mean symptom scores of Close Contacts who developed PCR-proven RTI under observation are presented.

A

	Cough	Fever	Headache	Malaise	Nasal Congestion	Nasal Discharge	Sneezing	Sore Throat	Total Symptoms
Influenza*	3.2	1.9	2.4	3.0	2.5	2.4	1.8	2.7	20.0
RSV	2.1	0.7	1.6	2.9	2.6	2.6	1.5	2.6	16.6
Human Metapneumovirus	2.6	0.8	1.5	2.5	2.4	2.5	1.9	2.2	16.3
Rhinovirus	2.2	0.5	1.1	2.1	2.3	2.3	1.5	2.1	14.0
Coxsackie/Echovirus	2.1	0.5	1.1	2.2	2.4	2.4	1.6	1.8	14.0
Coronavirus	1.8	0.7	1.0	2.1	2.3	2.5	1.6	1.8	13.8
Parainfluenzavirus	2.3	0.5	1.2	2.3	1.8	1.7	1.3	2.3	13.4
Adenovirus	2.2	1.2	1.4	2.2	1.6	1.6	0.2	2.2	12.6
Bocavirus	1.0	0.0	0.0	1.0	2.0	2.0	1.0	1.0	8.0

B

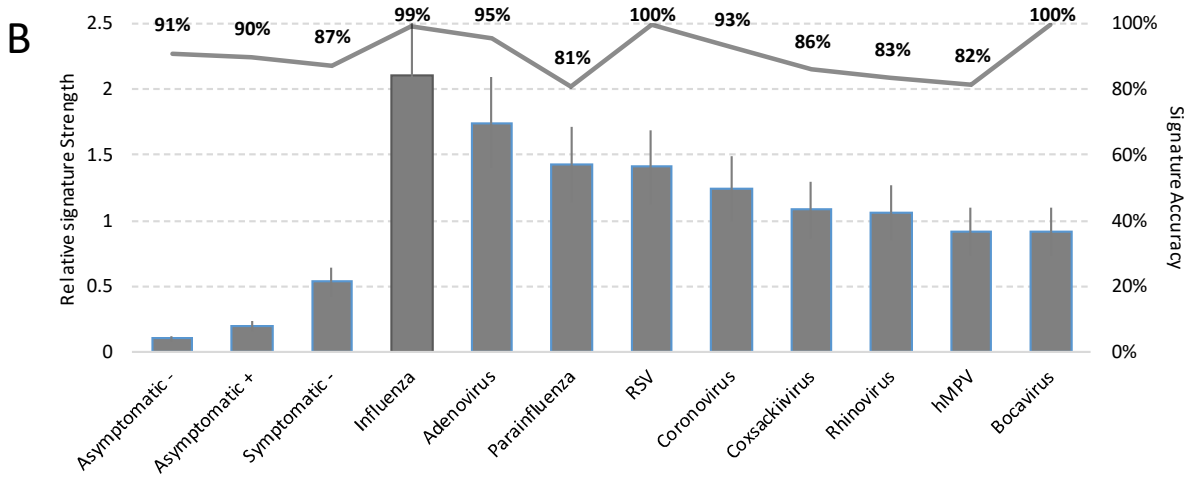


Figure 2. Clinical and genomic variation amongst viruses. For each type of viral infection, mean scores for each symptom measured at the time of maximal illness are presented (Panel A). The relative quantitative transcriptomic signature strength (bars) and accuracy (black line) at the time of maximal illness for each virus, as well as for Asymptomatic nonshedders (-), Asymptomatic shedders (+), and Symptomatic nonshedders (-) are presented in Panel B.

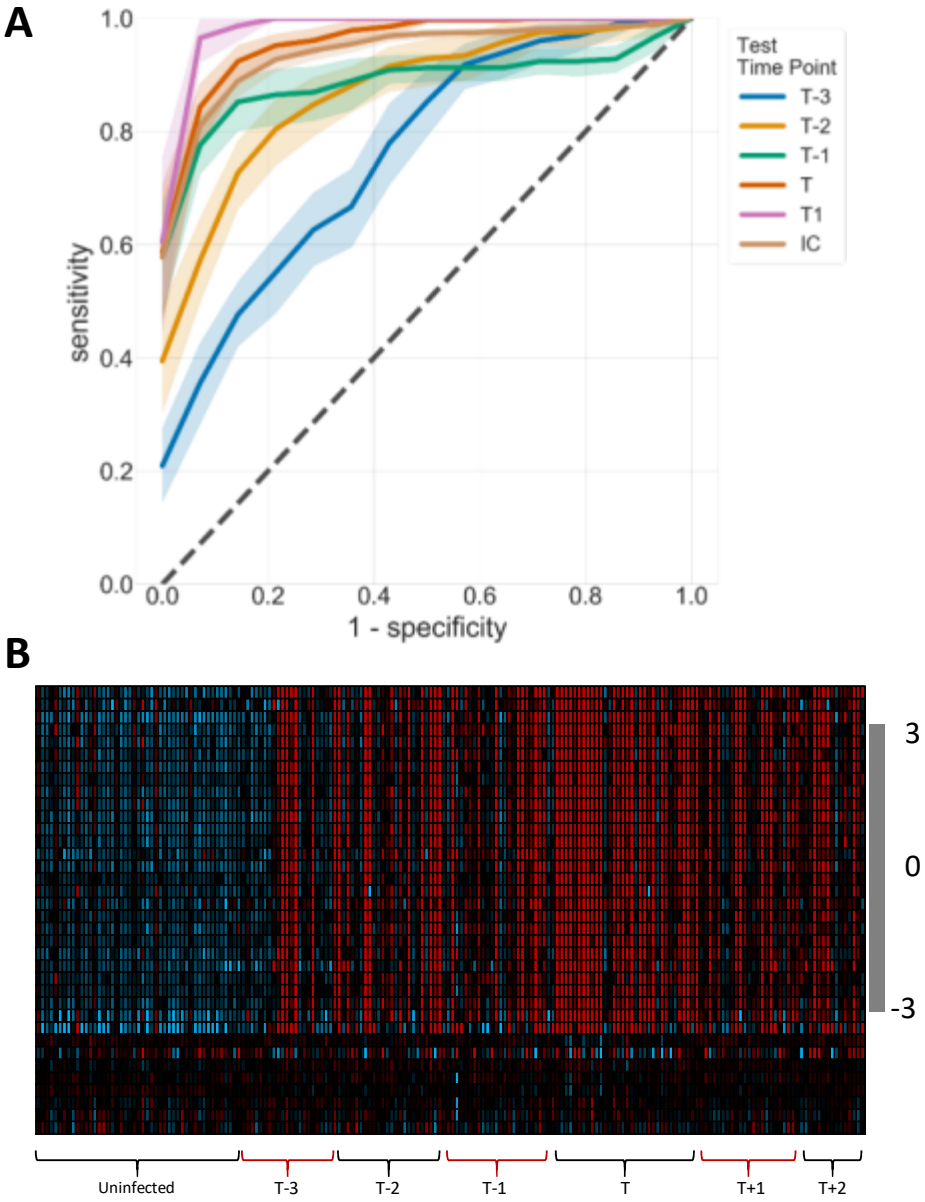


Figure 3: Signature performance across time. An RT-PCR-based 36-gene signature accurately discriminates subjects with naturally-acquired respiratory viral infection at both symptomatic and presymptomatic stages of infection (A). Heatmap of z-scores for each gene at each timepoint in subjects with proven symptomatic viral infection (Panel B), where time 'T' is the day of maximal illness.

Table 1. Viral etiologies of RTI in the Student Cohort

	2009-2010	2010-2011	2011-2012	2013-2014[a]	2014-2015	Total
Adenovirus	1	1	0	1	2	**5**
Bocavirus	0	0	0	0	2	**2**
Coronavirus	11	23	8	3	9	**54**
Coxsackie/Echovirus[b]	21	38	12	0	0	**71**
Human Metapneumovirus	2	1	3	4	1	**11**
Influenza	9	7	1	1	1	**19**
Parainfluenzavirus	3	10	1	0	7	**21**
RSV	3	1	3	0	3	**10**
Rhinovirus	20	23	29	3	37	**112**
Total	**70**	**104**	**57**	**12**	**62**	**305**

[a]truncated season, Spring enrollment only
[b]not present in 2013-2015 testing

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

Structural and Functional Analysis of the D614G SARS-CoV-2 Spike Protein Variant

Leonid Yurkovetskiy[1,12], Xue Wang[2,12], Kristen E. Pascal[3], Christopher Tomkins-Tinch[4,5], Thomas Nyalile[1], Yetao Wang[1], Alina Baum[3], William E. Diehl[1], Ann Dauphin[1], Claudia Carbone[1], Kristen Veinotte[1], Shawn B. Egri[1], Stephen F. Schaffner[4,5], Jacob E. Lemieux[4,8], James Munro[6,7], Ashique Rafique[3], Abhi Barve[2], Pardis C. Sabeti[4,5,9,10,11,13], Christos A. Kyratsous[3,13], Natalya Dudkina[2,13], Kuang Shen[1,13], Jeremy Luban[1,4,7,11,13,14]

[1]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA
[2]Thermo Fisher Scientific, Achtseweg Noord 5, 5651 GG Eindhoven, Netherlands
[3]Regeneron Pharmaceutical, Inc., 777 Old Saw Mill River Rd, Tarrytown, NY 10591
[4]Broad Institute of Harvard and MIT, 75 Ames Street, Cambridge, MA 02142, USA
[5]Harvard University, 52 Oxford Street, Cambridge, MA 02138, USA
[6]Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA 01605, USA
[7]Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01605, USA
[8]Massachusetts General Hospital, 55 Fruit Street, Boston, MA, 02114
[9]Harvard T.H. Chan School of Public Health, 677 Huntington Avenue, 02115 Boston, MA
[10]Howard Hughes Medical Institute, 4000 Jones Bridge Rd, Chevy Chase, MD 20815
[11]Massachusetts Consortium on Pathogen Readiness, Boston, MA, 02115

[12]These authors contributed equally to this work.

[13]Correspondence:
Pardis.Sabeti@broadinstitute.org (P.C.S.), christos.kyratsous@regeneron.com (C.A.K.), Natalya.Dudkina@thermofisher.com (N.D.), Kuang.Shen@umassmed.edu (K.S.), jeremy.luban@umassmed.edu (J.L.)

[14]Lead contact

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

SUMMARY

The SARS-CoV-2 spike (S) protein variant D614G supplanted the ancestral virus worldwide in a matter of months. Here we show that D614G was more infectious than the ancestral form on human lung cells, colon cells, and cells rendered permissive by ectopic expression of various mammalian ACE2 orthologs. Nonetheless, D614G affinity for ACE2 was reduced due to a faster dissociation rate. Assessment of the S protein trimer by cryo-electron microscopy showed that D614G disrupts a critical interprotomer contact and that this dramatically shifts the S protein trimer conformation toward an ACE2-binding and fusion-competent state. Consistent with the more open conformation, neutralization potency of antibodies targeting the S protein receptor-binding domain was not attenuated. These results indicate that D614G adopts conformations that make virion membrane fusion with the target cell membrane more probable but that D614G retains susceptibility to therapies that disrupt interaction of the SARS-CoV-2 S protein with the ACE2 receptor.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

INTRODUCTION

Next-generation sequencing permits real-time detection of genetic variants that appear in pathogens during disease outbreaks. Tracking viral variants now constitutes a requisite component of the epidemiologist's toolkit, one that can pinpoint the origin of a zoonotic virus and the trajectory it takes from one susceptible host to another (Hadfield et al., 2018; Shu and McCauley, 2017). Lagging behind sequence-based modeling of virus phylogenies and transmission chains is the ability to understand the effect of viral variants on the efficiency of transmission between hosts or on the clinical severity of infection. Most sequence variants that arise during virus replication are either detrimental to the fitness of the virus or without consequence. Even so, such variants can increase in frequency over the course of an outbreak by chance (Grubaugh et al., 2020). More rarely, though, increasing frequency of a variant can reflect competitive advantage due to higher intrinsic replication capacity, with increased viral load and transmissibility.

In December 2019, an outbreak of unexplained fatal pneumonia became apparent in Wuhan City, Hubei Province, China. By early January 2020, SARS-CoV-2 was identified as the virus causing the disease (Huang et al., 2020; Lu et al., 2020; Wu et al., 2020a, 2020b; Zhou et al., 2020b; Zhu et al., 2020). After SARS-CoV (Drosten et al., 2003; Ksiazek et al., 2003) and MERS-CoV (Zaki et al., 2012), SARS-CoV-2 is the third human coronavirus this century known to cause pneumonia with a significant case-fatality rate (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020). Hundreds of coronaviruses have been identified in bats, including at least 50 SARS-like *Sarbecoviruses (Lu et al., 2020; Zhou et al., 2020a).* The virus closest in sequence to SARS-CoV-2 observed to date was isolated from a bat

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

(Zhou et al., 2020b) though the most proximal animal reservoir for SARS-CoV-2 remains unknown (Andersen et al., 2020; Lam et al., 2020).

Over the course of the SARS-CoV-2 pandemic, there have been reports of super-spreaders and transmission chains that have been more difficult to interrupt in some locations than in others (Hu et al., 2020). As few as 10% of SARS-CoV-2 infected people account for the majority of virus transmission events (Endo et al., 2020). Reported case fatality rates have varied by more than 10-fold (Center for Systems Science and Engineering at Johns Hopkins University; COVID-19 National Emergency Response Center, Epidemiology and Case Management Team, Korea Centers for Disease Control and Prevention, 2020; Grasselli et al., 2020; Onder et al., 2020). Such differences may reflect regional differences in the age of susceptible populations or the prevalence of comorbidities such as hypertension. Regional variation in diagnostic testing frequency, or in rate of reporting, is another likely explanation for these differences, since a wider net of diagnostic testing will capture milder cases (Zhao et al., 2020). Understanding the cause of these observed differences is critical for rational utilization of limited medical resources to counteract the pandemic.

Coronaviruses have the largest genomes of any known RNA viruses (Saberi et al., 2018) and they encode a 3'-to-5'-exoribonuclease required for high-fidelity replication by the viral RNA-dependent RNA polymerase (Denison et al., 2011; Smith et al., 2014). By preventing otherwise lethal mutagenesis (Smith et al., 2013), the repair function of the coronavirus exonuclease is thought necessary for the coronavirus genome size to extend beyond the theoretical limit imposed by error rates of viral RNA polymerases (Holmes, 2003). Though the rate of sequence variation among SARS-CoV-2 isolates is modest, over the course of the pandemic the virus has had opportunity to generate numerous sequence variants, many of which have been identified among the thousands of SARS-CoV-2 genomes posted for public

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

access (https://www.gisaid.org/) (Hadfield et al., 2018). Here we investigate potential functional consequences of one of these variants, the Spike protein variant D614G.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

RESULTS

The SARS-CoV-2 D614G S protein variant supplanted the ancestral virus

Minimal genetic variation among SARS-CoV-2 genomes sequenced from the earliest COVID-19 cases suggests that a recent common ancestor gave rise to all SARS-CoV-2 genomes seen during the outbreak (Rambaut et al., 2020). Over the course of the pandemic, a total of 12,379 distinct single nucleotide polymorphisms (SNPs) have been identified in genomic data (relative to the ancestral genome NC_045512.2; GISAID download on 25 June 2020), 99.5% of which are within viral genes, and 63.3% of which encode amino acid changes. 6,077 of the SNPs were seen only once in the dataset and only four SNPs rose to high frequency. Among the latter, A23403G was first reported at the end of January 2020 in virus genomes from China (hCoV-19/Zhejiang/HZ103/2020; 24 January 2020) and Germany (hCoV-19/Germany/BavPat1-ChVir929/2020; 28 January 2020). Given how few SARS-CoV-2 genomes have been sequenced from early in the outbreak, the geographic origin of A23403G cannot be determined.

The frequency of A23403G has increased steadily over time. It is now present in approximately 74% of all published sequences (Figure 1A). Sequences that have become available over recent weeks indicate that it has nearly reached fixation globally (Figure 1A), as well as separately within Europe, North America, Oceania, and Asia (Figure 1B). A23403G emerged at higher frequency in Africa and South America, suggesting a founder effect where a plurality of introductions carried it to these regions. Although recent data for South America are sparse, available sequences suggest A23403G is approaching fixation in this continent as well. Thus, A23403G is now universally common on every continent except Antarctica.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

The SARS-CoV-2 D614G S protein variant increases infectivity in cell culture

The non-synonymous nucleotide change A23403G encodes the SARS-CoV-2 spike (S) protein variant D614G. The ability of the D614G S protein variant to target virions for infection of ACE2-positive cells was assessed using single-cycle lentiviral vector pseudotypes in tissue culture. Mammalian expression plasmids were engineered to encode the ancestral S protein D614 or the D614G variant. Each S protein expression plasmid was separately transfected into HEK-293 cells with plasmids encoding HIV-1 structural proteins and enzymes. Separate plasmids were transfected that encode RNAs with HIV-1 *cis*-acting signals for packaging and replication, and either GFP or luciferase (Luc) reporter cassettes. For each condition tested, multiple virus stocks were produced and tested individually after vector particle normalization using reverse transcriptase activity. 48 hours after challenge with the vectors, the transduction efficiency of each virion preparation was assessed by measuring the percent GFP-positive cells using flow cytometry, or by quantitating target cell-associated luciferase activity.

When Calu-3 human lung epithelial cells were used as targets, challenge with virus bearing D614G resulted in 6-fold more GFP-positive cells, or 5-fold more bulk luciferase activity, than did particles bearing D614 S protein (Figure 2A). When Caco-2 human colon epithelial cells were used as target cells, 4-fold higher infectivity was observed with D614G (Figure 2A). Additionally, when HEK-293 cells or SupT1 cells had been rendered infectable by stable expression of exogenous ACE2 and TMPRSS2, D614G was 9-fold more infectious than D614 (Figure 2A).

D614G increases infectivity on target cells bearing different ACE2 orthologs

The likely zoonotic origin of SARS-CoV-2 raises the question whether D614G was selected during the pandemic as a result of human-to-human transmission. Structural and biochemical studies (Letko et al., 2020; Walls et al., 2020; Wrapp et al., 2020; Zhou et al., 2020b) have

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

shown that SARS-CoV-2 bears a receptor binding domain with high affinity for ACE2 from humans, ferrets, cats, and other species that encode similar ACE2 (Wan et al., 2020). In a heterologous expression system, human, civet, horse-shoe bat, and pig orthologs each conferred susceptibility to infection, while the mouse ortholog did not (Zhou et al., 2020b). To determine whether the increased infectivity of D614G is specific for certain ACE2 orthologs, HEK-293 were transfected separately with plasmids encoding ACE2 orthologs from human (*Homo sapiens*), Malayan pangolin (*Manis javanica*), pig (*Sus scrofa*), cat (*Felis catus*), dog (*Canis lupis*), rat (*Rattus norvegicus*), or mouse (*Mus musculus*). Each ACE2 transfectant was then challenged with luciferase reporter viruses bearing SARS-CoV-2 Spike protein, either D614 or D614G. Relative increase in infectivity due to D614G was comparable in cells expressing human, pangolin, pig, cat, or dog ACE2 orthologs (Figure 2B). The relative increase in infectivity due to D614G on cells bearing rat or mouse ACE2 orthologs could not be quantitated since, in both cases, the luciferase activity with D614 was too close to background. These results suggest that increased infectivity due to D614G is not specific for human ACE2.

D614G does not alter S protein synthesis, processing, and incorporation into SARS-CoV-2 particles

The effect of D614G on S protein synthesis, processing, and incorporation into virion particles produced by SARS-CoV-2 structural proteins was assessed next (Figure 3A). HEK-293T cells were transfected with plasmids expressing each of the four SARS-CoV-2 virion-associated structural proteins (Figure 3A). Western blots were then performed on the cell lysate and on proteins from the pellet after ultracentrifugation of the transfection cell supernatant. The minimal requirement for virion assembly by other coronaviruses is the membrane protein (M) and the envelope protein (E) (for mouse hepatitis virus (Bos et al., 1996; Vennema et al., 1996)), or the

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

M and nucleocapsid protein (N) (for SARS-CoV (Huang et al., 2004)). In the case of SARS-CoV-2, M protein production was sufficient to release particles from transfected cells, though particle release by M was increased by co-production of N (Figure 3B). The S proteins D614 and D614G were produced to comparable levels, processed to S1 and S2 with comparable efficiency, and incorporated into SARS-CoV-2 virus-like particles at similar levels (Figure 3B). These results suggest that increased infectivity due to D614G is primarily manifest after virion assembly, during entry into target cells.

D614G decreases the affinity for ACE2 by increasing the rate of dissociation

The receptor-binding domain of the S protein interacts directly with ACE2, the primary entry receptor for SARS-CoV and SARS-CoV-2 on susceptible target cells (Hoffmann et al., 2020; Letko et al., 2020; Tian et al., 2020; Wrapp et al., 2020; Zhou et al., 2020b). Though D614G is located outside of the receptor-binding domain, this non-conservative amino acid change might alter ACE2-binding properties via allosteric effects. Surface plasmon resonance (SPR) was used to determine if the kinetics of SARS-CoV-2 S protein binding to human ACE2 is changed by D614G. Human ACE2 was immobilized and the binding of soluble, trimeric SARS-CoV-2 S protein, either D614 or D614G, was detected. At 25°C, the rate of association with ACE2 was little different between D614G and D614, but D614G dissociated from ACE2 at a rate four-fold faster than D614, resulting in a 5.7-fold reduction in binding affinity (Figure 4). At 37°C, the association rate between D614G and ACE2 was slower than between D614 and ACE2, and the dissociation rate of D614G was faster, again resulting in five-fold reduction in binding affinity (Figure 4). These data demonstrate that the increased infectivity of D614G is not explained by greater ACE2 binding strength.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

D614G and the ancestral S protein are equally sensitive to neutralization by monoclonal antibodies targeting the receptor binding domain

The global spread and enhanced infectivity of the SARS-CoV-2 D614G variant raises the question of whether this change in S protein structure may compromise the effectiveness of antiviral therapies targeting this viral protein, especially if they were designed to target D614. To determine if this is the case, the neutralization potency of four monoclonal antibodies that target the SARS-CoV-2 Spike protein receptor-binding domain was assessed. These fully human monoclonal antibodies are currently under evaluation in clinical trials (NCT04425629, NCT04426695) as therapeutics for COVID-19 (Hansen et al., 2020). Each of these monoclonal antibodies, whether tested individually or in various combinations, demonstrated similar neutralization potency against D614G as they did against D614 (Figure 5).

D614G changes the conformation of the S1 domain in the SARS-CoV-2 S protein

Structural studies of the trimeric S protein ectodomain demonstrate that the receptor-binding domain of each protomer can independently adopt either a closed or an open conformation, giving rise to asymmetric trimers (Walls et al., 2020; Wrapp et al., 2020). The open conformation is believed to be required for ACE2-binding and to be on-pathway for S protein-mediated fusion of the virion membrane with the target cell membrane (Tortorici and Veesler, 2019). Since the increased infectivity of D614G was not explained by increased affinity for ACE2 (Figure 4), cryo-electron microscopy (cryo-EM) was used to illuminate potential structural features that distinguish D614G from D614. Both S protein variants, D614 and D614G, were expressed in mammalian cells as soluble trimers. When enriched from culture media, and eluted from a size exclusion column, single peaks were observed for each variant protein at ~500 kD, the predicted mass of the homotrimer (Figure 6A, blue line). Enrichment of the protein complexes, and integrity of the full-length protomers, was additionally confirmed by SDS-PAGE (Figure 6B).

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

Well-defined particles of S protein trimers were evident by cryo-EM, and reference-free, two-dimensional clustering revealed structural details from different orientations (Figure 6C). Three-dimensional clustering and refinement generated the final density map for D614G (Figure 6D, EMD-22301), which showed a similar overall architecture to the published map of D614 (Figure 6E), but with distinct features, as discussed below. Unmasked Fourier shell correlation (FSC) analysis indicated that the D614G map had a mean resolution of 3.7 Å (gold-standard criteria, Figure 6F), which is sufficient to reveal fine differences from D614.

The SARS-CoV-2 S protein consists of S1 and S2 subunits, with multiple domains within S1 (Figure 6G). Comparing the map of D614G with that of D614, the N-terminal domain (NTD), the intermediary domain (INT), and the C-terminal domain (CTD) of the S1 subunit were clearly identified. However, the density corresponding to the receptor binding domain (RBD) that was well-resolved in D614 (arrows, Figure 6E), was missing in the D614G map, suggesting that the RBD is flexible and adopts multiple conformations (see section below).

Based on the resolved ensemble cryo-EM density map and the primary sequence, an atomic model of D614G without the RBD was built (Figure 6H, PDB: 6xs6). In the structural model, the S2 subunit of D614G overlapped well with the published structures for D614. In contrast, there was significant deviation within the S1 subunit (Figure 6I and 6J). When the S1 subunit of D614G was superimposed on the closed conformation of D614, the S1-NTD and S1-INT shifted away from each other by 6Å and 4Å, respectively (Figure 6I), revealing a wider space between these two domains. When the S1 subunit of D614G was superimposed on D614 in the open conformation, the S1-NTD was shifted outwards by 3Å, while the S1-INT overlapped well with that of D614 (Figure 6J). With respect to the ancestral protein, then, the D614G variant induces interdomain conformational changes, and likely leads to a more open conformation within the S1 subunit.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

D614 localizes to the interface between two protomers where its side chain forms hydrogen bonds with the T859 side-chain in the adjacent protomer (Figure 6K). The effect of D614G on this interaction could be assessed since local resolution in the map generated here reaches 3.2Å. The atomic model showed that D614G has two consequences. First, D614G disrupts the inter-protomer hydrogen bond with Thr859 (Figure 6L and M), weakening the stability of the trimer. In effect, D614 acts as a "latch" that secures two protomers together, and D614G loosens this latch (Figure 6L and M). Second, the backbone amine of the glycine residue shortens the hydrogen bond with the carboxyl group of Ala647 within the same protomer, from 3.4 Å to 2.7 Å, and thus strengthens it. This interaction might internally stabilize the S1-CTD.

D614G shifts the probability that SARS-CoV-2 S protein trimers occupy open conformations

The RBD cannot be visualized in the ensemble-averaged map for D614G. To better assess the conformation of the RBD, the flexible region of S1 was subjected to masked 3D classification and refinement, with the aim of resolving the conformational heterogeneity in that region. Two distinct classes arose from this analysis of the dataset. 58% of the protomers adopted an open conformation in which the RBD is positioned to interact with ACE2, and 42% were in the closed conformation in which the RBD is buried (Fig. 7A and 7B). This ratio of the two conformations contrasted dramatically with assessment of D614 structural data (Walls et al., 2020), in which only 18% of the RBDs were in the open conformation and 82% in the closed conformation. The closed conformation of D614G overlapped well with the closed conformation of D614, while the open conformation of D614G showed significant deviation of all S1 domains away from the S2 subunit when compared with the open conformation of D614 (Fig. 7C). This suggests that the

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

D614-Thr859 hydrogen bond latches adjacent protomers together when one protomer is in the open conformation.

Further classification of D614G particles to determine the distribution of RBD conformations within trimers revealed four different categories (Figure 7D). 5% of the particles had all three protomers in the closed conformation. 36% had one open protomer. 39% had two open protomers. 20% of the particles had all three protomers in the open conformation (Fig. 7D). The same analysis of the D614 dataset (Walls et al., 2020) showed that 46% of particles were in the all closed conformation and 54% in the one open conformation. These data further emphasize the contrast between D614G conformational space and that of D614, and suggest that the greater flexibility of the D614G RBD confers increased infectivity to D614G.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

DISCUSSION

The SARS-CoV-2 S protein variant D614G is one of only four SNPs, out of the more than 12,000 to be reported in GISAID, that has risen to high frequency (Figure 1). This suggests that D614G confers a replication advantage to SARS-CoV-2, such that it increases the likelihood of human-to-human transmission. Data in which the presence of D614G correlates with increased rates of transmission through human populations would support this hypothesis. To date, though, datasets that are powered sufficiently, and that are appropriately controlled for age and other important variables, have not been forthcoming. Prospective comparison of D614G transmission to that of D614 will be unlikely given that D614G has nearly gone to fixation world-wide (Figure 1), with only rare reports of D614 over recent weeks. It should be noted, however, that the roughly 53,000 high-quality SARS-CoV-2 genomic sequences available from GISAID as of 25 June 2020 are only a narrow snapshot of the pandemic, constituting approximately 0.5% of the 9.6 million cases confirmed as of that date (Dong et al., 2020). Additional sequencing of archived samples might provide opportunity to pinpoint the origin of D614G or to improve understanding of its trajectory.

Indirect evidence that D614G is more transmissible was provided here by experiments with pseudotyped viruses showing that D614G transduces 3 to 9-fold more efficiently than does the ancestral S protein (Figure 2A). This effect was seen with a range of cellular targets, including lung and colon epithelial cells, and required ACE2 on the target cell. The increased infectivity of D614G was evident whether or not TMPRSS2 was expressed on the target cells (Figure 2A). Efforts are underway to compare the replication efficiency of D614G with that of D614 in the context of the nearly 30,000 nucleotide SARS-CoV-2 genome. Such reverse genetic experiments, though, are technically difficult and potentially confounded by acquisition of unnatural, tissue culture-adapted mutations during genome rescue and expansion in

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

transformed cell lines, as has occurred during similar assessments of Ebola virus variants (Marzi et al., 2018; Ruedas et al., 2017). Consistent with the increased ability of D614G to infect cells in tissue culture, several studies suggest that D614G is associated with increased viral load in people with COVID-19 (Korber et al., 2020; McNamara et al., 2020; Wagner et al.), though these studies quantitated SARS-CoV-2 RNA and did not measure infectious virus.

If SARS-CoV-2 Spike D614G is an adaptive variant that was selected for increased human-to-human transmission, following spillover from an animal reservoir, one might expect that increased infectivity would only be evident on cells bearing ACE2 orthologs similar to the human. In contrast to the primate-specific increase in infectivity that was reported for the major clade-forming Ebola virus glycoprotein variant from the 2013-2016 West African outbreak (Diehl et al., 2016; Urbanowicz et al., 2016), the increased infectivity of D614G was equally evident on cells bearing ACE2 orthologs from a range of mammalian species (Figure 2B). Though absolute infectivity differed based on the affinity of the RBD for the particular ACE2 ortholog, the relative increase in infectivity due to D614G was the same (Figure 2B). The fact that mouse and rat ACE2 orthologs were non-permissive for D614, but gave detectable infection with D614G, underlines this point. Examination of D614G infectivity using ACE2 orthologs encoded by other mammalian species may provide further insight into the structure and function of the D614G variant.

Insight into the mechanism by which D614G increases infectivity was gleaned from cryo-EM studies of the SARS-CoV-2 S protein trimer. D614G exhibited striking conformational changes within individual S protein protomers (Figure 6I and J, Figure 7C) and within the trimers (Figure 7D), all of which may be attributable to disruption of the interprotomer latch between D614 in S1 and T859 in S2 (Figure 7K-M). Weakening of the interprotomer contacts results in increased distance between the protomers and a dramatic flip in the ratio of open to closed S

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

protein particles, from 82% closed and 18% open for D614, to 42% closed and 58% open for D614G. Perhaps the most remarkable conformational change was that, when the S protein trimers were examined, in addition to the all closed conformation and the one open conformation previously reported for D614 (Walls et al., 2020), a significant fraction of D614G trimers populated a two open conformation (39%) and an all open state (20%). Previous structural studies of SARS-CoV and MERS-CoV S proteins identified similar conformations, with only MERS-CoV S protein exhibiting an all open state like that reported here (Gui et al., 2017; Pallesen et al., 2017; Walls et al., 2019; Yuan et al., 2017). These studies suggested that only upon transition of an RBD to the open conformation is the protomer capable of binding the ACE2 receptor. Models of coronavirus S-mediated membrane fusion describe ACE2 binding to all three RBD domains in the open conformation as destabilizing the pre-fusion S trimer, leading to dissociation of S1 from S2 and promoting transition to the post-fusion conformation (Pallesen et al., 2017; Walls et al., 2019). Thus, the open conformation likely reflects an intermediate that is on-pathway to S-mediated membrane fusion. The well-populated all open conformation of D614G (Figure 7D) therefore suggests that this S protein variant increases infectivity by making virion membrane fusion with the target cell membrane more probable.

The D614G and D614 variants are equally sensitive to neutralization by four human monoclonal antibodies targeting the S protein RBD that are currently in clinical trials for people with COVID-19 (Figure 5). These results are in keeping with the fact that the location of D614G within the S protein is remote from the receptor binding domain and that D614G affinity for ACE2 is less than that of D614 (Figure 4). To date, no studies have demonstrated a worse clinical outcome for infection with D614G, and given the more open conformation of its receptor-binding domain, one might predict that D614G will be at least as immunogenic as the ancestral form.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

Methods

Analysis of D614G frequency in published data

The frequency of the SARS-CoV-2 D614G S protein variant in published genomic data was examined using the full Nextstrain-curated set of sequences available from GISAID as of 25 June 2020 (Hadfield et al., 2018; Shu and McCauley, 2017). Sequences were aligned to the ancestral reference sequence (NCBI GenBank accession NC_045512.2) using mafft v7.464 (Katoh and Standley, 2013) with the "--keeplength" and "--addfragments" parameters, which preserve the coordinate space of the reference sequence. To remove lower-quality sequences from the dataset, all sequences in the alignment were masked with ambiguous bases ('N') in the regions spanning the first 100bp and the last 50bp, as well as at error-prone sites located at the (1-indexed, NC_045512.2 coordinate space) positions 13402, 24389, 24390. Sequences shorter than 28kb or with >2% ambiguous bases were removed from the alignment. The frequency of D614G was calculated in the resulting data by extracting the sequence region corresponding to the gene for the S protein, spanning 21563-25384bp. These sequences were processed using a script importing biopython (Cock et al., 2009) to remove any gaps introduced by the alignment process and translate the sequence to protein space. The identity of the variant at amino acid position 614 was tabulated for the full dataset and reported as frequency by date using the collection dates reported in the Nextstrain-curated metadata file available from GISAID (Hadfield et al., 2018; Shu and McCauley, 2017). The frequency was calculated as (# sequences with D614G)/(# sequences). Frequency within the six continental regions was calculated based on the "region" geographic classification associated with each sample in the metadata. Frequency values were linearly interpolated for dates surrounded by valid data. The frequency of the last date with data was carried forward where recent dates lack data. The bresulting values were rendered as plots using matplotlib (Hunter, 2007). The script for

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

analyzing and plotting D614G variant frequency is available via GitHub: https://github.com/broadinstitute/sc2-variation-scripts

The diversity of SNPs and their functional effects based on the same GISAID sequences and MAFFT alignment used to plot the frequency of D614G over time, with the 5' and 3' ends not masked. In the alignment, ambiguous nucleotide codes (R,Y,W,S,M,K,H,B,V,D) were all masked with "N" values. SNPs were calculated from the alignment using the *snp-sites* tool (Page et al., 2016). The resulting VCF-format file was normalized using *bcftools* (Li et al., 2009) to include only SNPs. The VCF file with SNPs was annotated for functional effects using *SnpEff* (Cingolani et al., 2012).

Plasmids

The plasmids used here were either previously described or were generated using standard cloning methods. The full list of plasmids used here is provided in the Key Resources Table. All newly engineered plasmids are available from public repositories, along with full sequences, at either https://www.addgene.org/Jeremy_Luban/ or https://www.aidsreagent.org (search under Jeremy Luban).

Cell culture

All cells were cultured in humidified incubators with 5% CO_2 at 37º C, and monitored for mycoplasma contamination using the Mycoplasma Detection kit (Lonza LT07-318). HEK293 cells (ATCC CRL-1573), and HEK-293T cells (CRL-3216 or CRL-11268) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, 20 mM GlutaMAX, 1× MEM non-essential amino acids, and 25 mM HEPES, pH 7.2. Calu3 cells (ATCC HTB-55) were maintained in EMEM supplemented with 10% FBS. Caco2 cells (ATCC HTB-37)

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

were maintained in EMEM supplemented with 20% FBS. SUP-T1 [VB] cells (ATCC CRL-1942) were cultured in RPMI supplemented with 10% heat-inactivated FBS, 1mM sodium pyruvate, 20mM GlutaMAX, 1xMEM non-essential amino acids, and 25 mM HEPES, pH7.2. Vero cells (ATCC CCL-81) were cultured in DMEM high glucose media containing 10% heat-inactivated fetal bovine serum, and 1X Penicillin/Streptomycin/L-Glutamine.

Virus production

24 hrs prior to transfection, 6 × 10^5 HEK-293 cells were plated per well in 6 well plates. All transfections used 2.49 µg plasmid DNA with 6.25 µL TransIT LT1 transfection reagent (Mirus, Madison, WI) in 250 µL Opti-MEM (Gibco).

Single-cycle HIV-1 vectors pseudotyped with SARS-CoV-2 Spike protein, either D614 or D614G, were produced by transfection of either HIV-1 pNL4-3 Δenv Δvpr luciferase reporter plasmid (pNL4-3.Luc.R-E-), or pUC57mini NL4-3 Δenv eGFP reporter plasmid, in combination with the indicated Spike expression plasmid, at a ratio of 4:1.

ACE2 expression vectors were produced by transfecting cells with one of the pscALPSpuro-ACE2 plasmids, along with the HIV-1 *gag-pol* expression plasmid psPAX2, and the VSV glycoprotein expression plasmid pMD2.G (4:3:1 ratio of plasmids).. 16 hrs post-transfection, culture media was changed. Viral supernatant was harvested 48 hours after media change, passed through a 0.45 µm filter, and stored at 4ºC. TMPRSS2 expression transfer vector was produced similarly but with pscALPSblasti-TMPRSS2.

Exogenous reverse transcriptase assay

5 µL transfection supernatant was mixed with 5 µL 0.25% Triton X-100, 50 mM KCl, 100 mM Tris-HCl pH 7.4, and 0.4 U/µL RiboLock RNase inhibitor, and then diluted 1:100 in 5 mM

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

$(NH_4)_2SO_4$, 20 mM KCl, and 20 mM Tris-HCl pH 8.3. 10 µL of this was then added to a single-step, RT-PCR assay with 35 nM MS2 RNA (IDT) as template, 500 nM of each primer (5'-TCCTGCTCAACTTCCTGTCGAG-3' and 5'-CACAGGTCAAACCTCCTAGGAATG-3'), and 0.1 µL hot-start Taq DNA polymerase (Promega, Madison, WI) in 20 mM Tris-Cl pH 8.3, 5 mM $(NH_4)_2SO_4$, 20 mM KCl, 5 mM $MgCl_2$, 0.1 mg/ml BSA, 1/20,000 SYBR Green I (Invitrogen), and 200 µM dNTPs in total 20 µL reaction. The RT-PCR reaction was carried out in a Biorad CFX96 real-time PCR detection system with the following parameters: 42ºC for 20 min, 95ºC for 2 min, and 40 cycles [95ºC for 5 sec, 60ºC for 5 sec, 72ºC for 15 sec, and acquisition at 80ºC for 5 sec].

Generation of cell lines expressing ACE2 and TMPRSS2

2.5 x 10^5 HEK-293 cells were plated per well in a 12 well plate. The next day cells were transduced with 250 uL of supernatant containing TMPRSS2-encoding lentivirus for 16 hr at 37°C, after which fresh media was added to cells. 48 hrs after transduction cells were replated and selected with blasticidin (InvivoGen, catalogue #ant-bl-1) at 10 ug/ml. After selection, cells were transduced similarly with supernatant containing ACE2-encoding lentivirus and selected with 1 ug/mL of puromycin (InvivoGen, San Diego, CA, catalogue #ant-pr-1).

1 x 10^6 SupT1 cells were transduced with 400 uL of supernatant containing TMPRSS2-encoding virus followed by selection with 10 ug/mL blasticidin 48 hrs later. TMPRSS2 expressing SupT1 cells were then transduced with a second vector expressing ACE2, followed by puromycin selection at 1 ug/mL.

Virus Infectivity Assays

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

16 hours prior to transduction, adherent cells were seeded in 96 well plates. HEK-293 cells were plated at 5 x 10^4 cells per well. Calu3 and Caco2 were plated at 3 x 10^4 per well. Cells were incubated in virus-containing media for 16 hrs at 37°C when fresh medium was added to cells. 48 to 72 hours after transduction cells were assessed for luciferase activity or for GFP by flow cytometry. For transfection of SupT1 cells, 1 x 10^5 cells were plated in a U-bottom 96 well plate and spinfected at 30ºC with 100 uL of virus-containing media for 2 hours at 1,200 x g. Fresh media was added after spinfection and cells were incubated 48-72 hours prior to analysis. Cells transduced with GFP virus were fixed with BD Cytofix (BD Biosciences, San Jose, CA, Cat number 554655) and analyzed using the Accuri C6system. Data was analyzed using FlowJo 10.5 (FlowJo, LLC, Ashland, OR). Cells transduced with luciferase expressing virus were assessed using Promega Steady-Glo system (Promega Madison, WI)

Neutralizations assays with human monoclonal antibodies targeting the SARS-CoV-2 S protein receptor binding domain

VSV-SARS-CoV-2-S pseudoparticle generation and neutralization assays were performed as previously described (Baum et al., 2020; Hansen et al., 2020). HEK293T cells were seeded overnight in DMEM high glucose media (Life Technologies) containing 10% heat-inactivated fetal bovine serum (Life Technologies), and Penicillin/- Streptomycin-L-Glutamine (Life Technologies). The following day, Spike expression plasmids were transfected with Lipofectamine LTX (Life Technologies) following the manufacturer's protocol. At 24 hours post transfection, the cells were washed with phosphate buffered saline (PBS) and infected at an MOI of 1 with the VSVΔG:mNeon/VSV-G virus diluted in 10 mL Opti-MEM (Life Technologies). The cells were incubated 1 hour at 37°C with 5% CO2. Cells were washed three times with PBS to remove residual input virus and overlaid with DMEM high glucose media (Life Technologies)

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

with 0.7% low IgG BSA (Sigma), sodium pyruvate (Life Technologies), and gentamicin (Life Technologies). After 24 hours at 37° C with 5% CO2, the supernatant containing pseudoparticles was collected, centrifuged at 3,000 x g for 5 minutes to clarify, aliquoted, and frozen at -80° C.

For neutralization assays, Vero cells were seeded in 96-well plates 24 hours prior to assay and grown to 85% confluence before challenge. Antibodies were diluted in DMEM high 3 glucose media containing 0.7% Low IgG BSA (Sigma), 1X Sodium Pyruvate, and 0.5% Gentamicin (this will be referred to as “Infection Media”) to 2X assay concentration and diluted 3-fold down in Infection Media, for an 11-point dilution curve in the assay beginning at 10 ug/mL (66.67 nM). pVSV-SARS-CoV-2-S pseudoparticles were diluted 1:1 in Infection Media for a fluorescent focus (ffu) count in the assay of ~1000 ffu. Antibody dilutions were mixed 1:1 with pseudoparticles for 30 minutes at room temperature prior to addition onto Vero cells. Cells were incubated at 37° C, 5% CO2 for 24 hours. Supernatant was removed from cells and replaced with 100 uL PBS, and fluorescent foci were quantitated using the SpectraMax i3 plate reader with MiniMax imaging cytometer.

Production of SARS-CoV-2 virus-like particles (VLPs)

HEK-293T cells were cultured in DMEM supplemented with 10% heat-inactivated bovine serum, and transfected with pcDNA3.1 plasmids encoding the SARS-CoV-2 M, E, N, and S proteins, in different combinations, as indicated. 1 ug of each plasmid was used, with 5 ug of total plasmid in each transfection, normalized using empty vectors, in 400 ul Opti-MEM and 18 ul of PEI. The transfection mixture was incubated at room temperature for 15 minutes and dropped into a 50% confluent 10 cm plate of HEK-293T cells. The media and cell lysate were collected after 60 hours. 10 ml of supernatant was passed through a 0.45 um syringe filter, layered on top of a

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

20% sucrose cushion in PBS, and spun at 30,000 rpm in an SW41 rotor (111,000 x g, average) for two hours. The pellet was washed once with ice cold PBS, resuspended in 150 ul of 2x SDS loading buffer (Morris formulation), and sonicated in an ice-water bath (Branson 2800) for 15 minutes. After removal of the supernatant, the transfected cells were lysed in 800 ul PBS with 1% Triton and protease inhibitor (Roche cOmplete EDTA-free tablets), and cleared of debris by spinning in a table-top centrifuge (Eppendorf 5424R) at top speed (20,000 x g, average) for 10 minutes.

Immunoblots of SARS-CoV-2 VLPs

Proteins in VLP and cell lysate samples were separated by SDS-PAGE, as follows: 20 ul of unboiled VLP and cell lysate samples on a 10-20% Tris-Glycine gel to probe for the M protein; 2 ul of boiled cell lysate and 5 ul of boiled VLP samples on a 12% Tris-Glycine gel to probe for the N protein; 20 ul of boiled lysate and VLP samples on a 10% Tris-Glycine gel to probe for the S protein; 5 ul of boiled lysate on a 10% Tris-Glycine gel to probe for Raptor, as a loading control. Proteins were electro-transferred from the gels to PVDF membrane, which was blocked with 5% milk in Tris-Buffered Saline, pH 8.0, with 0.1% Tween-20, and detected with the indicated antibodies.

Surface plasmon resonance analysis

Binding kinetics and affinities for ACE2.Fc were assessed using surface plasmon resonance technology on a Biacore T200 instrument (GE Healthcare, Marlborough, MA) using a Series S CM5 sensor chip in filtered and degassed HBS-EP running buffer (10 mM HEPES, 150 mM NaCl, 3mM EDTA, 0.05% (v/v) polysorbate 20, pH 7.4). Capture sensor surfaces were prepared by covalently immobilizing with a mouse anti-human Fc mAb (REGN2567) on the chip

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

surface using the standard amine coupling chemistry, reported previously (Johnsson et al., 1991). Following surface activation, the remaining active carboxyl groups on the CM5 chip surface were blocked by injecting 1 M ethanolamine, pH 8.0 for 7 minutes. A typical resonance unit (RU) signal of ~12,000 RU was achieved after the immobilization procedure. At the end of each cycle, the anti-human Fc surface was regenerated using a 12 second injection of 20 mM phosphoric acid. Following the capture of the ACE2.Fc on the anti-human Fc mAb immobilized surface, 0.78 nM - 50 nM, two-fold serial dilutions, in duplicate, of soluble SARS-CoV-2 spike trimer protein, D614 or D614G, were injected for 3 minutes at a flow rate of 50 mL/min, with a 2 minute dissociation phase in the running buffer. All specific SPR binding sensorgrams were double-reference subtracted as reported previously (Myszka, 1999) and the kinetic parameters were obtained by globally fitting the double-reference subtracted data to a 1:1 binding model with mass transport limitation using Biacore T200 Evaluation software v 3.1 (GE Healthcare). The dissociation rate constant (k_d) was determined by fitting the change in the binding response during the dissociation phase and the association rate constant (k_a) was determined by globally fitting analyte binding at different concentrations. The equilibrium dissociation constant (K_D) was calculated from the ratio of the k_d and k_a. The dissociative half-life ($t_{½}$) in minutes was calculated as ln2/(k_d*60). The steady state analysis was performed using Scrubber software and the K_D value was determined.

Production and enrichment of soluble SARS-CoV-2 S Protein trimers

FreeStyle 293-F cells were cultured in SMM-293 TII serum-free media (SinoBiological) and maintained in a 37°C shaker with 8% CO_2 and 80% humidity. 600 ug of plasmid encoding His-tagged SARS-CoV-2 S protein was transfected into 400 ml of 293 FreeStyle cells at 10^6 cells/ml, using 25 ml Opti-MEM and 1.8 ml PEI. 60 hours later, the media was collected and

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

applied to 3 ml of Ni-NTA resin (Qiagen). The resin was collected and washed with PBS supplemented with 20 mM imidazole. Soluble S protein was eluted in PBS supplemented with 200 mM imidazole, and further enriched using a Superose 6 gel-filtration column (GE healthcare), in a buffer containing 25 mM NaHEPES (pH 7.4) and 150 mM NaCl. Yield was roughly 600 ug of soluble S protein homotrimer per liter of culture.

CryoEM sample preparation and data collection

3 µL of enriched D614G trimers, at 2.5 mg/mL, was deposited on an UltrAuFoil R1.2/1.3 300 mesh grid that had been glow discharged for 30 seconds in a GloQube Plus Glow Discharge System. Plunge freezing was performed with a Vitrobot Mark IV using a blot force 0 and 5s blot time at 100% humidity and 4℃. Frozen grids were imaged with a Thermo Scientific Krios transmission electron microscope operated at 300 kV. Movies were acquired using EPU 2 software. 10,264 micrographs were automatically collected with a defocus ranging between 0.6 and 1.8 µm, at a nominal magnification of 155,000x with a pixel size of 0.52Å. 7,653 micrographs were collected with a tilt-angle of 30° to overcome the preferred orientation. The dose rate was set at 20.3 electron/Å^2/s and total exposure time was 1.81s. With a dose fraction set at 62 frames, each movie series contained 62 frames and each frame received a dose of 0.6 electron/Å^2.

CryoEM data processing

Movie frame alignment, estimation of microscope contrast-transfer function parameters, particle picking, 2D classification, and homogeneous refinement using the published structure EMD-21452 as initial model were carried out in cryoSPARC. Ensemble averaging of 266,356 particles resulted in a 3.7Å map with C3 symmetry imposed according to “gold standard” Fourier

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

shell correlation of 0.143. Local resolution was estimated using cryoSPARC to extend from 3Å to 6 Å. Particle stacks with well-refined orientation parameters were imported in Relion3.1. Focused 3D classifications with a soft mask on the S1 subunit of the protomer were performed on the C3 symmetry expanded particles. Two monomer conformations, namely the closed state (a) and the open state (b) were identified after 3 rounds of classification. Four trimer classes were identified by 3D classification on the trimer particles: class_3a (13,555 particles), class_3b (51,600 particles), class_2a1b (96,029 particles), class_1a2b (105,118 particles). Homogeneous refinements were then performed on these 4 classes, with EMD-21452 as the initial model in Relion3.1. C3 symmetry was imposed on the refinement of particles from class_3a and class_3b; C1 symmetry was imposed on the refinement of particles from class_2a1b and class_1a2b. The final resolutions were class_1a2b with 4Å, class_2a1b with 3.9Å, class_3a with 4.2Å and class_3b with 3.9Å. The final map was deposited with the accession code EMD-22301.

Model building

Atomic models were prepared with Coot based on the resolved structure of D614 SARS-CoV-2 Spike (PDB: 6vxx and 6vsb). Real-space refinements were performed using PHENIX with secondary structure restraints. MolProbity was used to evaluate the geometries of the structural model. Corrected Fourier shell correlation curves were calculated using the refined atomic model and the cryo-EM density map. The coordinates were deposited with the accession code 6xs6.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

ACKNOWLEDGEMENTS

We thank all the members of the Sabeti, Shen, and Luban labs, and Thermo Fisher Scientific Workflow solutions team for technical assistance and helpful discussions. This work was supported by NIH grants R37AI147868 and R01AI148784 to J.L., NIH/NIAID U19AI110818 to P.C.S., 7DP2AI124384-02 to J.B.M., NCI K22CA241362 to K.S., a grant from the Evergrande COVID-19 Response Fund Award from the Massachusetts Consortium on Pathogen Readiness to J.L., a grant from the Worcester Foundation to K.S., a Sara Elizabeth O'Brien Fellowship Award/King Trust to L.Y., and a National Science Foundation Graduate Research Fellowship (Grant No. 1745303) to C.T.-T. A portion of this project has been funded in whole or in part with Federal funds from the Department of Health and Human Services; Office of the Assistant Secretary for Preparedness and Response; Biomedical Advanced Research and Development Authority, under OT number: HHSO100201700020C.

AUTHOR CONTRIBUTIONS

P.S., C.A.K., K.S., and J.L. conceived the experiments. C.T.-T., S.F.S, J.E.L., and P.C.S. analyzed the SARS-CoV-2 sequence data. L.Y., T.N., Y.W., W.E.D., A.D., C.A.K., K.S., and J.L. designed and cloned the expression plasmids. L.Y., C.C., A.D., K.E.P., and A.B. performed the virology experiments. L.Y., A.D., and K.S. performed the virus biochemistry. A.R. performed the SPR experiments. K.V., S.B.E., and K.S. purified the proteins. X.W., A.B., and N.D. collected and processed cryo-EM data. S.B.E. and K.S. built the structural model. L.Y., C.H.T.-T., S.F.S, C.A.K., A.B., A.R., J.B.M., K.S., and J.L. wrote and edited the manuscript with input from all authors.

DECLARATION OF INTERESTS

P.C.S. is a co-founder and shareholder of Sherlock Biosciences, and a Board member and shareholder of Danaher Corporation. J.E.L. consulted for Sherlock Biosciences. C.A.K., K.E.P., and A.B. are employed by Regeneron Pharmaceuticals and own stock options in the company. C.A.K. is an officer at Regeneron. X.W., A.B., and N.D. are employees of Thermo Fisher Scientific.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

References

Andersen, K.G., Rambaut, A., Lipkin, W.I., Holmes, E.C., and Garry, R.F. (2020). The proximal origin of SARS-CoV-2. Nat. Med. *26*, 450–452.

Baum, A., Fulton, B.O., Wloga, E., Copin, R., Pascal, K.E., Russo, V., Giordano, S., Lanza, K., Negron, N., Ni, M., et al. (2020). Antibody cocktail to SARS-CoV-2 spike protein prevents rapid mutational escape seen with individual antibodies. Science.

Bos, E.C., Luytjes, W., van der Meulen, H.V., Koerten, H.K., and Spaan, W.J. (1996). The production of recombinant infectious DI-particles of a murine coronavirus in the absence of helper virus. Virology *218*, 52–60.

Center for Systems Science and Engineering at Johns Hopkins University Coronavirus COVID-19 Global Cases.

Cingolani, P., Platts, A., Wang, L.L., Coon, M., Nguyen, T., Wang, L., Land, S.J., Lu, X., and Ruden, D.M. (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly *6*, 80–92.

Cock, P.J.A., Antao, T., Chang, J.T., Chapman, B.A., Cox, C.J., Dalke, A., Friedberg, I., Hamelryck, T., Kauff, F., Wilczynski, B., et al. (2009). Biopython: freely available Python tools for computational molecular biology and bioinformatics. Bioinformatics *25*, 1422–1423.

Coronaviridae Study Group of the International Committee on Taxonomy of Viruses (2020). The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. Nat Microbiol *5*, 536–544.

COVID-19 National Emergency Response Center, Epidemiology and Case Management Team, Korea Centers for Disease Control and Prevention (2020). Coronavirus Disease-19: The First 7,755 Cases in the Republic of Korea. Osong Public Health Res Perspect *11*, 85–90.

Denison, M.R., Graham, R.L., Donaldson, E.F., Eckerle, L.D., and Baric, R.S. (2011). Coronaviruses: an RNA proofreading machine regulates replication fidelity and diversity. RNA Biol. *8*, 270–279.

Diehl, W.E., Lin, A.E., Grubaugh, N.D., Carvalho, L.M., Kim, K., Kyawe, P.P., McCauley, S.M., Donnard, E., Kucukural, A., McDonel, P., et al. (2016). Ebola Virus Glycoprotein with Increased Infectivity Dominated the 2013-2016 Epidemic. Cell *167*, 1088–1098.e6.

Dong, E., Du, H., and Gardner, L. (2020). An interactive web-based dashboard to track COVID-19 in real time. Lancet Infect. Dis. *20*, 533–534.

Drosten, C., Günther, S., Preiser, W., van der Werf, S., Brodt, H.-R., Becker, S., Rabenau, H., Panning, M., Kolesnikova, L., Fouchier, R.A.M., et al. (2003). Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. *348*, 1967–1976.

Endo, A., Centre for the Mathematical Modelling of Infectious Diseases COVID-19 Working

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

Group, Abbott, S., Kucharski, A.J., and Funk, S. (2020). Estimating the overdispersion in COVID-19 transmission using outbreak sizes outside China. Wellcome Open Res *5*, 67.

Grasselli, G., Pesenti, A., and Cecconi, M. (2020). Critical Care Utilization for the COVID-19 Outbreak in Lombardy, Italy: Early Experience and Forecast During an Emergency Response. JAMA.

Grubaugh, N.D., Petrone, M.E., and Holmes, E.C. (2020). We shouldn't worry when a virus mutates during disease outbreaks. Nat Microbiol *5*, 529–530.

Gui, M., Song, W., Zhou, H., Xu, J., Chen, S., Xiang, Y., and Wang, X. (2017). Cryo-electron microscopy structures of the SARS-CoV spike glycoprotein reveal a prerequisite conformational state for receptor binding. Cell Res. *27*, 119–129.

Hadfield, J., Megill, C., Bell, S.M., Huddleston, J., Potter, B., Callender, C., Sagulenko, P., Bedford, T., and Neher, R.A. (2018). Nextstrain: real-time tracking of pathogen evolution. Bioinformatics *34*, 4121–4123.

Hansen, J., Baum, A., Pascal, K.E., Russo, V., Giordano, S., Wloga, E., Fulton, B.O., Yan, Y., Koon, K., Patel, K., et al. (2020). Studies in humanized mice and convalescent humans yield a SARS-CoV-2 antibody cocktail. Science.

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., Schiergens, T.S., Herrler, G., Wu, N.-H., Nitsche, A., et al. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell *181*, 271–280.e8.

Holmes, E.C. (2003). Error thresholds and the constraints to RNA virus evolution. Trends Microbiol. *11*, 543–546.

Hu, K., Zhao, Y., Wang, M., Zeng, Q., Wang, X., Wang, M., Zheng, Z., Li, X., Zhang, Y., Wang, T., et al. (2020). Identification of a super-spreading chain of transmission associated with COVID-19 (medRxiv).

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., Zhang, L., Fan, G., Xu, J., Gu, X., et al. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet *395*, 497–506.

Huang, Y., Yang, Z.-Y., Kong, W.-P., and Nabel, G.J. (2004). Generation of synthetic severe acute respiratory syndrome coronavirus pseudoparticles: implications for assembly and vaccine production. J. Virol. *78*, 12557–12565.

Hunter, J.D. (2007). Matplotlib: A 2D Graphics Environment. Comput. Sci. Eng. *9*, 90–95.

Johnsson, B., Löfås, S., and Lindquist, G. (1991). Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. Analytical Biochemistry *198*, 268–277.

Katoh, K., and Standley, D.M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. *30*, 772–780.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

Korber, B., Fischer, W.M., Gnanakaran, S., Yoon, H., Theiler, J., Abfalterer, W., Hengartner, N., Giorgi, E.E., Bhattacharya, T., Foley, B., et al. (2020). Tracking changes in SARS-CoV-2 Spike: evidence that D614G increases infectivity of the COVID-19 virus. Cell.

Ksiazek, T.G., Erdman, D., Goldsmith, C.S., Zaki, S.R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J.A., Lim, W., et al. (2003). A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. *348*, 1953–1966.

Lam, T.T.-Y., Jia, N., Zhang, Y.-W., Shum, M.H.-H., Jiang, J.-F., Zhu, H.-C., Tong, Y.-G., Shi, Y.-X., Ni, X.-B., Liao, Y.-S., et al. (2020). Identifying SARS-CoV-2-related coronaviruses in Malayan pangolins. Nature.

Letko, M., Marzi, A., and Munster, V. (2020). Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. Nat Microbiol *5*, 562–569.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., and 1000 Genome Project Data Processing Subgroup (2009). The Sequence Alignment/Map format and SAMtools. Bioinformatics *25*, 2078–2079.

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., Wang, W., Song, H., Huang, B., Zhu, N., et al. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. Lancet *395*, 565–574.

Marzi, A., Chadinah, S., Haddock, E., Feldmann, F., Arndt, N., Martellaro, C., Scott, D.P., Hanley, P.W., Nyenswah, T.G., Sow, S., et al. (2018). Recently Identified Mutations in the Ebola Virus-Makona Genome Do Not Alter Pathogenicity in Animal Models. Cell Rep. *23*, 1806–1816.

McNamara, R.P., Caro-Vegas, C., Landis, J.T., Moorad, R., Pluta, L.J., Eason, A.B., Thompson, C., Bailey, A., Villamor, F.C.S., Lange, P.T., et al. (2020). High-density amplicon sequencing identifies community spread and ongoing evolution of SARS-CoV-2 in the Southern United States.

Myszka, D.G. (1999). Improving biosensor analysis. J. Mol. Recognit. *12*, 279–284.

Onder, G., Rezza, G., and Brusaferro, S. (2020). Case-Fatality Rate and Characteristics of Patients Dying in Relation to COVID-19 in Italy. JAMA.

Page, A.J., Taylor, B., Delaney, A.J., Soares, J., Seemann, T., Keane, J.A., and Harris, S.R. (2016). SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. Microb Genom *2*, e000056.

Pallesen, J., Wang, N., Corbett, K.S., Wrapp, D., Kirchdoerfer, R.N., Turner, H.L., Cottrell, C.A., Becker, M.M., Wang, L., Shi, W., et al. (2017). Immunogenicity and structures of a rationally designed prefusion MERS-CoV spike antigen. Proc. Natl. Acad. Sci. U. S. A. *114*, E7348–E7357.

Rambaut, A., Holmes, E.C., Hill, V., O'Toole, Á., McCrone, J.T., Ruis, C., du Plessis, L., and Pybus, O.G. (2020). A dynamic nomenclature proposal for SARS-CoV-2 to assist genomic epidemiology.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

Ruedas, J.B., Ladner, J.T., Ettinger, C.R., Gummuluru, S., Palacios, G., and Connor, J.H. (2017). Spontaneous Mutation at Amino Acid 544 of the Ebola Virus Glycoprotein Potentiates Virus Entry and Selection in Tissue Culture. J. Virol. *91*, ii: e00392–17.

Saberi, A., Gulyaeva, A.A., Brubacher, J.L., Newmark, P.A., and Gorbalenya, A.E. (2018). A planarian nidovirus expands the limits of RNA genome size. PLoS Pathog. *14*, e1007314.

Shu, Y., and McCauley, J. (2017). GISAID: Global initiative on sharing all influenza data – from vision to reality. Eurosurveillance *22*, ii=30494.

Smith, E.C., Blanc, H., Surdel, M.C., Vignuzzi, M., and Denison, M.R. (2013). Coronaviruses lacking exoribonuclease activity are susceptible to lethal mutagenesis: evidence for proofreading and potential therapeutics. PLoS Pathog. *9*, e1003565.

Smith, E.C., Sexton, N.R., and Denison, M.R. (2014). Thinking Outside the Triangle: Replication Fidelity of the Largest RNA Viruses. Annu Rev Virol *1*, 111–132.

Tian, X., Li, C., Huang, A., Xia, S., Lu, S., Shi, Z., Lu, L., Jiang, S., Yang, Z., Wu, Y., et al. (2020). Potent binding of 2019 novel coronavirus spike protein by a SARS coronavirus-specific human monoclonal antibody. Emerg. Microbes Infect. *9*, 382–385.

Tortorici, M.A., and Veesler, D. (2019). Structural insights into coronavirus entry. Adv. Virus Res. *105*, 93–116.

Urbanowicz, R.A., McClure, C.P., Sakuntabhai, A., Sall, A.A., Kobinger, G., Müller, M.A., Holmes, E.C., Rey, F.A., Simon-Loriere, E., and Ball, J.K. (2016). Human Adaptation of Ebola Virus during the West African Outbreak. Cell *167*, 1079–1087.e5.

Vennema, H., Godeke, G.J., Rossen, J.W., Voorhout, W.F., Horzinek, M.C., Opstelten, D.J., and Rottier, P.J. (1996). Nucleocapsid-independent assembly of coronavirus-like particles by co-expression of viral envelope protein genes. The EMBO Journal *15*, 2020–2028.

Wagner, C., Roychoudhury, P., Hadfield, J., Hodcroft, E.B., Lee, J., Moncla, L.H., Müller, N.F., Behrens, C., Huang, M.L., Mathias, P., et al. Comparing viral load and clinical outcomes in Washington State across D614G mutation in spike protein of SARS-CoV-2. Github.

Walls, A.C., Xiong, X., Park, Y.-J., Tortorici, M.A., Snijder, J., Quispe, J., Cameroni, E., Gopal, R., Dai, M., Lanzavecchia, A., et al. (2019). Unexpected Receptor Functional Mimicry Elucidates Activation of Coronavirus Fusion. Cell *176*, 1026–1039.e15.

Walls, A.C., Park, Y.-J., Tortorici, M.A., Wall, A., McGuire, A.T., and Veesler, D. (2020). Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. Cell *181*, 281–292.e6.

Wan, Y., Shang, J., Graham, R., Baric, R.S., and Li, F. (2020). Receptor Recognition by the Novel Coronavirus from Wuhan: an Analysis Based on Decade-Long Structural Studies of SARS Coronavirus. J. Virol. *94*.

Wrapp, D., Wang, N., Corbett, K.S., Goldsmith, J.A., Hsieh, C.-L., Abiona, O., Graham, B.S., and McLellan, J.S. (2020). Cryo-EM structure of the 2019-nCoV spike in the prefusion

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

conformation. Science *367*, 1260–1263.

Wu, A., Peng, Y., Huang, B., Ding, X., Wang, X., Niu, P., Meng, J., Zhu, Z., Zhang, Z., Wang, J., et al. (2020a). Genome Composition and Divergence of the Novel Coronavirus (2019-nCoV) Originating in China. Cell Host Microbe *27*, 325–328.

Wu, F., Zhao, S., Yu, B., Chen, Y.-M., Wang, W., Song, Z.-G., Hu, Y., Tao, Z.-W., Tian, J.-H., Pei, Y.-Y., et al. (2020b). A new coronavirus associated with human respiratory disease in China. Nature *579*, 265–269.

Yuan, Y., Cao, D., Zhang, Y., Ma, J., Qi, J., Wang, Q., Lu, G., Wu, Y., Yan, J., Shi, Y., et al. (2017). Cryo-EM structures of MERS-CoV and SARS-CoV spike glycoproteins reveal the dynamic receptor binding domains. Nat. Commun. *8*, 15092.

Zaki, A.M., van Boheemen, S., Bestebroer, T.M., Albert D M, and Fouchier, R.A.M. (2012). Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. New England Journal of Medicine *367*, 1814–1820.

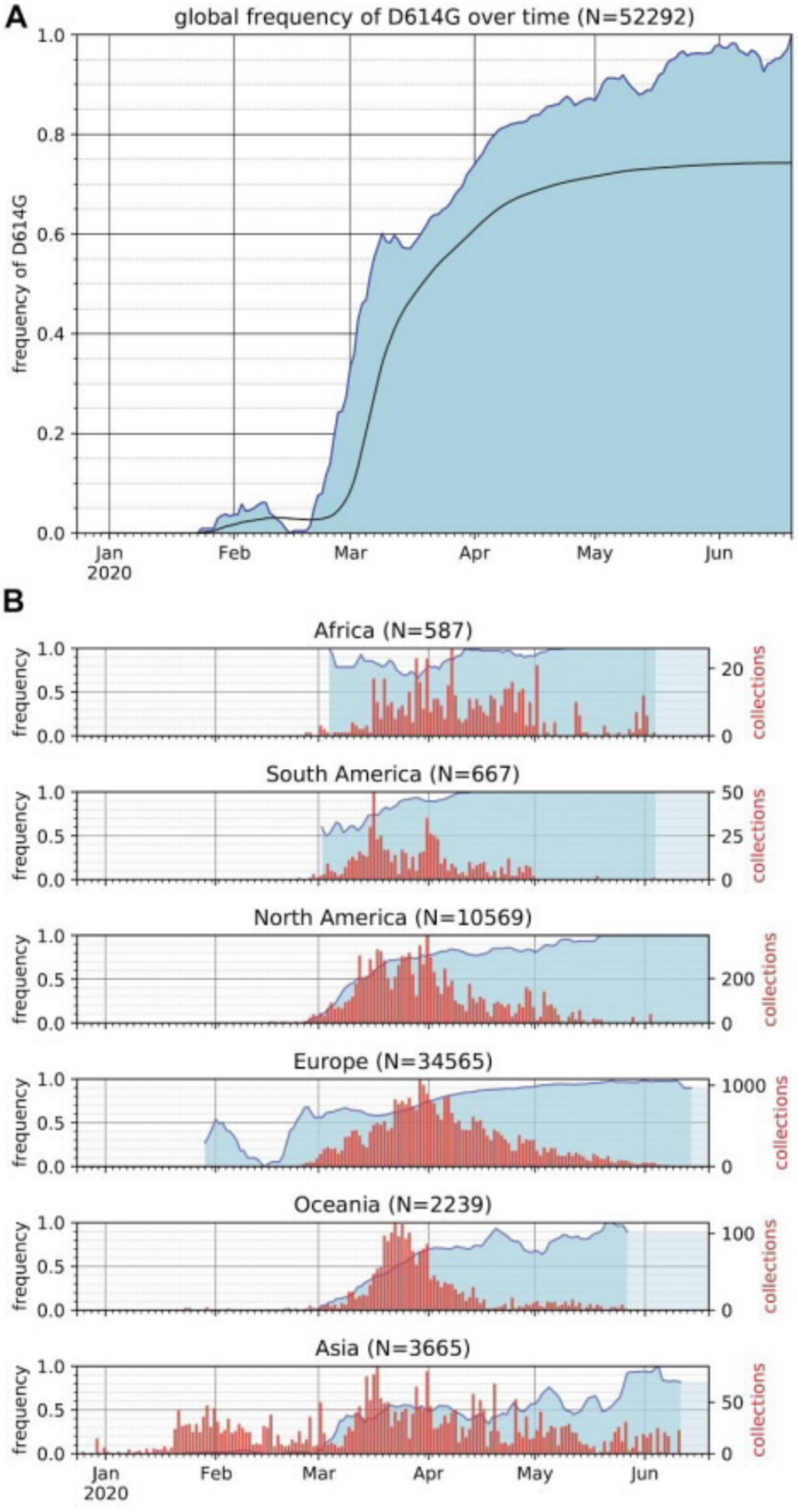
Zhao, S., Lin, Q., Ran, J., Musa, S.S., Yang, G., Wang, W., Lou, Y., Gao, D., Yang, L., He, D., et al. (2020). Preliminary estimation of the basic reproduction number of novel coronavirus (2019-nCoV) in China, from 2019 to 2020: A data-driven analysis in the early phase of the outbreak. Int. J. Infect. Dis. *92*, 214–217.

Zhou, H., Chen, X., Hu, T., Li, J., Song, H., Liu, Y., Wang, P., Liu, D., Yang, J., Holmes, E.C., et al. (2020a). A Novel Bat Coronavirus Closely Related to SARS-CoV-2 Contains Natural Insertions at the S1/S2 Cleavage Site of the Spike Protein. Current Biology *30*, 2196–2203.e3.

Zhou, P., Yang, X.-L., Wang, X.-G., Hu, B., Zhang, L., Zhang, W., Si, H.-R., Zhu, Y., Li, B., Huang, C.-L., et al. (2020b). A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature *579*, 270–273.

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Zhao, X., Huang, B., Shi, W., Lu, R., et al. (2020). A Novel Coronavirus from Patients with Pneumonia in China, 2019. N. Engl. J. Med. *382*, 727–733.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.



bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

Figure 1. Over the course of the COVID-19 pandemic the frequency of the SARS-CoV-2 S protein D614G variant has risen to near fixation

(A) The global frequency of the S protein D614G variant over time in sequences published via the GISAID SARS-CoV-2 database, as of 25 June 2020. The filled plot (blue) represents a 7-day rolling average of the fraction of sequences bearing the D614G variant for each collection date. Dates without published sequences are linearly interpolated. The cumulative frequency of the D614G variant (black line) is overlaid, showing the frequency of D614G in sequences from samples collected up to and including each date.

(B) The frequency of the D614G variant over time (blue) in sequences collected from six continental regions, using the same dataset as in (A), plotted as a 7-day rolling average. Frequencies are interpolated for dates without published sequence. The frequency of the last date with data is carried forward where recent dates lack data to indicate the most recent calculated frequency (light blue). The number of samples collected, sequenced, and published is overlaid (red bars) to provide the denominator used in calculating the frequency for each date, and to illustrate the non-uniformity of sampling.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

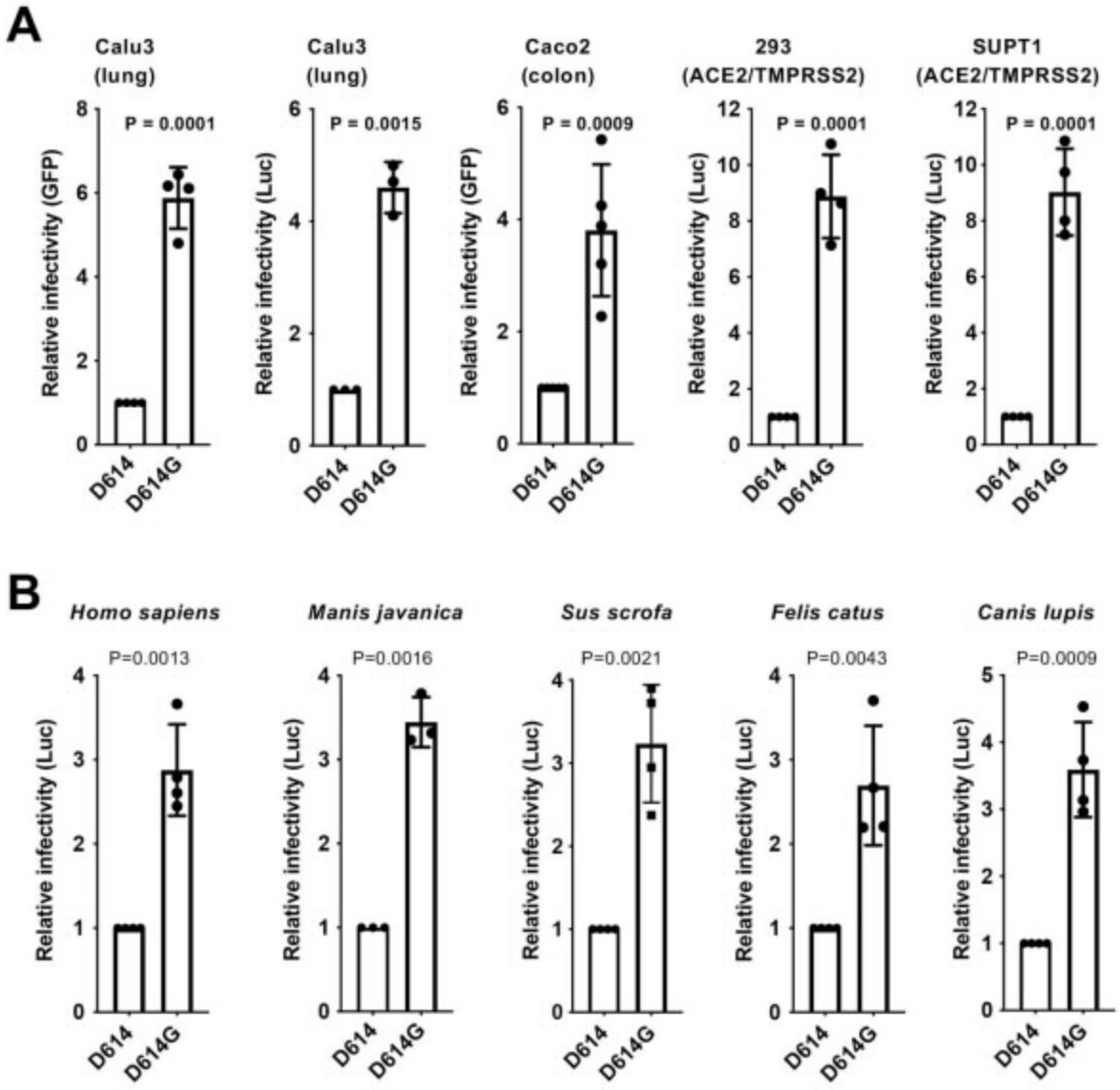


Figure 2. SARS-CoV-2 D614G S protein variant enhances infectivity

A. Lentiviral virions bearing either GFP or Luciferase transgenes, and pseudotyped with either SARS-CoV-2 D614 or D614G S proteins, were produced by transfection of HEK293 cells, and used to transduce human Calu3 lung cells, Caco2 colon cells, and either HEK293 or SupT1 cells stably expressing ACE2 and TMPRSS2. Relative infectivity of D614G vs D614, with D614 set at 1, was determined based on flow cytometry for percent GFP-positivity or bulk luciferase activity. Each point represents transduction with a lentiviral stock that was derived from an independent transfection. Shown are the mean +/- SD.

B. Lentiviral virions bearing a luciferase transgene, pseudotyped with either SARS-CoV-2 D614 or D614G S proteins, were produced by transfection of HEK293 cells, and used to transduce human HEK293 cells transiently transfected with plasmids encoding the indicated ACE2 orthologs. Relative infectivity of D614G vs D614, with D614 set at 1, was determined based on bulk luciferase activity. Each point represents transduction using lentiviral stock derived from an independent transfection. Shown are the mean +/- SD.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

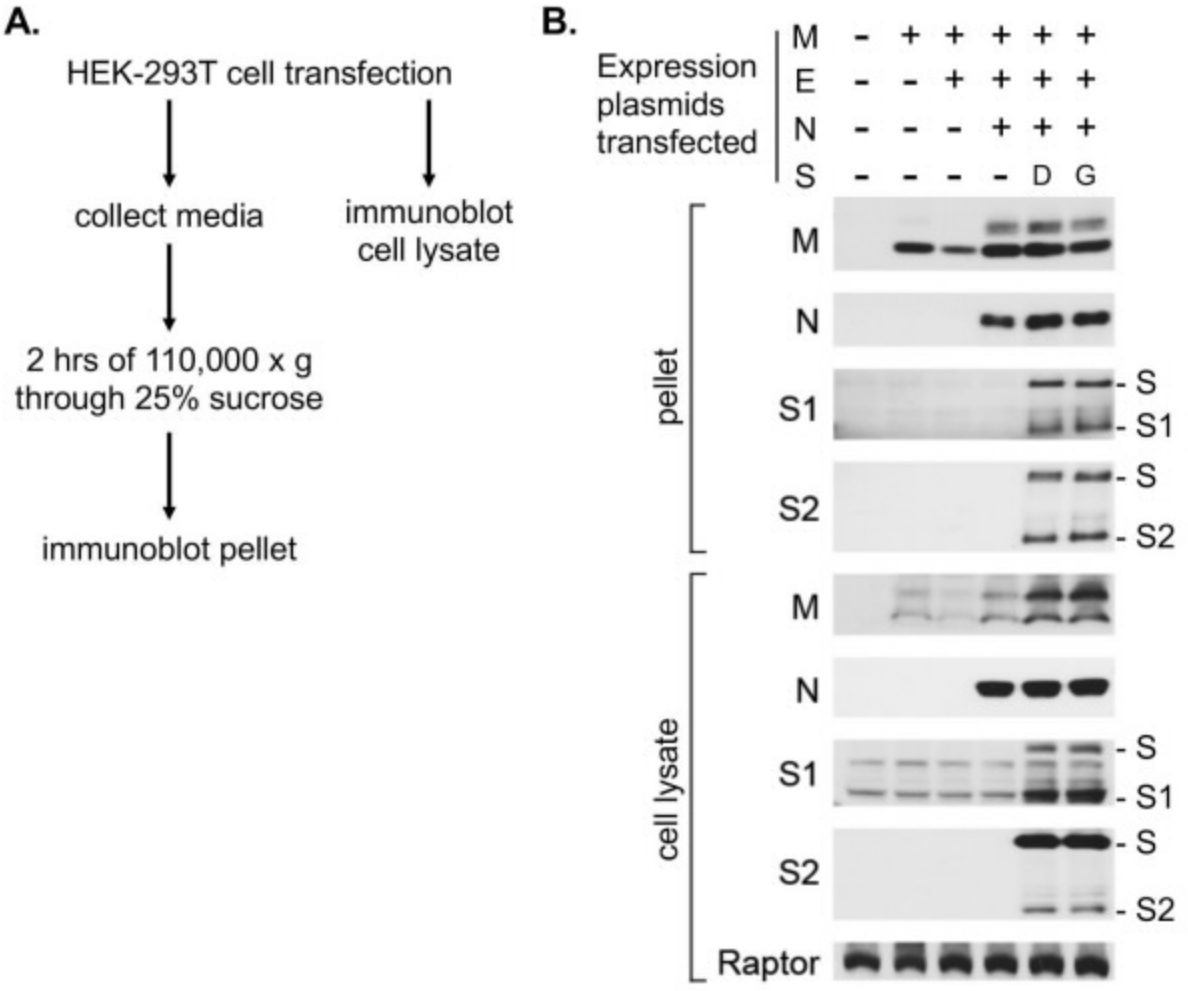
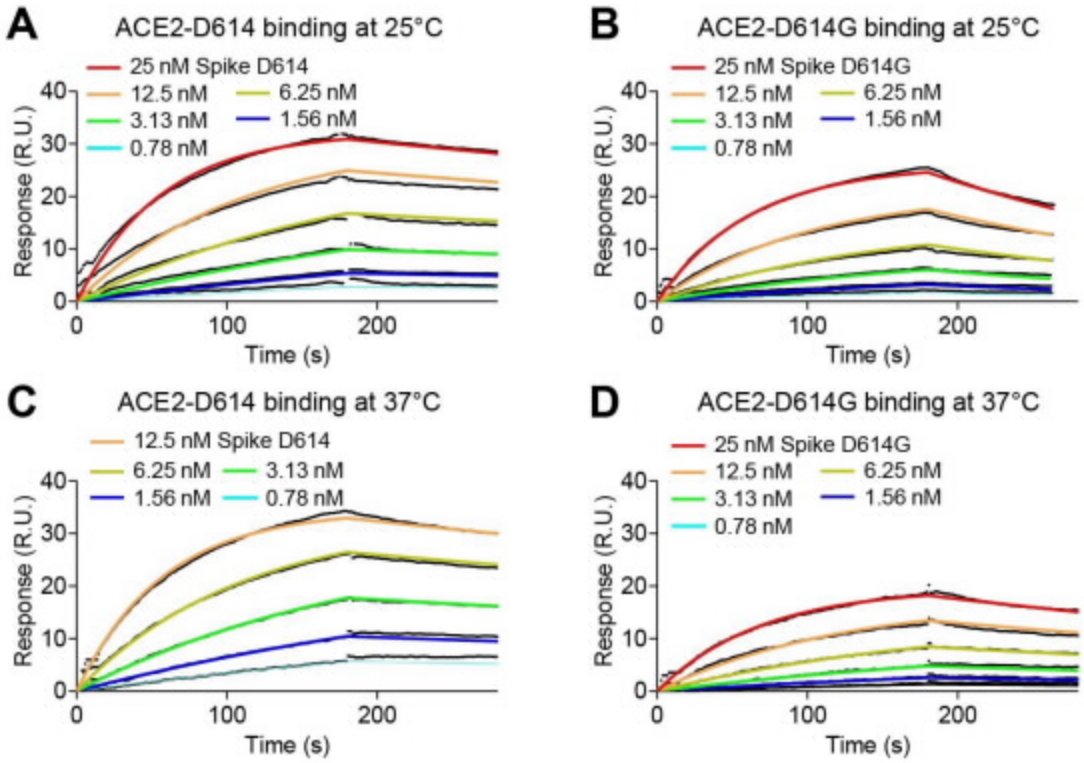


Figure 3. Synthesis, processing, and incorporation of S protein variants into SARS-CoV-2 virus-like particles

(A) Schematic showing how SARS-CoV-2 structural proteins were produced in HEK-293T cells and virus-like particles were enriched from the supernatant by ultracentrifugation.

(B) HEK-293T cells were transfected with plasmids encoding the proteins indicated at the top. Western blots were then performed on cell lysate and on ultracentrifuge pellets from cell culture supernatant with the primary antibodies indicated on the left of the blots. Anti-Raptor antibody was used as a loading control for the cell lysate. Uncleaved S protein, as well as the S1 and S2 cleavage products, are indicated on the right.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.



E **Binding kinetics between ACE2 and Spike**

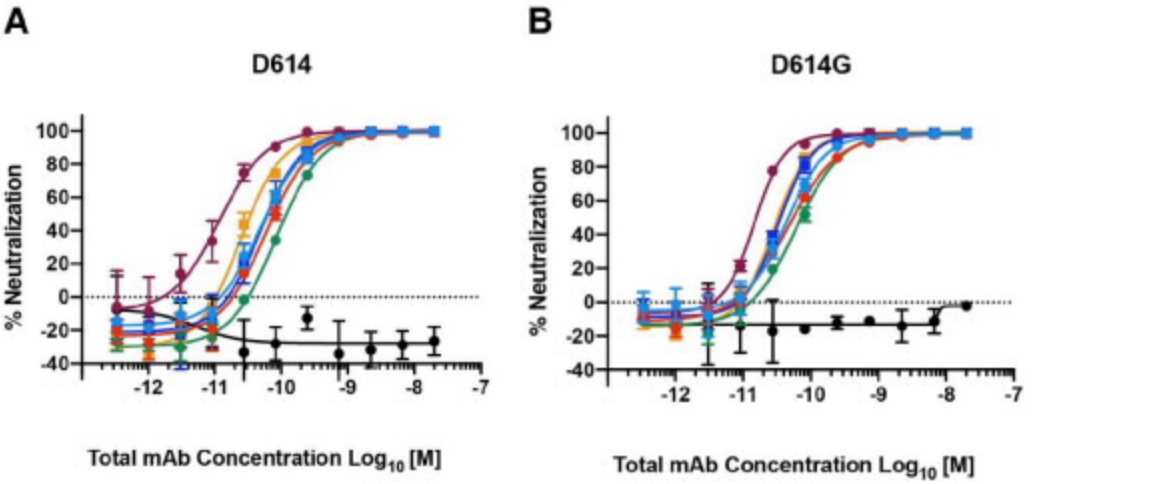
Captured receptor	Temperature (°C)	hsACE2 level (R.U.)	Spike variant	Spike bound at 50 nM (R.U.)	k_A ($M^{-1}s^{-1}$)	k_D (s^{-1})	K_D (nM)
hsACE2.hFc	25	47	D614	40	6.72×10^{5}	9.27×10^{-4}	1.38
		46	D614G	36	4.93×10^{5}	39.3×10^{-4}	7.97
	37	63	D614	47	13.3×10^{5}	9.44×10^{-4}	0.71
		61	D614G	26	5.06×10^{5}	19.0×10^{-4}	3.76

Figure 4. SARS-CoV-2 D614G S protein variant binds ACE2 weaker than the ancestral protein

(A-D) SPR measurement of D614-ACE2 binding (A and C) and D614G-ACE2 binding (B and D), at 25°C (A and B) or at 37°C (C and D).

(E) Summary of kinetic parameters measured in A-D. D614G binds ACE2 five-fold weaker than D614 at both temperatures tested.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.



C

Antibody	D614 IC_{50} (M)	D614G IC_{50} (M)
REGN10989	1.17E-11	1.47E-11
REGN10987	5.76E-11	4.57E-11
REGN10933	4.81E-11	4.34E-11
REGN10934	8.35E-11	6.04E-11
REGN10933+REGN10987	4.82E-11	3.28E-11
REGN10989+REGN10987	2.33E-11	2.46E-11

Figure 5. Neutralization potency of monoclonal antibodies targeting the SARS-CoV-2 S protein receptor binding domain.

(A and B) Vero cells were challenged with pVSV-SARS-CoV-2-S-mNeon pseudoparticles encoding either D614 (A) or D614G S protein variants, in the presence of serial dilutions of the indicated human monoclonal antibodies targeting the SARS-CoV-2 S protein receptor binding domain, or IgG1 isotype control. mNeon protein fluorescence was measured 24 hours post-infection as a read-out for virus infectivity. Data is graphed as percent neutralization relative to virus only infection control.

(C) Neutralization potency (IC_{50}) of individual monoclonals and of combinations of monoclonals, against the SARS-CoV-2 D614G and D614G S protein variants, as indicated.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

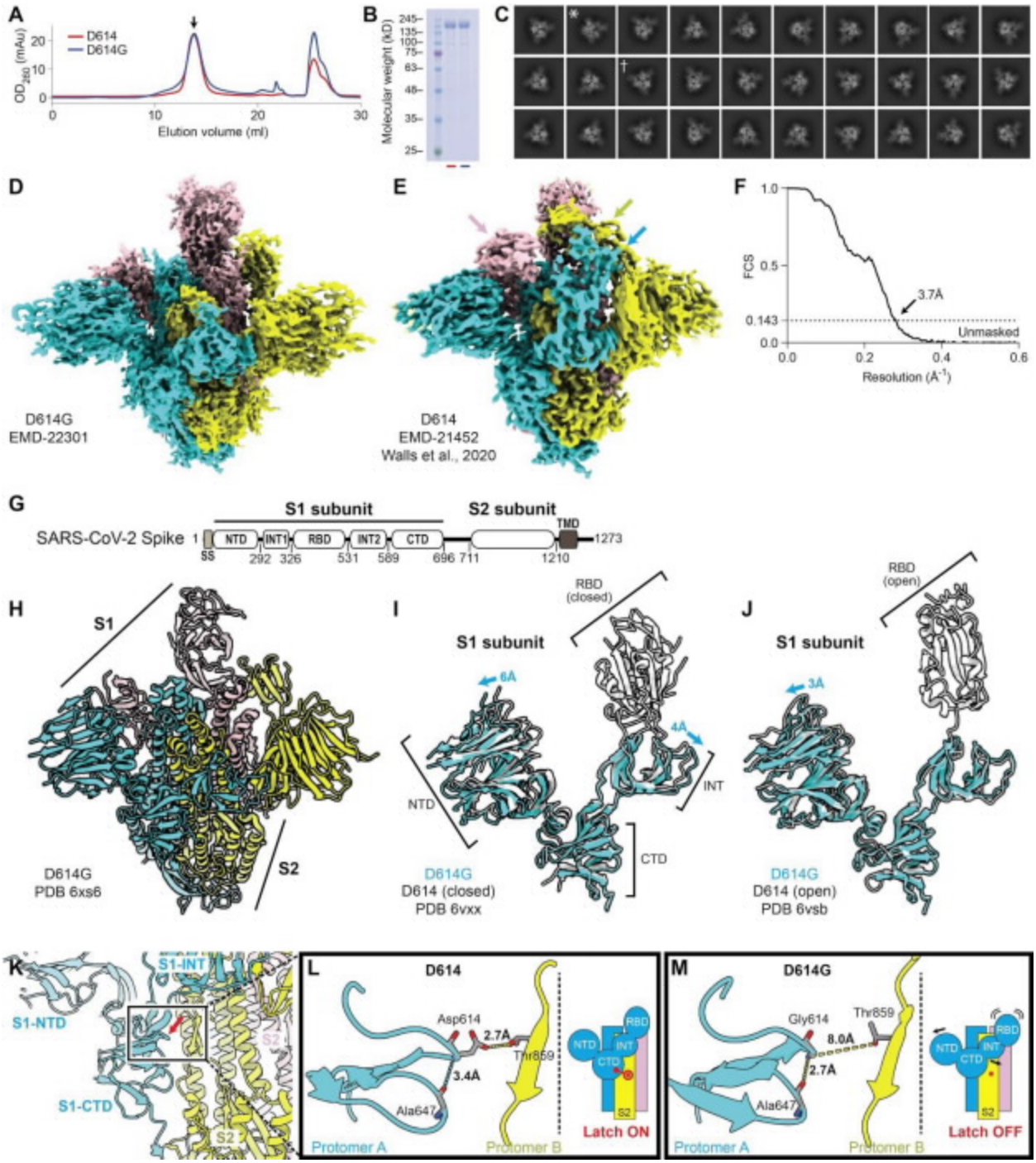


Figure 6. Structural determination of Spike D614G.

(A) Size exclusion column elution profiles of D614G (blue line) and D614 (red line).

(B) Coomassie-stained SDS-PAGE of the peaks collected in (A) shows equivalent, full-length monomeric S proteins at around 180 kD.

(C) 2D-clustering of trimeric D614G particles. Dagger denotes a top-view. Asterisk denotes a side-view.

(D) D614G envelope from the three-dimensional reconstruction. EMDB: EMD-22301.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

(E) Published D614 envelope (EMD-21452). Arrows point to the density corresponding to the receptor binding domains, which is missing in the corresponding positions in (D).
(F) Gold-standard Fourier shell correlation of the density map in (D).
(G) Domain arrangement of the SARS-CoV-2 S protein.
(H) Atomic model for D614G without the receptor binding domain. PDB: 6XS6.
(I-J) Comparison of the D614G S1 subunit with the closed conformation (I) and open conformation (J) of the D614 S1 subunit. Arrows indicate the relative movement of the S1 subunit of D614G.
(K) Position of amino acid 614 on the S protein.
(L-M) Substitution of Asp614 with glycine changes hydrogen bonding around residue 614. In the case of D614 (L), an inter-protomer hydrogen bond is detected. For D614G (M), the Asp614-Thr859 hydrogen bond is eliminated, and interaction with intradomain Ala647 is strengthened.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

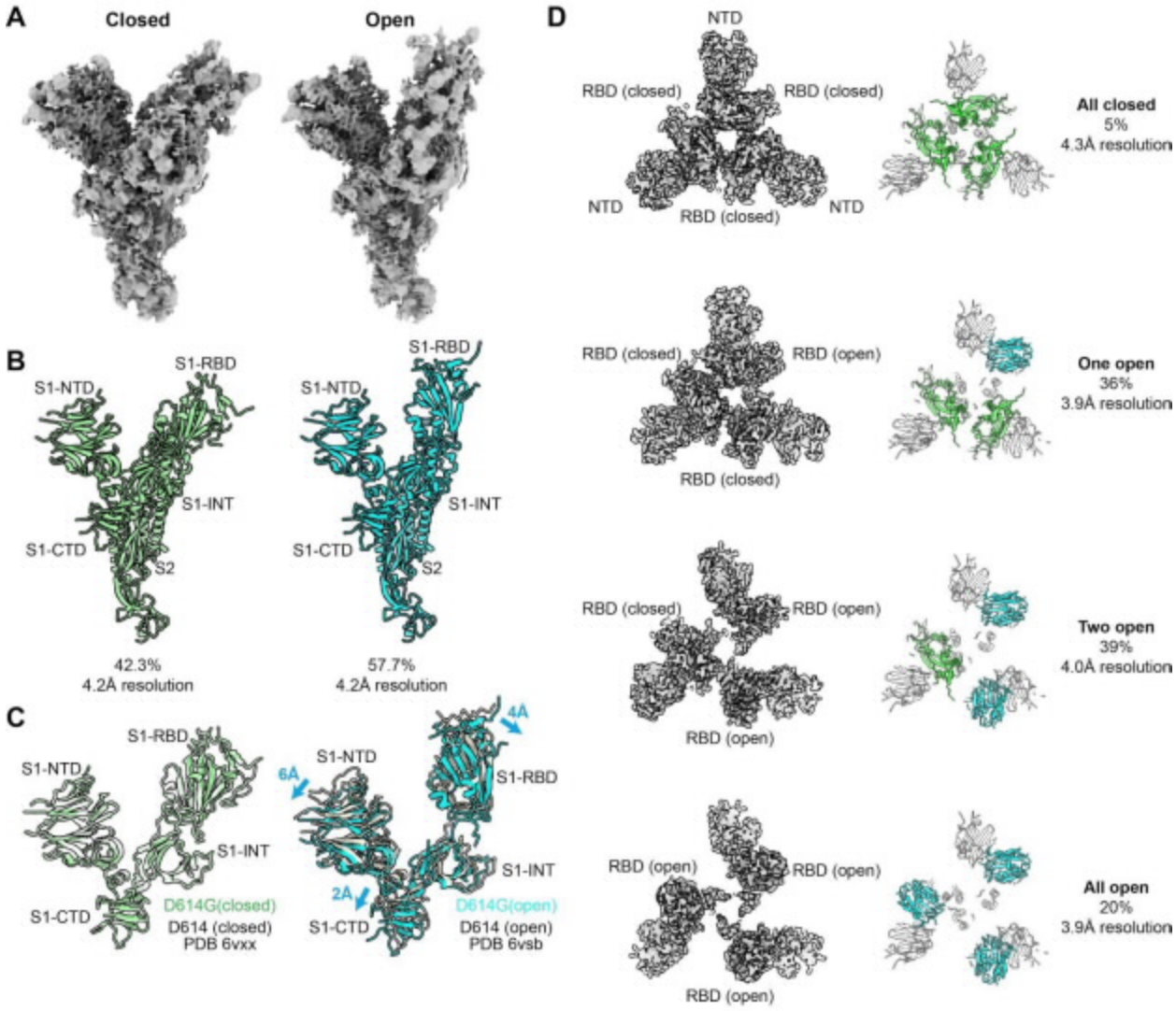


Figure 7. D614G populates more open conformations than does the ancestral S protein

(A) Cryo-EM density maps of the two conformations of D614G protomer. The first is a closed conformation with a buried RBD. The second is an open conformation with the RBD standing up.

(B) Atomic models for the closed (left) and open (right) conformations for the two D614G protomers shown in (A).

(C) Comparison of the two D614G protomer S1 subunit conformations with the corresponding conformations of the D614 protomer S1 subunit.

(D) The D614G S protein trimer adopts four conformations. In addition to the all closed and one open conformation detected with the D614 S protein trimer, the D614G S protein trimer adopts two-open and three-open conformations.

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

Table 1. Summary of cryo-EM data collection, 3D reconstruction, and model refinement

	SARS-Covid19 Spike (D614G)
Imaging parameters and 3D reconstruction	
Cryo-EM grids	UltrAuFoil R1.2/1.3
Calibrated magnification	155000
Acceleration voltage [kV]	300
Pixel size [Å]	0.52
Total dose [$e^-/Å^2$]	37
Exposure time [s]	1.81
Defocus range [μm]	-0.6 ~ -1.8
Particles in final 3D reconstruction	266356
Resolution ('Gold-standard' at FSC 0.143) [Å]	3.7
Model refinement	
Resolution in phenix.real_space_refine [Å]	4.0
No. atoms: protein	18294
Ligands / ions	N/A
r.m.s. deviations: bond lengths [Å]	0.004
r.m.s. deviations: bond angles [°]	0.808
MolProbity score	4.49
Rotamer outliers [%]	0.00
Ramachandran angles: favored [%]	93.88
Ramachandran angles: allowed [%]	6.12
Ramachandran angles: outliers [%]	0.00
PDB	6xs6
EMDB	EMD-22301

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

Table 2. List of Reagents and Resources used in this manuscript

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-SARS-CoV-2 M protein	ThermoFisher	PA1-41160
Rabbit polyclonal anti-SARS-CoV-2 E protein	Antibodies-online.com	ABIN1031551
Rabbit polyclonal anti-SARS-CoV-2 N protein	Rockland Immunochemicals	200-401-A50
Rabbit monoclonal anti-SARS-CoV-2 S protein (S1-specific)	GeneTex	GTX635656
Rabbit monoclonal anti-SARS-CoV-2 S protein (S1-specific)	GeneTex	GTX635672
Mouse/human monoclonal anti-SARS-CoV-2 S protein (S2-specific)	Sino Biological	40590-D001
Rabbit polyclonal anti-Raptor	EMD Millipore	09-217
Goat anti-rabbit antibody 800CW	Licor	925-32211
Goat anti-mouse 680RD	Licor	925-68070

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

Goat anti-rabbit, HRP-linked antibody	Cell Signaling Technology	7074
Goat anti-mouse, HRP-linked antibody	Cell Signaling Technology	7076
Human monoclonal antibody targeting the receptor binding domain of the SARS-CoV-2 S protein	A. Baum et al., Science10.1126/science.abd0831 (2020) J. Hansen et al., Science10.1126/science.abd0827 (2020)	REGN10987
Human monoclonal antibody targeting the receptor binding domain of the SARS-CoV-2 S protein	A. Baum et al., Science10.1126/science.abd0831 (2020) J. Hansen et al., Science10.1126/science.abd0827 (2020)	REGN10989
Human monoclonal antibody targeting the receptor binding domain of the SARS-CoV-2 S protein	A. Baum et al., Science10.1126/science.abd0831 (2020) J. Hansen et al., Science10.1126/science.abd0827 (2020)	REGN10933
Human monoclonal antibody targeting the receptor binding domain of the SARS-CoV-2 S protein	A. Baum et al., Science10.1126/science.abd0831 (2020) J. Hansen et al., Science10.1126/science.abd0827 (2020)	REGN10934
Mouse monoclonal, anti-human Fc antibody	A. Baum et al., Science10.1126/science.abd0831 (2020) J. Hansen et al., Science10.1126/science.abd0827 (2020)	REGN2567
Viruses		

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

VSVΔG:mNeon/VSV-G virus	A. Baum et al., Science10.1126/science.abd0831 (2020) J. Hansen et al., Science10.1126/science.abd0827 (2020)	N/A
Chemicals, Peptides, Commerical Assays, and Recombinant Proteins		
hsACE2.hFc	A. Baum et al., Science10.1126/science.abd0831 (2020) J. Hansen et al., Science10.1126/science.abd0827 (2020)	N/A
blasticidin	InvivoGen	ant-bl-1
puromycin	InvivoGen	ant-pr-1
gentamicin	Life Technologies	15750078
Ni-NTA resin	EMD Millipore	30230
Superose 6 gel-filtration column	GE Healthcare	29091596
Mycoplasma detection kit	Lonza	LT07-318
PEI	Polysciences, Inc.	23966-1
TransIT LT1 transfection reagent	Mirus Bio	MIR 2304
Lipofectamine LTX	Life Technologies	15338100
Steady-Glo Luciferase Assay System	Promega	E2550
Cytofix	BD Biosciences	554655
SMM-293 TII serum-free media	SinoBiological	M293TI

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

Deposited Data		
PDB	6xs6	N/A
EMDB	EMD-22301	
Cell Lines		
Human: HEK-293 cells	ATCC	CRL-1573
Human: HEK-293T cells	ATCC	CRL-3216
Human: HEK-293T/17 cells	ATCC	CRL-11268
Human: FreeStyle 293-F cells	ThermoFisher	R79007
Human: Calu-3 cells	ATCC	HTB-55
Human: Caco-2 cells	ATCC	HTB-37
Human: SUPT1 [VB] cells	ATCC	CRL-1942
Cercophithecus aethiops: Vero cells	ATCC	CCL-81
Recombinant DNA		
pcDNA3.1 SARS-CoV-2 S D614	This publication	Addgene #158074
pcDNA3.1 SARS-CoV-2 S D614G	This publication	Addgene #158075

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

pcDNA3.1 SARS-CoV-2 S D614-trunc	This publication	Addgene #158076
pcDNA3.1 SARS-CoV-2 S D614G-trunc	This publication	Addgene #158077
pcDNA3.1 SARS-CoV-2 M	This publication	Addgene #158078
pcDNA3.1 SARS-CoV-2 N	This publication	Addgene #158079
pcDNA3.1 SARS-CoV-2 E	This publication	Addgene #158080
pscALPSpuro	Addgene	Cat# 128504
pscALPSpuro-*Hs*ACE2 (human)	This publication NP_001358344.1	Addgene #158081
pscALPSpuro-*Fc*ACE2 (Cat)	This publication XP_023104564.1	Addgene #158082
pscALPSpuro-*Cl*ACE2 (Dog)	This publication NP_001158732.1	Addgene #158083
pscALPSpuro-*Mj*ACE2 (Pangolin)	This publication XP_017505752	Addgene #158084
pscALPSpuro-*Ss*ACE2 (Pig)	This publication NP_001116542.1	Addgene #158085
pscALPSpuro-*Rn*ACE2 (Rat)	This publication NP_001116542.1	Addgene #158086
pscALPSpuro-*Mm*ACE2 (Mouse)	This publication NP_001116542.1	Addgene #158087
pscALPSblasti-GFP	Addgene	Cat# 115804
pscALPSblasti-TMPRSS2 Blasti	This publication	Addgene #158088
HIV-1 pNL4-3 Δenv Δvpr luciferase reporter vector (pNL4-3.Luc.R-E-)	NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: from Dr. Nathaniel Landau	Cat #3418

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

pUC57mini NL4-3 Δenv eGFP	This publication	Pending NIH AIDS Reagent Program
psPAX2	Addgene	Cat #12260
pMD2.G	Addgene	Cat# 12259
pCAGGS-ACE2-his	This publication	Addgene #158089
Software and Algorithms		
FlowJo	FlowJo LLC; BD	N/A
Prism 8.4.3	GraphPad	N/A
T200 Evaluation software v 3.1	Biacore	N/A
Scrubber 2.0	BioLogic Software	N/A
Coot	https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/	Version 0.9
PHENIX	https://www.phenix-online.org/	Version 1.18.2
MolProbity	http://molprobity.biochem.duke.edu/	Version 4.5.1
MAFFT v7.464	Katoh and Standley, 2013	https://mafft.cbrc.jp/alignment/software/
Biopython v1.76	Cock et al., 2009	https://biopython.org/
Matplotlib v3.2.2	Hunter, 2007	https://matplotlib.org/
SnpEff v4.5covid19	Cingolani, et al. 2012	http://snpeff.sourceforge.net/
bcftools v1.9	Li et al., 2009	http://samtools.github.io/bcftools/bcftools.html
snp-sites v2.5.1	Page, et al. 2016	https://sanger-pathogens.github.io/snp-sites/
EPU 2 software for single particle analysis	Thermo Fisher Scientific	N/A
Hardware, Machines, and Other		

bioRxiv preprint doi: https://doi.org/10.1101/2020.07.04.187757 . this version posted July 16, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. It is made available under a CC-BY-NC-ND 4.0 International license.

Quantifoil Au grid	Quantifoil, Großlöbichau Germany	NA
GloQube Plus Glow Discharge System	Quorumtech	N/A
Vitrobot Mark IV	Thermo Fisher Scientific	N/A
300 kV Krios transmission electron microscope	Thermo Fisher Scientific	N/A

From:	Baric, Toni C[/O=EXCHANGELABS/OU=EXCHANGE ADMINISTRATIVE GROUP (FYDIBOHF23SPDLT)/CN=RECIPIENTS/CN=6A7390851CF045E8BE4BB68C16F4F916-TONI C BARI]
Location:	Microsoft Teams Meeting
Subject:	FW: TC: R. Baric + A. Pruijssers + M. Denison + BMGF
Importance:	Normal
Start Time:	Tue 7/28/2020 5:00:00 PM (UTC-04:00)
End Time:	Tue 7/28/2020 6:00:00 PM (UTC-04:00)
Required Attendees:	Baric, Ralph S; Baric, Ralph S; Pruijssers, Ardina; Denison, Mark; Tse, Long Ping Victor
Optional Attendees:	Miskel, Suzanne M; Baric, Toni C; isanajera; Isabel Najera

-----Original Appointment-----
From: Monalisa Chatterji <MONALISA.CHATTERJI@gatesfoundation.org>
Sent: Monday, July 27, 2020 7:50 PM
To: Monalisa Chatterji; Baric, Ralph S; Pruijssers, Ardina; Denison, Mark; Tse, Long Ping Victor
Cc: Miskel, Suzanne M; Baric, Toni C; isanajera; Isabel Najera
Subject: TC: R. Baric + A. Pruijssers + M. Denison + BMGF
When: Tuesday, July 28, 2020 2:00 PM-3:00 PM (UTC-08:00) Pacific Time (US & Canada).
Where: Microsoft Teams Meeting

Join Microsoft Teams Meeting

+1 206-316-9202 United States, Seattle (Toll)
(833) 696-7489 United States (Toll-free)
Conference ID: 594 739 674#
Local numbers | Reset PIN | Learn more about Teams | Meeting options
Join with a video conferencing device
311204904@teams.bjn.vc VTC Conference ID: 1195720743
Alternate VTC dialing instructions

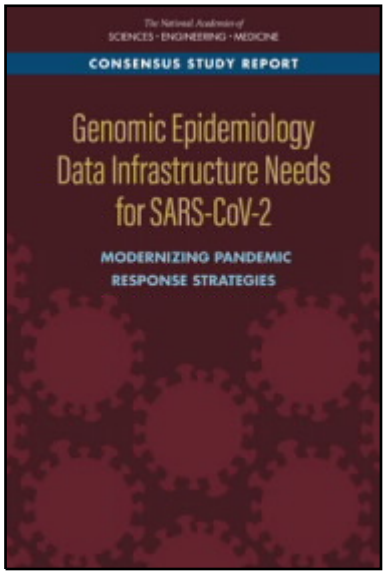
Consensus Study Report

JULY 2020

HIGHLIGHTS

Genomic Epidemiology Data Infrastructure Needs for SARS-CoV-2

Modernizing Pandemic Response Strategies



At the time of writing this report, the virus that causes COVID-19, SARS-CoV-2, has spread worldwide, infecting at least 10 million people and leading to an estimated 500,000 deaths. To best respond to this ongoing threat, policy makers and public health officials need access to cutting-edge pandemic, epidemic, and global preparedness and response strategies. Particularly, the ability to examine the genome of the virus may help public health officials more precisely understand transmission patterns, virus evolution, and potential treatment and prevention interventions. If policy makers and public health officials have access to these data, it may increase the possibility of breaking or delaying virus transmission and in turn reduce illness and deaths from COVID-19.

Recognizing the need to capitalize on these advances during the current COVID-19 pandemic, the Department of Health and Human Services' Office of the Assistant Secretary for Preparedness and Response (HHS/ASPR) and Office of Science and Technology Policy (OSTP) asked the National Academies of Sciences, Engineering, and Medicine to convene an ad hoc committee that would lay out a framework to help define and describe the data needs for a system that can track and correlate viral genome sequences with clinical and epidemiological data. The resulting system, outlined in this report, is designed to help integrate data sharing and data analysis on the evolution of SARS-CoV-2 into public health practice and decision making.

EXISTING DEFICIENCIES IN GENOMIC EPIDEMIOLOGY EFFORTS FOR SARS-COV-2

Several ongoing data projects are using genomic epidemiology to aid the public health response to COVID-19, including federal and nonprofit initiatives. In the United States, the Centers for Disease Control and Prevention's SARS-CoV-2 Sequencing for Public Health Emergency Response, Epidemiology, and Surveillance (SPHERES) consortium is coordinating a nationwide genomic sequencing effort. The National Institutes of Health is supporting two initiatives: the National COVID Cohort Collaborative (N3C), a secure portal for patient-level COVID-19 clinical data, and the National Center for Biotechnology Information's Reference Sequence (RefSeq) database. Several regional initiatives have emerged as well, integrating data sharing through existing global efforts like the Global Initiative on Sharing All Influenza Data (GISAID) and Nextstrain.

Due to a number of factors including poor funding, coordination, and capacity, the committee concluded that existing data sources and integration efforts for SARS-CoV-2 genome sequence data are patchy, typically passive, and reactive in the United States. As a result, available data do not represent key population features of individuals who are infected with SARS-CoV-2 and cannot adequately answer many pressing questions about the evolution and transmission of this virus.

To better collect necessary viral sequence data and other associated data, the committee recommended that HHS implement genome sequencing of SARS-CoV-2 on a national scale and ensure that this effort is fully representative of every segment of the population. Particularly, the committee highlighted the need to account for race and ethnicity, gender, age, geography, housing type, displayed symptoms, and transmissibility. To read the full text of this recommendation, see the Recommendations Insert.

BUILDING A FRAMEWORK TO TRACK AND CORRELATE VIRAL GENOME SEQUENCES WITH CLINICAL AND EPIDEMIOLOGICAL DATA

When combined with other types of data, researchers and public health officials may be able to use viral genome sequence data to inform questions related to transmission of a virus, virus evolution, and how the virus manifests as a disease.

However, in order to fully understand these issues, public officials need access to effectively integrated data. Currently, no central repository exists for the collection and curation of such infectious disease outbreak data from federal, state, and local health agencies, health care networks, and public health and clinical laboratories. To address this gap, the committee recommended that HHS develop and invest in a national data infrastructure system that can link genomic, clinical, and epidemiological data. This system should:

- Allow for linkage between these types of data without overburdening laboratories
- Create and foster safe data-sharing practices to protect personally identifying information
- Ensure shared data are standardized, interoperable, flexible, and practical
- Promote effective data collection
- Conduct annual reviews to identify gaps and potential improvements in data collection and sharing

To read the full text of this recommendation, see the Recommendations Insert.

DATA GOVERNANCE AND LEADERSHIP

In the United States, no current federal or state laws protect or mandate the sharing of sequence data from virus samples. Any such data sharing is done voluntarily and generally without concerns about possible regulatory barriers. In contrast, there are federal and state laws protecting clinical and epidemiological data, including the Health Insurance Portability and Accountability Act (HIPAA). Without funding to support the costs associated with gathering and preparing data to be shared with public health authorities and researchers, and without clear regulatory pathways and established infrastructure to support sharing, the sharing of virus sequencing data is suboptimal.

Given the national and interstate threat posed by a pandemic like COVID-19, data sharing needs to happen on a national scale through formalized agreements with federal authorities, rather than purely within state borders or with the federal government on an ad hoc basis. These data sharing and reporting processes should be clearly established and funded prior to any public health emergency. To standardize and encourage the practice of quality data sharing for public health practice during a pandemic, the committee recommended that HHS establish a national leadership structure to facilitate comprehensive data sharing. This structure should include science-driven leadership and governance for the use of SARS-CoV-2 genome sequences. Key components of this structure should also include

- leadership that has the authority and responsibility to identify and prioritize key issues related to data needs;
- a national strategy for using SARS-CoV-2 genome sequences that clearly articulates goals, priorities, and a path for achieving them; and
- a governing board with diverse, relevant expertise that has broad authority to oversee and advise the national strategy for SARS-CoV-2 genome sequencing and the delivery of actionable data for related investigations.

To read the full text of this recommendation, see the Recommendations Insert.

Committee on Data Needs to Monitor the Evolution of SARS-CoV-2

Diane Griffin (*Chair*)
Johns Hopkins Bloomberg School of Public Health

Ralph Baric
University of North Carolina at Chapel Hill

Kent Kester
Sanofi Pasteur

Deven McGraw
Ciitizen Corporation

Alexandra Phelan
Georgetown University Center for Global Health Science and Security

Saskia Popescu
HonorHealth
George Mason University
University of Arizona

Stuart Ray
Johns Hopkins University School of Medicine

David Relman
Stanford University
Palo Alto Health Care System

Julie Segre
National Institutes of Health National Human Genome Research Institute

Mark Smolinski
Ending Pandemics

Paul Turner
Yale University

Deborah Zarin
Multi-Regional Clinical Trials Center of Brigham and Women's Hospital and Harvard University

Study Sponsor

U.S. Department of Health and Human Services' Office of the Assistant Secretary for Preparedness and Response

Office of Science and Technology Policy

Study Staff

Lisa Brown
Study Director

Emma Fine
Associate Program Officer

Benjamin Kahn
Associate Program Officer

Steven Moss
Associate Program Officer

Andrew M. Pope
Senior Director, Board on Health Sciences Policy

Liaison to the Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats

Harvey Fineberg
Gordon and Betty Moore Foundation

Chair, Standing Committee on Emerging Infectious Diseases and 21st Century Health Threats

To read the full report, please visit
nationalacademies.org/SARS-CoV-2-Data-Infrastructure-Needs

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

The nation turns to the National Academies of Sciences, Engineering, and Medicine for independent, objective advice on issues that affect people's lives worldwide.
www.nationalacademies.org

Copyright 2020 by the National Academy of Sciences. All rights reserved.

RECOMMENDATIONS

JULY 2020 • GENOMIC EPIDEMIOLOGY DATA INFRASTRUCTURE NEEDS FOR SARS-COV-2 MODERNIZING PANDEMIC RESPONSE STRATEGIES

RECOMMENDATION 1

The U.S. Department of Health and Human Services should ensure the generation of representative, high-quality full genome sequences of SARS-CoV-2 across the United States, and in the future, from emerging epidemic or pandemic pathogens, in order that these data can be used to meet key needs for genomic surveillance

- Pathogen samples must be obtained from individuals who represent broad diversity of factors such as race and ethnicity, gender, age, geography, and other demographic features such as housing type, clinical manifestations and outcomes, and transmissibility.
- Capacity for genomic sequencing should be developed and supported at many geographically distributed sites performing testing, including public health laboratories, academic and medical centers.
- Representative SARS-CoV-2 clinical samples from across the United States should be collected and sequenced on an ongoing basis to provide baseline data and facilitate near real-time transmission tracking.

Genome sequences should be shared openly on publicly accessible databases, such as the National Center for Biotechnology Information linked to the Global Initiative on Sharing All Influenza Data.

RECOMMENDATION 2

The U.S. Department of Health and Human Services should develop and invest in a national data infrastructure system that constructively builds upon existing programmatic infrastructure with the ability to accurately, efficiently, and safely link genomic data, clinical data, epidemiological data, and other relevant data across multiple sources critical to a public health response such as the current SARS-CoV-2 outbreak. Such a system should:

- Allow for the linkage of genomic data, clinical data, epidemiological data, and other relevant data in a way that is not overly burdensome to laboratories that collect data regularly.
- Create and foster safe data sharing practices to ensure that individuals' personal identifying information remains unexposed when data are being used and shared across the system.
- Be grounded in the pursuit of standardization, interoperability, flexibility, and the practical linkage of data, including consideration of a potential national patient identifier.
- Consider not only the data required to create such a system, but also investment in mechanisms supporting the collection and analysis of such data, including promoting formal education in "data wrangling" at the intersection of data science and infectious disease epidemiology.
- Conduct regular annual reviews—including scenario-based simulations—to identify capacity gaps, promote process improvement (based on existing U.S. infrastructure to assess the annual risk of seasonal influenza, work could improve usability and coverage of health information exchanges, and other initiatives) and ensure inclusion of entities with supporting functions across scales—including private health care systems that provide data or state and local public health laboratories that collect data—in ongoing system development and evaluation.

RECOMMENDATION 3

The U.S. Department of Health and Human Services should establish an effective and sustainable science-driven leadership and governance structure for the use of SARS-CoV-2 genome sequences in addressing critical national public health and basic science issues, develop a national strategy, and ensure the funding needed for successful execution of the strategy.

- Leaders of this effort must have sufficient authorities and responsibilities to ensure that key issues are identified and prioritized, representative data are generated, and barriers to data sharing are diminished.
- A national strategy for SARS-CoV-2 genome sequences linked to clinical and epidemiological data should be developed that articulates goals, priorities, and a path for achieving them.
- A board with diverse relevant expertise should be established with broad authority to oversee and advise the national strategy for SARS-CoV-2 genome sequences linked to clinical and epidemiological data, and the delivery of actionable data for related investigations.

To read the full report, please visit
nationalacademies.org/SARS-CoV-2-Data-Infrastructure-Needs

The National Academies of
SCIENCES • ENGINEERING • MEDICINE

The nation turns to the National Academies of Sciences, Engineering, and Medicine for independent, objective advice on issues that affect people's lives worldwide.
www.nationalacademies.org

Copyright 2020 by the National Academy of Sciences. All rights reserved.

From: Peter Daszak[daszak@ecohealthalliance.org]
Sent: Mon 8/10/2020 12:04:01 PM (UTC-04:00)
To: EID Search[eidsearch@ecohealthalliance.org]
Cc: Robert Kessler[kessler@ecohealthalliance.org]
Subject: Press release for EID SEARCH, our NIAID-funded CREID contract
Attachment: EID SEARCH CREID Grant Kickoff Press Release FINAL.docx

We got our press release approved by NIAID. Please see the attached.

We're expecting the NIAID CREID website (www.niaidcreid.org) to go live on Wednesday 12th August, and we've been told that the news will be released to the press next week (beginning 17th Aug). NIAID will confirm this and we'll be back in touch. Please don't speak to reporters until we get that date from NIAID.

One other thing, please make sure (as per our Notice of Award) that NIAID and the grant number are referenced in any press release, publication, or presentation. It's really important that we people recognize NIAID are funding this sort of international collaboration – I want to make sure the US Govt continues to support our global efforts to prevent pandemics!!!

Thanks everyone and speak to you all soon

Cheers,

Peter

Peter Daszak
President

EcoHealth Alliance
520 Eighth Avenue, Suite 1200
New York, NY 10018-6507
USA

Tel.: +1-212-380-4474
Website: www.ecohealthalliance.org
Twitter: @PeterDaszak

EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation

Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Rapid recovery of a SARS-CoV-2 e infected X-linked agammaglobulinemia patient after infusion of COVID-19 convalescent plasma

Estefanía Mira, MD[a], Oscar A. Yarce, MD[b], Consuelo Ortega, MD, PhD[c], Silvia Fernández, PhD[c], Natalia M. Pascual, MD[a], Cristina Gómez, MD[a], Miguel A. Alvarez, MD[d], Ignacio J. Molina, MD, PhD[e], Rafael Lama, MD, PhD[a], and Manuel Santamaria, MD, PhD[b,c]

Clinical Implications

A patient with X-linked agammaglobulinemia was infected with severe acute respiratory syndrome coronavirus 2 and presented a 40-day long and stagnant clinical evolution refractory to treatment. The patient received a single dose of coronavirus disease 2019 convalescent plasma and experienced a rapid clinical improvement within 1 week of infusion.

The high infectivity of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the lack of preexisting immunity among the population, and the complexity of the coronavirus disease 2019 (COVID-19) clinical presentation are some of the underlying reasons of the ongoing worldwide health crisis. Antibodies play relevant roles in antiviral responses,[1] and hence, patients with compromised specific humoral immunity may be at increased risk to suffer from severe forms of the disease. In fact, 3 recent studies described the clinical evolution of 6 patients with common variable immunodeficiency (CVID), 3 patients with x-linked agammaglobulinemia (XLA) (OMIM# 300755; Bruton's disease), and 1 patient with autosomal agammaglobulinemia.[2-4] However, the mild clinical presentation in agammaglobulinemic patients, as opposed to the former, prompted speculations that anti–SARS-CoV-2 antibodies may be dispensable in clearing the disease.[4] This would highlight the relevance of T-cell responses and the significance of anti–SARS-CoV-2 T-cell exhaustion in the ultimate progression of the disease.[5] However, administration of hyperimmune plasma was beneficial in some immunocompetent patients with COVID-19.[6]

We report the case of a 39-year-old male patient with XLA, receiving monthly immunoglobulin replacement therapy, admitted to our hospital with dyspnea, dry cough, and fever of 2-week duration. Chest X-rays showed unilateral alveolar condensation, and quantitative PCR (qPCR) amplification of SARS-CoV-2 from a nasopharyngeal exudate sample confirmed the diagnosis of COVID-19. The patient received hydroxychloroquine (400 mg/12 h, the first day; 200 mg/12 h, 4 days, orally), azithromycin (500 mg/24 h, 5 days, orally), and ceftriaxone (2 g/24 h, 7 days, intravenously [IV]) and a single dose (0.3 g/kg) of IV immunoglobulins. No supplemental oxygen was required.

In spite of a favorable initial clinical evolution, fever persisted and intense asthenia appeared. Therefore, empirical therapy against nosocomial germs was started with piperacillin/tazobactam (4 g/0.5 g/8 h, IV) and linezolid (600 mg/12 h, IV). SARS-CoV-2 qPCR test result remained positive on day 11 after admission. On day 15 computed tomography showed extensive bilateral pulmonary involvement in the form of nodular consolidations in ground glass with underlying interstitial pattern (Figure 1, upper panel). Blood, sputum, stool, and urine cultures were negative. Infections by other respiratory viruses (adenovirus, metapneumovirus, inovirus, and respiratory syncytial virus), as well as the presence of opportunistic germs such as Pneumocystis jiroveci, cytomegalovirus, EBV, Mycoplasma pneumoniae, aspergillus, leishmania, and brucella, were all excluded. A third SARS-CoV-2 qPCR was carried out on day 21 after admission and the result remained positive.

Stagnancy in the patient's evolution, as represented by the lack of response to any of the treatments dispensed (including a second pulse of nonspecific immunoglobulins at 0.8 g/kg/24 h and aggressive antibiotic therapy), together with persistent fever and bilateral pneumonia, prompted us to consider the possibility that natural killer (NK)-cell and T-cell exhaustion might be an underlying cause for immunologic failure, as shown elsewhere.[5] We assessed this possibility in lung lymphoid populations obtained by bronchoalveolar lavage on day 21 after admission, and they consisted of $CD3^b$ $CD8^b$ (86%) and $CD3^b$ $CD4^b$ T lymphocytes (11.5%) and $CD56^b$ $CD16^b$ NK cells (2.5%). Staining of intracellular cytokines in stimulated $CD8^b$ cells showed negligible production of TNF-α or IFN-γ (Figure 2, A, middle and bottom panels).

Because of the resulting lymphocyte phenotype, we reasoned that, in the absence of functional B cells, viral clearance would not be possible. Hence, we administered on day 23 after admission compassionate-use approved COVID-19 convalescent donor plasma (200 mL, single dose, IV). The plasma was analyzed by an ELISA (COVID-19 ELISA IgG, Vircell SL, Granada, Spain), which detected IgG antibodies against either the spike or nucleocapsid viral proteins with a titer greater than or equal to 1/320. After 24 hours of infusion, fever ceased without subsequent reappearance and with progressive improvement of asthenia. After 48 hours of infusion, qPCR from nasopharyngeal exudate failed to amplify SARS-CoV-2 sequences.

On day 30 after admission, that is, 1 week after administration of COVID-19 convalescent donor plasma, the patient remained asymptomatic and a chest tomography showed almost complete resolution of bilateral lung lesions (Figure 1, lower panel). Furthermore, a vigorous recovery in the peripheral T-lymphocyte subpopulations ($CD3^b$, $CD4^b$, and $CD8^b$) was observed, whereas NK-cell counts slightly increased (Figure 2, B). Hence, the patient was subsequently discharged and remains COVID-19 free.

Although cumulative cases of SARS-CoV-2–infected patients suffering from primary antibody immunodeficiencies are limited, it appears that individuals devoid of B cells (agammaglobulinemia) have a milder presentation and course than those with a partial B-cell repertoire (CVID).[2-4] This observation is

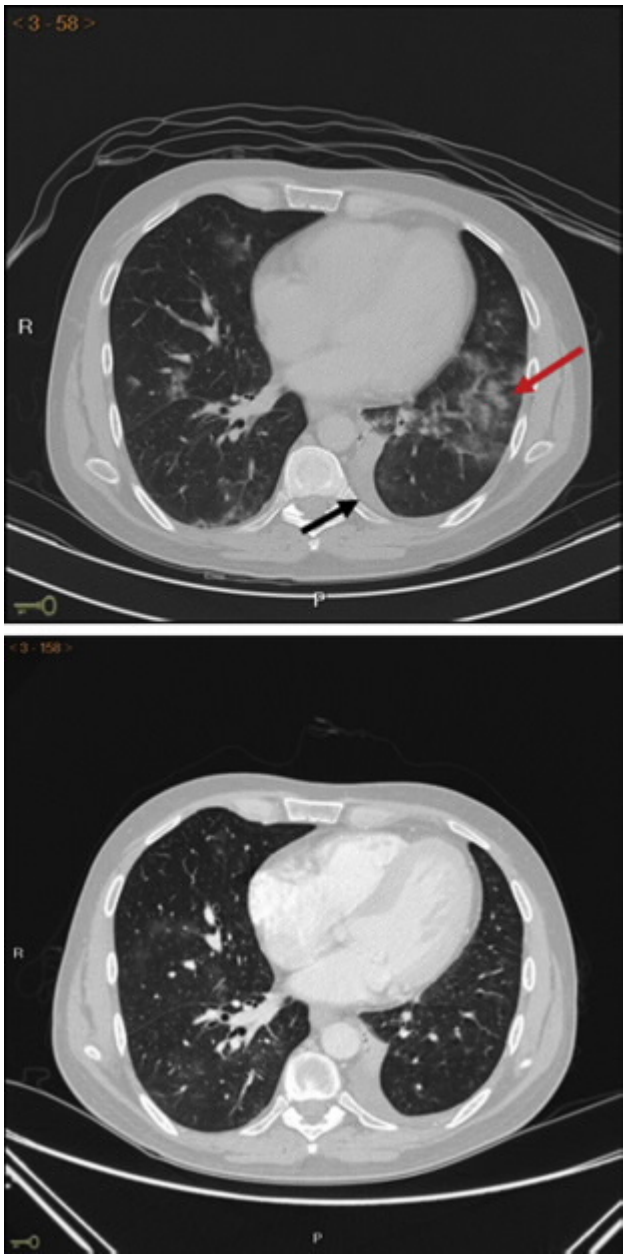


FIGURE 1. Upper panel: CT displaying bilateral pulmonary involvement in the form of nodular consolidations and ground-glass opacities primarily in the left lower lobe (red arrow) and small-size left pleural effusion (black arrow). Lower panel: CT of the chest 6 days after COVID-19 convalescent donor plasma transfusion showing almost complete resolution of bilateral lung lesions pattern. CT, Computed tomography.

strengthened by the finding that pharmacological blockade of Bruton tyrosine kinase results in diminished lung inflammatory response by inhibiting overproduction of macrophage-mediated cytokines.[7] The evolution of our patient was consistent with those observations until day 15 of admission, when he suffered a rapid deterioration in his condition accompanied by a lack of cytokine production by lung T and NK cells. If this lack of functional response was the consequence of cell exhaustion, we should bear in mind that T-cell response involve triggering of multiple epitope-specific clones, and not all may be equally prone to functional blockade.[8] Therefore, local silencing of immunodominant clones may hamper the overall immune response.

The remarkable and prompt recovery observed after infusion of convalescent plasma suggests that certain patients may have stringent dependency on specific antibodies to achieve viral clearance. Although this is a single case and therefore the observation could be coincidental and we have not formally established a cause-effect relationship, we nevertheless propose that administration of COVID-19 hyperimmune plasma should be further explored as first line of treatment for patients with congenital agammaglobulinemia, especially while SARS-CoV-2–specific antiviral drugs remain unavailable. Previous clinical cases suggest that B cells may have a prominent role in triggering lung inflammation[3]; in this scenario, defective germinal center formation due to the primary immunodeficiency would divert IL-6 intended for that process toward lung inflammation, thus raising the possibility of a B-cell interference as therapeutic intervention.[3] However, our data suggest a critical role of B cells in clearing the disease when certain immunologic conditions are present.

In summary, even though administration of passive immunity for patients with primary immunodeficiency has just been proposed,[9] this is the first report showing a rapid recovery of a patient with a primary antibody immunodeficiency after administration of hyperimmune plasma. Although it should be kept in mind whether the patient reported here was idiosyncratic and might not represent a prototypical patient with XLA (despite carrying a common Bruton tyrosine kinase gene mutation), our data merit further investigations to determine the precise clinical benefit of hyperimmune plasma in other antibody-deficient patients, as well as optimal dose and timing of its administration.

Acknowledgments

We thank Professor J.L. Martinez for invaluable help with microbiology studies. We gratefully acknowledge Red Andaluza de Medicina Transfusional, Tejidos y Células, Servicio Andaluz de Salud for supplying and analyzing the COVID-19 convalescent plasma.

[a]Service of Pneumology, Reina Sofía University Hospital, Córdoba, Spain
[b]Service of Immunology and Allergy, Reina Sofía University Hospital, Córdoba, Spain
[c]Unit of Immunology, Córdoba Medical School, University of Córdoba, Córdoba, Spain
[d]Service of Haematology Reina Sofía University Hospital, Córdoba, Spain
[e]Biopathology and Regenerative Medicine Institute, Biomedical Research Center, Health Sciences Technology Park, University of Granada, Granada, Spain
This work was supported by Consejería de Universidades, Junta de Andalucía (grant no. CT208 to M.S.) and Action for A-T (Haslemere, UK) (grant no. AAT-8GRA02 to I.J.M.).
Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.
Received for publication May 22, 2020; revised manuscript received and accepted for publication June 25, 2020.
Available online July 8, 2020.
Corresponding author: Manuel Santamaria, MD, PhD, Unit of Immunology, Cordoba Medical School, University of Córdoba, Avda Menéndez Pidal S/N, Cordoba 14004, Spain. E-mail: msantamaria@uco.es
2213-2198
ff 2020 American Academy of Allergy, Asthma & Immunology
https://doi.org/10.1016/j.jaip.2020.06.046

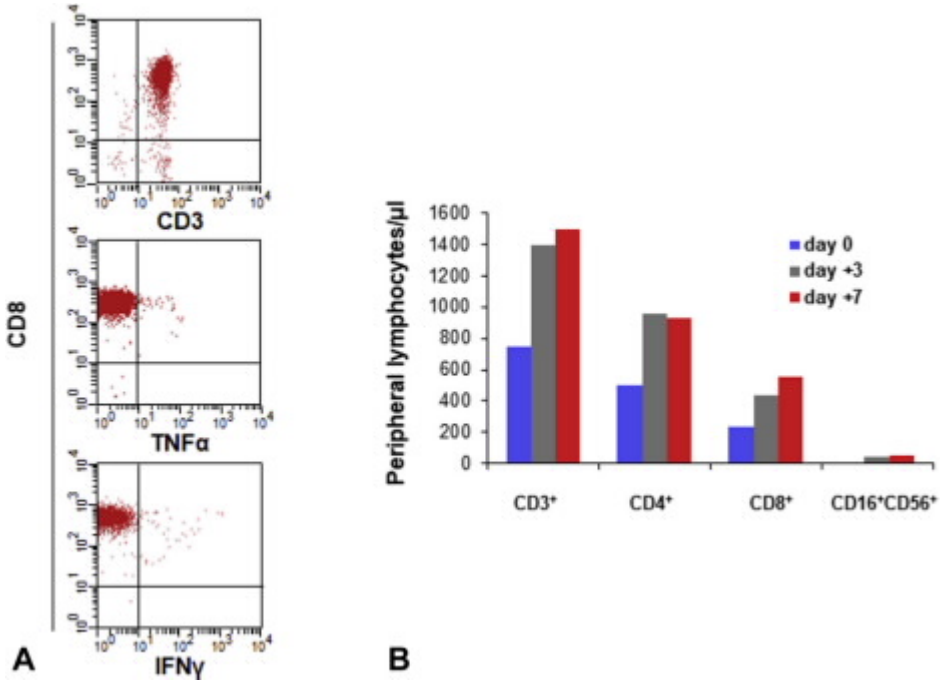


FIGURE 2. (A) Flow cytometric double fluorescence analysis of CD8 cells showing coexpression of surface CD3 (upper panel) and intracytoplasmic TNF-a (middle panel) or IFN-g (lower panel) after cell stimulation. (B) Blood peripheral T lymphocytes and NK-cell counts determined on days 0, 3, and 7 postinfusion of COVID-19 convalescent plasma.

REFERENCES

1. Burton DR. Antibodies, viruses and vaccines. Nat Rev Immunol 2002;2:706-13
2. Fill L, Hadney L, Graver K, Persaud R, Hostoffer R. The clinical observation of a CVID patient infected with COVID-19. Ann Allergy Asthma Immunol 2020;125:112-4
3. Quinti I, Lougaris V, Milito C, Cinetto F, Pecoraro A, Mezzaroma I, et al. A possible role for B cells in COVID-19? Lesson from patients with agammaglobulinemia. J Allergy Clin Immunol 2020;146:211-213.e4
4. Soresina A, Moratto D, Chiarini M, Paolillo C, Baresi G, Focà E, et al. Two X-linked agammaglobulinemia patients develop pneumonia as COVID-19 manifestation but recover. Pediatr Allergy Immunol 2020;31:565-9
5. Zheng M, Gao Y, Wang G, Song G, Liu S, Sun D, et al. Functional exhaustion of antiviral lymphocytes in COVID-19 patients. Cell Mol Immunol 2020;17:533-5
6. Zhang B, Liu S, Tan T, Huang W, Dong Y, Chen L, et al. Treatment with convalescent plasma for critically ill patients with severe acute respiratory syndrome coronavirus 2 infection. Chest 2020;158:e9-13
7. Roschewski M, Lionakis MS, Sharman JP, Roswarski J, Goy A, Monticelli MA, et al. Inhibition of Bruton tyrosine kinase in patients with severe COVID-19. Sci Immunol 2020;5:eabd0110
8. Kahan SM, Wherry EJ, Zajac AJ. T cell exhaustion during persistent viral infections. Virology 2015;479-80:180-93
9. Hammarstrom L, Abolhassani H, Baldanti F, Marcotte H, Pan-Hammarstrom Q. Development of passive immunity against SARS-CoV-2 for management of immunodeficient patients—a perspective. J Allergy Clin Immunol 2020;146:58-60

Received:15 July 2020 | Revised:23 August 2020 | Accepted: 24 August 2020

DOI: 10.1002/pbc.28693

Pediatric Blood & Cancer

aspho The American Society of Pediatric Hematology/Oncology

WILEY

PSYCHOSOCIALAND SUPPORTIVECARE: BRIEFREPORT

Convalescentplasmafor pediatricpatientswith SARS-CoV-2-associatedacuterespiratorydistresssyndrome

CarolineDiorio[1,2] | ElizabethM. Anderson[3] | KevinO. McNerney[1,2] | EileenC.Goodwin[3] | JulieC.Chase[1,4] | MarcusJ.Bolton[3] | ClaudiaP.Arevalo[3] | MadisonE.Weirick[3] | SigridGouma[3] | LauraA. Vella[1,5] | SarahE.Henrickson[1,6] | KathleenChiotos[7] | JulieC.Fitzgerald[1,7] | ToddJ.Kilbaugh[7] | AudreyR.OdomJohn[5] | AllisonM. Blatz[5] | Michele P.Lambert[1,8] | KathleenE.Sullivan[1,6] | Margaret R.Tartaglione[2] | DanielleZambrano[2] | MeghanMartin[2] | JessicaH. Lee[1] | PampeeYoung[9] | DavidFriedman[8,10] | DeborahA.Sesok-Pizzini[10] | ScottE.Hensley[3] | EdwardM. Behrens[1,4] | HamidBassiri[1,5] | David T.Teachey[1,2]

[1] Immune Dysregulation Frontier Program,Department of Pediatrics,Children's Hospital of Philadelphia,University of PennsylvaniaPerelman School of Medicine, Philadelphia,Pennsylvania

[2] Division of Oncology,Department of Pediatrics,Children's Hospital of Philadelphia,University of PennsylvaniaPerelman Schoolof Medicine, Philadelphia, Pennsylvania

[3] Department of Microbiology, PerelmanSchoolof Medicine, University of Pennsylvania,Philadelphia,Pennsylvania

[4] Division of Rheumatology,Department of Pediatrics,Children's Hospital of Philadelphia,University of PennsylvaniaPerelman Schoolof Medicine, Philadelphia, Pennsylvania

[5] Division of Infectious Diseases,Department of Pediatrics,Children's Hospital of Philadelphia,University of PennsylvaniaPerelman Schoolof Medicine,Philadelphia, Pennsylvania

[6] Division of Allergy and Immunology, Department of Pediatrics,Children's Hospital of Philadelphia,University of PennsylvaniaPerelman Schoolof Medicine, Philadelphia,Pennsylvania

[7] Division of Critical Care Medicine, Department of Anesthesiology and Critical Care Medicine, Children's Hospital of Philadelphia,University of Pennsylvania Perelman Schoolof Medicine, Philadelphia,Pennsylvania

[8] Division of Hematology, Children's Hospital of Philadelphia,University of PennsylvaniaPerelman Schoolof Medicine, Philadelphia,Pennsylvania

[9] American Red Cross,Washington, District of Columbia

[10] Pathology and Laboratory Medicine, University of PennsylvaniaPerelman Schoolof Medicine, Philadelphia,Pennsylvania

Correspondence
David T.Teachey,Department of Pediatrics, Division of Oncology,The Children's Hospital of Philadelphia,University of Pennsylvania PerelmanSchoolof Medicine,3008 CTRB,3501 Civic Center Blvd,Philadelphia, PA 19104.
Email:teacheyd@email.chop.edu

Abstract

There are no proven safe and effective therapies for children who develop life-threatening complications of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)Convalescentplasma(CP)hasdemonstrated potential benefit in adults with SARS-CoV-2,but has theoretical risks.We present the first report of CP in children with life-threatening coronavirus disease2019 (COVID-19), providing data on

Abbreviations:ADE,antibody-dependent enhancement;CHOP,Children's Hospital of Philadelphia; COVID-19, coronavirus disease2019; CP,convalescentplasma;ELISA,enzyme-linked immunosorbent assay;FDA,Food and Drug Administration; MIS-C,multisystem inflammatory syndrome in children; N protein, nucleocapsidprotein; RBD, receptor-binding domain; Sprotein, spike protein; SARS-CoV-2,severe acute respiratory syndrome coronavirus 2

This is an open accessarticle under the terms of the Creative Commons Attribution-NonCommercial License,which permits use,distribution and reproduction in any medium,provided the original work is properly cited and is not used for commercial purposes.
© 2020 Wiley PeriodicalsLLC

Caroline Diorio, Elizabeth M. Anderson, Edward M. Behrens, Hamid Bassiri, and David T. Teachey contributed equally to this work.

Funding information
Leukemia and Lymphoma Society; University of Pennsylvania; Kate Amato Foundation; American Academy of Allergy Asthma and Immunology; Cookies for Kids Cancer; Children's Oncology Group; Stand Up To Cancer; Alex's Lemonade Stand Foundation for Childhood Cancer; National Institutes of Health, Grant/Award Numbers: K08AI135091, K08AI136660, K12HS026393, R01A1123538, R01AI103280, R01AI121250, R01AI123433, R01CA193776, R21AI144472, T32AI007324, UG1CA233249, X01HD100702; TeamConnor; Clinical Immunology Society; Children's Hospital of Philadelphia, Grant/Award Number: Immune Dysregulation Frontier Program; Burroughs Wellcome Fund

four pediatric patients with acute respiratory distress syndrome. We measured donor antibody levels and recipient antibody response prior to and following CP infusion. Infusion of CP was not associated with antibody-dependent enhancement (ADE) and did not suppress endogenous antibody response. We found CP was safe and possibly efficacious. Randomized pediatric trials are needed.

KEYWORDS
convalescent plasma, COVID-19, pediatrics, SARS-CoV-2

1 | INTRODUCTION

Early reports from the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) global pandemic suggested that children were less severely affected by coronavirus disease 2019 (COVID-19) compared to older adults.[1–3] Nevertheless, some children with COVID-19 are critically ill.[4,5] Currently, no therapies have been proven effective in the treatment of COVID-19 in children.

Convalescent plasma (CP) derived from patients that have recovered from SARS-CoV-2 can be infused into currently ill patients. The proposed mechanism of action is via neutralizing antibodies binding to virus, rendering it inert.[6] Specific to SARS-CoV-2, antibodies against the receptor-binding domain (RBD) have been identified as surrogates for neutralization.[7–11] CP also contains antibodies against the immunogenic nucleocapsid (N) protein, which are nonneutralizing and present in both actively infected and recovered patients.[7] The protective function of N antibodies remains unclear.[7,12] Antibodies against the full-length SARS-CoV-2 spike (S) protein include both nonneutralizing antibodies and neutralizing antibodies.[13]

Initial series of CP in adults with COVID-19 demonstrated potential benefits without apparent side effects.[14–17] In a recent clinical trial of adults with COVID-19, there was a nonstatistically significant trend toward clinical improvement in CP-treated patients versus controls, without improvement in time to discharge or mortality.[18] This trial was halted early due to poor accrual.[18] To date, there have been no reports on the use of CP in children.

CP infusion is associated with the side effects of any blood product: allergic reaction, transfusion-associated circulatory overload, and infection with blood-borne pathogens. There is a theoretical risk that infusion of donor antibodies may impede the recipient's endogenous production of antibodies, and of antibody-dependent enhancement (ADE), where antibodies developed during a previous infection cause a worsened clinical response with subsequent infection. ADE has been described in dengue fever, and in preclinical models of other coronaviruses.[19–21] ADE has not been reported in adult patients receiving CP for SARS-CoV-2.[6,17,18]

We present the first report of pediatric patients receiving CP for life-threatening COVID-19-associated respiratory disease with correlative measurements of the associated pre- and posttransfusion antibody response.

2 | METHODS

We treated four critically ill children actively infected with SARS-CoV-2 with CP at the Children's Hospital of Philadelphia (CHOP) under emergency Investigational New Drug applications (eINDs) through the Food and Drug Administration (FDA). Patients were considered for CP if they met FDA guidance for life-threatening disease (Appendix S1). Per the American Red Cross Guidelines, donors were eligible to provide CP if they met the following criteria: (a) they were initially proven positive for SARS-CoV-2 by a laboratory test; and either (b1) at least 14 days from symptom resolution with a repeat documented negative test for SARS-CoV-2, or (b2) at least 28 days from symptom resolution without a documented repeat test results at the time of plasma collection.

We prospectively screened and enrolled these patients into a larger SARS-CoV-2 biobanking protocol. The objective of the SARS-CoV-2 biorepository protocol is to collect samples on patients with confirmed SARS-CoV-2 infection or multisystem inflammatory syndrome in children (MIS-C). An initial report from this biorepository has been published.[22] Patients were enrolled on the biobank protocol if they had evidence of SARS-CoV-2 infection on reverse-transcriptase polymerase chain reaction (RT-PCR) testing from respiratory tract mucosa. This protocol was approved by the CHOP Institutional Review Board (IRB). Consent was obtained for both protocols from a legal authorized representative per the Declaration of Helsinki (written informed consent for CP; verbal consent for biobanking).

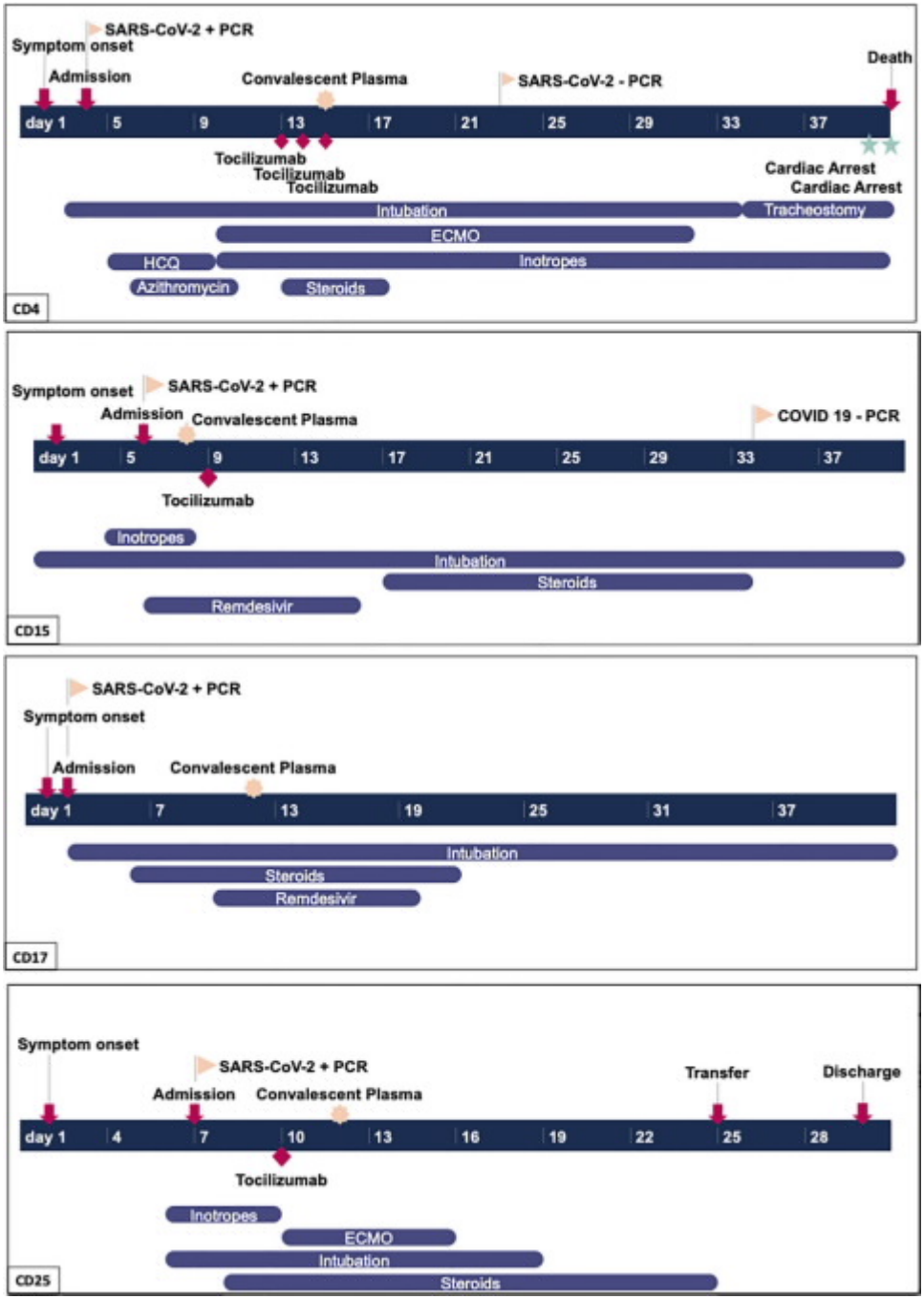


FIGURE 1 Graphical representation of clinical course of four patients included in the caseseries. Timeline includes up to day 40 of illness, or until death or discharge, whatever occurred first. ECMO, extracorporeal membrane oxygenation; HCQ, hydroxychloroquine; PCR, polymerase chain reaction

Verbal consent for biobanking was obtained based on IRB recommendations to prevent exposure to research staff and to avoid unintentional transmission of SARS-CoV-2via fomites such as consent forms.

Clinical data were abstracted from patient charts to standardized forms by a research assistant using a REDCap database, and verified by a physician. Blood sampleswere obtained from participants prior to CP infusion, following CP infusion, and weekly thereafter until death or discharge. Remnant donor CP was retained, per institutional protocol in case of transfusion reaction. Remnant donor CP was then collected and stored.

SARS-CoV-2-specificantibodies were measured by enzyme-linked immunosorbent assay (ELISA) as previously described.[8] Antibodies in serially diluted plasma (starting at 1:50) were measured against RBD of the SARS-CoV-2S protein,[9] full-length S protein, and the N protein (Sino Biological, Wayne, PA). Reciprocal plasma dilutions were calculated from an optical density threshold from a set concentration. Standard curves were generated by diluting an RBD-specific monoclonal

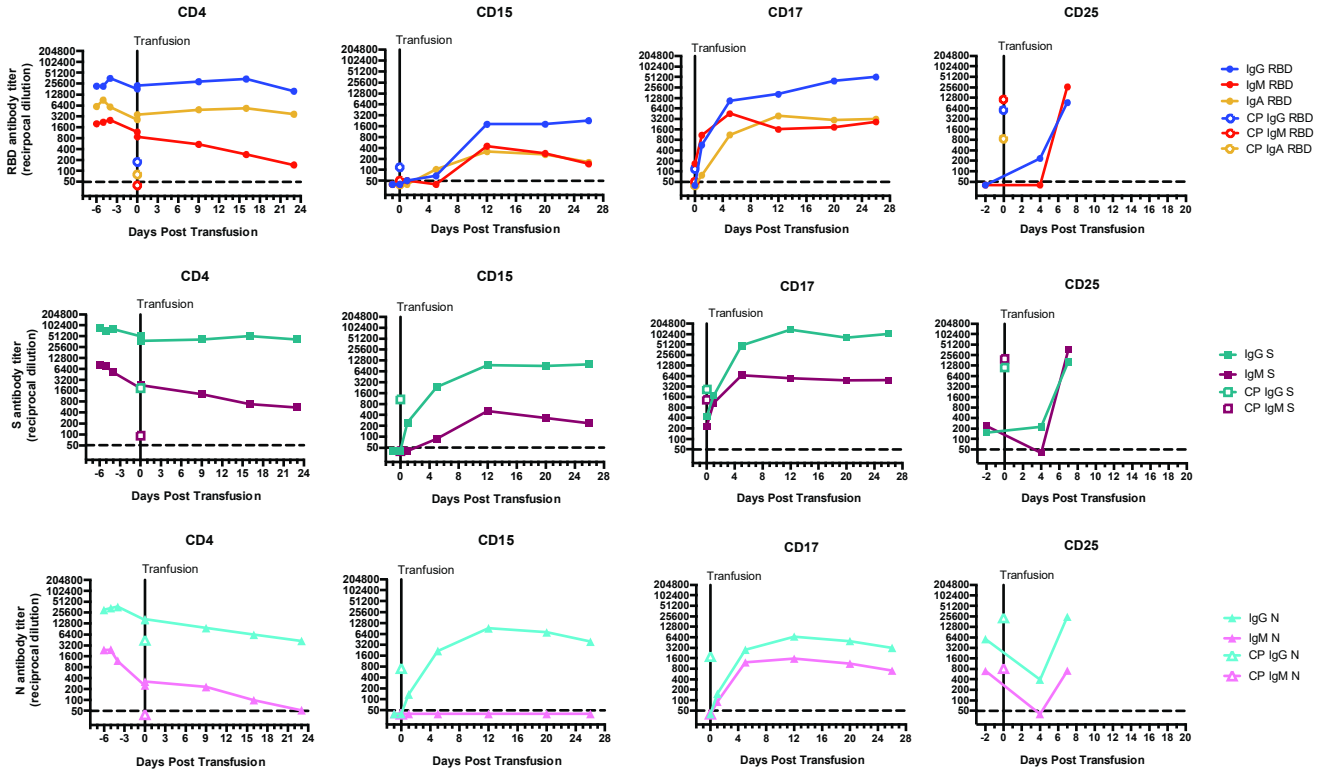


FIGURE 2 Longitudinal serum SARS-CoV-2 antibody titers in pediatric patients receiving convalescent plasma (CP) transfusion therapy. Antibody titers expressed as reciprocal serum dilution against SARS-CoV-2 antigens in four pediatric patients (CD4, CD15, CD17, and CD25), and corresponding CP donors (open symbols) before and after plasma transfusion. Antibody titers against (A-D) the SARS-CoV-2 receptor-binding domain of the spike (S) protein, (E-H) the full-length SARS-CoV-2 S protein (FL), and (I-L) the nucleoprotein of SARS-CoV-2 (N). Dashed lines denote the lower limit of detection (LLOD) of the assay (1:50 serum dilution)

antibody (CR3022) starting at 0.5 μg/mL (RBD and S ELISAs), or serially diluted pooled serum from actively SARS-CoV-2-infected adults (N ELISAs). See Appendix S2 for detailed methods.

3 | RESULTS AND DISCUSSION

Four patients, 14–18 years, were treated with CP during the study period. Patients received all available plasma from the donors, in the range of 200–220 mL. Patient CD4 received 2 mL/kg, and patients CD15, CD17, and CD25 received 4 mL/kg. All patients were critically ill with COVID-19-associated acute respiratory distress syndrome. All patients required intubation and ventilation; two required extracorporeal membrane oxygenation (ECMO). None of the patients had MIS-C. All patients received other SARS-CoV-2-directed therapies in addition to CP. Graphical case summaries are presented in Figure 1. Further details are presented in Appendix S3. No patients experienced any treatment-emergent adverse events (TEAE) related to CP infusion.

Longitudinal antibody titers are presented in Figure 2. Corresponding antibody titers for CP donors are displayed. At the time of CP infusion, patient CD4 had extremely high endogenous antibody titers (against SARS-CoV-2 RBD, S, and N). Antibody titers in this participant were sustained post transfusion. This individual showed transient clinical improvement, decannulating from ECMO; however, died from cardiac causes by day 25 post transfusion. An autopsy was performed, which demonstrated diffuse alveolar damage and hypertrophic cardiomyopathy with associated myocardial fibrosis. Spleen and lung cultures were positive for *Candida glabrata*. There was no evidence of myocarditis.

Patients CD15, CD17, and CD25 had low or undetectable RBD-specific antibody titers at the time of infusion. Serum antibodies against the SARS-CoV-2 RBD correlate with neutralizing activity, likely due to inhibition of the virus' ability to bind and enter target cells.[8–10] Of note, the donor plasma infused into patients CD15 and CD17 had relatively low RBD-specific antibody titer levels (< 1:160). Both CP donors for CD15 and CD17 had substantial full-length IgG S titers (> 1:1000), and these may have conferred some neutralizing activity. It is unclear if CP with low RBD-specific and high full-length IgG S antibody titers would provide benefit. CD15 and CD17 both remain in intensive care and did not have significant clinical improvement following CP infusion, although CD15 did wean off inotropic support shortly after receiving CP. CD17 did not require inotropic support. Both patients continued to require intubation and ventilatory support despite CP. As of August 22, 2020 both CD15 and CD17 remain in hospital and have had the placement of tracheostomies. They both remain on ventilatory support, although at a lower level than required during

acute illness. The donor for patient CD25 had higher SARS-CoV-2 RBD antibody titers (> 1:6000) than the donor for any of the other patients, making it more likely that this donor plasma could be beneficial. CD25 had the shortest period of admission and has been discharged from the hospital after being critically ill and on ECMO.

Prior to transfusion, three of the four recipients (CD15, CD17, and CD25) had undetectable IgG RBD antibody levels (< 1:50 serum dilution). Upon transfusion, all three participants eventually experienced a boost in IgG, IgM, and IgA antibodies against RBD that was sustained for 7-26 days post transfusion. In one participant (CD15), we detected antibodies against the full-length S protein before we detected antibodies against the RBD of the S protein. The boost in antibodies against N post transfusion was similar to that of RBD and S-specific serum antibodies for patients CD15, CD17, and CD25. In all three patients, the serum levels of SARS-CoV-2-specific antibodies eventually surpassed the amount transfused, indicating that these patients mounted a post transfusion de novo response and that CP did not eliminate the endogenous serological response.

In summary, CP was safely administered to four pediatric patients with severe COVID-19. There were no CP-related TEAE. The theoretical risks of ADE or elimination of endogenous antibody response did not occur. CP may be of greatest benefit for patients who are early in their illness and have not yet generated endogenous antibodies, and when the infused plasma has a high antibody titer. The patient who received CP with the highest antibody titer had the best clinical response, although the total antibody response mounted did not differ markedly from the other patients. The small sample size of our study precludes definitive conclusions about efficacy, however, the excellent clinical response in this patient is encouraging. The other patients had significant comorbidities that may have contributed to their clinical status. It is difficult to determine if CD15 and CD17 benefited, as it is possible without CP they could have continued to worsen. Future research should include randomized controlled trials to definitively examine the efficacy of CP in children.

ACKNOWLEDGMENTS

Financial support for this project was provided by CHOP Frontiers Program Immune Dysregulation Team (David T. Teachey, Edward M. Behrens, Hamid Bassiri), National Institute of Allergy and Infectious Diseases (NIAID) (US): R01AI121250 (Edward M. Behrens), R01AI103280 (Audrey R. Odom John), R01AI123433 (Audrey R. Odom John), R21AI144472 (Audrey R. Odom John), K08 AI136660 (Laura A. Vella), and K08AI135091 (Sarah Henrickson); Agency for Healthcare Research and Quality (AHRQ): 5K12HS026393 (Kathleen Chiotos); National Cancer Institute (NCI) (US): R01CA193776 (David T. Teachey), X01HD100702-01 (David T. Teachey), 5UG1CA233249 (David T. Teachey), and R01A1123538 (David T. Teachey); NIH Training in Virology: T32-AI-007324 (Elizabeth M. Anderson); Leukemia and Lymphoma Society (David T. Teachey); Cookies for Kids Cancer (David T. Teachey); Alex's Lemonade Stand Foundation for Childhood Cancer (David T. Teachey); Children's Oncology Group (David T. Teachey); Stand UP 2 Cancer (David T. Teachey); Team Connor (Hamid Bassiri); and Kate Amato Foundations (Hamid Bassiri). Burroughs Wellcome Fund CAMS (SH) and PATH (Audrey R. Odom John); Clinical Immunology Society (SH); and American Academy of Allergy, Asthma, and Immunology (SH). Serological studies were supported by institutional funds from the University of Pennsylvania. Caroline Diorio was supported by the Institute for Translational Medicine and Therapeutics of the Perelman School of Medicine at the University of Pennsylvania. We gratefully acknowledge the gift of plasmids of the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein from Florian Krammer, Mt. Sinai, and the gift of the RBD-specific monoclonal antibody CR3022 from Ian Wilson, Scripps Research Institute. We gratefully acknowledge the providers who cared for these patients, and the generosity of the patients and their families.

CONFLICT OF INTEREST

The authors declare that there is no relevant conflict of interest. David T. Teachey serves on advisory boards for Janssen, Amgen, La Roche, Sobi, and Humanigen. Hamid Bassiri has stock ownership in CSL Behring. Sarah Henrickson serves on the advisory board for Horizon Pharma. Audrey R. Odom John serves on the advisory board of Pluton Biosciences. Michele P. Lambert is an advisory board member for Octapharma, Novartis, Aregenix, and Shionogi, a consultant for Amgen, Novartis, Shionogi, Dova, Bayer, and has received research funding from Sysmex, Novartis, and Astra Zeneca. Kathleen E. Sullivan received personal fees from Elsevier and Immune Deficiency Foundation. Sarah Henrickson has received consultancy fee from Sanofi Pasteur, Lumen, Novavax, and Merck for work unrelated to this report.

AUTHOR CONTRIBUTIONS

Caroline Diorio and Elizabeth M. Anderson equally contributed to this work and are senior first authors. Edward M. Behrens, David T. Teachey, and Hamid Bassiri equally contributed to this work and are co-senior authors. Caroline Diorio, Elizabeth M. Anderson, Sarah E. Henrickson, Laura A. Vella, Kevin O. McNerney, Julie C. Chase, Scott E. Hensley, Edward M. Behrens, Hamid Bassiri, and David T. Teachey designed the study, analyzed data, and wrote the manuscript; Kathleen Chiotos, Michele P. Lambert, Allison M. Blatz, Julie C. Fitzgerald, Todd J. Kilbaugh, Audrey R. Odom John, Kathleen E. Sullivan, David Friedman, Deborah A. Sesok-Pizzini, Margaret R. Tartaglione, Danielle Zambrano, Meghan Martin, Jessica H. Lee, and Pampee Young contributed to the study design, analyzed data, and helped write the manuscript. Elizabeth M. Anderson, Madison E. Weirick, Sigrid Gouma, Eileen C. Goodwin, Marcus J. Bolton, Claudia P. Arevalo performed experiments, provided data, and edited manuscript. Caroline Diorio, Julie C. Chase, and Kevin O. McNerney enrolled subjects. Pampee Young, Deborah A. Sesok-Pizzini, and David Friedman also provided specimens. All authors contributed intellectually and reviewed and revised the manuscript.

FUNDING INFORMATION

Children's Hospital of Philadelphia Frontiers Program Immune Dysregulation Team, National Institute of Allergy and Infectious Diseases (NIAID) (US): R01AI121250, R01AI103280, R01AI123433, R21AI144472, K08 AI136660, and K08AI135091; Agency for Healthcare Research and Quality (AHRQ): 5K12HS026393; National

Cancer Institute (NCI) (US): R01CA193776, X01HD100702-01, 5UG1CA233249, and R01A1123538; NIH Training in Virology: T32-AI-007324; Leukemia and Lymphoma Society; Cookies for Kids Cancer; Alex's Lemonade Stand Foundation for Childhood Cancer; Children's Oncology Group; Stand UP 2 Cancer; Team Connor; and Kate Amato Foundations. Burroughs Wellcome Fund CAMS and PATH; Clinical Immunology Society; and American Academy of Allergy, Asthma, and Immunology; Institutional funds from the University of Pennsylvania; Institute for Translational Medicine and Therapeutics of the Perelman School of Medicine at the University of Pennsylvania

ORCID

Elizabeth M. Anderson https://orcid.org/0000-0002-1168-4773
Kevin O. McNerney https://orcid.org/0000-0002-4359-3569
Allison M. Blatz https://orcid.org/0000-0003-2525-1212
Michele P. Lambert https://orcid.org/0000-0003-0439-402X
David T. Teachey https://orcid.org/0000-0001-7373-8987

REFERENCES

1. Dong Y, Mo X, Hu Y, et al. Epidemiological characteristics of 2143 pediatric patients with 2019 coronavirus diseasein China. *Pediatrics* 2020;146:e20200702.
2. Liu W, Zhang Q, Chen J, et al. Detection of Covid-19 in children in early January 2020 in Wuhan, China. *N Engl J Med*. 2020;382(14):1370-1371.
3. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020;395(10223):497-506.
4. Zachariah P, Johnson CL, Halabi KC, et al. Epidemiology, clinical features, and diseaseseverity in patients with coronavirus disease2019 (COVID-19) in a children's hospital in New York City, New York. *JAMA Pediatr.* 2020:e202430. https://doi.org/10.1001/jamapediatrics.2020.2430
5. Chao JY, Derespina KR, Herold BC, et al. Clinical characteristics and outcomes of hospitalized and critically ill children and adolescents with coronavirus disease2019 (COVID-19) at a tertiary care medical center in New York City. *J Pediatr.* 2020;223:14-19.e2.
6. Bloch EM, Shoham S, Casadevall A, et al. Deployment of convalescent plasma for the prevention and treatment of COVID-19. *J Clin Invest* 2020;130(6):2757-2765.
7. Hachim A, Kavian N, Cohen CA, et al. Beyond the spike: identification of viral targets of the antibody response to SARS-CoV-2in COVID-19 patients. *medRxiv* 2020. https://doi.org/10.1101/2020.04.30.20085670
8. Flannery D, Gouma S, Dhudasia M, et al. SARS-CoV-2 seroprevalence among parturient women. 2020. https://www.researchsquare.com/article/rs-27402/v1 . Accessed August 23, 2020.
9. Amanat F, Stadlbauer D, Strohmeier S, et al. A serological assay to detect SARS-CoV-2 seroconversion in humans. *Nat Med.* 2020;26:1033-1036.
10. Okba NM, Müller MA, Li W, et al. Severe acute respiratory syndrome coronavirus 2-specific antibody responsesin coronavirus diseasepatients. *Emerg Infect Dis.* 2020;26(7):1478-1488.
11. Premkumar L, Segovia-Chumbez B, Jadi R, et al. The receptor-binding domain of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients. *Sci Immunol* 2020;5(48):eabc8413.
12. Leung DT, Tam FC, Ma CH, et al. Antibody response of patients with severe acute respiratory syndrome (SARS) targets the viral nucleocapsid. *J Infect Dis.* 2004;190(2):379-386.
13. Lv H, Wu NC, Tsang OT, et al. Cross-reactive antibody response between SARS-CoV-2 and SARS-CoV infections. *Cell Rep* 2020;31(9):107725.
14. Shen C, Wang Z, Zhao F, et al. Treatment of 5 critically ill patients with COVID-19 with convalescent plasma. *JAMA* 2020;323(16):1582-1589.
15. Zhang B, Liu S, Tan T, et al. Treatment with convalescent plasma for critically ill patients with severe acute respiratory syndrome coronavirus 2 infection. *Chest* 2020;158(1):e9-e13.
16. Duan K, Liu B, Li C, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc Natl Acad Sci U S A.* 2020;117(17):9490-9496.
17. Joyner MJ, Wright RS, Fairweather D, et al. Early safety indicators of COVID-19 convalescent plasma in 5000 patients [published online ahead of print August 10, 2020]. *J Clin Invest* 2020:140200. https://doi.org/10.1172/JCI140200
18. Li L, Zhang W, Hu Y, et al. Effect of convalescent plasma therapy on time to clinical improvement in patients with severe and life-threatening COVID-19: a randomized clinical trial. *JAMA* 2020;324(5):1-11.
19. Yip MS, Leung NHL, Cheung CY, et al. Antibody-dependent infection of human macrophages by severe acute respiratory syndrome coronavirus. *Virol J.* 2014;11(1):82.
20. Wang S-F, Tseng S-P, Yen C-H, et al. Antibody-dependent SARS coronavirus infection is mediated by antibodies against spike proteins. *Biochem Biophys Res Commun* 2014;451(2):208-214.
21. Agrawal AS, Tao X, Algaissi A, et al. Immunization with inactivated Middle East Respiratory Syndrome coronavirus vaccine leads to lung immunopathology on challenge with live virus. *Hum Vaccin Immunother* 2016;12(9):2351-2356.
22. Diorio C, Henrickson SE, Vella LA, et al. Multisystem inflammatory syndrome in children and COVID-19 are distinct presentations of SARS-CoV-2 [published online ahead of print July 30, 2020]. *J Clin Invest* 2020:140970. https://doi.org/10.1172/JCI140970

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Diorio C, Anderson EM, McNerney KO, et al. Convalescent plasma for pediatric patients with SARS-CoV-2-associated acute respiratory distress syndrome. *Pediatr Blood Cancer* 2020;e28693. https://doi.org/10.1002/pbc.28693

EXCEPTIONAL CASE REPORT

blood advances

Check for updates

COVID-19 convalescent plasma clears SARS-CoV-2 refractory to remdesivir in an infant with congenital heart disease

Zahidee Rodriguez,[1] Andi L. Shane,[2] Hans Verkerke,[3] Christopher Lough,[4] Matthew G. Zimmerman,[2,5,6] Mehul Suthar,[2,5,6] Jens Wrammert,[2,5] Heather MacDonald,[7] Michael Wolf,[1] Shanelle Clarke,[1] John D. Roback,[3] Connie M. Arthur,[3] Sean R. Stowell,[3,*] and Cassandra D. Josephson[3,8,*]

[1]Division of Cardiology, [2]Division of Infectious Disease, Department of Pediatrics, Children's Healthcare of Atlanta, and [3]Department of Pathology and Laboratory Medicine, Center for Transfusion and Cellular Therapies, Emory University School of Medicine, Atlanta, GA; [4]LifeSouth Community Blood Centers, Inc., Gainesville, FL; [5]Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA; [6]Yerkes National Primate Research Center, Atlanta, GA; [7]Department of Pathology, Children's Healthcare of Atlanta, Atlanta, GA; and [8]Department of Pediatrics, Aflac Cancer Center and Blood Disorders Service, Emory University School of Medicine and Children's Healthcare of Atlanta, Atlanta, GA

Key Points

- ü COVID-19 convalescent plasma (CP) may be a safe and effective treatment option in SARS-CoV-2 infection refractory to remdesivir.
- ü Infants may benefit from CP despite developing immune systems and donor variability emphasizes the need for characterization prior to use.

Introduction

Although , 2% of coronavirus disease 2019 (COVID-19) infections are reported in the pediatric population, children with comorbidities such as congenital heart disease and those at a young age are more likely to become critically ill.[1-3] Remdesivir has been reported to be efficacious in adults with COVID-19[4]; however, there are no studies in children. Convalescent plasma (CP) can contain neutralizing antibodies to viruses,[5] and has been used during previous viral epidemics with clinical improvement.[6-11] COVID-19 CP (C19-CP) may be useful in critically ill adults, resulting in improvement in inflammatory markers, pulmonary lesions, and mortality.[12] However, the impact of C19-CP in pediatric patients, particularly infants with developing immune systems and significant comorbidities, is completely unknown.

We present an infant with cardiopulmonary failure secondary to unrepaired congenital heart disease exacerbated by COVID-19. Given postsurgical complications of children with viral respiratory infection,[13-17] the patient required clearance of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) for surgical candidacy. We hypothesized that C19-CP administration may clear SARS-CoV-2 following failure of remdesivir.

Case description

A 3.1-kg term 9-week-old female with trisomy 21 and unrepaired balanced complete atrioventricular canal defect with baseline mild to moderate common atrioventricular valve regurgitation (AVVR) presented in acute hypoxemic respiratory failure secondary to progressive decompensated heart failure. Notable findings included lymphopenia, b-natriuretic peptide of 530 pg/mL (reference range , 100 pg/mL), worsened AVVR (moderate to severe), and cardiomegaly and pulmonary edema on chest X-ray. Initial treatment, including bilevel noninvasive positive pressure ventilation, milrinone infusion, and IV diuretics, resulted in restoration of organ function. By hospital day 8, her common AVVR returned to baseline; she transitioned from milrinone to captopril, carvedilol, and IV diuretics and had weaned to high-flow nasal cannula; and she was transferred to the general cardiology floor.

On hospital day 12, she developed acute hypoxemic respiratory failure and decompensated heart failure, with atelectasis on chest X-ray and new rhinorrhea requiring bilevel noninvasive positive pressure ventilation. The result of SARS-CoV-2 nucleic acid testing (NAT) (DiaSorin Molecular Simplexa direct real-time PCR of viral RNA, Cypress, CA) on a nasopharyngeal (NP) swab was positive; a residual sample from a respiratory viral panel performed on hospital day 5 for intermittent desaturations was retested for SARS-CoV-2 and likewise had a positive result (Figure 1A). The source of the infection remains unclear. While the mother and siblings were asymptomatic, they were quarantined for 14 days after infection was identified in the patient but never tested. Nosocomial infection is certainly possible

Submitted 29 May 2020; accepted 6 July 2020; published online 11 September 2020.
DOI 10.1182/bloodadvances.2020002507.

*S.R.S. and C.D.J. contributed equally to this study.

Requests for data sharing should be e-mailed to the corresponding author at rodriguezz@kidsheart.com.

© 2020 by The American Society of Hematology

Downloaded from https://ashpublications.org/bloodadvances/article-pdf/4/18/4278/1757955/advancesadv2020002507.pdf by guest on 12 September 2020

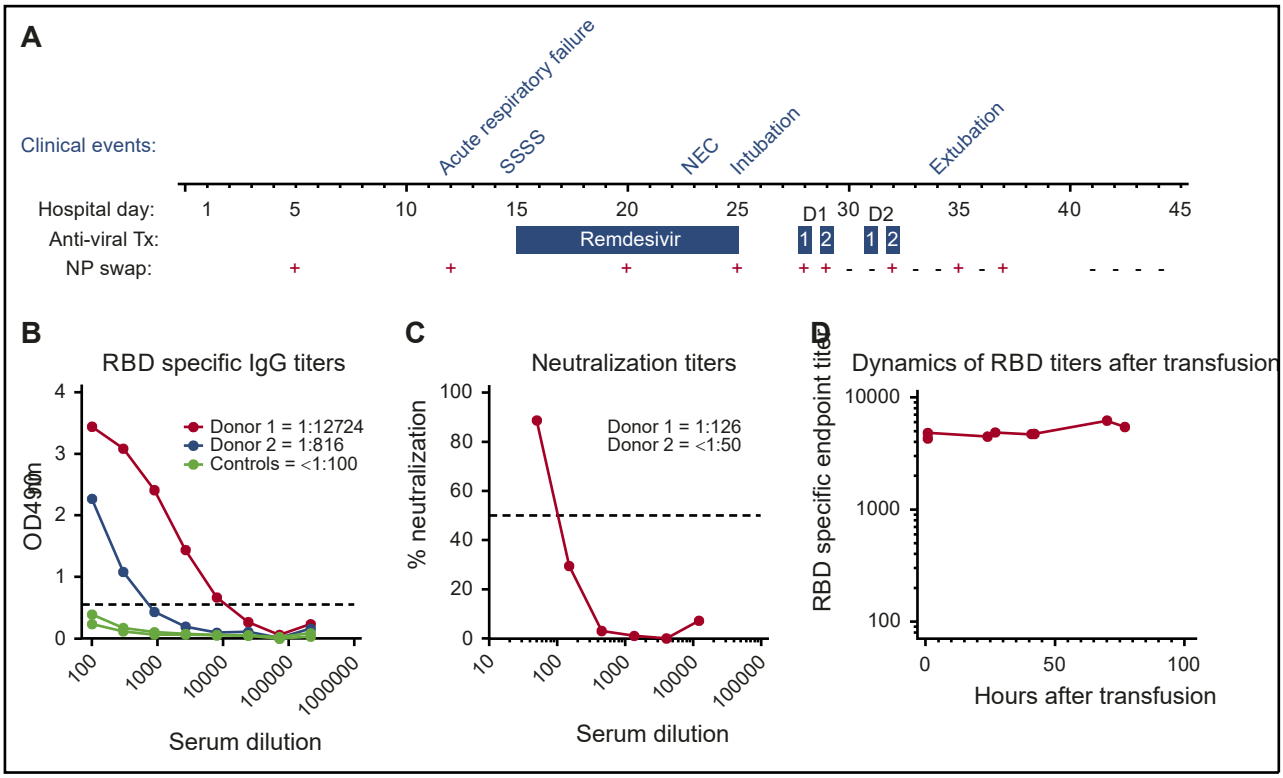


Figure 1. Hospital course summary and donor and patient plasma characterization. (A) Hospital course by clinical event, antiviral therapy (Tx), and C19-CP transfusion in relation to NP swab SARS-CoV-2 RNA results. Clinical events are as follows: acute respiratory failure on hospital day 12, staphylococcal scalded skin syndrome (SSSS) on hospital day 14, necrotizing enterococcus (NEC) on hospital day 23, intubation on hospital day 25, and extubation on hospital day 34. Dosages of antiviral therapy are as follows: remdesivir: 5 mg/kg per day on hospital day 15 and 2.5 mg/kg/day on hospital days 16 to 25; C19-CP donor 1: 10 mL/kg per day over 4 hours; 20 hours apart on hospital days 28 and 29; and C19-CP donor 2: 10 mL/kg per day over 4 hours; 20 hours apart on hospital days 31 and 32. (B) Enzyme-linked immunosorbent assays showing titration of samples from the 2 C19-CP donors and 3 negative controls collected before 2019, against SARS-CoV-2 spike RBD. End point titers were calculated using a cutoff of the average of the control samples, plus 3 times standard deviation value, marked with a dotted line. End points were calculated as the intersection of samples with this cutoff. Titers are shown numerically in the legend. (C) Neutralization activity of C19-CP donor samples against SARS-CoV-2. The focus reduction neutralization test $(FRNT)_{50}$ titers of the C19-CP donor samples were determined by an FRNT assay using an immunostain to detect infected foci. The dotted line represents the maximum concentrations of the serum tested (1/50). (D) Dynamics of RBD-specific end-point titers in the recipient after transfusion.

but less likely, as the hospital had very few infected patients, none of those infected were in the cardiac intensive care unit, and there was no shortage of personal protective gear at the hospital prior or following the patient's infection. Additional investigation did not reveal macrophage activation syndrome or new bacterial or viral infection. As her prolonged QTc prohibited use of hydroxychloroquine, remdesivir (5 mg/kg per day on hospital day 15 and 2.5 mg/kg per day on hospital days 16-25) was given. Her hospital course was complicated by staphylococcal scalded skin syndrome, treated with nafcillin, and stage II necrotizing enterocolitis (Bell criteria), treated with piperacillin-tazobactam (Figure 1A). Throughout this time, she continued to have persistent lymphopenia, a C-reactive protein (CRP) level of 5 mg/dL (reference range, , 1.0 mg/dL) with normal fibrinogen, and a common AVVR that remained unchanged with preserved biventricular function managed with IV diuretics.

Despite completion of remdesivir, on hospital day 25, she required endotracheal intubation for worsening respiratory failure. Repeat investigation was notable for persistent lymphopenia, persistent SARS-CoV-2, CRP 7.7 mg/dL, but no evidence of new secondary infection or macrophage activation syndrome was found. Milrinone was reinitiated in addition to IV diuretics. Given her lack of response to remdesivir and deteriorating clinical status, 2 aliquots of CP (10 mL/kg per aliquot) from 2 COVID-19 recovered donors (C19-CP) were transfused in accordance with the World Health Organization guidelines for Ebola CP treatment in children[9] (Figure 1A). She received increased IV diuretics, daily monitoring of serum chemistries, and intermittent monitoring of coagulopathy. Daily surveillance of SARS-CoV-2 NAT by NP swab was performed, and the results were negative following the second aliquot from donor 1 (Figure 1A) with concurrent reduced ventilator support. She was extubated on hospital day 34. The infant tolerated all transfusions without anaphylaxis, transfusion-related circulatory overload, transfusion-related acute lung injury, or hypercoagulability. The results of daily NP swabs were intermittently positive before becoming persistently negative (Figure 1A). Other notable findings post–C19-CP transfusion include CRP of 1.3 mg/dL and intermittent normalization of absolute lymphocyte count. The patient had an uneventful complete repair 47 days (hospital day 91) after SARS-CoV-2 infection resolution and is doing well.

Downloaded from https://ashpublications.org/bloodadvances/article-pdf/4/18/4278/1757955/advancesadv2020002507.pdf by guest on 12 September 2020

Methods

The patient is blood group O RhD negative. Donor 1 is a 63-year-old male who donated 35 days following COVID-19 symptom resolution and is blood type A RhD positive. Donor 2 is a 56-year-old male who donated 55 days following COVID-19 symptom resolution and is blood type O RhD negative. Both had mild COVID-19 infection, and neither was hospitalized. The plasma donor units were transfused without prior characterization per emergency use. To understand the impact of C19-CP treatment, both donor units were tested for titers of IgG against the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein (Emory University and Children's Healthcare of Atlanta, Atlanta, GA), which has been shown to correlate with neutralizing titers,[18] and subsequent microneutralization assays were performed in a biosafety level 3 laboratory.[19] Donor 1 had an immunoglobulin G (IgG) titer of 1:12 724 and neutralizing titer of 1:126. Donor 2 had an IgG titer of 1:816 and a neutralizing titer of , 1:50 (Figure 1B-C, respectively). Following the first aliquot from donor 1, the recipient showed robust IgG anti-SARS-CoV-2 antibody levels of ; 1:5000, which was sustained after all subsequent transfusions (Figure 1D).

Results and discussion

Young age, unrepaired congenital heart disease, trisomy 21, persistent lymphopenia, and naive immune system likely increased this patient's SARS-CoV-2 vulnerability. While this represents only 1 case, her improved respiratory status, increase in lymphocytes, and decrease in CRP after C19-CP transfusion suggest a positive clinical correlation, especially in the setting of subsequent SARS-CoV-2 clearance.

Logistical constraints prevented anti-SARS-CoV-2 antibody characterization of C19-CP prior to transfusion, which likely reflects common current approaches to emergent C19-CP administration. C19-CP from donor 1 likely provided the most significant impact on SARS-CoV-2 eradication given the higher neutralizing titer (Figure 1C), which is comparable to US Food and Drug Administration recommendations[20] and may be sufficient when treating COVID-19 in adults.[10-21] This case suggests that such titers may likewise be sufficient in infants. The delay in viral clearance may reflect the time required for C19-CP therapy to effectively eliminate the virus and is consistent with reports in adults with persistent SARS-CoV-2 NAT by NP samples beyond symptom resolution.[22,23]

Factors influencing high anti-SARS-CoV-2 titers with effective characteristics remain incompletely understood. Severe illness would be presumed to correlate with higher anti-SARS-CoV-2 antibodies. However, even though neither C19-CP donor was hospitalized, donor 1 possessed higher titers than donor 2, whose titers were below the limit of detection. This variability illustrates the challenge of current C19-CP transfusion practice, making it difficult to determine the potential benefit without adequate testing prior to C19-CP transfusion in place.

While neutralizing antibodies are critical for antibody-mediated protection against viral infection, the impact of CP on resolution of active infection remains incompletely understood. However, beyond direct neutralization of the virus, other mechanisms, such as antibody-dependent cellular cytotoxicity, may also play a role in the ability of C19-CP to aid in the clearance of an active infection. Such considerations may be especially important in infants with developing or suppressed immune systems that may attenuate the ability of other antibody effector systems to effectively clear COVID-19 following C19-CP therapy. While we were unfortunately unable to acquire neutralizing titer data from the patient prior to C19-CP administration to determine potential changes in antibody levels prior to and following administration of the first dose of C19-CP, data obtained following serial SARS-CoV-2 NAT evaluation provides uncommon insight into the temporal relationship between C19-CP and its potential impact on SARS-CoV-2 infection. However, this is a single case, and understandable uncertainties remain. Future studies will certainly be needed to fully define the efficacy of C19-CP in this vulnerable patient population.

Acknowledgments

The authors thank their colleagues at LifeSouth for collection of donor plasma. Parental consent was obtained for treatment with C19-CP. Review of medical records, publication of results, and research use of biospecimens per the Declaration of Helsinki under the protocols were approved by the Institutional Review Board of Emory University School of Medicine and the Department of Health and Human Services.

Authorship

Contribution: Z.R., S.R.S., and C.D.J. conceived of and wrote the manuscript; S.R.S., C.D.J., Z.R., M.S., and J.W. prepared the figures; A.L.S., H.V., C.L., M.G.Z., M.S., J.W., H.M., M.W., S.C., J.D.R.C., M.A., S.R.S., and C.D.J. critically reviewed, analyzed, and interpreted the data; M.G.Z., J.W., M.S., H.M., C.M.A., and S.R.S. performed the immunologic assays; and C.L. collected the donor units and information on blood donors.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: Z.R., 0000-0002-0804-7132; A.L.S., 0000-0003-0606-7188.

Correspondence: Zahidee Rodriguez, Emory University, Children's Healthcare of Atlanta–Egleston, 1405 Clifton Rd, 2nd Floor, Tower 1, Atlanta, GA 30322; e-mail: rodriguezz@kidsheart.com.

References

1. Dong Y, Mo X, Hu Y, et al. Epidemiology of COVID-19 among children in China. Pediatrics. 2020;145(6):e20200702.
2. CDC COVID-19 Response Team. Coronavirus disease 2019 in children: United States, February 12-April 2, 2020. MMWR Morb Mortal Wkly Rep. 2020;69(14):422-426.
3. DeBiasi RL, Song X, Delaney M, et al. Severe COVID-19 in children and young adults in the Washington, DC metropolitan region. J Pediatr. 2020;223:199-203.e1.

Downloaded from https://ashpublications.org/bloodadvances/article-pdf/4/18/4278/1757955/advancesadv2020002507.pdf by guest on 12 September 2020

4. Ledford H. Hopes rise for coronavirus drug remdesivir [published online ahead of print 29 April 2020]. Nature. doi:10.1038/d41586-020-01295-8.
5. Zhang JS, Chen JT, Liu YX, et al. A serological survey on neutralizing antibody titer of SARS convalescent sera. J Med Virol. 2005;77(2):147-150.
6. Luke TC, Kilbane EM, Jackson JL, Hoffman SL. Meta-analysis: convalescent blood products for Spanish influenza pneumonia: a future H5N1 treatment? Ann Intern Med. 2006;145(8):599-609.
7. Hung IF, To KK, Lee CK, et al. Convalescent plasma treatment reduced mortality in patients with severe pandemic influenza A (H1N1) 2009 virus infection. Clin Infect Dis. 2011;52(4):447-456.
8. Hung IFN, To KKW, Lee CK, et al. Hyperimmune IV immunoglobulin treatment: a multicenter double-blind randomized controlled trial for patients with severe 2009 influenza A(H1N1) infection. Chest. 2013;144(2):464-473.
9. van Griensven J, Edwards T, de Lamballerie X, et al; Ebola-Tx Consortium. Evaluation of convalescent plasma for Ebola virus disease in Guinea. N Engl J Med. 2016;374(1):33-42.
10. Cheng Y, Wong R, Soo YO, et al. Use of convalescent plasma therapy in SARS patients in Hong Kong. Eur J Clin Microbiol Infect Dis. 2005;24(1):44-46.
11. Yeh KM, Chiueh TS, Siu LK, et al. Experience of using convalescent plasma for severe acute respiratory syndrome among healthcare workers in a Taiwan hospital. J Antimicrob Chemother. 2005;56(5):919-922.
12. Shen C, Wang Z, Zhao F, et al. Treatment of 5 critically ill patients with COVID-19 with convalescent plasma. JAMA. 2020;323(16):1582.
13. Malviya S, Voepel-Lewis T, Siewert M, Pandit UA, Riegger LQ, Tait AR. Risk factors for adverse postoperative outcomes in children presenting for cardiac surgery with upper respiratory tract infections. Anesthesiology. 2003;98(3):628-632.
14. Tait AR, Malviya S, Voepel-Lewis T, Munro HM, Seiwert M, Pandit UA. Risk factors for perioperative adverse respiratory events in children with upper respiratory tract infections. Anesthesiology. 2001;95(2):299-306.
15. Mallory MD, Travers C, McCracken CE, Hertzog J, Cravero JP. Upper respiratory infections and airway adverse events in pediatric procedural sedation. Pediatrics. 2017;140(1):e20170009.
16. Parnis SJ, Barker DS, Van Der Walt JH. Clinical predictors of anaesthetic complications in children with respiratory tract infections. Paediatr Anaesth. 2001;11(1):29-40.
17. Tanne JH. Covid-19: FDA approves use of convalescent plasma to treat critically ill patients. BMJ. 2020;368:m1256.
18. To KK, Tsang OT, Leung WS, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. Lancet Infect Dis. 2020;20(5):565-574.
19. Suthar MS, Zimmerman M, Kauffman R, et al. Rapid generation of neutralizing antibody responses in COVID-19 patients. medRxiv. 2020;1(3):100040.
20. FDA. Recommendations for investigational COVID-19 convalescent plasma. https://www.fda.gov/vaccines-blood-biologics/investigational-new-drug-ind-or-device-exemption-ide-process-cber/recommendations-investigational-covid-19-convalescent-plasma. Accessed 1 May 2020.
21. Bloch EM, Shoham S, Casadevall A, et al. Deployment of convalescent plasma for the prevention and treatment of COVID-19. J Clin Invest. 2020;130(6):2757-2765.
22. Sethuraman N, Jeremiah SS, Ryo A. Interpreting diagnostic tests for SARS-CoV-2. JAMA. 2020;323(22):2249.
23. Wajnberg A, Mansour M, Leven E, et al. Humoral immune response and prolonged PCR positivity in a cohort of 1343 SARS-CoV2 patients in the New York City region [published online ahead of print 5 May 2020]. medRxiv. doi:10.1101/2020.04.30.20085613.

Downloaded from https://ashpublications.org/bloodadvances/article-pdf/4/18/4278/1757955/advancesadv2020002507.pdf by guest on 12 September 2020

Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Rapid recovery of a SARS-CoV-2 e infected X-linked agammaglobulinemia patient after infusion of COVID-19 convalescent plasma

Estefanía Mira, MD[a], Oscar A. Yarce, MD[b], Consuelo Ortega, MD, PhD[c], Silvia Fernández, PhD[c], Natalia M. Pascual, MD[a], Cristina Gómez, MD[a], Miguel A. Alvarez, MD[d], Ignacio J. Molina, MD, PhD[e], Rafael Lama, MD, PhD[a], and Manuel Santamaria, MD, PhD[b,c]

Clinical Implications

A patient with X-linked agammaglobulinemia was infected with severe acute respiratory syndrome coronavirus 2 and presented a 40-day long and stagnant clinical evolution refractory to treatment. The patient received a single dose of coronavirus disease 2019 convalescent plasma and experienced a rapid clinical improvement within 1 week of infusion.

The high infectivity of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the lack of preexisting immunity among the population, and the complexity of the coronavirus disease 2019 (COVID-19) clinical presentation are some of the underlying reasons of the ongoing worldwide health crisis. Antibodies play relevant roles in antiviral responses,[1] and hence, patients with compromised specific humoral immunity may be at increased risk to suffer from severe forms of the disease. In fact, 3 recent studies described the clinical evolution of 6 patients with common variable immunodeficiency (CVID), 3 patients with x-linked agammaglobulinemia (XLA) (OMIM# 300755; Bruton's disease), and 1 patient with autosomal agammaglobulinemia.[2-4] However, the mild clinical presentation in agammaglobulinemic patients, as opposed to the former, prompted speculations that anti–SARS-CoV-2 antibodies may be dispensable in clearing the disease.[4] This would highlight the relevance of T-cell responses and the significance of anti–SARS-CoV-2 T-cell exhaustion in the ultimate progression of the disease.[5] However, administration of hyperimmune plasma was beneficial in some immunocompetent patients with COVID-19.[6]

We report the case of a 39-year-old male patient with XLA, receiving monthly immunoglobulin replacement therapy, admitted to our hospital with dyspnea, dry cough, and fever of 2-week duration. Chest X-rays showed unilateral alveolar condensation, and quantitative PCR (qPCR) amplification of SARS-CoV-2 from a nasopharyngeal exudate sample confirmed the diagnosis of COVID-19. The patient received hydroxychloroquine (400 mg/12 h, the first day; 200 mg/12 h, 4 days, orally), azithromycin (500 mg/24 h, 5 days, orally), and ceftriaxone (2 g/24 h, 7 days, intravenously [IV]) and a single dose (0.3 g/kg) of IV immunoglobulins. No supplemental oxygen was required.

In spite of a favorable initial clinical evolution, fever persisted and intense asthenia appeared. Therefore, empirical therapy against nosocomial germs was started with piperacillin/tazobactam (4 g/0.5 g/8 h, IV) and linezolid (600 mg/12 h, IV). SARS-CoV-2 qPCR test result remained positive on day 11 after admission. On day 15 computed tomography showed extensive bilateral pulmonary involvement in the form of nodular consolidations in ground glass with underlying interstitial pattern (Figure 1, upper panel). Blood, sputum, stool, and urine cultures were negative. Infections by other respiratory viruses (adenovirus, metapneumovirus, inovirus, and respiratory syncytial virus), as well as the presence of opportunistic germs such as Pneumocystis jiroveci, cytomegalovirus, EBV, Mycoplasma pneumoniae, aspergillus, leishmania, and brucella, were all excluded. A third SARS-CoV-2 qPCR was carried out on day 21 after admission and the result remained positive.

Stagnancy in the patient's evolution, as represented by the lack of response to any of the treatments dispensed (including a second pulse of nonspecific immunoglobulins at 0.8 g/kg/24 h and aggressive antibiotic therapy), together with persistent fever and bilateral pneumonia, prompted us to consider the possibility that natural killer (NK)-cell and T-cell exhaustion might be an underlying cause for immunologic failure, as shown elsewhere.[5] We assessed this possibility in lung lymphoid populations obtained by bronchoalveolar lavage on day 21 after admission, and they consisted of $CD3^b$ $CD8^b$ (86%) and $CD3^b$ $CD4^b$ T lymphocytes (11.5%) and $CD56^b$ $CD16^b$ NK cells (2.5%). Staining of intracellular cytokines in stimulated $CD8^b$ cells showed negligible production of TNF-α or IFN-γ (Figure 2, A, middle and bottom panels).

Because of the resulting lymphocyte phenotype, we reasoned that, in the absence of functional B cells, viral clearance would not be possible. Hence, we administered on day 23 after admission compassionate-use approved COVID-19 convalescent donor plasma (200 mL, single dose, IV). The plasma was analyzed by an ELISA (COVID-19 ELISA IgG, Vircell SL, Granada, Spain), which detected IgG antibodies against either the spike or nucleocapsid viral proteins with a titer greater than or equal to 1/320. After 24 hours of infusion, fever ceased without subsequent reappearance and with progressive improvement of asthenia. After 48 hours of infusion, qPCR from nasopharyngeal exudate failed to amplify SARS-CoV-2 sequences.

On day 30 after admission, that is, 1 week after administration of COVID-19 convalescent donor plasma, the patient remained asymptomatic and a chest tomography showed almost complete resolution of bilateral lung lesions (Figure 1, lower panel). Furthermore, a vigorous recovery in the peripheral T-lymphocyte subpopulations ($CD3^b$, $CD4^b$, and $CD8^b$) was observed, whereas NK-cell counts slightly increased (Figure 2, B). Hence, the patient was subsequently discharged and remains COVID-19 free.

Although cumulative cases of SARS-CoV-2–infected patients suffering from primary antibody immunodeficiencies are limited, it appears that individuals devoid of B cells (agammaglobulinemia) have a milder presentation and course than those with a partial B-cell repertoire (CVID).[2-4] This observation is

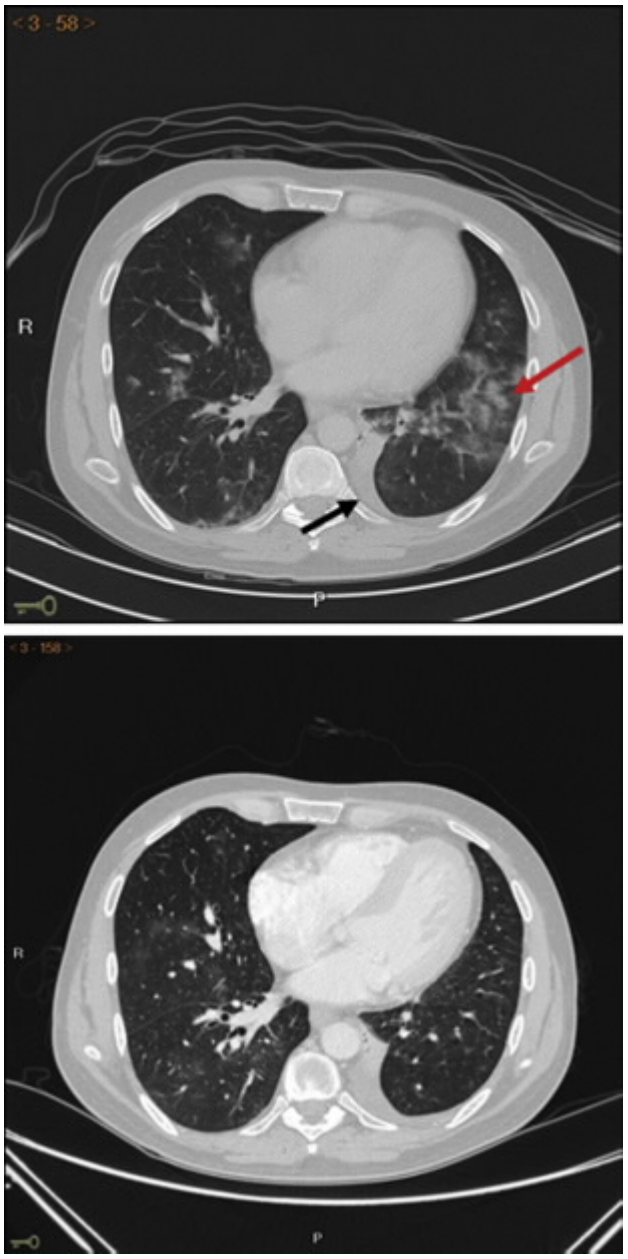


FIGURE 1. Upper panel: CT displaying bilateral pulmonary involvement in the form of nodular consolidations and ground-glass opacities primarily in the left lower lobe (red arrow) and small-size left pleural effusion (black arrow). Lower panel: CT of the chest 6 days after COVID-19 convalescent donor plasma transfusion showing almost complete resolution of bilateral lung lesions pattern. CT, Computed tomography.

strengthened by the finding that pharmacological blockade of Bruton tyrosine kinase results in diminished lung inflammatory response by inhibiting overproduction of macrophage-mediated cytokines.[7] The evolution of our patient was consistent with those observations until day 15 of admission, when he suffered a rapid deterioration in his condition accompanied by a lack of cytokine production by lung T and NK cells. If this lack of functional response was the consequence of cell exhaustion, we should bear in mind that T-cell response involve triggering of multiple epitope-specific clones, and not all may be equally prone to functional blockade.[8] Therefore, local silencing of immunodominant clones may hamper the overall immune response.

The remarkable and prompt recovery observed after infusion of convalescent plasma suggests that certain patients may have stringent dependency on specific antibodies to achieve viral clearance. Although this is a single case and therefore the observation could be coincidental and we have not formally established a cause-effect relationship, we nevertheless propose that administration of COVID-19 hyperimmune plasma should be further explored as first line of treatment for patients with congenital agammaglobulinemia, especially while SARS-CoV-2–specific antiviral drugs remain unavailable. Previous clinical cases suggest that B cells may have a prominent role in triggering lung inflammation[3]; in this scenario, defective germinal center formation due to the primary immunodeficiency would divert IL-6 intended for that process toward lung inflammation, thus raising the possibility of a B-cell interference as therapeutic intervention.[3] However, our data suggest a critical role of B cells in clearing the disease when certain immunologic conditions are present.

In summary, even though administration of passive immunity for patients with primary immunodeficiency has just been proposed,[9] this is the first report showing a rapid recovery of a patient with a primary antibody immunodeficiency after administration of hyperimmune plasma. Although it should be kept in mind whether the patient reported here was idiosyncratic and might not represent a prototypical patient with XLA (despite carrying a common Bruton tyrosine kinase gene mutation), our data merit further investigations to determine the precise clinical benefit of hyperimmune plasma in other antibody-deficient patients, as well as optimal dose and timing of its administration.

Acknowledgments

We thank Professor J.L. Martinez for invaluable help with microbiology studies. We gratefully acknowledge Red Andaluza de Medicina Transfusional, Tejidos y Células, Servicio Andaluz de Salud for supplying and analyzing the COVID-19 convalescent plasma.

[a]Service of Pneumology, Reina Sofía University Hospital, Córdoba, Spain
[b]Service of Immunology and Allergy, Reina Sofía University Hospital, Córdoba, Spain
[c]Unit of Immunology, Córdoba Medical School, University of Córdoba, Córdoba, Spain
[d]Service of Haematology Reina Sofía University Hospital, Córdoba, Spain
[e]Biopathology and Regenerative Medicine Institute, Biomedical Research Center, Health Sciences Technology Park, University of Granada, Granada, Spain
This work was supported by Consejería de Universidades, Junta de Andalucía (grant no. CT208 to M.S.) and Action for A-T (Haslemere, UK) (grant no. AAT-8GRA02 to I.J.M.).
Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.
Received for publication May 22, 2020; revised manuscript received and accepted for publication June 25, 2020.
Available online July 8, 2020.
Corresponding author: Manuel Santamaria, MD, PhD, Unit of Immunology, Cordoba Medical School, University of Córdoba, Avda Menéndez Pidal S/N, Cordoba 14004, Spain. E-mail: msantamaria@uco.es
2213-2198
ff 2020 American Academy of Allergy, Asthma & Immunology
https://doi.org/10.1016/j.jaip.2020.06.046

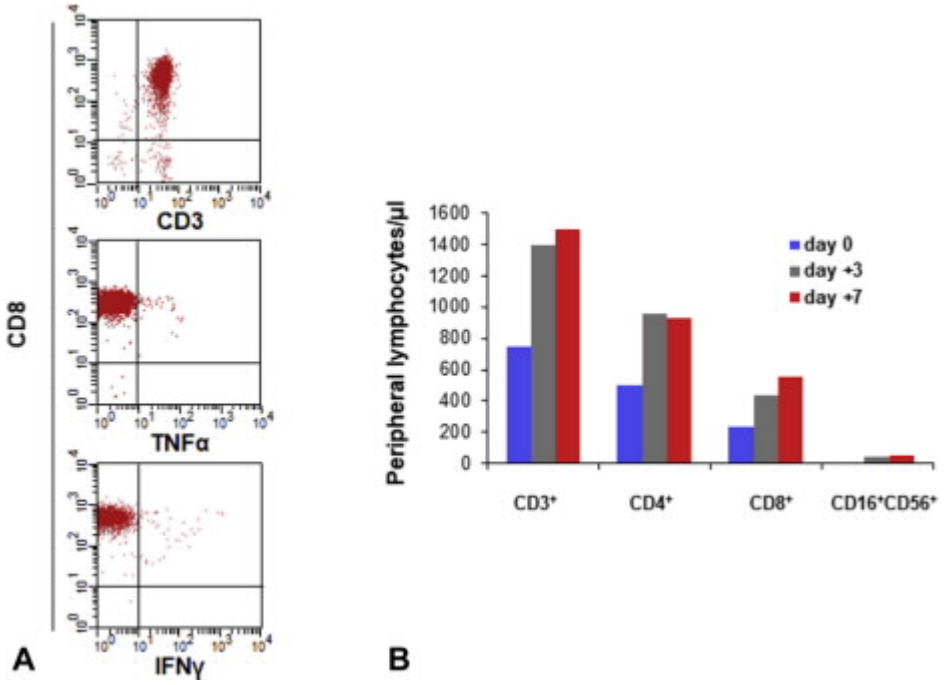


FIGURE 2. (A) Flow cytometric double fluorescence analysis of CD8 cells showing coexpression of surface CD3 (upper panel) and intracytoplasmic TNF-a (middle panel) or IFN-g (lower panel) after cell stimulation. (B) Blood peripheral T lymphocytes and NK-cell counts determined on days 0, 3, and 7 postinfusion of COVID-19 convalescent plasma.

REFERENCES

1. Burton DR. Antibodies, viruses and vaccines. Nat Rev Immunol 2002;2:706-13
2. Fill L, Hadney L, Graver K, Persaud R, Hostoffer R. The clinical observation of a CVID patient infected with COVID-19. Ann Allergy Asthma Immunol 2020;125:112-4
3. Quinti I, Lougaris V, Milito C, Cinetto F, Pecoraro A, Mezzaroma I, et al. A possible role for B cells in COVID-19? Lesson from patients with agammaglobulinemia. J Allergy Clin Immunol 2020;146:211-213.e4
4. Soresina A, Moratto D, Chiarini M, Paolillo C, Baresi G, Focà E, et al. Two X-linked agammaglobulinemia patients develop pneumonia as COVID-19 manifestation but recover. Pediatr Allergy Immunol 2020;31:565-9
5. Zheng M, Gao Y, Wang G, Song G, Liu S, Sun D, et al. Functional exhaustion of antiviral lymphocytes in COVID-19 patients. Cell Mol Immunol 2020;17:533-5
6. Zhang B, Liu S, Tan T, Huang W, Dong Y, Chen L, et al. Treatment with convalescent plasma for critically ill patients with severe acute respiratory syndrome coronavirus 2 infection. Chest 2020;158:e9-13
7. Roschewski M, Lionakis MS, Sharman JP, Roswarski J, Goy A, Monticelli MA, et al. Inhibition of Bruton tyrosine kinase in patients with severe COVID-19. Sci Immunol 2020;5:eabd0110
8. Kahan SM, Wherry EJ, Zajac AJ. T cell exhaustion during persistent viral infections. Virology 2015;479-80:180-93
9. Hammarstrom L, Abolhassani H, Baldanti F, Marcotte H, Pan-Hammarstrom Q. Development of passive immunity against SARS-CoV-2 for management of immunodeficient patients—a perspective. J Allergy Clin Immunol 2020;146:58-60

Received:15 July 2020 | Revised:23 August 2020 | Accepted: 24 August 2020

DOI: 10.1002/pbc.28693

Pediatric Blood & Cancer

aspho The American Society of Pediatric Hematology/Oncology

WILEY

PSYCHOSOCIALAND SUPPORTIVECARE: BRIEFREPORT

Convalescentplasmafor pediatricpatientswith SARS-CoV-2-associatedacuterespiratorydistresssyndrome

CarolineDiorio[1,2] | ElizabethM. Anderson[3] | KevinO. McNerney[1,2] | EileenC.Goodwin[3] | JulieC.Chase[1,4] | MarcusJ.Bolton[3] | ClaudiaP.Arevalo[3] | MadisonE.Weirick[3] | SigridGouma[3] | LauraA. Vella[1,5] | SarahE.Henrickson[1,6] | KathleenChiotos[7] | JulieC.Fitzgerald[1,7] | ToddJ.Kilbaugh[7] | AudreyR.OdomJohn[5] | AllisonM. Blatz[5] | Michele P.Lambert[1,8] | KathleenE.Sullivan[1,6] | Margaret R.Tartaglione[2] | DanielleZambrano[2] | MeghanMartin[2] | JessicaH. Lee[1] | PampeeYoung[9] | DavidFriedman[8,10] | DeborahA.Sesok-Pizzini[10] | ScottE.Hensley[3] | EdwardM. Behrens[1,4] | HamidBassiri[1,5] | David T.Teachey[1,2]

[1] Immune Dysregulation Frontier Program,Department of Pediatrics,Children's Hospital of Philadelphia,University of PennsylvaniaPerelman School of Medicine, Philadelphia,Pennsylvania

[2] Division of Oncology,Department of Pediatrics,Children's Hospital of Philadelphia,University of PennsylvaniaPerelman Schoolof Medicine, Philadelphia, Pennsylvania

[3] Department of Microbiology, PerelmanSchoolof Medicine, University of Pennsylvania,Philadelphia,Pennsylvania

[4] Division of Rheumatology,Department of Pediatrics,Children's Hospital of Philadelphia,University of PennsylvaniaPerelman Schoolof Medicine, Philadelphia, Pennsylvania

[5] Division of Infectious Diseases,Department of Pediatrics,Children's Hospital of Philadelphia,University of PennsylvaniaPerelman Schoolof Medicine,Philadelphia, Pennsylvania

[6] Division of Allergy and Immunology, Department of Pediatrics,Children's Hospital of Philadelphia,University of PennsylvaniaPerelman Schoolof Medicine, Philadelphia,Pennsylvania

[7] Division of Critical Care Medicine, Department of Anesthesiology and Critical Care Medicine, Children's Hospital of Philadelphia,University of Pennsylvania Perelman Schoolof Medicine, Philadelphia,Pennsylvania

[8] Division of Hematology, Children's Hospital of Philadelphia,University of PennsylvaniaPerelman Schoolof Medicine, Philadelphia,Pennsylvania

[9] American Red Cross,Washington, District of Columbia

[10] Pathology and Laboratory Medicine, University of PennsylvaniaPerelman Schoolof Medicine, Philadelphia,Pennsylvania

Correspondence
David T.Teachey,Department of Pediatrics, Division of Oncology,The Children's Hospital of Philadelphia,University of Pennsylvania PerelmanSchoolof Medicine,3008 CTRB,3501 Civic Center Blvd,Philadelphia, PA 19104.
Email:teacheyd@email.chop.edu

Abstract

There are no proven safe and effective therapies for children who develop life-threatening complications of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)Convalescentplasma(CP)hasdemonstrated potential benefit in adults with SARS-CoV-2,but has theoretical risks.We present the first report of CP in children with life-threatening coronavirus disease2019 (COVID-19), providing data on

Abbreviations:ADE,antibody-dependent enhancement;CHOP,Children's Hospital of Philadelphia; COVID-19, coronavirus disease2019; CP,convalescentplasma;ELISA,enzyme-linked immunosorbent assay;FDA,Food and Drug Administration; MIS-C,multisystem inflammatory syndrome in children; N protein, nucleocapsidprotein; RBD, receptor-binding domain; Sprotein, spike protein; SARS-CoV-2,severe acute respiratory syndrome coronavirus 2

This is an open accessarticle under the terms of the Creative Commons Attribution-NonCommercial License,which permits use,distribution and reproduction in any medium,provided the original work is properly cited and is not used for commercial purposes.
© 2020 Wiley PeriodicalsLLC

Caroline Diorio, Elizabeth M. Anderson, Edward M. Behrens, Hamid Bassiri, and David T. Teachey contributed equally to this work.

Funding information
Leukemia and Lymphoma Society; University of Pennsylvania; Kate Amato Foundation; American Academy of Allergy Asthma and Immunology; Cookies for Kids Cancer; Children's Oncology Group; Stand Up To Cancer; Alex's Lemonade Stand Foundation for Childhood Cancer; National Institutes of Health, Grant/Award Numbers: K08AI135091, K08AI136660, K12HS026393, R01A1123538, R01AI103280, R01AI121250, R01AI123433, R01CA193776, R21AI144472, T32AI007324, UG1CA233249, X01HD100702; TeamConnor; Clinical Immunology Society; Children's Hospital of Philadelphia, Grant/Award Number: Immune Dysregulation Frontier Program; Burroughs Wellcome Fund

four pediatric patients with acute respiratory distress syndrome. We measured donor antibody levels and recipient antibody response prior to and following CP infusion. Infusion of CP was not associated with antibody-dependent enhancement (ADE) and did not suppress endogenous antibody response. We found CP was safe and possibly efficacious. Randomized pediatric trials are needed.

KEYWORDS
convalescent plasma, COVID-19, pediatrics, SARS-CoV-2

1 | INTRODUCTION

Early reports from the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) global pandemic suggested that children were less severely affected by coronavirus disease 2019 (COVID-19) compared to older adults.[1–3] Nevertheless, some children with COVID-19 are critically ill.[4,5] Currently, no therapies have been proven effective in the treatment of COVID-19 in children.

Convalescent plasma (CP) derived from patients that have recovered from SARS-CoV-2 can be infused into currently ill patients. The proposed mechanism of action is via neutralizing antibodies binding to virus, rendering it inert.[6] Specific to SARS-CoV-2, antibodies against the receptor-binding domain (RBD) have been identified as surrogates for neutralization.[7–11] CP also contains antibodies against the immunogenic nucleocapsid (N) protein, which are nonneutralizing and present in both actively infected and recovered patients.[7] The protective function of N antibodies remains unclear.[7,12] Antibodies against the full-length SARS-CoV-2 spike (S) protein include both nonneutralizing antibodies and neutralizing antibodies.[13]

Initial series of CP in adults with COVID-19 demonstrated potential benefits without apparent side effects.[14–17] In a recent clinical trial of adults with COVID-19, there was a nonstatistically significant trend toward clinical improvement in CP-treated patients versus controls, without improvement in time to discharge or mortality.[18] This trial was halted early due to poor accrual.[18] To date, there have been no reports on the use of CP in children.

CP infusion is associated with the side effects of any blood product: allergic reaction, transfusion-associated circulatory overload, and infection with blood-borne pathogens. There is a theoretical risk that infusion of donor antibodies may impede the recipient's endogenous production of antibodies, and of antibody-dependent enhancement (ADE), where antibodies developed during a previous infection cause a worsened clinical response with subsequent infection. ADE has been described in dengue fever, and in preclinical models of other coronaviruses.[19–21] ADE has not been reported in adult patients receiving CP for SARS-CoV-2.[6,17,18]

We present the first report of pediatric patients receiving CP for life-threatening COVID-19-associated respiratory disease with correlative measurements of the associated pre- and posttransfusion antibody response.

2 | METHODS

We treated four critically ill children actively infected with SARS-CoV-2 with CP at the Children's Hospital of Philadelphia (CHOP) under emergency Investigational New Drug applications (eINDs) through the Food and Drug Administration (FDA). Patients were considered for CP if they met FDA guidance for life-threatening disease (Appendix S1). Per the American Red Cross Guidelines, donors were eligible to provide CP if they met the following criteria: (a) they were initially proven positive for SARS-CoV-2 by a laboratory test; and either (b1) at least 14 days from symptom resolution with a repeat documented negative test for SARS-CoV-2, or (b2) at least 28 days from symptom resolution without a documented repeat test results at the time of plasma collection.

We prospectively screened and enrolled these patients into a larger SARS-CoV-2 biobanking protocol. The objective of the SARS-CoV-2 biorepository protocol is to collect samples on patients with confirmed SARS-CoV-2 infection or multisystem inflammatory syndrome in children (MIS-C). An initial report from this biorepository has been published.[22] Patients were enrolled on the biobank protocol if they had evidence of SARS-CoV-2 infection on reverse-transcriptase polymerase chain reaction (RT-PCR) testing from respiratory tract mucosa. This protocol was approved by the CHOP Institutional Review Board (IRB). Consent was obtained for both protocols from a legal authorized representative per the Declaration of Helsinki (written informed consent for CP; verbal consent for biobanking).

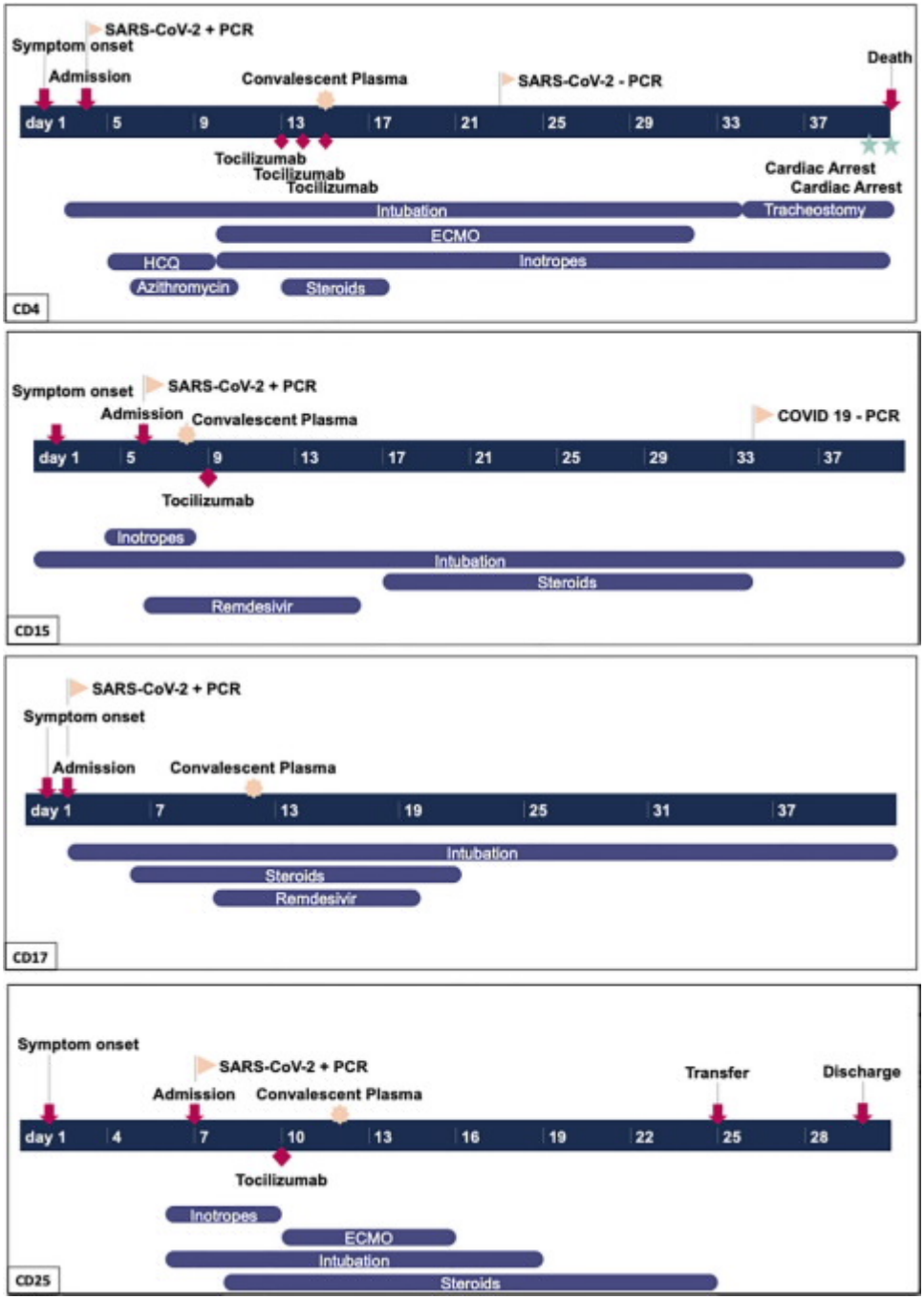


FIGURE 1 Graphical representation of clinical course of four patients included in the caseseries. Timeline includes up to day 40 of illness, or until death or discharge, whatever occurred first. ECMO, extracorporeal membrane oxygenation; HCQ, hydroxychloroquine; PCR, polymerase chain reaction

Verbal consent for biobanking was obtained based on IRB recommendations to prevent exposure to research staff and to avoid unintentional transmission of SARS-CoV-2via fomites such as consent forms.

Clinical data were abstracted from patient charts to standardized forms by a research assistant using a REDCap database, and verified by a physician. Blood sampleswere obtained from participants prior to CP infusion, following CP infusion, and weekly thereafter until death or discharge. Remnant donor CP was retained, per institutional protocol in case of transfusion reaction. Remnant donor CP was then collected and stored.

SARS-CoV-2-specificantibodies were measured by enzyme-linked immunosorbent assay (ELISA) as previously described.[8] Antibodies in serially diluted plasma (starting at 1:50) were measured against RBD of the SARS-CoV-2S protein,[9] full-length S protein, and the N protein (Sino Biological, Wayne, PA). Reciprocal plasma dilutions were calculated from an optical density threshold from a set concentration. Standard curves were generated by diluting an RBD-specific monoclonal

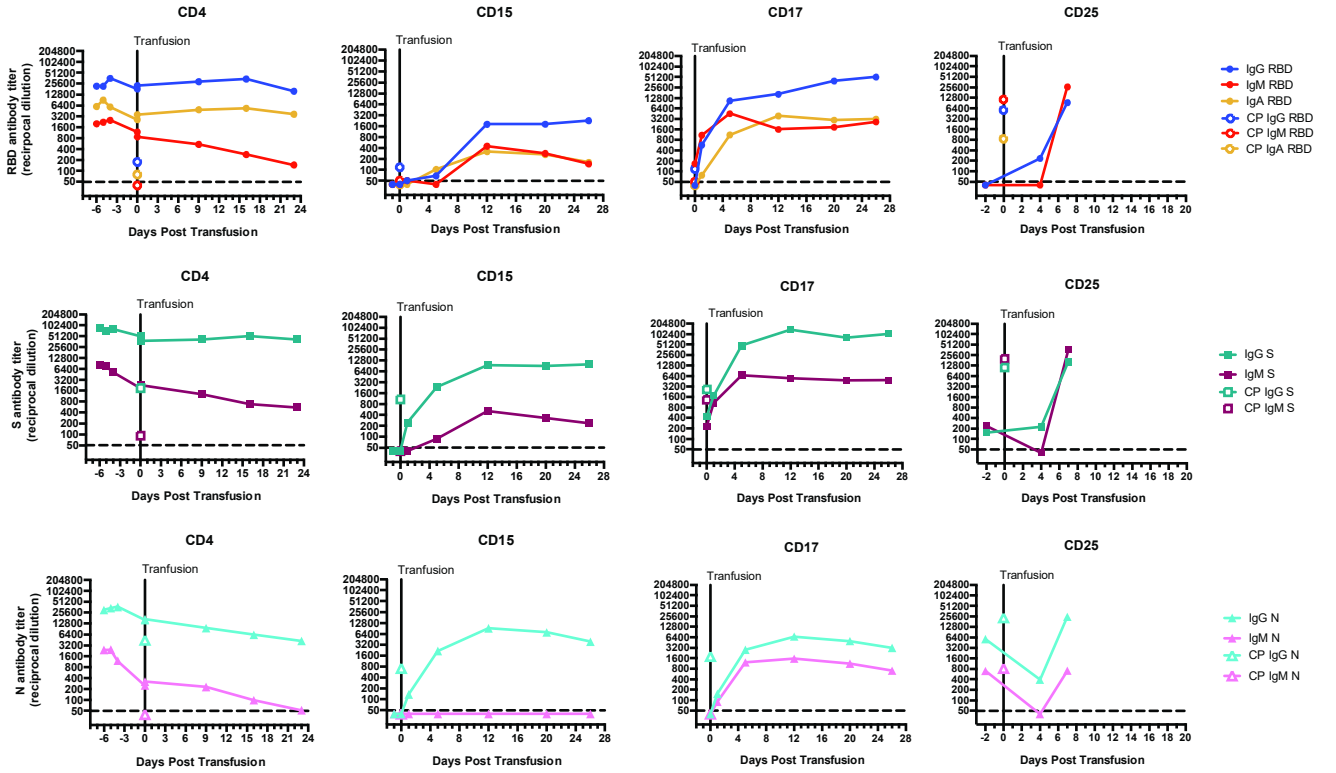


FIGURE 2 Longitudinal serum SARS-CoV-2 antibody titers in pediatric patients receiving convalescent plasma (CP) transfusion therapy. Antibody titers expressed as reciprocal serum dilution against SARS-CoV-2 antigens in four pediatric patients (CD4, CD15, CD17, and CD25), and corresponding CP donors (open symbols) before and after plasma transfusion. Antibody titers against (A-D) the SARS-CoV-2 receptor-binding domain of the spike (S) protein, (E-H) the full-length SARS-CoV-2 S protein (FL), and (I-L) the nucleoprotein of SARS-CoV-2 (N). Dashed lines denote the lower limit of detection (LLOD) of the assay (1:50 serum dilution)

antibody (CR3022) starting at 0.5 μg/mL (RBD and S ELISAs), or serially diluted pooled serum from actively SARS-CoV-2-infected adults (N ELISAs). See Appendix S2 for detailed methods.

3 | RESULTS AND DISCUSSION

Four patients, 14–18 years, were treated with CP during the study period. Patients received all available plasma from the donors, in the range of 200–220 mL. Patient CD4 received 2 mL/kg, and patients CD15, CD17, and CD25 received 4 mL/kg. All patients were critically ill with COVID-19-associated acute respiratory distress syndrome. All patients required intubation and ventilation; two required extracorporeal membrane oxygenation (ECMO). None of the patients had MIS-C. All patients received other SARS-CoV-2-directed therapies in addition to CP. Graphical case summaries are presented in Figure 1. Further details are presented in Appendix S3. No patients experienced any treatment-emergent adverse events (TEAE) related to CP infusion.

Longitudinal antibody titers are presented in Figure 2. Corresponding antibody titers for CP donors are displayed. At the time of CP infusion, patient CD4 had extremely high endogenous antibody titers (against SARS-CoV-2 RBD, S, and N). Antibody titers in this participant were sustained post transfusion. This individual showed transient clinical improvement, decannulating from ECMO; however, died from cardiac causes by day 25 post transfusion. An autopsy was performed, which demonstrated diffuse alveolar damage and hypertrophic cardiomyopathy with associated myocardial fibrosis. Spleen and lung cultures were positive for *Candida glabrata*. There was no evidence of myocarditis.

Patients CD15, CD17, and CD25 had low or undetectable RBD-specific antibody titers at the time of infusion. Serum antibodies against the SARS-CoV-2 RBD correlate with neutralizing activity, likely due to inhibition of the virus' ability to bind and enter target cells.[8–10] Of note, the donor plasma infused into patients CD15 and CD17 had relatively low RBD-specific antibody titer levels (< 1:160). Both CP donors for CD15 and CD17 had substantial full-length IgG S titers (> 1:1000), and these may have conferred some neutralizing activity. It is unclear if CP with low RBD-specific and high full-length IgG S antibody titers would provide benefit. CD15 and CD17 both remain in intensive care and did not have significant clinical improvement following CP infusion, although CD15 did wean off inotropic support shortly after receiving CP. CD17 did not require inotropic support. Both patients continued to require intubation and ventilatory support despite CP. As of August 22, 2020 both CD15 and CD17 remain in hospital and have had the placement of tracheostomies. They both remain on ventilatory support, although at a lower level than required during

acute illness. The donor for patient CD25 had higher SARS-CoV-2 RBD antibody titers (> 1:6000) than the donor for any of the other patients, making it more likely that this donor plasma could be beneficial. CD25 had the shortest period of admission and has been discharged from the hospital after being critically ill and on ECMO.

Prior to transfusion, three of the four recipients (CD15, CD17, and CD25) had undetectable IgG RBD antibody levels (< 1:50 serum dilution). Upon transfusion, all three participants eventually experienced a boost in IgG, IgM, and IgA antibodies against RBD that was sustained for 7-26 days post transfusion. In one participant (CD15), we detected antibodies against the full-length S protein before we detected antibodies against the RBD of the S protein. The boost in antibodies against N post transfusion was similar to that of RBD and S-specific serum antibodies for patients CD15, CD17, and CD25. In all three patients, the serum levels of SARS-CoV-2-specific antibodies eventually surpassed the amount transfused, indicating that these patients mounted a post transfusion de novo response and that CP did not eliminate the endogenous serological response.

In summary, CP was safely administered to four pediatric patients with severe COVID-19. There were no CP-related TEAE. The theoretical risks of ADE or elimination of endogenous antibody response did not occur. CP may be of greatest benefit for patients who are early in their illness and have not yet generated endogenous antibodies, and when the infused plasma has a high antibody titer. The patient who received CP with the highest antibody titer had the best clinical response, although the total antibody response mounted did not differ markedly from the other patients. The small sample size of our study precludes definitive conclusions about efficacy, however, the excellent clinical response in this patient is encouraging. The other patients had significant comorbidities that may have contributed to their clinical status. It is difficult to determine if CD15 and CD17 benefited, as it is possible without CP they could have continued to worsen. Future research should include randomized controlled trials to definitively examine the efficacy of CP in children.

ACKNOWLEDGMENTS

Financial support for this project was provided by CHOP Frontiers Program Immune Dysregulation Team (David T. Teachey, Edward M. Behrens, Hamid Bassiri), National Institute of Allergy and Infectious Diseases (NIAID) (US): R01AI121250 (Edward M. Behrens), R01AI103280 (Audrey R. Odom John), R01AI123433 (Audrey R. Odom John), R21AI144472 (Audrey R. Odom John), K08 AI136660 (Laura A. Vella), and K08AI135091 (Sarah Henrickson); Agency for Healthcare Research and Quality (AHRQ): 5K12HS026393 (Kathleen Chiotos); National Cancer Institute (NCI) (US): R01CA193776 (David T. Teachey), X01HD100702-01 (David T. Teachey), 5UG1CA233249 (David T. Teachey), and R01A1123538 (David T. Teachey); NIH Training in Virology: T32-AI-007324 (Elizabeth M. Anderson); Leukemia and Lymphoma Society (David T. Teachey); Cookies for Kids Cancer (David T. Teachey); Alex's Lemonade Stand Foundation for Childhood Cancer (David T. Teachey); Children's Oncology Group (David T. Teachey); Stand UP 2 Cancer (David T. Teachey); Team Connor (Hamid Bassiri); and Kate Amato Foundations (Hamid Bassiri). Burroughs Wellcome Fund CAMS (SH) and PATH (Audrey R. Odom John); Clinical Immunology Society (SH); and American Academy of Allergy, Asthma, and Immunology (SH). Serological studies were supported by institutional funds from the University of Pennsylvania. Caroline Diorio was supported by the Institute for Translational Medicine and Therapeutics of the Perelman School of Medicine at the University of Pennsylvania. We gratefully acknowledge the gift of plasmids of the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein from Florian Krammer, Mt. Sinai, and the gift of the RBD-specific monoclonal antibody CR3022 from Ian Wilson, Scripps Research Institute. We gratefully acknowledge the providers who cared for these patients, and the generosity of the patients and their families.

CONFLICT OF INTEREST

The authors declare that there is no relevant conflict of interest. David T. Teachey serves on advisory boards for Janssen, Amgen, La Roche, Sobi, and Humanigen. Hamid Bassiri has stock ownership in CSL Behring. Sarah Henrickson serves on the advisory board for Horizon Pharma. Audrey R. Odom John serves on the advisory board of Pluton Biosciences. Michele P. Lambert is an advisory board member for Octapharma, Novartis, Aregenix, and Shionogi, a consultant for Amgen, Novartis, Shionogi, Dova, Bayer, and has received research funding from Sysmex, Novartis, and Astra Zeneca. Kathleen E. Sullivan received personal fees from Elsevier and Immune Deficiency Foundation. Sarah Henrickson has received consultancy fee from Sanofi Pasteur, Lumen, Novavax, and Merck for work unrelated to this report.

AUTHOR CONTRIBUTIONS

Caroline Diorio and Elizabeth M. Anderson equally contributed to this work and are senior first authors. Edward M. Behrens, David T. Teachey, and Hamid Bassiri equally contributed to this work and are co-senior authors. Caroline Diorio, Elizabeth M. Anderson, Sarah E. Henrickson, Laura A. Vella, Kevin O. McNerney, Julie C. Chase, Scott E. Hensley, Edward M. Behrens, Hamid Bassiri, and David T. Teachey designed the study, analyzed data, and wrote the manuscript; Kathleen Chiotos, Michele P. Lambert, Allison M. Blatz, Julie C. Fitzgerald, Todd J. Kilbaugh, Audrey R. Odom John, Kathleen E. Sullivan, David Friedman, Deborah A. Sesok-Pizzini, Margaret R. Tartaglione, Danielle Zambrano, Meghan Martin, Jessica H. Lee, and Pampee Young contributed to the study design, analyzed data, and helped write the manuscript. Elizabeth M. Anderson, Madison E. Weirick, Sigrid Gouma, Eileen C. Goodwin, Marcus J. Bolton, Claudia P. Arevalo performed experiments, provided data, and edited manuscript. Caroline Diorio, Julie C. Chase, and Kevin O. McNerney enrolled subjects. Pampee Young, Deborah A. Sesok-Pizzini, and David Friedman also provided specimens. All authors contributed intellectually and reviewed and revised the manuscript.

FUNDING INFORMATION

Children's Hospital of Philadelphia Frontiers Program Immune Dysregulation Team, National Institute of Allergy and Infectious Diseases (NIAID) (US): R01AI121250, R01AI103280, R01AI123433, R21AI144472, K08 AI136660, and K08AI135091; Agency for Healthcare Research and Quality (AHRQ): 5K12HS026393; National

Cancer Institute (NCI) (US): R01CA193776, X01HD100702-01, 5UG1CA233249, and R01A1123538; NIH Training in Virology: T32-AI-007324; Leukemia and Lymphoma Society; Cookies for Kids Cancer; Alex's Lemonade Stand Foundation for Childhood Cancer; Children's Oncology Group; Stand UP 2 Cancer; Team Connor; and Kate Amato Foundations. Burroughs Wellcome Fund CAMS and PATH; Clinical Immunology Society; and American Academy of Allergy, Asthma, and Immunology; Institutional funds from the University of Pennsylvania; Institute for Translational Medicine and Therapeutics of the Perelman School of Medicine at the University of Pennsylvania

ORCID

Elizabeth M. Anderson https://orcid.org/0000-0002-1168-4773
Kevin O. McNerney https://orcid.org/0000-0002-4359-3569
Allison M. Blatz https://orcid.org/0000-0003-2525-1212
Michele P. Lambert https://orcid.org/0000-0003-0439-402X
David T. Teachey https://orcid.org/0000-0001-7373-8987

REFERENCES

1. Dong Y, Mo X, Hu Y, et al. Epidemiological characteristics of 2143 pediatric patients with 2019 coronavirus diseasein China. *Pediatrics* 2020;146:e20200702.
2. Liu W, Zhang Q, Chen J, et al. Detection of Covid-19 in children in early January 2020 in Wuhan, China. *N Engl J Med*. 2020;382(14):1370-1371.
3. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020;395(10223):497-506.
4. Zachariah P, Johnson CL, Halabi KC, et al. Epidemiology, clinical features, and diseaseseverity in patients with coronavirus disease2019 (COVID-19) in a children's hospital in New York City, New York. *JAMA Pediatr.* 2020:e202430. https://doi.org/10.1001/jamapediatrics.2020.2430
5. Chao JY, Derespina KR, Herold BC, et al. Clinical characteristics and outcomes of hospitalized and critically ill children and adolescents with coronavirus disease2019 (COVID-19) at a tertiary care medical center in New York City. *J Pediatr.* 2020;223:14-19.e2.
6. Bloch EM, Shoham S, Casadevall A, et al. Deployment of convalescent plasma for the prevention and treatment of COVID-19. *J Clin Invest* 2020;130(6):2757-2765.
7. Hachim A, Kavian N, Cohen CA, et al. Beyond the spike: identification of viral targets of the antibody response to SARS-CoV-2in COVID-19 patients. *medRxiv* 2020. https://doi.org/10.1101/2020.04.30.20085670
8. Flannery D, Gouma S, Dhudasia M, et al. SARS-CoV-2 seroprevalence among parturient women. 2020. https://www.researchsquare.com/article/rs-27402/v1 . Accessed August 23, 2020.
9. Amanat F, Stadlbauer D, Strohmeier S, et al. A serological assay to detect SARS-CoV-2 seroconversion in humans. *Nat Med.* 2020;26:1033-1036.
10. Okba NM, Müller MA, Li W, et al. Severe acute respiratory syndrome coronavirus 2-specific antibody responsesin coronavirus diseasepatients. *Emerg Infect Dis.* 2020;26(7):1478-1488.
11. Premkumar L, Segovia-Chumbez B, Jadi R, et al. The receptor-binding domain of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients. *Sci Immunol* 2020;5(48):eabc8413.
12. Leung DT, Tam FC, Ma CH, et al. Antibody response of patients with severe acute respiratory syndrome (SARS) targets the viral nucleocapsid. *J Infect Dis.* 2004;190(2):379-386.
13. Lv H, Wu NC, Tsang OT, et al. Cross-reactive antibody response between SARS-CoV-2 and SARS-CoV infections. *Cell Rep* 2020;31(9):107725.
14. Shen C, Wang Z, Zhao F, et al. Treatment of 5 critically ill patients with COVID-19 with convalescent plasma. *JAMA* 2020;323(16):1582-1589.
15. Zhang B, Liu S, Tan T, et al. Treatment with convalescent plasma for critically ill patients with severe acute respiratory syndrome coronavirus 2 infection. *Chest* 2020;158(1):e9-e13.
16. Duan K, Liu B, Li C, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc Natl Acad Sci U S A.* 2020;117(17):9490-9496.
17. Joyner MJ, Wright RS, Fairweather D, et al. Early safety indicators of COVID-19 convalescent plasma in 5000 patients [published online ahead of print August 10, 2020]. *J Clin Invest* 2020:140200. https://doi.org/10.1172/JCI140200
18. Li L, Zhang W, Hu Y, et al. Effect of convalescent plasma therapy on time to clinical improvement in patients with severe and life-threatening COVID-19: a randomized clinical trial. *JAMA* 2020;324(5):1-11.
19. Yip MS, Leung NHL, Cheung CY, et al. Antibody-dependent infection of human macrophages by severe acute respiratory syndrome coronavirus. *Virol J.* 2014;11(1):82.
20. Wang S-F, Tseng S-P, Yen C-H, et al. Antibody-dependent SARS coronavirus infection is mediated by antibodies against spike proteins. *Biochem Biophys Res Commun* 2014;451(2):208-214.
21. Agrawal AS, Tao X, Algaissi A, et al. Immunization with inactivated Middle East Respiratory Syndrome coronavirus vaccine leads to lung immunopathology on challenge with live virus. *Hum Vaccin Immunother* 2016;12(9):2351-2356.
22. Diorio C, Henrickson SE, Vella LA, et al. Multisystem inflammatory syndrome in children and COVID-19 are distinct presentations of SARS-CoV-2 [published online ahead of print July 30, 2020]. *J Clin Invest* 2020:140970. https://doi.org/10.1172/JCI140970

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Diorio C, Anderson EM, McNerney KO, et al. Convalescent plasma for pediatric patients with SARS-CoV-2-associated acute respiratory distress syndrome. *Pediatr Blood Cancer* 2020;e28693. https://doi.org/10.1002/pbc.28693

EXCEPTIONAL CASE REPORT

blood advances

Check for updates

COVID-19 convalescent plasma clears SARS-CoV-2 refractory to remdesivir in an infant with congenital heart disease

Zahidee Rodriguez,[1] Andi L. Shane,[2] Hans Verkerke,[3] Christopher Lough,[4] Matthew G. Zimmerman,[2,5,6] Mehul Suthar,[2,5,6] Jens Wrammert,[2,5] Heather MacDonald,[7] Michael Wolf,[1] Shanelle Clarke,[1] John D. Roback,[3] Connie M. Arthur,[3] Sean R. Stowell,[3,*] and Cassandra D. Josephson[3,8,*]

[1]Division of Cardiology, [2]Division of Infectious Disease, Department of Pediatrics, Children's Healthcare of Atlanta, and [3]Department of Pathology and Laboratory Medicine, Center for Transfusion and Cellular Therapies, Emory University School of Medicine, Atlanta, GA; [4]LifeSouth Community Blood Centers, Inc., Gainesville, FL; [5]Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA; [6]Yerkes National Primate Research Center, Atlanta, GA; [7]Department of Pathology, Children's Healthcare of Atlanta, Atlanta, GA; and [8]Department of Pediatrics, Aflac Cancer Center and Blood Disorders Service, Emory University School of Medicine and Children's Healthcare of Atlanta, Atlanta, GA

Key Points

- ü COVID-19 convalescent plasma (CP) may be a safe and effective treatment option in SARS-CoV-2 infection refractory to remdesivir.
- ü Infants may benefit from CP despite developing immune systems and donor variability emphasizes the need for characterization prior to use.

Introduction

Although , 2% of coronavirus disease 2019 (COVID-19) infections are reported in the pediatric population, children with comorbidities such as congenital heart disease and those at a young age are more likely to become critically ill.[1-3] Remdesivir has been reported to be efficacious in adults with COVID-19[4]; however, there are no studies in children. Convalescent plasma (CP) can contain neutralizing antibodies to viruses,[5] and has been used during previous viral epidemics with clinical improvement.[6-11] COVID-19 CP (C19-CP) may be useful in critically ill adults, resulting in improvement in inflammatory markers, pulmonary lesions, and mortality.[12] However, the impact of C19-CP in pediatric patients, particularly infants with developing immune systems and significant comorbidities, is completely unknown.

We present an infant with cardiopulmonary failure secondary to unrepaired congenital heart disease exacerbated by COVID-19. Given postsurgical complications of children with viral respiratory infection,[13-17] the patient required clearance of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) for surgical candidacy. We hypothesized that C19-CP administration may clear SARS-CoV-2 following failure of remdesivir.

Case description

A 3.1-kg term 9-week-old female with trisomy 21 and unrepaired balanced complete atrioventricular canal defect with baseline mild to moderate common atrioventricular valve regurgitation (AVVR) presented in acute hypoxemic respiratory failure secondary to progressive decompensated heart failure. Notable findings included lymphopenia, b-natriuretic peptide of 530 pg/mL (reference range , 100 pg/mL), worsened AVVR (moderate to severe), and cardiomegaly and pulmonary edema on chest X-ray. Initial treatment, including bilevel noninvasive positive pressure ventilation, milrinone infusion, and IV diuretics, resulted in restoration of organ function. By hospital day 8, her common AVVR returned to baseline; she transitioned from milrinone to captopril, carvedilol, and IV diuretics and had weaned to high-flow nasal cannula; and she was transferred to the general cardiology floor.

On hospital day 12, she developed acute hypoxemic respiratory failure and decompensated heart failure, with atelectasis on chest X-ray and new rhinorrhea requiring bilevel noninvasive positive pressure ventilation. The result of SARS-CoV-2 nucleic acid testing (NAT) (DiaSorin Molecular Simplexa direct real-time PCR of viral RNA, Cypress, CA) on a nasopharyngeal (NP) swab was positive; a residual sample from a respiratory viral panel performed on hospital day 5 for intermittent desaturations was retested for SARS-CoV-2 and likewise had a positive result (Figure 1A). The source of the infection remains unclear. While the mother and siblings were asymptomatic, they were quarantined for 14 days after infection was identified in the patient but never tested. Nosocomial infection is certainly possible

Submitted 29 May 2020; accepted 6 July 2020; published online 11 September 2020.
DOI 10.1182/bloodadvances.2020002507.

*S.R.S. and C.D.J. contributed equally to this study.

Requests for data sharing should be e-mailed to the corresponding author at rodriguezz@kidsheart.com.

© 2020 by The American Society of Hematology

Downloaded from https://ashpublications.org/bloodadvances/article-pdf/4/18/4278/1757955/advancesadv2020002507.pdf by guest on 12 September 2020

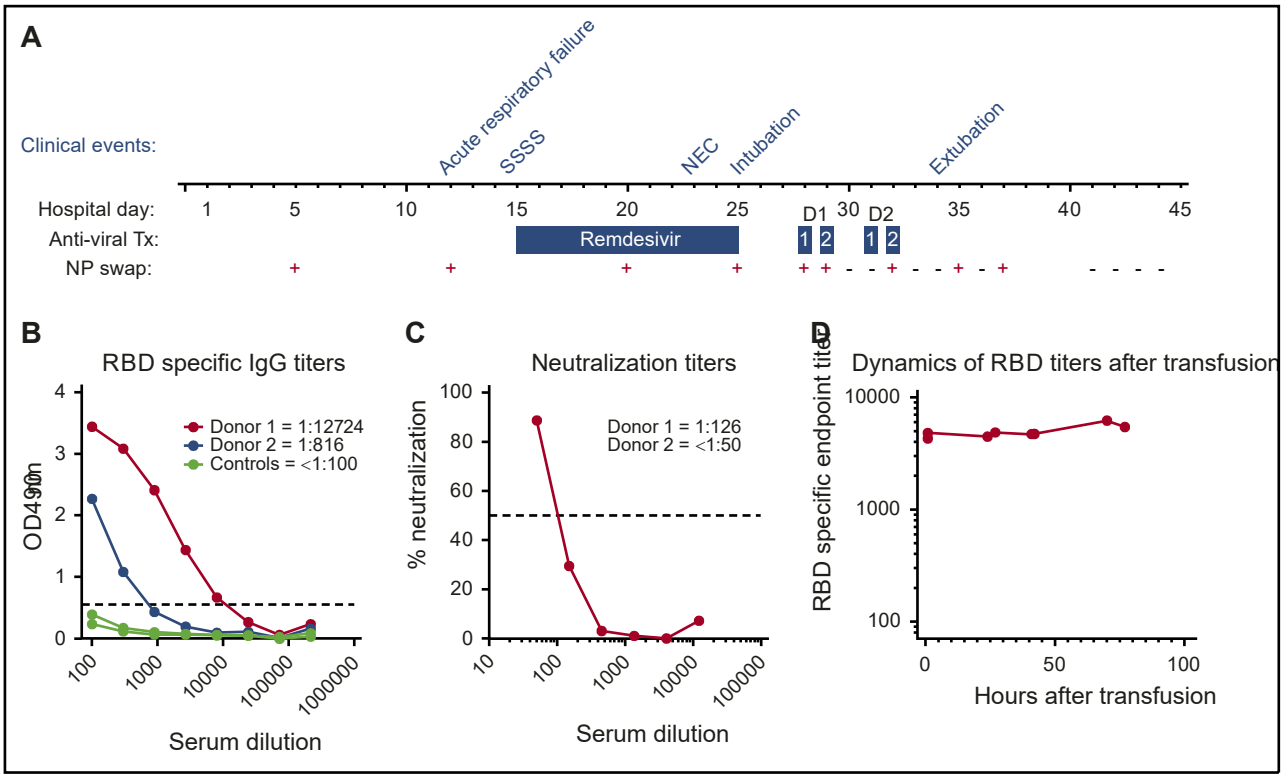


Figure 1. Hospital course summary and donor and patient plasma characterization. (A) Hospital course by clinical event, antiviral therapy (Tx), and C19-CP transfusion in relation to NP swab SARS-CoV-2 RNA results. Clinical events are as follows: acute respiratory failure on hospital day 12, staphylococcal scalded skin syndrome (SSSS) on hospital day 14, necrotizing enterococcus (NEC) on hospital day 23, intubation on hospital day 25, and extubation on hospital day 34. Dosages of antiviral therapy are as follows: remdesivir: 5 mg/kg per day on hospital day 15 and 2.5 mg/kg/day on hospital days 16 to 25; C19-CP donor 1: 10 mL/kg per day over 4 hours; 20 hours apart on hospital days 28 and 29; and C19-CP donor 2: 10 mL/kg per day over 4 hours; 20 hours apart on hospital days 31 and 32. (B) Enzyme-linked immunosorbent assays showing titration of samples from the 2 C19-CP donors and 3 negative controls collected before 2019, against SARS-CoV-2 spike RBD. End point titers were calculated using a cutoff of the average of the control samples, plus 3 times standard deviation value, marked with a dotted line. End points were calculated as the intersection of samples with this cutoff. Titers are shown numerically in the legend. (C) Neutralization activity of C19-CP donor samples against SARS-CoV-2. The focus reduction neutralization test $(FRNT)_{50}$ titers of the C19-CP donor samples were determined by an FRNT assay using an immunostain to detect infected foci. The dotted line represents the maximum concentrations of the serum tested (1/50). (D) Dynamics of RBD-specific end-point titers in the recipient after transfusion.

but less likely, as the hospital had very few infected patients, none of those infected were in the cardiac intensive care unit, and there was no shortage of personal protective gear at the hospital prior or following the patient's infection. Additional investigation did not reveal macrophage activation syndrome or new bacterial or viral infection. As her prolonged QTc prohibited use of hydroxychloroquine, remdesivir (5 mg/kg per day on hospital day 15 and 2.5 mg/kg per day on hospital days 16-25) was given. Her hospital course was complicated by staphylococcal scalded skin syndrome, treated with nafcillin, and stage II necrotizing enterocolitis (Bell criteria), treated with piperacillin-tazobactam (Figure 1A). Throughout this time, she continued to have persistent lymphopenia, a C-reactive protein (CRP) level of 5 mg/dL (reference range, , 1.0 mg/dL) with normal fibrinogen, and a common AVVR that remained unchanged with preserved biventricular function managed with IV diuretics.

Despite completion of remdesivir, on hospital day 25, she required endotracheal intubation for worsening respiratory failure. Repeat investigation was notable for persistent lymphopenia, persistent SARS-CoV-2, CRP 7.7 mg/dL, but no evidence of new secondary infection or macrophage activation syndrome was found. Milrinone was reinitiated in addition to IV diuretics. Given her lack of response to remdesivir and deteriorating clinical status, 2 aliquots of CP (10 mL/kg per aliquot) from 2 COVID-19 recovered donors (C19-CP) were transfused in accordance with the World Health Organization guidelines for Ebola CP treatment in children[9] (Figure 1A). She received increased IV diuretics, daily monitoring of serum chemistries, and intermittent monitoring of coagulopathy. Daily surveillance of SARS-CoV-2 NAT by NP swab was performed, and the results were negative following the second aliquot from donor 1 (Figure 1A) with concurrent reduced ventilator support. She was extubated on hospital day 34. The infant tolerated all transfusions without anaphylaxis, transfusion-related circulatory overload, transfusion-related acute lung injury, or hypercoagulability. The results of daily NP swabs were intermittently positive before becoming persistently negative (Figure 1A). Other notable findings post–C19-CP transfusion include CRP of 1.3 mg/dL and intermittent normalization of absolute lymphocyte count. The patient had an uneventful complete repair 47 days (hospital day 91) after SARS-CoV-2 infection resolution and is doing well.

Downloaded from https://ashpublications.org/bloodadvances/article-pdf/4/18/4278/1757955/advancesadv2020002507.pdf by guest on 12 September 2020

Methods

The patient is blood group O RhD negative. Donor 1 is a 63-year-old male who donated 35 days following COVID-19 symptom resolution and is blood type A RhD positive. Donor 2 is a 56-year-old male who donated 55 days following COVID-19 symptom resolution and is blood type O RhD negative. Both had mild COVID-19 infection, and neither was hospitalized. The plasma donor units were transfused without prior characterization per emergency use. To understand the impact of C19-CP treatment, both donor units were tested for titers of IgG against the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein (Emory University and Children's Healthcare of Atlanta, Atlanta, GA), which has been shown to correlate with neutralizing titers,[18] and subsequent microneutralization assays were performed in a biosafety level 3 laboratory.[19] Donor 1 had an immunoglobulin G (IgG) titer of 1:12 724 and neutralizing titer of 1:126. Donor 2 had an IgG titer of 1:816 and a neutralizing titer of , 1:50 (Figure 1B-C, respectively). Following the first aliquot from donor 1, the recipient showed robust IgG anti-SARS-CoV-2 antibody levels of ; 1:5000, which was sustained after all subsequent transfusions (Figure 1D).

Results and discussion

Young age, unrepaired congenital heart disease, trisomy 21, persistent lymphopenia, and naive immune system likely increased this patient's SARS-CoV-2 vulnerability. While this represents only 1 case, her improved respiratory status, increase in lymphocytes, and decrease in CRP after C19-CP transfusion suggest a positive clinical correlation, especially in the setting of subsequent SARS-CoV-2 clearance.

Logistical constraints prevented anti-SARS-CoV-2 antibody characterization of C19-CP prior to transfusion, which likely reflects common current approaches to emergent C19-CP administration. C19-CP from donor 1 likely provided the most significant impact on SARS-CoV-2 eradication given the higher neutralizing titer (Figure 1C), which is comparable to US Food and Drug Administration recommendations[20] and may be sufficient when treating COVID-19 in adults.[10-21] This case suggests that such titers may likewise be sufficient in infants. The delay in viral clearance may reflect the time required for C19-CP therapy to effectively eliminate the virus and is consistent with reports in adults with persistent SARS-CoV-2 NAT by NP samples beyond symptom resolution.[22,23]

Factors influencing high anti-SARS-CoV-2 titers with effective characteristics remain incompletely understood. Severe illness would be presumed to correlate with higher anti-SARS-CoV-2 antibodies. However, even though neither C19-CP donor was hospitalized, donor 1 possessed higher titers than donor 2, whose titers were below the limit of detection. This variability illustrates the challenge of current C19-CP transfusion practice, making it difficult to determine the potential benefit without adequate testing prior to C19-CP transfusion in place.

While neutralizing antibodies are critical for antibody-mediated protection against viral infection, the impact of CP on resolution of active infection remains incompletely understood. However, beyond direct neutralization of the virus, other mechanisms, such as antibody-dependent cellular cytotoxicity, may also play a role in the ability of C19-CP to aid in the clearance of an active infection. Such considerations may be especially important in infants with developing or suppressed immune systems that may attenuate the ability of other antibody effector systems to effectively clear COVID-19 following C19-CP therapy. While we were unfortunately unable to acquire neutralizing titer data from the patient prior to C19-CP administration to determine potential changes in antibody levels prior to and following administration of the first dose of C19-CP, data obtained following serial SARS-CoV-2 NAT evaluation provides uncommon insight into the temporal relationship between C19-CP and its potential impact on SARS-CoV-2 infection. However, this is a single case, and understandable uncertainties remain. Future studies will certainly be needed to fully define the efficacy of C19-CP in this vulnerable patient population.

Acknowledgments

The authors thank their colleagues at LifeSouth for collection of donor plasma. Parental consent was obtained for treatment with C19-CP. Review of medical records, publication of results, and research use of biospecimens per the Declaration of Helsinki under the protocols were approved by the Institutional Review Board of Emory University School of Medicine and the Department of Health and Human Services.

Authorship

Contribution: Z.R., S.R.S., and C.D.J. conceived of and wrote the manuscript; S.R.S., C.D.J., Z.R., M.S., and J.W. prepared the figures; A.L.S., H.V., C.L., M.G.Z., M.S., J.W., H.M., M.W., S.C., J.D.R.C., M.A., S.R.S., and C.D.J. critically reviewed, analyzed, and interpreted the data; M.G.Z., J.W., M.S., H.M., C.M.A., and S.R.S. performed the immunologic assays; and C.L. collected the donor units and information on blood donors.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: Z.R., 0000-0002-0804-7132; A.L.S., 0000-0003-0606-7188.

Correspondence: Zahidee Rodriguez, Emory University, Children's Healthcare of Atlanta–Egleston, 1405 Clifton Rd, 2nd Floor, Tower 1, Atlanta, GA 30322; e-mail: rodriguezz@kidsheart.com.

References

1. Dong Y, Mo X, Hu Y, et al. Epidemiology of COVID-19 among children in China. Pediatrics. 2020;145(6):e20200702.
2. CDC COVID-19 Response Team. Coronavirus disease 2019 in children: United States, February 12-April 2, 2020. MMWR Morb Mortal Wkly Rep. 2020;69(14):422-426.
3. DeBiasi RL, Song X, Delaney M, et al. Severe COVID-19 in children and young adults in the Washington, DC metropolitan region. J Pediatr. 2020;223:199-203.e1.

Downloaded from https://ashpublications.org/bloodadvances/article-pdf/4/18/4278/1757955/advancesadv2020002507.pdf by guest on 12 September 2020

4. Ledford H. Hopes rise for coronavirus drug remdesivir [published online ahead of print 29 April 2020]. Nature. doi:10.1038/d41586-020-01295-8.
5. Zhang JS, Chen JT, Liu YX, et al. A serological survey on neutralizing antibody titer of SARS convalescent sera. J Med Virol. 2005;77(2):147-150.
6. Luke TC, Kilbane EM, Jackson JL, Hoffman SL. Meta-analysis: convalescent blood products for Spanish influenza pneumonia: a future H5N1 treatment? Ann Intern Med. 2006;145(8):599-609.
7. Hung IF, To KK, Lee CK, et al. Convalescent plasma treatment reduced mortality in patients with severe pandemic influenza A (H1N1) 2009 virus infection. Clin Infect Dis. 2011;52(4):447-456.
8. Hung IFN, To KKW, Lee CK, et al. Hyperimmune IV immunoglobulin treatment: a multicenter double-blind randomized controlled trial for patients with severe 2009 influenza A(H1N1) infection. Chest. 2013;144(2):464-473.
9. van Griensven J, Edwards T, de Lamballerie X, et al; Ebola-Tx Consortium. Evaluation of convalescent plasma for Ebola virus disease in Guinea. N Engl J Med. 2016;374(1):33-42.
10. Cheng Y, Wong R, Soo YO, et al. Use of convalescent plasma therapy in SARS patients in Hong Kong. Eur J Clin Microbiol Infect Dis. 2005;24(1):44-46.
11. Yeh KM, Chiueh TS, Siu LK, et al. Experience of using convalescent plasma for severe acute respiratory syndrome among healthcare workers in a Taiwan hospital. J Antimicrob Chemother. 2005;56(5):919-922.
12. Shen C, Wang Z, Zhao F, et al. Treatment of 5 critically ill patients with COVID-19 with convalescent plasma. JAMA. 2020;323(16):1582.
13. Malviya S, Voepel-Lewis T, Siewert M, Pandit UA, Riegger LQ, Tait AR. Risk factors for adverse postoperative outcomes in children presenting for cardiac surgery with upper respiratory tract infections. Anesthesiology. 2003;98(3):628-632.
14. Tait AR, Malviya S, Voepel-Lewis T, Munro HM, Seiwert M, Pandit UA. Risk factors for perioperative adverse respiratory events in children with upper respiratory tract infections. Anesthesiology. 2001;95(2):299-306.
15. Mallory MD, Travers C, McCracken CE, Hertzog J, Cravero JP. Upper respiratory infections and airway adverse events in pediatric procedural sedation. Pediatrics. 2017;140(1):e20170009.
16. Parnis SJ, Barker DS, Van Der Walt JH. Clinical predictors of anaesthetic complications in children with respiratory tract infections. Paediatr Anaesth. 2001;11(1):29-40.
17. Tanne JH. Covid-19: FDA approves use of convalescent plasma to treat critically ill patients. BMJ. 2020;368:m1256.
18. To KK, Tsang OT, Leung WS, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. Lancet Infect Dis. 2020;20(5):565-574.
19. Suthar MS, Zimmerman M, Kauffman R, et al. Rapid generation of neutralizing antibody responses in COVID-19 patients. medRxiv. 2020;1(3):100040.
20. FDA. Recommendations for investigational COVID-19 convalescent plasma. https://www.fda.gov/vaccines-blood-biologics/investigational-new-drug-ind-or-device-exemption-ide-process-cber/recommendations-investigational-covid-19-convalescent-plasma. Accessed 1 May 2020.
21. Bloch EM, Shoham S, Casadevall A, et al. Deployment of convalescent plasma for the prevention and treatment of COVID-19. J Clin Invest. 2020;130(6):2757-2765.
22. Sethuraman N, Jeremiah SS, Ryo A. Interpreting diagnostic tests for SARS-CoV-2. JAMA. 2020;323(22):2249.
23. Wajnberg A, Mansour M, Leven E, et al. Humoral immune response and prolonged PCR positivity in a cohort of 1343 SARS-CoV2 patients in the New York City region [published online ahead of print 5 May 2020]. medRxiv. doi:10.1101/2020.04.30.20085613.

Downloaded from https://ashpublications.org/bloodadvances/article-pdf/4/18/4278/1757955/advancesadv2020002507.pdf by guest on 12 September 2020

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME:

eRA COMMONS USER NAME (credential, e.g., agency login):

POSITION TITLE:

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY

A. Personal Statement

B. Positions and Honors

C. Contributions to Science

D. Additional Information: Research Support and/or Scholastic Performance

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES**.

NAME: Hunt, Morgan Casey

eRA COMMONS USER NAME (credential, e.g., agency login): huntmc

POSITION TITLE: Associate Professor of Psychology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of California, Berkeley	B.S	05/1990	Psychology
University of Vermont	Ph.D.	05/1996	Experimental Psychology
University of California, Berkeley	Postdoctoral	08/1998	Public Health and Epidemiology

A. Personal Statement

I have the expertise, leadership, training, expertise and motivation necessary to successfully carry out the proposed research project. I have a broad background in psychology, with specific training and expertise in ethnographic and survey research and secondary data analysis on psychological aspects of drug addiction. My research includes neuropsychological changes associated with addiction. As PI or co-Investigator on several university- and NIH-funded grants, I laid the groundwork for the proposed research by developing effective measures of disability, depression, and other psychosocial factors relevant to the aging substance abuser, and by establishing strong ties with community providers that will make it possible to recruit and track participants over time as documented in the following publications. In addition, I successfully administered the projects (e.g. staffing, research protections, budget), collaborated with other researchers, and produced several peer-reviewed publications from each project. As a result of these previous experiences, I am aware of the importance of frequent communication among project members and of constructing a realistic research plan, timeline, and budget. The current application builds logically on my prior work. During 2005-2006 my career was disrupted due to family obligations. However, upon returning to the field I immediately resumed my research projects and collaborations and successfully competed for NIH support.

1. Merryle, R.J. & Hunt, M.C. (2004). Independent living, physical disability and substance abuse among the elderly. Psychology and Aging, 23(4), 10-22.
2. Hunt, M.C., Jensen, J.L. & Crenshaw, W. (2007). Substance abuse and mental health among community-dwelling elderly. International Journal of Geriatric Psychiatry, 24(9), 1124-1135.
3. Hunt, M.C., Wiechelt, S.A. & Merryle, R. (2008). Predicting the substance-abuse treatment needs of an aging population. American Journal of Public Health, 45(2), 236-245. PMCID: PMC9162292 Hunt, M.C., Newlin, D.B. & Fishbein, D. (2009). Brain imaging in methamphetamine abusers across the life-span. Gerontology, 46(3), 122-145.

SAMPLE

B. Positions and Honors

Positions and Employment

1998-2000	Fellow, Division of Intramural Research, National Institute of Drug Abuse, Bethesda, MD
2000-2002	Lecturer, Department of Psychology, Middlebury College, Middlebury, VT
2001-	Consultant, Coastal Psychological Services, San Francisco, CA
2002-2005	Assistant Professor, Department of Psychology, Washington University, St. Louis, MO
2007-	Associate Professor, Department of Psychology, Washington University, St. Louis, MO

Other Experience and Professional Memberships

1995-	Member, American Psychological Association
1998-	Member, Gerontological Society of America
1998-	Member, American Geriatrics Society
2000-	Associate Editor, Psychology and Aging
2003-	Board of Advisors, Senior Services of Eastern Missouri
2003-05	NIH Peer Review Committee: Psychobiology of Aging, ad hoc reviewer
2007-11	NIH Risk, Adult Addictions Study Section, members

Honors

2003	Outstanding Young Faculty Award, Washington University, St. Louis, MO
2004	Excellence in Teaching, Washington University, St. Louis, MO
2009	Award for Best in Interdisciplinary Ethnography, International Ethnographic Society

C. Contribution to Science

1. My early publications directly addressed the fact that substance abuse is often overlooked in older adults. However, because many older adults were raised during an era of increased drug and alcohol use, there are reasons to believe that this will become an increasing issue as the population ages. These publications found that older adults appear in a variety of primary care settings or seek mental health providers to deal with emerging addiction problems. These publications document this emerging problem but guide primary care providers and geriatric mental health providers to recognize symptoms, assess the nature of the problem and apply the necessary interventions. By providing evidence and simple clinical approaches, this body of work has changed the standards of care for addicted older adults and will continue to provide assistance in relevant medical settings well into the future. I served as the primary investigator or co-investigator in all of these studies.
    a. Gryczynski, J., Shaft, B.M., Merryle, R., & Hunt, M.C. (2002). Community based participatory research with late-life addicts. American Journal of Alcohol and Drug Abuse, 15(3), 222-238.
    b. Shaft, B.M., Hunt, M.C., Merryle, R., & Venturi, R. (2003). Policy implications of genetic transmission of alcohol and drug abuse in female nonusers. International Journal of Drug Policy, 30(5), 46-58.
    c. Hunt, M.C., Marks, A.E., Shaft, B.M., Merryle, R., & Jensen, J.L. (2004). Early-life family and community characteristics and late-life substance abuse. Journal of Applied Gerontology, 28(2),26-37.
    d. Hunt, M.C., Marks, A.E., Venturi, R., Crenshaw, W. & Ratonian, A. (2007). Community-based intervention strategies for reducing alcohol and drug abuse in the elderly. Addiction, 104(9), 1436-1606. PMCID: PMC9000292

2. In addition to the contributions described above, with a team of collaborators, I directly documented the effectiveness of various intervention models for older substance abusers and demonstrated the importance of social support networks. These studies emphasized contextual factors in the etiology and maintenance of addictive disorders and the disruptive potential of networks in substance abuse treatment. This body of work also discusses the prevalence of alcohol, amphetamine, and opioid abuse in older adults and how networking approaches can be used to mitigate the effects of these disorders.
    a. Hunt, M.C., Merryle, R. & Jensen, J.L. (2005). The effect of social support networks on morbidity among elderly substance abusers. Journal of the American Geriatrics Society, 57(4), 15-23.
    b. Hunt, M.C., Pour, B., Marks, A.E., Merryle, R. & Jensen, J.L. (2005). Aging out of methadone treatment. American Journal of Alcohol and Drug Abuse, 15(6), 134-149.

SAMPLE

c. Merryle, R. & Hunt, M.C. (2007). Randomized clinical trial of cotinine in older nicotine addicts. Age and Ageing, 38(2), 9-23. PMCID: PMC9002364

3. Methadone maintenance has been used to treat narcotics addicts for many years but I led research that has shown that over the long-term, those in methadone treatment view themselves negatively and they gradually begin to view treatment as an intrusion into normal life. Elderly narcotics users were shown in carefully constructed ethnographic studies to be especially responsive to tailored social support networks that allow them to eventually reduce their maintenance doses and move into other forms of therapy. These studies also demonstrate the policy and commercial implications associated with these findings.

   a. Hunt, M.C. & Jensen, J.L. (2003). Morbidity among elderly substance abusers. Journal of the Geriatrics, 60(4), 45-61.
   b. Hunt, M.C. & Pour, B. (2004). Methadone treatment and personal assessment. Journal Drug Abuse, 45(5), 15-26.
   c. Merryle, R. & Hunt, M.C. (2005). The use of various nicotine delivery systems by older nicotine addicts. Journal of Ageing, 54(1), 24-41. PMCID: PMC9112304
   d. Hunt, M.C., Jensen, J.L. & Merryle, R. (2008). The aging addict: ethnographic profiles of the elderly drug user. NY, NY: W. W. Norton & Company.

Complete List of Published Work in MyBibliography:
http://www.ncbi.nlm.nih.gov/sites/myncbi/collections/public/1PgT7IEFIAJBtGMRDdWFmjWAO/?sort=date&direction=ascending

D. Additional Information: Research Support and/or Scholastic Performance

Ongoing Research Support

R01 DA942367 Hunt (PI) 09/01/08-08/31/16
Health trajectories and behavioral interventions among older substance abusers
The goal of this study is to compare the effects of two substance abuse interventions on health outcomes in an urban population of older opiate addicts.
Role: PI

R01 MH922731 Merryle (PI) 12/15/07-11/30/15
Physical disability, depression and substance abuse in the elderly
The goal of this study is to identify disability and depression trajectories and demographic factors associated with substance abuse in an independently-living elderly population.
Role: Co-Investigator

Faculty Resources Grant, Washington University 08/15/09-08/14/15
Opiate Addiction Database
The goal of this project is to create an integrated database of demographic, social and biomedical information for homeless opiate abusers in two urban Missouri locations, using a number of state and local data sources.
Role: PI

Completed Research Support

R21 AA998075 Hunt (PI) 01/01/11-12/31/13
Community-based intervention for alcohol abuse
The goal of this project was to assess a community-based strategy for reducing alcohol abuse among older individuals.
Role: PI

SAMPLE

[Insert Letterhead here]

XX September 2020

Dr. Peter Daszak
President
EcoHealth Alliance
520 Eighth Avenue, Ste. 1200
New York, NY 10018

Dear Dr. Daszak,

This letter expresses the high interest that staff at [insert name of institution] has in collaborative research with EcoHealth Alliance and other partners on the proposed NIAID R01 research entitled "Analyzing the potential for future bat coronavirus emergence in Southeast Asia".

The [insert name of institution] recognizes the mutual benefits to be gained through research cooperation. The successful partnership on the proposed research will advance our understanding of the ability of bat-origin coronaviruses to spillover into high-risk human populations and cause illnesses that may have been previously unreported, or undiagnosed. We believe the results will be scientifically important, and have great public health value for [Insert country name] and the region by identifying key pandemic threats.

We at [insert name of institution] look forward to working with EcoHealth Alliance on this important research. Our work will include managing [Insert sentence on key roles in project, e.g. "human surveillance, wildlife surveillance, sample collection, testing, and development of laboratory diagnostics"]. We will also actively take part in knowledge sharing and research cross-training with other partners, and are willing to work together with other partner to strengthen the ability of [Insert country name] and other countries in the region to respond to epidemic disease outbreaks.

Sincerely,

[Insert signature]

[Insert full name, title, institution, address, and email]

EXCEPTIONAL CASE REPORT

blood advances

Successful transfer of anti–SARS-CoV-2 immunity using convalescent plasma in an MM patient with hypogammaglobulinemia and COVID-19

Tim Luetkens,[1,2] Ryan Metcalf,[3,4] Vicente Planelles,[3] Yue Zheng,[3] Erin T. Larragoite,[3] Emily S. Spivak,[5] Adam M. Spivak,[5] Mary Steinbach,[1] Robert C. Blaylock,[3,4] Stephanie V. Avila,[1] Kim G. Hankey,[2] Thomas B. Martins,[3,4] Patricia R. Slev,[3,4] Heather D. Mannuel,[6,7] Mohammad Sajadi,[8] Aaron P. Rapoport,[2] and Djordje Atanackovic[1,2]

[1]Hematology and Hematologic Malignancies, Huntsman Cancer Institute, University of Utah, Salt Lake City, UT; [2]Blood and Marrow Transplantation Program, University of Maryland Marlene and Stewart Greenebaum Comprehensive Cancer Center, Baltimore, MD; [3]Department of Pathology, University of Utah, Salt Lake City, UT; [4]ARUP Laboratories, Salt Lake City, UT; [5]Department of Medicine, University of Utah School of Medicine, Salt Lake City, UT; [6]Hematology/Oncology, University of Maryland Marlene and Stewart Greenebaum Comprehensive Cancer Center, Baltimore, MD; [7]Baltimore Veterans Affairs Medical Center, Baltimore, MD; and [8]Institute of Human Virology, University of Maryland, Baltimore, MD

Key Points

- A severely immunocompromised patient with MM and COVID19 who received a convalescent plasma product showed SARS-CoV-2 clearance.
- The convalescent plasma showed humoral immunity against all structural SARS-CoV-2 proteins, which was successfully transferred to the patient.

Introduction

Coronavirus disease 2019 (COVID-19) has become the greatest global health challenge.[1] Importantly, patients at an advanced age and with preexisting medical conditions,[2-5] in particular cancer patients on immunosuppressive treatment, show a more severe disease and increased mortality.[6-8] COVID-19 infection is caused by a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[9] The virus consists of various nonstructural proteins and 4 major structural proteins: the surface-exposed spike (S), membrane (M), and envelope (E) proteins and the internal nucleocapsid (N) protein.[9,10] The SARS-CoV-2 virus enters cells, such as pneumocytes in the lung,[11] via binding of the receptor-binding domain (RBD) within its S1 protein[12] to the angiotensin-converting enzyme-2 receptor.[10,13]

Recent findings indicate that infected patients develop spontaneous antibody-mediated immune responses against viral particles,[10,14,15] and an apparent improvement in the clinical status was observed upon treatment with convalescent plasma containing anti–SARS-CoV-2 antibodies.[16] Indeed, it was recently shown that patient-derived antibodies directed against the viral S protein are able to neutralize the SARS-CoV-2 virus.[12] However, the exact targets of the clinically relevant antibodies within the polyclonal plasma remain unclear, the potential immunogenicity and clinical relevance of other viral proteins has not been investigated, and individual epitopes of the antibodies present in convalescent plasma and in the blood of COVID-19 patients have not been identified. In addition, it has never been demonstrated whether the complete antiviral immunity can be transferred and detected in the recipient after transfusing a convalescent plasma product.

Case description

Our patient ID359 is a 72-year-old female who was diagnosed with immunoglobulin G (IgG) k multiple myeloma (MM) 10 years prior to her admission for COVID-19. She had received 4 prior lines of treatment, including 3 autologous stem cell transplants, steroids, cytotoxic chemotherapy, proteasome inhibitors, immunomodulatory drugs, and the anti-CD38 monoclonal antibody daratumumab. She was in partial remission after 8 cycles of carfilzomib/pomalidomide/dexamethasone and had received her most recent dose of carfilzomib; 3 weeks prior to this admission; she still had active disease with a serum IgG k monoclonal protein measuring 0.36 g/dL (Figure 1A), serum free k light chains of 30.6 mg/L, and an elevated k/l ratio.

The patient underwent testing of a nasopharyngeal sample by SARS-CoV-2 polymerase chain reaction (PCR), following continuous exposure to an individual with known COVID-19 for ; 1 week, and was diagnosed with the infection. At the time of testing (day 2 3), she was asymptomatic; however, on the following day (day 2 2), she developed diarrhea and nausea. One day later (day 2 1), she developed substantial dyspnea, cough, wheezing, and, from home, she reported hypoxia with an oxygen saturation of 75% to 85%. The patient was admitted to the emergency room at the University of Utah. On

Submitted 8 June 2020; accepted 14 August 2020; published online 8 October 2020.
DOI 10.1182/bloodadvances.2020002595.

Data sharing requests should be sent to Djordje Atanackovic (e-mail: djordje.atanackovic@hci.utah.edu).

Downloaded from http://ashpublications.org/bloodadvances/article-pdf/4/19/4864/1760926/advancesadv2020002595.pdf by guest on 11 October 2020

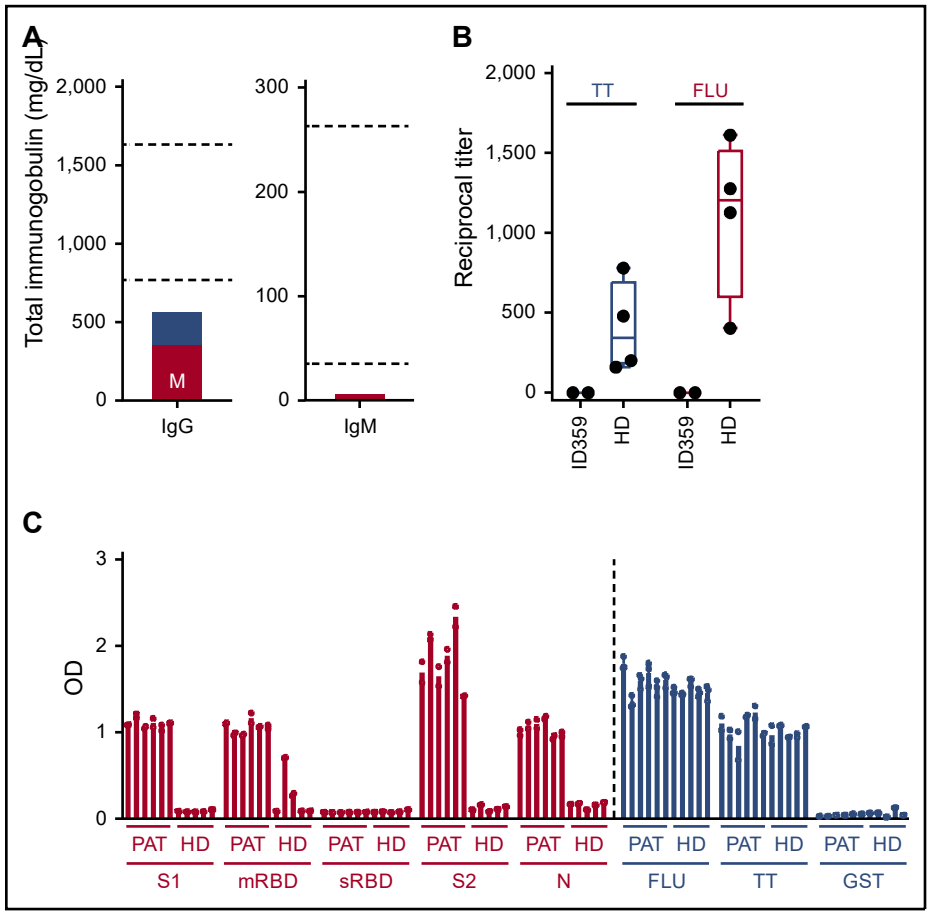


Figure 1. Compromised antiviral immunity in an MM patient with hypogammaglobulinemia. (A) Total IgG and IgM levels of patient ID359 on the day of COVID-19 diagnosis, as determined by immunoelectrophoresis. M indicates the level of total monoclonal protein in the g region, and dotted lines indicate the reference range. (B) Reciprocal IgG titers against tetanus toxoid (TT; Millipore-Sigma, cat. no. 582231) and influenza H1N1 nucleoprotein (FLU; Sino Biological, cat. no. 11675-V08B) in patient ID359 plasma at COVID-19 diagnosis, as well as in 4 healthy donors (HD), as determined by ELISA. (C) IgG responses, expressed as optical density (OD) readings, of 6 COVID-19 patients (PAT) and 5 healthy donors (HD) against SARS-CoV-2 proteins S1 (ACRO Biosystems; cat. no. S1N-C52H3), S2 (expressed in Expi293 cells), and N (BioVision; cat. no. P1523-50), the S1 RBD expressed in mammalian cells (mRBD; RayBiotech; cat. no. 230-30162) or produced synthetically (sRBD; LifeTein), positive-control proteins TT and FLU, and negative-control glutathione S-transferase (GST; expressed in Expi293 cells), as determined by ELISA. Data represent the mean, and circles indicate technical replicates.

admission, she was found to be in respiratory distress and was hypoxic and disoriented. Venous blood gas analysis showed hypoxia and hypercapnia. She was placed on oxygen via nasal cannula, and a chest radiograph showed streaky left basilar opacities pointing to viral pneumonia. Other abnormal laboratory results included leukopenia and lymphopenia and worsening of her chronic renal disease. The patient was diagnosed with acute hypoxic respiratory failure due to COVID-19 infection, without any clinical signs of cytokine storm, and viral pneumonia. She was admitted to the medical COVID unit where she received 2 to 3 L of oxygen via nasal cannula for the next 24 hours.

Evaluating her humoral immune system, the patient was found to have severe hypogammaglobulinemia with very low absolute levels of normal IgG and IgM (Figure 1A), consistent with her long-standing MM and several lines of immunosuppressive treatments, including a monoclonal antibody targeting CD38-expressing plasma cells. Remarkably, the low amount of total IgG that she did have consisted largely of myeloma-related monoclonal M protein (Figure 1A). Accordingly, she showed nonexistent antibody titers against influenza A virus and tetanus toxoid (Figure 1B). A routine ELISA screening for anti–COVID-19 antibodies demonstrated a complete absence of antiviral serological reactivity.

On day 0, based on the lack of clinical improvement, the US Food and Drug Administration clinical criteria for convalescent plasma administration under the expanded access program, and her severe immunosuppression, we transfused 1 200-mL unit of convalescent plasma from a donor who had recovered from COVID-19. Importantly, the patient did not receive any other treatment potentially having an effect on the course of COVID-19, such as steroids or antivirals. After transfusion of the convalescent plasma, the patient showed a dramatic clinical improvement, became asymptomatic, and was discharged home only 2 days later (day 1 3). The patient continued to be asymptomatic; a SARS-CoV-2 PCR performed on a nasopharyngeal sample on day 1 19 was negative, indicating complete viral clearance.

Methods

Serum samples

Patient and healthy donor samples were collected under protocol #45880, which was approved by the Institutional Review Board at the University of Utah. Written informed consent was obtained from participants prior to inclusion in the study.

Serum ELISA and titration

Ninety-six-well plates were coated with 5 mg/mL recombinant protein or peptides. ELISAs were performed as described previously.[17]

Pseudotyped virus-neutralization assay

Assays were performed as described, using plasmid HIV-1-d-env-luc with pSARS-Cov-2-S for patient samples and MLV-C (2)Env(-), L-luc-SN and pSARS-Cov-2-S for donor plasma.[18]

Downloaded from http://ashpublications.org/bloodadvances/article-pdf/4/19/4864/1760926/advancesadv2020002595.pdf by guest on 11 October 2020

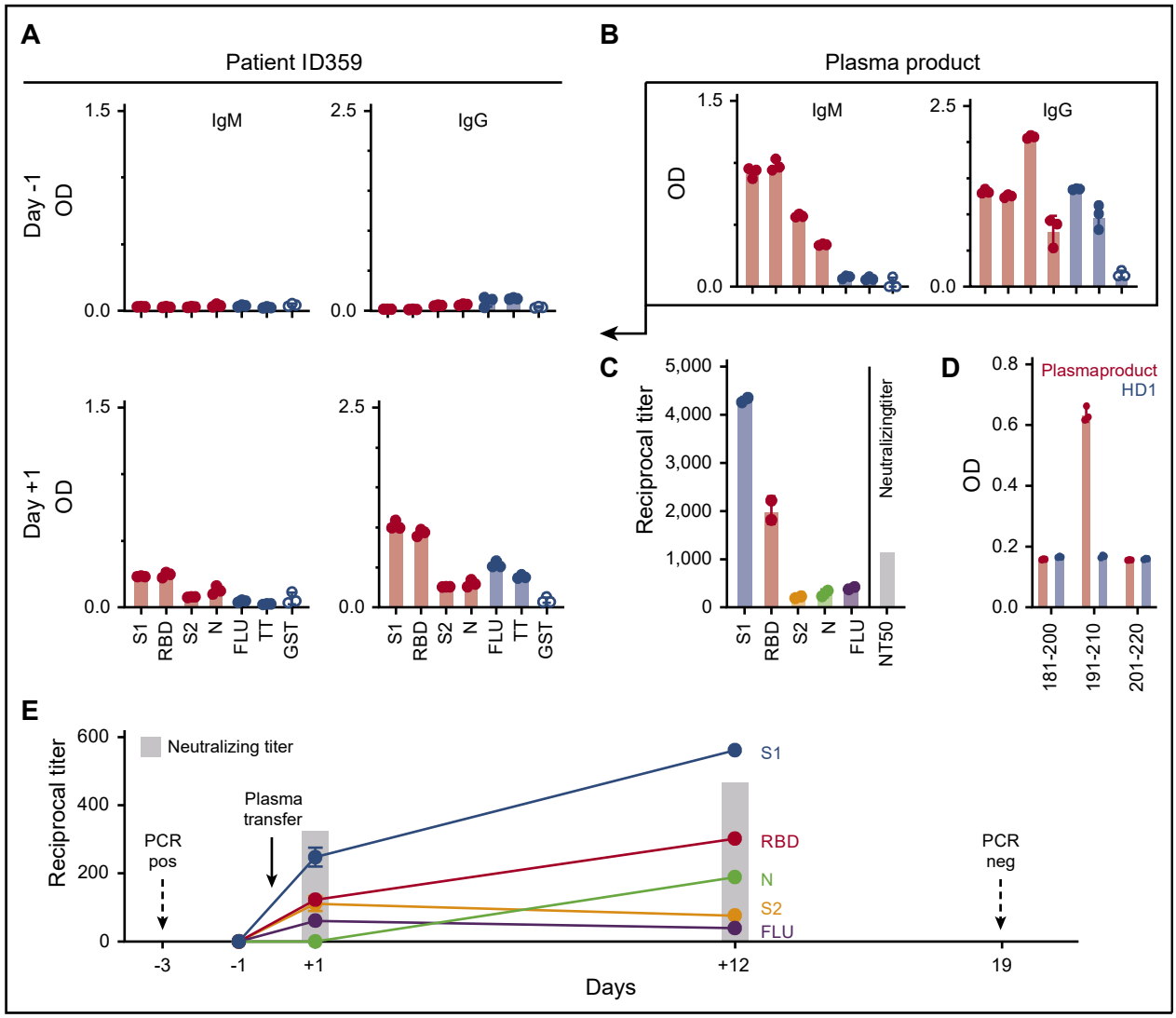


Figure 2. Transfer of SARS-CoV-2–specific immunity to MM patient by convalescent plasma infusion and subsequent viral clearance. (A) IgM and IgG antibody responses in patient ID359 plasma against SARS-CoV-2 proteins and control proteins at 1:20 serum dilution before and after convalescent plasma infusion, as determined by ELISA. (B) IgM and IgG responses at 1:20 plasma dilution against SARS-CoV-2 and control proteins in convalescent plasma product, as determined by ELISA. (C) Reciprocal IgG antibody titers against SARS-CoV-2 and control proteins and neutralizing antibody titers in the convalescent plasma product. (D) Epitope mapping of convalescent plasma product and plasma from a healthy donor (HD1) using synthetic S1 peptides, as determined by ELISA. 20mer peptides overlapping by 10 aa for SARS-CoV-2 S1, S2, M, and E sequences were synthetized (peptides & elephants, Hennigsdorf, Germany). Data represent the mean, and circles indicate technical replicates. (E) Reciprocal IgG titers against SARS-CoV-2 proteins and FLU, as well as neutralizing antibody activity in patient ID359 plasma, before and after convalescent plasma infusion. Antibody titers represent mean 6 standard deviation from 2 technical replicates. GST, glutathione S-transferase; neg, negative.

Results and discussion

To perform the first detailed analysis of convalescent plasma product and its effect on the anti–SARS-CoV-2 immunity in the recipient, we established an ELISA measuring antibody titers against the SARS-CoV-2 structural proteins. We obtained full-length recombinant proteins for the S1 protein, the RBD within the S1 protein, and the S2 and nucleocapsid (N) proteins. S1, S2, and RBD used in this study were expressed in human 293 cells to preserve structure and glycosylation patterns. Peptide libraries consisting of 20mers overlapping by 10 aa were synthesized for these SARS-CoV-2 proteins and for the M and E proteins. In addition, we obtained a 69-aa synthetic peptide comprising a highly conserved cryptic epitope in the RBDs of SARS-CoV-2 and severe acute respiratory syndrome coronavirus.[19]

We performed assay validation assessing sera from 6 patients with PCR-confirmed and symptomatic COVID-19 and from 5 healthy donors for antibodies against the different SARS-CoV-2 proteins and peptides, positive-control antigens influenza H1N1 nucleoprotein (FLU) and tetanus toxoid (TT), and the negative-control protein glutathione S-transferase. We found that all patients showed strong antibody responses against S1 and the RBD (Figure 1C). In addition, all 5 patients showed antibody responses against the S2 and N proteins. Interestingly, the RBD produced synthetically was not recognized by any of the patient-derived antibodies,

Downloaded from http://ashpublications.org/bloodadvances/article-pdf/4/19/4864/1760926/advancesadv2020002595.pdf by guest on 11 October 2020

pointing to glycosylation-dependent and/or conformational epitopes as targets for the antiviral immune responses. Accordingly, we did not observe antibody responses against pooled M and E peptides (data not shown). With the exception of the mammalian cell–expressed RBD, which was recognized by serum from 1 healthy donor, none of the other SARS-CoV-2 proteins were recognized by sera from non–COVID-19 individuals (Figure 1C).

As expected, when screening the patient's blood on the day of admission, we did not detect antibodies against any of the viral proteins (Figure 2A) or any neutralizing antibody activity (Figure 2E). It has previously been shown that, at least in the case of severe acute respiratory syndrome coronavirus, lung injury is associated with neutralizing antibody development[20] and, it may be noteworthy that, in our patient, respiratory distress occurred despite a severely suppressed humoral immune system and the absence of anti–SARS-CoV-2 antibodies. In contrast, a , 1-mL leftover from the frozen and thawed convalescent plasma product administered to the patient contained IgM and IgG antibodies (Figure 2B) and a low level of IgA antibodies (data not shown) against S1, RBD, S2, and N proteins, as well as IgG antibodies against positive-control proteins FLU and TT (Figure 2B-C). Importantly, the donor plasma also contained significant neutralizing activity (reciprocal 50% neutralizing titer [NT_{50}]; Figure 2C). Analyzing the convalescent plasma product using overlapping synthetic peptide libraries we were able to identify 1 linear epitope within the S1 protein, N-terminal from the RBD, at aa position 191 to 210 (Figure 2D).

On the morning after treatment with convalescent plasma (day 1 1), we were able to detect IgM and IgG antibody responses (Figure 2A), but no IgA antibodies (data not shown), against all tested viral proteins in the patient's blood, albeit at a lower level compared with the plasma product. The plasma product evidenced the highest IgG antibody titers against SARS-CoV-2 proteins S1 and RBD and lower antibody titers against S2 and N (Figure 2D). Following plasma transfer, the patient showed a similar distribution pattern (Figure 2E), confirming that the humoral immunity had indeed been passively transferred to the recipient. Importantly, the transfer of the donor-derived antiviral immunity also resulted in neutralizing antibody activity in the patient's blood (Figure 2E). The antibodies against the different viral proteins were still detectable in the patient 12 days after transfusion of the convalescent plasma, at titers even higher than on the day after treatment, with a higher neutralizing activity (Figure 2E) and new low-level antiviral IgA antibodies (data not shown), indicating the development of at least some autologous SARS-CoV-2–specific immunity.

In summary, our data indicate that transfer of convalescent plasma is safe, even in severely immunocompromised patients with hematologic malignancies who are under treatment for their malignancy. We also show here for the first time that the complete humoral immunity against the different viral target proteins can successfully be transferred and detected in the recipient and that antibody levels are maintained over time. Finally, our observations indicate that these patients may derive clinical benefit from such a treatment, although this will need to be confirmed by randomized clinical trials.

Acknowledgments

This work was supported by a grant from the CrowdCare Foundation (D.A.) and by a seed grant from the University of Utah Vice President for Research and the Immunology, Inflammation, and Infectious Disease Initiative (V.P.). This research was supported by a grant from the CrowdCare Foundation Inc.

Authorship

Contribution: T.L. designed the study, performed analyses, analyzed data, and wrote the manuscript; R.M. performed analyses, provided samples, and wrote the manuscript; V.P. performed analyses and wrote the manuscript; Y.Z., E.T.L., and S.V.A. performed analyses; E.S.S. provided samples and performed analyses; A.M.S., R.C.B., and H.D.M. wrote the manuscript; M. Steinbach obtained clinical data and wrote the manuscript; K.G.H. analyzed data and wrote the manuscript; T.B.M. and M. Sajadi provided samples and wrote the manuscript; P.R.S. provided samples and analyzed data; A.P.R. analyzed data and wrote the manuscript; and D.A. designed the study, provided samples, obtained clinical data, analyzed data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profile: A.M.S., 0000-0003-1815-7893.

Correspondence: Djordje Atanackovic, Department of Medicine/Bone Marrow Transplant, University of Maryland Greenebaum Comprehensive Cancer Center, Bressler Research Building, Room 9-011, 655 W Baltimore St, Baltimore, MD 21201; email: datanackovic@som.umaryland.edu.

References

1. World Health Organization. Coronavirus disease (COVID-19) pandemic. https://www.who.int/emergencies/diseases/novel-coronavirus-2019. Accessed 30 June 2020.
2. Li Q, Guan X, Wu P, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. N Engl J Med. 2020;382(13):1199-1207.
3. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020;395(10223):497-506.
4. Chen R, Liang W, Jiang M, et al; Medical Treatment Expert Group for COVID-19. Risk factors of fatal outcome in hospitalized subjects with coronavirus disease 2019 from a nationwide analysis in China. Chest. 2020;158(1):97-105.
5. Guan WJ, Liang WH, Zhao Y, et al; China Medical Treatment Expert Group for COVID-19. Comorbidity and its impact on 1590 patients with COVID-19 in China: a nationwide analysis. Eur Respir J. 2020;55(5):2000547.
6. He W, Chen L, Chen L, et al. COVID-19 in persons with haematological cancers. Leukemia 2020;34(6):1637-1645.
7. Yang F, Shi S, Zhu J, Shi J, Dai K, Chen X. Clinical characteristics and outcomes of cancer patients with COVID-19 [published online ahead of print 5 May 2020]. J Med Virol. doi:10.1002/jmv.25972.

Downloaded from http://ashpublications.org/bloodadvances/article-pdf/4/19/4864/1760926/advancesadv2020002595.pdf by guest on 11 October 2020

8. Zhang L, Zhu F, Xie L, et al. Clinical characteristics of COVID-19-infected cancer patients: a retrospective case study in three hospitals within Wuhan, China. Ann Oncol. 2020;31(7):P894-901.

9. Zhu N, Zhang D, Wang W, et al; China Novel Coronavirus Investigating and Research Team. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. 2020;382(8):727-733.

10. Zhou P, Yang XL, Wang XG, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020;579(7798):270-273.

11. Chu H, Chan JF-W, Wang Y, et al. Comparative replication and immune activation profiles of SARS-CoV-2 and SARS-CoV in human lungs: an ex vivo study with implications for the pathogenesis of COVID-19. Clin Infect Dis. 2020;71(6):1400-1409.

12. Ou X, Liu Y, Lei X, et al. Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. Nat Commun. 2020;11(1):1620.

13. Ge XY, Li JL, Yang XL, et al. Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. Nature. 2013;503(7477):535-538.

14. Du Z, Zhu F, Guo F, Yang B, Wang T. Detection of antibodies against SARS-CoV-2 in patients with COVID-19 [published online ahead of print 3 April 2020]. J Med Virol. doi:10.1002/jmv.25820.

15. Thevarajan I, Nguyen THO, Koutsakos M, et al. Breadth of concomitant immune responses prior to patient recovery: a case report of non-severe COVID-19. Nat Med. 2020;26(4):453-455.

16. Shen C, Wang Z, Zhao F, et al. Treatment of 5 critically ill patients with COVID-19 with convalescent plasma. JAMA. 2020;323(16):1582-1589.

17. Atanackovic D, Yousef S, Shorter C, et al. In vivo vaccination effect in multiple myeloma patients treated with the monoclonal antibody isatuximab. Leukemia. 2020;34(1):317-321.

18. Zheng Y, Larragoite ET, Lama J, et al Neutralization assay with SARS-CoV-1 and SARS-CoV-2 spike pseudotyped murine leukemia virions. https://www.biorxiv.org/content/10.1101/2020.07.17.207563v1.full.pdf Accessed 30 July 2020.

19. Yuan M, Wu NC, Zhu X, et al. A highly conserved cryptic epitope in the receptor binding domains of SARS-CoV-2 and SARS-CoV. Science. 2020;368(6491):630-633.

20. Liu L, Wei Q, Lin Q, et al. Anti-spike IgG causes severe acute lung injury by skewing macrophage responses during acute SARS-CoV infection. JCI Insight. 2019;4(4):e123158.

Downloaded from http://ashpublications.org/bloodadvances/article-pdf/4/19/4864/1760926/advancesadv2020002595.pdf by guest on 11 October 2020

[Please insert letterhead here]

To: NSF Ecology and Evolution of Infectious Diseases (EEID) Program (NSF-19-592)

From:
Ralph S Baric, PhD
William R. Kenan, Jr. Distinguished Professor. Department of Epidemiology
Professor, Department of Microbiology and Immunology

By signing below, I acknowledge that I am listed as a collaborator on this proposal, entitled "**US-China Collab: Spillover, co-infection, and control of bat-origin livestock coronaviruses**" with Peter Daszak as the Principal Investigator. I agree to undertake the tasks assigned to me or my organization, as described in the project description of the proposal, and I commit to provide or make available the resources specified therein.

Sincerely,

Organization:
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599

Date: 29 October, 2020

REGENERON

Back

October 30, 2020 at 9:12 AM EDT

REGN-COV2 INDEPENDENT DATA MONITORING COMMITTEE RECOMMENDS HOLDING ENROLLMENT IN HOSPITALIZED PATIENTS WITH HIGH OXYGEN REQUIREMENTS AND CONTINUING ENROLLMENT IN PATIENTS WITH LOW OR NO OXYGEN REQUIREMENTS

TARRYTOWN, N.Y., Oct. 30, 2020 /PRNewswire/ --

The IDMC also recommends continuation of enrollment in the REGN-COV2 outpatient trial

Regeneron Pharmaceuticals, Inc. (NASDAQ: REGN) received today a recommendation from the independent data monitoring committee (IDMC) for the REGN-COV2 antibody cocktail treatment trials for COVID-19 that the current hospitalized patient trial be modified. Specifically, based on a potential safety signal and an unfavorable risk/benefit profile at this time, the IDMC recommends further enrollment of patients requiring high-flow oxygen or mechanical ventilation be placed on hold pending collection and analysis of further data on patients already enrolled. The IDMC also recommends continuing enrollment of hospitalized patients requiring either no or low-flow oxygen as the risk/benefit remains acceptable in these cohorts. Finally, the IDMC recommends continuation of the outpatient trial without modification.

Regeneron remains blinded to the data and is implementing the IDMC recommendations. Regeneron is also informing the U.S. Food and Drug Administration, which is currently evaluating REGN-COV2 for a potential Emergency Use Authorization in mild-to-moderate outpatients at high risk for poor outcomes. Regeneron is also sharing the recommendation with the independent committee monitoring the RECOVERY trial in the UK, which is evaluating REGN-COV2 in hospitalized patients.

About the REGN-COV2 Trial in Hospitalized Patients

The trial is designed to enroll patients in four independently randomized cohorts:

- Cohort 1: patients on low-flow oxygen
- Cohort 1A: patients not requiring oxygen
- Cohort 2: patients on high-flow oxygen
- Cohort 3: patients on mechanical ventilation

About REGN-COV2
REGN-COV2 is a combination of two monoclonal antibodies (REGN10933 and REGN10987) and was designed specifically to block infectivity of SARS-CoV-2, the virus that causes COVID-19.

To develop REGN-COV2, Regeneron scientists evaluated thousands of fully-human antibodies produced by the company's *VelocImmune®* mice, which have been genetically modified to have a human immune system, as well as antibodies identified from humans who have recovered from COVID-19. The two potent, virus-neutralizing antibodies that form REGN-COV2 bind non-competitively to the critical receptor binding domain of the virus's spike protein, which diminishes the ability of mutant viruses to escape treatment and protects against spike variants that have arisen in the human population, as detailed in *Science*.

REGN-COV2's development and manufacturing has been funded in part with federal funds from the Biomedical Advanced Research and Development Authority (BARDA), part of the Office of the Assistant Secretary for Preparedness and Response at the U.S. Department of Health and Human Services under OT number: HHSO100201700020C. Regeneron has partnered with Roche to increase the global supply of REGN-COV2 beginning in 2021. If REGN-COV2 proves safe and effective in clinical trials and regulatory approvals are granted, Regeneron will manufacture and distribute it in the U.S. and Roche will develop, manufacture and distribute it outside the U.S.

About Regeneron

Regeneron (NASDAQ: REGN) is a leading biotechnology company that invents life-transforming medicines for people with serious diseases. Founded and led for over 30 years by physician-scientists, our unique ability to repeatedly and consistently translate science into medicine has led to eight FDA-approved treatments and numerous product candidates in development, all of which were homegrown in our laboratories. Our medicines and pipeline are designed to help patients with eye diseases, allergic and inflammatory diseases, cancer, cardiovascular and metabolic diseases, pain, infectious diseases and rare diseases.

Regeneron is accelerating and improving the traditional drug development process through our proprietary *VelociSuite* technologies, such as *VelocImmune®*, which uses unique genetically-humanized mice to produce optimized fully-human antibodies and bispecific antibodies, and through ambitious research initiatives such as the Regeneron Genetics Center, which is conducting one of the largest genetics sequencing efforts in the world.

For additional information about the company, please visit www.regeneron.com or follow @Regeneron on Twitter.

Forward-Looking Statements and Use of Digital Media

This press release includes forward-looking statements that involve risks and uncertainties relating to future events and the future performance of Regeneron Pharmaceuticals, Inc. ("Regeneron" or the "Company"), and actual events or results may differ materially from these forward-looking statements. Words such as "anticipate," "expect," "intend," "plan," "believe," "seek," "estimate," variations of such words, and similar expressions are intended to identify such forward-looking statements, although not all forward-looking statements contain these identifying words. These statements concern, and these risks and uncertainties include, among others, the impact of SARS-CoV-2 (the virus that has caused the COVID-19 pandemic) on Regeneron's business and its employees, collaborators, and suppliers and other third parties on which Regeneron relies, Regeneron's and its collaborators' ability to continue to conduct research and clinical programs (including those discussed in this press release), Regeneron's ability to manage its supply chain, net product sales of products marketed by Regeneron and/or its collaborators (collectively, "Regeneron's Products"), and the global economy; the nature, timing, and possible success and therapeutic applications of Regeneron's Products and product candidates and research and clinical programs now underway or planned, including without limitation the development program relating to REGN-COV2 (Regeneron's investigational dual antibody for the treatment and prevention of COVID-19); safety issues resulting from the administration of Regeneron's Products and product candidates (such as REGN-COV2) in patients, including serious complications or side effects in connection with the use of Regeneron's Products and product candidates in clinical trials; the impact of the recommendation of the independent data monitoring committee for the REGN-COV2 trials and the potential safety signal discussed in this press release on further clinical development of REGN-COV2, the decision by the U.S. Food and Drug Administration ("FDA") with respect to a potential Emergency Use Authorization ("EUA") for REGN-COV2, and possible regulatory approval of REGN-COV2; whether the FDA will grant an EUA for REGN-COV2 and, if an EUA is granted, the scope and terms of such EUA and how long such EUA would remain in effect for REGN-COV2; the likelihood, timing, and scope of possible regulatory approval and commercial launch of Regeneron's product candidates (such as REGN-COV2) and new indications for Regeneron's Products; the ability of

Regeneron to manufacture in anticipated quantities Regeneron's Products and product candidates, including REGN-COV2; the ability of Regeneron to manufacture and manage supply chains for multiple products and product candidates; the ability of Regeneron's collaborators, suppliers, or other third parties (as applicable) to perform manufacturing, filling, finishing, packaging, labeling, distribution, and other steps related to Regeneron's Products and product candidates; uncertainty of market acceptance and commercial success of Regeneron's Products and product candidates and the impact of studies (whether conducted by Regeneron or others and whether mandated or voluntary), including the studies evaluating REGN-COV2 discussed in this press release, on any potential regulatory approval (including with respect to REGN-COV2) and/or the commercial success of Regeneron's Products and product candidates; determinations by regulatory and administrative governmental authorities which may delay or restrict Regeneron's ability to continue to develop or commercialize Regeneron's Products and product candidates, including without limitation REGN-COV2; ongoing regulatory obligations and oversight impacting Regeneron's Products, research and clinical programs, and business, including those relating to patient privacy; the availability and extent of reimbursement of Regeneron's Products from third-party payers, including private payer healthcare and insurance programs, health maintenance organizations, pharmacy benefit management companies, and government programs such as Medicare and Medicaid; coverage and reimbursement determinations by such payers and new policies and procedures adopted by such payers; competing drugs and product candidates that may be superior to, or more cost effective than, Regeneron's Products and product candidates; the extent to which the results from the research and development programs conducted by Regeneron and/or its collaborators may be replicated in other studies and/or lead to advancement of product candidates to clinical trials, therapeutic applications, or regulatory approval; unanticipated expenses; the costs of developing, producing, and selling products; the ability of Regeneron to meet any of its financial projections or guidance and changes to the assumptions underlying those projections or guidance; the potential for any license or collaboration agreement, including Regeneron's agreements with Sanofi, Bayer, and Teva Pharmaceutical Industries Ltd. (or their respective affiliated companies, as applicable), as well as Regeneron's collaboration with Roche relating to REGN-COV2, to be cancelled or terminated; and risks associated with intellectual property of other parties and pending or future litigation relating thereto (including without limitation the patent litigation and other related proceedings relating to EYLEA® (aflibercept) Injection, Dupixent® (dupilumab), and Praluent® (alirocumab)), other litigation and other proceedings and government investigations relating to the Company and/or its operations, the ultimate outcome of any such proceedings and investigations, and the impact any of the foregoing may have on Regeneron's business, prospects, operating results, and financial condition. A more complete description of these and other material risks can be found in Regeneron's filings with the U.S. Securities and Exchange Commission, including its Form 10-K for the year ended December 31, 2019 and its Form 10-Q for the quarterly period ended June 30, 2020. Any forward-looking statements are made based on management's current beliefs and judgment, and the reader is cautioned not to rely on any forward-looking statements made by Regeneron. Regeneron does not undertake any obligation to update (publicly or otherwise) any forward-looking statement, including without limitation any financial projection or guidance, whether as a result of new information, future events, or otherwise.

Regeneron uses its media and investor relations website and social media outlets to publish important information about the Company, including information that may be deemed material to investors. Financial and other information about Regeneron is routinely posted and is accessible on Regeneron's media and investor relations website (http://newsroom.regeneron.com) and its Twitter feed (http://twitter.com/regeneron).

Contacts:

Media Relations
Alexandra Bowie
Tel: +1 (914) 847-3407
alexandra.bowie@regeneron.com

Investor Relations
Mark Hudson
Tel: +1 (914) 847-3482
mark.hudson@regeneron.com

View original content:http://www.prnewswire.com/news-releases/regn-cov2-independent-data-monitoring-committee-recommends-holding-enrollment-in-hospitalized-patients-with-high-oxygen-requirements-and-continuing-enrollment-in-patients-with-low-or-no-oxygen-requirements-301163876.html

SOURCE Regeneron Pharmaceuticals, Inc.

Investor Relations
914.847.7741
invest@regeneron.com

Media Relations
media@regeneron.com

Investor email alerts

DOWNLOAD CENTER

- 2020 Proxy Statement
- 2019 Annual Report
- 2019 Responsibility Report
- 2019 Form 10-K
- Senior Management Biographies
- Corporate Fact Sheet
  Regeneron Genetics Center Backgrounder

Investor Relations
914.847.7741
invest@regeneron.com

Media Relations
media@regeneron.com

Investor email alerts

MAYO
CLINIC

Ecology and Evolution of Infectious Diseases (EEID)

PROGRAM SOLICITATION
NSF 20-585

REPLACES DOCUMENT(S):
NSF 19-592

National Science Foundation

Directorate for Biological Sciences

Directorate for Social, Behavioral and Economic Sciences

Directorate for Geosciences

National Institutes of Health

John E. Fogarty International Center

National Institute of General Medical Sciences

National Institute of Allergy and Infectious Diseases

National Institute of Food and Agriculture

United Kingdom Research and Innovation

Council

Medical Research Council (part of UK Research and Innovation)

U.K. Biotechnology and Biological Sciences Research Council

Natural Environment Research Council

U.K. Economic and Social Research Council

UK Engineering and Physical Science Research Council

United States-Israel Binational Science Foundation

National Natural Science Foundation of China

Full Proposal Deadline(s) (due by 5 p.m. submitter's local time):

November 18, 2020

Third Wednesday in November, Annually Thereafter

IMPORTANT INFORMATION AND REVISION NOTES

This revision includes new details about what needs to be included in the Data Management Plan.

Any proposal submitted in response to this solicitation should be submitted in accordance with the revised*NSF Proposal & Award Policies & Procedures Guide* (PAPPG) (NSF 20-1), which is effective for proposals submitted, or due, on or after June 1, 2020.

SUMMARY OF PROGRAM REQUIREMENTS

General Information

Program Title:

Ecology and Evolution of Infectious Diseases (EEID)

Synopsis of Program:

The multi-agency Ecology and Evolution of Infectious Diseases program supports research on the ecological, evolutionary, and social drivers that influence the transmission dynamics of infectious diseases. The central theme of submitted projects must be the quantitative or computational understanding of pathogen transmission dynamics. The intent is discovery of principles of infectious disease transmission and testing mathematical or computational models that elucidate infectious disease systems. Projects should be broad, interdisciplinary efforts that go beyond the scope of typical studies. They should focus on the determinants and interactions of transmission among any host species, including but not limited to humans, non-human animals, and/or plants. This includes, for example, the spread of pathogens; the influence of environmental factors such as climate; the population dynamics and genetics of reservoir species or hosts; the feedback between ecological transmission and evolutionary dynamics; and the cultural, social, behavioral, and economic dimensions of pathogen transmission. Research may be on zoonotic, environmentally-borne, vector-borne, or enteric pathogens of either terrestrial or aquatic systems and organisms, including diseases of animals and plants, at any scale from specific pathogens to inclusive environmental systems. Proposals for research on disease systems of public health concern to developing countries are strongly encouraged, as are disease systems of concern in agricultural systems. Investigators are encouraged to develop the appropriate multidisciplinary team, including for example, anthropologists, modelers, ecologists, bioinformaticians, genomics researchers, social scientists, economists, oceanographers, mathematical scientists, epidemiologists, evolutionary biologists, entomologists, parasitologists, microbiologists, bacteriologists, virologists, pathologists or veterinarians, with the goal of integrating knowledge across disciplines to enhance our ability to predict and control infectious diseases.

Cognizant Program Officer(s):

Please note that the following information is current at the time of publishing. See program website for any updates to the points of contact.

- Katharina Dittmar, Program Director, NSF/BIO, telephone: (703) 292-7799, email: kdittmar@nsf.gov
- Christine Jessup, Program Director, NIH/FIC, telephone: (301) 496-1653, fax: (301) 402-0779, email: christine.jessup@nih.gov
- Timothy Sullivan, National Program Leader, USDA/NIFA, telephone: (816) 527-5434, email: timothy.sullivan@usda.gov
- Iain Templeman, Portfolio Manager, UKRI/BBSRC, telephone: 44(0)1793-413368, email: iain.templeman@bbsrc.ukri.org
- Rachel (Heni) Haring, Deputy Executive Director, BSF, telephone: 972 2 5828239, email: heni@bsf.org.il
- Xiuping Liu, Division Director, NSFC, telephone: 86 10-62325377, email: liuxp@nsfc.gov.cn
- Daniel Janes, Program Director, NIH/NIGMS, telephone: (301) 594-0943, email: daniel.janes@nih.gov
- Stephanie Coomes, Health Scientist Administrator, NIH/NIAID, telephone: (301) 761-6855, email: stephanie.coomes@nih.gov
- Jing Chen, Program Manager, NSFC, telephone: 86 10-62326877, email: chenjing@nsfc.gov.cn
- Rebecca Ferrell, Program Director, NSF/SBE, telephone: (703) 292-7850, email: rferrell@nsf.gov
- Siobhan M. Mattison, NSF/SBE, telephone: (703) 292-2967, email: smattiso@nsf.gov
- Daniel J. Thornhill, Program Director, NSF/GEO, telephone: (703) 292-8143, email: dthornhi@nsf.gov
- Joaquin Martinez Martinez, NSF/GEO, telephone: (703) 292-8580, email: jmartine@nsf.gov
- Samuel M. Scheiner, Program Director, NSF/BIO, telephone: (703) 292-7175, email: sscheine@nsf.gov

Applicable Catalog of Federal Domestic Assistance (CFDA) Number(s):

- 10.310 --- USDA-NIFA Agriculture and Food Research Initiative
- 47.050 --- Geosciences
- 47.074 --- Biological Sciences
- 47.075 --- Social Behavioral and Economic Sciences
- 93.856 --- Microbiology and Infectious Diseases Research
- 93.859 --- National Institute of General Medical Sciences
- 93.989 --- John E. Fogarty International Center

Award Information

Anticipated Type of Award: Standard Grant or Continuing Grant

Estimated Number of Awards: 12

Anticipated Funding Amount: $24,000,000

in FY 2021, pending the availability of funds. That amount includes approximately $11.0M from NSF for new standard or continuing awards, approximately $7.5M from NIH for new or continuing awards, and $5.5M from USDA for new awards. The expected funding from the UKRI for the UK component of the US-UK Collaborative Projects will be a maximum of £2M. The expected funding from the BSF for the Israeli component of the US-Israel Collaborative Projects will be a maximum of $720,000. The expected funding from the NSFC for the Chinese component of the US-China Collaborative Projects will be a maximum of ¥9M.

Eligibility Information

Who May Submit Proposals:

The categories of proposers eligible to submit proposals to the National Science Foundation are identified in the *NSF Proposal & Award Policies & Procedures Guide* (PAPPG), Chapter I.E. Unaffiliated individuals are not eligible to submit proposals in response to this solicitation.

Who May Serve as PI:

There are no restrictions or limits.

Limit on Number of Proposals per Organization:

There are no restrictions or limits.

Limit on Number of Proposals per PI or Co-PI: 2

In a given year, an individual may participate as a PI, co-PI, or subaward lead on no more than **two** proposals submitted in response to this solicitation. **This limit does not include Research Coordination Networks (RCN) proposals.** In addition, an individual from Israel may participate in no more than **one** US-BSF proposal and an individual from China may participate in no more than **one** US-China proposal. Proposals in excess of the limit for any person will be returned without review in the reverse order received. Participating in a proposal as other senior personnel does not count in this limit. Changes in investigator roles post-submission to meet the eligibility limits will not be allowed. **It is the responsibility of the submitters to confirm that the entire team is within the eligibility guidelines.**

Proposal Preparation and Submission Instructions

A. Proposal Preparation Instructions

- **Letters of Intent:** Not required
- **Preliminary Proposal Submission:** Not required

Full Proposals:

- Full Proposals submitted via FastLane: *NSF Proposal and Award Policies and Procedures Guide* (PAPPG) guidelines apply. The complete text of the PAPPG is available electronically on the NSF website at: https://www.nsf.gov/publications/pub_summ.jsp?ods_key=pappg
- Full Proposals submitted via Research.gov: *NSF Proposal and Award Policies and Procedures Guide* (PAPPG) guidelines apply. The complete text of the PAPPG is available electronically on the NSF website at: https://www.nsf.gov/publications/pub_summ.jsp?ods_key=pappg
- Full Proposals submitted via Grants.gov: *NSF Grants.gov Application Guide: A Guide for the Preparation and Submission of NSF Applications via Grants.gov* guidelines apply (Note: The *NSF Grants.gov Application Guide* is available on the Grants.gov website and on the NSF website at: https://www.nsf.gov/publications/pub_summ.jsp?ods_key=grantsgovguide).

B. Budgetary Information

- **Cost Sharing Requirements:**

  Inclusion of voluntary committed cost sharing is prohibited.

- **Indirect Cost (F&A) Limitations:**

  Not Applicable

- **Other Budgetary Limitations:**

  Other budgetary limitations apply. Please see the full text of this solicitation for further information.

C. Due Dates

Full Proposal Deadline(s) (due by 5 p.m. submitter's local time):

November 18, 2020

Third Wednesday in November, Annually Thereafter

Proposal Review Information Criteria

Merit Review Criteria:

National Science Board approved criteria. Additional merit review criteria apply. Please see the full text of this solicitation for further information.

Award Administration Information

Award Conditions:

Additional award conditions apply. Please see the full text of this solicitation for further information.

Reporting Requirements:

Additional reporting requirements apply. Please see the full text of this solicitation for further information.

TABLE OF CONTENTS

Summary of Program Requirements

I. **Introduction**
II. **Program Description**
III. **Award Information**
IV. **Eligibility Information**
V. **Proposal Preparation and Submission Instructions**
  A. Proposal Preparation Instructions
  B. Budgetary Information
  C. Due Dates
  D. FastLane/Research.gov/Grants.gov Requirements
VI. **NSF Proposal Processing and Review Procedures**
  A. Merit Review Principles and Criteria
  B. Review and Selection Process
VII. **Award Administration Information**
  A. Notification of the Award
  B. Award Conditions
  C. Reporting Requirements
VIII. **Agency Contacts**
IX. **Other Information**

I. INTRODUCTION

The past twenty-five years have seen a dramatic increase in our awareness of the need to understand the ecological and evolutionary drivers of disease emergence and transmission dynamics. While our knowledge has increased about specific systems and the basic principles of simple systems, understanding of complex systems and translation of those principles into ecosystem, public health, and agricultural health management tools remains challenging. System complexity includes such factors as multiple interacting species of hosts, pathogens and/or vectors; interactions among pathogenic and non-pathogenic microbes; interactions between biological, cultural and social factors; effects of spatial and temporal structure; and evolutionary dynamics.

The emergence and the re-emergence of numerous infectious diseases around the world have coincided with unprecedented rates of change in the structure and diversity of the environment and human social and economic systems. Nearly all of the world's terrestrial and aquatic ecosystems have undergone dramatic changes due to a variety of human activities such as habitat transformation, human displacement and relocation, urbanization, rapid long-distance transport and increased international trade, species invasions, deliberate introduction of infectious diseases for biological control, wildlife trafficking, chemical waste contamination, use of antimicrobial agents in agriculture and medicine, and climate change. The coincidence of broad scale environmental changes, the expansion of human social and economic networks, and the emergence and re-emergence of infectious diseases may point to underlying predictable ecological and eco-evolutionary relationships.

We have improved our ability to define the molecular identity and dynamics of pathogens and have greatly increased our understanding of host defense systems. We are able to apply genetic knowledge to understand the evolutionary dynamics of infectious diseases. These improvements have contributed significantly to our knowledge of the epidemiology and transmission patterns of diseases. However, the relationship of these factors to the biotic and structural complexity of ecological, agricultural, and social systems in which transmission occurs remains poorly understood. For example, little is known about the transmission dynamics of interacting pathogens and non-pathogens within a common host. In addition, although these dynamics take place over evolutionary time for pathogens and in the context of social systems, insufficient attention has been given to integrating ecological, epidemiological, evolutionary, and socio-economic dynamics.

At present, basic and applied research in infectious disease ecology and evolution are not well integrated. The potential benefits of an interdisciplinary research program in this area include:

- development of disease transmission theory,
- improved understanding of how diseases (re)emerge,
- improved understanding of host population and ecosystem effects on disease transmission,
- increased capacity to forecast and respond to outbreaks,
- improved understanding of unintended health effects of development projects affecting terrestrial, freshwater, and coastal marine systems,
- enhanced safety of food supplies, and
- improved strategies to control or prevent infectious diseases and enhance biosecurity.

An understudied aspect of disease transmission is the importance of socio-ecological factors and processes. Important new insights into the drivers and control of infectious diseases in humans and other species can only be achieved by integrated approaches that take into account the ways in which the natural and social environments affect the emergence and spread of infectious disease. This concept, often called "one health," links medical, veterinary, social and environmental sciences by drawing on a common pool of knowledge between the three sectors in order to exploit the potential of animal disease research to provide insights into ecosystem, agricultural, and human health.

This activity is a continuation of the previous joint National Science Foundation/National Institutes of Health/United States Department of Agriculture (NSF/NIH/USDA) Ecology of Infectious Disease competition. Information on past awards can be found atEEID Awards. Additional information for NIH can be found at http://www.fic.nih.gov/Programs/Pages/ecology-infectious-diseases.aspxand for USDA at https://nifa.usda.gov/funding-opportunity/ecology-and-evolution-infectious-diseases.

II. PROGRAM DESCRIPTION

The goal of the Ecology and Evolution of Infectious Diseases (EEID) program is to support important and innovative research on the ecological, evolutionary, and socio-ecological principles that influence the transmission dynamics of infectious diseases. The program's focus is on the discovery of general principles and processes and on building and testing models that elucidate these principles.**Projects must address the quantitative or computational understanding of pathogen transmission dynamics.** Research in EEID is expected to be an interdisciplinary effort that goes beyond the scope of typical studies funded by the standing programs of the partner agencies. Projects should bring together such areas as anthropology, bioinformatics, computational science, ecology, economics, epidemiology, evolution, food science, genomics, geography, global health, mathematics, medicine, microbiology, oceanography, plant science, population biology, sociology, physical environmental sciences, systems science, and veterinary medicine. Research within EEID is expected to generate rigorously characterized and tested models that are of value to the scientific community, but also may be useful in decision making.**The history of the EEID program has shown that the most competitive proposals are those that advance broad, conceptual knowledge that reaches beyond the specific system under study and that may be useful for understanding public, agricultural or ecosystem health, natural resource use and wildlife management, and/or economic development. Such proposals are typically interdisciplinary in their approach and/or the nature of the question(s) being addressed.**

Infectious disease transmission reflects complex, dynamic relationships that occur on varying spatial and temporal landscapes, are created by both ecological and evolutionary processes, and are revealed in genome architecture, physiological systems, population dynamics, and community structure, as well as behavioral and social dynamics. The interactions between disease-causing organisms, their vectors, and their host(s) are embedded within much larger networks of interacting systems, including other microorganisms that may or may not cause disease, one or more vector species, and multiple host species. Analysis of environmental influences (biological, geophysical, economic, and social) on individual and population susceptibility is fundamental to understanding these complex systems of infectious diseases. Research into the ecology (population, community, evolutionary, and social) of infectious diseases will contribute to a deeper understanding of these complex infectious disease systems, to the development of well characterized and tested models, and to the elucidation of general ecological and evolutionary principles.

Insights into the dynamics of infectious disease systems may require integration across several temporal, spatial, and functional scales including molecular, individual, population, societal, and ecosystem levels. Similarly, they may require integration across biological, socio-economic, and geophysical domains. The field of evolutionary ecology, which focuses on both the importance of ecological context in studies of evolution and the importance of evolutionary change for ecological systems, may also provide important insights into infectious disease systems. The interplay of evolution and ecology has implications for understanding how infectious agents emerge as pathogens, adapt to one or more hosts, interact with other microbial communities (e.g., microbiome), and are transmitted among hosts.

A critical goal of research supported by this program is the generation of principles and conceptual frameworks that organize and inform the research and that lead to mathematical, computational, and statistical models of infectious disease dynamics. Diverse modeling approaches are appropriate, including, but not limited to, mathematical equations, computational simulations, geospatial algorithms, and statistical models.**For the EEID program, the most competitive proposals are organized around an overarching conceptual framework that leads to such a model.** Models should aim to be explanatory beyond the specific system under study and must be well-characterized and rigorously tested. Proposals must describe how models will be developed, evaluated, and disseminated. Proposals must identify which individual(s) will oversee the quantitative approaches and provide evidence of demonstrated expertise in mathematical, computational, or statistical modeling and/or data analysis. Likewise, strategies for data collection must be well designed to contribute to and test model design. Proposals must include plans for dissemination of data, models, and tools developed by this program.

A variety of *topics, questions, systems and approaches*are appropriate. Among the areas of particular interest are: the role of social influences on the susceptibility of individuals or populations; multiway interactions between pathogenic and non-pathogenic organisms and their mutual hosts; the role of medical, agricultural or environmental practices on pathogen emergence and transmission; emergence of pathogens from non-pathogenic populations; host switching; evolutionary dynamics in an ecological context such as disease control interventions and drug resistance. These topics have significant ecological and evolutionary components that should be studied as a system, not in isolation. Depending on the hypotheses or research questions being addressed, investigations might entail some combination of laboratory experiments, field observations or manipulations, public health interventions (although clinical trials are beyond the scope of the EEID program), analysis of social and cultural processes, or ethnographic studies. Research may also focus on novel analyses of existing data and/or theoretical investigations of ecological and evolutionary dynamics. Investigations may focus on model infectious disease systems in natural (terrestrial, freshwater, or marine) or laboratory settings where those systems elucidate general principles.

Research may use a variety of study systems. The organism(s) or system(s) selected for study should be justified with respect to its suitability to study questions of ecology and/or evolutionary ecology. Research may involve a variety of infectious agents, individual diseases, or groups of diseases, and might involve one or more social systems, regions, habitats, or groups of organisms. Proposals may focus on terrestrial or aquatic systems and organisms and may include infectious diseases of humans, non-human animals, or plants.**Regardless of the system or approach taken, a proposal must have a significant focus on the ecology of pathogen transmission to be eligible for funding.**

Because of the complexity of studies on the ecology and evolutionary ecology of infectious diseases, multidisciplinary teams of domestic and international collaborators with expertise from diverse disciplines are likely to be most effective. Investigators are encouraged to develop collaborations with public health research communities where that is appropriate. Collaborative teams could include, for example: ecologists, epidemiologists, medical scientists, veterinary scientists, oceanographers, evolutionary biologists, social and behavioral scientists, entomologists, food scientists, microbiologists, pathologists, and parasitologists, geologists, hydrologists, geospatial analysts, and mathematical scientists. The research plan should indicate how multiple disciplines will be integrated and how new investigators in U.S. and collaborating foreign institutions will be prepared to further this research.

The EEID program is not intended to be the only avenue of support by the participating agencies for supporting research on infectious diseases. Specifically, proposals submitted in response to this solicitation must address ecological dynamics and among-host transmission, even when evolutionary studies are a substantive part of the proposal. Investigations that are outside the scope of this EEID announcement include:

- those limited solely to genetic patterns of evolutionary change (e.g., comparative genomics),
- those that focus solely on human diseases without considering the broader ecological context,
- those that focus solely on pathogen discovery,
- those that focus solely on within-host biological processes,
- those that focus solely on vector species ecology, and
- those that have not pre-identified at least one pathogenic organism that will be the focus of the study,
- those that focus on antimicrobial resistance without considering pathogen transmission dynamics.

The EEID competition broadly welcomes, but does not require, that projects include international collaborators. Three specific forms of collaboration (US-UK Collaborative Projects, US-Israel Collaborative Projects, and US-China Collaborative Projects) are described below. These specific activities do not preclude other international collaborations.

EEID Partner Interests

Fogarty International Center

The Fogarty International Center (FIC) is dedicated to advancing the mission of the National Institutes of Health (NIH) by supporting and facilitating global health research conducted by U.S. and international investigators, building partnerships between health research institutions in the U.S. and abroad, and training the next generation of scientists to address global health needs. The FIC is interested in EEID applications that include explicit plans for capacity building in Low- or Middle-Income Countries (LMICs), as defined by the World Bank and encourages applications that are focused on significant and/or emerging infectious disease threats, including zoonotic disease threats, to human health in LMICs. The FIC will consider supporting meritorious EEID research applications that address the above priorities and/or meritorious EEID Research Coordination Network (RCN) applications aimed at capacity building in LMICs.

National Institute of General Medical Sciences

The National Institute of General Medical Sciences (NIGMS) supports basic research that improves understanding of biological processes and lays the foundation for advances in disease diagnoses, prevention, and treatment. NIGMS also has a strong interest in training and support of the nation's scientific workforce. NIGMS is interested in EEID applications that address the evolution of hosts, pathogens and their interactions as well as basic biology and population genetics of hosts and pathogens as they relate to disease transmission and prevention. NIGMS will consider supporting meritorious EEID research applications as single-PI or multi-PI research program grants.

National Institute of Allergy and Infectious Diseases

The National Institute of Allergy and Infectious Diseases (NIAID) conducts and supports basic and applied research to better understand, treat, and ultimately prevent infectious, immunologic, and allergic diseases. NIAID supports research on nearly 300 infectious agents and investigates the biological properties of these pathogens and the immune system's responses to them. Findings from this research are vital to NIAID efforts to create vaccines, drugs, and diagnostic tools to better diagnose, prevent, and treat infectious diseases.

USDA/NIFA

The National Institute of Food and Agriculture (NIFA) supports research, education, and extension work that addresses key problems of national, regional, and multi-state importance in sustaining all components of food and agriculture, including farm efficiency and profitability, ranching, bioenergy, forestry (both urban and agroforestry), aquaculture, rural communities and entrepreneurship, human nutrition, food safety, physical and social sciences, home economics and rural human ecology, biotechnology, and conventional breeding, and including both conventional and organic food production systems.

Applicants may submit proposals that support one or more of the seven USDA's strategic goals for FY2018-2022 (https://www.usda.gov/our-agency/about-usda/strategic-goals). Any such activity proposed (e.g., partnerships, exchanges, training, and/or travel), must first and foremost support NIFA's domestic program goals. Applicants must clearly describe and demonstrate how international activities proposed in applications will contribute to and support advances in U.S. agriculture.

US-UK Collaborative Proposals

Recognizing the potential for international collaboration to advance EEID research and education objectives, NSF has partnered for this solicitation with the UK Research and Innovation. This partnership will facilitate coordinated funding of U.S. and U.K. research collaboration within the EEID program. UKRI encourages proposals with focus on animal and human Coronaviruses including SARS-CoV-2, although other topics are also eligible.

UK researchers applying under this heading must meet UKRI eligibility requirements for managed mode calls and must apply through an institution eligible to receive UKRI funding. Please see UKRI funding rules: http://www.bbsrc.ac.uk/funding/apply/grants-guide.aspx.

PIs are **strongly encouraged** to contact the relevant Cognizant Program Officer to confirm that the UK component fits UKRI requirements. Applications with non-eligible UK partners will not be considered for funding as a US-UK Collaborative Projects.

US-Israel Collaborative Proposals

Recognizing the potential for international collaboration to advance EEID research and education objectives, NSF has partnered for this solicitation with the U.S.-Israel Binational Science Foundation (BSF), which is an organization owned by the two governments with the aim of facilitating scientific relations between them. The Israeli component of the US-Israel Collaborative Projects will be funded by the BSF using special funds provided by the Israeli government.

Israeli researchers applying under this heading must meet BSF eligibility requirements and must apply through an institution eligible to receive BSF funding. Please see BSF eligibility rules: http://www.bsf.org.il/ElectronicSubmission/GatewayFormsAndGuidelines.aspx?PageId=7&innerTextID=0

US-UK-Israel Collaborative Proposals

Multinational collaborative research projects that involve researchers from the US, UK and Israel are also welcome. Any such projects must meet the requirements of each of the NSF, the UKRI, and the BSF.

US-China Collaborative Proposals

Recognizing the potential for international collaboration to advance EEID research and education objectives, NSF has partnered for this solicitation with the National Natural Science Foundation of China (NSFC). The Chinese component of the US-China Collaborative Projects will be funded by the NSFC using funds provided by the Chinese government.

Chinese researchers applying under this heading must meet NSFC eligibility requirements and must apply through an institution eligible to receive NSFC funding. Please see NSFC eligibility rules: http://www.nsfc.gov.cn/nsfc/cen/xmzn/2017xmzn/sqxz.html. Applications with non-eligible China partners will not be considered for funding as a US-China Collaborative Project.

US-China-UK Collaborative Proposals

Multinational collaborative research projects that involve researchers from the US, China and the UK are also welcome. Any such projects must meet the requirements of each of the NSF, the NSFC, and the UKRI.

US-China-Israel Collaborative Proposals

Multinational collaborative research projects that involve researchers from the US, China and Israel are also welcome. Any such projects must meet the requirements of each of the NSF, the NSFC, and the BSF.

Research Coordination Network (RCN) Proposals

The EEID program will accept proposals to establish Research Coordination Networks that focus on issues involving infectious disease ecology, socio-ecology, and evolution. RCN projects are also eligible to be submitted as US-UK, US-China, or US-UK-China Collaborative Projects. Information on the scope of RCN projects and the format of those proposals can be found at (https://www.nsf.gov/funding/pgm_summ.jsp?pims_id=11691&org=BIO&from=home). Such RCN proposals should be submitted under the EEID solicitation and deadline.

III. AWARD INFORMATION

Award size: Under this solicitation, the maximum award size for all years for the US component is $2.5 million, including indirect costs. The minimum award size is $1.5 million total project costs for all years, except for international collaborative proposals (US-UK, US-Israel, and US-China Collaborative Projects) that have a minimum award size of $1.0 million for the US component. Those collaborative projects can request additional funding for the international component of the project. For US-Israel Collaborative Projects, the maximum award size for the Israeli portion is $720,000. For US-China Collaborative Projects, the maximum award size for the Chinese portion is ¥4.5M total project costs for all years.

Award duration: The maximum award duration is five years.

RCN proposals: The maximum award size for RCN proposals is $500,000 as per the RCN solicitation. For international Collaborative RCN proposals, the maximum award size for the US component is $500,000.

Award number: Approximately 12 new awards are anticipated in FY 2021, depending on the quality of submissions and the availability of funds; the expected funding will be $24.0 million. That amount includes approximately $11.0M from the NSF for new standard or continuing awards, approximately $7.5M from the NIH for new or continuing awards, and $5.5M from the USDA for new awards. Of those 12 awards, up to 4 are anticipated to be US-UK Collaborative Projects, depending on the quality of submissions and the availability of funds; the expected funding from the UKRI for this call is up to £2M. This amount reflects 80% of the full economic costs in the U.K. Of those 12 awards, up to 1 is anticipated to be a US-Israel Collaborative Project, depending on the quality of submissions and the availability of funds; the expected funding from the BSF for the Israeli component of the US-Israel Collaborative Projects will be a maximum of $80,000/year. Of those 12 awards, up to 2 are anticipated to be US-China Collaborative Projects for a total of ¥9M, depending on the quality of the submissions and the availability of funds.

Upon conclusion of the review process, meritorious proposals may be recommended for funding by either NSF, NIH, or USDA, at the option of the agencies, not the proposing organizations. Proposals selected for funding by NIH or USDA will need to be reformatted and resubmitted to that agency. Subsequent submission and grant administration procedures will be in accordance with the individual policies of the awarding agency. Further information will be provided to these applicants after selection.

Proposals selected for funding consideration by the NIH will be invited to resubmit to the Division of Receipt and Referral (DRR) in NIH's Center for Scientific Review (CSR) (see Section VI.B). PIs submitting to the NIH must be registered in eRA Commons and the applicant's organization must be registered with SAM, Grants.gov and eRA Commons. PIs should therefore ensure that all registrations required for NIH submission are in place before the NIH receipt deadline.

USDA/NIFA Legislative Authority: The USDA authority for this RFA is contained in Section 7406 of the Food, Conservation, and Energy Act of 2008 (FCEA) (Pub. L. 110-246) which amends section 2(b) of the Competitive, Special, and Facilities Research Grant Act (7 U.S.C. 450i(b)) to authorize the Secretary of Agriculture to establish the Agriculture and Food Research Initiative (AFRI); a new competitive grant program to provide funding for fundamental and applied research, extension, and education to address food and agricultural sciences. AFRI is subject to the provision found at 7 CFR Part 3430.

For US-UK, US-UK-Israel, or US-UK-China Collaborative Projects, the UK component of the collaboration will be awarded through the UKRI in accordance with its policies. If the UKRI selects an application for funding, the applicant will be required to submit the costs for the UK element of the proposal via the Je-S application submission system before final sign-off. UK collaborators should therefore ensure they are registered Je-S users before the proposal is submitted.

For US-Israel, US-Israel-UK, or US-Israel-China Collaborative Projects, the Israeli component of the collaboration will be awarded through the BSF in accordance with its policies and regulations. The collaborative proposal must be submitted to the BSF by the Israeli scientist by the BSF-established deadline.

For US-China, US-China-UK, or US-China-Israel Collaborative Projects, the Chinese component of the collaboration will be awarded through the NSFC in accordance with its policies and regulations. The collaborative proposal must be submitted to the NSFC application submission system by the Chinese scientists by the NSFC-established deadline.

IV. ELIGIBILITY INFORMATION

Who May Submit Proposals:

The categories of proposers eligible to submit proposals to the National Science Foundation are identified in the *NSF Proposal & Award Policies & Procedures Guide* (PAPPG), Chapter I.E. Unaffiliated individuals are not eligible to submit proposals in response to this solicitation.

Who May Serve as PI:

There are no restrictions or limits.

Limit on Number of Proposals per Organization:

There are no restrictions or limits.

Limit on Number of Proposals per PI or Co-PI: 2

In a given year, an individual may participate as a PI, co-PI, or subaward lead on no more than **two** proposals submitted in response to this solicitation. **This limit does not include Research Coordination Networks (RCN) proposals.** In addition, an individual from Israel may participate in no more than **one** US-BSF proposal and an individual from China may participate in no more than **one** US-China proposal. Proposals in excess of the limit for any person will be returned without review in the reverse order received. Participating in a proposal as other senior personnel does not count in this limit. Changes in investigator roles post-submission to meet the eligibility limits will not be allowed. **It is the responsibility of the submitters to confirm that the entire team is within the eligibility guidelines.**

Additional Eligibility Info:

NIH Eligibility: Clinical trials are not allowed. The NIH will only consider applications that do not propose clinical trials. Individuals who are considering submitting a proposal in response to this solicitation should review https://grants.nih.gov/ct-decision/ in determining whether the project meets the NIH definition of a Clinical Trial. Institutions eligible for awards by the NIH's Fogarty International Center (FIC) include foreign institutions in low- and middle-income countries (LMICs), as defined by the World Bank (foreign institutions in high-income countries are not eligible for FIC awards).

USDA/NIFA Eligibility: Eligible entities for award include, (1) State agricultural experiment stations; (2) colleges and universities (including junior colleges offering associate degrees or higher); (3) university research foundations; (4) other research institutions and organizations; (5) Federal agencies, (6) national laboratories; (7) private organizations or corporations; (8) individuals who are U.S. citizens, nations, or permanent residents; and (9) any group consisting of 2 or more entities identified in (1) through (8). Eligible institutions do not include foreign and international organizations.

V. PROPOSAL PREPARATION AND SUBMISSION INSTRUCTIONS

A. Proposal Preparation Instructions

Full Proposal Preparation Instructions: Proposers may opt to submit proposals in response to this Program Solicitation via FastLane, Research.gov, or Grants.gov.

- Full proposals submitted via FastLane: Proposals submitted in response to this program solicitation should be prepared and submitted in accordance with the general guidelines contained in the *NSF Proposal & Award Policies & Procedures Guide* (PAPPG). The complete text of the PAPPG is available electronically on the NSF website at: https://www.nsf.gov/publications/pub_summ.jsp?ods_key=pappg. Paper copies of the PAPPG may be obtained from the NSF Publications Clearinghouse, telephone (703) 292-8134 or by e-mail from nsfpubs@nsf.gov. Proposers are reminded to identify this program solicitation number in the program solicitation block on the NSF Cover Sheet For Proposal to the National Science Foundation. Compliance with this requirement is critical to determining the relevant proposal processing guidelines. Failure to submit this information may delay processing.
- Full Proposals submitted via Research.gov: Proposals submitted in response to this program solicitation should be prepared and submitted in accordance with the general guidelines contained in the *NSF Proposal and Award Policies and Procedures Guide* (PAPPG). The complete text of the PAPPG is available electronically on the NSF website at: https://www.nsf.gov/publications/pub_summ.jsp?ods_key=pappg. Paper copies of the PAPPG may be obtained from the NSF Publications Clearinghouse, telephone (703) 292-8134 or by e-mail from nsfpubs@nsf.gov. The Prepare New Proposal setup will prompt you for the program solicitation number.
- Full proposals submitted via Grants.gov: Proposals submitted in response to this program solicitation via Grants.gov should be prepared and submitted in accordance with the *NSF Grants.gov Application Guide: A Guide for the Preparation and Submission of NSF Applications via Grants.gov*. The complete text of the *NSF Grants.gov Application Guide* is available on the Grants.gov website and on the NSF website at: (https://www.nsf.gov/publications/pub_summ.jsp?ods_key=grantsgovguide). To obtain copies of the Application Guide and Application Forms Package, click on the Apply tab on the Grants.gov site, then click on the Apply Step 1: Download a Grant Application Package and Application Instructions link and enter the funding opportunity number, (the program solicitation number without the NSF prefix) and press the Download Package button. Paper copies of the Grants.gov Application Guide also may be obtained from the NSF Publications Clearinghouse, telephone (703) 292-8134 or by e-mail from nsfpubs@nsf.gov.

See PAPPG Chapter II.C.2 for guidance on the required sections of a full research proposal submitted to NSF. Please note that the proposal preparation instructions provided in this program solicitation may deviate from the PAPPG instructions.

Special Information and Supplementary Documentation:

Proposals Involving Multiple Institutions. Of the two types of collaborative proposal formats described in the Proposal & Award Policies & Procedures Guide, this solicitation allows only a single proposal submission with subawards administered by that lead organization. In the case of proposals involving multiple organizations, a single organization **must** be identified as the lead, and **a single proposal** describing the entire project must be submitted by that organization. Funds may be distributed among partner organizations via subawards from the lead organization. A budget utilizing the standard NSF budget format should be submitted for each subawardee. The requirement for a single organization to submit the sole proposal for a project is designed to facilitate effective coordination among participating organizations and to avoid difficulties that ensue in funded projects when individuals change organizations and/or cease to fulfill project responsibilities.

- **Research Experiences for Undergraduates**. Funds to support Research Experiences for Undergraduates (REUs) should be included in the original proposal. A limited number of post-award supplements may be available if such activities were unforeseen at the time of submission and the request broadens participation in STEM fields. Eligibility for post-award educational supplements for REU, RET, RAHSS, and ROA projects is described on the DEB supplement request website: https://www.nsf.gov/bio/deb/suppopp.jsp. **Submission Guidance:** The descriptions of proposed REU activities should be included in the Supplementary Documents. For REUs, follow the guidelines for "REU supplement requests as part of a proposal" in the REU solicitation. REU projects must involve students in meaningful ways in ongoing research programs or in research projects specifically designed for the REU student. If the intent is to engage students as technicians, then an REU is not the appropriate support mechanism; instead, salary support should be entered on the Undergraduate Students line of the proposal budget. The description of these activities is limited to 3 pages. If multiple institutions on a collaborative proposal are requesting funds for REUs, all REU activities should be included in one supplementary document, limited to 3 pages. All person related costs, including stipends and/or travel should be placed in Participant Support Costs on the budget. A detailed breakdown of the budget must be included in the budget justification. Budgets for REUs are generally $6,000-8,000 per student.

- **Senior Personnel List Spreadsheet.** An additional spreadsheet listing all senior personnel involved in the project **must** be submitted. This spreadsheet is separate from the spreadsheet that lists collaborators and other affiliations (COA) information. The spreadsheet template can be found at https://www.nsf.gov/bio/deb/debpersonnellist.xlsx. Please read the instructions carefully. Using the template, compile an Excel file that provides information for all persons identified in the proposal as: "PI or co-PI" (i.e., those listed on the Cover Sheet); "Other Senior Personnel/Subawardee"; or "Other Personnel" who have a biographical sketch included in the proposal, including all international collaborators. Only one spreadsheet should be submitted per project. The file **must** include the FastLane proposal ID assigned after submission of your proposal (i.e., not the Temporary ID # or Grants.gov ID #). Once completed, the file should be submitted by email to debtemplate@nsf.gov within one business day of proposal submission.

- **Data Management Plan.** The PAPPG (II.C.2.j) requires the inclusion of a Data Management Plan (DMP) with all full proposal submissions. The DMP can be no longer than two pages and must be inclusive of the entire project. It must include two sections: (1) *Data Plans*, and (2) *Intellectual Property Plans*. Those sections should address the following points:

    1. *Data Plans*. All projects must ensure that data and biological materials are collected, archived, digitized, and made available using methods that allow current and future investigators to access data and material. Funded projects must disseminate project data broadly in a timely and responsible manner, using widely accepted electronic data standards, a named community-accepted, publicly-accessible data repository and with as few restrictions as possible. Data and digital products should be identified, and the following described for *each* of them:
        - Format and standard of primary data;
        - Metadata to be collected and disseminated with the primary data;
        - Timetable of release of ALL data, consistent with privacy and other concerns regarding sensitive information;
        - Public repository to be used;
        - License for use, with an emphasis on open source licenses such as MIT and GPL;
        - Any constraints on release, which must be clearly justified; and
        - Person(s) responsible for the release.

        All software and code must be in a versioned code repository (e.g., GitHub, BitBucket). We strongly encourage release of ready-to-use software and code through integration with computing resources (e.g., Galaxy, CyVerse), in Virtual Machines (e.g., AWS, JetStream), and/or in Containers (e.g., Docker/DockerHub). Published results should always include information on how to access the supporting data.

        Additional guidance about the development of Data Management plans, including domain-specific guidance, is provided by the Directorate for Biological Sciences, the Directorate for Social, Behavioral and Economic Sciences, the Division of Ocean Sciences, and the UKRI.

    2. *Intellectual Property Plans*. The DMP MUST provide a protocol and timeline for the development of intellectual property agreements. The agreement should indicate:
        - Who are the owners of any data or other intellectual property;
        - How financial benefits of the intellectual property will be allocated;
        - How authorship of publications will be determined; and
        - How IP disputes will be adjudicated.

    A reasonable charge for community resources is permissible, but the fee structure must be outlined clearly in the IP plan. If a Material Transfer Agreement is required, the terms must be described in detail. No reach-through rights are allowed. Data or materials resulting from NSF-funded research obtained with proprietary materials must be readily available without any restrictions to the users. For this reason, the terms of any usage agreements should be stated clearly in the IP plan.

    For multi-institutional projects, the lead institution is responsible for coordinating and managing the intellectual property resulting from the award. A complete IP agreement MUST be included with the first annual report of the project.

    - **Letters of Collaboration.** Supplementary Documents may include letters of collaboration from individuals or organizations that are integral parts of the proposed project but are not listed as PI, co-PI, or other senior personnel on the main proposal or any subaward. Such involvement may include subsidiary involvement in some aspect of the project, cooperation on outreach efforts, or documentation of permission to access materials or data. Letters of collaboration should focus solely on affirming that the individual or organization is willing to collaborate on the project as specified in the project description. No additional text, especially elaboration of the nature of activities to be undertaken by the collaborator and endorsements of the potential value or significance of the project for the collaborator, may be included. The template that **must** be used for the preparation of letters of collaboration is provided below.

    Letters of collaboration should not be provided from any individual designated as a principal investigator or a senior personnel, nor are letters of collaboration required from any organization that will be a subawardee in the proposal budget.

    Each letter of collaboration **must** be signed by the designated collaborator. Requests to collaborators for letters of collaboration should be made by the PI well in advance of the proposal submission deadline, because they **must** be included at the time of proposal submission. **Letters deviating from**

this template will not be accepted and may be grounds for returning the proposal without review.

Template to be used for letters of collaboration

To: NSF _____(*Program Title*)_____ Program

From: _____

(*Printed name of the individual collaborator or name of the organization and name and position of the official submitting this memo*)

By signing below (or transmitting electronically), I acknowledge that I am listed as a collaborator on this proposal, entitled "_____(*proposal title*)_____," with _____(*PI name*)_____ as the Principal Investigator. I agree to undertake the tasks assigned to me or my organization, as described in the project description of the proposal, and I commit to provide or make available the resources specified therein.

Signed: _____

Organization: _____

Date: _____

Research Coordination Network proposals

These proposals should begin the title with "**RCN:**" and follow the proposal preparation instructions in the RCN solicitation (https://www.nsf.gov/funding/pgm_summ.jsp?pims_id=11691&org=BIO&from=home).

US-UK Collaborative proposals

For full application: These proposals should begin the title with "**US-UK Collab:**". Collaborative RCN proposals should begin the title with "**US-UK Collab: RCN**:" Information for the UK portion of US-UK Collaborative Proposals should be included as Supplementary Documents or as a single copy document as specified below. That information should include the following, and only the following:

1. Biographical sketches of UK senior personnel: Those biographical sketches must conform to NSF format and limitations.

2. UK budget: Costs for the UK component of the project should be entered onto the Je-S system but the completed form **SHOULD NOT** be submitted electronically to the UKRI at this stage. Instead, a PDF version of the form should be saved and sent to the US lead PI for inclusion as a supplementary document in the proposal. Also, an electronic copy of this document should be sent to UKRI Cognizant Programme Officer no later than the NSF submission deadline. Full details on what is required can be obtained at (http://www.bbsrc.ac.uk/eeid). Applicants should ensure that they contact the main UK Cognizant Programme Officer at UKRI to discuss the remit of their proposal and to confirm whether they should complete a Je-S form. The researchers will be asked to attend a meeting to be held at either the National Science Foundation or an alternate location. Include the necessary travel costs for attendance at the meeting in the proposed budget.

3. Letters of collaboration: Letters of collaboration from UK scientists are required. These letters must be restricted to a statement of intent to collaborate only as described above. Additional information on the nature of the collaboration and the roles of the investigators should be included in the Project Description.

4. Institutional endorsement: An institutional certification of the submission must be a signed letter from an authorized U.K. institutional representative and included in supplemental documents with the following text:

"I confirm on behalf of [*insert name of institution*] that the U.S.-U.K.Collaborative proposal between [*insert name of US PI and institution*] and [*insert name of UK PI*] is endorsed and has been submitted by [*name of Research Office*]."

5. Sharing of unattributed reviews: Unattributed reviews will be shared with UKRI. The following text must be included in a **Single Copy Document** and signed by the lead US investigator, confirming that the investigators involved in the proposal acknowledge and confirm this fact.

On behalf of the proposal investigators, I, _____ (*insert US Lead PI Name*), consent that the proposal as well as its unattributed reviews will be shared with the EEID partner-funding agencies.

Signed: _____

Organization: _____

Date: _____

6. Collaborators and other affiliations. A COA document must be submitted for each senior personnel for whom a biographical sketch is included.

US-Israel Collaborative proposals

These proposals should begin the title with "**US-Israel Collab**:". Information for the Israel portion of the US-Israel Collaborative Proposals should be included as Supplementary Documents or as a single copy document as specified below. That information should include the following, and only the following:

1. Biographical sketches of Israeli PIs: Those biographical sketches must conform to NSF format and limitations.

2. Israeli budget: Costs for the Israeli component should be copied from the BSF submission of the proposal using the BSF format: http://www.bsf.org.il/data/FormsToDownload/BSF-NSF_EEID_Program_Call_for_Proposal.pdf

3. Letters of collaboration: Letters of collaboration from scientists on the Israeli component are required. These letters must be restricted to a statement of intent to collaborate only as described above. Additional information on the nature of the collaboration and the roles of the investigators should be included in the Project Description.

4. Institutional endorsement: An institutional certification of the submission must be a signed letter from an authorized Israeli institutional

representative and included in supplemental documents with the following text:

"I confirm on behalf of [*insert name of institution*] that the U.S.-Israel Collaborative proposal between [*insert name of US PI and institution*] and [*insert name of Israeli PI*] is endorsed and has been submitted by [*name of Research Office*]."

5. Sharing of unattributed reviews: Unattributed reviews will be shared with the BSF. The following text must be included in a**Single Copy Document** and signed by the lead US investigator, confirming that the investigators involved in the proposal acknowledge and confirm this fact.

On behalf of the proposal investigators, I, _____ (*insert US Lead PI Name)*, consent that the proposal as well as its unattributed reviews will be shared with the EEID partner-funding agencies.

Signed: _____

Organization: _____

Date: _____

6. Collaborators and other affiliations. A COA document must be submitted for each senior personnel for whom a biographical sketch is included.

Full proposals that include an Israeli collaboration must be also submitted to the BSF by the Israeli partner, using the BSF submission system: http://www.bsf.org.il/ElectronicSubmission/GatewayFormsAndGuidelines.aspx?PageId=7&innerTextID=0 Additional information can be found at: http://www.bsf.org.il/data/FormsToDownload/BSF-NSF_Ecology_and_Evolution_Infectious_Diseases_Program_Call_for_Proposal.pdf

US-UK-Israel Collaborative proposals

These proposals should begin the title with '**US-UK-Israel Collab:**". Information for the UK and Israel portion should be included as Supplementary Documents as described above.

US-China Collaborative proposals

These proposals should begin the title with '**US-China Collab**:". Collaborative RCN proposals should begin the title with '**US-China Collab: RCN**:" Information for the China portion of the US-China Collaborative Proposals should be included as Supplementary Documents or as a single copy document as specified below. That information should include the following, and only the following:

1. Biographical sketches of Chinese PIs: Those biographical sketches must conform to NSF format and limitations.

2. Chinese budget: Costs for the Chinese component should be copied from the Chinese submission of the proposal using the NSFC format.

3. Letters of collaboration: Letters of collaboration from scientists on the Chinese component are required. These letters must be restricted to a statement of intent to collaborate only as described above. Additional information on the nature of the collaboration and the roles of the investigators should be included in the Project Description.

4. Sharing of unattributed reviews: Unattributed reviews will be shared with the NSFC. The following text must be included in a**Single Copy Document** and signed by the lead US investigator, confirming that the investigators involved in the proposal acknowledge and confirm this fact.

On behalf of the proposal investigators, I, (*insert US Lead PI Name*), consent that the proposal as well as its unattributed reviews will be shared with the EEID partner-funding agencies.

Signed: _____

Organization: _____

Date: _____

5. Collaborators and other affiliations. A COA document must be submitted for each senior personnel for whom a biographical sketch is included.

Full proposals that include an Chinese collaboration must be also submitted to the NSFC by the Chinese partner as supplementary documents, including the Letters of Collaboration, to their online applications, using the NSFC application submission system: http://isisn.nsfc.gov.cn/egrantweb/

US-UK-China Collaborative proposals

These proposals should begin the title with '**US-UK-China Collab:**". Collaborative RCN proposals should begin the title with '**US-UK-China Collab: RCN**:" Information for the UK and China portion should be included as Supplementary Documents as described above.

US-China-Israel Collaborative proposals

These proposals should begin the title with '**US-China-Israel Collab:**". Information for the China and Israel portion should be included as Supplementary Documents as described above.

B. Budgetary Information

Cost Sharing:

Inclusion of voluntary committed cost sharing is prohibited.

Other Budgetary Limitations:

EEID projects must have a minimum budget of $1,500,000 in total project costs for all years, except that US-UK Collaborative, US-Israel, and US-China Collaborative projects must have a minimum budget of $1,000,000 in total project costs for all years. Research that falls within the scope of the EEID initiative

but with project aims that do not require budgets of this magnitude should be directed to the appropriate core program.

Budget Preparation Instructions:

Subawards

In accordance with the applicable award terms and conditions, proposers are reminded of their responsibilities with regard to subawardees. Should an award be made, the primary awardee is responsible for ensuring compliance with the appropriate terms and conditions to, as well as the management and oversight of, any subawardees on the project, including any foreign subawardees.

C. Due Dates

- **Full Proposal Deadline(s)** (due by 5 p.m. submitter's local time):

  November 18, 2020

  Third Wednesday in November, Annually Thereafter

D. FastLane/Research.gov/Grants.gov Requirements

For Proposals Submitted Via FastLane or Research.gov:

To prepare and submit a proposal via FastLane, see detailed technical instructions available at: https://www.fastlane.nsf.gov/a1/newstan.htm. To prepare and submit a proposal via Research.gov, see detailed technical instructions available at: https://www.research.gov/research-portal/appmanager/base/desktop?_nfpb=true&_pageLabel=research_node_display&_nodePath=/researchGov/Service/Desktop/ProposalPreparationandSubmission.html. For FastLane or Research.gov user support, call the FastLane and Research.gov Help Desk at 1-800-673-6188 or e-mail fastlane@nsf.gov or rgov@nsf.gov. The FastLane and Research.gov Help Desk answers general technical questions related to the use of the FastLane and Research.gov systems. Specific questions related to this program solicitation should be referred to the NSF program staff contact(s) listed in Section VIII of this funding opportunity.

For Proposals Submitted Via Grants.gov:

Before using Grants.gov for the first time, each organization must register to create an institutional profile. Once registered, the applicant's organization can then apply for any federal grant on the Grants.gov website. Comprehensive information about using Grants.gov is available on the Grants.gov Applicant Resources webpage: https://www.grants.gov/web/grants/applicants.html. In addition, the NSF Grants.gov Application Guide (see link in Section V.A) provides instructions regarding the technical preparation of proposals via Grants.gov. For Grants.gov user support, contact the Grants.gov Contact Center at 1-800-518-4726 or by email: support@grants.gov. The Grants.gov Contact Center answers general technical questions related to the use of Grants.gov. Specific questions related to this program solicitation should be referred to the NSF program staff contact(s) listed in Section VIII of this solicitation.

Submitting the Proposal: Once all documents have been completed, the Authorized Organizational Representative (AOR) must submit the application to Grants.gov and verify the desired funding opportunity and agency to which the application is submitted. The AOR must then sign and submit the application to Grants.gov. The completed application will be transferred to the NSF FastLane system for further processing.

Proposers that submitted via FastLane or Research.gov may use Research.gov to verify the status of their submission to NSF. For proposers that submitted via Grants.gov, until an application has been received and validated by NSF, the Authorized Organizational Representative may check the status of an application on Grants.gov. After proposers have received an e-mail notification from NSF, Research.gov should be used to check the status of an application.

VI. NSF PROPOSAL PROCESSING AND REVIEW PROCEDURES

Proposals received by NSF are assigned to the appropriate NSF program for acknowledgement and, if they meet NSF requirements, for review. All proposals are carefully reviewed by a scientist, engineer, or educator serving as an NSF Program Officer, and usually by three to ten other persons outside NSF either as *ad hoc* reviewers, panelists, or both, who are experts in the particular fields represented by the proposal. These reviewers are selected by Program Officers charged with oversight of the review process. Proposers are invited to suggest names of persons they believe are especially well qualified to review the proposal and/or persons they would prefer not review the proposal. These suggestions may serve as one source in the reviewer selection process at the Program Officer's discretion. Submission of such names, however, is optional. Care is taken to ensure that reviewers have no conflicts of interest with the proposal. In addition, Program Officers may obtain comments from site visits before recommending final action on proposals. Senior NSF staff further review recommendations for awards. A flowchart that depicts the entire NSF proposal and award process (and associated timeline) is included in PAPPG Exhibit III-1.

A comprehensive description of the Foundation's merit review process is available on the NSF website at: https://www.nsf.gov/bfa/dias/policy/merit_review/.

Proposers should also be aware of core strategies that are essential to the fulfillment of NSF's mission, as articulated in *Building the Future: Investing in Discovery and Innovation - NSF Strategic Plan for Fiscal Years (FY) 2018 – 2022* These strategies are integrated in the program planning and implementation process, of which proposal review is one part. NSF's mission is particularly well-implemented through the integration of research and education and broadening participation in NSF programs, projects, and activities.

One of the strategic objectives in support of NSF's mission is to foster integration of research and education through the programs, projects, and activities it supports at academic and research institutions. These institutions must recruit, train, and prepare a diverse STEM workforce to advance the frontiers of science and participate in the U.S. technology-based economy. NSF's contribution to the national innovation ecosystem is to provide cutting-edge research under the guidance of the Nation's most creative scientists and engineers. NSF also supports development of a strong science, technology, engineering, and mathematics (STEM) workforce by investing in building the knowledge that informs improvements in STEM teaching and learning.

NSF's mission calls for the broadening of opportunities and expanding participation of groups, institutions, and geographic regions that are underrepresented in STEM disciplines, which is essential to the health and vitality of science and engineering. NSF is committed to this principle of diversity and deems it central to the programs, projects, and activities it considers and supports.

A. Merit Review Principles and Criteria

The National Science Foundation strives to invest in a robust and diverse portfolio of projects that creates new knowledge and enables breakthroughs in understanding across all areas of science and engineering research and education. To identify which projects to support, NSF relies on a merit review process that incorporates consideration of both the technical aspects of a proposed project and its potential to contribute more broadly to advancing NSF's mission "to promote the progress of science; to advance the national health, prosperity, and welfare; to secure the national defense; and for other purposes." NSF makes every effort to conduct a fair, competitive, transparent merit review process for the selection of projects.

1. Merit Review Principles

These principles are to be given due diligence by PIs and organizations when preparing proposals and managing projects, by reviewers when reading and evaluating proposals, and by NSF program staff when determining whether or not to recommend proposals for funding and while overseeing awards. Given that NSF is the primary federal agency charged with nurturing and supporting excellence in basic research and education, the following three principles apply:

- All NSF projects should be of the highest quality and have the potential to advance, if not transform, the frontiers of knowledge.
- NSF projects, in the aggregate, should contribute more broadly to achieving societal goals. These "Broader Impacts" may be accomplished through the research itself, through activities that are directly related to specific research projects, or through activities that are supported by, but are complementary to, the project. The project activities may be based on previously established and/or innovative methods and approaches, but in either case must be well justified.
- Meaningful assessment and evaluation of NSF funded projects should be based on appropriate metrics, keeping in mind the likely correlation between the effect of broader impacts and the resources provided to implement projects. If the size of the activity is limited, evaluation of that activity in isolation is not likely to be meaningful. Thus, assessing the effectiveness of these activities may best be done at a higher, more aggregated, level than the individual project.

With respect to the third principle, even if assessment of Broader Impacts outcomes for particular projects is done at an aggregated level, PIs are expected to be accountable for carrying out the activities described in the funded project. Thus, individual projects should include clearly stated goals, specific descriptions of the activities that the PI intends to do, and a plan in place to document the outputs of those activities.

These three merit review principles provide the basis for the merit review criteria, as well as a context within which the users of the criteria can better understand their intent.

2. Merit Review Criteria

All NSF proposals are evaluated through use of the two National Science Board approved merit review criteria. In some instances, however, NSF will employ additional criteria as required to highlight the specific objectives of certain programs and activities.

The two merit review criteria are listed below. **Both** criteria are to be given **full consideration** during the review and decision-making processes; each criterion is necessary but neither, by itself, is sufficient. Therefore, proposers must fully address both criteria. (PAPPG Chapter II.C.2.d(i). contains additional information for use by proposers in development of the Project Description section of the proposal). Reviewers are strongly encouraged to review the criteria, including PAPPG Chapter II.C.2.d(i), prior to the review of a proposal.

When evaluating NSF proposals, reviewers will be asked to consider what the proposers want to do, why they want to do it, how they plan to do it, how they will know if they succeed, and what benefits could accrue if the project is successful. These issues apply both to the technical aspects of the proposal and the way in which the project may make broader contributions. To that end, reviewers will be asked to evaluate all proposals against two criteria:

- **Intellectual Merit:** The Intellectual Merit criterion encompasses the potential to advance knowledge; and
- **Broader Impacts:** The Broader Impacts criterion encompasses the potential to benefit society and contribute to the achievement of specific, desired societal outcomes.

The following elements should be considered in the review for both criteria:

1. What is the potential for the proposed activity to
    a. Advance knowledge and understanding within its own field or across different fields (Intellectual Merit); and
    b. Benefit society or advance desired societal outcomes (Broader Impacts)?
2. To what extent do the proposed activities suggest and explore creative, original, or potentially transformative concepts?
3. Is the plan for carrying out the proposed activities well-reasoned, well-organized, and based on a sound rationale? Does the plan incorporate a mechanism to assess success?
4. How well qualified is the individual, team, or organization to conduct the proposed activities?
5. Are there adequate resources available to the PI (either at the home organization or through collaborations) to carry out the proposed activities?

Broader impacts may be accomplished through the research itself, through the activities that are directly related to specific research projects, or through activities that are supported by, but are complementary to, the project. NSF values the advancement of scientific knowledge and activities that contribute to achievement of societally relevant outcomes. Such outcomes include, but are not limited to: full participation of women, persons with disabilities, and underrepresented minorities in science, technology, engineering, and mathematics (STEM); improved STEM education and educator development at any level; increased public scientific literacy and public engagement with science and technology; improved well-being of individuals in society; development of a diverse, globally competitive STEM workforce; increased partnerships between academia, industry, and others; improved national security; increased economic competitiveness of the United States; and enhanced infrastructure for research and education.

Proposers are reminded that reviewers will also be asked to review the Data Management Plan and the Postdoctoral Researcher Mentoring Plan, as appropriate.

Additional Solicitation Specific Review Criteria

Reviewers may be requested to provide numeric NIH overall impact scores from 1 (highest) to 9 (lowest) based upon their evaluations of the intellectual merits and broader impacts of the applications.

Overall Impact is the likelihood for the project to exert a sustained, powerful influence on the research field(s) involved and is based on the following five core review criteria and relevant additional review criteria. An application does not need to be strong in all categories to be judged likely to have major scientific impact.

The five core review criteria for NIH are:

- **Significance.** Does the applicant discuss the strengths and weaknesses of the rigor of the prior research used to support the proposed project?(Rigor of the prior research, which includes preliminary and published results, concerns the quality and strength of the research being cited by the applicant as crucial to support the application; this is distinct from the hypothesis or justification.) How will the proposed research address weaknesses, gaps, important problems, or critical barriers to progress in the field? If the project aims are achieved, how will scientific knowledge, technical capability, and/or clinical practice be improved? Will successful completion of the aims change the concepts, methods, treatments, technologies, services, or preventative interventions that drive this field?
- **Investigator(s)**. Are the principal investigator (PI), project director (PD), collaborators, and other researchers well suited to the project? For those in the early stages of independent careers, do they have appropriate experience and training? For more established investigators, have they demonstrated an ongoing record of accomplishments that have advanced their field(s)? If the project is collaborative or multi-PD/PI, do the investigators have complementary and integrated expertise; are their leadership approach, governance, and organizational structure appropriate for the project?
- **Innovation**. Does the application challenge and seek to shift current research or clinical practice paradigms by utilizing novel theoretical concepts, approaches or methodologies, instrumentation, or interventions? Are the concepts, approaches or methodologies, instrumentation, or interventions novel to one field of research or novel in a broad sense? Is a refinement, improvement, or new application of theoretical concepts, approaches or methodologies, instrumentation, or interventions proposed?
- **Approach**. Are the overall strategy, methodology, and analyses well-reasoned, appropriate, and supported by rigor in the prior research to accomplish the project's specific aims? Is there scientific rigor in the proposed study? (Scientific rigor is defined as the strict application of the scientific method to ensure robust and unbiased experimental design, methodology, analysis, interpretation, and reporting of results.) Are potential problems, alternative strategies, and benchmarks for success presented? If the project is in the early stages of development, will the strategy establish feasibility, and will particularly risky aspects be managed? Have the investigators presented adequate plans to address relevant biological variables (e.g. sex, age, weight, health condition, human ethnicity, animal species and strains, etc.) for studies in vertebrate animals or human subjects? If the project involves human subjects, are there plans for the protection of human subjects from research risks, and the inclusion (or exclusion) of individuals on the basis of sex/gender, race, and ethnicity, as well as the inclusion (or exclusion) of individuals of all ages (including children and older adults), justified in terms of the scientific goals and research strategy proposed?
- **Environment**. Will the scientific environment in which the work will be done contribute to the probability of success? Are the institutional support, equipment, and other physical resources available to the investigators adequate for the project proposed? Will the project benefit from unique features of the scientific environment, subject populations, or collaborative arrangements?

As relevant to the proposed project, the following additional review criteria will be addressed and considered in the determination of scientific merit and rating as part of the Overall Impact score.

- **Protections for Human Subjects.** To be considered human subjects research, the participating individuals must be living and identifiable, and the data and/or specimens are specifically obtained for the proposed research. For research that involves human subjects but does not involve one of the categories of research that are exempt under 45 CFR Part 46.104, the committee will evaluate the justification for involvement of human subjects and the proposed protections from research risk relating to their participation according to the following review criteria: 1) risk to subjects, 2) adequacy of protection against risks, 3) potential benefits to the subjects and others, and 4) importance of the knowledge to be gained. For research that involves human subjects and meets the criteria for one or more of the eight categories of research that are exempt under 45 CFR Part 46.104, the committee will evaluate: 1) the justification for the exemption, 2) human subjects involvement and characteristics, and 3) sources of materials.
- **Inclusion of Women, Minorities, and Individuals Across the Lifespan**. When the proposed project involves human subjects and/or NIH-defined clinical research, the committee will evaluate the proposed plans for the inclusion (or exclusion) of individuals on the basis of sex/gender, race/ethnicity or if non-US residents, as well as the inclusion (or exclusion) of individuals of all ages including children (under 18 years old), adults (18 to 64 years old) and older adults (over 64 years old) to determine if it is justified in terms of the scientific goals and research strategy proposed.
- **Vertebrate Animals**. The committee will evaluate the involvement of live vertebrate animals as part of the scientific assessment according to the following criteria: (1) a concise description of proposed procedures involving animals, including identifying the species, strains, ages, sex, and total number to be used and if involved, the sources of dogs or cats; (2) the justifications that the species are appropriate for the proposed research and explaining why the research goals cannot be accomplished using an alternative model (e.g., computational, human, invertebrate, in vitro); (3) the interventions to minimize discomfort, distress, pain and injury; and (4) the justification for euthanasia method if NOT consistent with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals. Reviewers will assess the use of chimpanzees as they would any other application proposing the use of vertebrate animals.
- **Biohazards**. Reviewers will identify potential biohazards (biological organisms or their products, such as toxins, that pose a threat to human health) and other hazards (such as radioactivity, dangerous chemicals, or recombinant DNA) that are known in their professional community to pose a particularly significant risk to research personnel and/or the environment. Reviewers will assess whether materials or procedures proposed are potentially hazardous to research personnel and/or the environment, and if needed, determine whether adequate protection is proposed.

As applicable for the proposed project, reviewers will address each of the following review considerations, but will not consider them in providing an NIH Overall Impact score.

- **Applications from Foreign Organizations.** If the organization of the contact principal investigator/project director is not US-based, reviewers will assess whether the project presents special opportunities for furthering research programs through the use of unusual talent, resources, populations, or environmental conditions that exist in other countries and either are not readily available in the United States or augment existing U.S. resources.
- **Select Agent Research.** If the study involves a Select Agent (refer to Ecology and Evolution of Infectious Diseases (EEID) (http://www.selectagents.gov/SelectAgentsandToxinsList.html), reviewers will assess the information provided in this section of the application, including 1) the identification of the Select Agent(s) to be used in the proposed research, 2) the registration status of all entities where the Select Agent(s) will be used, 3) the procedures that will be used to monitor possession use and transfer of Select Agent(s), and 4) plans for appropriate biosafety, biocontainment, and security of the Select Agent(s).
- **Budget and Period of Support**. Reviewers will consider whether the budget and the requested period of support are fully justified and reasonable in relation to the proposed research.

International Collaborations and Expenditures

For all proposals involving international collaborations, reviewers will consider: mutual benefits, true intellectual collaboration with the foreign partner(s), benefits

to be realized from the expertise and specialized skills, facilities, sites and/or resources of the international counterpart, and active research engagement of U.S. students and early-career researchers, where such individuals are engaged in the research. Furthermore, the justification for funding for an international branch campus of a US IHE, or involvement of a foreign organization (e.g., through use of subawards or consultant arrangements), as described in PAPPG Chapter I.E, will be specifically reviewed to assure that conditions for the justification of such expenditures have been met, as detailed inPAPPG Chapter I.E.6.

US-UK, US-Israel and US-China Collaborative Projects will also be reviewed with respect to the extent which they demonstrate a substantial collaboration between the US and foreign partners and enhance research on infectious disease transmission.

B. Review and Selection Process

NSF will manage the review of proposals in consultation with NIH and USDA, and in the case of US-UK, US-Israel or US-China Collaborative Projects, the UKRI, BSF or NSFC, respectively. Copies of proposals and unattributed reviews will be shared with the partner funding organizations, as appropriate. Upon conclusion of the review process, meritorious projects may be recommended for funding by any of the partner funding organizations at the option of the agencies, not the proposing organizations.

Proposals submitted in response to this program solicitation will be reviewed by Ad hoc Review and/or Panel Review.

Reviewers will be asked to evaluate proposals using two National Science Board approved merit review criteria and, if applicable, additional program specific criteria. A summary rating and accompanying narrative will generally be completed and submitted by each reviewer and/or panel. The Program Officer assigned to manage the proposal's review will consider the advice of reviewers and will formulate a recommendation.

NSF Process: Those proposals selected for funding by NSF will be handled in accordance with standard NSF procedures. After scientific, technical and programmatic review and consideration of appropriate factors, the NSF Program Officer recommends to the cognizant Division Director whether the proposal should be declined or recommended for award. NSF is striving to be able to tell applicants whether their proposals have been declined or recommended for funding within six months. The time interval begins on the deadline or target date, or receipt date, whichever is later. The interval ends when the Division Director accepts the Program Officer's recommendation.

A summary rating and accompanying narrative will be completed and submitted by each reviewer. In all cases, reviews are treated as confidential documents. Verbatim copies of reviews, excluding the names of the reviewers, are sent to the Principal Investigator/Project Director by the Program OfficerIn addition, the proposer will receive an explanation of the decision to award or decline funding.

In all cases, after programmatic approval has been obtained, the proposals recommended for funding will be forwarded to the Division of Grants and Agreements for review of business, financial, and policy implications and the processing and issuance of a grant or other agreement. Proposers are cautioned that only a Grants and Agreements Officer may make commitments, obligations or awards on behalf of NSF or authorize the expenditure of funds. No commitment on the part of NSF should be inferred from technical or budgetary discussions with a NSF Program Officer. A Principal Investigator or organization that makes financial or personnel commitments in the absence of a grant or cooperative agreement signed by the NSF Grants and Agreements Officer does so at their own risk.

NIH Process: Proposals selected for funding consideration by NIH will be invited to submit reformatted applications to the Division of Receipt and Referral (DRR) in NIH's Center for Scientific Review (CSR). A receipt date of approximately March 15 annually is in effect for the NIH formatted applications.Following the initial peer review, recommended applications that have been resubmitted to the NIH are required to go to second level review by the Advisory Council or Advisory Board of the awarding Institute or Center. The following will be considered in making funding decisions:

- Scientific and technical merit of the proposed project as determined by scientific peer review.
- Availability of funds.
- Relevance of the proposed project to program priorities.

Subsequent award processing and grant administration procedures will be in accordance with NIH policies and procedures.

USDA/NIFA Process: Applicants submitting proposals selected for funding by USDA/NIFA will receive specific instructions outlining what additional information is needed in accordance with the policies of the agency. USDA/NIFA will make final funding decisions based on the results of the peer review process. Applications selected for funding by NIFA will be forwarded to the USDA/NIFA Awards Management Division for award processing in accordance with the USDA/NIFA procedures. All proposals selected for funding by USDA/NIFA, in FY 2020 will be limited to 30 percent indirect cost rate. Therefore, the recovery of indirect costs on awards made by NIFA under this program area may not exceed the lesser of the institution's official negotiated indirect cost rate or the equivalent of 30 percent of total Federal funds awarded. If the limitation of indirect costs changes, the applicant will be notified.

US-UK Collaborative Projects: The UK component of the collaboration will be awarded through the UKRI in accordance with the policies of that agency. If the UKRI selects an application for funding, the costs for the UK element of the proposal must be submitted via the UKRI's Je-S application submission system before final sign-off. UK collaborators should therefore ensure they are registered Je-S users before the proposal is submitted.

US-Israel Collaborative Projects: The Israeli component of the collaboration will be awarded through the BSF in accordance with its policies and regulations. The collaborative proposal must also be submitted to the BSF by the Israeli scientists and include the US scientists' information, after being submitted to the NSF.

US-China Collaborative Projects: The Chinese component of the collaboration will be awarded through the NSFC in accordance with its policies and regulations. The collaborative proposal must be submitted by the Chinese scientists to the NSFC as a supplementary document to their online applications by the NSFC-established deadline.

VII. AWARD ADMINISTRATION INFORMATION

A. Notification of the Award

NSF Process: Notification of the award is made to *the submitting organization* by a Grants Officer in the Division of Grants and Agreements. Organizations whose proposals are declined will be advised as promptly as possible by the cognizant NSF Program administering the program. Verbatim copies of reviews, not including the identity of the reviewer, will be provided automatically to the Principal Investigator. (See Section VI.B. for additional information on the review process.)

NIH Process: If the application is under consideration for NIH funding, NIH will request "just-in-time" information from the applicant as described in the NIH Grants Policy Statement.

A formal notification in the form of a Notice of Award (NoA) will be provided to the applicant organization for successful applications. The NoA signed by the grants management officer is the authorizing document and will be sent via email to the grantee's business official.

NIFA Process: Notification of a recommended award is made to the submitting PI by a National Program Leader at NIFA. Verbatim copies of reviews, not including the identity of the reviewers, will be provided to the Principal Investigator at the time of the award recommendation notification.

B. Award Conditions

An NSF award consists of: (1) the award notice, which includes any special provisions applicable to the award and any numbered amendments thereto; (2) the budget, which indicates the amounts, by categories of expense, on which NSF has based its support (or otherwise communicates any specific approvals or disapprovals of proposed expenditures); (3) the proposal referenced in the award notice; (4) the applicable award conditions, such as Grant General Conditions (GC-1)*; or Research Terms and Conditions* and (5) any announcement or other NSF issuance that may be incorporated by reference in the award notice. Cooperative agreements also are administered in accordance with NSF Cooperative Agreement Financial and Administrative Terms and Conditions (CA-FATC) and the applicable Programmatic Terms and Conditions. NSF awards are electronically signed by an NSF Grants and Agreements Officer and transmitted electronically to the organization via e-mail.

*These documents may be accessed electronically on NSF's Website at https://www.nsf.gov/awards/managing/award_conditions.jsp?org=NSF. Paper copies may be obtained from the NSF Publications Clearinghouse, telephone (703) 292-8134 or by e-mail from nsfpubs@nsf.gov.

More comprehensive information on NSF Award Conditions and other important information on the administration of NSF awards is contained in the NSF *Proposal & Award Policies & Procedures Guide* (PAPPG) Chapter VII, available electronically on the NSF Website at https://www.nsf.gov/publications/pub_summ.jsp?ods_key=pappg.

Special Award Conditions:

Proposals funded by NIH:

Selection of an application for award is not an authorization to begin performance. Any costs incurred before receipt of the Notice of Award are at the recipient's risk. These costs may be reimbursed only to the extent considered allowable pre-award costs.

Any application awarded by NIH in response to this solicitation will be subject to the DUNS, CCR Registration, and Transparency Act requirements as noted on the Award Conditions and Information for NIH Grants website.

All NIH grant and cooperative agreement awards include the *NIH Grants Policy Statement* as part of the NoA. For these terms of award, see the NIH Grants Policy Statement Part II: Terms and Conditions of NIH Grant Awards, Subpart A: General and Part II: Terms and Conditions of NIH Grant Awards, Subpart B: Terms and Conditions for Specific Types of Grants, Grantees, and Activities. More information is provided at Award Conditions and Information for NIH Grants.

Proposals funded by USDA/NIFA:

Awards issued as a result of this RFA will have designated the Automated Standard Applications for Payment System (ASAP), operated by the Department of Treasury's Bureau of the Fiscal Service, as the payment system for funds. For more information see https://www.fiscal.treasury.gov/.

Several federal statutes and regulations apply to grant applications considered for review and to project grants awarded under this program. These may include, but are not limited to, the ones listed on the NIFA web page - http://nifa.usda.gov/federal-regulations.

NIFA Federal Assistance Policy Guide—a compendium of basic NIFA policies and procedures that apply to all NIFA awards, unless there are statutory, regulatory, or award-specific requirements to the contrary is available at http://nifa.usda.gov/policy-guide.

Other Requirements

USDA/NIFA:

1. Delegation of Fiscal Responsibility

Unless the terms and conditions of the grant state otherwise, the grantee may not, in whole or in part, delegate or transfer to another person, institution, or organization the responsibility for use or expenditure of grant funds.

2. Changes in Project Plans

The permissible changes by the grantee, PD(s), or other key project personnel in the approved project grant shall be limited to changes in methodology, techniques, or other similar aspects of the project to expedite achievement of the project's approved goals. If the grantee or the PD(s) is uncertain as to whether a change complies with this provision, the question must be referred to the Authorized Departmental Officer (ADO) for a final determination. The ADO is the signatory of the award document, not the program contact.

- Changes in approved goals or objectives shall be requested by the grantee and approved in writing by the ADO prior to effecting such changes. In no event shall requests for such changes be approved which are outside the scope of the original approved project.
- Changes in approved project leadership or the replacement or reassignment of other key project personnel shall be requested by the grantee and approved in writing by the ADO prior to effecting such changes.
- Transfers of actual performance of the substantive programmatic work in whole or in part and provisions for payment of funds, whether or not Federal funds are involved, shall be requested by the grantee and approved in writing by the ADO prior to effecting such transfers, unless prescribed otherwise

in the terms and conditions of the grant.
- Changes in Project Period: The project period may be extended by USDA/NIFA without additional financial support, for such additional period(s) as the ADO determines may be necessary to complete or fulfill the purposes of an approved project, but in no case shall the total project period exceed ten years. Any extension of time shall be conditioned upon prior request by the grantee and approval in writing by the ADO, unless prescribed otherwise in the terms and conditions of a grant.
- Changes in Approved Budget: Changes in an approved budget must be requested by the grantee and approved in writing by the ADO prior to instituting such changes if the revision will involve transfers or expenditures of amounts requiring prior approval as set forth in the applicable Federal cost principles, Departmental regulations, or grant award.

US-UK Collaborative projects:

UKRI Awardees are subject to UKRI reporting and administration requirements as appropriate and outlined in the Research Funding Guide at http://www.bbsrc.ac.uk/funding/apply/grants-guide.aspx. US-UK Collaborative Projects should report on activities of the entire collaborative effort and submit that information to both NSF and UKRI as part of the annual and final reports.

US-Israel Collaborative projects:

BSF Awardees are subject to BSF reporting and administration requirements as appropriate and outlined in the BSF website: http://www.bsf.org.il/BSFPublic/DefaultPage1.aspx?PageId=41&innerTextID=41. US-Israel Collaborative Projects should report on activities of the entire collaborative effort and submit that information to both NSF and BSF as part of the annual and final reports.

US-China Collaborative projects:

NSFC Awardees are subject to NSFC reporting and administration requirements as appropriate and outlined in the NSFC website: http://www.nsfc.gov.cn/publish/portal2/tab193/info24527.htm. US-China Collaborative Projects should report on activities of the entire collaborative effort and submit that information to both NSF and NSFC as part of the annual and final reports.

C. Reporting Requirements

For all multi-year grants (including both standard and continuing grants), the Principal Investigator must submit an annual project report to the cognizant Program Officer no later than 90 days prior to the end of the current budget period. (Some programs or awards require submission of more frequent project reports). No later than 120 days following expiration of a grant, the PI also is required to submit a final project report, and a project outcomes report for the general public.

Failure to provide the required annual or final project reports, or the project outcomes report, will delay NSF review and processing of any future funding increments as well as any pending proposals for all identified PIs and co-PIs on a given award. PIs should examine the formats of the required reports in advance to assure availability of required data.

PIs are required to use NSF's electronic project-reporting system, available through Research.gov, for preparation and submission of annual and final project reports. Such reports provide information on accomplishments, project participants (individual and organizational), publications, and other specific products and impacts of the project. Submission of the report via Research.gov constitutes certification by the PI that the contents of the report are accurate and complete. The project outcomes report also must be prepared and submitted using Research.gov. This report serves as a brief summary, prepared specifically for the public, of the nature and outcomes of the project. This report will be posted on the NSF website exactly as it is submitted by the PI.

More comprehensive information on NSF Reporting Requirements and other important information on the administration of NSF awards is contained in the *NSF Proposal & Award Policies & Procedures Guide* (PAPPG) Chapter VII, available electronically on the NSF Website at https://www.nsf.gov/publications/pub_summ.jsp?ods_key=pappg.

Reporting on NIH Awards:

Awardees will be required to submit the Research Performance Progress Report (RPPR) annually and financial statements as required in the NIH Grants Policy Statement.

A final progress report, invention statement, and the expenditure data portion of the Federal Financial Report are required for closeout of an award, as described in the *NIH Grants Policy Statement*.

The Federal Funding Accountability and Transparency Act of 2006 (Transparency Act), includes a requirement for awardees of Federal grants to report information about first-tier subawards and executive compensation under Federal assistance awards issued in FY2011 or later. All awardees of applicable NIH grants and cooperative agreements are required to report to the Federal Subaward Reporting System (FSRS) available at www.fsrs.gov on all subawards over $25,000. See the NIH Grants Policy Statement for additional information on this reporting requirement.

Reporting on USDA Awards:
Grantees are to submit initial project information and annual summary reports to NIFA's electronic, Web-based inventory system that facilitates both grantee submissions of project outcomes and public access to information on Federally-funded projects. The details of these reporting requirements are included in the award terms and conditions.

Any additional reporting requirements will be identified in the terms and conditions of the award (see Part VII, B. above for a link to view the NIFA award terms and conditions).

For informational purposes, the "Federal Financial Report," Form SF-425, consolidates into a single report the former Financial Status Report (SF-269 and SF-269A) and the Federal Cash Transactions Report (SF-272 and SF-272A). The NIFA Agency-specific Terms and Conditions include the requirement that Form SF-425 is due on a **annual basis no later than 90 days following the award's anniversary date *(i.e.*, one year following the month and day of which the project period begins and each year thereafter up until a final report is required). A final "Federal Financial Report," Form SF-425, is due 90 days after the expiration date of this award.**

US-UK Collaborative projects:

UKRI Awardees are subject to UKRI reporting requirements as outlined in the Research Funding Guide at http://www.bbsrc.ac.uk/funding/apply/grants-

guide.aspx. US-UK Collaborative Projects should report on activities of the entire collaborative effort and submit that information to both NSF and UKRI as part of the annual and final reports.

US-Israel Collaborative projects:

BSF Awardees are subject to BSF reporting and administration requirements as appropriate and outlined in the BSF website: http://www.bsf.org.il/BSFPublic/DefaultPage1.aspx?PageId=41&innerTextID=41 US-Israel Collaborative Projects should report on activities of the entire collaborative effort and submit that information to both NSF and BSF as part of the annual and final reports.

US-China Collaborative projects:

NSFC Awardees are subject to NSFC reporting and administration requirements as appropriate and outlined in the NSFC website: http://www.nsfc.gov.cn/publish/portal2/tab193/info24527.htm US-China Collaborative Projects should report on activities of the entire collaborative effort and submit that information to both NSF and NSFC as part of the annual and final reports.

VIII. AGENCY CONTACTS

Please note that the program contact information is current at the time of publishing. See program website for any updates to the points of contact.

General inquiries regarding this program should be made to:

- Katharina Dittmar, Program Director, NSF/BIO, telephone: (703) 292-7799, email: kdittmar@nsf.gov
- Christine Jessup, Program Director, NIH/FIC, telephone: (301) 496-1653, fax: (301) 402-0779, email: christine.jessup@nih.gov
- Timothy Sullivan, National Program Leader, USDA/NIFA, telephone: (816) 527-5434, email: timothy.sullivan@usda.gov
- Iain Templeman, Portfolio Manager, UKRI/BBSRC, telephone: 44(0)1793-413368, email: iain.templeman@bbsrc.ukri.org
- Rachel (Heni) Haring, Deputy Executive Director, BSF, telephone: 972 2 5828239, email: heni@bsf.org.il
- Xiuping Liu, Division Director, NSFC, telephone: 86 10-62325377, email: liuxp@nsfc.gov.cn
- Daniel Janes, Program Director, NIH/NIGMS, telephone: (301) 594-0943, email: daniel.janes@nih.gov
- Stephanie Coomes, Health Scientist Administrator, NIH/NIAID, telephone: (301) 761-6855, email: stephanie.coomes@nih.gov
- Jing Chen, Program Manager, NSFC, telephone: 86 10-62326877, email: chenjing@nsfc.gov.cn
- Rebecca Ferrell, Program Director, NSF/SBE, telephone: (703) 292-7850, email: rferrell@nsf.gov
- Siobhan M. Mattison, NSF/SBE, telephone: (703) 292-2967, email: smattiso@nsf.gov
- Daniel J. Thornhill, Program Director, NSF/GEO, telephone: (703) 292-8143, email: dthornhi@nsf.gov
- Joaquin Martinez Martinez, NSF/GEO, telephone: (703) 292-8580, email: jmartine@nsf.gov
- Samuel M. Scheiner, Program Director, NSF/BIO, telephone: (703) 292-7175, email: sscheine@nsf.gov

For questions related to the use of FastLane or Research.gov, contact:

- FastLane and Research.gov Help Desk: 1-800-673-6188

  FastLane Help Desk e-mail: fastlane@nsf.gov.

  Research.gov Help Desk e-mail: rgov@nsf.gov

For questions relating to Grants.gov contact:

- Grants.gov Contact Center: If the Authorized Organizational Representatives (AOR) has not received a confirmation message from Grants.gov within 48 hours of submission of application, please contact via telephone: 1-800-518-4726; e-mail:support@grants.gov.

IX. OTHER INFORMATION

The NSF website provides the most comprehensive source of information on NSF Directorates (including contact information), programs and funding opportunities. Use of this website by potential proposers is strongly encouraged. In addition, "NSF Update" is an information-delivery system designed to keep potential proposers and other interested parties apprised of new NSF funding opportunities and publications, important changes in proposal and award policies and procedures, and upcoming NSF Grants Conferences. Subscribers are informed through e-mail or the user's Web browser each time new publications are issued that match their identified interests. "NSF Update" also is available onNSF's website.

Grants.gov provides an additional electronic capability to search for Federal government-wide grant opportunities. NSF funding opportunities may be accessed via this mechanism. Further information on Grants.gov may be obtained athttps://www.grants.gov.

A notice on the Ecology and Evolution of Infectious Disease research initiative and this announcement is also posted in the NIH Guide to Grants and Contracts https://grants.nih.gov/grants/guide/index.html along with all NIH opportunities.

ABOUT THE NATIONAL SCIENCE FOUNDATION

The National Science Foundation (NSF) is an independent Federal agency created by the National Science Foundation Act of 1950, as amended (42 USC 1861-75). The Act states the purpose of the NSF is "to promote the progress of science; [and] to advance the national health, prosperity, and welfare by supporting research and education in all fields of science and engineering."

NSF funds research and education in most fields of science and engineering. It does this through grants and cooperative agreements to more than 2,000 colleges, universities, K-12 school systems, businesses, informal science organizations and other research organizations throughout the US. The Foundation accounts for about one-fourth of Federal support to academic institutions for basic research.

NSF receives approximately 55,000 proposals each year for research, education and training projects, of which approximately 11,000 are funded. In addition, the Foundation receives several thousand applications for graduate and postdoctoral fellowships. The agency operates no laboratories itself but does support National Research Centers, user facilities, certain oceanographic vessels and Arctic and Antarctic research stations. The Foundation also supports cooperative research between universities and industry, US participation in international scientific and engineering efforts, and educational activities at every academic level.

Facilitation Awards for Scientists and Engineers with Disabilities(FASED) provide funding for special assistance or equipment to enable persons with disabilities to work on NSF-supported projects. See the *NSF Proposal & Award Policies & Procedures Guide* Chapter II.E.6 for instructions regarding preparation of these types of proposals.

The National Science Foundation has Telephonic Device for the Deaf (TDD) and Federal Information Relay Service (FIRS) capabilities that enable individuals with hearing impairments to communicate with the Foundation about NSF programs, employment or general information. TDD may be accessed at (703) 292-5090 and (800) 281-8749, FIRS at (800) 877-8339.

The National Science Foundation Information Center may be reached at (703) 292-5111.

The National Science Foundation promotes and advances scientific progress in the United States by competitively awarding grants and cooperative agreements for research and education in the sciences, mathematics, and engineering.

To get the latest information about program deadlines, to download copies of NSF publications, and to access abstracts of awards, visit the NSF Website at https://www.nsf.gov

- **Location:** 2415 Eisenhower Avenue, Alexandria, VA 22314
- **For General Information** (NSF Information Center)**:** (703) 292-5111
- **TDD (for the hearing-impaired):** (703) 292-5090
- **To Order Publications or Forms:**

  Send an e-mail to: nsfpubs@nsf.gov

  or telephone: (703) 292-8134
- **To Locate NSF Employees:** (703) 292-5111

PRIVACY ACT AND PUBLIC BURDEN STATEMENTS

The information requested on proposal forms and project reports is solicited under the authority of the National Science Foundation Act of 1950, as amended. The information on proposal forms will be used in connection with the selection of qualified proposals; and project reports submitted by awardees will be used for program evaluation and reporting within the Executive Branch and to Congress. The information requested may be disclosed to qualified reviewers and staff assistants as part of the proposal review process; to proposer institutions/grantees to provide or obtain data regarding the proposal review process, award decisions, or the administration of awards; to government contractors, experts, volunteers and researchers and educators as necessary to complete assigned work; to other government agencies or other entities needing information regarding applicants or nominees as part of a joint application review process, or in order to coordinate programs or policy; and to another Federal agency, court, or party in a court or Federal administrative proceeding if the government is a party. Information about Principal Investigators may be added to the Reviewer file and used to select potential candidates to serve as peer reviewers or advisory committee members. See System of Record Notices, NSF-50, "Principal Investigator/Proposal File and Associated Records," and NSF-51, "Reviewer/Proposal File and Associated Records." Submission of the information is voluntary. Failure to provide full and complete information, however, may reduce the possibility of receiving an award.

An agency may not conduct or sponsor, and a person is not required to respond to, an information collection unless it displays a valid Office of Management and Budget (OMB) control number. The OMB control number for this collection is 3145-0058. Public reporting burden for this collection of information is estimated to average 120 hours per response, including the time for reviewing instructions. Send comments regarding the burden estimate and any other aspect of this collection of information, including suggestions for reducing this burden, to:

Suzanne H. Plimpton
Reports Clearance Officer
Office of the General Counsel
National Science Foundation
Alexandria, VA 22314

Policies and Important Links | Privacy | FOIA | Help | Contact NSF | Contact Web Master | SiteMap

National Science Foundation, 2415 Eisenhower Avenue, Alexandria, Virginia 22314, USA
Tel: (703) 292-5111, FIRS: (800) 877-8339 | TDD: (703) 292-5090 or (800) 281-8749

Text Only

GACETILLA

Convalescent plasma with high antibody titers applied in the first days of disease turns COVID-19 into a bad cold in older people.

A randomized, double-blind, placebo-controlled study conducted by the INFANT Foundation evaluated the efficacy of early administration of convalescent plasma with high titers of antibodies in older adults and observed that its application **decreases the possibility that these patients** require oxygen during illness.

The study, conducted by a team of scientists led by Dr. Fernando Polack (Director of the INFANT Foundation), demonstrated that convalescent plasma administered to people infected patients over 65 years of age with comorbidities and in all those over 75 years of age it is effective to prevent COVID-19 from becoming a serious respiratory illness, provided you **give it to you within 72 hours of symptom onset.**

In this way, and in line with the use of plasma derivatives - such as gamma globulin hyperimmune- and antiviral drugs in other diseases-, antibody-rich plasma acts at the beginning of the disease preventing it from progressing, unlike trials performed in critically ill patients where stopping the progression of COVID-19 becomes more difficult and

no conclusive benefits have been found in its use.

"Plasma is just a vehicle that carries antibodies. E **l 28% of people have, as our study, the amount of antibodies needed to donate plasma for this treatment.** By restricting donors to those with higher antibody concentrations, it is possible to improve plasma performance even further. Although this leaves out the donation to a group of convalescent, between 4 and 8 recovered patients have the antibodies needed to be a donor. Patients who had pneumonia or needed hospitalization they are the ones who usually produce the most antibodies, "Polack explained.

The researchers identified the desirable amounts of protective antibodies in the plasma bags using the COVIDAR antibody test developed by the Institute Leloir in Buenos Aires. This information is an extremely important tool for the design of public policies, since it will allow the design of donation campaigns that aim directly to identify the correct donors, optimizing time, effort and resources.

Page 2

"This study describes the first strategy in the world to stop the progression of SARS-COV2 with an economic intervention, universal, not subject to patents, proven safe and that it can be administered on an outpatient basis in care units without the need for hospitalization, "Polack said.

The trial, which was carried out between the months of June and last October, involved 160 adults older than 65 years with at least one comorbidity (hypertension, diabetes, obesity, kidney failure and / or chronic obstructive pulmonary disease), and to all those over 75 years. The program coordinated efforts from the public and private sectors, bringing together hospitals from the Province of Buenos Aires and sanatoriums of the Federal Capital and had the support of the Ministry and the respective Ministry of Health, PAMI, the Military Hospital, various actors from the health and hundreds of volunteers.

An independent Data and Safety Monitoring Committee (DSMB) supervised the study. monitoring its quality and the well-being of patients. The study was funded by a grant from the Bill & Melinda Gates Foundation and the INFANT Foundation Pandemic Fund that brings together numerous national companies and private contributors.

The research was carried out with the collaboration of the Institute of Clinical Effectiveness and Sanitaria (IECS) and the Sarmiento Hematology Foundation at the Dr. Carlos Bocalandro Hospital, the

Hospital San Juan de Dios, Hospital Simply Evita, Hospital Central de San Isidro Dr. Melchor Ángel Posse, the Olivos Clinic and a network of geriatric care units linked to the PAMI in the Province of Buenos Aires, and in the Central Military Hospital, the Galician Center, the Sanatorio de los Arcos, CEMIC University Hospital, Sanatorio Sagrado Corazón, Sanatorio Anchorena and Sanatorio Finochietto in the Autonomous City of Buenos Aires.

About the study

The design of this study was randomized, double-blind, and placebo-controlled. This means that half of the patients randomly received high antibody titer plasma and half remaining placebo (physiological solution), without doctors or participants knowing which substance was administered to each individual. This method is used to ensure fairness in the evaluation of the results.

Once all recruited patients received plasma or placebo, the evolution of the disease in both groups. **Of the patients actually treated with plasma only 9 required oxygen versus 23 who received placebo treatment.** I know estimates that one in 6 people treated with plasma who would have had disease severe if he had not received it, he did not develop it.

"This is the only study in the world against SARS-COV2 that was done with this methodology rigorous early comparison of one group against another and thus provides evidence that the disease did not progress due to the administration of plasma and not other reasons ", explained Dr. Romina Libster, one of the main authors of the work, medical pediatrician specialized in vaccines and researcher at the INFANT Foundation.

For this study to be possible, the work required the detection of donors of plasma, the identification of patients in the nursing homes of the Province and Capital of the elderly with early mild symptoms, his rapid home swab test for SARS CoV2 in the laboratory of the Central Military Hospital, and the transfer of those infected who wish participate in the study institutions in charge of a mobile group provided free of charge by Swiss Medical who toured the region for months.

There, a team from the Sarmiento Hematology Foundation coordinated the administration of plasma

or placebo taking care that nobody knew who received each product. This continued with a daily monitoring of participants for up to 25 days duration and analysis of the entire information by a group of experts in research and statistics from INFANT and the IECS.

In the first weeks of May, against the clock and in the midst of a pandemic with enormous uncertainty, the **INFANT** team of researchers **trained more than 200 physicians** from participating health centers. The goal was to reduce the chances of error in all procedures.

At the same time, a large group of volunteers were actively working in search of donors. Every day they called a hundred people recovered from COVID-19 to invite them to participate in the program and make them aware of the importance that this contribution could have for new patients.

More than 100 volunteers were trained who helped us day and night in identifying possible patients to enroll in the study, coming from their homes and nursing homes from the Buenos Aires suburbs, referred through the collaboration of PAMI, or arrived at consultations in the clinics of Capital Federal.

"During all stages, the project, which involved around 400 people, required meticulousness to assemble the different links and cover both scientific needs as logistics ", said Gonzalo Pérez Marc, pediatrician, deputy director of the Department Maternal and Child of the Central Military Hospital and also one of the main authors of the job.

This study, carried out with Argentine scientists, **is the first evidence in the world of a universal, accessible and safe treatment** that can save lives until vaccines are available available.

About Fundación INFANT

INFANT is a non-profit organization, whose mission is to investigate the causes of respiratory diseases that affect the population such as bronchiolitis, asthma, pneumonia and the flu. In its 17 years of existence, the institution carried out important scientific discoveries of great health impact.

Faced with the arrival of SARS CoV2 in Argentina, the INFANT Foundation catalyzed a project collaborative with public and private sector actors, supported by experts in respiratory diseases and clinical research, epidemiologists, physicians, biologists and more than 400 volunteers to devise and explore a possible solution against COVID-19 with the support generous of international and national entities and private contributors.

Contact

INFANT Foundation
press@infant.org.ar
www.infant.org.ar

FUNDACIÓN INFANT
TRADUCIENDO CIENCIA EN SALUD INFANTIL

GACETILLA

El plasma de convaleciente con altos títulos de anticuerpos aplicado en los primeros días de enfermedad transforma el COVID-19 en un mal catarro en personas mayores.

Un estudio randomizado, doble-ciego y contra placebo realizado por la Fundación INFANT evaluó la eficacia de la administración temprana de plasma de convaleciente con altos títulos de anticuerpos en adultos mayores y observó que su aplicación **disminuye un 61 % la posibilidad de que estos pacientes** necesiten oxígeno durante la enfermedad.

El estudio, a cargo de un equipo de científicos liderados por el Dr. Fernando Polack (Director de la Fundación INFANT), demostró que el plasma de convalescientes administrado en personas infectadas mayores de 65 años con comorbilidades y en todos los mayores de 75 años es eficaz para evitar que el COVID-19 se transforme en una enfermedad respiratoria grave, siempre que **se lo administre dentro de las primeras 72 horas de la aparición de síntomas.**

De esta manera, y en línea con el uso de derivados del plasma -como la gammaglobulina hiperinmune- y las drogas antivirales en otras enfermedades-, el plasma rico en anticuerpos actúa al comienzo de la enfermedad evitando que avance, a diferencia de los ensayos realizados en pacientes graves donde detener la progresión del COVID-19 se hace más difícil y no se han encontrado beneficios concluyentes en su utilización.

“El plasma es sólo un vehículo que lleva anticuerpos. E**l 28% de las personas tiene, según nuestro estudio, la cantidad de anticuerpos necesarios para donar plasma para este tratamiento.** Restringiendo los donantes a los de mayores concentraciones de anticuerpos, es posible mejorar el rendimiento del plasma aún más. Si bien esto deja afuera de la donación a un grupo de convalecientes, enrtre 4 y 8 enfermos recuperados tiene los anticuerpos necesarios para ser donante. Los pacientes que tuvieron neumonía o necesitaron internarse son quienes más anticuerpos suelen producir”, explicó Polack.

Los investigadores identificaron las cantidades deseables de anticuerpos protectores en las bolsas de plasma utilizando el ensayo de anticuerpos COVIDAR desarrollado por el Instituto Leloir en Buenos Aires. Esta información es una herramienta de suma importancia para el diseño de políticas públicas, ya que permitirá diseñar campañas de donación que apunten directamente a identificar a los donantes correctos, optimizando tiempo, esfuerzo y recursos.

“Este estudio describe la primera estrategia en el mundo para detener la progresión del SARS-COV2 con una intervencion económica, universal, no sujeta a patentes, probadamente segura y que puede admnistrarse en forma ambulatoria en unidades de atención sin necesidad de hospitalizacion”, dijo Polack.

El ensayo, que se realizó entre los meses de junio y octubre últimos, involucró a 160 adultos mayores de 65 años con al menos una comorbilidad (hipertensión, diabetes, obesidad, insuficiencia renal y/o enfermedad pulmonar obstructiva crónica), y a todos los mayores de 75 años. El programa articuló esfuerzos del sector público y privado, congregando hospitales de la Provincia de Buenos Aires y sanatorios de la Capital Federal y contó con el apoyo del Ministerio y Secretaría de Salud respectivos, el PAMI, el Hospital Militar, diversos actores del área de salud y centenares de voluntarios.

Un Comité de Monitoreo de Seguridad y Datos (DSMB) independiente supervisó el estudio monitoreando su calidad y el bienestar de los pacientes. El estudio se financió mediante un subsidio de la Fundación Bill & Melinda Gates y el Fondo de Pandemia de la Fundación INFANT que congrega a numerosas empresas nacionales y aportantes privados.

La investigación se llevó a cabo con la colaboración del Instituto de Efectividad Clínica y Sanitaria (IECS) y la Fundación Hematológica Sarmiento en el Hospital Dr. Carlos Bocalandro, el Hospital San Juan de Dios, el Hospital Simplemente Evita, el Hospital Central de San Isidro Dr. Melchor Ángel Posse, la Clínica Olivos y una red de unidades geriátricas de atención ligadas al PAMI en la Provincia de Buenos Aires, y en el Hospital Militar Central, el Centro Gallego, el Sanatorio de los Arcos, el Hospital Universitario CEMIC, el Sanatorio Sagrado Corazón, el Sanatorio Anchorena y el Sanatorio Finochietto en la Ciudad Autónoma de Buenos Aires.

Acerca del estudio

El diseño de este estudio fue aleatorio, doble ciego y controlado con placebo. Esto significa que la mitad de los pacientes recibió al azar plasma de alto título de anticuerpos y la mitad restante placebo (solución fisiológica), sin que médicos ni participantes supieran qué sustancia se administró a cada individuo. Este método se usa para garantizar la imparcialidad en la evaluación de los resultados.

Una vez que todos los pacientes reclutados recibieron plasma o placebo se comparó la evolución de la enfermedad en ambos grupos. **De los pacientes efectivamente tratados con plasma sólo 9 necesitaron oxígeno contra 23 que recibieron tratamiento con placebo.** Se estima que una de cada 6 personas tratadas con plasma que hubiera tenido enfermedad severa de no haberlo recibido, no llegó a desarrollarla.

“Este es el único estudio en el mundo contra el SARS-COV2 que se hizo con esta metodología rigurosa de comparación temprana de un grupo contra otro y, por lo tanto, otorga evidencia de que la enfermedad no avanzó debido a la administración del plasma y no por otras razones”, explicó la Dra. Romina Libster, una de las autoras principales del trabajo, médica pediatra especialista en vacunas e investigadora de la Fundación INFANT.

Para que este estudio fuera posible, el trabajo requiriró de la detección de donantes de plasma, la identificacionpacientes en los geriátricos de Provincia y Capital de personas mayores con síntomas leves tempranos, su testeo rápido en domicilio por hisopado para SARS CoV2 en el laboratorio del Hospital Militar Central, y el traslado de los infectados que deseasen participar a las instituciones del estudio a cargo de un grupo móvil provisto en forma gratuita por Swiss Medical que recorrió durante meses la región.

Allí, un equipo de la Fundación Hematológica Sarmiento coordinó la administración de plasma o placebo cuidando que nadie supiese quién recibió cada producto. Esto continuó con un monitoreo diario de los participantes de hasta 25 días de duración y el análisis de toda la información por un grupo de expertos en investigación y estadistíca de INFANT y el IECS.

En las primeras semanas de mayo, contrarreloj y en plena pandemia con enorme incertidumbre, el equipo de investigadores de **INFANT entrenó en el protocolo a más de 200 médicos** de los centros de salud participantes. El objetivo fue reducir las posibilidades de error en todos los procedimientos.

Al mismo tiempo, un nutrido grupo de voluntarios trabajaba activamente en busca de donantes. Todos los días llamaban a un centenar de personas recuperadas de COVID-19 para invitarlas a participar del programa y concientizarlas sobre la importancia que este aporte podía tener para nuevos enfermos.

Se capacitaron **a más de 100 voluntarios** que nos ayudaron día y noche en la identificación de posibles pacientes para enrolar en el estudio, provenientes de sus hogares y geriátricos del conurbano bonaerense, referidos a través de la colaboración del PAMI, o llegados a consultas en las clínicas de Capital Federal.

“Durante todas las etapas, el proyecto, que involucró a alrededor de 400 personas, requirió de minuciosidad para ensamblar los distintos eslabones y cubrir tanto las necesidades científicas como logísticas”, contó Gonzalo Pérez Marc, médico pediatra subdirector del Departamento Materno Infantil del Hospital Militar Central y también uno de los principales autores del trabajo.

Este estudio, realizado con científicos argentinos, **es la primera evidencia en el mundo de un tratamiento universal, accesible y seguro** que puede salvar vidas hasta que haya vacunas disponibles.

Acerca de Fundación INFANT

INFANT es una organización sin fines de lucro, cuya misión es investigar las causas de enfermedades respiratorias que afectan a la población como la bronquiolitis, el asma, la neumonía y la gripe. En sus 17 años de existencia, la institución realizó importantes descubrimientos científicos de gran impacto sanitario.

Frente a la llegada del SARS CoV2 a la Argentina, la Fundación INFANT catalizó un proyecto colaborativo con actores del sector público y el sector privado, sostenido por expertos en enfermedades respiratorias e investigación clínica, epidemiólogos, médicos, biólogos y más de 400 voluntarios para idear y explorar una posible solución contra el COVID-19 con el apoyo generoso de entidades internacionales, nacionales y aportantes particulares .

Contacto

Fundación INFANT
prensa@infant.org.ar
www.infant.org.ar

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

Therapeutic use of convalescent plasma in COVID-19 patients with immunodeficiency

Jonathon W. Senefeld[1#], PhD, Stephen A. Klassen[1#], PhD, Shane K. Ford[1#], BS, Chad C. Wiggins[1], PhD, Bruce C. Bostrom[2], MD, Michael A. Thompson, MD, PhD[3], Sarah E. Baker[1], PhD, Wayne T. Nicholson[1], MD, Patrick W. Johnson[4], BS, Rickey E. Carter[4], PhD, Jeffrey P. Henderson[5], MD, PhD, William R. Hartman[6], MD, Liise-anne Pirofski[7], MD, R. Scott Wright[8], MD, DeLisa Fairweather[9], PhD, Katelyn A. Bruno[9], PhD, Nigel S. Paneth[10†], MD, Arturo Casadevall[11†], MD, PhD and Michael J. Joyner[1†], MD*

1 | Department of Anesthesiology and Perioperative Medicine, Mayo Clinic, Rochester, Minnesota
2 | Pediatric Oncology & Hematology, Children's Hospital of Minnesota, Minneapolis, Minnesota
3 | Aurora Cancer Care, Advocate Aurora Health, Milwaukee, Wisconsin
4 | Department of Health Sciences Research, Mayo Clinic, Jacksonville, Florida
5 | Department of Medicine and Department of Molecular Microbiology, Washington University School of Medicine in St. Louis, St. Louis, Missouri
6 | Department of Anesthesiology, University of Wisconsin-Madison School of Medicine and Public Health, Madison, Wisconsin
7 | Division of Infectious Diseases, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York
8 | Department of Cardiovascular Medicine and Director Human Research Protection Program, Mayo Clinic, Rochester, Minnesota
9 | Department of Cardiovascular Medicine, Mayo Clinic, Jacksonville, Florida
10 | Department of Epidemiology and Biostatistics and Department of Pediatrics and Human Development, Michigan State University, East Lansing, Michigan
11 | Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland

NOTE: This preprint reports new research that has not been certified by peer review and should not be used to guide clinical practice.

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
All rights reserved. No reuse allowed without permission.

0 | Abstract

In the absence of effective countermeasures, human convalescent plasma has been widely used to treat severe acute respiratory syndrome coronavirus 2 including among patients with innate or acquired immunodeficiency. However, the association between COVID-19-associated mortality in patients with immunodeficiency and therapeutic use of convalescent plasma is unknown. We review clinical features and treatment protocols of COVID-19 patients with immunodeficiency after treatment with human convalescent plasma. We also discuss the time course and clinical features of recovery. These insights provide evidence for the need to develop a clear treatment protocol for COVID-19 patients with immunodeficiency and support the efficacy of convalescent plasma in patients with primary or secondary immunodeficiency.

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

1 | Introduction

Convalescent plasma represents a passive antibody therapy that has been used to prevent or treat infectious diseases for more than a century.[1,2] In the context of the novel coronavirus disease 2019 (COVID-19) pandemic, convalescent plasma has received full or conditional regulatory authorization in the United States for therapeutic use in adults and children hospitalized with suspected or laboratory confirmed SARS-CoV-2 positive COVID-19.[3–5] Although early signals for efficacy and optimal use of convalescent plasma have emerged,[6–10] additional large randomized controlled trials are needed to garner clinical consensus regarding the therapeutic effectiveness of convalescent plasma.

Inasmuch as convalescent plasma confers antiviral properties for the recipient, evidence of therapeutic effectiveness may be confounded by endogenous anti-SARS-CoV-2 antibody responses, particularly when convalescent plasma transfusion occurs late in the disease course.[11] Hence, evaluation of the clinical responses to convalescent plasma transfusion in immunosuppressed patients who cannot generate innate immune responses may provide an optimal opportunity to assess the effect of convalescent plasma *per se.*[12] Thus, herein we summarize the literature detailing the clinical experiences of patients with primary and secondary immunosuppression who were transfused with COVID-19 convalescent plasma. We hypothesize that patients with immunodeficiency syndromes, or patients with diseases associated with blunted endogenous antibody responses to COVID-19, may provide evidence of improved clinical status when given plasma containing anti-SARS-CoV-2 antibodies, with improvements over and above expectation in light of their underlying clinical conditions and the severity of COVID-19.

This review highlights 40 reports including 104 COVID-19 patients with primary immunosuppression due to Agammaglobulinemia (X-linked or autosomal) or Common Variable Immunodeficiency, and secondary immunodeficiencies related to hematological malignancies and solid organ transplants who were transfused with convalescent plasma. We also highlight additional cases of patients with COVID-19, including but not limited to patients with other forms of immunosuppression, transfused with convalescent plasma. We provide a summary overview of patient characteristics, COVID-19 therapies used, including convalescent plasma, and clinical symptomology (**table**).

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

Search strategy and selection criteria

References for this Review were identified through searches of PubMed for articles published from Jan 1, 2020 to Nov 1, 2020, using the Medical Subject Headings terms "COVID-19", "convalescent plasma", "convalescent serum", "immunosuppression", "cancer", "transplant", "agammaglobulinemia", "malignancy", and any relevant entry terms and supplementary concepts (**figure**). Relevant articles and data were also identified through searches in Google Scholar, medRxiv, and other websites. Articles resulting from these searches and relevant references cited in those articles were reviewed. Articles published in English were identified and included. All procedures accessed public information and did not require ethical review as determined by the Mayo Clinic Institutional Review Board in accordance with the Code of Federal Regulations, 45 CFR 46.102, and the Declaration of Helsinki.

2 | Primary Immunodeficiency

2.1 | Patients with Agammaglobulinemia

This narrative includes one patient with Autosomal Agammaglobulinemia and six patients with X-linked Agammaglobulinemia (XLA), six of whom were reported in peer-reviewed articles and one patient who was identified in a media report.[13–16] Patients with Agammaglobulinemia do not produce endogenous antibodies and require regular intravenous infusions (monthly) or subcutaneous injections (bi-weekly) of immunoglobulins to avoid serial infections from various common pathogens.[17] However, immunoglobulin replacement therapy cannot protect patients against pathogens for which antibodies are uncommon or absent in the immunoglobulin donor pool, such as the SARS-CoV-2 virus.[17] The clinical narratives describing patients with Agammaglobulinemias all demonstrate a prolonged course of disease and absent antibody response to COVID-19. However, all patients with Agammaglobulinemia demonstrated clinical improvement and symptom resolution following convalescent plasma transfusion, with three patients improving rapidly and demonstrating SARS-CoV-2 serum antibodies after transfusion. In addition to immunoglobulin therapy, some patients also received the experimental COVID-19 therapies remdesivir and hydroxychloroquine.

2.2 | Patients with Common Variable Immunodeficiency

We identified four patients with Common Variable Immunodeficiency transfused with convalescent plasma for COVID-19 therapy.[16] Common Variable Immunodeficiency represents a heterogeneous collection of immunodeficiencies commonly characterized by intrinsic B-cell defects and suppressed antibody production.[18] Patients with Common Variable Immunodeficiency often present with inflammatory and autoimmune disorders, which are suspected to elevate these patients risk for progression to severe COVID-19.[16,18] The four patients described here were all antibody deficient and diagnosed with severe or life-threatening COVID-19. Notably, three of these patients survived following convalescent plasma transfusion, including two patients whose clinical symptomatology required mechanical ventilation or extracorporeal membrane oxygenation.

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

3 | Secondary Immunodeficiency

3.1 | Patients with Hematological Malignancies

We identified 54 patients with hematological malignancies transfused with convalescent plasma in 18 peer-reviewed reports.[19,20,28,29,29–36,21–24,24–27] The largest cohort of patients with hematological malignancies was a group of 17 patients with B-cell depletion whose immunodeficiency was secondary to therapies for various types of lymphoma or leukemia.[19] These patients experienced protracted severe COVID-19 symptoms and absent endogenous SARS-CoV-2 antibody responses due to cancer-directed therapies that suppress B-cell proliferation, such as rituximab. Within 48 hours of convalescent plasma transfusion the majority of patients demonstrated improved clinical status and viral clearance. Similarly, in a separate cohort of 14 patients with hematological malignancies, most patients exhibited improvements in clinical symptomatology, including reduced oxygen requirements,[20] after treatment with convalescent plasma. Other case-reports are of lymphoma (n = 11), leukemia (n = 5), multiple myeloma (n = 3), and myelodysplastic syndrome (n = 1) and include one four year old pediatric patient with leukemia who received two convalescent plasma doses over two days.[28] A majority of patients with hematological malignancies recovered following convalescent plasma transfusion, with many demonstrating rapid clinical improvements shortly after transfusion. Notably, a patient with protracted COVID-19, evidenced by three separate COVID-19-related hospitalisations over a 100+ day period, and with lymphoma-associated B-cell immunodeficiency demonstrated rapid reductions in fever, oxygen requirements, and lung infiltrates (via chest computed tomography) forthwith after two separate convalescent plasma transfusions separated by ~90 days.[22]

3.2 | Solid Organ Transplant Patients

Among nine peer-reviewed articles and two media reports we identified a total of 29 COVID-19 patients transfused with convalescent plasma whom were receiving immunosuppressive therapies for previous solid organ transplants.[37–47] In a cohort of 13 transplant recipients transfused with convalescent plasma concomitant to hydroxychloroquine, steroids and anticoagulants therapies for COVID-19, eight patients demonstrated improved oxygen requirements and were promptly discharged [37]. In the other nine reports, a majority of the 17 patients with COVID-19 demonstrated improved clinical symptomatology following convalescent plasma transfusion. Improvement was even seen in a liver transplant patient discharged home after a convalescent plasma transfusion that occurred during a seventeen day medically-induced coma due to COVID-19 complications.[44]

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

4 | Conclusion

These data provide evidence and encouraging anecdotal statements (**panel**) supporting the efficacy of convalescent plasma in patients with primary or secondary immunodeficiency, and are consistent with the historical evidence demonstrating that passive antibody therapies for infectious diseases are especially effective when given early in the course of disease prior to an endogenous antibody response.[2,48,49] In contrast to ongoing studies of convalescent plasma efficacy in clinical trials where the majority of patients are not immunosuppressed and thus mount their own protective antibody responses,[6,11,50] convalescent plasma use in these immunosuppressed patients represents a situation where exogenous antibody is provided in the setting of an immune deficit.[17] This is particularly true in the case for patients with Agammaglobulinemia, who represent in effect a natural 'immunological knockout' providing the opportunity to evaluate the value of adding specific antibody to a host unable to make antibody to SARS-CoV-2.

The results for patients with Agammaglobulinemia are striking for the beneficial effects of convalescent plasma, and even though the reports lack a control group, the rapid time course of clinical improvement following administration of specific antibody in a situation where no endogenous antibody was present is impressive. Convalescent plasma was also found to be safe and efficacious in COVID-19 patients with common variable immunodeficiency, myasthenia gravis, and Sjögren's syndrome, in addition to COVID-19 patients with heart rhythm abnormalities and gravida.[51–57] Hence, from the viewpoint of establishing the efficacy of convalescent plasma administration in COVID-19 therapy, the experience with this patient set provides the important criterion that addition of specific antibody to a host with no antibodies resulted in a favorable therapeutic effect.

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
All rights reserved. No reuse allowed without permission.

Contributors

MJJ is the guarantor. SAK, JWS, SKF, AC and MJJ conceptualised the paper, extracted the data and established the writing consortium. All authors contributed to the reviewing and editing of the report and approved the final version.

Declaration of interests

We declare no competing interests.

Acknowledgements

We thank the members of the National COVID-19 Convalescent Plasma Project (http://ccpp19.org) for their support and collation of key scientific papers.

Funding/Support

This study was supported in part by National Heart, Lung, and Blood Institute (NHLBI) grant 5R35HL139854 (to MJJ), grant F32HL154320 (to JWS), and grant RO1 HL059842 (to AC); Natural Sciences and Engineering Research Council of Canada (NSERC) PDF-532926-2019 (to SAK); National Institute of Allergy and Infectious Disease (NIAID) grants R21 AI145356, R21 AI152318 and R21 AI154927 (to DF), and grant R01 AI152078 9 (to AC); and National Institute on Aging (NIA) U54AG044170 (to SEB).

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

References

1 Casadevall A, Pirofski L. The convalescent sera option for containing COVID-19. *J Clin Invest* 2020; **130**: 1545–8.

2 Luke TC, Kilbane EM, Jackson JL, Hoffman SL. Meta-analysis: convalescent blood products for Spanish influenza pneumonia: a future H5N1 treatment? *Ann Intern Med* 2006; **145**: 599–609.

3 Bloch EM, Shoham S, Casadevall A, *et al.* Deployment of convalescent plasma for the prevention and treatment of COVID-19. *J Clin Invest* 2020.

4 US Food and Drug Administration. Recommendations for Investigational COVID-19 Convalescent Plasma. 2020. https://www.fda.gov/vaccines-blood-biologics/investigational-new-drug-ind-or-device-exemption-ide-process-cber/recommendations-investigational-covid-19-convalescent-plasma.

5 US Food and Drug Administration. Clinical Memorandum for the Emergency Use Authorization of COVID-19 Convalescent Plasma. 2020 https://www.fda.gov/media/141480/download.

6 Li L, Zhang W, Hu Y, *et al.* Effect of Convalescent Plasma Therapy on Time to Clinical Improvement in Patients With Severe and Life-threatening COVID-19: A Randomized Clinical Trial. *JAMA* 2020.

7 Abolghasemi H, Eshghi P, Cheraghali AM, *et al.* Clinical Efficacy of Convalescent Plasma for Treatment of COVID-19 Infections: Results of a Multicenter Clinical Study. *Transfus Apher Sci* 2020; : 102875.

8 Salazar E, Christensen PA, Graviss EA, *et al.* Early transfusion of a large cohort of COVID-19 patients with high titer anti-SARS-CoV-2 spike protein IgG convalescent plasma confirms a signal of significantly decreased mortality. *medRxiv* 2020.

9 Joyner MJ, Senefeld JW, Klassen SA, *et al.* Effect of convalescent plasma on mortality among hospitalized patients with COVID-19: initial three-month experience. *MedRxiv* 2020.

10 Joyner MJ, Bruno K, Klassen SA, *et al.* Safety Update: COVID-19 Convalescent Plasma in 20,000 Hospitalized Patients. *Mayo Clin Proc* 2020; **95**.

11 Agarwal A, Mukherjee A, Kumar G, Chatterjee P, Bhatnagar T, Malhotra P. Convalescent plasma in the management of moderate covid-19 in adults in India: open label phase II multicentre randomised controlled trial (PLACID Trial). *bmj* 2020; **371**.

12 Patel SY, Carbone J, Jolles S. The expanding field of secondary antibody deficiency: causes, diagnosis, and management. *Front Immunol* 2019; **10**: 33.

13 Jin H, Reed JC, Liu STH, *et al.* Three patients with X-linked agammaglobulinemia hospitalized for COVID-19 improved with convalescent plasma. *J Allergy Clin Immunol Pract* 2020.

14 Mira E, Yarce OA, Ortega C, *et al.* Rapid recovery of a SARS-CoV-2–infected X-linked agammaglobulinemia patient after infusion of COVID-19 convalescent plasma. *J Allergy Clin Immunol Pract* 2020; **8**: 2793–5.

15 Flaherty C. Man leaves hospital after three month battle with Covid-19. Impartial Report. 2020. https://www.impartialreporter.com/news/18599490.man-leaves-hospital-three-month-battle-covid-19/ (accessed Oct 25, 2020).

16 Meyts I, Bucciol G, Quinti I, *et al.* Coronavirus Disease 2019 in patients with inborn errors of immunity: an international study. *J Allergy Clin Immunol* 2020.

17 Mazhar M, Waseem M. Agammaglobulinemia. In: StatPearls [Internet]. StatPearls Publishing, 2020.

18 Abbott JK, Gelfand EW. Common variable immunodeficiency: diagnosis, management, and treatment. *Immunol Allergy Clin* 2015; **35**: 637–58.

19 Hueso T, Pouderoux C, Péré H, *et al.* Convalescent plasma therapy for B-cell depleted patients with protracted COVID-19 disease. *Blood* 2020.

20 Tremblay D, Seah C, Schneider T, *et al.* Convalescent Plasma for the Treatment of Severe COVID 19 Infection in Cancer Patients. *Cancer Med* 2020.

21 Hatzl S, Eisner F, Schilcher G, *et al.* Response to “COVID-19 in persons with haematological

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

cancers". *Leukemia* 2020; **34**: 2265–70.

22 Baang JH, Smith C, Mirabelli C, *et al.* Prolonged SARS-CoV-2 replication in an immunocompromised patient. *medRxiv* 2020.

23 Lancman G, Mascarenhas J, Bar-Natan M. Severe COVID-19 virus reactivation following treatment for B cell acute lymphoblastic leukemia. *J Hematol Oncol* 2020; **13**: 1–3.

24 Moore JL, Ganapathiraju P V, Kurtz CP, Wainscoat B. A 63-Year-Old Woman with a History of Non-Hodgkin Lymphoma with Persistent SARS-CoV-2 Infection Who Was Seronegative and Treated with Convalescent Plasma. *Am J Case Rep* 2020; **21**: e927812.

25 Çınar OE, Sayınalp B, Karakulak EA, *et al.* Covalescent (immune) plasma treatment in a myelodysplastic COVID-19 patient with disseminated Tuberculosis. *Transfus Apher Sci* 2020; : 102821.

26 Karataş A, İnkaya AÇ, Demiroğlu H, *et al.* Prolonged viral shedding in a lymphoma patient with COVID-19 infection receiving convalescent plasma. *Transfus Apher Sci* 2020; : 102871.

27 Luetkens T, Metcalf R, Planelles V, *et al.* Successful transfer of anti–SARS-CoV-2 immunity using convalescent plasma in an MM patient with hypogammaglobulinemia and COVID-19. *Blood Adv* 2020; **4**: 4864–8.

28 Shankar R, Radhakrishnan N, Dua S, *et al.* Convalescent plasma to aid in recovery of COVID-19 pneumonia in a child with acute lymphoblastic leukemia. *Transfus Apher Sci* 2020; : 102956.

29 Wright Z, Bersabe A, Eden R, Cap A. Successful use of COVID-19 convalescent plasma in a patient recently treated for follicular lymphoma. *Clin Lymphoma Myeloma Leuk* 2020.

30 Clark E, Guilpain P, Filip IL, *et al.* Convalescent plasma for persisting Covid 19 following therapeutic lymphocyte depletion: a report of rapid recovery. *Br J Haematol* 2020; **190**: e154–6.

31 Niu A, McDougal A, Ning B, *et al.* COVID-19 in allogeneic stem cell transplant: high false-negative probability and role of CRISPR and convalescent plasma. *Bone Marrow Transplant* 2020; : 1–3.

32 Pal P, Ibrahim M, Niu A, *et al.* Safety and efficacy of COVID-19 convalescent plasma in severe pulmonary disease: A report of 17 patients. *Transfus Med* 2020.

33 Abid MB, Chhabra S, Buchan B, *et al.* Bronchoalveolar lavage-based COVID-19 testing in patients with cancer. *Hematol Oncol Stem Cell Ther* 2020.

34 Malsy J, Veletzky L, Heide J, *et al.* Sustained response after remdesivir and convalescent plasma therapy in a B-cell depleted patient with protracted COVID-19. *Clin Infect Dis* 2020.

35 Wang B, Van Oekelen O, Mouhieddine T, *et al.* A tertiary center experience of multiple myeloma patients with COVID-19: lessons learned and the path forward. *medRxiv* 2020.

36 Zhang LL, Liu Y, Guo YG, *et al.* Convalescent Plasma Rescued a Severe COVID-19 Patient with Chronic Myeloid Leukemia Blast Crisis and Myelofibrosis. *Turkish J Haematol Off J Turkish Soc Haematol* 2020.

37 Rahman F, Liu STH, Taimur S, *et al.* Treatment with convalescent plasma in solid organ transplant recipients with COVID 19: Experience at large transplant center in New York City. *Clin Transplant* 2020; : e14089.

38 Fung M, Nambiar A, Pandey S, *et al.* Treatment of Immunocompromised COVID 19 patients with Convalescent Plasma. *Transpl Infect Dis* 2020.

39 Trimarchi H, Gianserra R, Lampo M, Monkowski M, Lodolo J. Eculizumab, SARS-CoV-2 and atypical hemolytic uremic syndrome. 2020.

40 Naeem S, Gohh R, Bayliss G, *et al.* Successful recovery from COVID 19 in three kidney transplant recipients who received convalescent plasma therapy. *Transpl Infect Dis* 2020; : e13451.

41 Lima B, Gibson GT, Vullaganti S, *et al.* COVID 19 in recent heart transplant recipients: Clinicopathologic features and early outcomes. *Transpl Infect Dis* 2020; : e13382.

42 Jiang J, Miao Y, Zhao Y, *et al.* Convalescent plasma therapy: Helpful treatment of COVID 19 in a kidney transplant recipient presenting with severe clinical manifestations and complex complications. *Clin Transplant* 2020; **34**: e14025.

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

43 WDRB Media and Fox 59. Plasma donation helps save Indiana man diagnosed with COVID-19. 2020. https://www.wdrb.com/news/coronavirus/plasma-donation-helps-save-indiana-man-diagnosed-with-covid-19/article_540bc9b2-ac1d-11ea-958d-abf6e203c27a.html.

44 NHS Blood and Transplant. One of the first COVID-19 convalescent plasma recipients supports donor appeal. Give Blood. 2020. https://www.blood.co.uk/news-and-campaigns/news-and-statements/one-of-the-first-covid-19-convalescent-plasma-recipients-supports-donor-appeal/.

45 Bartosch J. Lung transplant patient with COVID-19 recovers following plasma clinical trial. Univ. Chicago Med. 2020. https://www.uchicagomedicine.org/forefront/coronavirus-disease-covid-19/lung-transplant-patient-with-covid-19-recovers-following-plasma-clinical-trial.

46 Choudhury A, Reddy GS, Venishetty S, *et al.* COVID-19 in Liver Transplant Recipients-A Series with Successful Recovery. *J Clin Transl Hepatol* 2020; **8**: 1–7.

47 Christensen J, Kumar D, Moinuddin I, *et al.* Coronavirus Disease 2019 Viremia, Serologies, and Clinical Course in a Case Series of Transplant Recipients. In: Transplantation proceedings. Elsevier, 2020.

48 Cheng Y, Wong R, Soo YOY, *et al.* Use of convalescent plasma therapy in SARS patients in Hong Kong. *Eur J Clin Microbiol Infect Dis* 2005; **24**: 44–6.

49 Hung IFN, To KKW, Lee C-K, *et al.* Convalescent plasma treatment reduced mortality in patients with severe pandemic influenza A (H1N1) 2009 virus infection. *Clin Infect Dis* 2011; **52**: 447–56.

50 Gharbharan A, Jordans CCE, GeurtsvanKessel C, *et al.* Convalescent Plasma for COVID-19. A randomized clinical trial. *medRxiv* 2020.

51 Diorio C, Anderson EM, McNerney KO, *et al.* Convalescent plasma for pediatric patients with SARS CoV 2 associated acute respiratory distress syndrome. *Pediatr Blood Cancer* 2020; **67**: e28693.

52 Aksoy E, Oztutgan T. COVID-19 presentation in association with myasthenia gravis: a case report and review of the literature. *Case Rep Infect Dis* 2020; **2020**.

53 Hartman W, Hess AS, Connor JP. Hospitalized COVID-19 patients treated with Convalescent Plasma in a mid-size city in the midwest. *medRxiv* 2020.

54 Jafari R, Jonaidi-Jafari N, Dehghanpoor F, Saburi A. Convalescent plasma therapy in a pregnant COVID-19 patient with a dramatic clinical and imaging response: A case report. *World J Radiol* 2020; **12**: 137.

55 Pelayo J, Pugliese G, Salacup G, *et al.* Severe COVID-19 in Third Trimester Pregnancy: Multidisciplinary Approach. *Case Reports Crit Care* 2020; **2020**.

56 Rodriguez Z, Shane AL, Verkerke H, *et al.* COVID-19 convalescent plasma clears SARS-CoV-2 refractory to remdesivir in an infant with congenital heart disease. *Blood Adv* 2020; **4**: 4278.

57 Ye M, Fu D, Ren Y, *et al.* Treatment with convalescent plasma for COVID 19 patients in Wuhan, China. *J Med Virol* 2020.

Condition	No. Patients	WHO Disease Severity Scale*	Illness onset to Treatment (days)*	Mortality (n, %)	Rapid Improvement in Supplemental Oxygen (≤5 days) (n, %)*	Discharge (days)*
Primary Immunosuppresion						
Agammaglobulinemia	7	3·4 (2 - 5)	26·3 (16 - 44)	0, 0%	2, 50%	4·3 (1-7)
Common Variable Immune Deficiency	4	2·8 (2 - 5)	··	1, 25%	··	··
Secondary Immunosuppression						
Hematological Malignancies	54	3·2 (2 - 5)	23·8 (2 - 90)	7, 13·0%	22, 61·1%	17·1 (2 - 50)
Solid Organ Transplants	29	3·9 (2 - 5)	10·6 (2 - 31)	4, 13·8%	14, 56·0%	14·0 (2 - 58)
Other[a]						
	10	4·0 (3 - 5)	14·3 (6 - 39)	1, 10·0%	4, 40·0%	18·1 (1·5 - 60)

Data are presented as mean (range) or count (n), mean (%). *Data were not reported for all patients, please see Supplement 1 for additional information. [a]Other includes pediatric patients, patients with congenital heart disease, gravida patients, and patients with Sjögren's syndrome

Table: **Summary of each patient's hospital course and disposition after transfusion of COVID-19 convalescent plasma.**

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
All rights reserved. No reuse allowed without permission.

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

Panel: Anecdotal statements supporting the efficacy of convalescent plasma

- ∀ In the present case, the rapid clinical improvement followed by viral clearance after administration of hyperimmune plasma argue that passively transferred antibodies played a key role in COVID-19 recovery.[23]
- ∀ One day later [after convalescent plasma transfusion], the patient was afebrile for the first time in 3 weeks and had improved energy.[13]
- ∀ On day 122 (of illness), due to worsening symptoms, the patient was given convalescent plasma. He defervesced within 24 hours and was discharged nine days later.[31]
- ∀ …she was transferred to the intensive care unit for intubation. In the meantime…the patient received convalescent therapy instead and did not undergo intubation following the immediate improvement after plasma therapy infusion.[52]
- ∀ Based on the lack of clinical improvement…we transfused 1 unit of convalescent plasma…Importantly, the patient did not receive any other treatment potentially having an effect on the course of COVID-19…After transfusion of the convalescent plasma, the patient showed a dramatic clinical improvement, became asymptomatic, and was discharged home only 2 days later.[36]
- ∀ The patient was discharged after 2 weeks [convalescent plasma transfusion] with a dramatic response to therapy. Both newborns had no COVID-19 symptoms and negative PCR results.[54]
- ∀ 36 hours after [convalescent plasma] transfusion, the patient was discharged from the hospital reporting that he felt improved.[53]
- ∀ COVID-19 antibody testing showed complete lack of COVID-19 antibodies…She received 2 units of convalescent plasma…with rapid improvement in oxygen requirements. She was weaned off high-flow nasal cannula within 48 h and within a few days was discharged home in stable condition.[32]
- ∀ Intravenous convalescent plasma…was administered…Her health condition quickly improved, allowing [withdrawal of oxygen supplementation]…[23]
- ∀ Within a day of receiving her first transfusion of convalescent plasma, she reported improvement in shortness of breath and cough, had fever resolution, and decreasing oxygen requirements.[24]
- ∀ She received COVID convalescent plasma…She showed remarkable improvement [the next day]…with reduction of respiratory rate…and oxygen requirements.[21]
- ∀ …the patient received a transfusion of convalescent plasma…one day later her [arterial oxygen saturation] increased to 98%...clinical symptoms and pathological criteria improved rapidly within 3 days.[29]
- ∀ Within hours after receiving the convalescent plasma… [The patient's] fever started going down. Days later, his breathing and kidney function improved.[45]
- ∀ [The patient]…received a transfusion of convalescent plasma…He is recovering at home after spending two and a half weeks in a coma fighting for his life...[44]
- ∀ …received 217 mL of convalescent plasma…24 hours later, his heart rate had improved to 60-70 bpm with less frequent premature atrial contractions and premature ventricular contractions and he was breathing comfortably on room air…36 hours after transfusion the patient was discharged from the hospital…[53]
- ∀ Stagnancy in the patient's evolution, as represented by the lack of response to any of the treatments dispensed....we administered on day 23 COVID-19 convalescent plasma...after 24 hours of infusion, fever ceased without subsequent reappearance and with progressive improvement of asthenia.[14]

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
All rights reserved. No reuse allowed without permission.

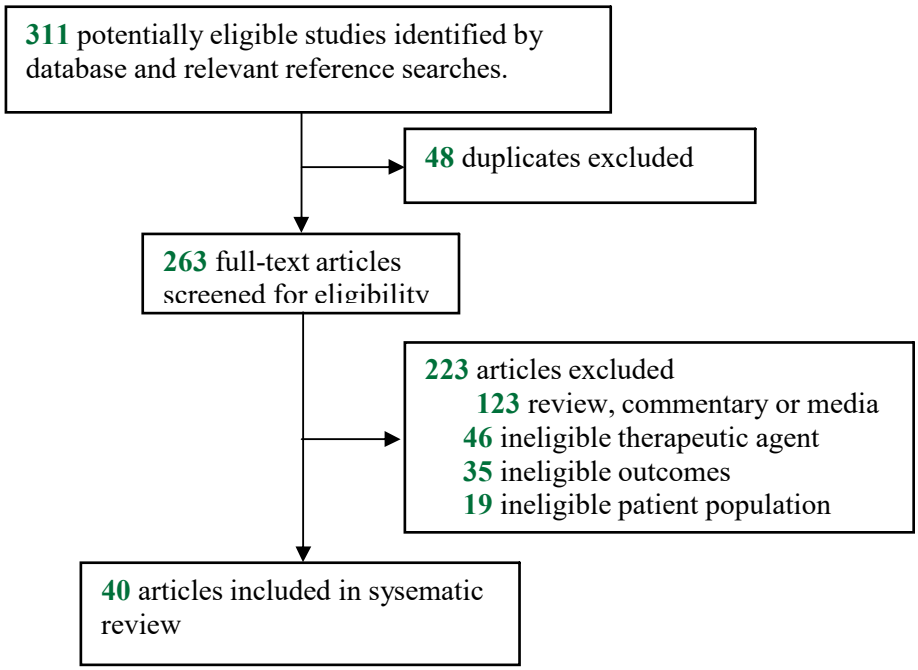


Figure: **Flowchart of the study selection.**

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Therapeutic use of convalescent plasma in COVID-19 patients with immunodeficiency

Most Recent Update: November 8, 2020

Study	Patient #	Condition	Age (years)	Sex	WHO Disease Severity Scale
Primary Immunosuppresion					
Agammaglobulinemia					
Jin et al.	1	X-Linked Agammaglobulinemia	10	Male	4
	2	X-Linked Agammaglobulinemia	24	Male	3
	3	X-Linked Agammaglobulinemia	40	Male	3
Meyts et al.	1	(Pt. #11) Autosomal Agammaglobulinemia	35-44	Male	5
	2	(Pt. #18) X-Linked Agammaglobulinemia	3-12	Male	3-4
Flaherty	1	X-Linked Agammaglobulinemia	32	Male	..
Mira et al.	1	X-Linked Agammaglobulinemia	39	Male	2
Common Variable Immune Deficiency					
Meyts et al.	1	(Pt. #2) Common Variable Immune Deficiency	35-44	Female	2
	2	(Pt. #13) Common Variable Immune Deficiency	13-18	Male	5
	3	(Pt. #46) Common Variable Immune Deficiency	25-34	Male	2
	4	(Pt. #69) Common Variable Immune Deficiency	65-74	Female	2
Secondary Immunosuppression					
Hematological Malignancies					
Hueso et al.	1-17	Diffuse large B-Cell lymphoma, 4 | Mantle cell lymphoma, 3 | Follicular lymphoma, 3 | Chronic lymphocytic leukemia, 3 | Marginal zone lymphoma, 1 | Waldentröm Macroglubulinemia, 1 | Multiple sclerosis, 1 | Common variable immune deficiency, 1	(35-77)	Male, 12 | Female, 5	2-5
Tremblay et al.	1-14	Non-Hodgkin lymphoma, 5 | Multiple myeloma, 4 | Acute lymphoblastic leukemia, 2 | Hodgkin lymphoma, 1 | Myelofibrosis, 1 | Chronic lymphocytic leukemia, 1	(31-88)	Male, 8 | Female, 6	3-5
Pal et al.	1	(Pt. #1) Diffuse large B-cell lymphoma/central nervous system lymphoma	70	Female	3
	2	(Pt. #2) Acute Myeloid Lymphoma	30	Male	2
	3	(Pt. #4) Acute Lymphoblastic Leukemia	53	Female	3
	4	(Pt. #11) Acute Lymphoblastic Leukemia	64	Male	3
	5	(Pt. #12) Follicular Lymphoma	68	Female	4
	6	(Pt. #13) Acute Myeloid Lymphoma	24	Male	2
Abid et al.	1	(Pt. #1) Myelodysplastic Syndrome	76	Male	5

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

	2	(Pt. #2) Multiple Myeloma	88	Male	3
Hatzl et al.	1	Diffuse large B-Cell lymphoma	52	Male	5
	2	Follicular lymphoma	55	Male	5
Baang et al.	1	Refractory mantle cell lymphoma	60	Male	3
Cinar et al.	1	Myelodysplastic syndrome	55	Male	3
Clark et al.	1	Orbital and Meningeal Marginal Zone Lymphoma	76	Female	4
Karatas et al.	1	Mixed Cellularity Classical Hodgkin Lymphoma, Peripheral T-cell lymphoma, Autologous Stem-Cell Transplant	61	Male	3
Lancman et al.	1	B-Cell acute lymphoblastic leukemia	55	Female	4
Luetkens et al.	1	Immunoglobulin G k multiple myeloma	72	Female	3
Malsy et al.	1	Follicular lymphoma	53	Female	2
Moore et al.	1	Non-Hodgkin lymphoma	63	Female	2
Niu et al.	1	B-cell acute lymphoblastic leukemia, Allogenic stem-cell transplant	53	Female	3
Shankar et al.	1	B-Lineage Acute Lymphoblastic Leukemia	4	Female	3
Wang et al.	1	Multiple Myeloma	..	..	..
Wright et al.	1	Follicular Lymphoma	54	Male	3
Zhang et al.	1	Myeloid Leukemia	46	Female	2
Organ Transplants					
Rahman et al.	1-13		51 (20-75)	Male, 8 | Female, 5	3-5
	1	Kidney Transplant	..	..	3
	2	Kidney Transplant	..	..	3
	3	Kidney Transplant	..	..	4
	4	Kidney Transplant	..	..	4
	5	Liver & Kidney Transplant	..	..	4
	6	Kidney Transplant	..	..	3
	7	Kidney Transplant	..	..	4
	8	Heart Transplant	..	..	3
	9	Liver Transplant	..	..	3
	10	Liver Transplant	..	..	3
	11	Liver Transplant	..	..	3
	12	Liver Transplant	..	..	4
	13	Kidney & Pancreas Transplant	..	..	3
Fung et al.	1	Kidney Transplant	44	Male	5
	2	Kidney Transplant	44	Male	4
	3	Bilateral Lung Transplant & Sjögrens Syndrome	65	Female	3
	4	Chronic myelogenous leukemia	62	Male	4
Naeem et al.	1	Kidney Transplant	65	Female	2
	2	Kidney Transplant	35	Female	5
	3	Kidney Transplant	36	Male	5
Lima et al.	1	(Pt #4) Heart Transplant	62	Male	5
	2	(Pt #5) Heart Transplant	68	Male	5
Bartosch	1	Lung Transplant	30	Male	..

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
All rights reserved. No reuse allowed without permission.

Choudhury et al.	1	Liver Transplant	52	Male	4
Christensen et al.	1	(Pt. #3) Kidney transplant	64	Female	5
Jiang et al.	1	Kidney Transplant	70	Female	4
NHS Blood and Transplant	1	Liver Transplant	49	Male	5
Trimarchi et al.	1	Kidney Transplant	24	Male	3
WDRB Media and Fox 59	1	Kidney Transplant	64	Female	5
Other					
Diorio et al.	1	(CD4) Pediatric Patient	18	Female	5
	2	(CD15) Pediatric Patient	15	Female	4
	3	(CD17) Pediatric Patient	17	Female	4
	4	(CD25) Pediatric Patient	14	Female	5
Aksoy et al.	1	Myasthenia Gravis	46	Female	4
Hartman et al.	1	Asthma, Chronic Obstructive Pulmonary Disease, Sinus Bradycardia	62	Male	3
Jafari et al.	1	Gravida (36 weeks)	26	Female	3
Pelayo et al.	1	Gravida (36 weeks)	35	Female	4
Rodriguez et al.	1	Congenital Heart Disease, Trisomy 21	9 weeks	Female	5
Ye et al.	1	Sjögrens Syndrome	63	Female	3

Supplementary Table 1: **Summary of patient's characteristics prior to transfusion of COVID-19 convalescent plasma.**

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

Study	Patient #	Illness onset to Treatment (days)	Mortality, n, %	Improvement in Supplemental Oxygen (≤5 days), n, %	Treatment to Discharge (days)
Primary Immunosuppresion					
Agammaglobulinemia					
Jin et al.	1	22	0, 0%	0, 0%	6
	2	16	0, 0%	0, 0%	3
	3	44	0, 0%	1, 100%	1
Meyts et al.	1	..	0, 0%	..	..
	2	..	0, 0%	..	..
Flaherty	1	..	..	..	..
Mira et al.	1	23	0, 0%	1, 100%	7
Common Variable Immune Deficiency					
Meyts et al.	1	..	1, 100%	..	..
	2	..	0, 0%	..	..
	3	..	0, 0%	..	..
	4	..	0, 0%	..	..
Secondary Immunosuppression					
Hematological Malignancies					
Hueso et al.	1-17	56 (7-83)	1, 5·9%	..	7 [2-14]
Tremblay et al.	1-14	3 [2-7]	5, 35·7%	4, 28·6%	..
Pal et al.	1	4	0, 0%	1, 100%	4
	2	5	0, 0%	0, 0%	5
	3	5	0, 0%	1, 100%	12
	4	10	0, 0%	1, 100%	12
	5	18	0, 0%	1, 100%	24
	6	5	0, 0%	0, 0%	41
Abid et al.	1	10	1, 100%	0, 0%	..
	2	5	0, 0%	1, 100%	7
Hatzl et al.	1	14	0, 0%	1, 100%	46
	2	19	0, 0%	1, 100%	38
Baang et al.	1	31	0, 0%	1, 100%	8
Cinar et al.	1	5	0, 0%	1, 100%	10
Clark et al.	1	50	0, 0%	1, 100%	19
Karatas et al.	1	40	0, 0%	1, 100%	38
Lancman et al.	1	81	0, 0%	1, 100%	~5
Luetkens et al.	1	3	0, 0%	1, 100%	2
Malsy et al.	1	~90	0, 0%	0, 0%	~50
Moore et al.	1	88	0, 0%	1, 100%	2
Niu et al.	1	4 & 13	0, 0%	1, 100%	10
Shankar et al.	1	8 & 9	0, 0%	1, 100%	10
Wang et al.	1	..	..	..	RH
Wright et al.	1	24	0, 0%	1, 100%	7
Zhang et al.	1	5	0, 0%	1, 100%	9
Organ Transplants					
Rahman et al.	1-13	10·2	3, 23·1%	8, 61·5%	≤ 7 days, 6
	1	7	0, 0%	1, 100%	..
	2	5	0, 0%	1, 100%	..
	3	6	0, 0%	1, 100%	..
	4	15	1, 100%	0, 0%	..
	5	9	1, 100%	0, 0%	..
	6	11	0, 0%	0, 0%	..
	7	31	1, 100%	0, 0%	..
	8	12	0, 0%	1, 100%	..

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

	9	7	0, 0%	1, 100%	··
	10	11	0, 0%	1, 100%	··
	11	4	0, 0%	1, 100%	··
	12	7	0, 0%	0, 0%	··
	13	8	0, 0%	1, 100%	··
Fung et al.	1	27	0, 0%	0, 0%	46
	2	4	0, 0%	1, 100%	11
	3	8	0, 0%	1, 100%	2
	4	3	0, 0%	0, 0%	58
Naeem et al.	1	7	0, 0%	1, 100%	2
	2	3	0, 0%	0, 0%	21
	3	2	0, 0%	1, 100%	13
Lima et al.	1	··	0, 0%	··	RH
	2	··	0, 0%	··	RH
Bartosch	1	··	0, 0%	1, 100%	6
Choudhury et al.	1	8	0, 0%	1, 100%	6
Christensen et al.	1	··	1, 100%	0, 0%	··
Jiang et al.	1	26	0, 0%	0, 0%	26
NHS Blood and Transplant	1	24	0, 0%	··	··
Trimarchi et al.	1	9	0, 0%	1, 100%	5
WDRB Media and Fox 59	1	··	0, 0%	··	··
Other					
Diorio et al.	1	14	1, 100%	0, 0%	··
	2	7	0, 0%	0, 0%	RH
	3	11	0, 0%	0, 0%	RH
	4	11	0, 0%	0, 0%	17
Aksoy et al.	1	6	0, 0%	1, 100%	16
Hartman et al.	1	7	0, 0%	1, 100%	1.5
Jafari et al.	1	6	0, 0%	0, 0%	8
Pelayo et al.	1	··	0, 0%	0, 0%	··
Rodriguez et al.	1	28-32	0, 0%	1, 100%	~60
Ye et al.	1	39	0, 0%	1, 100%	6

Supplementary Table 2: **Summary of patient's hospital course and disposition after transfusion of COVID-19 convalescent plasma.**

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

		Concomitant therapies				
Study	**Patient #**	**IVIG**	**Steroids**	**remdesivir**	**(hydoxy)chloroquine**	**antibiotics**
Primary Immunosuppresion						
Agammaglobulinemia						
Jin et al.	1	Y		Y		
	2	Y				Y
	3					Y
Meyts et al.	1	Y			Y	Y
	2			Y		Y
Flaherty	1			Y		
Mira et al.	1	Y			Y	Y
Common Variable Immune Deficiency						
Meyts et al.	1	Y	Y		Y	Y
	2		Y	Y		Y
	3	Y	Y		Y	Y
	4				Y	Y
Secondary Immunosuppression						
Hematological Malignancies						
Hueso et al.	1-17		Y	Y	Y	Y
Tremblay et al.	1-14			Y	Y	
Pal et al.	1			Y		
	2			Y		
	3					
	4					
	5			Y		
	6					
Abid et al.	1			Y		
	2					
Hatzl et al.	1		Y		Y	Y
	2		Y		Y	Y
Baang et al.	1			Y		
Cinar et al.	1					
Clark et al.	1		Y			
Karatas et al.	1				Y	Y
Lancman et al.	1		Y		Y	Y
Luetkens et al.	1		Y			
Malsy et al.	1			Y		
Moore et al.	1					
Niu et al.	1	Y				Y
Shankar et al.	1	Y	Y			Y
Wang et al.	1	..	..	..	..	..
Wright et al.	1				Y	Y
Zhang et al.	1					Y
Organ Transplants						
Rahman et al.	1-13		Y		Y	
	1	..	..	..	..	..
	2	..	..	..	..	..
	3	..	..	..	..	..
	4	..	..	..	..	..
	5	..	..	..	..	..
	6	..	..	..	..	..
	7	..	..	..	..	..
	8	..	..	..	..	..
	9	..	..	..	..	..
	10	..	..	..	..	..

medRxiv preprint doi: https://doi.org/10.1101/2020.11.08.20224790 ; this version posted November 10, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. All rights reserved. No reuse allowed without permission.

	11	..	..	..	..	..	
	12	..	..	..	..	..	
	13	..	..	..	..	..	
Fung et al.	1		Y		Y	Y	
	2						
	3			Y			
	4						
Naeem et al.	1		Y				
	2		Y	Y		Y	
	3		Y	Y		Y	
Lima et al.	1		Y				
	2		Y				
Bartosch	1	..	..	..	..	..	
Choudhury et al.	1		Y	Y	Y	Y	
Christensen et al.	1				Y		
Jiang et al.	1		Y			Y	
NHS Blood and Transplant	1	..	..	..	..	..	
Trimarchi et al.	1		Y			Y	
WDRB Media and Fox 59	1	..	..	..	..	..	
Other							
Diorio et al.	1		Y		Y		
	2			Y			
	3		Y	Y			
	4		Y				
Aksoy et al.	1		Y		Y	Y	
Hartman et al.	1		Y				
Jafari et al.	1				Y	Y	
Pelayo et al.	1		Y	Y			
Rodriguez et al.	1			Y	Y		
Ye et al.	1					Y	

Supplementary Table 3: **Summary of patient's concomitant COVID-19 therapeutics prior to transfusion of COVID-19 convalescent plasma.**

-Author name in bold denotes the presenting author
-Asterisk * with author name denotes a Non-ASH member
denotes an abstract that is clinically relevant.

denotes that this is a recommended PHD Trainee Session.

denotes that this is a ticketed session.

103 A National Collaborative Multicenter Phase II Study for Potential Safety Efficacy of Convalescent Plasma to Treat Severe COVID-19 Patients

Program: Oral and Poster Abstracts
Type: Oral
Session: 401 Basic Science and Clinical Practice in Blood Transfusion: COVID-19 Convalescent Plasma, Antigen Typing and the Prothrombin Complex II
Hematology Disease Topics & Pathways:
Coronaviruses, Biological, Therapies, Clinically relevant, transfusion

Saturday, December 5, 2020: 10:30 AM

Hani Al Hashmi, MD[1], Deia Alawami, MD[1*], Syed Ziauddin A Zaidi, FRCP, FRCPath, MBBS[2], Nawal F Alshehry, MD[3*], Hazza A. Alzahrani, MD[4*], Abdul Rehman Z Zaidi, MBBS[5], Ahmed Al Sagheir, MD[6*], Osamah Khojah, MD[7], Ahmed Alaskar, MD, FRCPC[8], Noura AlMozain, MD[9*], Rehab Al-Ansari, MD[10*], Mona Alfaraj, MD[11*], Ghada ElGohary, MD, PhD, MBBS[12*], Afra Dayel, MD[1*], Ahmed Bahrani, MD[1*], Arwa Nabhan Abdulhamid, MD[13*], Hind Alhumaidan, MD[14*], Mohammed Albalawi, MD[15*], Alia AlFraedhi, MD[16*], Jawaher Al-Otaibi, MD[17*] and Rania Abdallah, MD[18*]

[1]King Fahad Specialist Hospital, Dammam, Saudi Arabia
[2]Department of Adult Hematology/BMT, Comprehensive Cancer Center, King Fahad Medical City, Riyadh, Saudi Arabia
[3]King Fahad Medical City, Department of Adult Hematology/BMT, Riyadh, Saudi Arabia
[4]Hematology/HSCT Section, King Faisal Cancer Center, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia
[5]Department of Medicine, King Fahad Medical City, Riyadh, Saudi Arabia
[6]Department of Hematology and Oncology, Johns Hopkins Aramco Healthcare, Dhahran, Saudi Arabia
[7]Pathology Department, Hematology Unit, King Saud University, Riaydh, Saudi Arabia
[8]King Abdullah International Medical Research Center, Riyadh, Saudi Arabia
[9]King Saud University Medical College, King Saud University, Riyadh, Saudi Arabia
[10]King Fahad Military Medical Complex, Dhahran, Saudi Arabia
[11]Hematology Laboratory Section of Medical Laboratory Department, Qatif Central Hospital, Qatif, Saudi Arabia
[12]Hematology Department, King Khaled University Hospital, Riyadh, Saudi Arabia
[13]Imam Abdulrahman Bin Faisal University, AlKhobar, Saudi Arabia
[14]Blood Bank & Transfusion Services, Department of Pathology and Laboratory Medicine, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia
[15]Internal medicine department, College of Medicine, Taibah University, Madinah, Saudi Arabia
[16]Department of Hematopathology and Blood Bank, King Saud University, Riyadh, Saudi Arabia
[17]Infectious diseases, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia
[18]Pulmonary and Critical Care Medicine, King Fahad Medical City, Riyadh, Saudi Arabia

Introduction

Coronavirus disease (COVID-19) pandemic has started to affect Saudi Arabia in the beginning of March 2020 and is expected to cause significant morbidity to many patients, especially to elderly, who might require intensive care unit (ICU) support to survive as its lethality increases with the increasing age. Recent publications suggested the benefit of utilizing convalescent plasma from recovered donors as a therapeutic approach in treating COVID-19 patients. Convalescent plasma could provide our first-line defense for people with COVID-19, especially those who are older and at a much higher risk for complications.. therefore, we developed a national protocol to investigate the safety, benefit and applicability at larger scale and at different health care

complications; therefore, we developed a national protocol to investigate the safety, benefit and applicability at larger scale and at different health care facilities in Saudi Arabia (KSA).

Objectives

Primary endpoints are:

1. ICU (or designated area for critical patients) length of stay
2. Safety of convalescent plasma

Secondary endpoints included:

1. 30 days mortality
2. Number of days on mechanical ventilation
3. Days to clinical recovery

Method

Eligible convalescent plasma donors will be invited to participate in trial. The arrangement for plasmapheresis will start after obtaining donor informed consent. The collected plasma will be treated with pathogen reduction system. The convalescent plasma units will be labelled, stored and shipped as per the standard transfusion medicine protocols. It will be used only for eligible patients' "recipients" as per the following eligibility criteria:

1- Inclusion criteria:

- Confirmed case of SARS-CoV-2 infection with POSITIVE rRT PCR test
- 18 or older
- Must have been requiring ICU care or severe or immediately life-threatening care (any one of the following):
    1. Patient requiring ICU care/admission.
    2. Severe disease is defined as:
        a. Dyspnea
        b. Respiratory frequency ≥ 30/min
        c. Blood oxygen saturation ≤ 93%
        d. Partial pressure of arterial oxygen to fraction of inspired oxygen ratio < 300, and/or Lung infiltrates > 50% within 24 to 48 hours
    3. Life-threatening disease is defined as:
        a. Respiratory failure
        b. Septic shock, and/or
        c. Multiple organ dysfunction or failure

2- Exclusion criteria:

- Negative or non-conclusive test COVID-19 rRT PCR test
- Mild symptoms
- Hospitalization not requiring ICU care/admission

Eligible Patients will be infused with the convalescent plasma (200-400 ml / treatment dose) at least once & if possible, daily, for up to 5 sessions. Other supportive and therapeutic measures should continue according to the locally approved protocols with due diligence.

Sample size was calculated with 80% power and 5% level of significance based on the recently published data to detect statistical difference in the study outcome. Therefore, we plan to recruit total of 575 patients.

- Convalescent plasma Recipient Group:
    - 115 patients (recipients) who have COVID 19 as per the inclusion criteria.
- Comparative control Group:
    - 460 Patients who are eitherCOVID 19 historical control or only consent for sharing their clinical and laboratory data

Matching for age, gender, Hypertension, Diabetes and intubation status were done based on the propensity score. Continuous variables will be presented as the median and interquartile range (IQR). Statistical software SPSS 24.0 will be used. Demographic, Clinical, imaging and laboratory information of all enrolled patients will be retrieved from the hospital electronic/paper records system to be used for the outcomes analysis.

Results

22 sites across KSA that participated in the study. Tertiary, secondary, academic and non-academic centers participated (real world data). There were no unusual safety issues related to convalescent plasma infusion since all mortalities in the plasma group were not related to plasma infusion which represent similar finding from other the published international reports. Keeping in-mind that our data is still maturing, 30 survival probability in the plasma group was 69% compared to 56% in the comparative group (p value = 0.066) (figure-1). This benefit to seem to be more noticeable in the COVID-19 cases who did not meet the criteria for life-threatening disease (figure-2).

Conclusion

Conclusion

Our study supports the safety of convalescent plasma in treating COVID-19 patients. Patients who are in the category of life-threating/end organs failure do not seem to benefit. There might be a benefit in the other subgroups.

Figure-1: Survival Probability between Convalescent Plasma to Treat Severe COVID-19 and matched comparative patients

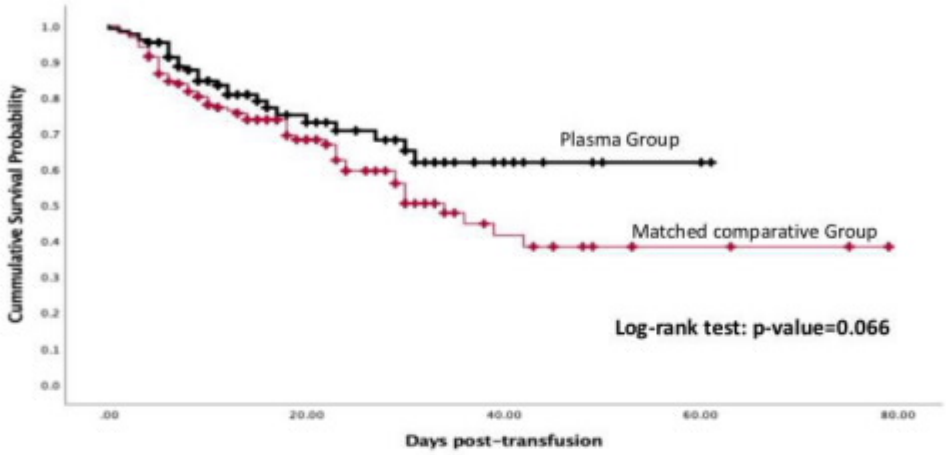
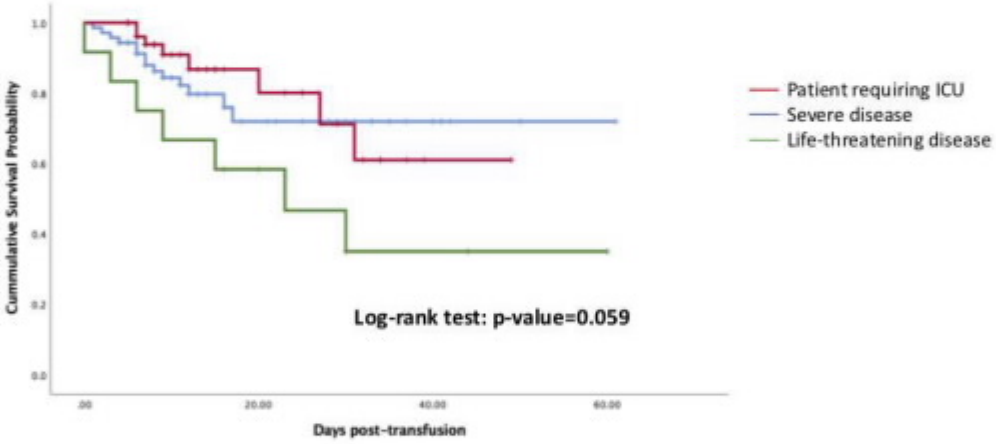


Figure-2: Survival Probability for Convalescent Plasma recipients patients among one of the three inclusion criteria (3 subgroups)



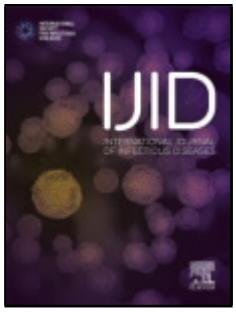
Disclosures: No relevant conflicts of interest to declare.

See more of: 401 Basic Science and Clinical Practice in Blood Transfusion: COVID-19 Convalescent Plasma, Antigen Typing and the Prothrombin Complex II
See more of: Oral and Poster Abstracts

<< Previous Abstract | Next Abstract >>

*signifies non-member of ASH

Journal Pre-proof



COVID-19 Convalescent Plasma Treatment of Moderate and Severe Cases of SARS-CoV-2 Infection: A Multicenter Interventional Study

Sondas Alsharidah, Mariam Ayed, Reem M. Ameen, Fatma Alhuraish, Najat A. Rouheldeen, Farah R. Alshammari, Alia Embaireeg, Mariam Almelahi, Maitham Adel, Mohammed E. Dawoud, Mohammad A. Aljasmi, Nashmi Alshammari, Abdullah Alsaeedi, Wasel Al-Adsani, Hussan Arian, Husain Awad, Humoud A. Alenezi, Azeez Alzafiri, Enas F. Gouda, Mohammad Almehanna, Salem Alqahtani, Abdulrahman Alshammari, Medhat Z. Askar

PII: S1201-9712(20)32513-3

DOI: https://doi.org/10.1016/j.ijid.2020.11.198

Reference: IJID 4908

To appear in: *International Journal of Infectious Diseases*

Received Date: 1 October 2020

Revised Date: 25 November 2020

Accepted Date: 26 November 2020

Please cite this article as: Alsharidah S, Ayed M, Ameen RM, Alhuraish F, Rouheldeen NA, Alshammari FR, Embaireeg A, Almelahi M, Adel M, Dawoud ME, Aljasmi MA, Alshammari N, Alsaeedi A, Al-Adsani W, Arian H, Awad H, Alenezi HA, Alzafiri A, Gouda EF, Almehanna M, Alqahtani S, Alshammari A, Askar MZ, COVID-19 Convalescent Plasma Treatment of Moderate and Severe Cases of SARS-CoV-2 Infection: A Multicenter Interventional Study, *International Journal of Infectious Diseases* (2020), doi: https://doi.org/10.1016/j.ijid.2020.11.198

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Published by Elsevier.

Journal Pre-proof

COVID-19 Convalescent Plasma Treatment of Moderate and Severe Cases of SARS-CoV-2 Infection: A Multicenter Interventional Study

Sondas Alsharidah,MD[1]; Mariam Ayed,MD[2];Reem M Ameen,PhD[3];Fatma Alhuraish,MD[4];Najat A Rouheldeen,MD[5];Farah R Alshammari,MD[6];Alia Embaireeg,MD[7]; Mariam Almelahi,MD[8]; Maitham Adel,MD[9]; Mohammed E Dawoud,MD[10];Mohammad A Aljasmi,MD[9]; Nashmi Alshammari,MD[11];Abdullah Alsaeedi,MD[6];Wasel Al-Adsani,MD[8]; Hussan Arian, MD[9];Husain Awad,MD[9]; Humoud A Alenezi,MD[12];Azeez Alzafiri,MD[6];Enas F Gouda,MD[13]; Mohammad Almehanna,MD[9];Salem Alqahtani,MD[14];Abdulrahman Alshammari,MD[6];Medhat Z Askar,MD.[15,16]

[1]Stem Cell Transplant Unit, Pediatric Hematology Oncology Department, National Bank Kuwait Specialized Children Hospital, Ministry of Health, Kuwait.

[2] Neonatology Department, Farwaniya Hospital, Ministry of Health, Kuwait.

[3] Medical Laboratory Sciences, Health Sciences Center, Kuwait University, Kuwait.

[4] Respiratory Unit, Department of Internal Medicine, Sabah Hospital, Ministry of Health, Kuwait.

[5] Hematology Department, Maternity Hospital, Ministry of Health, Kuwait.

[6] Department of Internal Medicine, Jahra Hospital, Ministry of Health, Kuwait.

[7] Department of Pediatrics, Amiri Hospital, Ministry of Health, Kuwait.

[8] Department of Internal Medicine, Mubarak Hospital, Ministry of Health, Kuwait.

[9]Jaber Stadium COVID-19 Quarantine Center, Ministry of Health, Kuwait.

[10]Department of Emergency Medicine, Amiri Hospital, Ministry of Health, Kuwait.

[11]Department of Intensive Care, Jaber Al-Ahmad Hospital, COVID-19 Center, Ministry of Health, Kuwait.

[12]COVID-19 Military Quarantine Center, Ministry of Defense, Kuwait.

[13]Hematology Department, Jahra Hospital, Ministry of Health, Kuwait.

Journal Pre-proof

[14]Department of Internal Medicine, Farwaniya Hospital, Ministry of Health, Kuwait.

[15] Baylor University Medical Center, Dallas, Texas, USA.

[16] Texas A&M Health Science Center College of Medicine, Bryan, Texas, USA.

Corresponding author:

Sondas I. Alsharidah,MBBCH, BOA, MRCPCH, Stem Cell Transplant Unit, Pediatric Hematology Oncology Department. National Bank of Kuwait Specialized Children Hospital. Ministry of Health, State of Kuwait.

salsharida@moh.gov.kw

Tell: (+965) 66691663

P.O: (5) 13001

State - Kuwait

7540 HA 0008997

Highlights

- Convalescent plasma is adjunct treatment in COVID-19 moderate and severe disease.
- Clinical improvement is noticed in COVID-19 disease after convalescent plasma.
- Mortality is significantly reduced after COVID-19 convalescent plasma treatment.

Abstract

Objective: To study the effectiveness of CCP therapy for moderate and severe COVID-19 disease patients in Kuwait.

Methods: This non-randomized prospective cohort study was conducted from May 21, 2020, to June 30, 2020, at four major tertiary hospitals in Kuwait. CCP was administered to 135 patients. The control group comprised 233 patients who received standard treatment. All patients (N=368, median age 54 [range 15-82] years) had laboratory-confirmed SARS-CoV-2 infection and either moderate or severe COVID-19 disease.

Journal Pre-proof

Results: CCP treatment was significantly associated with a higher rate of clinical improvement in patients with moderate or severe disease. Among those with moderate COVID-19 disease, time to clinical improvement was 7 days in the CCP group versus 8 days in the control group (p=0·006). For severe COVID-19 disease, time to clinical improvement was 7 days in the CCP group versus 15.5 days in the control group (p=0·003). In the adjusted analysis, the CCP-treated patients with moderate disease had significantly lower 30-day mortality. Compared to the control group, oxygen saturation improved within 3 days of CCP transfusion, and lymphocyte counts improved from day 7 in patients with moderate disease and day 11 in patients with severe disease. Furthermore, C-reactive protein declined throughout the first 14 days after CCP transfusion. None of the CCP patients developed a serious transfusion reaction.

Conclusions: The data show that administration of CCP is a safe treatment option for patients with COVID-19 disease, with a favorable outcome in the rate and time to clinical improvement.

Keywords: convalescent plasma, COVID-19, SARS-CoV-2, pneumonia, clinical improvement, mortality.

Introduction

The novel coronavirus disease of 2019 (COVID-19) pandemic is considered one of the greatest global public health crises since the 1918 influenza pandemic.[1] COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a single-stranded RNA virus that belongs to *coronaviridae*.[2,3] The clinical spectrum of infection is broad, ranging from asymptomatic to severe pneumonia, multiorgan failure, and death. Due to the absence of established antiviral treatment for COVID-19, the historical choice of therapeutic convalescent plasma (CP) was globally appealing, especially with its prior success against RNA viruses.[1] CP has been proposed not only to neutralize the pathogen, but provide passive immunomodulatory properties that allow the recipient to control the

Journal Pre-proof

exaggerated inflammatory cascade.[4,5] However, the mechanism of action of therapeutic CP remains unclear. Reports from open label trials and case series suggest that plasma collected from convalescent COVID-19 patients is safe to administer and may be effective in treating patients with COVID-19.[6–8] Therefore, the World Health Organization (WHO) and United States Food and Drug Administration (FDA) have issued guidelines for the usage of COVID-19 convalescent plasma (CCP) and standardized donor selection, which was further supported by an Emergency Use Authorization (EUA) from the FDA.[9,10]

A number of reports have demonstrated promising results of CCP in severe and critically ill COVID-19 patients, albeit not consistently.[8,11-12] The effectiveness of CCP in less than severe cases remains unclear. The aim of the current study was to assess the effectiveness of CCP in both moderate and severe COVID-19 cases compared to the standard treatment alone. The CCP effectiveness was evaluated in terms of time to clinical improvement and hospital mortality, the changes in oxygen saturation and laboratory markers (lymphocytes, neutrophils counts, lactate dehydrogenase [LDH], and C-reactive protein [CRP]).

Patients and Methods

Study design

This prospective multicenter interventional study was conducted in four major tertiary hospitals in Kuwait (Al-Sabah, Farwaniya, Mubarak Al-Kabeer, and Jahra) from May 21 to June 30, 2020, with the last follow-up data collected July 12, 2020. All patients and CCP donors had confirmed SARS-CoV-2 infection by an EUA-approved real-time reverse transcriptase-polymerase chain reaction (RT-PCR) using nasopharyngeal swabs (Cobas 6800 Systems, Roche, Switzerland and Taq Path, Thermo-Fisher Scientific, USA). The CCP donors were tested for SARS-CoV-2 IgG using a CE-marked rapid test (BIOZEK COVID-19 IgG/IgM qualitative test: Inzek International Trading, Apeldoom, the Netherlands).

The study protocol was approved by the Ethics and Research Committee of Kuwait Ministry of Health (#2020/1417). Written informed consent was obtained from all patients or next of

Journal Pre-proof

kin. If the patient was unconscious and next of kin not available, the healthcare proxy provided written informed consent.

Inclusion criteria

We included patients aged ≥18 years with confirmed laboratory diagnosis of SARS-CoV-2 infection and admission diagnosis of COVID-19. Patients <18 years old were enrolled after thorough evaluation and discussion among an interdisciplinary team. Patients were eligible for CCP if they had moderate or severe COVID-19 as determined according to the WHO classification at admission by the treating physician.[9] Patients with moderate disease exhibited clinical signs of pneumonia (fever, cough, dyspnea, fast breathing) and had SpO2 >90% in room air. Patients with severe disease exhibited clinical signs of pneumonia and at least one of the following: respiratory rate >30 breaths/minutes, SpO2<90% in room air, or admission to intensive care unit (ICU) for respiratory support (i.e., non-invasive mechanical ventilation and intubation).

Exclusion criteria

Patients were excluded if they had a contraindication to transfusion (volume overload, history of anaphylaxis to blood products), acute severe multiorgan failure, hemodynamic instability, severe disseminated intravascular coagulopathy (DIC), septic shock, or expected survival of less than 48 hours.

Control sampling procedure

After screening for the inclusion and exclusion criteria, we applied a systematic stratified sampling technique to identify control patients from the COVID-19 national registry. For

Journal Pre-proof

each patient who received CCP, we selected the first two patients with the same disease severity strata admitted on that calendar date from the same participating center.

Standard treatment

Standard treatment was based on our institutional protocol and international guidelines.[13] The majority of patients received antibiotics and low molecular weight heparin. None of our patients received antiviral therapy or hydroxychloroquine. Special therapy (i.e., steroids and/or tocilizumab) was prescribed at the discretion of treating physicians.

Data collection

Clinical and laboratory data were extracted from patient medical records using a standardized data collection sheet. We captured pre-existing medical conditions, including diabetes mellitus, hypertension, and obesity. Specific therapies, such as anticoagulants, antibiotics, steroids, and tocilizumab, was also collected. Complete blood counts (CBCs), CRP, D-dimer, and LDH were also collected on days 1, 3, 7, 11, and 14 of hospitalization. We followed the patients' progress daily from day 1 through day 30 or until discharge, whichever came first. Clinical improvement was defined as a two-grade decrease from hospitalization by assessing the clinical status based on the WHO ordinal scale.[9]

Selection of CCP donors

The CP was collected from individuals who had recovered from RT-PCR-confirmed COVID-19 disease and tested positive for IgG antibodies against SARS-CoV-2. All donors tested positive for SARS-CoV-2 by an EUA-approved RT-PCR test on a specimen collected by nasopharyngeal swab at the time of illness and a positive EUA-approved qualitative serological test for SARS-CoV-2 IgG antibodies after recovery. Donors who tested negative or were positive only for SARS-CoV-2 IgM were excluded. All donors were negative for HLA antibodies. Additional donor eligibility criteria and qualification were based on the American Association of Blood Banks (AABB) standards and the FDA guidance on CCP

Journal Pre-proof

administration, including questionnaires and testing for relevant transfusion-transmitted infections.[10,14] CCP was only collected from individuals who met all donor eligibility requirements according to the United States Code of Federal Regulations (21 CFR 630.10 and 21 CFR 630.15). Pathogen inactivation was also performed for each unit.

CCP administration

A total of 135 patients with moderate to severe COVID-19 were enrolled in this cohort (89 patients with moderate disease and 46 patients with severe disease). All patients were pre-medicated (paracetamol, antihistamine, steroids) according to an institutional protocol. Overall, 107 (79·3%) patients received 2 units of ABO-compatible CCP (each unit containing 200 ml of CCP) 12 hours apart and 28 (20·7%) received 1 unit of CCP according to the treating physician and protocol dosage range (200-400 mL). All patients received CCP within 24 hours of admission. Three patients (2%) had allergic skin reactions that were completely resolved after transfusion.

Statistical analysis

Categorical variables are presented as number and percentage (%). Continuous variables are presented as median and interquartile range (IQR). Pairwise comparisons were performed using Pearson's chi-squared test for categorical variables and Kruskal-Wallis test or Mann-Whitney U test for continuous variables. For the primary end point, time to clinical improvement between the groups before 30 days was right censored at day 30. We used the Kaplan Meier analysis to assess the time to clinical improvement and the log rank test to compare the endpoint among groups. Mixed ordinal logistic regression modeling was used to measure the effect size (odds ratio [OR] and 95% confidence interval [CI]) of CCP on the WHO ordinal scale, stratified by disease severity.

A Cox proportional hazards model was used to assess the overall 30-day clinical improvement. Hazard ratios (HRs) with 95% CIs were reported in the Cox model. For the

Journal Pre-proof

secondary endpoint, we calculated the OR and 95% CI using logistic regression analysis. Variables included in the multivariate Cox proportional hazard and logistic regression models were age, baseline SpO2 < 88%, lymphocyte count <1 x10^9, and CRP.

We also examined whether an interaction occurred between CCP and adjunct therapy (i.e., steroids and/or tocilizumab) that affected the outcomes. We tracked laboratory results, including lymphocyte and neutrophil counts, CRP, D-dimer, and LDH, in addition to oxygen saturation over time. For the analysis of repeated measures, we used a linear fixed model (regression coefficient β with 95% CI) to analyze the effect of CCP on laboratory markers and oxygen saturation at baseline and during the first 14 days of hospitalization.

Missing data were not handled in a specific manner. Statistical analyses were carried out using Stata 14 software (StataCorp, College Station, Texas). Two-sided p-values <0·05 were considered significant.

Results

A total of 368 patients were included in the cohort; 135 received CCP and 233 were in the control group (Figure 1). Demographic and baseline characteristics are shown in Table 1. The median age was 54 (range 15-82) years, and the majority of patients were male (n=304, 82·6%). We found no differences among groups in the baseline characteristics or admission laboratory values, with the exception of baseline CRP, which was significantly higher in the CCP-treated patients with severe disease than other groups.

Primary outcomes

Overall 30-day clinical improvement was observed in 77 (86·5%) of the CCP-treated patients with moderate disease and 106 (68·4%) of corresponding controls (p=0·001). Among those with severe disease, clinical improvement was observed in 28 (60·8%) of the CCP-treated patients and 27 (34·6%) of their control counterparts (p=0·001). The median time to clinical improvement among those with moderate disease was 7 (IQR 4-9) days with CCP treatment and 8 (IQR 6-12) days in the control group (p=0·006), and among those with severe disease

Journal Pre-proof

was 7 (IQR 5-12) days with CCP treatment and 15.5 (IQR 10-20) days in the control group (p=0·003, Figure 2). The distribution of patients in the WHO ordinal scale categories over the 30 days of hospitalization are shown in eFigure1 and eFigure 2 in Supplement 2. For both moderate and severe disease, CCP treatment was associated with a significantly lower score on the WHO ordinal scale compared to the control group on days 7, 14, and 30. On day 7, the score reduction was significant in both the moderate (OR, 0·25 [95% CI 0·13-0·47]) and severe disease groups (OR, 0·33 [95% CI 0·14-0·82]). We also noted a score reduction in both moderate and severe disease on day 14 (OR, 0·17 [95% CI 0·08-0·34] and OR, 0·28 [95% CI 0·11-0·73], respectively) and day 30 (OR, 0·17 [95% CI 0·07-0·36] and OR, 0·11 [95% CI 0·04-0·31], respectively).

In the adjusted Cox model, the overall CCP group had a significantly higher rate of clinical improvement than the control group (HR, 1·9 [95% CI 1·4-2·7]). The effect was seen among both the moderate (HR, 1·9 [95% CI 1·3-2·8]) and severe disease (HR, 2·5 [95% CI 1·2-5·2]) groups (Table 2). There was no significant interaction effects between CCP and steroids (p=0·40), tocilizumab (p=0·12), or steroids plus tocilizumab (p=0·44) on the clinical improvement.

Secondary outcome

Effect of CCP on Mortality

In univariate analysis, the CCP-treated patients in both severity groups had lower mortality rates than the control groups (n=10, 11·4% and n=14, 30·4% versus n=46, 29·7% and n=44, 57·1%; p=0·001 and p=0·006, respectively). In the adjusted model, the risk of mortality was significantly lower in the CCP-treated patients with moderate disease (adjusted OR, 0·27 [95% CI 0·12–0·62]) compared to the control group, but not significantly different in the CCP-treated patients with severe disease (adjusted OR, 0·38 [95% CI 0·14–1·02]) compared to the control group. no significant interaction effects between CCP and steroids (p=0·36 for interaction), CCP and tocilizumab (p=0·56 for interaction), or CCP and steroids plus tocilizumab (p=0·75 for interaction) on mortality.

Journal Pre-proof

Effect of CCP on oxygen saturation and laboratory value kinetics

The effects of CCP on patients' oxygen saturation and laboratory markers over the first 14 days of the hospital stay are shown in Table 3. Relative to baseline, CCP treatment improved oxygen saturation by 5·4% [95% CI 3·3–7·4] on day 1 and 4·1% [95% CI 2·3-5·9] on day 3 in patients with moderate disease, but not among those with severe disease (eFigure 3 in Supplement). Lymphocyte counts increased significantly on days 7 (β=0·46 [95% CI 0·15-0·78]) and 14 (β=0·59 [95% CI 0·11-1·1]) post-hospital admission in the moderate CCP group. Similarly, lymphocyte counts increased on days 11 (β=0·76 [95% CI 0·03–1·5]) and 14 (β=0·98 [95% CI 0·04–1·9]) in the severe disease CCP group (eFigure 4 in Supplement). Total neutrophil counts were not significantly different among the groups at baseline or any other time point (eFigure 5 in Supplement). Relative to baseline, CRP significantly decreased in both the moderate and severe groups at every time point after CCP treatment (p=0·001; eFigure 6 in Supplement). D-dimer was not significantly different between the groups at baseline or in the first 11 days, but the levels were lower 14 days post-admission after CCP in the moderate disease group (eFigure 7 in Supplement). Furthermore, LDH was not significantly different among moderate and severe disease groups at baseline or in the first 14 days, but was lower at day 14 in the overall CCP group (eFigure 8 in Supplement).

Discussion

Currently, no vaccine or specific antiviral treatments are approved for COVID-19 patients. CP is a widely available non-pharmaceutical treatment that has been used with varying degrees of success in prior pandemics, including SARS, MERS, and Ebola.[15–18] In our study, we investigated the effect of CCP on time to clinical improvement, 30-day mortality, changes in oxygen saturation and laboratory values within 14 days of hospital admission in both moderate and severe COVID-19 patients. Many prior studies focused on severe and critically ill patients whereas we included severe cases as well as moderate cases where the risk benefit balance is more delicate. Improved clinical symptoms and mortality among

Journal Pre-proof

patients with severe and critical COVID-19 after CCP transfusion has been previously reported.[8,11] In a randomized clinical trial of CCP in severe and life-threatening COVID-19, Li et al. could not detect a significant reduction in time to clinical improvement within 28 days after CCP treatment.[7] However, the study was terminated early after enrollment of 52 patients who received CCP and 51 in the control group and may have been underpowered to detect significant differences. In contrast, our study included 135 patients in the CCP arm and 233 controls. Another report, concluded that CCP treatment did not reduce mortality and suggested that earlier adminstration of CCP might be beneficial.[12] The fact that patients in our study received CCP within 24 hours of hospital admission may have contributed to improving outcomes. In our study, CCP administration was associated with both a higher rate of and faster time to clinical improvement among patients with moderate and severe disease. The clinical improvement was observed in 80·6% of the CCP group compared to 58·6% of the control group. In addition, median time to clinical improvement in the CCP group was 7 days compared to 10 days in the control group. This is consistent with a prior case series of 10 patients, in which symptoms resolved within the first 72 hours with complete improvement on radiological examination within 7 days post-CCP administration.[19] In the H1N1 pandemic, CP was associated with a significant reduction in mortality, especially if administered early.[20] An exploratory post hoc meta-analysis revealed a significant reduction in the pooled odds of mortality following CP treatment of SARS coronavirus infection and severe influenza compared to placebo or no therapy.[21] Li et al. did not detect a significant reduction in 28-day mortality among CCP-treated patients. [7] In our study, 30-day mortality was significantly reduced in moderate, but not severe, COVID-19 cases. This may imply that CCP is more effective in reducing 30-day mortality in moderate disease, or may be attributed to inclusion of a relatively small number of severe cases. Our results are also consistent with those of a single arm study of 46 patients with moderate to severe disease where 7-day mortality was 6.5% in patients who received CCP compared to the 15% average national statistics and 30% in a concurrent cohort of 23 patients.[22]

Journal Pre-proof

CCP in our study was also associated with significantly improved oxygen saturation and recovery in lymphocyte counts and CRP within 14 days post-CCP treatment. These findings are consistent with recent reports of the effect of CCP.[8,11,23]

Prior studies have associated lower lymphocyte counts and high CRP levels with adverse outcomes in COVID-19 patients.[1,19] In our study, lymphocyte counts started to increase 1 day after CCP transfusion, with a significant increase 7 days post-admission in the CCP group compared to the control group. Furthermore, CRP was remarkably decreased starting day 1 in the CCP group. The improvement of both lymphocyte counts and CRP in our study may be attributed in part to a previously postulated immunomodulatory effect of CCP such as providing passive immunity by blocking inflammatory cytokines, autoantibodies, and complement pathways.[24] CCP may also inhibit inflammatory cascades and immune cell infiltration within the lungs.[25] These effects can clinically be seen as improvement in oxygen saturation and pulmonary radiological lesions.[19]

In our study, oxygen saturation significantly increased in CCP-treated patients during the first 72 hours. This finding is consistent with earlier studies reporting associations between CCP and improved oxygen saturation.[8,23]

All CCP recipients tolerated the transfusion except for 3 (2%) who had minor allergic skin reactions, which resolved completely without preventing administration of the full CCP dose. A recent study of early safety indicators for CCP reported 36 cases (0·72%) with serious adverse effects (SAEs).[26] The incidence of severe allergic reaction in that study was 0·06%, and the incidence of less than severe allergic reactions was not reported.

Our study has a number of limitations, including a lack of randomization. However, randomization was not feasible during the pandemic in our region. The administration of CCP within 24 hours of hospital admission was intended to limit selection bias. Another limitation is that at the time of analysis some patients were still hospitalized which may have biased the results. The clinical management of a potentially life-threatening illness with an unpredictable clinical course was the main contributor to this limitation. In addition, there was not an approved standard protocol for the treatment during our study, especially regarding

Journal Pre-proof

steroid and tocilizumab use. However, this stems from the lack of universally established treatments for COVID-19. We did not observe an interaction between the administration of either/both of the drugs and the observed effect of CCP in our patients. Finally, we were not able to determine the titer of SARS-CoV-2 IgG antibodies in CCP donors or whether the detected antibodies were neutralizing antibodies due to a lack of these tests in Kuwait at the time of the study. We did exclude any donors who were negative for IgG antibodies. Not performing additional antibody characterizing tests could only lead to underestimation of the benefit of this therapeutic modality due to potential inclusion of CCP units with low IgG levels or antibodies of limited neutralizing capacity. In addition, in many parts of the world, including some developed countries, there is a striking shortage of testing for COVID-19 in general, and particularly for more specialized tests, such as neutralizing antibodies.

To the best of our knowledge, this study is the first to investigate the effectiveness and feasibility of CCP in COVID-19 patients in the Middle East. Although we did not perform an a priori sample size calculation, 135 patients in the CCP group demonstrated significant differences. The involvement of multiple centers supports the generalizability of the results. Throughout the worldwide lockdown, the supply of CCP was consistently available in Kuwait, which offers free access to this therapeutic intervention. This contrasts pharmaceuticals subject to supply chain limitations. Availability is crucial in the Middle East, which relies on pharmaceutical manufacturing that is currently concentrated in Western countries.

Conclusion

In our prospective interventional study including patients with moderate and severe COVID-19, CCP administration was significantly associated with improved clinical outcomes. Thirty-day survival was significantly improved in the moderate group. In addition, administration of CCP in both moderate and severe cases was also associated with improved oxygen saturation and recovery of lymphocytes and CRP. Larger multicenter controlled randomized trials to further evaluate the effectiveness of CCP in COVID-19 patients with particular emphasis on CCP donor qualification based on neutralizing antibody levels are warranted.

Journal Pre-proof

Contributors

SA conceptualized and planned the study design, planned data collection,oversaw data collection with MA,literature review, performed the text mining analysis, drafted and revised the final version of the manuscript. MA also performed the statistical analysis. MZA and RA conducted literature review, contributed to the writing and reviewing of the manuscript. All other co-authors contributed to data collection and oversaw the manuscript.

Funding: Kuwait Ministry Of Health.

Trial registration: Clinicaltrials.gov NCT04474340.

Declaration of interests

We declare no competing interests.

Acknowledgments

This work was supported by Ministry Of Health in Kuwait. We thank Jeethu Anu Geo and Jibi Roshan for the technical support and reviewing the data, Kuwait University. We also thank Dr Mohammed Buhamra, for the technical support from the COVID-19 dispatch team,Ministry Of Health, Kuwait.

Journal Pre-proof

Journal Pre-proof

References

1. Brown BL, McCullough J. Treatment for emerging viruses: Convalescent plasma and COVID-19. *Transfus Apher Sci* 2020; **59**(3): 102790.

2. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**(10223): 497-506.

3. Zhu N, Zhang D, Wang W, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N Engl J Med* 2020; **382**(8): 727-33.

4. Garraud O, Heshmati F, Pozzetto B, et al. Plasma therapy against infectious pathogens, as of yesterday, today and tomorrow. *Transfus Clin Biol* 2016; **23**(1): 39-44.

5. Shakir EM, Cheung DS, Grayson MH. Mechanisms of immunotherapy: a historical perspective. *Ann Allergy Asthma Immunol* 2010; **105**(5): 340-7; quiz 8, 68.

6. Joyner MJ, Wright RS, Fairweather D, et al. Early safety indicators of COVID-19 convalescent plasma in 5,000 patients. *J Clin Invest* 2020.

7. Li L, Zhang W, Hu Y, et al. Effect of Convalescent Plasma Therapy on Time to Clinical Improvement in Patients With Severe and Life-threatening COVID-19: A Randomized Clinical Trial. *JAMA* 2020.

8. Shen C, Wang Z, Zhao F, et al. Treatment of 5 Critically Ill Patients With COVID-19 With Convalescent Plasma. *JAMA* 2020.

9. Integrated Management of Adolescent and Adult Illness (IMAI) District Clinician Manual: Hospital Care for Adolescents and Adults. GUIDELINES FOR THE MANAGEMENT OF COMMON ILLNESSES WITH LIMITED RESOURCES. Geneva, Switzerland: World Health Organization; 2011.

10. Investigational COVID-19 Convalescent Plasma Guidance for Industry. Food and Drug Administration Center for Biologics Evaluation and Research 2020.

11. Xia X, Li K, Wu L, et al. Improved clinical symptoms and mortality among patients with severe or critical COVID-19 after convalescent plasma transfusion. *Blood* 2020; **136**(6): 755-9.

Journal Pre-proof

12. Zeng QL, Yu ZJ, Gou JJ, et al. Effect of Convalescent Plasma Therapy on Viral Shedding and Survival in Patients With Coronavirus Disease 2019. *J Infect Dis* 2020; **222**(1): 38-43.

13. R&D Blueprint and COVID-19. World Health Organization; 2020.

14. Donor Eligibility (Screening and Testing). American Association of Blood Banks (AABB); 2020.

15. Soo YO, Cheng Y, Wong R, et al. Retrospective comparison of convalescent plasma with continuing high-dose methylprednisolone treatment in SARS patients. *Clin Microbiol Infect* 2004; **10**(7): 676-8.

16. Arabi Y, Balkhy H, Hajeer AH, et al. Feasibility, safety, clinical, and laboratory effects of convalescent plasma therapy for patients with Middle East respiratory syndrome coronavirus infection: a study protocol. *Springerplus* 2015; **4**: 709.

17. Ko JH, Seok H, Cho SY, et al. Challenges of convalescent plasma infusion therapy in Middle East respiratory coronavirus infection: a single centre experience. *Antivir Ther* 2018; **23**(7): 617-22.

18. van Griensven J, Edwards T, de Lamballerie X, et al. Evaluation of Convalescent Plasma for Ebola Virus Disease in Guinea. *N Engl J Med* 2016; **374**(1): 33-42.

19. Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. *Intensive Care Medicine* 2020; **46**(5): 846-8.

20. Hung IF, To KK, Lee CK, et al. Convalescent plasma treatment reduced mortality in patients with severe pandemic influenza A (H1N1) 2009 virus infection. *Clin Infect Dis* 2011; **52**(4): 447-56.

21. Mair-Jenkins J, Saavedra-Campos M, Baillie JK, et al. The effectiveness of convalescent plasma and hyperimmune immunoglobulin for the treatment of severe acute respiratory infections of viral etiology: a systematic review and exploratory meta-analysis. *J Infect Dis* 2015; **211**(1): 80-90.

Journal Pre-proof

22. Perotti C, Baldanti F, Bruno R, et al. Mortality reduction in 46 severe Covid-19 patients treated with hyperimmune plasma. A proof of concept single arm multicenter trial. *Haematologica* 2020.

23. Duan K, Liu B, Li C, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc Natl Acad Sci U S A* 2020; **117**(17): 9490-6.

24. Lunemann JD, Nimmerjahn F, Dalakas MC. Intravenous immunoglobulin in neurology--mode of action and clinical efficacy. *Nat Rev Neurol* 2015; **11**(2): 80-9.

25. Gralinski LE, Sheahan TP, Morrison TE, et al. Complement Activation Contributes to Severe Acute Respiratory Syndrome Coronavirus Pathogenesis. *mBio* 2018; **9**(5).

26. Eckhardt CM, Cummings MJ, Rajagopalan KN, et al. Evaluating the efficacy and safety of human anti-SARS-CoV-2 convalescent plasma in severely ill adults with COVID-19: A structured summary of a study protocol for a randomized controlled trial. *Trials* 2020; **21**(1): 499.

Table 1. Demographic and baseline characteristics by disease severity.

Characteristic	Control			CCP			P-value
	All N= 233	Moderate N= 155	Severe N= 78	All N= 135	Moderate N= 89	Severe N= 46	
Age (years)	54 [45 - 62]	52 [43 - 61]	57 [51 - 65]	54 [48 - 60]	54 [49 - 60]	53·5 [45 - 58]	**0·74**
Male	198 (85%)	136 (87·7 %)	62 (79·5%)	105 (77·8 %)	63 (70·8%)	42 (91·3%)	**0·27**
Diabetes	94 (45·4%)	58 (43·3 %)	36 (49·3%)	57 (42·5 %)	43 (48·3%)	14 (31·1%)	**0·21**
Hypertension	80 (39%)	44 (33·3 %)	36 (49·3%)	60 (45%)	43 (48·8%)	17 (37·8%)	**0·15**
Obesity (BMI>40)	20 (8·9%)	14 (9·5%)	6 (8%)	16 (12%)	8 (9·1%)	8 (17·8%)	**0·21**
Laboratory Findings							
Lymphocytes x10^9	0·93 [0·7 - 1·4]	1 [0·72 - 1·4]	0·85 [0·6 - 1·3]	1·06 [0·8 - 1·38]	1·1 [0·9 - 1·4]	1 [0·8 - 1·3]	**0·59**
Lymphocytes <1 x10^9	101 (47%)	68 (43·9 %)	46 (59%)	73 (57%)	35 (39·3%)	20 (43·5%)	**0·63**

Journal Pre-proof

Neutrophils x10^9	5·3 [4 - 7·6]	5·2 [4 - 7·8]	5·7 [4 - 10]	5·2 [4 - 9]	5·4 [4 - 7·7]	5·8 [4 - 7·5]	**0·68**
Platelets	226 [170 - 292]	226 [168 - 305]	224 [186 - 264]	219 [175 - 283]	309 [175 - 284]	229 [171 - 273]	**0·69**
CRP (mg/L)	81·4 [34·1 - 136]	82·4 [38 - 141]	78 [25 - 121]	96 [73·5 - 154]	94 [73 - 148]	98 [82·4 - 179]	**0·04**
LDH (IU/L)	429 [333 - 598]	410 [331 - 567]	480 [347 - 710]	391 [325 - 605]	360 [289 - 467]	514 [387 - 686]	**0·84**
D-dimer (ng/mL)	459 [289 - 800]	431 [288 - 796]	507 [292 - 820]	384 [296 - 588]	345 [235 - 562]	477 [348 - 919]	**0·96**
Ferritin (ng/mL)	668 [362 - 1889]	665 [360 - 1243]	678 [401 - 1050]	638 [334 - 1130]	624 [332 - 940]	824 [412 - 1178]	**0·63**
PT (seconds)	14·1 [13 - 16]	13·9 [12·6 - 15·3]	15 [13·5 - 17]	15·1 [14 - 16·8]	15 [13·8 - 16·7]	15·7 [13·7 - 17]	**0·36**
APTT (seconds)	36·4 [32 - 40·8]	36·4 [32 - 40·6]	37 [32 - 42·4]	35·6 [32·6 - 38·5]	35·5 [33 - 38·4]	36 [32 - 39]	**0·46**
Clinical Characteristics							
Baseline Spo2 (%)	91 [88 - 94]	92 [90 - 95]	90 [85 - 92]	91 [89 - 94]	92 [90 - 94]	90 [87 - 92]	**0·06**
WHO ordinal scale on admission							
	47 (20·2%)	46 (29·1 %)	1 (1·3%)	13 (9·6%)	12 (13·5%)	1 (2·2%)	
	123 (52·7%)	94 (60·6 %)	29 (23·2%)	72 (53·3 %)	60 (67·4%)	12 (26·1%)	
	59 (25·3%)	15 (9·7%)	0	45 (33·3 %)	17 (19·2%)	28 (60·8%)	
	4 (1·7%)	0	4 (5·1%)	5 (3·7%)	0	5 (10·9%)	
Treatment received							
Steroid	113 (48·5%)	76 (49%)	37 (47·4%)	77 (57%)	46 (51·7%)	31 (67·4%)	**0·14**
Tocilizumab	153 (66·7%)	100 (64·5 %)	53 (67·9%)	65 (67·7 %)	43 (63·2%)	22 (78·5%)	**0·45**

Data are given as median [IQR] or n (%). Abbreviations: IQR, interquartile; BMI, body mass index (as calculated in kilograms divided by height in meters squared); LDH, lactate dehydrogenase; CRP, C-reactive protein; PT, prothrombin time; APTT, activated partial prothrombin time.
SI conversion factors: to convert lactate dehydrogenase to microkatal per liter, multiply by 0·167; to convert D-dimer to nanomoles per liter, divide by 5·476; to convert CRP to nanomoles per liter, multiply by 9·524.
Moderate COVID-19: defined as the presence of clinical signs of pneumonia (fever, cough, dyspnea) and oxygen saturation (SpO2) of more than 90% in room air.
Severe COVID-19: defined as clinical signs of pneumonia plus SpO2 less than 90% in room air or admission to intensive care unit (ICU) for respiratory support (i.e., high flow nasal cannula, non-invasive mechanical ventilation and intubation).
WHO 7-category ordinal scale that ranges from 1 (discharged with normal activity) to 7 (death). Scale 2=Not hospitalized, but unable to resume normal activities; scale 3=hospitalized, not requiring supplemental oxygen; scale

Journal Pre-proof

4= hospitalized, requiring supplemental oxygen, scale 5= hospitalized, requiring high-flow nasal oxygen (HFNC) and/or non-invasive mechanical ventilation (IMV); scale 6= hospitalized, requiring ECMO and/or IMV; scale 7= death.

Table 2. Primary and secondary outcomes

Primary outcome	**Overall control**	**Overall CCP**	**P-value**	**Adjusted HR* (95% CI); p-value**
Clinical improvement	133 (58·6%)	100 (80·6%)	<0·001	1·9 (1·4-2·7) ;<0·001
Days to clinical improvements, median (IQR)	10 (6-15)	7 (5-9)	<0·001	
Primary outcome	**Moderate control N (%)**	**Moderate CCP N (%)**	**P-value**	**Adjusted HR* (95% CI); p-value**
Clinical improvement	106 (68·4%)	77 (86·5%)	0·001	1·9 (1·3-2·8); 0·001
Days to clinical improvements, median (IQR)	8 (6-12)	7 (4-9)	0·006	
Primary outcome	**Severe control N (%)**	**Severe CCP N (%)**	**P-value**	**Adjusted HR* (95% CI); p-value**
Clinical improvement	27 (34·6%)	28 (60·8%)	0·006	2·5 (1·2-5·2); 0·012
Days to clinical improvements, median (IQR)	15·5 (10-20)	7 (5-12)	0·003	
Secondary outcome	**Overall control**	**Overall CCP**	**P-value**	**Adjusted OR* (95% CI); *P*p-value**
Death	90 (38·8%)	24 (17·8%)	<0·001	0·32 (0·18- 0·58); 0·001

Journal Pre-proof

Secondary outcome	Moderate control N (%)	Moderate CCP N (%)	P-value	Adjusted OR* (95% CI); p-value
Death	46 (29·7%)	10 (11·4%)	0·001	0·27 (0·12- 0·62); 0·002
Secondary outcome	**Severe control**	**Severe CCP**	**P-value**	**Adjusted OR* (95% CI); p-value**
Death	44 (57·1%)	14 (30·4%)	0·006	0·38 (0·14-1·02); 0·06

*Adjusted for age, baseline oxygen saturation < 88%, lymphocytes <1x10^9, and C-reactive protein

Table 3. Changes in oxygen saturation and laboratory markers

Marker	Day 1	Day 3	Day 7	Day 11	Day 14
All patients	4·4 (2·2 to 6·6)**	3·6 (1·8 to 5·3)**	2·1 (-0·22 to 4·5)	-0·2 (-4·1 to 3·6)	0·98 (-1·3 to 3·3)
Moderate patients	5·4 (3·3 to 7·4) **	4·1 (2·3 to 5·9)**	1·9 (-0·55 to 4·4)	0·27 (-3·9 to 4·5)	1·2 (-1·1 to 3·4)
Severe patients	5·3 (-0·8 to 11·4)	2·8 (-1·1 to 6·7)	0·50 (-4·4 to 5·4)	-1·02 (-7·8 to 5·7)	0·92 (-5·9 to 7·7)
All patients	0·18 (-0·27 to 0·63)	042 (-0·26 to 1·04)	0·55 (0·22 to 0·88)**	0·68 (0·21 to 1·1)*	0·92 (0·41 to 1·4) **
Moderate disease	0·06 (-0·37 to 0·48)	0·21 (-0·23 to 0·66)	0·46 (0·15 to 0·78)*	0·52 (-0·11 to 1·2)	0·59 (0·11 to 1·1) *
Severe disease	0·49 (-0·59 to 1·5)	0·85 (-0·58 to 2·2)	0·57 (-0·05 to 1·2)	0·76 (0·03 to 1·5)*	0·98 (0·04 to 1·9) *
All patients	4·3 (-8·6 to 17·4)	1·6 (-0·81 to 3·9)	1·9 (-0·19 to 4·1)	-1·8 (-6·04 to 2·3)	-3·8 (-7·9 to 0·17)
Moderate disease	0	0·85 (-1·87 to 3·5)	2·2 (-0·5 to 4·8)	- 7·9 (-13·7 to 2·1)	-3·8 (-8·04 to 0·39)

Journal Pre-proof

Severe disease	8·5 (-5·04 to 22·1)	3·6 (-0·34 to 6·8)	1·7 (-1·7 to 5·1)	4·5 (-1·4 to 10·4)	-1·8 (-9·2 to 5·6)
All patients	-128·8 (-160·9 to -96·7) **	-125 (-161·4 to -88·7) **	-86·6 (-113·5 to -59·7) **	-115 (-162 to -68·1) **	-90·7 (-134·6 to -46·8) **
Moderate disease	-112·2 (-147·8 to -76·6) **	-143·9 (-188·5 to -99·4) **	-76·1 (-109·7 to -42·5) **	-99·3 (-141·4 to -57·2) **	-57·7 (-87·7 to -27·7) **
Severe disease	-176·5 [-234 to -119] **	-91·3 [-173 to -9·5] *	-104·2 [-163·2 to -45·3] **	-171 [-265·8 to -76·3] **	-99·8 [-190·4 to -9·3] *
All patients	NA	109 (-557 to 775·4);0·749	-253·1 (-938·5 to 432·4);0·469	-327·3 (-2578·2 to 1923·6);0·776	-718·3 (-1580·5 to 143·9);0·103
Moderate disease	NA	240·3 (-572·5 to 1053)	-348·7 (-1441·1 to 743)	0	-535·7 (-981·7 to -89·7)*
Severe disease	NA	297 (-1805·6 to 2399·5)	-541·9 (-1064·4 to 980·5)	1777·5 (-1625 to 5180)	-1162·1 (-3565·7 to 1241·5)
All patients	NA	-77·2 (-361·4 to 207·1)	61·8 (-109·5 to 233·1)	NA	-393·4(-637·4 to -149·5)*
Moderate disease	NA	-89·2 (-789·4 to 610·9)	7·04 (-212·9 to 226·9)	NA	-272·7 (-641·6 to 96·2)
Severe disease	NA	122·4 (-870·2 to 1115)	-78·4 (-568·2 to 411·4)	NA	-272·2 (-989·2 to 444·9)

All values are compared to laboratory values at baseline (before CCP or at admission in control group), CCP compared to control NA=not available
Values are expressed as β coefficient (95% CI) *p-value<0·05 **p-value<0·001

Journal Pre-proof

Effectiveness of Convalescent Plasma in Indian Patients with COVID-19

Sandeep Budhiraja[1,6], Arun Dewan[2], Ritesh Aggarwal[2], Omender Singh[2], Deven Juneja[2], Sangeeta Pathak[3], Y P Singh[2], Ajay Gupta[2], Reeta Rai[3], Abhaya Indrayan[4], Vinitaa Jha[1], Rahul Naithani[5]

[1]Clinical Directorate, Max Healthcare, New Delhi; [2]Critical Care, Max Healthcare, New Delhi; [3]Transfusion Medicine, Max Healthcare, New Delhi; [4]Division of Bio Statistics, Max Healthcare, New Delhi; [5]Hematology & Bone Marrow Transplant Division, Max Healthcare, New Delhi; [6]Institute of Internal Medicine, Max Healthcare, New Delhi

Corresponding author:

Dr Sandeep Budhiraja, Clinical Directorate, Max Healthcare, 1,2 Press Enclave Marg, Saket, New Delhi 110 017

Email: sbudhiraja@maxhealthcare.com

Abstract

Background: Convalescent plasma (CP) is being used as a treatment option in hospitalized patients with COVID-19. Till date, there is conflicting evidence on efficacy of CP in reducing COVID-19 related mortality.

Objective: to evaluate the effect of CP on 28-day mortality reduction in patients with COVID-19.

Methods: We did a multi-center, retrospective case control observational study from 1st May 2020 to 31st August 2020. A total of 1079 adult patients with moderate and severe COVID-19 requiring oxygen, were reviewed. Of these, 694 patients were admitted to ICU. Out of these, 333 were given CP along with best supportive care and remaining 361 received best supportive care only.

Results: In the overall group of 1079 patients, mortality in plasma vs no plasma group was statistically not significant (22.4% vs 18.5%; p = 0.125). However, in patients with COVID-19 admitted to ICU, mortality was significantly lower in plasma group (25.5% vs 33.2%; p = 0.026). This benefit of reduced mortality was most seen in age group 60 to 74 years (26.7% vs 43.0%; p = 0.004), driven mostly by females of this age group (23.1% vs 53.5%; p = 0.013). Significant difference in mortality was observed in patients with one comorbidity (22.3% vs 36.5%; p = 0.004). Moreover, patients on ventilator had significantly lower mortality in the plasma arm (37.2% vs 49.3%; p = 0.009); particularly so for patients on invasive mechanical ventilation (63.9% vs 82.9%; p = 0.014).

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Conclusion: The use of CP reduced mortality in COVID-19 elderly patients admitted in ICU, above 60 years of age, particularly females, those with comorbidities and especially those who required some form of ventilation.

Key words: Convalescent plasma therapy, COVID-19, ICU cases, Mortality, Subgroup analysis

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-COV-2), the novel coronavirus causing COVID-19, originated from Wuhan, China and has spread rapidly across the globe[1]. As no definitive treatment options are currently available for COVID-19, researchers the world over have been investigating a variety of drugs like azithromycin, hydroxychloroquine, remdesivir, Tociluzimab and dexamethasone[2-6]. Some of these are repurposed drugs and have been approved by regulators of various countries to be used as "Emergency Use Approval" (EUA) or "off label" medication[7].

Convalescent plasma (CP) has been used as a passive source of antibodies against various bacterial (tetanus and diphtheria) and viral diseases (poliomyelitis, measles, mumps)[8] and influenza A H1N1[9]. CP was also considered in earlier pandemics of Spanish flu, West Nile Virus, Middle East Respiratory Syndrome (MERS), Severe Acute Respiratory Syndrome (SARS), and more recently Ebola virus[10,11,12]. The convalescent plasma therapy (CPT) for COVID-19 has also been recently approved by US FDA and Indian Central Drugs Standard Control Organization[13]. It has been approved by the Ministry of Health and Family Welfare (MoHFW), Govt. of India, as "off label" use in patients with moderate and severe COVID-19 who are not improving and have increasing oxygen requirement despite use of steroids[7].

Evidence suggests that CP contains receptor binding domain specific antibodies which have potent antiviral activity[14-15]. Use of convalescent plasma, is known to be a well-tolerated with only a few easily managed adverse effects[16].

There have been, till date, few larger randomized controlled trials (RCT)[17,18], some retrospective observational studies[16,19,20] and many small case reports[21,22,23] on the benefits of CPT in COVID-19 patients with conflicting results. There is still no clarity whether CPT offers mortality benefit and, if yes, in which category of COVID-19 patients. The present study was designed to answer some of these questions.

Patients and Methods

Study Design

This is a multicentre, retrospective, observational case control study. COVID-19 patients admitted at various tertiary care teaching hospitals in Delhi of the same network, with electronic heath records (EHR), were included. Clinical and laboratory data were retrieved from EHR. Patients admitted from 01 May 2020 to 31 Aug 2020, with moderate and severe and / or

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

life threatening COVID-19, who needed oxygen therapy, were included. Of a total of 1249 such patients admitted during this period, 170 patients were excluded due to unavailability of sufficient clinical data. Remaining 1079 patients were included in the final analysis. All patients in either arm received various treatments as per physician discretion, institutional protocol or national guidelines for the management of COVID-19,issued by the government from time to time. A variety of medicines were used in these patients and included hydroxychloroquine (HCQ), azithromycin, doxycycline, ivermectin, remdesivir, other broad spectrum antibiotics, steroids (methylprednisolone or dexamethasone), tocilizumab and oxygen support/ventilation as required. Figure 1 shows the CONSORT diagram. All were followed for 28 days after discharge by tele-calling. The detailed analysis was performed on 694 patients admitted to ICU – 333 of these received plasma in addition to best supportive care and the other 361 were on best supportive care only.

Majority of COVID-19 patients who were given CP came from one major hospital complex, which contributed to 239 out of 333 (71.8%) patients. The physicians in this hospital had started using CP from very early days of COVID-19 outbreak in India and, because of this, significant use was observed in this hospital complex. Other hospitals, where either the usage of CP or availability of CP, was low, contributed 28.2% of plasma cases and a greater number of no plasma cases. Otherwise, the standard of care in all hospitals, across the network remained uniform.

Inclusion Criteria

Eligible patients included adults (> 18 years) with evidence of SARS-COV-2 infection by RT-PCR test of nasopharyngeal or oropharyngeal swab. Criteria for classifying patients into moderate and severe category were as per the clinical management protocols of MoHFW[7].

Moderate disease: pneumonia with no signs of severe disease, and having one or more:

- Dyspnoea, fever and cough
- SPO2 < 94% on room air
- Respiratory rate > 24 / minute

Severe disease: patient having one or more of the following conditions:

- Clinical signs of pneumonia plus one of the following: respiratory rate > 30/min or severe respiratory distress requiring ventilation or SPO2 < 90% on room air.
- ARDS (new onset bilateral opacities & SpO2 / FiO2 ≤ 315)
- Sepsis
- Septic shock

Based on these criteria, we selected only patients who needed oxygen therapy.

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Donor Selection Criteria[24]

Donors had to meet the following criteria: 1) prior diagnosis of COVID-19 documented by a laboratory test, 2.a) complete resolution of symptoms at least 28 days prior to donation or 2.b) complete resolution of symptoms at least 14 days prior to donation. In such cases, if the patient has been discharged without negative RT-PCR for COVID-19, 2 samples of naso- and oropharyngeal swab have to be sent for RT-PCR for COVID-19, collected 24 hours apart and negative result ascertained before considering the recovered patients as a potential donor. 3) only males and nulliparous female donors of weight > 55 kg, 4) Donor with Hb>12.5g/dL and platelet count > 150 000 per microliter of blood and TLC within normal limits, 5) HIV, HBV and HCV by serology and NAT negative patients, 6) syphilis and malaria negative donors and 7) total serum protein > 6 g/dL all donor selection criteria for blood donation were followed as per Drugs and Cosmetics (second Amendment) Rules, 2020[25] .

In the absence of a standard viral neutralization test, and limited possibility of assessment of neutralizing antibody titres of > 1:640, we adopted a policy of the use of an assay to assess quantitative antibodies of anti-SARS COV2 antibodies prior to harvest of CP from the eligible donors. Antibody testing was done prospectively in donors from mid June onwards, prior to which donor samples were stored at -80° C and were retrospectively tested. For the 36 donors who's assays were done retrospectively, 12% were found have inadequate antibodies. In our larger centre, the available platform which we used was an FDA approved (Diaosorin), Quantitative IgG antibody assay against the S1&S2 protein of SARS COVID-2 virus. The cut-off of 15 AU/ml was taken for a positive result which is equivalent to 1:40 Plaque Neutralization antibody & 80 AU/ml is equivalent to 1:160 plaque neutralizing antibody assay. In the other two smaller centres, Ortho Clinical Diagnostic Vitros Anti SARS-COV-2 IgG Chemiluminescent Immuno Assay was used. The Ortho Clinical IgG is a qualitative assay. Result of this assay are based on the sample signal-to-cut-off (S/CO) ratio, with values <1.0 and ≥ 1.0 corresponding the Negative and Positive results.

Volume of donated plasmas was 400 ml. The first 200 ml (one unit) plasma was infused over one to one and half hours and if the patient did not show any improvement after 24 h, based on the decision of responsible physician, another unit of plasma was administered.

Exclusion Criteria

Patients not requiring oxygen therapy were excluded. Pregnant and lactating mothers were also excluded.

Objectives

Primary objective of the study was to study 28-day mortality in patients who received convalescent plasma therapy versus those who did not.

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Secondary objectives were to assess if CP reduced the chances of patients going on to invasive ventilation and whether it offered any mortality benefits in critically ill patients on ventilator and vasopressors.

Statistical Analysis

Baseline characteristics such as age, gender, and comorbidities in the plasma and no plasma group were compared by chi-square test. The number of patients on invasive and non-invasive ventilators and mortality in plasma and no plasma group in various subgroup of patients were also compared with chi-square test. P-value < 0.05 was considered statistically significant. SPSS21 was used for statistical calculations. The sample size in the two groups was adequate to detect a difference of at least 10% in overall mortality between plasma and no plasma group with power of 80% when the mortality in ICU cases in the standard care group is 25%.

Results

The mean (SD) age of patients was 60.1 $\pm$ 12.1 years vs 58.9 $\pm$ 13.8 years in plasma vs no plasma group (p = 0.231). However, the age-gender distribution and the comorbidity-gender distribution was different for the following subgroups (Table I).

Males were significantly higher in the plasma group (80.2% vs 72.3%) (p = 0.015) and less of younger age (12.3% vs 19.2%) (p = 0.023). There were more females in the plasma group with multiple comorbidities, than in the no plasma group. Specific comorbidities examined were hypertension (HTN), diabetes mellitus (DM), coronary artery disease (CAD) and hypothyroidism. These were the most commonly occurring comorbidities in these cases. All other comorbidities (CKD, cancer, stroke, etc.) were reported in less than 10% cases. Hypertension was more common in plasma group (54.7% vs 43.5%, p = 0.003) and CAD less common (7.5% vs 14.1%, p = 0.005). However, the difference in mortality in these subgroups was not statistically significant.

The percentage of patients on ventilator, invasive and non-invasive, and on vasopressor were not significantly different (minimum p = 0.166) between the two groups (Table 2).

For the total of 1079 patients on oxygen, overall mortality in patients was 22.4% in the plasma group and 18.5% in the no plasma group (p = 0.125). Total 694 (64.3%) patients required transfer to intensive care unit (ICU). Three hundred thirty-three (48.0%) patients received convalescent plasma in addition to the best supportive care and the remaining 361 (52.0%) were on best supportive care only. In the ICU patients, mortality was 25.5% vs 33.2% in plasma vs no plasma group (p = 0.026). Reduced mortality in plasma group was particularly seen in patients of age 60–74 years (26.7% vs 43.0%, p = 0.004), driven mostly by females of this age group (23.1% vs 53.5%, p = 0.013).

In one of the main hospitals, where the experience with the use of CP was maximum, out of a total of 342 patients in ICU, 239 (69.8%) received plasma while 103 (30.1%) did not. Overall

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

mortality in the plasma group was 49/239 (20.5%) as compared to 36/103 (36.9%) in the no plasma group (p = 0.0014).

Significant difference in mortality was observed in patients with one comorbidity (22.3% vs 36.5%, p = 0.004). This also was primarily due to females in this subgroup (mortality 6.7% vs 49.1%, p < 0.001) in the plasma vs no plasma group (Table 3). We observed a similar reduction in mortality in patients with 2 comorbidities (35.1% vs 49.2%) but this did not reach statistical significance (p=0.099). The mortality in patients with specific comorbidity such as hypertension, diabetes and coronary artery disease were not different (least p = 0.099) but was significantly different in patients with hypothyroidism (19.0% vs 42.4%, p = 0.027). Of 38 women of age 60–74 years with one comorbidity, 12 were in plasma group and 26 in the no plasma group. Only one of these (8.3%) died in the plasma group and 13 (50.0%) in the no plasma group (p = 0.013).

Out of a total of 694 patients with severe COVID-19 in ICU, 101 (14.6%) patients worsened on non-invasive ventilation (NIV) and needed to be shifted to invasive ventilator. Fifty-four (53.46%) of these patients were given CP. There were 316 (45.5%) patients, who remained on NIV only and out of these 157 (49.6%) received CP. Overall, CP did not reduce the chances of being put on invasive ventilator from NIV (p = 0.508).

Patients on ventilator had significantly lower mortality in plasma arm (37.2% vs 49.3%, p=0.009) (Table 4). This was particularly so for patients on invasive ventilator (IV) with mortality 63.9% vs 82.9% (p = 0.014), respectively in plasma vs no plasma group. Patients on an invasive ventilator and on vasopressor had high (almost 90%) mortality in both the groups (Table 4).

Twenty-six out of 333 (7.8%) received second unit of CP, as per the physician's decision. Ten of these patients (38%) died.

Safety of CPT

Convalescent plasma infusion was largely safe and no major adverse effects were observed in any of our patients. Two patients with minor allergic reactions (rashes) were reported to the Blood Bank Officer.

Discussion

The current study was undertaken to evaluate the effectiveness of CP in patients with moderate and severe COVID-19 disease. To date, this is the largest retrospective case control study from India, presently reporting world's second largest number of COVID-19 cases.

There are a number of reports comprising small number of patients treated with CP[21,22,23] from different countries across the world. Duan et al. were first to report benefit of CP in a small cohort of 10 patients with improvement in all 10 patients and undetectable viral load in 7/10 patients[21]. In contrast, Zeng et al. reported mortality in 5/6 patients who received CP[26]. Table 5

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

summarizes the literature review of relevant studies on CP, with more than 100 patients and / or RCTs.

The results of our study are quite different from that of the only other large-scale study from India - a multicentre prospective RCT PLACID trial, which included 464 patients of moderate severity COVID-19, from 39 different hospitals across India[17]. This study did not find any difference in mortality between plasma vs no plasma group (15% vs 14%), respectively. However, this study did not include very severe or life threatening COVID-19 patients. Even in our study, if we see the overall cohort of 1079 patients (moderate and severe), CP did not have any mortality benefit (22.4% vs 18.5%; p = 0.125). The benefit emerged only in more critically ill patients of COVID-19 who were in ICU (25.5% vs 33.2%; p = 0.026). This happened despite plasma group had more elderly males, where anyway the mortality is expected to be higher[27]. Also, the main hospital, which had maximum experience with the use of CP, showed a highly significant mortality benefit in severe COVID-19 (20.5% vs 36.9%; p = 0.0014).

A similar study involving moderately ill COVID-19 patients by Abolghasemi et. al, showed that significantly higher number of patients who received CP were discharged from the hospital versus control (98.2% vs 78.7%)[28]. They reported that CP significantly reduced need for mechanical ventilation in treatment versus control group (7% vs 20.3%). All-cause mortality in CP group was 14.8% versus 24.3% in control arm in the study but this was not statistically significant (p = 0.09). RCT by Ling Li et al. also showed no significant difference in time to clinical improvement or in 28-day mortality between patients who received CP vs control[18].

Study by Xia et al. also showed that there was less likelihood of patient who received CP to be transferred to ICU as compared to those who did not receive CP (2.4% vs 5.1%)[20]. Our study did not show any benefit with CP, in terms of reducing the chances of patients going onto invasive ventilation from NIV. The Indian PLACID trial also showed no benefit of CP in terms of total and post enrolment duration of respiratory support, and proportion of patients who had to be put on invasive ventilation and those needing vasopressor support[17]. In the study by Altuntas et al., the duration of stay in ICU, rate of mechanical ventilation and vasopressor requirement were lower in the CP group versus control (p = 0.001, p = 0.02, p = 0.001 respectively)[29]. They however, found no statistically significant difference in the fatality rate between the CP and control groups (24.7% vs 27.7%, p = 0.150). In our study, however patients on ventilator had significantly lower mortality in plasma arm as compared to no plasma arm (37.2% vs 49.3%, p = 0.009). This was particularly so for patients on invasive ventilator with mortality 63.9% and 82.9% (p = 0.014) in plasma and no plasma arm, respectively.

The patient outcome in the current study was quite similar to the study by Salazar et al. where they found significant mortality reduction in severe and life threatening COVID-19 patients (p = 0.047) especially when CP was given within 72 hours of admission[30].

In our study, we found maximum benefit of CP in ICU patients, in the age group 60-74 years, where the mortality in the plasma group was 26.7% versus 43% in the no plasma group (p=

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

0.004). Similar findings were reported by Rogers et al. wherein a subgroup analysis of patients above 65-years-old or greater who received CP demonstrated a significantly increased hospital discharge rate among these patients (RR 1.86, 95% CI 1.03-3.36)[31].

Significant difference in mortality between plasma and no plasma group was also observed in patients in our study with one comorbidity largely driven by females. This finding has not been reported in literature so far. The mortality in patients with specific comorbidity such as HTN, DM and CAD was not different (minimum p = 0.099) but was significantly different in patients with hypothyroidism (19% vs 42.4%, p = 0.027). This needs further investigation.

Klassen et al. aggregated patient outcome data from various RCTs, matched-control, case series and case report studies and found that CP exhibited a 51% reduction in mortality rate compared to patients receiving standard treatments (19% vs. 29% mortality; OR:0.49, 95% CI: 0.37, 0.64, p< 0.001)[32].

Our study showed mortality benefit of CP in severely ill COVID-19 patients requiring some form of ventilation and in the age group >60 years, especially women with 1-2 medical comorbidities. We do not know the exact mechanism of action of CP in COVID-19 but it seems that plasma therapy played a significant role in reduction of mortality in the highly vulnerable patient population of elderly and on ventilator. Perhaps a little push by convalescent plasma in this segment of patients was able to tilt the balance towards recovery.

Limitations of the study

It is a retrospective study. During the study duration, patients received several treatments such as HCQs, remdesevir, ivermectin, azithromycin, steroids (dexamethasone or methylprednisolone), tociluzimab depending on prevailing guidelines at those time points and at treating physician's discretion. Majority of patients in plasma arm were recruited at one centre while no plasma arm patients were at other hubs. We also do not have information if some of the patients had already developed adequate antibodies when CP was performed. These limitations notwithstanding, we are able to identify a subset of patients with COVID-19 who are likely to benefit from CP.

Conclusions

The use of convalescent plasma reduced mortality in severe COVID-19 elderly patients, above 60 years of age, particularly females, those with comorbidities and especially those who require some form of ventilation. This beneficial effect was lost when the entire cohort of patients across varying severity of illness was compared. Plasma did not seem to offer any mortality benefit in patients of moderate severity or those who were terminally ill. Further research into the mechanism of actions of CP in COVID-19 may help predict the good responders.

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Authors Contributions

SB provided overall guidance, was involved in patient care and management , data analysis and interpretation and in writing the manuscript; AD, RA, OS, DJ, YPS, AG were involved in clinical care including ICU management; SP and RR were the transfusion medicine experts and contributed in selection of plasma donor and in apheresis and maintaining records of donations and adverse events; AI was the chief bio statistician and helped in data analysis and interpretation and drafting the manuscript; VJ provided data collation oversight and drafting manuscript; RN was involved in data interpretation and writing the manuscript. All authors have seen and approved the final draft.

Acknowledgements

We wish to thank Dr. Vibha Jain, Ms. Shruti Singh, Dr. Menka Loomba, Ms. Taruna Sharma, Ms. Ratnam Shukla, Ms. Aditi Saini. Mr. Rahul Arya, Mr. Rajat Kumar Sharma, Mr. Pushpender Sharma and Ms. Kritika Chadha, for their help towards data collection and validation.

References

1. World Health Organization. 2020. WHO Director-General's Remarks at the Media Briefing on 2019-nCoV on 11 February 2020.https://www.who.int/dg/speeches/detail/who-director-general-s-remarks-at-the-media-briefing-on-2019-ncov-on-11-february-2020

2. Beigel J, Tomashek K, Dodd L et al. Remdesivir for the treatment of Covid-19 — Final Report. N Eng J Med. 2020 Oct 8; NEJMoa2007764.doi:10.1056NEJMoa2007764

3. Gautret P, Lagier JC, Parola P, et al. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. Int J Antimicrob Agents. 2020 Jul; 56(1):105949. doi: 10.1016/j.ijantimicag.2020.105949

4. Bhimraj A, Morgan RL, Shumaker AH, et al. Infectious Diseases Society of America Guidelines on the Treatment and Management of Patients with COVID-19. Clin Infect Dis. 2020 Apr:ciaa478.doi: 10.1093/cid/ciaa478

5. Villar J, Ferrando C, Domingo M, et al. Dexamethasone treatment for the acute respiratory distress syndrome: a multicentre, randomised controlled trial. Lancet Resp Med. 2020 Mar 1; 8(3):267-276.

6. Guaraldi G, Meschiari M, Cozzi-Lepri A, et al. Tocilizumab in patients with severe COVID-19: a retrospective cohort study. Lancet Rheumatol. 2020 Aug;2 (8):e474-e484

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

7. Ministry of Health & family Welfare. Clinical Management Protocol: COVID-19 version 5 [Internet]. Mohfw.gov.in. 2020 [cited 23 October 2020]. Available from: https://www.mohfw.gov.in/pdf/UpdatedClinicalManagementProtocolforCOVID19dated03072020.pdf.

8. Yongzhi Xi. Convalescent plasma therapy for COVID – 19: a tried-and-true old strategy? Signal Transduct Target Ther 2020; 5(1):203.

9. Hung IF, To KK, Lee CK, et al. Convalescent plasma treatment reduced mortality in patients with severe pandemic influenza A (H1N1) 2009 virus infection. Clin Infect Dis. 2011 Feb 15; 52(4):447-456.

10. Van Griensven, Johan et al. Evaluation of convalescent plasma for Ebola virus disease in Guinea. N Engl J Med 2016; 374(1)): 33-42.

11. Luke TC, Kilbane EM, Jackson JL, Hoffman SL. Meta-analysis: convalescent blood products for Spanish influenza pneumonia: a future H5N1 treatment? Ann Intern Med. 2006 Oct 17; 145(8):599-609.

12. Marson P, Cozza A, De Silvestro G. The true historical origin of convalescent plasma therapy. Transfus Apher Sci. 2020; 102847. doi: 10.1016/j.transci.2020.102847

13. Tanne JH. Covid-19: FDA approves use of convalescent plasma to treat critically ill patients. BMJ 2020; 368:m1256. doi: 10.1136/bmj.m1256

14. Zeng QL, Yu ZJ, Gou JJ, et al. Effect of convalescent plasma therapy on viral shedding and survival in COVID-19 patients. J Infect Dis. 2020; 222(1):38 -43.

15. Chen L, Xiong J, Bao L, Shi Y. Convalescent plasma as a potential therapy for COVID-19. Lancet Infect Dis, 2020;20(4):398- 400.

16. Joyner MJ, Bruno KA, Klassen SA, et al. Safety Update: COVID-19 Convalescent plasma in 20,000 hospitalized patients. Mayo Clin Proc. 2020;95(9):1888-1897.

17. Agarwal A, Mukherjee A, Kumar G, Chatterjee P, Bhatnagar T, Malhotra P, on behalf of PLACID Trial Collaborators. Convalescent plasma in the management of moderate Covid-19 in adults in India: open label phase II multicentre randomised controlled trial (PLACID Trial). BMJ 2020;371: m3939.http://dx.doi.org/10.1136/bmj.m3939

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

18. Li L, Zhang W, Hu Y, et al. Effect of convalescent plasma therapy on time to clinical improvement in patients with severe and life-threatening COVID-19. A Randomized Clinical Trial. JAMA 2020; 324(5): 460-470.

19. Cantore I, Valente P. Convalescent plasma from COVID 19 patients enhances intensive care unit survival rate. A preliminary report. Transfus Apher Sci. 2020:102848. doi: 10.1016/j.transci.2020.102848

20. Xia X, Li K, WU L et al. Improved clinical symptoms and mortality among patients with severe or critical COVID-19 after convalescent plasma transfusion. Blood 2020; 136(6):755–759.

21. Duan K, Liu B, Li C, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. Proc Natl Acad Sci U S A 2020; 117(17):9490-9496.

22. Shen C, Wang Z, Zhao F, et al. Treatment of 5 critically ill patients with COVID-19 with convalescent plasma. JAMA 2020; 323(16):1582-1589.

23. Hartman WR, Hess AS, Connor JP. Hospitalized COVID-19 patients treated with convalescent plasma in a mid-size city in the Midwest. Transl Med Commun 2020;5(1): 17. doi: 10.1186/s41231-020-00068-9

24. ICMR PLACID trial. A Phase II, Open Label, Randomized Controlled Trial to Assess the Safety and Efficacy of Convalescent Plasma to Limit COVID-19 Associated Complications in Moderate Disease; Convalescent Plasma in COVID-19 Version 1.5 Dated 11th May, 2020 (Cited 2020 July 23) Available from: https://cdsco.gov.in/opencms/resources/UploadCDSCOWeb/2018/UploadPublic_NoticesFiles/ICMR%20Convalescent%20plasma%20_protocol_v1.5.pdf

25. Final G.S.R. 166(E)_Amendment in Part X B & Part XII B pertains to Blood center and Blood components, Drugs and Cosmetics Act 1940 and Rules 1945, amended 11th March 2020 (accessed 2020 March 18, Cited 2020 July 23) available from : https://cdsco.gov.in/opencms/opencms/en/Notifications/Gazette-Notifications

26. Zeng F, Chen X, Deng G. Convalescent plasma for patients with COVID-19 (Letter). Proc Natl Acad Scie U S A 2020; 117(23): 12528

27. Jin JM, Bai P, He W, et al. Gender Differences in Patients With COVID-19: Focus on Severity and Mortality. Front Public Health 2020;8:152

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

28. Abolghasemi H, Eshghi P, Cheraghali AM, et al. Clinical efficacy of convalescent plasma for treatment of COVID-19 infections: Results of a multicenter clinical study. Transfus Apher Sci., 2020 Jul 15;102875. https://doi.org/10.1016/j.transci.2020.102875

29. Altuntas F, Ata N, Yigenoglu TN, et al. Convalescent plasma therapy in patients with COVID-19. Transfus Apher Sci., https://doi.org/10.1016/j.transci.2020.102955

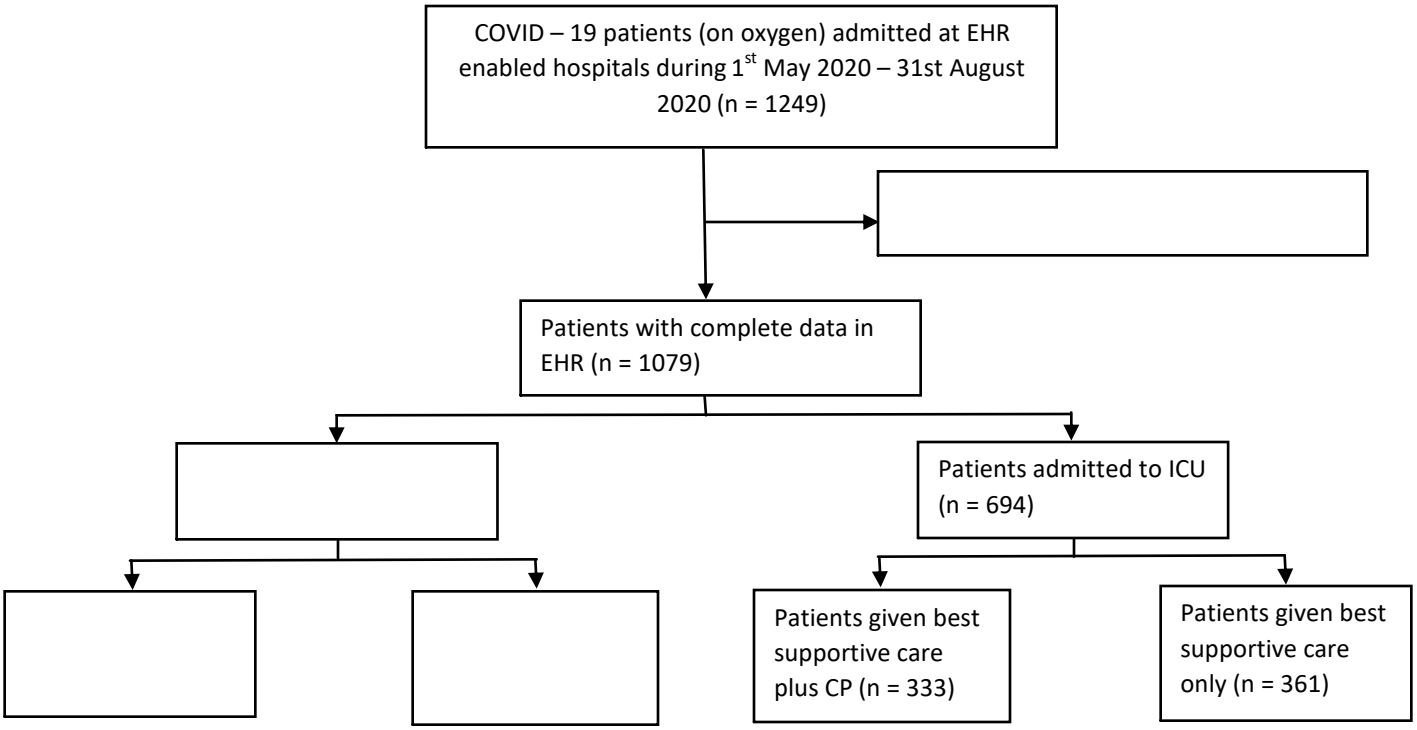
30. Salazar E, Christensen PA, Graviss EA, et al. Treatment of coronavirus disease 2019 patients with convalescent plasma reveals a signal of significantly decreased mortality. Am J Pathol. 2020;190(11):2290-2303.

31. Rogers R, Shehadeh F, Eng M, et al. Convalescent plasma for patients with severe COVID-19: a matched cohort study. https://doi.org/10.1101/2020.08.18.20177402

32. Klassen SA, Senefeld JW, Johnson PW, et al. Evidence favouring the efficacy of convalescent plasma for COVID-19 therapy. https://doi.org/10.1101/2020.07.29.20162917

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Figure 1: CONSORT Chart for the study



This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Table 1. Baseline comparison of cases in the Best Supportive Care Only Group and Plasma with Best Supportive Care Group

Characteristic	Best supportive Care Only		Plasma with Best Supportive Care		p-value
	Count	Percent	Count	Percent	
All in ICU	361	52.6%	333	84.7%	<0.001
<45	62	17.2%	40	12.0%	0.055
45-59	115	31.9%	122	36.6%	0.185
60-74	149	41.3%	135	40.5%	0.844
75+	35	9.7%	36	10.8%	0.628
Males	261	72.3%	267	80.2%	0.015
<45	50	19.2%	32	12.3%	0.023
45-59	80	30.7%	96	36.8%	0.196
60-74	106	40.6%	109	41.8%	0.961
75+	25	9.6%	30	11.5%	0.533
Females	100	27.7%	66	19.8%	Same as for males
<45	12	12.0%	8	12.1%	0.981
45-59	35	35.0%	26	39.4%	0.566
60-74	43	43.0%	26	39.4%	0.645
75+	10	10.0%	6	9.1%	0.846
No: of Comorbidities	361	52.6%	333	84.7%	<0.001
None	92	25.5%	72	21.6%	0.231
1	181	50.1%	166	49.8%	0.939
2	61	16.9%	74	22.2%	0.077
3+	27	7.5%	21	6.3%	0.543
Males	261	72.3%	267	80.2%	0.015
None	72	27.6%	63	23.6%	0.293
1	128	49.0%	136	50.9%	0.663
2	45	17.2%	53	19.9%	0.441
3+	16	6.1%	15	5.6%	0.802
Females	100	27.7%	66	19.8%	Same as for males
None	20	20.0%	9	13.6%	0.291
1	53	53.0%	30	45.5%	0.341
2	16	16.0%	21	31.8%	0.017
3+	11	11.0%	6	9.1%	0.691
Specific Comorbidity M+F					
HTN	157	43.5%	182	54.7%	0.003
DM	66	18.3%	61	18.3%	0.990
CAD	51	14.1%	25	7.5%	0.005
Hypothyroidism	33	9.1%	42	12.6%	0.141

HTN: Hypertension; DM: Diabetes mellitus; CAD: Coronary artery disease

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Table 2. Need for ventilation and / or vasopressors in Best Supportive Care Only Group and Plasma with Best Supportive Care Group

Characteristic	Best Supportive Care Only (*n* =361)		Plasma with Best Supportive Care (*n* = 333)		p-value
	Count	Percent	Count	Percent	
No Ventilator	132	36.6%	115	34.5%	0.577
Any Ventilator	229	63.4%	218	65.5%	
Non-Invasive (NIV)	159	69.4%	157	72.0%	0.412
Invasive (IV)	70	30.6%	61	28.0%	0.548
Vasopressor in IV group	36	51.4%	24	39.3%	0.166
NIV to IV (out of IV group)	47	20.5%	54	24.8%	0.283
Vasopressor in NIV to IV group	23	48.9%	23	42.6%	0.523

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Table 3. Mortality in various groups in cases on Best Supportive Care Only and cases on Plasma with Best Supportive Care

Characteristic	Best Supportive Care Only (*n* = 361)		Plasma with Best Supportive Care (*n* = 333)		p-value
	Deaths*	Mortality	Deaths*	Mortality	
All in ICU	120	33.2%	85	25.5%	0.026
<45	14	22.6%	7	17.5%	0.536
45-59	27	23.5%	28	23.0%	0.923
60-74	64	43.0%	36	26.7%	0.004
75+	15	42.9%	14	38.9%	0.734
Male	73	28.0%	72	27.0%	0.796
<45	9	18.0%	6	18.8%	0.932
45-59	13	16.3%	24	25.0%	0.156
60-74	41	38.7%	30	27.5%	0.082
75+	10	40.0%	12	40.0%	1.000
Female	47	47.0%	13	19.7%	<0.001
<45	5	41.7%	1	12.5%	0.325
45-59	14	40.0%	4	15.4%	0.037
60-74	23	53.5%	6	23.1%	0.013
75+	5	50.0%	2	33.3%	0.633
No: of Comorbidities					
None	16	17.4%	14	19.4%	0.736
1	66	36.5%	37	22.3%	0.004
2	30	49.2%	26	35.1%	0.099
3+	8	29.6%	8	38.1%	0.537
Males	73	28.0%	72	27.0%	0.796
None	8	11.1%	12	19.0%	0.195
1	40	31.3%	35	25.7%	0.321
2	19	42.2%	20	37.7%	0.651
3+	6	37.5%	5	33.3%	0.809
Females	47	47.0%	13	19.7%	<0.001
None	8	40.0%	2	22.2%	0.351
1	26	49.1%	2	6.7%	<0.001
2	11	68.8%	6	28.6%	0.015
3+	2	18.2%	3	50.0%	0.169
Specific Comorbidity M+F					
HTN	59	37.6%	53	29.1%	0.099
DM	17	25.8%	18	29.5%	0.636
CAD	24	47.1%	11	44.0%	0.802
Hypothyroidism	14	42.4%	8	19.0%	0.027

*Deaths out of cases mentioned in Table 1 for the respective subgroups
HTN: Hypertension; DM: Diabetes mellitus; CAD: Coronary artery disease

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Table 4. Comparative Mortality of patients on ventilation & vasopressor between Best Supportive Care Only Group & Plasma with Best Supportive Care Group

Characteristic	Best Supportive Care Only (*n* = 361)		Plasma with Best Supportive Care (*n* = 333)		p-value
	Deaths*	Mortality	Deaths*	Mortality	
No Ventilator	7	5.3%	4	3.5%	0.488
Any Ventilator	113	49.3%	81	37.2%	0.009
Non-Invasive (NIV)	55	34.6%	42	26.8%	0.131
Invasive (IV)	58	82.9%	39	63.9%	0.014
Vasopressor with invasive ventilation (IV)	33	91.7%	21	87.5%	0.675

*Deaths out of cases mentioned in Table 2 in the respective rows

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Table 5. Review of various studies (larger RCTs and/or > 100 matched cases) on effectiveness of Convalescent Plasma in COVID-19

Ref	Number of patients	Study	Patient population	Antibody titre used	Results	Adverse Effects	Author Conclusions	Our comments
Ling Li et al.[18]	103	Open label RCT, multicentre	23 vs 22 Severe 29 vs 29 Life-threatening COVID-19	1:640	No significant difference in time to clinical improvement or in 28 days mortality between patients who received CP versus control	Chills and rashes -1 shortness of breath, cyanosis, and severe dyspnea -1	CP not effective	Not powered to predict the difference
Abolghasemi H et al.[28]	189 115 in plasma group and 74 in control group	Multicentre nonrandomized clinical study	Moderate severity	Antibody titer cut off > 1.1 using semi quantitative ELISA and Rapid strip test	CP reduced all cause mortality in treatment group compared with control group (14.8% vs 24.3%); however this was not statistically significant (p=0.09). it had a significant impact of length of stay (p=0.002) and need for intubation (p=0.006)		CP effective	Control group was much smaller and had milder cases
Salazar E et al.[30]	136 cases and 251 controls	Prospective propensity matched study	Severe and/or life threatening COVID-19	Anti-RBD IgG titre of 1:1350	Significant reduction (p= 0.047) in mortality within 28 days, specifically in patients transfused within 72 hours of admission with CP with anti-RBD IgG titre of > 1:1350		Transfusion of high anti-RBD IgG titre CP early in hospitalisation reduces mortality	
Xia X et al.[20]	138 cases and 1568 controls	Retrospective observational study	Severe or critical COVID-19		Mortality in CP group was 2.2% as compared to 4.1% in non CP group also from CP group, only 2.4% got shifted to ICU whereas in non CP group, 5.1% patients got ICU transfer	3 patients had minor allergic reactions (pruritus or erythema) during transfusion	Patients with SCSS > 5 before therapy showed no improvement after CP.	Authors classified patients who were given CP into responders, partial responders and nonresponders.
Altuntas F et al.[29]	Cases 888 Control 888	Retrospective case control study	Severe and critically ill COVID-19	IgG antibody not routinely done	Duration in ICU, rate of mechanical ventilation support and vasopressor support were lower in CP group (p = 0.001, p = 0.02, p = 0.001, respectively).	Not available		Antibody titres of plasma donors were not available

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

					CFR was 24.7 in CP group and 27.7 in control group (p = 0.15)			
Agarwal A et al & PLACID trial collaborators[17]	Cases: 235 Controls: 229	Open label parallel are phase II Multicentre randomized controlled trial	Moderately ill COVID- 19 patients	Nearly 2 / 3rd of donor had NAb titre > 1:20 with a median titre of 1:40	Mortality in intervention arm was 14.5% vs 13.5% in control arm (odd ratios was 1.06). There was no difference in the 2 arms with respect to duration of respiratory support, proportion of patient requiring invasive ventilation and vasopressor support	Minor adverse event of pain, chills, nausea in one patient; fever and tachycardia in 3 patients; dyspnoea and IV catheter blockage in 2 patients each. Mortality was assessed as possibly related to CP in 3 patients (1.3%).	CP not effective	Three reported cases of mortality related to CP raised a significant concern regarding safety profile of CP.
OUR STUDY	1079 (total cases in ward and ICU) In ICU : 694 Plasma group in ICU :333 No plasma group in ICU : 361	Retrospective case control observational study	Moderate and Severe COVID-19	Quantitative IgG antibody assay with cut off 15 AU/ml equivalent to 1:40 plaque neutralization antibody	Overall no statistically significant difference in mortality between CP and non CP group (22.4% vs 18.5%; p=0.125. However, significantly reduced mortality in COVID-19 patients admitted in ICU (25.5% vs 33.2%; p=0.026)especially in age group 60-74 (26.7% vs 43%; p=0.004), females (23.1% vs 53.5%; p=0.013), those with one comorbid medical condition(22.3% vs 36.5%; p=0.004) and on invasive mechanical ventilation (63.9%vs 82.9%; p =0.014)	2 patients had mild allergic reaction (rashes) during plasma transfusion	Highest mortality benefit with CP seen in COVID-19 patients above 60 years of age, admitted in ICU, especially females and those on ventilator and having medical comorbid conditions.	

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

-Author name in bold denotes the presenting author
-Asterisk * with author name denotes a Non-ASH member
denotes an abstract that is clinically relevant.

denotes that this is a recommended PHD Trainee Session.

denotes that this is a ticketed session.

103 A National Collaborative Multicenter Phase II Study for Potential Safety Efficacy of Convalescent Plasma to Treat Severe COVID-19 Patients

Program: Oral and Poster Abstracts
Type: Oral
Session: 401 Basic Science and Clinical Practice in Blood Transfusion: COVID-19 Convalescent Plasma, Antigen Typing and the Prothrombin Complex II
Hematology Disease Topics & Pathways:
Coronaviruses, Biological, Therapies, Clinically relevant, transfusion

Saturday, December 5, 2020: 10:30 AM

Hani Al Hashmi, MD[1], Deia Alawami, MD[1*], Syed Ziauddin A Zaidi, FRCP, FRCPath, MBBS[2], Nawal F Alshehry, MD[3*], Hazza A. Alzahrani, MD[4*], Abdul Rehman Z Zaidi, MBBS[5], Ahmed Al Sagheir, MD[6*], Osamah Khojah, MD[7], Ahmed Alaskar, MD, FRCPC[8], Noura AlMozain, MD[9*], Rehab Al-Ansari, MD[10*], Mona Alfaraj, MD[11*], Ghada ElGohary, MD, PhD, MBBS[12*], Afra Dayel, MD[1*], Ahmed Bahrani, MD[1*], Arwa Nabhan Abdulhamid, MD[13*], Hind Alhumaidan, MD[14*], Mohammed Albalawi, MD[15*], Alia AlFraedhi, MD[16*], Jawaher Al-Otaibi, MD[17*] and Rania Abdallah, MD[18*]

[1]King Fahad Specialist Hospital, Dammam, Saudi Arabia
[2]Department of Adult Hematology/BMT, Comprehensive Cancer Center, King Fahad Medical City, Riyadh, Saudi Arabia
[3]King Fahad Medical City, Department of Adult Hematology/BMT, Riyadh, Saudi Arabia
[4]Hematology/HSCT Section, King Faisal Cancer Center, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia
[5]Department of Medicine, King Fahad Medical City, Riyadh, Saudi Arabia
[6]Department of Hematology and Oncology, Johns Hopkins Aramco Healthcare, Dhahran, Saudi Arabia
[7]Pathology Department, Hematology Unit, King Saud University, Riaydh, Saudi Arabia
[8]King Abdullah International Medical Research Center, Riyadh, Saudi Arabia
[9]King Saud University Medical College, King Saud University, Riyadh, Saudi Arabia
[10]King Fahad Military Medical Complex, Dhahran, Saudi Arabia
[11]Hematology Laboratory Section of Medical Laboratory Department, Qatif Central Hospital, Qatif, Saudi Arabia
[12]Hematology Department, King Khaled University Hospital, Riyadh, Saudi Arabia
[13]Imam Abdulrahman Bin Faisal University, AlKhobar, Saudi Arabia
[14]Blood Bank & Transfusion Services, Department of Pathology and Laboratory Medicine, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia
[15]Internal medicine department, College of Medicine, Taibah University, Madinah, Saudi Arabia
[16]Department of Hematopathology and Blood Bank, King Saud University, Riyadh, Saudi Arabia
[17]Infectious diseases, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia
[18]Pulmonary and Critical Care Medicine, King Fahad Medical City, Riyadh, Saudi Arabia

Introduction

Coronavirus disease (COVID-19) pandemic has started to affect Saudi Arabia in the beginning of March 2020 and is expected to cause significant morbidity to many patients, especially to elderly, who might require intensive care unit (ICU) support to survive as its lethality increases with the increasing age. Recent publications suggested the benefit of utilizing convalescent plasma from recovered donors as a therapeutic approach in treating COVID-19 patients. Convalescent plasma could provide our first-line defense for people with COVID-19, especially those who are older and at a much higher risk for complications.. therefore, we developed a national protocol to investigate the safety, benefit and applicability at larger scale and at different health care

complications; therefore, we developed a national protocol to investigate the safety, benefit and applicability at larger scale and at different health care facilities in Saudi Arabia (KSA).

Objectives

Primary endpoints are:

1. ICU (or designated area for critical patients) length of stay
2. Safety of convalescent plasma

Secondary endpoints included:

1. 30 days mortality
2. Number of days on mechanical ventilation
3. Days to clinical recovery

Method

Eligible convalescent plasma donors will be invited to participate in trial. The arrangement for plasmapheresis will start after obtaining donor informed consent. The collected plasma will be treated with pathogen reduction system. The convalescent plasma units will be labelled, stored and shipped as per the standard transfusion medicine protocols. It will be used only for eligible patients' "recipients" as per the following eligibility criteria:

1- Inclusion criteria:

- Confirmed case of SARS-CoV-2 infection with POSITIVE rRT PCR test
- 18 or older
- Must have been requiring ICU care or severe or immediately life-threatening care (any one of the following):
    1. Patient requiring ICU care/admission.
    2. Severe disease is defined as:
        a. Dyspnea
        b. Respiratory frequency ≥ 30/min
        c. Blood oxygen saturation ≤ 93%
        d. Partial pressure of arterial oxygen to fraction of inspired oxygen ratio < 300, and/or Lung infiltrates > 50% within 24 to 48 hours
    3. Life-threatening disease is defined as:
        a. Respiratory failure
        b. Septic shock, and/or
        c. Multiple organ dysfunction or failure

2- Exclusion criteria:

- Negative or non-conclusive test COVID-19 rRT PCR test
- Mild symptoms
- Hospitalization not requiring ICU care/admission

Eligible Patients will be infused with the convalescent plasma (200-400 ml / treatment dose) at least once & if possible, daily, for up to 5 sessions. Other supportive and therapeutic measures should continue according to the locally approved protocols with due diligence.

Sample size was calculated with 80% power and 5% level of significance based on the recently published data to detect statistical difference in the study outcome. Therefore, we plan to recruit total of 575 patients.

- Convalescent plasma Recipient Group:
    - 115 patients (recipients) who have COVID 19 as per the inclusion criteria.
- Comparative control Group:
    - 460 Patients who are eitherCOVID 19 historical control or only consent for sharing their clinical and laboratory data

Matching for age, gender, Hypertension, Diabetes and intubation status were done based on the propensity score. Continuous variables will be presented as the median and interquartile range (IQR). Statistical software SPSS 24.0 will be used. Demographic, Clinical, imaging and laboratory information of all enrolled patients will be retrieved from the hospital electronic/paper records system to be used for the outcomes analysis.

Results

22 sites across KSA that participated in the study. Tertiary, secondary, academic and non-academic centers participated (real world data). There were no unusual safety issues related to convalescent plasma infusion since all mortalities in the plasma group were not related to plasma infusion which represent similar finding from other the published international reports. Keeping in-mind that our data is still maturing, 30 survival probability in the plasma group was 69% compared to 56% in the comparative group (p value = 0.066) (figure-1). This benefit to seem to be more noticeable in the COVID-19 cases who did not meet the criteria for life-threatening disease (figure-2).

Conclusion

Conclusion

Our study supports the safety of convalescent plasma in treating COVID-19 patients. Patients who are in the category of life-threating/end organs failure do not seem to benefit. There might be a benefit in the other subgroups.

Figure-1: Survival Probability between Convalescent Plasma to Treat Severe COVID-19 and matched comparative patients

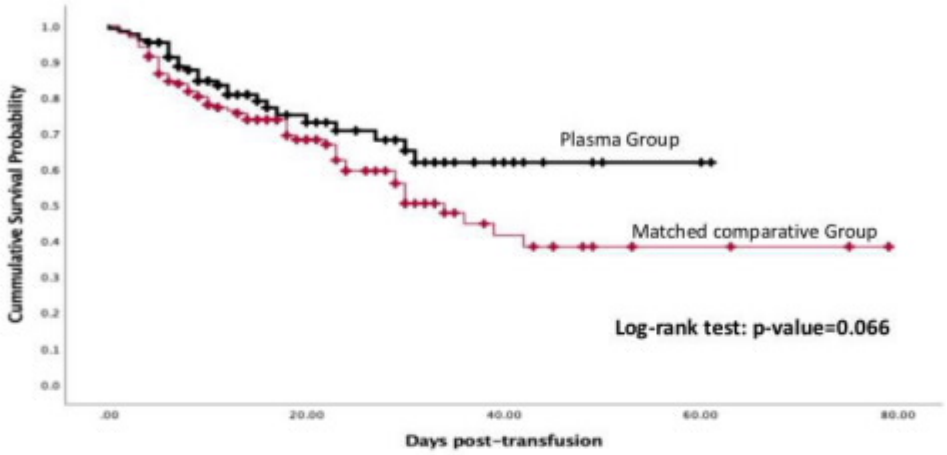
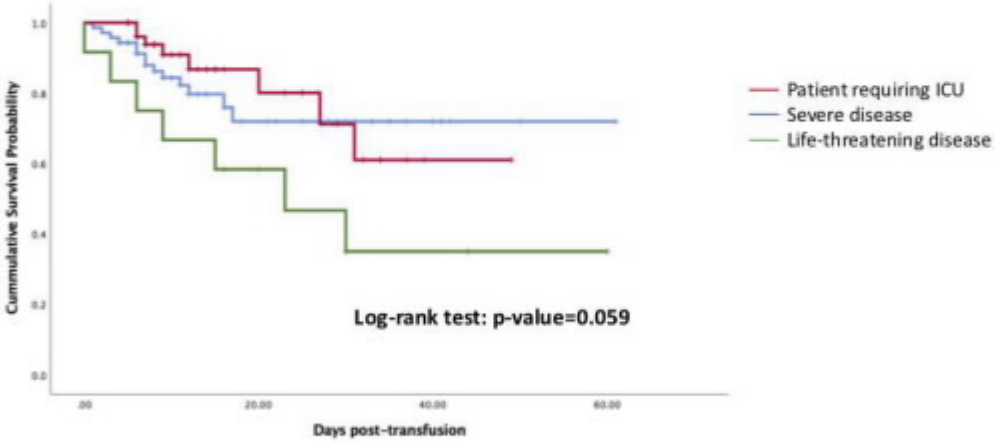


Figure-2: Survival Probability for Convalescent Plasma recipients patients among one of the three inclusion criteria (3 subgroups)



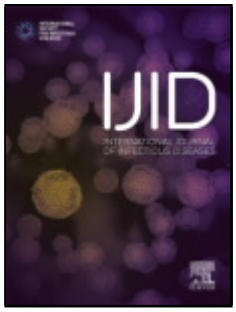
Disclosures: No relevant conflicts of interest to declare.

See more of: 401 Basic Science and Clinical Practice in Blood Transfusion: COVID-19 Convalescent Plasma, Antigen Typing and the Prothrombin Complex II
See more of: Oral and Poster Abstracts

<< Previous Abstract | Next Abstract >>

*signifies non-member of ASH

Journal Pre-proof



COVID-19 Convalescent Plasma Treatment of Moderate and Severe Cases of SARS-CoV-2 Infection: A Multicenter Interventional Study

Sondas Alsharidah, Mariam Ayed, Reem M. Ameen, Fatma Alhuraish, Najat A. Rouheldeen, Farah R. Alshammari, Alia Embaireeg, Mariam Almelahi, Maitham Adel, Mohammed E. Dawoud, Mohammad A. Aljasmi, Nashmi Alshammari, Abdullah Alsaeedi, Wasel Al-Adsani, Hussan Arian, Husain Awad, Humoud A. Alenezi, Azeez Alzafiri, Enas F. Gouda, Mohammad Almehanna, Salem Alqahtani, Abdulrahman Alshammari, Medhat Z. Askar

PII: S1201-9712(20)32513-3

DOI: https://doi.org/10.1016/j.ijid.2020.11.198

Reference: IJID 4908

To appear in: *International Journal of Infectious Diseases*

Received Date: 1 October 2020

Revised Date: 25 November 2020

Accepted Date: 26 November 2020

Please cite this article as: Alsharidah S, Ayed M, Ameen RM, Alhuraish F, Rouheldeen NA, Alshammari FR, Embaireeg A, Almelahi M, Adel M, Dawoud ME, Aljasmi MA, Alshammari N, Alsaeedi A, Al-Adsani W, Arian H, Awad H, Alenezi HA, Alzafiri A, Gouda EF, Almehanna M, Alqahtani S, Alshammari A, Askar MZ, COVID-19 Convalescent Plasma Treatment of Moderate and Severe Cases of SARS-CoV-2 Infection: A Multicenter Interventional Study, *International Journal of Infectious Diseases* (2020), doi: https://doi.org/10.1016/j.ijid.2020.11.198

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Published by Elsevier.

Journal Pre-proof

COVID-19 Convalescent Plasma Treatment of Moderate and Severe Cases of SARS-CoV-2 Infection: A Multicenter Interventional Study

Sondas Alsharidah,MD[1]; Mariam Ayed,MD[2];Reem M Ameen,PhD[3];Fatma Alhuraish,MD[4];Najat A Rouheldeen,MD[5];Farah R Alshammari,MD[6];Alia Embaireeg,MD[7]; Mariam Almelahi,MD[8]; Maitham Adel,MD[9]; Mohammed E Dawoud,MD[10];Mohammad A Aljasmi,MD[9]; Nashmi Alshammari,MD[11];Abdullah Alsaeedi,MD[6];Wasel Al-Adsani,MD[8]; Hussan Arian, MD[9];Husain Awad,MD[9]; Humoud A Alenezi,MD[12];Azeez Alzafiri,MD[6];Enas F Gouda,MD[13]; Mohammad Almehanna,MD[9];Salem Alqahtani,MD[14];Abdulrahman Alshammari,MD[6];Medhat Z Askar,MD.[15,16]

[1]Stem Cell Transplant Unit, Pediatric Hematology Oncology Department, National Bank Kuwait Specialized Children Hospital, Ministry of Health, Kuwait.

[2] Neonatology Department, Farwaniya Hospital, Ministry of Health, Kuwait.

[3] Medical Laboratory Sciences, Health Sciences Center, Kuwait University, Kuwait.

[4] Respiratory Unit, Department of Internal Medicine, Sabah Hospital, Ministry of Health, Kuwait.

[5] Hematology Department, Maternity Hospital, Ministry of Health, Kuwait.

[6] Department of Internal Medicine, Jahra Hospital, Ministry of Health, Kuwait.

[7] Department of Pediatrics, Amiri Hospital, Ministry of Health, Kuwait.

[8] Department of Internal Medicine, Mubarak Hospital, Ministry of Health, Kuwait.

[9]Jaber Stadium COVID-19 Quarantine Center, Ministry of Health, Kuwait.

[10]Department of Emergency Medicine, Amiri Hospital, Ministry of Health, Kuwait.

[11]Department of Intensive Care, Jaber Al-Ahmad Hospital, COVID-19 Center, Ministry of Health, Kuwait.

[12]COVID-19 Military Quarantine Center, Ministry of Defense, Kuwait.

[13]Hematology Department, Jahra Hospital, Ministry of Health, Kuwait.

Journal Pre-proof

[14]Department of Internal Medicine, Farwaniya Hospital, Ministry of Health, Kuwait.

[15] Baylor University Medical Center, Dallas, Texas, USA.

[16] Texas A&M Health Science Center College of Medicine, Bryan, Texas, USA.

Corresponding author:

Sondas I. Alsharidah,MBBCH, BOA, MRCPCH, Stem Cell Transplant Unit, Pediatric Hematology Oncology Department. National Bank of Kuwait Specialized Children Hospital. Ministry of Health, State of Kuwait.

salsharida@moh.gov.kw

Tell: (+965) 66691663

P.O: (5) 13001

State - Kuwait

7540 HA 0008997

Highlights

- Convalescent plasma is adjunct treatment in COVID-19 moderate and severe disease.
- Clinical improvement is noticed in COVID-19 disease after convalescent plasma.
- Mortality is significantly reduced after COVID-19 convalescent plasma treatment.

Abstract

Objective: To study the effectiveness of CCP therapy for moderate and severe COVID-19 disease patients in Kuwait.

Methods: This non-randomized prospective cohort study was conducted from May 21, 2020, to June 30, 2020, at four major tertiary hospitals in Kuwait. CCP was administered to 135 patients. The control group comprised 233 patients who received standard treatment. All patients (N=368, median age 54 [range 15-82] years) had laboratory-confirmed SARS-CoV-2 infection and either moderate or severe COVID-19 disease.

Journal Pre-proof

Results: CCP treatment was significantly associated with a higher rate of clinical improvement in patients with moderate or severe disease. Among those with moderate COVID-19 disease, time to clinical improvement was 7 days in the CCP group versus 8 days in the control group (p=0·006). For severe COVID-19 disease, time to clinical improvement was 7 days in the CCP group versus 15.5 days in the control group (p=0·003). In the adjusted analysis, the CCP-treated patients with moderate disease had significantly lower 30-day mortality. Compared to the control group, oxygen saturation improved within 3 days of CCP transfusion, and lymphocyte counts improved from day 7 in patients with moderate disease and day 11 in patients with severe disease. Furthermore, C-reactive protein declined throughout the first 14 days after CCP transfusion. None of the CCP patients developed a serious transfusion reaction.

Conclusions: The data show that administration of CCP is a safe treatment option for patients with COVID-19 disease, with a favorable outcome in the rate and time to clinical improvement.

Keywords: convalescent plasma, COVID-19, SARS-CoV-2, pneumonia, clinical improvement, mortality.

Introduction

The novel coronavirus disease of 2019 (COVID-19) pandemic is considered one of the greatest global public health crises since the 1918 influenza pandemic.[1] COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a single-stranded RNA virus that belongs to *coronaviridae*.[2,3] The clinical spectrum of infection is broad, ranging from asymptomatic to severe pneumonia, multiorgan failure, and death. Due to the absence of established antiviral treatment for COVID-19, the historical choice of therapeutic convalescent plasma (CP) was globally appealing, especially with its prior success against RNA viruses.[1] CP has been proposed not only to neutralize the pathogen, but provide passive immunomodulatory properties that allow the recipient to control the

Journal Pre-proof

exaggerated inflammatory cascade.[4,5] However, the mechanism of action of therapeutic CP remains unclear. Reports from open label trials and case series suggest that plasma collected from convalescent COVID-19 patients is safe to administer and may be effective in treating patients with COVID-19.[6–8] Therefore, the World Health Organization (WHO) and United States Food and Drug Administration (FDA) have issued guidelines for the usage of COVID-19 convalescent plasma (CCP) and standardized donor selection, which was further supported by an Emergency Use Authorization (EUA) from the FDA.[9,10]

A number of reports have demonstrated promising results of CCP in severe and critically ill COVID-19 patients, albeit not consistently.[8,11-12] The effectiveness of CCP in less than severe cases remains unclear. The aim of the current study was to assess the effectiveness of CCP in both moderate and severe COVID-19 cases compared to the standard treatment alone. The CCP effectiveness was evaluated in terms of time to clinical improvement and hospital mortality, the changes in oxygen saturation and laboratory markers (lymphocytes, neutrophils counts, lactate dehydrogenase [LDH], and C-reactive protein [CRP]).

Patients and Methods

Study design

This prospective multicenter interventional study was conducted in four major tertiary hospitals in Kuwait (Al-Sabah, Farwaniya, Mubarak Al-Kabeer, and Jahra) from May 21 to June 30, 2020, with the last follow-up data collected July 12, 2020. All patients and CCP donors had confirmed SARS-CoV-2 infection by an EUA-approved real-time reverse transcriptase-polymerase chain reaction (RT-PCR) using nasopharyngeal swabs (Cobas 6800 Systems, Roche, Switzerland and Taq Path, Thermo-Fisher Scientific, USA). The CCP donors were tested for SARS-CoV-2 IgG using a CE-marked rapid test (BIOZEK COVID-19 IgG/IgM qualitative test: Inzek International Trading, Apeldoom, the Netherlands).

The study protocol was approved by the Ethics and Research Committee of Kuwait Ministry of Health (#2020/1417). Written informed consent was obtained from all patients or next of

Journal Pre-proof

kin. If the patient was unconscious and next of kin not available, the healthcare proxy provided written informed consent.

Inclusion criteria

We included patients aged ≥18 years with confirmed laboratory diagnosis of SARS-CoV-2 infection and admission diagnosis of COVID-19. Patients <18 years old were enrolled after thorough evaluation and discussion among an interdisciplinary team. Patients were eligible for CCP if they had moderate or severe COVID-19 as determined according to the WHO classification at admission by the treating physician.[9] Patients with moderate disease exhibited clinical signs of pneumonia (fever, cough, dyspnea, fast breathing) and had SpO2 >90% in room air. Patients with severe disease exhibited clinical signs of pneumonia and at least one of the following: respiratory rate >30 breaths/minutes, SpO2<90% in room air, or admission to intensive care unit (ICU) for respiratory support (i.e., non-invasive mechanical ventilation and intubation).

Exclusion criteria

Patients were excluded if they had a contraindication to transfusion (volume overload, history of anaphylaxis to blood products), acute severe multiorgan failure, hemodynamic instability, severe disseminated intravascular coagulopathy (DIC), septic shock, or expected survival of less than 48 hours.

Control sampling procedure

After screening for the inclusion and exclusion criteria, we applied a systematic stratified sampling technique to identify control patients from the COVID-19 national registry. For

Journal Pre-proof

each patient who received CCP, we selected the first two patients with the same disease severity strata admitted on that calendar date from the same participating center.

Standard treatment

Standard treatment was based on our institutional protocol and international guidelines.[13] The majority of patients received antibiotics and low molecular weight heparin. None of our patients received antiviral therapy or hydroxychloroquine. Special therapy (i.e., steroids and/or tocilizumab) was prescribed at the discretion of treating physicians.

Data collection

Clinical and laboratory data were extracted from patient medical records using a standardized data collection sheet. We captured pre-existing medical conditions, including diabetes mellitus, hypertension, and obesity. Specific therapies, such as anticoagulants, antibiotics, steroids, and tocilizumab, was also collected. Complete blood counts (CBCs), CRP, D-dimer, and LDH were also collected on days 1, 3, 7, 11, and 14 of hospitalization. We followed the patients' progress daily from day 1 through day 30 or until discharge, whichever came first. Clinical improvement was defined as a two-grade decrease from hospitalization by assessing the clinical status based on the WHO ordinal scale.[9]

Selection of CCP donors

The CP was collected from individuals who had recovered from RT-PCR-confirmed COVID-19 disease and tested positive for IgG antibodies against SARS-CoV-2. All donors tested positive for SARS-CoV-2 by an EUA-approved RT-PCR test on a specimen collected by nasopharyngeal swab at the time of illness and a positive EUA-approved qualitative serological test for SARS-CoV-2 IgG antibodies after recovery. Donors who tested negative or were positive only for SARS-CoV-2 IgM were excluded. All donors were negative for HLA antibodies. Additional donor eligibility criteria and qualification were based on the American Association of Blood Banks (AABB) standards and the FDA guidance on CCP

Journal Pre-proof

administration, including questionnaires and testing for relevant transfusion-transmitted infections.[10,14] CCP was only collected from individuals who met all donor eligibility requirements according to the United States Code of Federal Regulations (21 CFR 630.10 and 21 CFR 630.15). Pathogen inactivation was also performed for each unit.

CCP administration

A total of 135 patients with moderate to severe COVID-19 were enrolled in this cohort (89 patients with moderate disease and 46 patients with severe disease). All patients were pre-medicated (paracetamol, antihistamine, steroids) according to an institutional protocol. Overall, 107 (79·3%) patients received 2 units of ABO-compatible CCP (each unit containing 200 ml of CCP) 12 hours apart and 28 (20·7%) received 1 unit of CCP according to the treating physician and protocol dosage range (200-400 mL). All patients received CCP within 24 hours of admission. Three patients (2%) had allergic skin reactions that were completely resolved after transfusion.

Statistical analysis

Categorical variables are presented as number and percentage (%). Continuous variables are presented as median and interquartile range (IQR). Pairwise comparisons were performed using Pearson's chi-squared test for categorical variables and Kruskal-Wallis test or Mann-Whitney U test for continuous variables. For the primary end point, time to clinical improvement between the groups before 30 days was right censored at day 30. We used the Kaplan Meier analysis to assess the time to clinical improvement and the log rank test to compare the endpoint among groups. Mixed ordinal logistic regression modeling was used to measure the effect size (odds ratio [OR] and 95% confidence interval [CI]) of CCP on the WHO ordinal scale, stratified by disease severity.

A Cox proportional hazards model was used to assess the overall 30-day clinical improvement. Hazard ratios (HRs) with 95% CIs were reported in the Cox model. For the

Journal Pre-proof

secondary endpoint, we calculated the OR and 95% CI using logistic regression analysis. Variables included in the multivariate Cox proportional hazard and logistic regression models were age, baseline SpO2 < 88%, lymphocyte count <1 x10^9, and CRP.

We also examined whether an interaction occurred between CCP and adjunct therapy (i.e., steroids and/or tocilizumab) that affected the outcomes. We tracked laboratory results, including lymphocyte and neutrophil counts, CRP, D-dimer, and LDH, in addition to oxygen saturation over time. For the analysis of repeated measures, we used a linear fixed model (regression coefficient β with 95% CI) to analyze the effect of CCP on laboratory markers and oxygen saturation at baseline and during the first 14 days of hospitalization.

Missing data were not handled in a specific manner. Statistical analyses were carried out using Stata 14 software (StataCorp, College Station, Texas). Two-sided p-values <0·05 were considered significant.

Results

A total of 368 patients were included in the cohort; 135 received CCP and 233 were in the control group (Figure 1). Demographic and baseline characteristics are shown in Table 1. The median age was 54 (range 15-82) years, and the majority of patients were male (n=304, 82·6%). We found no differences among groups in the baseline characteristics or admission laboratory values, with the exception of baseline CRP, which was significantly higher in the CCP-treated patients with severe disease than other groups.

Primary outcomes

Overall 30-day clinical improvement was observed in 77 (86·5%) of the CCP-treated patients with moderate disease and 106 (68·4%) of corresponding controls (p=0·001). Among those with severe disease, clinical improvement was observed in 28 (60·8%) of the CCP-treated patients and 27 (34·6%) of their control counterparts (p=0·001). The median time to clinical improvement among those with moderate disease was 7 (IQR 4-9) days with CCP treatment and 8 (IQR 6-12) days in the control group (p=0·006), and among those with severe disease

Journal Pre-proof

was 7 (IQR 5-12) days with CCP treatment and 15.5 (IQR 10-20) days in the control group (p=0·003, Figure 2). The distribution of patients in the WHO ordinal scale categories over the 30 days of hospitalization are shown in eFigure1 and eFigure 2 in Supplement 2. For both moderate and severe disease, CCP treatment was associated with a significantly lower score on the WHO ordinal scale compared to the control group on days 7, 14, and 30. On day 7, the score reduction was significant in both the moderate (OR, 0·25 [95% CI 0·13-0·47]) and severe disease groups (OR, 0·33 [95% CI 0·14-0·82]). We also noted a score reduction in both moderate and severe disease on day 14 (OR, 0·17 [95% CI 0·08-0·34] and OR, 0·28 [95% CI 0·11-0·73], respectively) and day 30 (OR, 0·17 [95% CI 0·07-0·36] and OR, 0·11 [95% CI 0·04-0·31], respectively).

In the adjusted Cox model, the overall CCP group had a significantly higher rate of clinical improvement than the control group (HR, 1·9 [95% CI 1·4-2·7]). The effect was seen among both the moderate (HR, 1·9 [95% CI 1·3-2·8]) and severe disease (HR, 2·5 [95% CI 1·2-5·2]) groups (Table 2). There was no significant interaction effects between CCP and steroids (p=0·40), tocilizumab (p=0·12), or steroids plus tocilizumab (p=0·44) on the clinical improvement.

Secondary outcome

Effect of CCP on Mortality

In univariate analysis, the CCP-treated patients in both severity groups had lower mortality rates than the control groups (n=10, 11·4% and n=14, 30·4% versus n=46, 29·7% and n=44, 57·1%; p=0·001 and p=0·006, respectively). In the adjusted model, the risk of mortality was significantly lower in the CCP-treated patients with moderate disease (adjusted OR, 0·27 [95% CI 0·12–0·62]) compared to the control group, but not significantly different in the CCP-treated patients with severe disease (adjusted OR, 0·38 [95% CI 0·14–1·02]) compared to the control group. no significant interaction effects between CCP and steroids (p=0·36 for interaction), CCP and tocilizumab (p=0·56 for interaction), or CCP and steroids plus tocilizumab (p=0·75 for interaction) on mortality.

Journal Pre-proof

Effect of CCP on oxygen saturation and laboratory value kinetics

The effects of CCP on patients' oxygen saturation and laboratory markers over the first 14 days of the hospital stay are shown in Table 3. Relative to baseline, CCP treatment improved oxygen saturation by 5·4% [95% CI 3·3–7·4] on day 1 and 4·1% [95% CI 2·3-5·9] on day 3 in patients with moderate disease, but not among those with severe disease (eFigure 3 in Supplement). Lymphocyte counts increased significantly on days 7 (β=0·46 [95% CI 0·15-0·78]) and 14 (β=0·59 [95% CI 0·11-1·1]) post-hospital admission in the moderate CCP group. Similarly, lymphocyte counts increased on days 11 (β=0·76 [95% CI 0·03–1·5]) and 14 (β=0·98 [95% CI 0·04–1·9]) in the severe disease CCP group (eFigure 4 in Supplement). Total neutrophil counts were not significantly different among the groups at baseline or any other time point (eFigure 5 in Supplement). Relative to baseline, CRP significantly decreased in both the moderate and severe groups at every time point after CCP treatment (p=0·001; eFigure 6 in Supplement). D-dimer was not significantly different between the groups at baseline or in the first 11 days, but the levels were lower 14 days post-admission after CCP in the moderate disease group (eFigure 7 in Supplement). Furthermore, LDH was not significantly different among moderate and severe disease groups at baseline or in the first 14 days, but was lower at day 14 in the overall CCP group (eFigure 8 in Supplement).

Discussion

Currently, no vaccine or specific antiviral treatments are approved for COVID-19 patients. CP is a widely available non-pharmaceutical treatment that has been used with varying degrees of success in prior pandemics, including SARS, MERS, and Ebola.[15–18] In our study, we investigated the effect of CCP on time to clinical improvement, 30-day mortality, changes in oxygen saturation and laboratory values within 14 days of hospital admission in both moderate and severe COVID-19 patients. Many prior studies focused on severe and critically ill patients whereas we included severe cases as well as moderate cases where the risk benefit balance is more delicate. Improved clinical symptoms and mortality among

Journal Pre-proof

patients with severe and critical COVID-19 after CCP transfusion has been previously reported.[8,11] In a randomized clinical trial of CCP in severe and life-threatening COVID-19, Li et al. could not detect a significant reduction in time to clinical improvement within 28 days after CCP treatment.[7] However, the study was terminated early after enrollment of 52 patients who received CCP and 51 in the control group and may have been underpowered to detect significant differences. In contrast, our study included 135 patients in the CCP arm and 233 controls. Another report, concluded that CCP treatment did not reduce mortality and suggested that earlier adminstration of CCP might be beneficial.[12] The fact that patients in our study received CCP within 24 hours of hospital admission may have contributed to improving outcomes. In our study, CCP administration was associated with both a higher rate of and faster time to clinical improvement among patients with moderate and severe disease. The clinical improvement was observed in 80·6% of the CCP group compared to 58·6% of the control group. In addition, median time to clinical improvement in the CCP group was 7 days compared to 10 days in the control group. This is consistent with a prior case series of 10 patients, in which symptoms resolved within the first 72 hours with complete improvement on radiological examination within 7 days post-CCP administration.[19] In the H1N1 pandemic, CP was associated with a significant reduction in mortality, especially if administered early.[20] An exploratory post hoc meta-analysis revealed a significant reduction in the pooled odds of mortality following CP treatment of SARS coronavirus infection and severe influenza compared to placebo or no therapy.[21] Li et al. did not detect a significant reduction in 28-day mortality among CCP-treated patients. [7] In our study, 30-day mortality was significantly reduced in moderate, but not severe, COVID-19 cases. This may imply that CCP is more effective in reducing 30-day mortality in moderate disease, or may be attributed to inclusion of a relatively small number of severe cases. Our results are also consistent with those of a single arm study of 46 patients with moderate to severe disease where 7-day mortality was 6.5% in patients who received CCP compared to the 15% average national statistics and 30% in a concurrent cohort of 23 patients.[22]

Journal Pre-proof

CCP in our study was also associated with significantly improved oxygen saturation and recovery in lymphocyte counts and CRP within 14 days post-CCP treatment. These findings are consistent with recent reports of the effect of CCP.[8,11,23]

Prior studies have associated lower lymphocyte counts and high CRP levels with adverse outcomes in COVID-19 patients.[1,19] In our study, lymphocyte counts started to increase 1 day after CCP transfusion, with a significant increase 7 days post-admission in the CCP group compared to the control group. Furthermore, CRP was remarkably decreased starting day 1 in the CCP group. The improvement of both lymphocyte counts and CRP in our study may be attributed in part to a previously postulated immunomodulatory effect of CCP such as providing passive immunity by blocking inflammatory cytokines, autoantibodies, and complement pathways.[24] CCP may also inhibit inflammatory cascades and immune cell infiltration within the lungs.[25] These effects can clinically be seen as improvement in oxygen saturation and pulmonary radiological lesions.[19]

In our study, oxygen saturation significantly increased in CCP-treated patients during the first 72 hours. This finding is consistent with earlier studies reporting associations between CCP and improved oxygen saturation.[8,23]

All CCP recipients tolerated the transfusion except for 3 (2%) who had minor allergic skin reactions, which resolved completely without preventing administration of the full CCP dose. A recent study of early safety indicators for CCP reported 36 cases (0·72%) with serious adverse effects (SAEs).[26] The incidence of severe allergic reaction in that study was 0·06%, and the incidence of less than severe allergic reactions was not reported.

Our study has a number of limitations, including a lack of randomization. However, randomization was not feasible during the pandemic in our region. The administration of CCP within 24 hours of hospital admission was intended to limit selection bias. Another limitation is that at the time of analysis some patients were still hospitalized which may have biased the results. The clinical management of a potentially life-threatening illness with an unpredictable clinical course was the main contributor to this limitation. In addition, there was not an approved standard protocol for the treatment during our study, especially regarding

Journal Pre-proof

steroid and tocilizumab use. However, this stems from the lack of universally established treatments for COVID-19. We did not observe an interaction between the administration of either/both of the drugs and the observed effect of CCP in our patients. Finally, we were not able to determine the titer of SARS-CoV-2 IgG antibodies in CCP donors or whether the detected antibodies were neutralizing antibodies due to a lack of these tests in Kuwait at the time of the study. We did exclude any donors who were negative for IgG antibodies. Not performing additional antibody characterizing tests could only lead to underestimation of the benefit of this therapeutic modality due to potential inclusion of CCP units with low IgG levels or antibodies of limited neutralizing capacity. In addition, in many parts of the world, including some developed countries, there is a striking shortage of testing for COVID-19 in general, and particularly for more specialized tests, such as neutralizing antibodies.

To the best of our knowledge, this study is the first to investigate the effectiveness and feasibility of CCP in COVID-19 patients in the Middle East. Although we did not perform an a priori sample size calculation, 135 patients in the CCP group demonstrated significant differences. The involvement of multiple centers supports the generalizability of the results. Throughout the worldwide lockdown, the supply of CCP was consistently available in Kuwait, which offers free access to this therapeutic intervention. This contrasts pharmaceuticals subject to supply chain limitations. Availability is crucial in the Middle East, which relies on pharmaceutical manufacturing that is currently concentrated in Western countries.

Conclusion

In our prospective interventional study including patients with moderate and severe COVID-19, CCP administration was significantly associated with improved clinical outcomes. Thirty-day survival was significantly improved in the moderate group. In addition, administration of CCP in both moderate and severe cases was also associated with improved oxygen saturation and recovery of lymphocytes and CRP. Larger multicenter controlled randomized trials to further evaluate the effectiveness of CCP in COVID-19 patients with particular emphasis on CCP donor qualification based on neutralizing antibody levels are warranted.

Journal Pre-proof

Contributors

SA conceptualized and planned the study design, planned data collection,oversaw data collection with MA,literature review, performed the text mining analysis, drafted and revised the final version of the manuscript. MA also performed the statistical analysis. MZA and RA conducted literature review, contributed to the writing and reviewing of the manuscript. All other co-authors contributed to data collection and oversaw the manuscript.

Funding: Kuwait Ministry Of Health.

Trial registration: Clinicaltrials.gov NCT04474340.

Declaration of interests

We declare no competing interests.

Acknowledgments

This work was supported by Ministry Of Health in Kuwait. We thank Jeethu Anu Geo and Jibi Roshan for the technical support and reviewing the data, Kuwait University. We also thank Dr Mohammed Buhamra, for the technical support from the COVID-19 dispatch team,Ministry Of Health, Kuwait.

Journal Pre-proof

Journal Pre-proof

References

1. Brown BL, McCullough J. Treatment for emerging viruses: Convalescent plasma and COVID-19. *Transfus Apher Sci* 2020; **59**(3): 102790.

2. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**(10223): 497-506.

3. Zhu N, Zhang D, Wang W, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N Engl J Med* 2020; **382**(8): 727-33.

4. Garraud O, Heshmati F, Pozzetto B, et al. Plasma therapy against infectious pathogens, as of yesterday, today and tomorrow. *Transfus Clin Biol* 2016; **23**(1): 39-44.

5. Shakir EM, Cheung DS, Grayson MH. Mechanisms of immunotherapy: a historical perspective. *Ann Allergy Asthma Immunol* 2010; **105**(5): 340-7; quiz 8, 68.

6. Joyner MJ, Wright RS, Fairweather D, et al. Early safety indicators of COVID-19 convalescent plasma in 5,000 patients. *J Clin Invest* 2020.

7. Li L, Zhang W, Hu Y, et al. Effect of Convalescent Plasma Therapy on Time to Clinical Improvement in Patients With Severe and Life-threatening COVID-19: A Randomized Clinical Trial. *JAMA* 2020.

8. Shen C, Wang Z, Zhao F, et al. Treatment of 5 Critically Ill Patients With COVID-19 With Convalescent Plasma. *JAMA* 2020.

9. Integrated Management of Adolescent and Adult Illness (IMAI) District Clinician Manual: Hospital Care for Adolescents and Adults. GUIDELINES FOR THE MANAGEMENT OF COMMON ILLNESSES WITH LIMITED RESOURCES. Geneva, Switzerland: World Health Organization; 2011.

10. Investigational COVID-19 Convalescent Plasma Guidance for Industry. Food and Drug Administration Center for Biologics Evaluation and Research 2020.

11. Xia X, Li K, Wu L, et al. Improved clinical symptoms and mortality among patients with severe or critical COVID-19 after convalescent plasma transfusion. *Blood* 2020; **136**(6): 755-9.

Journal Pre-proof

12. Zeng QL, Yu ZJ, Gou JJ, et al. Effect of Convalescent Plasma Therapy on Viral Shedding and Survival in Patients With Coronavirus Disease 2019. *J Infect Dis* 2020; **222**(1): 38-43.

13. R&D Blueprint and COVID-19. World Health Organization; 2020.

14. Donor Eligibility (Screening and Testing). American Association of Blood Banks (AABB); 2020.

15. Soo YO, Cheng Y, Wong R, et al. Retrospective comparison of convalescent plasma with continuing high-dose methylprednisolone treatment in SARS patients. *Clin Microbiol Infect* 2004; **10**(7): 676-8.

16. Arabi Y, Balkhy H, Hajeer AH, et al. Feasibility, safety, clinical, and laboratory effects of convalescent plasma therapy for patients with Middle East respiratory syndrome coronavirus infection: a study protocol. *Springerplus* 2015; **4**: 709.

17. Ko JH, Seok H, Cho SY, et al. Challenges of convalescent plasma infusion therapy in Middle East respiratory coronavirus infection: a single centre experience. *Antivir Ther* 2018; **23**(7): 617-22.

18. van Griensven J, Edwards T, de Lamballerie X, et al. Evaluation of Convalescent Plasma for Ebola Virus Disease in Guinea. *N Engl J Med* 2016; **374**(1): 33-42.

19. Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. *Intensive Care Medicine* 2020; **46**(5): 846-8.

20. Hung IF, To KK, Lee CK, et al. Convalescent plasma treatment reduced mortality in patients with severe pandemic influenza A (H1N1) 2009 virus infection. *Clin Infect Dis* 2011; **52**(4): 447-56.

21. Mair-Jenkins J, Saavedra-Campos M, Baillie JK, et al. The effectiveness of convalescent plasma and hyperimmune immunoglobulin for the treatment of severe acute respiratory infections of viral etiology: a systematic review and exploratory meta-analysis. *J Infect Dis* 2015; **211**(1): 80-90.

Journal Pre-proof

22. Perotti C, Baldanti F, Bruno R, et al. Mortality reduction in 46 severe Covid-19 patients treated with hyperimmune plasma. A proof of concept single arm multicenter trial. *Haematologica* 2020.

23. Duan K, Liu B, Li C, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc Natl Acad Sci U S A* 2020; **117**(17): 9490-6.

24. Lunemann JD, Nimmerjahn F, Dalakas MC. Intravenous immunoglobulin in neurology--mode of action and clinical efficacy. *Nat Rev Neurol* 2015; **11**(2): 80-9.

25. Gralinski LE, Sheahan TP, Morrison TE, et al. Complement Activation Contributes to Severe Acute Respiratory Syndrome Coronavirus Pathogenesis. *mBio* 2018; **9**(5).

26. Eckhardt CM, Cummings MJ, Rajagopalan KN, et al. Evaluating the efficacy and safety of human anti-SARS-CoV-2 convalescent plasma in severely ill adults with COVID-19: A structured summary of a study protocol for a randomized controlled trial. *Trials* 2020; **21**(1): 499.

Table 1. Demographic and baseline characteristics by disease severity.

Characteristic	Control			CCP			P-value
	All N= 233	Moderate N= 155	Severe N= 78	All N= 135	Moderate N= 89	Severe N= 46	
Age (years)	54 [45 - 62]	52 [43 - 61]	57 [51 - 65]	54 [48 - 60]	54 [49 - 60]	53·5 [45 - 58]	**0·74**
Male	198 (85%)	136 (87·7 %)	62 (79·5%)	105 (77·8 %)	63 (70·8%)	42 (91·3%)	**0·27**
Diabetes	94 (45·4%)	58 (43·3 %)	36 (49·3%)	57 (42·5 %)	43 (48·3%)	14 (31·1%)	**0·21**
Hypertension	80 (39%)	44 (33·3 %)	36 (49·3%)	60 (45%)	43 (48·8%)	17 (37·8%)	**0·15**
Obesity (BMI>40)	20 (8·9%)	14 (9·5%)	6 (8%)	16 (12%)	8 (9·1%)	8 (17·8%)	**0·21**
Laboratory Findings							
Lymphocytes x10^9	0·93 [0·7 - 1·4]	1 [0·72 - 1·4]	0·85 [0·6 - 1·3]	1·06 [0·8 - 1·38]	1·1 [0·9 - 1·4]	1 [0·8 - 1·3]	**0·59**
Lymphocytes <1 x10^9	101 (47%)	68 (43·9 %)	46 (59%)	73 (57%)	35 (39·3%)	20 (43·5%)	**0·63**

Journal Pre-proof

Neutrophils x10^9	5·3 [4 - 7·6]	5·2 [4 - 7·8]	5·7 [4 - 10]	5·2 [4 - 9]	5·4 [4 - 7·7]	5·8 [4 - 7·5]	**0·68**
Platelets	226 [170 - 292]	226 [168 - 305]	224 [186 - 264]	219 [175 - 283]	309 [175 - 284]	229 [171 - 273]	**0·69**
CRP (mg/L)	81·4 [34·1 - 136]	82·4 [38 - 141]	78 [25 - 121]	96 [73·5 - 154]	94 [73 - 148]	98 [82·4 - 179]	**0·04**
LDH (IU/L)	429 [333 - 598]	410 [331 - 567]	480 [347 - 710]	391 [325 - 605]	360 [289 - 467]	514 [387 - 686]	**0·84**
D-dimer (ng/mL)	459 [289 - 800]	431 [288 - 796]	507 [292 - 820]	384 [296 - 588]	345 [235 - 562]	477 [348 - 919]	**0·96**
Ferritin (ng/mL)	668 [362 - 1889]	665 [360 - 1243]	678 [401 - 1050]	638 [334 - 1130]	624 [332 - 940]	824 [412 - 1178]	**0·63**
PT (seconds)	14·1 [13 - 16]	13·9 [12·6 - 15·3]	15 [13·5 - 17]	15·1 [14 - 16·8]	15 [13·8 - 16·7]	15·7 [13·7 - 17]	**0·36**
APTT (seconds)	36·4 [32 - 40·8]	36·4 [32 - 40·6]	37 [32 - 42·4]	35·6 [32·6 - 38·5]	35·5 [33 - 38·4]	36 [32 - 39]	**0·46**
Clinical Characteristics							
Baseline Spo2 (%)	91 [88 - 94]	92 [90 - 95]	90 [85 - 92]	91 [89 - 94]	92 [90 - 94]	90 [87 - 92]	**0·06**
WHO ordinal scale on admission							
	47 (20·2%)	46 (29·1 %)	1 (1·3%)	13 (9·6%)	12 (13·5%)	1 (2·2%)	
	123 (52·7%)	94 (60·6 %)	29 (23·2%)	72 (53·3 %)	60 (67·4%)	12 (26·1%)	
	59 (25·3%)	15 (9·7%)	0	45 (33·3 %)	17 (19·2%)	28 (60·8%)	
	4 (1·7%)	0	4 (5·1%)	5 (3·7%)	0	5 (10·9%)	
Treatment received							
Steroid	113 (48·5%)	76 (49%)	37 (47·4%)	77 (57%)	46 (51·7%)	31 (67·4%)	**0·14**
Tocilizumab	153 (66·7%)	100 (64·5 %)	53 (67·9%)	65 (67·7 %)	43 (63·2%)	22 (78·5%)	**0·45**

Data are given as median [IQR] or n (%). Abbreviations: IQR, interquartile; BMI, body mass index (as calculated in kilograms divided by height in meters squared); LDH, lactate dehydrogenase; CRP, C-reactive protein; PT, prothrombin time; APTT, activated partial prothrombin time.
SI conversion factors: to convert lactate dehydrogenase to microkatal per liter, multiply by 0·167; to convert D-dimer to nanomoles per liter, divide by 5·476; to convert CRP to nanomoles per liter, multiply by 9·524.
Moderate COVID-19: defined as the presence of clinical signs of pneumonia (fever, cough, dyspnea) and oxygen saturation (SpO2) of more than 90% in room air.
Severe COVID-19: defined as clinical signs of pneumonia plus SpO2 less than 90% in room air or admission to intensive care unit (ICU) for respiratory support (i.e., high flow nasal cannula, non-invasive mechanical ventilation and intubation).
WHO 7-category ordinal scale that ranges from 1 (discharged with normal activity) to 7 (death). Scale 2=Not hospitalized, but unable to resume normal activities; scale 3=hospitalized, not requiring supplemental oxygen; scale

Journal Pre-proof

4= hospitalized, requiring supplemental oxygen, scale 5= hospitalized, requiring high-flow nasal oxygen (HFNC) and/or non-invasive mechanical ventilation (IMV); scale 6= hospitalized, requiring ECMO and/or IMV; scale 7= death.

Table 2. Primary and secondary outcomes

Primary outcome	**Overall control**	**Overall CCP**	**P-value**	**Adjusted HR* (95% CI); p-value**
Clinical improvement	133 (58·6%)	100 (80·6%)	<0·001	1·9 (1·4-2·7) ;<0·001
Days to clinical improvements, median (IQR)	10 (6-15)	7 (5-9)	<0·001	
Primary outcome	**Moderate control N (%)**	**Moderate CCP N (%)**	**P-value**	**Adjusted HR* (95% CI); p-value**
Clinical improvement	106 (68·4%)	77 (86·5%)	0·001	1·9 (1·3-2·8); 0·001
Days to clinical improvements, median (IQR)	8 (6-12)	7 (4-9)	0·006	
Primary outcome	**Severe control N (%)**	**Severe CCP N (%)**	**P-value**	**Adjusted HR* (95% CI); p-value**
Clinical improvement	27 (34·6%)	28 (60·8%)	0·006	2·5 (1·2-5·2); 0·012
Days to clinical improvements, median (IQR)	15·5 (10-20)	7 (5-12)	0·003	
Secondary outcome	**Overall control**	**Overall CCP**	**P-value**	**Adjusted OR* (95% CI); *P*p-value**
Death	90 (38·8%)	24 (17·8%)	<0·001	0·32 (0·18- 0·58); 0·001

Journal Pre-proof

Secondary outcome	Moderate control N (%)	Moderate CCP N (%)	P-value	Adjusted OR* (95% CI); p-value
Death	46 (29·7%)	10 (11·4%)	0·001	0·27 (0·12- 0·62); 0·002
Secondary outcome	**Severe control**	**Severe CCP**	**P-value**	**Adjusted OR* (95% CI); p-value**
Death	44 (57·1%)	14 (30·4%)	0·006	0·38 (0·14-1·02); 0·06

*Adjusted for age, baseline oxygen saturation < 88%, lymphocytes <1x10^9, and C-reactive protein

Table 3. Changes in oxygen saturation and laboratory markers

Marker	Day 1	Day 3	Day 7	Day 11	Day 14
All patients	4·4 (2·2 to 6·6)**	3·6 (1·8 to 5·3)**	2·1 (-0·22 to 4·5)	-0·2 (-4·1 to 3·6)	0·98 (-1·3 to 3·3)
Moderate patients	5·4 (3·3 to 7·4) **	4·1 (2·3 to 5·9)**	1·9 (-0·55 to 4·4)	0·27 (-3·9 to 4·5)	1·2 (-1·1 to 3·4)
Severe patients	5·3 (-0·8 to 11·4)	2·8 (-1·1 to 6·7)	0·50 (-4·4 to 5·4)	-1·02 (-7·8 to 5·7)	0·92 (-5·9 to 7·7)
All patients	0·18 (-0·27 to 0·63)	042 (-0·26 to 1·04)	0·55 (0·22 to 0·88)**	0·68 (0·21 to 1·1)*	0·92 (0·41 to 1·4) **
Moderate disease	0·06 (-0·37 to 0·48)	0·21 (-0·23 to 0·66)	0·46 (0·15 to 0·78)*	0·52 (-0·11 to 1·2)	0·59 (0·11 to 1·1) *
Severe disease	0·49 (-0·59 to 1·5)	0·85 (-0·58 to 2·2)	0·57 (-0·05 to 1·2)	0·76 (0·03 to 1·5)*	0·98 (0·04 to 1·9) *
All patients	4·3 (-8·6 to 17·4)	1·6 (-0·81 to 3·9)	1·9 (-0·19 to 4·1)	-1·8 (-6·04 to 2·3)	-3·8 (-7·9 to 0·17)
Moderate disease	0	0·85 (-1·87 to 3·5)	2·2 (-0·5 to 4·8)	- 7·9 (-13·7 to 2·1)	-3·8 (-8·04 to 0·39)

Journal Pre-proof

Severe disease	8·5 (-5·04 to 22·1)	3·6 (-0·34 to 6·8)	1·7 (-1·7 to 5·1)	4·5 (-1·4 to 10·4)	-1·8 (-9·2 to 5·6)
All patients	-128·8 (-160·9 to -96·7) **	-125 (-161·4 to -88·7) **	-86·6 (-113·5 to -59·7) **	-115 (-162 to -68·1) **	-90·7 (-134·6 to -46·8) **
Moderate disease	-112·2 (-147·8 to -76·6) **	-143·9 (-188·5 to -99·4) **	-76·1 (-109·7 to -42·5) **	-99·3 (-141·4 to -57·2) **	-57·7 (-87·7 to -27·7) **
Severe disease	-176·5 [-234 to -119] **	-91·3 [-173 to -9·5] *	-104·2 [-163·2 to -45·3] **	-171 [-265·8 to -76·3] **	-99·8 [-190·4 to -9·3] *
All patients	NA	109 (-557 to 775·4);0·749	-253·1 (-938·5 to 432·4);0·469	-327·3 (-2578·2 to 1923·6);0·776	-718·3 (-1580·5 to 143·9);0·103
Moderate disease	NA	240·3 (-572·5 to 1053)	-348·7 (-1441·1 to 743)	0	-535·7 (-981·7 to -89·7)*
Severe disease	NA	297 (-1805·6 to 2399·5)	-541·9 (-1064·4 to 980·5)	1777·5 (-1625 to 5180)	-1162·1 (-3565·7 to 1241·5)
All patients	NA	-77·2 (-361·4 to 207·1)	61·8 (-109·5 to 233·1)	NA	-393·4(-637·4 to -149·5)*
Moderate disease	NA	-89·2 (-789·4 to 610·9)	7·04 (-212·9 to 226·9)	NA	-272·7 (-641·6 to 96·2)
Severe disease	NA	122·4 (-870·2 to 1115)	-78·4 (-568·2 to 411·4)	NA	-272·2 (-989·2 to 444·9)

All values are compared to laboratory values at baseline (before CCP or at admission in control group), CCP compared to control NA=not available
Values are expressed as β coefficient (95% CI) *p-value<0·05 **p-value<0·001

Journal Pre-proof

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY-NC-ND 4.0 International license .

Treatment of Severe COVID-19 with Convalescent Plasma in the Bronx, NYC

Hyun ah Yoon[1], Rachel Bartash[1], Inessa Gendlina[1], Johanna Rivera[1,2], Antonio Nakouzi[1,2], Robert H. Bortz III[2], Ariel S. Wirchnianski[2,3], Monika Paroder[4], Karen Fehn[5], Leana Serrano-Rahman[4], Rachelle Babb[1,2], Uzma N. Sarwar[1], Denise Haslwanter[2], Ethan Laudermilch[2], Catalina Florez[2,7†], M. Eugenia Dieterle[2], Rohit K. Jangra[2], J. Maximilian Fels[2], Karen Tong[3], Margarette C. Mariano[3], Olivia Vergnolle[3], George I. Georgiev[3], Natalia G. Herrera[3], Ryan J. Malonis[3], Jose A. Quiroz[3], Nicholas C. Morano[3], Gregory J. Krause[8,9], Joseph M. Sweeney[10], Kelsie Cowman[1], Stephanie Allen[6], Jayabhargav Annam[6], Ariella Applebaum[6], Daniel Barboto[6], Ahmed Khokhar[6], Brianna J. Lally[6], Audrey Lee[6], Max Lee[6], Avinash Malaviya[6], Reise Sample[6], Xiuyi A. Yang[6], Yang Li[11], Rafael Ruiz[12,13], Raja Thota[12], Jason Barnhill[7†], Doctor Y. Goldstein[4], Joan Uehlinger[4], Scott J. Garforth[3], Steven C. Almo[3,10], Jonathan R. Lai[3], Morayma Reyes Gil[4], Amy S. Fox[4], Kartik Chandran[2¶], Tao Wang[11¶], Johanna P. Daily[1,2¶], Liise-anne Pirofski[1,2*]

1. Division of Infectious Diseases, Department of Medicine. Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY.
2. Department of Microbiology and Immunology. Albert Einstein College of Medicine, Bronx, NY.
3. Department of Biochemistry. Albert Einstein College of Medicine, Bronx, NY.
4. Department of Pathology. Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY.
5. Department of Oncology. Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

6. Albert Einstein College of Medicine, Bronx, NY.
7. Department of Chemistry and Life Science, United States Military Academy at West Point, NY.
8. Department of Developmental and Molecular Biology. Albert Einstein College of Medicine, Bronx, NY.
9. Institute of Aging Studies. Albert Einstein College of Medicine, Bronx, NY.
10. Department Physiology and Biophysics. Albert Einstein College of Medicine, Bronx, NY.
11. Department of Epidemiology and Population Health. Albert Einstein College of Medicine, Bronx, NY.
12. Network Performance Group. Montefiore Medical Center, Bronx, NY.
13. Division of Hospital Medicine, Department of Medicine. Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY.

¶ These senior authors contributed equally to this work.

*: The corresponding author

Liise-anne Pirofski, MD

Albert Einstein College of Medicine and Montefiore Medical Center

Phone: 718-430-2940

Email: l.pirofski@einsteinmed.org

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

† Disclaimer: The views expressed herein are those of the author and do not reflect the position of the United States Military Academy, the Department of the Army, or the Department of Defense.

Conflicts of Interest statement

KC is a member of the Scientific Advisory Board of Integrum Scientific, LLC. In addition, KC has a SARS-CoV-2 spike neutralization assay patent pending, and a SARS-CoV-2 spike antibody assay patent pending. JL reports grants from Adimab LLC, grants from Integrated BioTherapeutics, grants from Mapp Biopharmaceutical, personal fees from Johnson & Johnson, personal fees from Celdara Medical. In addition, JL has a COVID-19 antibody diagnostic patent pending. Other authors have declared that no conflict of interest exists.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

Abstract

Convalescent plasma with severe acute respiratory disease coronavirus 2 (SARS-CoV-2) antibodies (CCP) may hold promise as treatment for Coronavirus Disease 2019 (COVID-19). We compared the mortality and clinical outcome of patients with COVID-19 who received 200mL of CCP with a Spike protein IgG titer ≥1:2,430 (median 1:47,385) within 72 hours of admission to propensity score-matched controls cared for at a medical center in the Bronx, between April 13 to May 4, 2020. Matching criteria for controls were age, sex, body mass index, race, ethnicity, comorbidities, week of admission, oxygen requirement, D-dimer, lymphocyte counts, corticosteroids, and anticoagulation use. There was no difference in mortality or oxygenation between CCP recipients and controls at day 28. When stratified by age, compared to matched controls, CCP recipients <65 years had 4-fold lower mortality and 4-fold lower deterioration in oxygenation or mortality at day 28. For CCP recipients, pre-transfusion Spike protein IgG, IgM and IgA titers were associated with mortality at day 28 in univariate analyses. No adverse effects of CCP were observed. Our results suggest CCP may be beneficial for hospitalized patients <65 years, but data from controlled trials is needed to validate this finding and establish the effect of ageing on CCP efficacy.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

Introduction:

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (1), a highly transmissible enveloped positive-strand RNA virus (2), is the causative agent of the Coronavirus disease 2019 (COVID-19) pandemic (3). The first case was reported in December 2019, and by November 2020 more than 61 million infections were reported worldwide, with one fifth of the cases and deaths occurring in the United States (U.S.) (4). In April 2020, New York City (NYC), especially the borough of the Bronx, was an early epicenter of the COVID-19 pandemic in the U.S. (5, 6). Since then, an antiviral that reduced duration of illness (7), Remdesivir, received FDA approval on October 22, 2020, and corticosteroids, which reduced mortality in severely ill patients in a large randomized clinical trial and prospective meta-analysis (8, 9), have become standard of care. As of November 2020, there is no approved therapy for COVID-19 that reduces mortality of hospitalized patients with respiratory manifestations of severe or life-threatening disease.

Convalescent plasma (CP) obtained from recovered persons was deployed for previous respiratory pandemics, including 1918 and 2009 influenza and Severe Acute Respiratory Syndrome (SARS) (10-13). Given the lack of established therapies for COVID-19, CP containing SARS-CoV-2 antibodies (CCP) was proposed as a therapeutic option early in the pandemic (14). As of November 2020, it has shown a possible benefit in multiple studies. In early case series, CCP-treated patients exhibited viral clearance and reductions in inflammatory markers (15-19). Observational studies comparing CCP-treated patients to retrospective controls showed a reduction in mortality in non-intubated patients and/or those transfused within 72 hours of hospitalization with high titer CCP (20-24). Analysis of a subset of more than 3,000 CCP recipients in an open label study found a dose-response whereby non-intubated patients who

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

received high titer CCP had lower mortality than those who received low titer CCP (22). Several randomized controlled trials (RCT) have not shown a benefit of CCP, but were limited by premature termination due to a lack of patients to recruit (25, 26). One trial found CCP had an antiviral effect, but there was no effect on mortality (27), another found a reduction in mortality, albeit with a very small sample size (28), and another was terminated due to the presence of neutralizing antibodies in CCP recipients at the time of transfusion, despite being on track to meet the primary endpoint (29). A recent double-blind, placebo controlled, multicenter RCT did not show an effect of CCP on mortality (30). Evidence of safety and possible benefit led the FDA to issue an emergency use authorization (EUA) for CCP use in hospitalized patients with COVID-19 on August 23, 2020.

We treated 103 patients at Montefiore Medical Center (MMC), a 1,491-bed tertiary medical center in the Bronx, New York with serious or life-threatening COVID-19 with CCP between April 13 and May 4, 2020 and conducted a propensity score matched study. Herein, we report mortality and clinical and laboratory findings of CCP recipients compared to matched controls.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

Results

Baseline characteristics of CCP recipients and retrospective controls

One hundred and three (103) patients were enrolled in the Mayo Clinic expanded access protocol (EAP) (31) and treated with one 200 mL unit of CCP within 72 hours of hospital admission. Clinical status and mortality on day 28 post-transfusion was compared to retrospective propensity score matched controls identified by querying the electronic medical record (Figure 1). Analysis included 90 CCP recipients and 258 controls after exclusion of twelve patients who did not meet the eligibility criteria because of mechanical ventilation for > 24 hours or CCP transfusion > 72 hours after admission, and one patient with missing data (Figure 1). Compared to controls, CCP recipients were younger (median 66 vs 72 years, $P = 0.002$), had higher BMI (28 vs 27 kg/m^2, $P = 0.05$), and lower rates of congestive heart failure (18 vs 29%, $P = 0.03$), and chronic kidney disease (29 vs 41%, $P = 0.03$). At baseline, a lower proportion of CCP recipients were on low-flow oxygen support (68 vs 81%, $P < 0.0001$) and a higher proportion required mechanical ventilation (20 vs 6%, $P < 0.0001$), had lower baseline lymphocyte counts (0.8 vs 1.0 x 10^9/L, $P = 0.001$), and received systemic corticosteroids (93 vs 63%, $P < 0.0001$) (Supplemental Table 1). After propensity score matching, 73 CCP recipients and 73 control patients were well balanced for all matching variables except in a subgroup analysis stratified by age; CCP recipients ≥ 65 years had lower lymphocyte counts than controls (median, 0.8 vs 1.0 x10^9/L) (Table 1, Supplemental Figure 1).

Convalescent plasma SARS-CoV-2 spike protein and neutralizing antibody (NAb) titers

Of the 200 mL units of CCP administered in this study, 95 of 103 were obtained from 46 persons who donated CP at MMC in April 2020. SARS-CoV-2 spike protein IgG endpoint titers were

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

measured in CCP obtained from these donors using the MMC in-house research full-length spike protein ELISA (32). Median IgG, IgM and IgA titers were, respectively, 1:47,385 (interquartile range [IQR], 21,870 – 65,610; n = 46), 1:810 (IQR, 810 – 2,430; n = 43) and 1:90 (IQR, 90 – 270; n = 43). Median NAb titer by pseudovirus neutralization assay was 1:938 (IQR, 407 – 2,784; n = 42). There was a direct correlation between NAb titer and spike protein IgG (Spearman $r = 0.78$, $P < 0.0001$), IgM ($r = 0.58$, $P < 0.0001$) and weakly with IgA ($r = 0.29$, $P = 0.05$) titers (Supplemental Figure 2). For the eight patients who did not receive MMC donor CCP, 7 received units that tested as 'reactive' by the New York State Department of Health (NYSDOH) Wadsworth Center's SARS-CoV-2 Microsphere Immunoassay for anti-Nucleocapsid antibody detection (33), and one received CCP with a spike protein IgG titer of 1:320 measured by the in-house spike protein ELISA assay at Mount Sinai Hospital (34) .

Comparison of clinical outcomes of CCP recipients and controls

There was no difference in mortality between 73 CCP recipients and 73 propensity score matched controls by day 28 ($P = 0.47$, Kaplan-Meier log rank test) (Figure 2). To account for the potential interaction between age and CCP treatment ($P = 0.11$, interaction term), analysis was stratified by age. Compared to matched controls, CCP recipients < 65 years had lower mortality by day 28 ($P = 0.04$, Kaplan-Meier log rank test), whereas the mortality of CCP recipients and matched controls ≥ 65 years did not differ significantly ($P = 0.61$, Kaplan-Meier log rank test) (Figure 2). There was no difference in mortality between groups when CCP recipients and controls were stratified by baseline oxygen requirement (Supplemental Figure 3).

There was no significant difference between CCP recipients and matched controls in all-cause mortality at 28 days (31.5 vs 38.4%; odds ratio [OR], 0.74; 95% confidence interval [CI],

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

0.37 – 1.46; $P = 0.37$) (Figure 3). When stratified by age, CCP recipients < 65 years had a 4-fold decrease in mortality (8.8 vs 29.4%; OR, 0.23; 95% CI, 0.05 – 0.95; $P = 0.04$) and a 4-fold decrease in deterioration in oxygenation or mortality (11.8 vs 35.3%; OR, 0.24; 95% CI, 0.06 – 0.87; $P = 0.03$). There was no significant difference in mortality of CCP recipients ≥ 65 years (52.6 vs 45.9%; OR, 1.07; $P = 0.89$) (Figure 3, Table 1). Among the 103 CCP recipients, mortality at day 28 was associated with time from symptom onset to transfusion (OR, 1.12; 95% CI, 1.01 – 1.24; $P = 0.04$), earlier week of admission (OR, 2.22; 95% CI, 1.00 – 5.00; $P = 0.05$) and being Hispanic/LatinX in ethnicity compared to not being Hispanic/LatinX (OR, 8.33; 95% CI, 1.69 – 33.3; $P = 0.009$), adjusted for age, sex, BMI, race, ethnicity, comorbid conditions, week of admission, duration of symptoms, baseline oxygen requirement, corticosteroids, anticoagulation use, D-dimer and lymphocyte counts (Table 2). There was no significant association between CCP NAb or spike protein IgG titers and mortality or oxygenation status in CCP recipients.

Multivariable analysis of 90 CCP recipients and 258 controls adjusted for covariates age, sex, BMI, race, ethnicity, comorbid conditions, week of admission, baseline oxygen requirement, corticosteroids, anticoagulation use, D-dimer and lymphocyte counts did not show any difference in outcome between the two groups. When stratified by age, CCP recipients < 65 years had lower mortality or deterioration in oxygenation but this was not statistically significant (OR, 0.23; 95% CI, 0.04 – 1.19; $P = 0.08$) (Supplemental Figure 4). Additionally, multivariable analysis indicated that age, use of mechanical ventilation at baseline, use of systemic corticosteroids, not being on anticoagulation in patients ≥ 65 years and earlier week of admission were associated with mortality at day 28 adjusted for covariates (Supplemental Table 2). Corticosteroid use was

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

associated with mortality in patients receiving low-flow oxygen at baseline (adjusted OR, 2.68; 95% CI, 1.27 – 5.68; $P = 0.009$) when analysis was stratified by baseline oxygen requirement.

CCP safety and adverse events

There were no adverse reactions, including no instances of transfusion-related acute lung injury or transfusion-associated circulatory overload attributable to CCP administration.

CCP recipient SARS-CoV-2 spike protein antibody titers

We measured CCP recipient spike protein IgG, IgM, and IgA in remnant serum samples obtained before (D -1) and after transfusion (Figure 4). Baseline spike protein IgG, IgM and IgA were significantly higher in patients mechanically ventilated at enrollment ($P = 0.009$, $P = 0.01$, $P = 0.02$, respectively) and those who died by day 28 ($P = 0.02$, $P = 0.02$, $P = 0.002$, respectively) (Supplemental Table 3, Figure 4, Supplemental Figure 5). There was no association between baseline antibody titers and time from symptom onset to transfusion or time from hospital admission to transfusion. Plateau in median IgG after CCP administration was reached earlier in patients ≥ 65 than < 65 years, mechanically ventilated at baseline versus not, and those who died by versus those alive at day 28 (Figure 4). Baseline IgA was higher in patients ≥ 65 than those < 65 years ($P = 0.04$) (Supplemental Table 3).

There was a direct association between mortality at day 28 and baseline (D -1) IgG (OR, 1.4; 95% CI, 1.04 – 1.78; $P = 0.03$), IgM (OR, 1.39; 95% CI, 1.00 – 1.92; $P = 0.048$) and IgA (OR, 1.45; 95% CI, 1.11 – 1.91; $P = 0.007$) in the univariate analyses but not in the multivariable analysis adjusted for covariates (Table 2). Baseline spike protein IgG titer was significantly correlated with D-dimer ($r = 0.46$; $P = 0.0002$; n = 60) (Figure 4). In addition, there was a direct,

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

albeit weak correlation between baseline spike protein IgG titer and detected viral load measured by cycle threshold (Ct) value of nasopharyngeal SARS-CoV-2 reverse-transcriptase-polymerase-chain-reaction (RT-PCR) ($r = 0.39$; $P = 0.002$; n = 57) (Figure 4). There was no correlation between Ct value and age, duration of illness, or D-dimer.

CCP recipient inflammatory and hematology measures

There was no significant difference in change in lymphocyte counts, D-dimer, or CRP between day 0 and 28 in CCP recipients compared to controls (data not shown).

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

Discussion

CCP has been used as an investigational treatment for COVID-19 since the early days of the pandemic. Numerous observational studies report safety and signals of possible efficacy of CCP in hospitalized patients with COVID-19 (15, 20, 22-25, 35, 36). Here, we report the mortality and clinical outcomes of a cohort of 73 patients with severe to life-threatening COVID-19 who were transfused with one unit of CCP by 72 hours of hospitalization and 73 propensity score matched controls. There was no significant difference in mortality or improvement in oxygenation in CCP recipients compared to controls. Although treated within 72 hours of hospitalization, CCP treated patients had symptom duration of 5-9 days and multiple indicators of severe or life-threatening disease including lymphopenia, elevated D-dimer levels, and the need for supplemental oxygen. In this regard, our findings are similar to those of other studies in which there was no benefit of CCP in hospitalized patients with severe COVID-19 (25, 27, 30). Nonetheless in a subset of patients stratified by age, CCP recipients < 65 years had significantly lower mortality and deterioration in oxygenation by day 28 than controls. Age and duration of symptoms were independently associated with mortality at day 28 in CCP recipients. There were no adverse reactions directly attributable to CCP.

There was no evidence of benefit of CCP in patients ≥ 65 years. Age, a well-documented risk factor for COVID-19 severity and mortality (37, 38), was significantly associated with mortality in unadjusted and adjusted analyses. Patients ≥ 65 years had a higher frequency of co-morbid conditions, higher D-dimer, CRP and SARS-CoV-2 IgA values, and lower lymphocyte levels than those < 65 years, all of which are markers of severe disease (39-44). Notably in published case-control studies in which CCP was associated with reduced mortality, median ages of patients were < 60 years (21, 23, 45), and in the large open label Mayo Clinic study in which

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

there was a signal of reduced mortality in patients who received high titer CCP, 44% of the cohort was < 60, 70% was < 70 years, and CCP was less effective in those > 80 years (22). In addition, a small RCT comparing 80 patients randomized to CCP versus standard of care posted on the pre-print server on November 29th, 2020 found a benefit of CCP in patients < 67 years, but not in the entire cohort with a median age of 61 years (46). These data along with ours suggest that ageing may have a detrimental effect on CCP efficacy.

In a case-control study that also did not show a signal of CCP efficacy, Rogers et al. found a higher rate of hospital discharge in patients ≥ 65 years (47). In their study, 14% of CCP recipients were black/African American, 31% were Caucasian/white, 42% were Hispanic/LatinX, and 34% had hypertension, 25% had diabetes, and all received two units of CCP. Our cohort was older, more severely ill, and came from racial/ethnic populations at higher risk for severe COVID-19 and death (48): 26% of CCP recipients were black/African American, 9% were Caucasian/white, 51% were Hispanic/LatinX, which was associated with mortality in our multivariable analysis, and 82% had hypertension, 42% had diabetes, and all received one unit of CCP. This suggests social determinants of health may have adversely affected clinical outcomes of our cohort.

Reflecting practice at the peak of the pandemic at our center, the majority of patients in our cohort received corticosteroids and corticosteroid use was associated with mortality in those requiring low flow oxygen. In another propensity score matched study, corticosteroids use was also associated with higher mortality (49). Among CCP recipients ≥ 65 years in our study, 98% received corticosteroids concurrently with or before CCP. More CCP recipients than controls also received corticosteroids in the Rogers et al. study, which did not find evidence of CCP benefit (47). In addition, although not statistically significant, a higher proportion of CCP

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

recipients than controls received corticosteroids in the Li et al. RCT, in which there was not a signal of CCP efficacy and the median age was 70 years (25). Corticosteroid use has been associated with lower mortality in patients with COVID-19 who require mechanical ventilation (8, 9), and not in the early course of the disease (9, 50). Viral clearance was slower in patients with SARS and MERS who received corticosteroids (51, 52) and corticosteroid use was associated with lower anti-spike protein receptor binding domain (RBD) IgG and neutralization titers in COVID-19 patients (53). Thus, we wonder if corticosteroid use further impaired the immune status of elderly patients in our cohort, who already had lower lymphocyte counts. Data from ongoing RCTs is needed to evaluate the effect of covariates, including corticosteroids on CCP efficacy.

The ability of CCP to affect the course of COVID-19 is most likely a function of viral neutralization early in disease (14, 54). The Mayo Clinic study analysis found that high titer CCP, as defined by signal to cut off ratio on the OrthoV platform, reduced COVID-19 mortality relative to low titer CCP (22). In a propensity score matched study, compared to controls, CCP with a RBD IgG titer ≥1:1,350 reduced mortality in non-intubated patients transfused within 72 hours of hospital admission (21). Although titers in our study cannot be directly compared with titers in other studies, CCP used in our study had high titer spike protein IgG and a median neutralizing titer of 1:938 based, respectively, on a highly specific full length spike protein ELISA (55-57) and a pseudovirus neutralization assay that correlates with live virus (plaque reduction) neutralization (42, 57, 58). Nonetheless, although there was a possible signal of efficacy in the sub-group < 65 years who received CCP within 72 hours of hospitalization, this was not the case in patients ≥ 65 years, who were more severely ill based on baseline data. It is possible elderly patients may require more than one dose of 200 mL of CCP, but two units did

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

not mediate an effect in the Rogers et al. cohort (47). While there are theoretical concerns of antibody-dependent enhancement (ADE) in the presence of sub-neutralizing concentrations of anti-viral antibodies (59, 60), ADE was not reported in other CCP treatment studies and the levels of spike protein antibody with neutralizing capability in CCP in this study make it very unlikely. Given the high mortality of COVID-19 in patients ≥ 65 years and lack of evidence of CCP efficacy in this group in our study and others (25, 61) there is a need for more data on the effect of aging on CCP efficacy in COVID-19 .

Consistent with other reports associating SARS-CoV-2 antibody titer with disease severity and/or mortality (53, 62-64), spike protein IgG at enrollment was directly associated with mortality among 60 CCP recipients who had remnant sera available. Although we observed post-transfusion increases in spike protein IgG in patients < 65 years or who were not intubated, this cannot be distinguished from endogenous antibody without comparison to untreated controls. While we did not measure post-transfusion viral loads, pre-transfusion antibody titers correlated with RT-PCR Ct values (inversely correlated with viral load). Thus, as in other studies (53, 65), endogenous antibody may have already contributed to viral control in CCP recipients. The Gharbharan et al. RCT was terminated early when it was discovered majority of the study patients had neutralizing titers at enrollment equivalent to donor CCP (29), although the study appeared on track to meet its expected endpoint. Patients in the PLACID RCT (27) who received CCP had earlier conversion to viral RNA negativity, despite having low levels of neutralizing antibodies at enrollment. In the ConPlas-19 RCT (26), in which 49% of the patients had positive SARS-CoV-2 IgG at enrollment, CCP did not confer benefit, but there was a trend towards reduced mortality. Nonetheless, in our study as in others (27, 66), antibody titers and symptom duration associated with disease severity and a lack of evidence of CCP efficacy. This

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

underscores the longstanding principle that convalescent antibody therapy is most likely to be effective early in the course of viral respiratory diseases (14) as shown for COVID-19 in propensity score matched studies (23, 49) and a recently published outpatient RCT (66).

A strength of our study is that it includes patients over the age of 65 years, who represent a population with disproportionately higher COVID-19 mortality. To date, studies of CCP and other potential therapies for COVID-19 in patients negatively affected by social determinants of health are lacking. The Bronx has a higher poverty rate than the other New York City (NYC) boroughs (5, 67) and our cohort was comprised predominantly of Hispanic or Latinx and black or African American populations who were severely affected by the surge conditions in NYC and experienced higher COVID-19 mortality (68-71). We attempted to control for hospital surge capacity and social determinants of health that can significantly contribute to COVID-19 outcome (72) by including week of admission, race, and ethnicity as propensity scores.

A major limitation of this study is its retrospective and non-randomized study design. The retrospectively identified controls differed in baseline characteristics from the cases, most notably in baseline oxygen requirement, proportion of corticosteroid use and baseline lymphocyte counts, suggesting a mismatch in severity of illness due to selection bias. While propensity score matching and multivariable analysis with adjustments were done to correct for confounding variables, there likely were additional latent and unmeasured variables that were not adjusted for. In addition, time-dependent variables such as hospital bed capacity during surge conditions and advances in clinical practice, such as use of proning techniques, lung protective ventilation strategies, improvement in sedation, may not have been accounted for in our analysis, despite matching and adjusting for baseline week. In addition, poor or absent documentation of oxygen requirement and inconsistencies in obtaining inflammatory markers during the height of

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

the pandemic resulted in missing data, precluding a more complete analysis. Finally, since we could not obtain antibody data for controls, we cannot assess CCP effects on antibody levels.

In summary, we report that CCP administration within 72 hours of hospitalization demonstrated a possible signal of reduced mortality in patients < 65 years. Similar to others, we found CCP was safe with no adverse events directly attributable to transfusion (21, 73, 74). Although our data suggest possible effects of age, disease severity, and corticosteroid use on CCP efficacy, prospective, randomized, controlled trials are needed to establish CCP efficacy and the effect of these and other covariates. Antibody-based therapies, including CCP have now shown promise in outpatients with mild to moderate COVID-19 (66, 75). However, nearly a year into the pandemic, effective therapies for hospitalized patients are still urgently needed, particularly for those who are elderly and at high risk for mortality. If effective in any group of hospitalized patients, CCP will have immense impact on healthcare resources and public health during this ongoing pandemic (76). CCP is rapidly available compared to other pharmaceuticals or vaccines and may be a more feasible option in surge conditions and/or resource limited settings (24).

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

Methods

Patient enrollment

One hundred and three (103) adult patients with laboratory (nasopharyngeal PCR)-confirmed COVID-19 were enrolled in the Mayo Clinic expanded access treatment protocol (31) to receive CCP between April 13 and May 4, 2020. Hospitalized patients were referred to the study team by hospitalists and/or infectious diseases consultants and were deemed eligible to receive CCP if they had been hospitalized for ≤ 3 days or were symptomatic for 3 to 7 days prior to transfusion and had severe and/or life-threatening COVID-19 disease. Patients who were on mechanical ventilation for more than 24 hours were excluded. Severe disease was defined as respiratory symptoms with hypoxemia requiring ≥ 5 liters of nasal cannula oxygen support. Life-threatening disease was defined as respiratory failure requiring mechanical ventilation, shock, and/or multiple organ dysfunction or failure. Ninety-one patients received CCP by day three of hospitalization. Patients or their legally authorized representatives provided informed consent prior to treatment.

Convalescent plasma procurement and transfusion

After obtaining informed consent, blood was collected between March and April 2020 from otherwise healthy adult volunteers residing in Westchester County, Rockland County and the Bronx, NY who had recovered from COVID-19. Potential donors had a documented positive nasopharyngeal swab by PCR for SARS-CoV-2 during illness and had been asymptomatic for ≥14 days prior to sample collection. Serum was obtained by venipuncture (BD Vacutainer, serum), aliquoted, heat-inactivated at 56°C for 30 minutes and stored at -4°C prior to antibody screening by enzyme-linked immunosorbent assay (ELISA). Donors with SARS-CoV-2 spike

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY-NC-ND 4.0 International license .

protein titers >1:1,000 were referred for apheresis at the New York Blood Center (NYBC). CCP from 46 MMC donors and 8 donors from the general NYBC pool was administered to patients in the study.

Plasma recipients were transfused with 1 unit (~ 200 mL) of ABO-type matched CCP over 2-3 hours and monitored before, during and after infusion for signs of transfusion-related reactions per standard transfusion protocol.

Controls and data collection

We identified 1,347 non-CCP recipients with a positive SARS-CoV-2 PCR admitted to MMC between April 13 and May 4 by querying the electronic medical record (EMR). Since most CCP was administered by day 3 post-admission, baseline day was set at day +2 post-admission in non-CCP recipients. Of the 1,347 identified patients, the CCP recipients and 986 non-CCP recipients were excluded because they required < 5 L or no baseline oxygen support, or had a missing baseline oxygen value (n = 903), or were intubated for > 24 hours at baseline (n = 47), or had missing data (n = 36). This resulted in a retrospective control group of 258 non-CCP recipients (Figure 1). We collected age, sex, BMI, race, ethnicity, comorbidities, medications, laboratory findings, and day of death or discharge from CCP-treated and control patients. Additionally, we collected duration of symptoms and hospital day of transfusion from CCP-treated patients from the EMR. Specific laboratory values and clinical characteristics were obtained from EMR by using Structured Query Language.

For both control and CCP recipients, patient oxygen support was evaluated at day 0 and 28 post-CCP and the corresponding day post-admission for controls as follows: low-flow oxygen through nasal cannula or non-rebreather mask (5 - 15 liter), high-flow nasal cannula or non-

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY-NC-ND 4.0 International license .

invasive ventilation, and invasive mechanical ventilation. If a patient's oxygen requirement increased or the patient died prior to the time-point of interest, patient's oxygenation status was considered to have worsened. Initial cycle threshold (Ct) value from the SARS-CoV-2 RT-PCR assay performed on a single platform was queried retrospectively for CCP recipients.

SARS-CoV-2 spike protein IgG, IgM and IgA titers before and after transfusion of CCP

SARS-CoV-2 spike protein-binding IgG, IgM and IgA titers were determined by ELISA using remnant sera obtained from baseline (Day -1 (D-1)) and 1, 3, 7 days after patients received CCP (D1, D3 and D7, respectively). Briefly, microtiter plates (Costar, Corning, NY) were coated with 25 µl of 2 µg/ml purified spike protein (55, 56, 77) in phosphate-buffered saline (PBS) overnight at 4°C, washed with 1X PBS/0.1% Tween (PBS-T), blocked with 3% (v/v) milk (Bio-Rad)/PBS-T for 1 hr at room temperature (RT), washed, and incubated with heat inactivated sera for 2 hrs at RT. Plates were then washed, incubated with isotype-specific horseradish phosphatase (HRP) labeled goat anti-human (GAH) IgG (Thermo Fisher), IgM, or IgA (Millipore Sigma) for 1 hr at RT. Following final washes, plates were incubated with ultra-TMB ELISA substrate (Thermo Scientific) and color development was stopped by addition of 0.5 M sulfuric acid (Sigma Aldrich). Well absorbances at 450 nm (A_{450}) were determined using a Cytation 5 (BioTek). The endpoint titer was determined as the highest dilution to give a signal three times the background A_{450} (wells with no sera).

rVSV-SARS-CoV-2 S Neutralization assay

The neutralization assay was done as previously described (57). Briefly, CCP samples were serially diluted and incubated with pre-titrated amounts of virus for 1 hr at RT, plasma-virus

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

mixtures were added to 96-well plates (Corning) containing monolayers of Vero cells, incubated for 7 hr at 37°C/5% CO2, fixed with 4% paraformaldehyde (Sigma) in PBS, washed with PBS, and stored in PBS containing Hoechst-33342 (1:2,000 dilution; Invitrogen). Viral infectivity was measured by automated enumeration of green fluorescent protein (GFP)-positive cells from captured images using a Cytation 5 automated fluorescence microscope (BioTek) and analyzed using the Gen 5 data analysis software (BioTek). The serum half-maximal inhibitory concentration (NC_{50}) was calculated using a nonlinear regression analysis with GraphPad Prism software.

Study outcomes

The primary outcome was all-cause mortality at day 28 post-CCP. The secondary outcomes were improvement in oxygenation status or mortality at day 28 post-CCP. Exploratory outcomes were associations between pre-CCP SARS-CoV-2 antibody titers and mortality at day 28.

Statistics

Patient characteristics and outcomes were reported as frequencies and proportions for categorical variables, and median and interquartile range (IQR) for continuous variables. Differences between groups (e.g., CCP versus non-CCP) were determined by student t-test or Mann Whitney U test for continuous variables, and Chi-square or Fischer's exact tests for categorical variables, as appropriate.

For the outcome analysis, we performed 1:1 propensity score matching using the nearest neighbor matching without replacement on 90 case and 258 control patients to optimize balance of baseline characteristics for assessing the independent effect of CCP on oxygenation and

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

survival. The distribution of O2 requirement prior to matching showed that the cases had higher oxygen requirement ($P < 0.001$). The primary matching criteria included age, sex, race, ethnicity, BMI, week of admission, D-dimer, lymphocyte count, corticosteroids use, anticoagulation use, hypertension, diabetes, chronic pulmonary disease, chronic kidney disease, coronary artery disease, hyperlipidemia with exact matching on baseline oxygen requirement and age group (categorical, < 65 vs ≥ 65 years). Propensity scores were calculated using a logistic regression model. After 1:1 propensity score matching, the analysis included 73 cases and 73 controls and the variables were not significantly different between CCP recipients and controls based on an omnibus test ($P = 0.80$) (78). The all-cause mortality at day 28 post-CCP was depicted by Kaplan-Meier curves. Differences between groups were compared using the log-rank test. Stratification analyses were done by age < 65 vs ≥ 65 years and by baseline oxygen requirement. Corticosteroids and anticoagulation use were not well balanced in the subgroups and were further adjusted for in age-stratified analysis.

As a sensitivity analysis, factors associated with oxygenation status at day 28 were evaluated by proportional odds model, and mortality at day 28 was evaluated using logistic regression model. Adjusted OR and corresponding 95% confidence intervals were calculated. To identify variables that predicted mortality in CCP group, we performed a stepwise model selection using the Akaike Information Criterion (AIC) in a logistic regression model with age, sex, BMI, race, ethnicity, comorbidities, week of admission, time from symptom onset to transfusion, baseline oxygen requirement, anticoagulation, corticosteroid use, D-dimer, and lymphocyte count. Log-transformed SARS-CoV-2 spike protein antibody titers were individually added to select models to evaluate their association with each outcome. A two-sided *P*-value of <

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

0.05 was considered statistically significant. Prism Version 8.0, Stata/IC Version 16.1 and R was used for analysis.

Study approval

The retrospective cohort study, the donor plasma procurement protocol and the use of the expanded access protocol was approved by the Albert Einstein College of Medicine Institutional Review Board.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

Author contributions

HY, LP, JD, KC designed the study. HY, LP wrote the manuscript with input from all authors. JD, IG, KC, TW contributed to critical revision of the manuscript. HY, RB, KF enrolled patients. MP, LS, JU provided CCP from the blood bank. HY, UNS, KC, SA, JA, AA, DB, AK, BL, AL, ML, AM, XY collected clinical data. IG, RR, RT, PS retrospectively identified control patients and collected clinical data. MRG, ASF provided clinical specimens and critical data interpretation. JR, AN, R Babb collected clinical specimens and performed ELISAs. RHBIII, ASW curated clinical specimens, performed ELISAs, and analyzed ELISA data. EL, CF, DH, MED, JMF assisted with clinical specimen processing and performed ELISAs. DH, MED, RKJ, RHBIII performed and analyzed viral neutralization assays. KT, MCM, OV performed ELISAs. GIG and RJM expressed and purified spike protein. JAQ assisted with management of clinical information. NGH, NCM and SJG expressed, purified and performed quality control on the spike protein. GJK and JMS assisted with specimen collection and transport. RS assisted with specimen collection. DYG provided data. TW, YL, HY performed statistical analysis. HY, LP, JD, KC, TW, IG, UNS, DYG, ASF assisted with data analysis and interpretation. JB acquired funding. All authors revised and approved the final version.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

Acknowledgements

HY was supported by NIH/National Center for Advancing Translational Service (NCATS) Einstein-Montefiore CTSA Grant Number UL1TR001073. LP was supported in part by NIH grants AI 123664, AI 143453, NCATS 3UL1TR002556-04S1 and a grant from the Mathers Foundation. KC was supported in part by NIH grants U19AI142777 and R01AI32633 and a COVID-19 Pilot Award from Albert Einstein College of Medicine. JRL was supported by the NIH (R01-AI125462) and a COVID-19 Pilot Award from Albert Einstein College of Medicine. SCA was supported by R01AI145024, the Wollowick Family Foundation Chair in Multiple Sclerosis and Immunology, and Janet & Martin Spatz and the Helen & Irving Spatz Foundation. Additional support provided by the Albert Einstein Macromolecular Therapeutics Development Facility, the Einstein-Rockefeller-CUNY Center for AIDS Research (P30AI124414), and the Albert Einstein Cancer Center (P30CA013330). JPD was supported by NIAID R21AI141367. MCM and GIG were supported by an NIH Training Program in Cellular and Molecular Biology and Genetics (T32-GM007491). KT, JAQ, RJM, RHBIII and GK were supported by an NIH Medical Scientist Training Program (T32-GM007288) at Albert Einstein College of Medicine. This study was supported in part by a US Department of Health and Human Services (HHS), Biomedical Advanced Research and Development Authority (BARDA) grant contract 75A50120C00096, National Center for Advancing Translational Sciences (NCATS) grant UL1TR002377, Schwab Charitable Fund (Eric E Schmidt, Wendy Schmidt donors), United Health Group, National Basketball Association (NBA), Millennium Pharmaceuticals, Octopharma USA, Inc, and the Mayo Clinic.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

References

1. Gorbalenya AE, Baker SC, Baric RS, de Groot RJ, Drosten C, Gulyaeva AA, et al. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nature Microbiology.* 2020;5(4):536-44.
2. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet.* 2020;395(10223):497-506.
3. Guan W-j, Ni Z-y, Hu Y, Liang W-h, Ou C-q, He J-x, et al. Clinical Characteristics of Coronavirus Disease 2019 in China. *New England Journal of Medicine.* 2020;382(18):1708-20.
4. John Hopkins University Coronavirus Resource Center. COVID-19 Dashboard by the Center for Systems Science and Engineering at Johns Hopkins University. Accessed Nov 16. https://coronavirus.jhu.edu/map.html.
5. Wadhera RK, Wadhera P, Gaba P, Figueroa JF, Joynt Maddox KE, Yeh RW, et al. Variation in COVID-19 Hospitalizations and Deaths Across New York City Boroughs. *JAMA.* 2020.
6. New York City Department of Health and Mental Hygiene. COVID-19 Data. Accessed Nov 16. https://www1.nyc.gov/site/doh/covid/covid-19-data.page.
7. Beigel JH, Tomashek KM, Dodd LE, Mehta AK, Zingman BS, Kalil AC, et al. Remdesivir for the Treatment of Covid-19 — Final Report. *New England Journal of Medicine.* 2020;383(19):1813-26.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

8. Sterne JAC, Murthy S, Diaz JV, Slutsky AS, Villar J, Angus DC, et al. Association Between Administration of Systemic Corticosteroids and Mortality Among Critically Ill Patients With COVID-19: A Meta-analysis. *Jama.* 2020.

9. Horby P, Lim WS, Emberson JR, Mafham M, Bell JL, Linsell L, et al. Dexamethasone in Hospitalized Patients with Covid-19 - Preliminary Report. *N Engl J Med.* 2020.

10. Luke TC, Kilbane EM, Jackson JL, and Hoffman SL. Meta-analysis: convalescent blood products for Spanish influenza pneumonia: a future H5N1 treatment? *Ann Intern Med.* 2006;145(8):599-609.

11. Cheng Y, Wong R, Soo YO, Wong WS, Lee CK, Ng MH, et al. Use of convalescent plasma therapy in SARS patients in Hong Kong. *Eur J Clin Microbiol Infect Dis.* 2005;24(1):44-6.

12. Hung IF, To KK, Lee CK, Lee KL, Chan K, Yan WW, et al. Convalescent plasma treatment reduced mortality in patients with severe pandemic influenza A (H1N1) 2009 virus infection. *Clin Infect Dis.* 2011;52(4):447-56.

13. Mair-Jenkins J, Saavedra-Campos M, Baillie JK, Cleary P, Khaw FM, Lim WS, et al. The effectiveness of convalescent plasma and hyperimmune immunoglobulin for the treatment of severe acute respiratory infections of viral etiology: a systematic review and exploratory meta-analysis. *J Infect Dis.* 2015;211(1):80-90.

14. Casadevall A, and Pirofski LA. The convalescent sera option for containing COVID-19. *J Clin Invest.* 2020;130(4):1545-8.

15. Duan K, Liu B, Li C, Zhang H, Yu T, Qu J, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proceedings of the National Academy of Sciences.* 2020;117(17):9490-6.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

16. Shen C, Wang Z, Zhao F, Yang Y, Li J, Yuan J, et al. Treatment of 5 Critically Ill Patients With COVID-19 With Convalescent Plasma. *JAMA.* 2020;323(16):1582-9.

17. Ye M, Fu D, Ren Y, Wang F, Wang D, Zhang F, et al. Treatment with convalescent plasma for COVID-19 patients in Wuhan, China. *J Med Virol.* 2020.

18. Zhang B, Liu S, Tan T, Huang W, Dong Y, Chen L, et al. Treatment With Convalescent Plasma for Critically Ill Patients With Severe Acute Respiratory Syndrome Coronavirus 2 Infection. *Chest.* 2020.

19. Ahn JY, Sohn Y, Lee SH, Cho Y, Hyun JH, Baek YJ, et al. Use of Convalescent Plasma Therapy in Two COVID-19 Patients with Acute Respiratory Distress Syndrome in Korea. *J Korean Med Sci.* 2020;35(14):e149.

20. Xia X, Li K, Wu L, Wang Z, Zhu M, Huang B, et al. Improved Clinical Symptoms and Mortality on Severe/Critical COVID-19 Patients Utilizing Convalescent Plasma Transfusion. *Blood.* 2020.

21. Salazar E, Christensen PA, Graviss EA, Nguyen DT, Castillo B, Chen J, et al. Treatment of COVID-19 Patients with Convalescent Plasma Reveals a Signal of Significantly Decreased Mortality. *Am J Pathol.* 2020.

22. Joyner MJ, Senefeld JW, Klassen SA, Mills JR, Johnson PW, Theel ES, et al. Effect of Convalescent Plasma on Mortality among Hospitalized Patients with COVID-19: Initial Three-Month Experience. *medRxiv.* 2020:2020.08.12.20169359.

23. Liu STH, Lin H-M, Baine I, Wajnberg A, Gumprecht JP, Rahman F, et al. Convalescent plasma treatment of severe COVID-19: a propensity score–matched control study. *Nature Medicine.* 2020.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

24. Skrip L, Derra K, Kaboré M, Noori N, Gansané A, Valéa I, et al. Clinical management and mortality among COVID-19 cases in sub-Saharan Africa: A retrospective study from Burkina Faso and simulated case analysis. *Int J Infect Dis.* 2020.

25. Li L, Zhang W, Hu Y, Tong X, Zheng S, Yang J, et al. Effect of Convalescent Plasma Therapy on Time to Clinical Improvement in Patients With Severe and Life-threatening COVID-19: A Randomized Clinical Trial. *JAMA.* 2020.

26. Avendano-Sola C, Ramos-Martinez A, Munez-Rubio E, Ruiz-Antoran B, Malo de Molina R, Torres F, et al. Convalescent Plasma for COVID-19: A multicenter, randomized clinical trial. *medRxiv.* 2020:2020.08.26.20182444.

27. Agarwal A, Mukherjee A, Kumar G, Chatterjee P, Bhatnagar T, and Malhotra P. Convalescent plasma in the management of moderate covid-19 in adults in India: open label phase II multicentre randomised controlled trial (PLACID Trial). *BMJ.* 2020;371:m3939.

28. Rasheed AM, Ftak DF, Hashim HA, Maulood MF, Kabah KK, Almusawi YA, et al. The therapeutic effectiveness of Convalescent plasma therapy on treating COVID-19 patients residing in respiratory care units in hospitals in Baghdad, Iraq. *medRxiv.* 2020:2020.06.24.20121905.

29. Gharbharan A, Jordans CCE, GeurtsvanKessel C, den Hollander JG, Karim F, Mollema FPN, et al. Convalescent Plasma for COVID-19. A randomized clinical trial. *medRxiv.* 2020:2020.07.01.20139857.

30. Simonovich VA, Burgos Pratx LD, Scibona P, Beruto MV, Vallone MG, Vázquez C, et al. A Randomized Trial of Convalescent Plasma in Covid-19 Severe Pneumonia. *New England Journal of Medicine.* 2020.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY-NC-ND 4.0 International license .

31. Joyner M. Expanded Access to Convalescent Plasma for the Treatment of Patients with COVID-19. Unique Protocol Identification Number: 20-003312. Version 8.0. 16 June 2020. 2020.

32. Bortz RH, Florez C, Laudermilch E, Wirchnianski AS, Lasso G, Malonis RJ, et al. Development, clinical translation, and utility of a COVID-19 antibody test with qualitative and quantitative readouts. *medRxiv.* 2020.

33. FDA Coronavirus Disease 2019 Emergency Use Authorizations for Medical Devices. Accessed Oct. 21. https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/eua-authorized-serology-test-performance.

34. Wajnberg A, Amanat F, Firpo A, Altman DR, Bailey MJ, Mansour M, et al. Robust neutralizing antibodies to SARS-CoV-2 infection persist for months. *Science.* 2020:eabd7728.

35. Hegerova L, Gooley T, Sweerus KA, Maree CL, Bailey N, Bailey M, et al. Use of Convalescent Plasma in Hospitalized Patients with Covid-19 - Case Series. *Blood.* 2020.

36. Salazar E, Christensen PA, Graviss EA, Nguyen DT, Castillo B, Chen J, et al. Significantly Decreased Mortality in a Large Cohort of Coronavirus Disease 2019 (COVID-19) Patients Transfused Early with Convalescent Plasma Containing High-Titer Anti-Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Spike Protein IgG. *The American journal of pathology.* 2020:S0002-9440(20)30489-2.

37. Yang W, Kandula S, Huynh M, Greene SK, Van Wye G, Li W, et al. Estimating the infection-fatality risk of SARS-CoV-2 in New York City during the spring 2020 pandemic wave: a model-based analysis. *Lancet Infect Dis.* 2020.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

38. Ioannou GN, Locke E, Green P, Berry K, O'Hare AM, Shah JA, et al. Risk Factors for Hospitalization, Mechanical Ventilation, or Death Among 10 131 US Veterans With SARS-CoV-2 Infection. *JAMA Network Open.* 2020;3(9):e2022310-e.

39. Nikolich-Zugich J, Knox KS, Rios CT, Natt B, Bhattacharya D, and Fain MJ. SARS-CoV-2 and COVID-19 in older adults: what we may expect regarding pathogenesis, immune responses, and outcomes. *Geroscience.* 2020;42(2):505-14.

40. Tan L, Wang Q, Zhang D, Ding J, Huang Q, Tang Y-Q, et al. Lymphopenia predicts disease severity of COVID-19: a descriptive and predictive study. *Signal Transduction and Targeted Therapy.* 2020;5(1):33.

41. Diao B, Wang C, Tan Y, Chen X, Liu Y, Ning L, et al. Reduction and Functional Exhaustion of T Cells in Patients With Coronavirus Disease 2019 (COVID-19). *Front Immunol.* 2020;11:827.

42. Wang X, Guo X, Xin Q, Pan Y, Hu Y, Li J, et al. Neutralizing Antibodies Responses to SARS-CoV-2 in COVID-19 Inpatients and Convalescent Patients. *Clinical Infectious Diseases.* 2020.

43. Ma H, Zeng W, He H, Zhao D, Jiang D, Zhou P, et al. Serum IgA, IgM, and IgG responses in COVID-19. *Cellular & Molecular Immunology.* 2020;17(7):773-5.

44. Wu F, Wang A, Liu M, Wang Q, Chen J, Xia S, et al. Neutralizing antibody responses to SARS-CoV-2 in a COVID-19 recovered patient cohort and their implications. *medRxiv.* 2020:2020.03.30.20047365.

45. Donato M, Park S, Baker M, Korngold R, Morawski A, Geng X, et al. Clinical and laboratory evaluation of patients with SARS-CoV-2 pneumonia treated with high-titer convalescent plasma: a prospective study. *medRxiv.* 2020:2020.07.20.20156398.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

46. Ray Y, Paul SR, Bandopadhyay P, D'Rozario R, Sarif J, Lahiri A, et al. Clinical and immunological benefits of convalescent plasma therapy in severe COVID-19: insights from a single center open label randomised control trial. *medRxiv.* 2020:2020.11.25.20237883.

47. Rogers R, Shehadeh F, Mylona EK, Rich J, Neill M, Touzard-Romo F, et al. Convalescent plasma for patients with severe COVID-19: a matched cohort study. *Clinical Infectious Diseases.* 2020.

48. Izzy S, Tahir Z, Cote DJ, Al Jarrah A, Roberts MB, Turbett S, et al. Characteristics and Outcomes of Latinx Patients With COVID-19 in Comparison With Other Ethnic and Racial Groups. *Open Forum Infectious Diseases.* 2020;7(10).

49. Salazar E, Christensen PA, Graviss EA, Nguyen DT, Castillo B, Chen J, et al. Significantly decreased mortality in a large cohort of COVID-19 patients transfused early with convalescent plasma containing high titer anti-SARS-CoV-2 spike protein IgG. *The American Journal of Pathology.* 2020.

50. Keller MJ, Kitsis EA, Arora S, Chen JT, Agarwal S, Ross MJ, et al. Effect of Systemic Glucocorticoids on Mortality or Mechanical Ventilation in Patients With COVID-19. *J Hosp Med.* 2020;15(8):489-93.

51. Lee N, Allen Chan KC, Hui DS, Ng EKO, Wu A, Chiu RWK, et al. Effects of early corticosteroid treatment on plasma SARS-associated Coronavirus RNA concentrations in adult patients. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology.* 2004;31(4):304-9.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

52. Arabi YM, Hajeer AH, Luke T, Raviprakash K, Balkhy H, Johani S, et al. Feasibility of Using Convalescent Plasma Immunotherapy for MERS-CoV Infection, Saudi Arabia. *Emerg Infect Dis.* 2016;22(9):1554-61.

53. Garcia-Beltran WF, Lam EC, Astudillo MG, Yang D, Miller TE, Feldman J, et al. COVID-19 neutralizing antibodies predict disease severity and survival. *medRxiv.* 2020:2020.10.15.20213512.

54. Klingler J, Weiss S, Itri V, Liu X, Oguntuyo KY, Stevens C, et al. Role of IgM and IgA Antibodies in the Neutralization of SARS-CoV-2. *medRxiv.* 2020.

55. Bortz RH, Florez C, Laudermilch E, Wirchnianski AS, Lasso G, Malonis RJ, et al. Development, clinical translation, and utility of a COVID-19 antibody test with qualitative and quantitative readouts. *medRxiv.* 2020.

56. Herrera NG, Morano NC, Celikgil A, Georgiev GI, Malonis RJ, Lee JH, et al. Characterization of the SARS-CoV-2 S Protein: Biophysical, Biochemical, Structural, and Antigenic Analysis. *bioRxiv.* 2020:2020.06.14.150607.

57. Dieterle ME, Haslwanter, D., Bortz III., R.H., Wirchnianski, A.S., Lasso,, G. V, O., Abbasi, S.A., Fels, J.M., Laudermilch, E., Florez, C., Mengotto, A., Kimmel, D.,, Malonis RJ, Georgiev, G., Quiroz, J., Barnhill, J., Pirofski, L.-a., Daily, J.P., Dye, J.M., Lai, J.R.,, and Herbert AS, Chandran, K., Jangra, R.K. A replication-competent vesicular stomatitis virus for studies of SARS-CoV-2 spike-mediated cell entry and its inhibition. *Cell Host and Microbe (2020).* 2020.

58. Okba NMA, Müller MA, Li W, Wang C, GeurtsvanKessel CH, Corman VM, et al. Severe Acute Respiratory Syndrome Coronavirus 2-Specific Antibody Responses in Coronavirus Disease Patients. *Emerg Infect Dis.* 2020;26(7):1478-88.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

59. Fleming AB, and Raabe V. Current studies of convalescent plasma therapy for COVID-19 may underestimate risk of antibody-dependent enhancement. *J Clin Virol.* 2020;127:104388.

60. Jaume M, Yip MS, Cheung CY, Leung HL, Li PH, Kien F, et al. Anti-severe acute respiratory syndrome coronavirus spike antibodies trigger infection of human immune cells via a pH- and cysteine protease-independent FcγR pathway. *J Virol.* 2011;85(20):10582-97.

61. Pimenoff VN, Elfstrom M, and Dillner J. A systematic review of convalescent plasma treatment for COVID19. *medRxiv.* 2020:2020.06.05.20122820.

62. Long Q-X, Liu B-Z, Deng H-J, Wu G-C, Deng K, Chen Y-K, et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nature Medicine.* 2020;26(6):845-8.

63. Zhao J, Yuan Q, Wang H, Liu W, Liao X, Su Y, et al. Antibody responses to SARS-CoV-2 in patients of novel coronavirus disease 2019. *Clin Infect Dis.* 2020.

64. Wang Y, Zhang L, Sang L, Ye F, Ruan S, Zhong B, et al. Kinetics of viral load and antibody response in relation to COVID-19 severity. *J Clin Invest.* 2020.

65. Regeneron's REGN-COV2 Antibody Cocktail reduced viral levels and improved symptoms in non-hospitalized COVID-19 patients. September 29.

66. Libster R, Marc GP, Wappner D, Coviello S, Bianchi A, Braem V, et al. Prevention of severe COVID-19 in the elderly by early high-titer plasma. *medRxiv.* 2020:2020.11.20.20234013.

67. Ross J, Diaz CM, and Starrels JL. The Disproportionate Burden of COVID-19 for Immigrants in the Bronx, New York. *JAMA Internal Medicine.* 2020.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

68. Bhala N, Curry G, Martineau AR, Agyemang C, and Bhopal R. Sharpening the global focus on ethnicity and race in the time of COVID-19. *Lancet.* 2020.

69. Wortham JM LJ, Althomsons S, et al. . Characteristics of Persons Who Died with COVID-19 — United States, February 12–May 18, 2020. MMWR Morb Mortal Wkly Rep. ePub: 10 July 2020. 2020.

70. COVID-19 in Racial and Ethnic Minority Groups. CDC C-. https://www.cdc.gov/coronavirus/2019-ncov/need-extra-precautions/racial-ethnic-minorities.html.

71. Golestaneh L, Neugarten J, Fisher M, Billett HH, Gil MR, Johns T, et al. The association of race and COVID-19 mortality. *EClinicalMedicine.* 2020;25:100455.

72. Abrams EM, and Szefler SJ. COVID-19 and the impact of social determinants of health. *Lancet Respir Med.* 2020;8(7):659-61.

73. Michael J. Joyner KAB, Stephen A. Klassen, et al. . Safety Update: COVID-19 Convalescent Plasma in 20,000 Hospitalized Patients. Mayo Clinic Proceedings. 2020.

74. Joyner MJ, Wright RS, Fairweather D, Senefeld JW, Bruno KA, Klassen SA, et al. Early safety indicators of COVID-19 convalescent plasma in 5,000 patients. *The Journal of Clinical Investigation.* 2020.

75. Chen P, Nirula A, Heller B, Gottlieb RL, Boscia J, Morris J, et al. SARS-CoV-2 Neutralizing Antibody LY-CoV555 in Outpatients with Covid-19. *New England Journal of Medicine.* 2020.

76. Emanuel EJ, Persad G, Upshur R, Thome B, Parker M, Glickman A, et al. Fair Allocation of Scarce Medical Resources in the Time of Covid-19. *New England Journal of Medicine.* 2020;382(21):2049-55.

medRxiv preprint doi: https://doi.org/10.1101/2020.12.02.20242909 ; this version posted December 4, 2020. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

77. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh C-L, Abiona O, et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science.* 2020;367(6483):1260-3.

78. Hansen BB, and Bowers J. Covariate Balance in Simple, Stratified and Clustered Comparative Studies. *Statist Sci.* 2008;23(2):219-36.

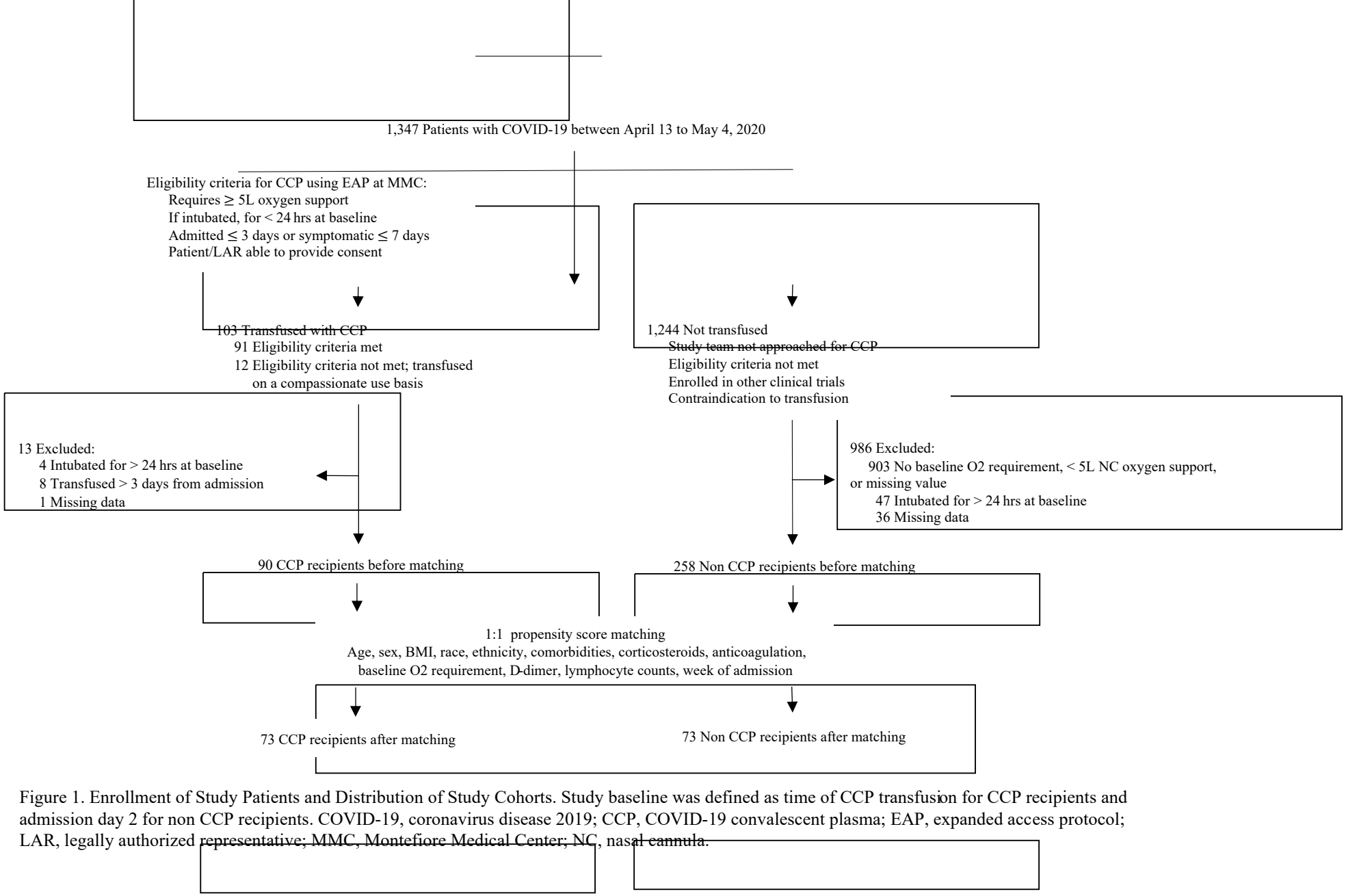


Figure 1. Enrollment of Study Patients and Distribution of Study Cohorts. Study baseline was defined as time of CCP transfusion for CCP recipients and admission day 2 for non CCP recipients. COVID-19, coronavirus disease 2019; CCP, COVID-19 convalescent plasma; EAP, expanded access protocol; LAR, legally authorized representative; MMC, Montefiore Medical Center; NC, nasal cannula.

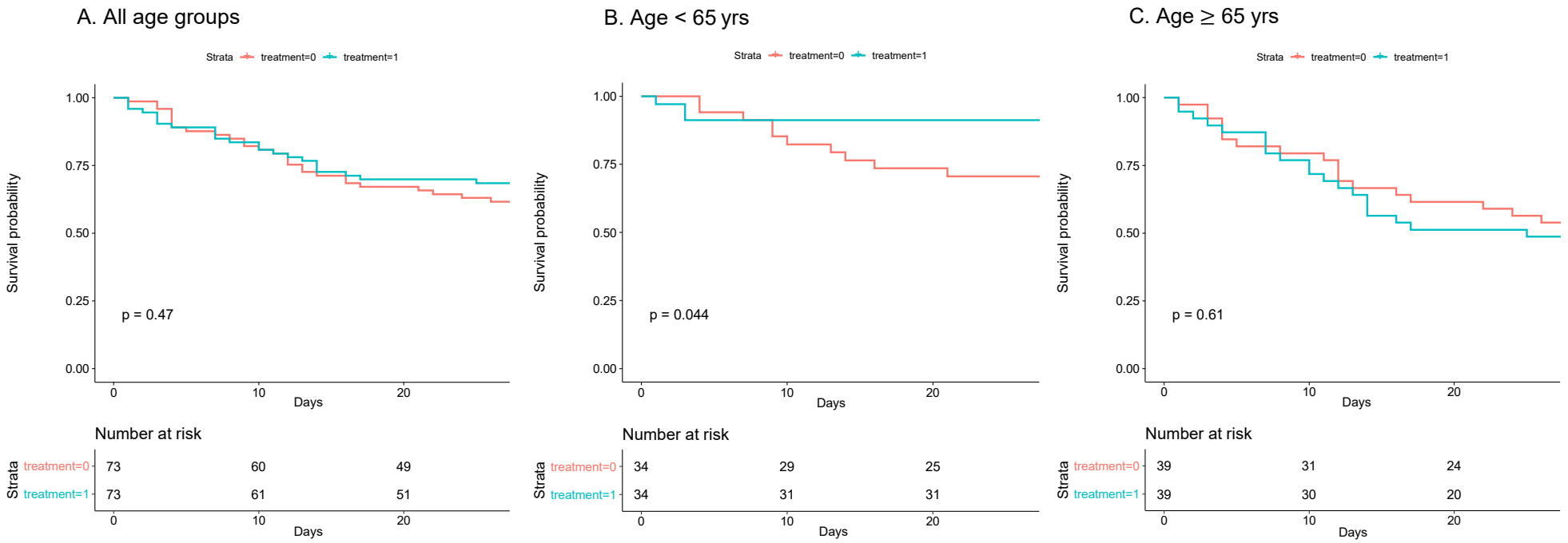


Figure 2. Kaplan–Meier Plot of the Probability of Survival from time of transfusion to day 28 in CCP recipients (n = 73) vs matched controls (n = 73). A. All age groups. B. Age < 65 years. C. Age ≥ 65 years.

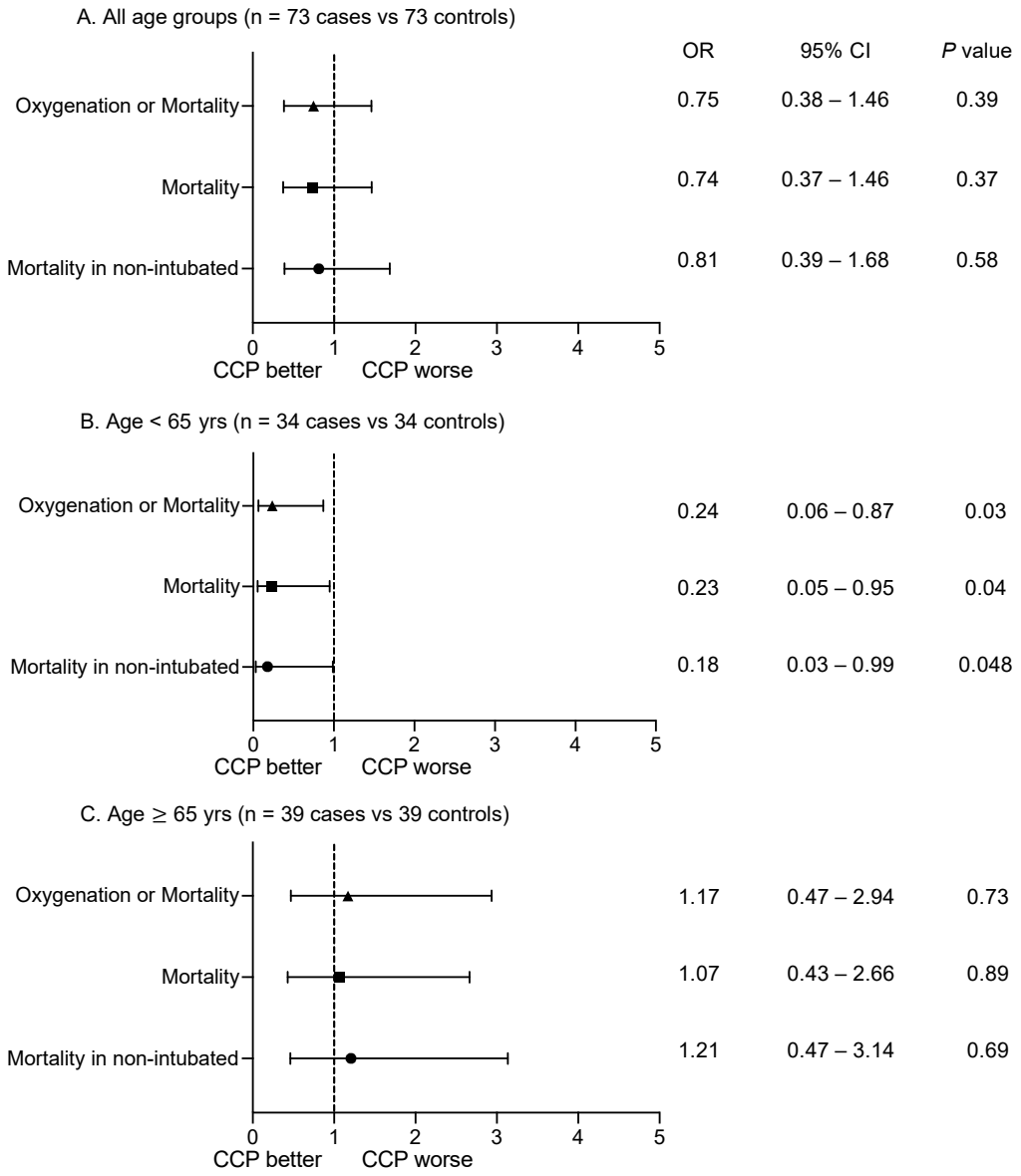


Figure 3. Day 28 outcomes for CCP recipients (n = 73) vs matched controls (n = 73) presented by odds ratio and 95% confidence intervals. A. All age groups (n = 73 cases vs 73 controls). B. Age < 65 years (n = 34 vs 34). C. Age ≥ 65 years (n = 39 vs 39). CCP, COVID-19 convalescent plasma; CI, confidence interval; OR, odds ratio.

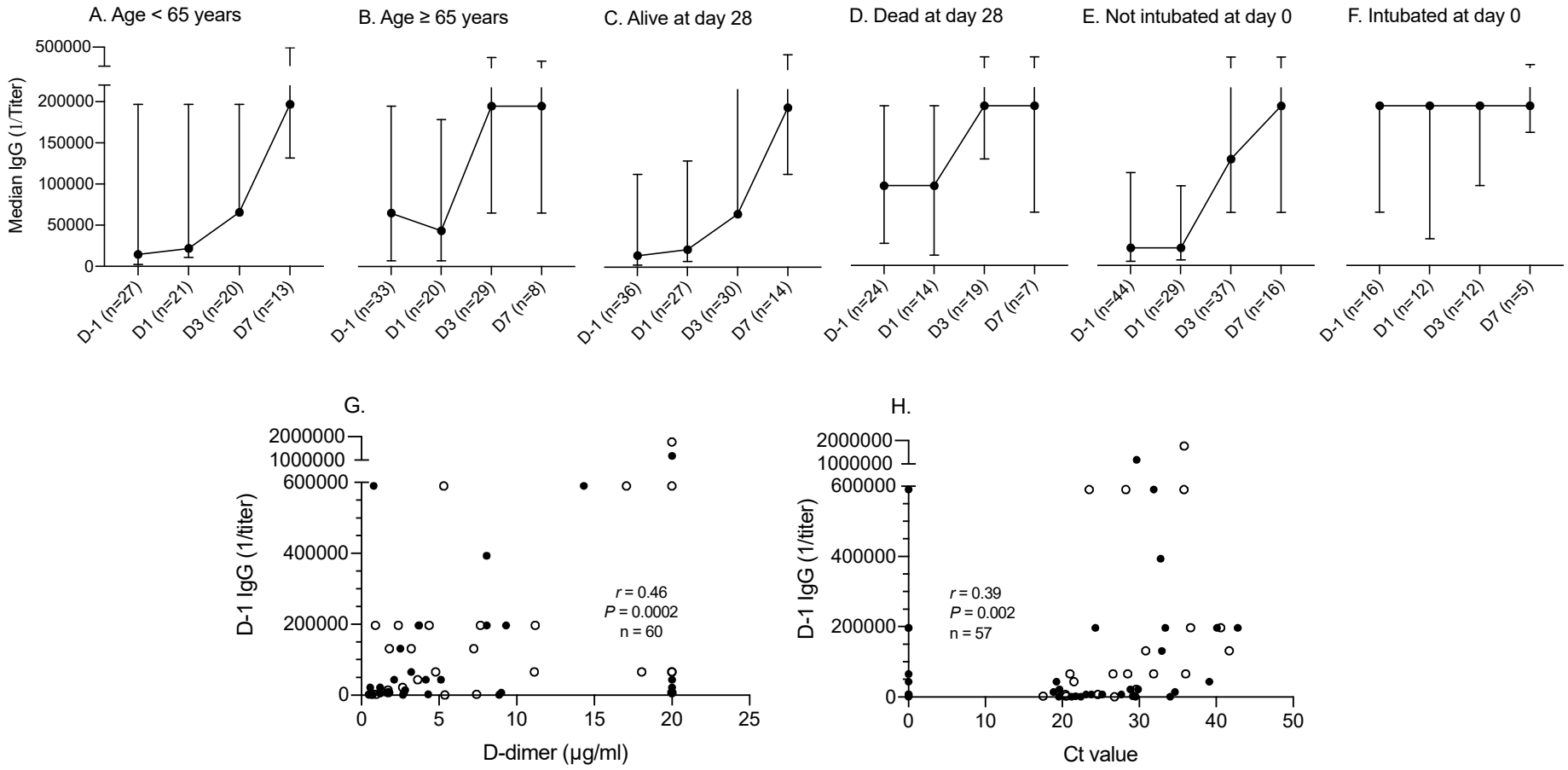


Figure 4. SARS-CoV-2 spike protein IgG titers determined by ELISA at baseline (Day-1 or D-1) and 1, 3 and 7 day after transfusion (D1, D3 and D7) in CCP recipients. (A) Age < 65 years. (B) Age ≥ 65 years. (C) Alive at day 28. (D) Died by day 28. (E) Not intubated on day of transfusion. (F) Intubated on day of transfusion. Correlation between baseline spike protein IgG titer and (G) D-dimer and (H) cycle threshold (Ct) value from initial nasopharyngeal SARS-CoV-2 RT-PCR in CCP recipients. The median titers and interquartile ranges are shown on the y-axis for each time point shown on the x-axis (A-F). X-axis shows days relative to convalescent plasma transfusion (A-F). Open circles show patients who died by day 28 (G, H). r: Spearman's correlation coefficient. Ct value, cycle threshold value.

Table 1. Baseline characteristics and outcomes in CCP recipients transfused by admission day 3 (n = 73) and propensity score matched controls (n = 73).

	All patients			**< 65 yrs**			**≥ 65 yrs**		
Characteristic	**Case (n=73; 64 not on MV, 9 on MV)**	**Controls (n=73; 64 not on MV, 9 on MV)**	***P* value**	**Case (n=34; 28 not on MV, 6 on MV)**	**Controls (n=34; 28 not on MV, 6 on MV)**	***P* value**	**Case (n=39; 36 not on MV, 3 on MV)**	**Controls (n=39; 36 not on MV, 3 on MV)**	***P* value**
Median age (IQR) - yr	67 (55-75)	66 (56-77)	0.80	55 (50-57)	55 (49-59)	0.84	75 (69-82)	76 (71-81)	0.63
Not on MV	68 (55-77)	67 (56-77.5)	0.87	54.5 (48.5-59)	55.5 (48.5-60)	0.86	75 (70-82)	76.5 (71-82)	0.80
On MV	56 (55-66)	59 (53-72)	0.93	55.5 (50-56)	53.5 (49-59)	0.87	69 (66-78)	75 (72-78)	0.38
Age category – no. (%), yr									
<45	7 (9.6)	6 (8.2)	0.97	7 (20.6)	6 (17.7)	0.76	0	0	
45 to <65	27 (36.9)	28 (38.4)		27 (79.4)	28 (82.4)		0	0	
65 to <75	19 (26.0)	17 (23.3)		0	0		19 (48.7)	17 (43.6)	0.82
75 to <85	14 (19.2)	14 (19.2)		0	0		14 (35.9)	14 (35.9)	

≥85	6 (8.2)	8 (10.9)		0	0		6 (15.4)	8 (20.5)	
Sex, BMI									
Male – no. (%)	41 (56.2)	47 (64.4)	0.31	21 (61.8)	22 (64.7)	0.80	20 (51.3)	25 (64.1)	0.25
Body-mass index, median (IQR)[A]	28.3 (23.7-33.5)	27.4 (23.2-31.6)	0.32	29.6 (27.6-34.7)	28.1 (24.8-34.3)	0.52	26.9 (21.9-32.8)	25.8 (22.9-28.5)	0.58
Ethnicity[B]– no. (%)									
Hispanic	38 (52.1)	36 (49.3)	0.45	16 (47.1)	18 (52.9)	0.39	22 (56.4)	18 (46.2)	0.68
Non-Hispanic	24 (32.9)	30 (41.1)		11 (32.4)	13 (38.2)		13 (33.3)	17 (43.6)	
Unknown	11 (15.1)	7 (9.6)		7 (20.6)	3 (8.8)		4 (10.3)	4 (10.3)	
Race[B]– no. (%)									
White	6 (8.2)	6 (8.2)	0.93	3 (8.8)	4 (11.8)	0.56	3 (7.7)	2 (5.1)	0.79
African American	19 (26.0)	17 (23.3)		12 (35.3)	8 (23.5)		7 (17.9)	9 (23.1)	
Others/Unknown	48 (65.8)	50 (68.5)		19 (55.9)	22 (64.7)		29 (74.4)	28 (71.8)	
Coexisting disorder – no. (%)									
Hyperlipidemia	42 (57.5)	44 (60.3)	0.74	17 (50.0)	15 (44.1)	0.63	25 (64.1)	29 (74.4)	0.33
Hypertension	60 (82.2)	62 (84.9)	0.66	24 (70.6)	27 (79.4)	0.40	36 (92.3)	35 (89.7)	0.69

Coronary artery disease	10 (13.7)	13 (17.8)	0.49	2 (5.9)	4 (11.8)	0.67	8 (20.5)	9 (23.1)	0.78
Congestive heart failure	15 (20.6)	19 (26.9)	0.43	4 (11.8)	5 (14.7)	0.72	11 (28.2)	14 (35.9)	0.47
Chronic pulmonary disease	26 (35.6)	22 (30.1)	0.48	10 (29.4)	7 (20.6)	0.40	16 (41.0)	15 (38.5)	0.82
Chronic kidney disease	24 (32.9)	31 (42.5)	0.23	8 (23.5)	9 (26.5)	0.78	16 (41.0)	22 (56.4)	0.17
Obesity (BMI ≥ 30)	29 (39.7)	22 (30.1)	0.22	16 (47.1)	15 (44.1)	0.81	13 (33.3)	7 (17.9)	0.12
Morbid obesity (BMI ≥ 35)	14 (19.2)	10 (13.7)	0.37	7 (20.6)	7 (20.6)	1.00	7 (17.9)	3 (7.7)	0.18
Diabetes	33 (45.2)	34 (46.6)	0.87	11 (32.4)	11 (32.4)	1.00	22 (56.4)	23 (58.9)	0.82
Pharmacologic interventions – no. (%)									
Corticosteroids	67 (91.8)	64 (87.7)	0.41	29 (85.3)	30 (88.2)	0.72	38 (97.4)	34 (87.2)	0.09
Therapeutic anticoagulation	56 (76.7)	47 (64.4)	0.10	18 (52.9)	26 (76.5)	**0.04**	29 (74.4)	30 (76.9)	0.79

Oxygen on the day of transfusion – no. (%)									
Low-flow oxygen, 5L - 15L	53 (72.6)	53 (72.6)	1.00	23 (67.7)	23 (67.7)	1.00	30 (76.9)	30 (76.9)	1.00
High-flow oxygen or NIV	11 (15.1)	11 (15.1)		5 (14.7)	5 (14.7)		6 (15.4)	6 (15.4)	
MV	9 (12.3)	9 (12.3)		6 (17.7)	7 (17.7)		3 (7.7)	5 (7.7)	
Laboratory values on the day of transfusion, median (IQR)									
Lymphocyte count, $x10^9$/L	0.8 (0.5-1.2)	0.9 (0.7-1.2)	0.97	0.9 (0.7-1.2)	0.9 (0.7-1.2)	0.44	0.8 (0.5-1.0)	1.0 (0.6-1.2)	**0.03**
Not on MV	0.8 (0.6-1.1)	0.9 (0.7-1.2)	0.12	0.9 (0.7-1.2)	0.9 (0.7-1.2)	0.90	0.8 (0.5-1.0)	1.0 (0.6-1.4)	**0.04**
On MV	1.2 (0.5-3.9)	1.2 (0.9-1.2)	0.72	1.3 (0.8-3.9)	1.1 (0.9-1.3)	0.47	0.5 (0.2-8.8)	1.2 (0.8-1.2)	0.51
D-dimer, µg/ml	2.8 (1.2-8.1)	2.9 (1.4-6.4)	0.83	1.5 (0.7-7.7)	2.3 (1.3-5.1)	0.27	3.7 (2.1-10.9)	3.2 (1.9-7.4)	0.41
Not on MV	2.8 (1.2-7.5)	3.1 (1.4-7.1)	0.51	1.2 (0.6-3.0)	2.3 (1.2-6.3)	0.09	3.8 (2.1-9.9)	3.3 (2.1-7.9)	0.43
On MV	7.7 (2.2-9.3)	2.1 (1.6-3.6)	0.20	7.9 (2.3-9.3)	2.2 (1.6-3.6)	0.15	2.2 (1.0-17.1)	2.1 (1.2-4.3)	0.83

C-reactive protein, mg/dL	17.4 (11.1-27.9)	17.8 (8.3-28.5)	0.68	16.2 (10.6-24.2)	17 (7.1-27.6)	0.97	18.8 (14.8-28.8)	18.2 (8.5-28.7)	0.52
Not on MV	18.1 (11-28.2)	18.2 (8.1-28.9)	0.86	16.2 (9.6-23.3)	14.5 (6.8-29.2)	0.92	19.5 (14.9-28.9)	18.5 (8.8-28.8)	0.68
On MV	14.9 (11.2-23.1)	12 (9.1-20.1)	0.56	16.5 (11.5-28.0)	18.6 (9.1-25)	0.87	11.9 (8.7-15)	9.6 (4.3-12.0)	0.56
Mortality Outcomes – no. (%)									
Day 28 mortality	23 (31.5)	28 (38.4)	0.39	3 (8.8)	10 (29.4)	**0.03**	20 (52.6)	17 (45.9)	0.56
Not on MV	21 (32.8)	24 (37.5)	0.58	2 (7.1)	8 (28.6)	**0.04**	19 (52.8)	16 (44.4)	0.48
On MV	2 (22.2)	4 (44.4)	0.32	1 (14.2)	3 (42.8)	0.23	1 (33.3)	2 (66.7)	0.41
Day 28 clinical status – no. (%)									
Stable/better	47 (64.4)	42 (57.5)	0.39	30 (88.2)	22 (64.7)	**0.02**	17 (43.6)	20 (51.3)	0.49
Worse/dead	26 (35.6)	31 (42.5)		4 (11.8)	12 (35.3)		22 (56.4)	19 (48.7)	

Abbreviations: BMI, body mass index; IQR, interquartile range; MV, mechanical ventilation; NIV, noninvasive ventilation

A. Body-mass index is the weight in kilograms divided by the square of the height in meters.

B. Information on race and ethnic group was obtained from entries in the medical record, as reported by the patients.

Table 2. Univariate and Multivariable analysis of mortality at day 28 in all CCP recipients (n = 103).

Univariate	**OR (95% CI)**	***P* value**
Pre-transfusion spike protein IgG (1/titer) (n = 60)	1.36 (1.04 – 1.78)	**0.03**
Pre-transfusion spike protein IgM (1/titer) (n = 59)	1.39 (1.00 – 1.92)	**0.048**
Pre-transfusion spike protein IgA (1/titer) (n = 59)	1.45 (1.10 – 1.91)	**0.007**
Multivariable[A]	**Adjusted OR (95% CI)**	***P* value**
Age (years)	1.13 (1.07 – 1.20)	**<0.0001**
Ethnicity		
Hispanic/Latinx	ref	
Not Hispanic/Latinx	0.12 (0.03 – 0.59)	**0.009**
Unknown	2.54 (0.36 – 17.8)	0.35
Time from symptom onset to transfusion (days)	1.12 (1.01 – 1.24)	**0.04**
Week of admission	0.45 (0.20 – 1.00)	**0.05**

Abbreviations: CI, confidence interval; OR, odds ratio

A. Covariates included in the model were age, sex, BMI, race, ethnicity, comorbid conditions, baseline oxygen requirement, time from symptom onset to transfusion, week of admission, anticoagulation, corticosteroids use, D-dimer, and lymphocyte count.

Effectiveness of Convalescent Plasma in Indian Patients with COVID-19

Sandeep Budhiraja[1,6], Arun Dewan[2], Ritesh Aggarwal[2], Omender Singh[2], Deven Juneja[2], Sangeeta Pathak[3], Y P Singh[2], Ajay Gupta[2], Reeta Rai[3], Abhaya Indrayan[4], Vinitaa Jha[1], Rahul Naithani[5]

[1]Clinical Directorate, Max Healthcare, New Delhi; [2]Critical Care, Max Healthcare, New Delhi; [3]Transfusion Medicine, Max Healthcare, New Delhi; [4]Division of Bio Statistics, Max Healthcare, New Delhi; [5]Hematology & Bone Marrow Transplant Division, Max Healthcare, New Delhi; [6]Institute of Internal Medicine, Max Healthcare, New Delhi

Corresponding author:

Dr Sandeep Budhiraja, Clinical Directorate, Max Healthcare, 1,2 Press Enclave Marg, Saket, New Delhi 110 017

Email: sbudhiraja@maxhealthcare.com

Abstract

Background: Convalescent plasma (CP) is being used as a treatment option in hospitalized patients with COVID-19. Till date, there is conflicting evidence on efficacy of CP in reducing COVID-19 related mortality.

Objective: to evaluate the effect of CP on 28-day mortality reduction in patients with COVID-19.

Methods: We did a multi-center, retrospective case control observational study from 1st May 2020 to 31st August 2020. A total of 1079 adult patients with moderate and severe COVID-19 requiring oxygen, were reviewed. Of these, 694 patients were admitted to ICU. Out of these, 333 were given CP along with best supportive care and remaining 361 received best supportive care only.

Results: In the overall group of 1079 patients, mortality in plasma vs no plasma group was statistically not significant (22.4% vs 18.5%; p = 0.125). However, in patients with COVID-19 admitted to ICU, mortality was significantly lower in plasma group (25.5% vs 33.2%; p = 0.026). This benefit of reduced mortality was most seen in age group 60 to 74 years (26.7% vs 43.0%; p = 0.004), driven mostly by females of this age group (23.1% vs 53.5%; p = 0.013). Significant difference in mortality was observed in patients with one comorbidity (22.3% vs 36.5%; p = 0.004). Moreover, patients on ventilator had significantly lower mortality in the plasma arm (37.2% vs 49.3%; p = 0.009); particularly so for patients on invasive mechanical ventilation (63.9% vs 82.9%; p = 0.014).

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Conclusion: The use of CP reduced mortality in COVID-19 elderly patients admitted in ICU, above 60 years of age, particularly females, those with comorbidities and especially those who required some form of ventilation.

Key words: Convalescent plasma therapy, COVID-19, ICU cases, Mortality, Subgroup analysis

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-COV-2), the novel coronavirus causing COVID-19, originated from Wuhan, China and has spread rapidly across the globe[1]. As no definitive treatment options are currently available for COVID-19, researchers the world over have been investigating a variety of drugs like azithromycin, hydroxychloroquine, remdesivir, Tociluzimab and dexamethasone[2-6]. Some of these are repurposed drugs and have been approved by regulators of various countries to be used as "Emergency Use Approval" (EUA) or "off label" medication[7].

Convalescent plasma (CP) has been used as a passive source of antibodies against various bacterial (tetanus and diphtheria) and viral diseases (poliomyelitis, measles, mumps)[8] and influenza A H1N1[9]. CP was also considered in earlier pandemics of Spanish flu, West Nile Virus, Middle East Respiratory Syndrome (MERS), Severe Acute Respiratory Syndrome (SARS), and more recently Ebola virus[10,11,12]. The convalescent plasma therapy (CPT) for COVID-19 has also been recently approved by US FDA and Indian Central Drugs Standard Control Organization[13]. It has been approved by the Ministry of Health and Family Welfare (MoHFW), Govt. of India, as "off label" use in patients with moderate and severe COVID-19 who are not improving and have increasing oxygen requirement despite use of steroids[7].

Evidence suggests that CP contains receptor binding domain specific antibodies which have potent antiviral activity[14-15]. Use of convalescent plasma, is known to be a well-tolerated with only a few easily managed adverse effects[16].

There have been, till date, few larger randomized controlled trials (RCT)[17,18], some retrospective observational studies[16,19,20] and many small case reports[21,22,23] on the benefits of CPT in COVID-19 patients with conflicting results. There is still no clarity whether CPT offers mortality benefit and, if yes, in which category of COVID-19 patients. The present study was designed to answer some of these questions.

Patients and Methods

Study Design

This is a multicentre, retrospective, observational case control study. COVID-19 patients admitted at various tertiary care teaching hospitals in Delhi of the same network, with electronic heath records (EHR), were included. Clinical and laboratory data were retrieved from EHR. Patients admitted from 01 May 2020 to 31 Aug 2020, with moderate and severe and / or

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

life threatening COVID-19, who needed oxygen therapy, were included. Of a total of 1249 such patients admitted during this period, 170 patients were excluded due to unavailability of sufficient clinical data. Remaining 1079 patients were included in the final analysis. All patients in either arm received various treatments as per physician discretion, institutional protocol or national guidelines for the management of COVID-19,issued by the government from time to time. A variety of medicines were used in these patients and included hydroxychloroquine (HCQ), azithromycin, doxycycline, ivermectin, remdesivir, other broad spectrum antibiotics, steroids (methylprednisolone or dexamethasone), tocilizumab and oxygen support/ventilation as required. Figure 1 shows the CONSORT diagram. All were followed for 28 days after discharge by tele-calling. The detailed analysis was performed on 694 patients admitted to ICU – 333 of these received plasma in addition to best supportive care and the other 361 were on best supportive care only.

Majority of COVID-19 patients who were given CP came from one major hospital complex, which contributed to 239 out of 333 (71.8%) patients. The physicians in this hospital had started using CP from very early days of COVID-19 outbreak in India and, because of this, significant use was observed in this hospital complex. Other hospitals, where either the usage of CP or availability of CP, was low, contributed 28.2% of plasma cases and a greater number of no plasma cases. Otherwise, the standard of care in all hospitals, across the network remained uniform.

Inclusion Criteria

Eligible patients included adults (> 18 years) with evidence of SARS-COV-2 infection by RT-PCR test of nasopharyngeal or oropharyngeal swab. Criteria for classifying patients into moderate and severe category were as per the clinical management protocols of MoHFW[7].

Moderate disease: pneumonia with no signs of severe disease, and having one or more:

- Dyspnoea, fever and cough
- SPO2 < 94% on room air
- Respiratory rate > 24 / minute

Severe disease: patient having one or more of the following conditions:

- Clinical signs of pneumonia plus one of the following: respiratory rate > 30/min or severe respiratory distress requiring ventilation or SPO2 < 90% on room air.
- ARDS (new onset bilateral opacities & SpO2 / FiO2 ≤ 315)
- Sepsis
- Septic shock

Based on these criteria, we selected only patients who needed oxygen therapy.

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Donor Selection Criteria[24]

Donors had to meet the following criteria: 1) prior diagnosis of COVID-19 documented by a laboratory test, 2.a) complete resolution of symptoms at least 28 days prior to donation or 2.b) complete resolution of symptoms at least 14 days prior to donation. In such cases, if the patient has been discharged without negative RT-PCR for COVID-19, 2 samples of naso- and oropharyngeal swab have to be sent for RT-PCR for COVID-19, collected 24 hours apart and negative result ascertained before considering the recovered patients as a potential donor. 3) only males and nulliparous female donors of weight > 55 kg, 4) Donor with Hb>12.5g/dL and platelet count > 150 000 per microliter of blood and TLC within normal limits, 5) HIV, HBV and HCV by serology and NAT negative patients, 6) syphilis and malaria negative donors and 7) total serum protein > 6 g/dL all donor selection criteria for blood donation were followed as per Drugs and Cosmetics (second Amendment) Rules, 2020[25] .

In the absence of a standard viral neutralization test, and limited possibility of assessment of neutralizing antibody titres of > 1:640, we adopted a policy of the use of an assay to assess quantitative antibodies of anti-SARS COV2 antibodies prior to harvest of CP from the eligible donors. Antibody testing was done prospectively in donors from mid June onwards, prior to which donor samples were stored at -80° C and were retrospectively tested. For the 36 donors who's assays were done retrospectively, 12% were found have inadequate antibodies. In our larger centre, the available platform which we used was an FDA approved (Diaosorin), Quantitative IgG antibody assay against the S1&S2 protein of SARS COVID-2 virus. The cut-off of 15 AU/ml was taken for a positive result which is equivalent to 1:40 Plaque Neutralization antibody & 80 AU/ml is equivalent to 1:160 plaque neutralizing antibody assay. In the other two smaller centres, Ortho Clinical Diagnostic Vitros Anti SARS-COV-2 IgG Chemiluminescent Immuno Assay was used. The Ortho Clinical IgG is a qualitative assay. Result of this assay are based on the sample signal-to-cut-off (S/CO) ratio, with values <1.0 and ≥ 1.0 corresponding the Negative and Positive results.

Volume of donated plasmas was 400 ml. The first 200 ml (one unit) plasma was infused over one to one and half hours and if the patient did not show any improvement after 24 h, based on the decision of responsible physician, another unit of plasma was administered.

Exclusion Criteria

Patients not requiring oxygen therapy were excluded. Pregnant and lactating mothers were also excluded.

Objectives

Primary objective of the study was to study 28-day mortality in patients who received convalescent plasma therapy versus those who did not.

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Secondary objectives were to assess if CP reduced the chances of patients going on to invasive ventilation and whether it offered any mortality benefits in critically ill patients on ventilator and vasopressors.

Statistical Analysis

Baseline characteristics such as age, gender, and comorbidities in the plasma and no plasma group were compared by chi-square test. The number of patients on invasive and non-invasive ventilators and mortality in plasma and no plasma group in various subgroup of patients were also compared with chi-square test. P-value < 0.05 was considered statistically significant. SPSS21 was used for statistical calculations. The sample size in the two groups was adequate to detect a difference of at least 10% in overall mortality between plasma and no plasma group with power of 80% when the mortality in ICU cases in the standard care group is 25%.

Results

The mean (SD) age of patients was 60.1 $\pm$ 12.1 years vs 58.9 $\pm$ 13.8 years in plasma vs no plasma group (p = 0.231). However, the age-gender distribution and the comorbidity-gender distribution was different for the following subgroups (Table I).

Males were significantly higher in the plasma group (80.2% vs 72.3%) (p = 0.015) and less of younger age (12.3% vs 19.2%) (p = 0.023). There were more females in the plasma group with multiple comorbidities, than in the no plasma group. Specific comorbidities examined were hypertension (HTN), diabetes mellitus (DM), coronary artery disease (CAD) and hypothyroidism. These were the most commonly occurring comorbidities in these cases. All other comorbidities (CKD, cancer, stroke, etc.) were reported in less than 10% cases. Hypertension was more common in plasma group (54.7% vs 43.5%, p = 0.003) and CAD less common (7.5% vs 14.1%, p = 0.005). However, the difference in mortality in these subgroups was not statistically significant.

The percentage of patients on ventilator, invasive and non-invasive, and on vasopressor were not significantly different (minimum p = 0.166) between the two groups (Table 2).

For the total of 1079 patients on oxygen, overall mortality in patients was 22.4% in the plasma group and 18.5% in the no plasma group (p = 0.125). Total 694 (64.3%) patients required transfer to intensive care unit (ICU). Three hundred thirty-three (48.0%) patients received convalescent plasma in addition to the best supportive care and the remaining 361 (52.0%) were on best supportive care only. In the ICU patients, mortality was 25.5% vs 33.2% in plasma vs no plasma group (p = 0.026). Reduced mortality in plasma group was particularly seen in patients of age 60–74 years (26.7% vs 43.0%, p = 0.004), driven mostly by females of this age group (23.1% vs 53.5%, p = 0.013).

In one of the main hospitals, where the experience with the use of CP was maximum, out of a total of 342 patients in ICU, 239 (69.8%) received plasma while 103 (30.1%) did not. Overall

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

mortality in the plasma group was 49/239 (20.5%) as compared to 36/103 (36.9%) in the no plasma group (p = 0.0014).

Significant difference in mortality was observed in patients with one comorbidity (22.3% vs 36.5%, p = 0.004). This also was primarily due to females in this subgroup (mortality 6.7% vs 49.1%, p < 0.001) in the plasma vs no plasma group (Table 3). We observed a similar reduction in mortality in patients with 2 comorbidities (35.1% vs 49.2%) but this did not reach statistical significance (p=0.099). The mortality in patients with specific comorbidity such as hypertension, diabetes and coronary artery disease were not different (least p = 0.099) but was significantly different in patients with hypothyroidism (19.0% vs 42.4%, p = 0.027). Of 38 women of age 60–74 years with one comorbidity, 12 were in plasma group and 26 in the no plasma group. Only one of these (8.3%) died in the plasma group and 13 (50.0%) in the no plasma group (p = 0.013).

Out of a total of 694 patients with severe COVID-19 in ICU, 101 (14.6%) patients worsened on non-invasive ventilation (NIV) and needed to be shifted to invasive ventilator. Fifty-four (53.46%) of these patients were given CP. There were 316 (45.5%) patients, who remained on NIV only and out of these 157 (49.6%) received CP. Overall, CP did not reduce the chances of being put on invasive ventilator from NIV (p = 0.508).

Patients on ventilator had significantly lower mortality in plasma arm (37.2% vs 49.3%, p=0.009) (Table 4). This was particularly so for patients on invasive ventilator (IV) with mortality 63.9% vs 82.9% (p = 0.014), respectively in plasma vs no plasma group. Patients on an invasive ventilator and on vasopressor had high (almost 90%) mortality in both the groups (Table 4).

Twenty-six out of 333 (7.8%) received second unit of CP, as per the physician's decision. Ten of these patients (38%) died.

Safety of CPT

Convalescent plasma infusion was largely safe and no major adverse effects were observed in any of our patients. Two patients with minor allergic reactions (rashes) were reported to the Blood Bank Officer.

Discussion

The current study was undertaken to evaluate the effectiveness of CP in patients with moderate and severe COVID-19 disease. To date, this is the largest retrospective case control study from India, presently reporting world's second largest number of COVID-19 cases.

There are a number of reports comprising small number of patients treated with CP[21,22,23] from different countries across the world. Duan et al. were first to report benefit of CP in a small cohort of 10 patients with improvement in all 10 patients and undetectable viral load in 7/10 patients[21]. In contrast, Zeng et al. reported mortality in 5/6 patients who received CP[26]. Table 5

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

summarizes the literature review of relevant studies on CP, with more than 100 patients and / or RCTs.

The results of our study are quite different from that of the only other large-scale study from India - a multicentre prospective RCT PLACID trial, which included 464 patients of moderate severity COVID-19, from 39 different hospitals across India[17]. This study did not find any difference in mortality between plasma vs no plasma group (15% vs 14%), respectively. However, this study did not include very severe or life threatening COVID-19 patients. Even in our study, if we see the overall cohort of 1079 patients (moderate and severe), CP did not have any mortality benefit (22.4% vs 18.5%; p = 0.125). The benefit emerged only in more critically ill patients of COVID-19 who were in ICU (25.5% vs 33.2%; p = 0.026). This happened despite plasma group had more elderly males, where anyway the mortality is expected to be higher[27]. Also, the main hospital, which had maximum experience with the use of CP, showed a highly significant mortality benefit in severe COVID-19 (20.5% vs 36.9%; p = 0.0014).

A similar study involving moderately ill COVID-19 patients by Abolghasemi et. al, showed that significantly higher number of patients who received CP were discharged from the hospital versus control (98.2% vs 78.7%)[28]. They reported that CP significantly reduced need for mechanical ventilation in treatment versus control group (7% vs 20.3%). All-cause mortality in CP group was 14.8% versus 24.3% in control arm in the study but this was not statistically significant (p = 0.09). RCT by Ling Li et al. also showed no significant difference in time to clinical improvement or in 28-day mortality between patients who received CP vs control[18].

Study by Xia et al. also showed that there was less likelihood of patient who received CP to be transferred to ICU as compared to those who did not receive CP (2.4% vs 5.1%)[20]. Our study did not show any benefit with CP, in terms of reducing the chances of patients going onto invasive ventilation from NIV. The Indian PLACID trial also showed no benefit of CP in terms of total and post enrolment duration of respiratory support, and proportion of patients who had to be put on invasive ventilation and those needing vasopressor support[17]. In the study by Altuntas et al., the duration of stay in ICU, rate of mechanical ventilation and vasopressor requirement were lower in the CP group versus control (p = 0.001, p = 0.02, p = 0.001 respectively)[29]. They however, found no statistically significant difference in the fatality rate between the CP and control groups (24.7% vs 27.7%, p = 0.150). In our study, however patients on ventilator had significantly lower mortality in plasma arm as compared to no plasma arm (37.2% vs 49.3%, p = 0.009). This was particularly so for patients on invasive ventilator with mortality 63.9% and 82.9% (p = 0.014) in plasma and no plasma arm, respectively.

The patient outcome in the current study was quite similar to the study by Salazar et al. where they found significant mortality reduction in severe and life threatening COVID-19 patients (p = 0.047) especially when CP was given within 72 hours of admission[30].

In our study, we found maximum benefit of CP in ICU patients, in the age group 60-74 years, where the mortality in the plasma group was 26.7% versus 43% in the no plasma group (p=

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

0.004). Similar findings were reported by Rogers et al. wherein a subgroup analysis of patients above 65-years-old or greater who received CP demonstrated a significantly increased hospital discharge rate among these patients (RR 1.86, 95% CI 1.03-3.36)[31].

Significant difference in mortality between plasma and no plasma group was also observed in patients in our study with one comorbidity largely driven by females. This finding has not been reported in literature so far. The mortality in patients with specific comorbidity such as HTN, DM and CAD was not different (minimum p = 0.099) but was significantly different in patients with hypothyroidism (19% vs 42.4%, p = 0.027). This needs further investigation.

Klassen et al. aggregated patient outcome data from various RCTs, matched-control, case series and case report studies and found that CP exhibited a 51% reduction in mortality rate compared to patients receiving standard treatments (19% vs. 29% mortality; OR:0.49, 95% CI: 0.37, 0.64, p< 0.001)[32].

Our study showed mortality benefit of CP in severely ill COVID-19 patients requiring some form of ventilation and in the age group >60 years, especially women with 1-2 medical comorbidities. We do not know the exact mechanism of action of CP in COVID-19 but it seems that plasma therapy played a significant role in reduction of mortality in the highly vulnerable patient population of elderly and on ventilator. Perhaps a little push by convalescent plasma in this segment of patients was able to tilt the balance towards recovery.

Limitations of the study

It is a retrospective study. During the study duration, patients received several treatments such as HCQs, remdesevir, ivermectin, azithromycin, steroids (dexamethasone or methylprednisolone), tociluzimab depending on prevailing guidelines at those time points and at treating physician's discretion. Majority of patients in plasma arm were recruited at one centre while no plasma arm patients were at other hubs. We also do not have information if some of the patients had already developed adequate antibodies when CP was performed. These limitations notwithstanding, we are able to identify a subset of patients with COVID-19 who are likely to benefit from CP.

Conclusions

The use of convalescent plasma reduced mortality in severe COVID-19 elderly patients, above 60 years of age, particularly females, those with comorbidities and especially those who require some form of ventilation. This beneficial effect was lost when the entire cohort of patients across varying severity of illness was compared. Plasma did not seem to offer any mortality benefit in patients of moderate severity or those who were terminally ill. Further research into the mechanism of actions of CP in COVID-19 may help predict the good responders.

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Authors Contributions

SB provided overall guidance, was involved in patient care and management , data analysis and interpretation and in writing the manuscript; AD, RA, OS, DJ, YPS, AG were involved in clinical care including ICU management; SP and RR were the transfusion medicine experts and contributed in selection of plasma donor and in apheresis and maintaining records of donations and adverse events; AI was the chief bio statistician and helped in data analysis and interpretation and drafting the manuscript; VJ provided data collation oversight and drafting manuscript; RN was involved in data interpretation and writing the manuscript. All authors have seen and approved the final draft.

Acknowledgements

We wish to thank Dr. Vibha Jain, Ms. Shruti Singh, Dr. Menka Loomba, Ms. Taruna Sharma, Ms. Ratnam Shukla, Ms. Aditi Saini. Mr. Rahul Arya, Mr. Rajat Kumar Sharma, Mr. Pushpender Sharma and Ms. Kritika Chadha, for their help towards data collection and validation.

References

1. World Health Organization. 2020. WHO Director-General's Remarks at the Media Briefing on 2019-nCoV on 11 February 2020.https://www.who.int/dg/speeches/detail/who-director-general-s-remarks-at-the-media-briefing-on-2019-ncov-on-11-february-2020

2. Beigel J, Tomashek K, Dodd L et al. Remdesivir for the treatment of Covid-19 — Final Report. N Eng J Med. 2020 Oct 8; NEJMoa2007764.doi:10.1056NEJMoa2007764

3. Gautret P, Lagier JC, Parola P, et al. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. Int J Antimicrob Agents. 2020 Jul; 56(1):105949. doi: 10.1016/j.ijantimicag.2020.105949

4. Bhimraj A, Morgan RL, Shumaker AH, et al. Infectious Diseases Society of America Guidelines on the Treatment and Management of Patients with COVID-19. Clin Infect Dis. 2020 Apr:ciaa478.doi: 10.1093/cid/ciaa478

5. Villar J, Ferrando C, Domingo M, et al. Dexamethasone treatment for the acute respiratory distress syndrome: a multicentre, randomised controlled trial. Lancet Resp Med. 2020 Mar 1; 8(3):267-276.

6. Guaraldi G, Meschiari M, Cozzi-Lepri A, et al. Tocilizumab in patients with severe COVID-19: a retrospective cohort study. Lancet Rheumatol. 2020 Aug;2 (8):e474-e484

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

7. Ministry of Health & family Welfare. Clinical Management Protocol: COVID-19 version 5 [Internet]. Mohfw.gov.in. 2020 [cited 23 October 2020]. Available from: https://www.mohfw.gov.in/pdf/UpdatedClinicalManagementProtocolforCOVID19dated03072020.pdf.

8. Yongzhi Xi. Convalescent plasma therapy for COVID – 19: a tried-and-true old strategy? Signal Transduct Target Ther 2020; 5(1):203.

9. Hung IF, To KK, Lee CK, et al. Convalescent plasma treatment reduced mortality in patients with severe pandemic influenza A (H1N1) 2009 virus infection. Clin Infect Dis. 2011 Feb 15; 52(4):447-456.

10. Van Griensven, Johan et al. Evaluation of convalescent plasma for Ebola virus disease in Guinea. N Engl J Med 2016; 374(1)): 33-42.

11. Luke TC, Kilbane EM, Jackson JL, Hoffman SL. Meta-analysis: convalescent blood products for Spanish influenza pneumonia: a future H5N1 treatment? Ann Intern Med. 2006 Oct 17; 145(8):599-609.

12. Marson P, Cozza A, De Silvestro G. The true historical origin of convalescent plasma therapy. Transfus Apher Sci. 2020; 102847. doi: 10.1016/j.transci.2020.102847

13. Tanne JH. Covid-19: FDA approves use of convalescent plasma to treat critically ill patients. BMJ 2020; 368:m1256. doi: 10.1136/bmj.m1256

14. Zeng QL, Yu ZJ, Gou JJ, et al. Effect of convalescent plasma therapy on viral shedding and survival in COVID-19 patients. J Infect Dis. 2020; 222(1):38 -43.

15. Chen L, Xiong J, Bao L, Shi Y. Convalescent plasma as a potential therapy for COVID-19. Lancet Infect Dis, 2020;20(4):398- 400.

16. Joyner MJ, Bruno KA, Klassen SA, et al. Safety Update: COVID-19 Convalescent plasma in 20,000 hospitalized patients. Mayo Clin Proc. 2020;95(9):1888-1897.

17. Agarwal A, Mukherjee A, Kumar G, Chatterjee P, Bhatnagar T, Malhotra P, on behalf of PLACID Trial Collaborators. Convalescent plasma in the management of moderate Covid-19 in adults in India: open label phase II multicentre randomised controlled trial (PLACID Trial). BMJ 2020;371: m3939.http://dx.doi.org/10.1136/bmj.m3939

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

18. Li L, Zhang W, Hu Y, et al. Effect of convalescent plasma therapy on time to clinical improvement in patients with severe and life-threatening COVID-19. A Randomized Clinical Trial. JAMA 2020; 324(5): 460-470.

19. Cantore I, Valente P. Convalescent plasma from COVID 19 patients enhances intensive care unit survival rate. A preliminary report. Transfus Apher Sci. 2020:102848. doi: 10.1016/j.transci.2020.102848

20. Xia X, Li K, WU L et al. Improved clinical symptoms and mortality among patients with severe or critical COVID-19 after convalescent plasma transfusion. Blood 2020; 136(6):755–759.

21. Duan K, Liu B, Li C, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. Proc Natl Acad Sci U S A 2020; 117(17):9490-9496.

22. Shen C, Wang Z, Zhao F, et al. Treatment of 5 critically ill patients with COVID-19 with convalescent plasma. JAMA 2020; 323(16):1582-1589.

23. Hartman WR, Hess AS, Connor JP. Hospitalized COVID-19 patients treated with convalescent plasma in a mid-size city in the Midwest. Transl Med Commun 2020;5(1): 17. doi: 10.1186/s41231-020-00068-9

24. ICMR PLACID trial. A Phase II, Open Label, Randomized Controlled Trial to Assess the Safety and Efficacy of Convalescent Plasma to Limit COVID-19 Associated Complications in Moderate Disease; Convalescent Plasma in COVID-19 Version 1.5 Dated 11th May, 2020 (Cited 2020 July 23) Available from: https://cdsco.gov.in/opencms/resources/UploadCDSCOWeb/2018/UploadPublic_NoticesFiles/ICMR%20Convalescent%20plasma%20_protocol_v1.5.pdf

25. Final G.S.R. 166(E)_Amendment in Part X B & Part XII B pertains to Blood center and Blood components, Drugs and Cosmetics Act 1940 and Rules 1945, amended 11th March 2020 (accessed 2020 March 18, Cited 2020 July 23) available from : https://cdsco.gov.in/opencms/opencms/en/Notifications/Gazette-Notifications

26. Zeng F, Chen X, Deng G. Convalescent plasma for patients with COVID-19 (Letter). Proc Natl Acad Scie U S A 2020; 117(23): 12528

27. Jin JM, Bai P, He W, et al. Gender Differences in Patients With COVID-19: Focus on Severity and Mortality. Front Public Health 2020;8:152

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

28. Abolghasemi H, Eshghi P, Cheraghali AM, et al. Clinical efficacy of convalescent plasma for treatment of COVID-19 infections: Results of a multicenter clinical study. Transfus Apher Sci., 2020 Jul 15;102875. https://doi.org/10.1016/j.transci.2020.102875

29. Altuntas F, Ata N, Yigenoglu TN, et al. Convalescent plasma therapy in patients with COVID-19. Transfus Apher Sci., https://doi.org/10.1016/j.transci.2020.102955

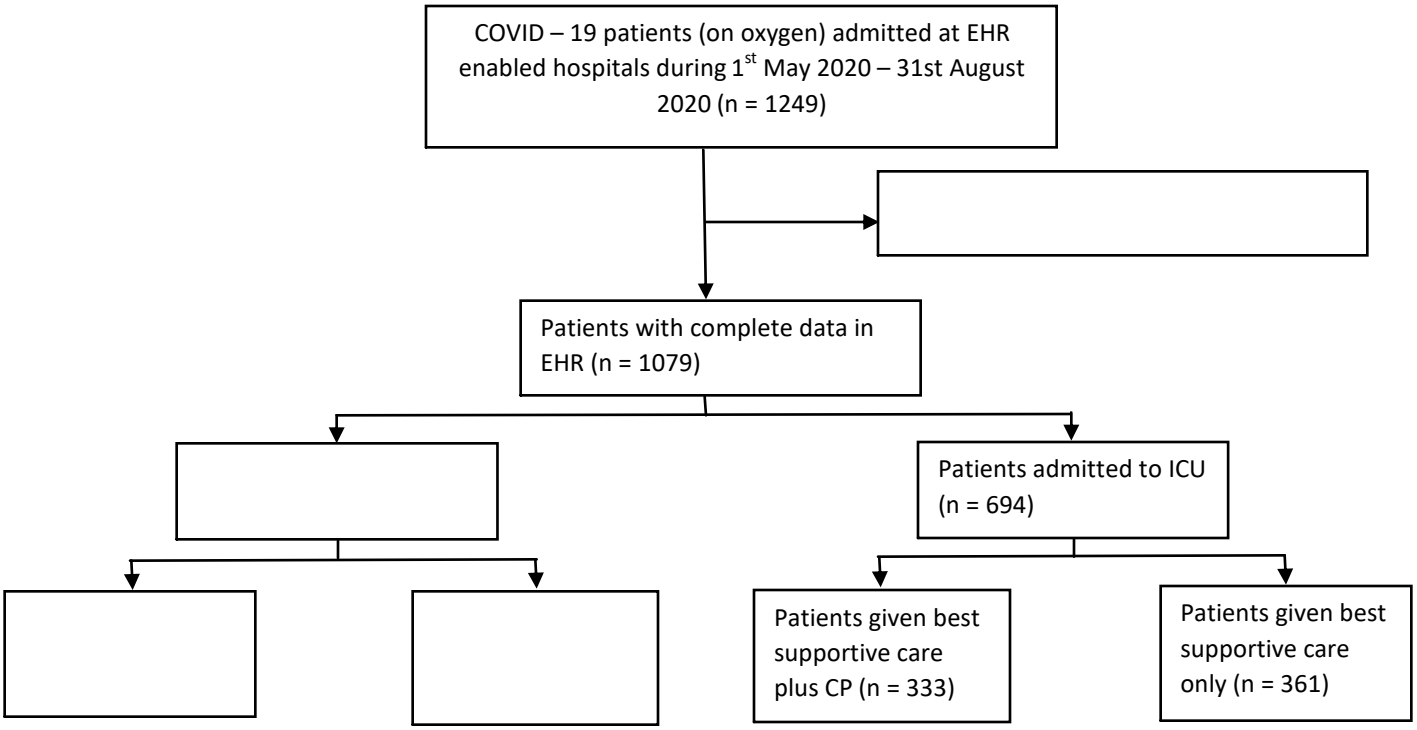
30. Salazar E, Christensen PA, Graviss EA, et al. Treatment of coronavirus disease 2019 patients with convalescent plasma reveals a signal of significantly decreased mortality. Am J Pathol. 2020;190(11):2290-2303.

31. Rogers R, Shehadeh F, Eng M, et al. Convalescent plasma for patients with severe COVID-19: a matched cohort study. https://doi.org/10.1101/2020.08.18.20177402

32. Klassen SA, Senefeld JW, Johnson PW, et al. Evidence favouring the efficacy of convalescent plasma for COVID-19 therapy. https://doi.org/10.1101/2020.07.29.20162917

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Figure 1: CONSORT Chart for the study



This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Table 1. Baseline comparison of cases in the Best Supportive Care Only Group and Plasma with Best Supportive Care Group

Characteristic	Best supportive Care Only		Plasma with Best Supportive Care		p-value
	Count	Percent	Count	Percent	
All in ICU	361	52.6%	333	84.7%	<0.001
<45	62	17.2%	40	12.0%	0.055
45-59	115	31.9%	122	36.6%	0.185
60-74	149	41.3%	135	40.5%	0.844
75+	35	9.7%	36	10.8%	0.628
Males	261	72.3%	267	80.2%	0.015
<45	50	19.2%	32	12.3%	0.023
45-59	80	30.7%	96	36.8%	0.196
60-74	106	40.6%	109	41.8%	0.961
75+	25	9.6%	30	11.5%	0.533
Females	100	27.7%	66	19.8%	Same as for males
<45	12	12.0%	8	12.1%	0.981
45-59	35	35.0%	26	39.4%	0.566
60-74	43	43.0%	26	39.4%	0.645
75+	10	10.0%	6	9.1%	0.846
No: of Comorbidities	361	52.6%	333	84.7%	<0.001
None	92	25.5%	72	21.6%	0.231
1	181	50.1%	166	49.8%	0.939
2	61	16.9%	74	22.2%	0.077
3+	27	7.5%	21	6.3%	0.543
Males	261	72.3%	267	80.2%	0.015
None	72	27.6%	63	23.6%	0.293
1	128	49.0%	136	50.9%	0.663
2	45	17.2%	53	19.9%	0.441
3+	16	6.1%	15	5.6%	0.802
Females	100	27.7%	66	19.8%	Same as for males
None	20	20.0%	9	13.6%	0.291
1	53	53.0%	30	45.5%	0.341
2	16	16.0%	21	31.8%	0.017
3+	11	11.0%	6	9.1%	0.691
Specific Comorbidity M+F					
HTN	157	43.5%	182	54.7%	0.003
DM	66	18.3%	61	18.3%	0.990
CAD	51	14.1%	25	7.5%	0.005
Hypothyroidism	33	9.1%	42	12.6%	0.141

HTN: Hypertension; DM: Diabetes mellitus; CAD: Coronary artery disease

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Table 2. Need for ventilation and / or vasopressors in Best Supportive Care Only Group and Plasma with Best Supportive Care Group

Characteristic	Best Supportive Care Only (*n* =361)		Plasma with Best Supportive Care (*n* = 333)		p-value
	Count	Percent	Count	Percent	
No Ventilator	132	36.6%	115	34.5%	0.577
Any Ventilator	229	63.4%	218	65.5%	
Non-Invasive (NIV)	159	69.4%	157	72.0%	0.412
Invasive (IV)	70	30.6%	61	28.0%	0.548
Vasopressor in IV group	36	51.4%	24	39.3%	0.166
NIV to IV (out of IV group)	47	20.5%	54	24.8%	0.283
Vasopressor in NIV to IV group	23	48.9%	23	42.6%	0.523

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Table 3. Mortality in various groups in cases on Best Supportive Care Only and cases on Plasma with Best Supportive Care

Characteristic	Best Supportive Care Only (*n* = 361)		Plasma with Best Supportive Care (*n* = 333)		p-value
	Deaths*	Mortality	Deaths*	Mortality	
All in ICU	120	33.2%	85	25.5%	0.026
<45	14	22.6%	7	17.5%	0.536
45-59	27	23.5%	28	23.0%	0.923
60-74	64	43.0%	36	26.7%	0.004
75+	15	42.9%	14	38.9%	0.734
Male	73	28.0%	72	27.0%	0.796
<45	9	18.0%	6	18.8%	0.932
45-59	13	16.3%	24	25.0%	0.156
60-74	41	38.7%	30	27.5%	0.082
75+	10	40.0%	12	40.0%	1.000
Female	47	47.0%	13	19.7%	<0.001
<45	5	41.7%	1	12.5%	0.325
45-59	14	40.0%	4	15.4%	0.037
60-74	23	53.5%	6	23.1%	0.013
75+	5	50.0%	2	33.3%	0.633
No: of Comorbidities					
None	16	17.4%	14	19.4%	0.736
1	66	36.5%	37	22.3%	0.004
2	30	49.2%	26	35.1%	0.099
3+	8	29.6%	8	38.1%	0.537
Males	73	28.0%	72	27.0%	0.796
None	8	11.1%	12	19.0%	0.195
1	40	31.3%	35	25.7%	0.321
2	19	42.2%	20	37.7%	0.651
3+	6	37.5%	5	33.3%	0.809
Females	47	47.0%	13	19.7%	<0.001
None	8	40.0%	2	22.2%	0.351
1	26	49.1%	2	6.7%	<0.001
2	11	68.8%	6	28.6%	0.015
3+	2	18.2%	3	50.0%	0.169
Specific Comorbidity M+F					
HTN	59	37.6%	53	29.1%	0.099
DM	17	25.8%	18	29.5%	0.636
CAD	24	47.1%	11	44.0%	0.802
Hypothyroidism	14	42.4%	8	19.0%	0.027

*Deaths out of cases mentioned in Table 1 for the respective subgroups
HTN: Hypertension; DM: Diabetes mellitus; CAD: Coronary artery disease

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Table 4. Comparative Mortality of patients on ventilation & vasopressor between Best Supportive Care Only Group & Plasma with Best Supportive Care Group

Characteristic	Best Supportive Care Only (*n* = 361)		Plasma with Best Supportive Care (*n* = 333)		p-value
	Deaths*	Mortality	Deaths*	Mortality	
No Ventilator	7	5.3%	4	3.5%	0.488
Any Ventilator	113	49.3%	81	37.2%	0.009
Non-Invasive (NIV)	55	34.6%	42	26.8%	0.131
Invasive (IV)	58	82.9%	39	63.9%	0.014
Vasopressor with invasive ventilation (IV)	33	91.7%	21	87.5%	0.675

*Deaths out of cases mentioned in Table 2 in the respective rows

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

Table 5. Review of various studies (larger RCTs and/or > 100 matched cases) on effectiveness of Convalescent Plasma in COVID-19

Ref	Number of patients	Study	Patient population	Antibody titre used	Results	Adverse Effects	Author Conclusions	Our comments
Ling Li et al.[18]	103	Open label RCT, multicentre	23 vs 22 Severe 29 vs 29 Life-threatening COVID-19	1:640	No significant difference in time to clinical improvement or in 28 days mortality between patients who received CP versus control	Chills and rashes -1 shortness of breath, cyanosis, and severe dyspnea -1	CP not effective	Not powered to predict the difference
Abolghasemi H et al.[28]	189 115 in plasma group and 74 in control group	Multicentre nonrandomized clinical study	Moderate severity	Antibody titer cut off > 1.1 using semi quantitative ELISA and Rapid strip test	CP reduced all cause mortality in treatment group compared with control group (14.8% vs 24.3%); however this was not statistically significant (p=0.09). it had a significant impact of length of stay (p=0.002) and need for intubation (p=0.006)		CP effective	Control group was much smaller and had milder cases
Salazar E et al.[30]	136 cases and 251 controls	Prospective propensity matched study	Severe and/or life threatening COVID-19	Anti-RBD IgG titre of 1:1350	Significant reduction (p= 0.047) in mortality within 28 days, specifically in patients transfused within 72 hours of admission with CP with anti-RBD IgG titre of > 1:1350		Transfusion of high anti-RBD IgG titre CP early in hospitalisation reduces mortality	
Xia X et al.[20]	138 cases and 1568 controls	Retrospective observational study	Severe or critical COVID-19		Mortality in CP group was 2.2% as compared to 4.1% in non CP group also from CP group, only 2.4% got shifted to ICU whereas in non CP group, 5.1% patients got ICU transfer	3 patients had minor allergic reactions (pruritus or erythema) during transfusion	Patients with SCSS > 5 before therapy showed no improvement after CP.	Authors classified patients who were given CP into responders, partial responders and nonresponders.
Altuntas F et al.[29]	Cases 888 Control 888	Retrospective case control study	Severe and critically ill COVID-19	IgG antibody not routinely done	Duration in ICU, rate of mechanical ventilation support and vasopressor support were lower in CP group (p = 0.001, p = 0.02, p = 0.001, respectively).	Not available		Antibody titres of plasma donors were not available

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

					CFR was 24.7 in CP group and 27.7 in control group (p = 0.15)			
Agarwal A et al & PLACID trial collaborators[17]	Cases: 235 Controls: 229	Open label parallel are phase II Multicentre randomized controlled trial	Moderately ill COVID- 19 patients	Nearly 2 / 3rd of donor had NAb titre > 1:20 with a median titre of 1:40	Mortality in intervention arm was 14.5% vs 13.5% in control arm (odd ratios was 1.06). There was no difference in the 2 arms with respect to duration of respiratory support, proportion of patient requiring invasive ventilation and vasopressor support	Minor adverse event of pain, chills, nausea in one patient; fever and tachycardia in 3 patients; dyspnoea and IV catheter blockage in 2 patients each. Mortality was assessed as possibly related to CP in 3 patients (1.3%).	CP not effective	Three reported cases of mortality related to CP raised a significant concern regarding safety profile of CP.
OUR STUDY	1079 (total cases in ward and ICU) In ICU : 694 Plasma group in ICU :333 No plasma group in ICU : 361	Retrospective case control observational study	Moderate and Severe COVID-19	Quantitative IgG antibody assay with cut off 15 AU/ml equivalent to 1:40 plaque neutralization antibody	Overall no statistically significant difference in mortality between CP and non CP group (22.4% vs 18.5%; p=0.125. However, significantly reduced mortality in COVID-19 patients admitted in ICU (25.5% vs 33.2%; p=0.026)especially in age group 60-74 (26.7% vs 43%; p=0.004), females (23.1% vs 53.5%; p=0.013), those with one comorbid medical condition(22.3% vs 36.5%; p=0.004) and on invasive mechanical ventilation (63.9%vs 82.9%; p =0.014)	2 patients had mild allergic reaction (rashes) during plasma transfusion	Highest mortality benefit with CP seen in COVID-19 patients above 60 years of age, admitted in ICU, especially females and those on ventilator and having medical comorbid conditions.	

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=3726179

NIBSC

Calibration of secondary standards to WHO International Standards

Peter Rigsby, Analytical & Biological Sciences, NIBSC

Medicines & Healthcare products Regulatory Agency

WHO International Standards (IS)

- World Health Organization International Standards: highest order standards used to enable biological & immunological assay results to be expressed in the same way worldwide
- WHO Expert Committee on Biological Standardization (ECBS) establishes and assigns a value to an IS
- “An international collaborative study must be carried out before any candidate biological reference standard can be considered for establishment by the WHO ECBS” *(WHO Technical Report Series, No. 932, 2006)*

WHO International Standards (IS)

- Most IS define International Units (IU) of biological activity
  - Arbitrary units representing content of ampoule or vial; no uncertainty assigned
  - Often not dependent on assay method used
- Often lyophilized, giving highly stable preparations

- Not intended for routine use
  - Secondary standards calibrated directly against (and traceable to) the relevant IS are required

Guidance documents

World Health Organization

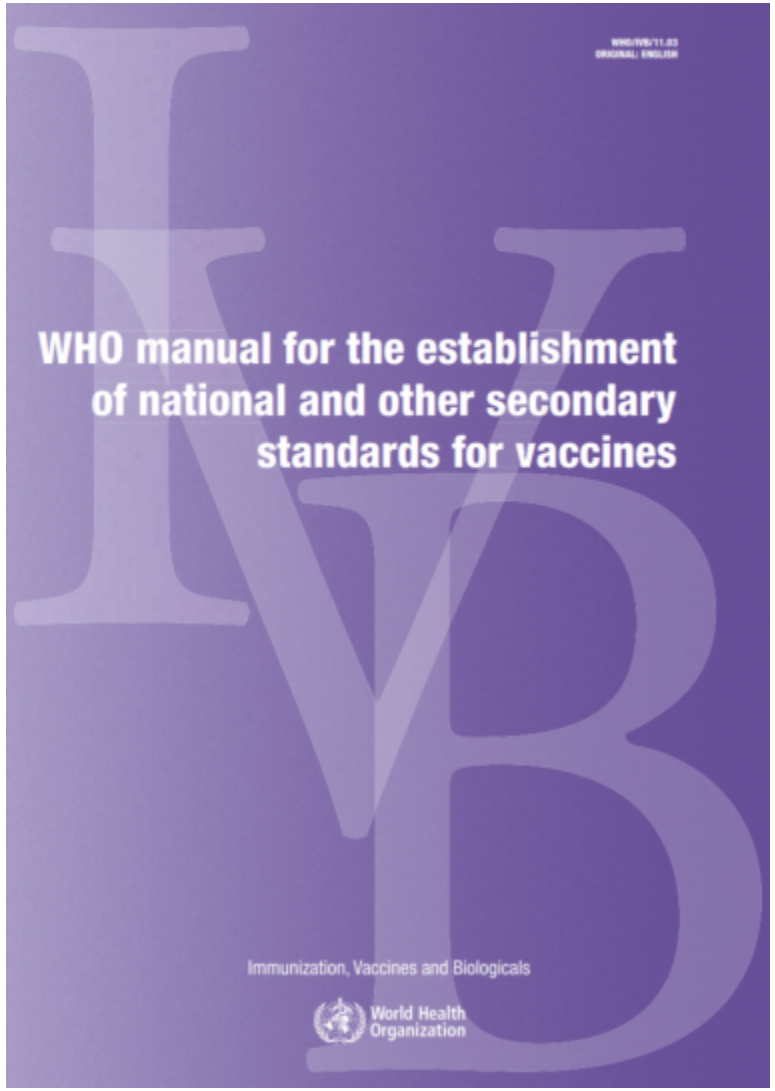
Post ECBS version

ENGLISH ONLY

MANUAL FOR THE PREPARATION OF SECONDARY REFERENCE MATERIALS FOR IN VITRO DIAGNOSTIC ASSAYS DESIGNED FOR INFECTIOUS DISEASE NUCLEIC ACID OR ANTIGEN DETECTION:

CALIBRATION TO WHO INTERNATIONAL STANDARDS

© World Health Organization 2016



Key properties of different standards

	WHO International Standard	Secondary standard	Tertiary standard
Alternate names	Highest order, International calibrator	Regional or national working reference materials, working reagents, manufacturer's working calibrator	Working reagents, in-house calibrators, in-house controls, manufacturer's product calibrator
Calibration	Established by WHO international collaborative study	Calibrated against the WHO International Standard	Calibrated against the secondary standard
Unitage	IU/mL	IU/mL	IU/mL
Traceability	N/A	Yes	Yes
Uncertainty of measurement	No	Yes (assay specific)	Yes (assay specific)
Final format	Lyophilised (generally)	Lyophilised or liquid	Liquid (generally)
Usage	Calibration of secondary standards, Initial validation of new assay/platform	Calibration of tertiary standards, working reagent, limited use as run control or calibrator	Working reagent, run control, calibrator
Establishment of standard	International agreement through WHO international collaborative study including laboratories worldwide, different assays, different types of test laboratories (approximately 15-30 participants)	May be calibrated in several ways: • In parallel with a study to establish the International Standard • Regional or national collaborative study similar to the WHO collaborative study but with fewer participants from regional laboratories • Small study by a single or limited number of laboratories with a single or limited number of different assays/platforms	Assay-specific study, normally by a single laboratory for use with a specific test/platform Small study by a single or limited number of laboratories with a single or limited number of different assays/platforms

(WHO Technical Report Series, No. 1004, Annex 6, 2017)

Calibration principles

- Test secondary standard multiple times on different occasions in parallel with the WHO IS under exact same test conditions
- Assess validity of individual assays e.g. linearity and parallelism
- Estimate potency (IU/mL) for secondary standard in all valid assays
- Combine estimates and assign combined estimate as potency

Notes on calibration

- Use optimal test system (e.g. commercial assay, validated laboratory test)
- Use only qualified operators, equipment etc.
- No general guidance regarding number of assay runs to perform
  - Decision will depend on various factors
  - E.g. sufficient testing may be performed to give Uncertainty of Measurement (UoM) that is negligible in comparison to the expected precision of the routine assay

Slide 7 Notes

Assuming appropriate assay conditions, UoM can be expressed as 95% Confidence Interval (CI) for combined estimate.
In such cases, this will account for imprecision but not any inherent bias so care should be taken with assay design etc.

Calibration example

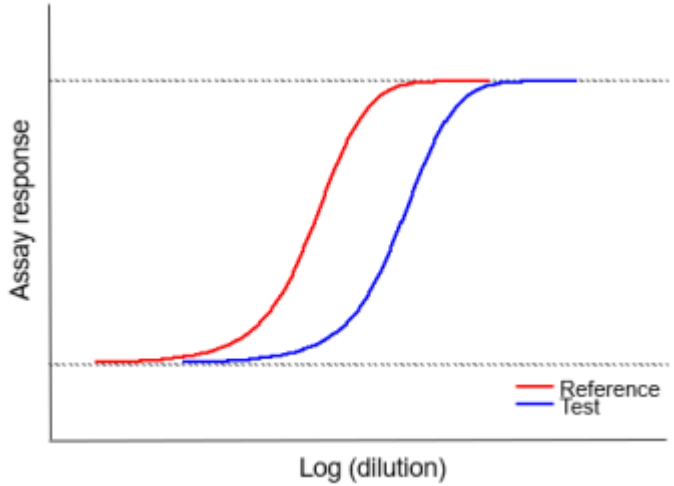
- Samples tested:
  - WHO IS for anti-SARS-CoV-2 immunoglobulin (20/136)
    Potency 250 IU/ampoule for neutralising antibody assays
    Reconstituted to concentration of 1000 IU/mL
  - In-House Standard (IHS)
- Aim: Calibrate IHS in IU/mL using IS
- Samples initially tested in 3 independent assay runs

Calibration example – assay 1

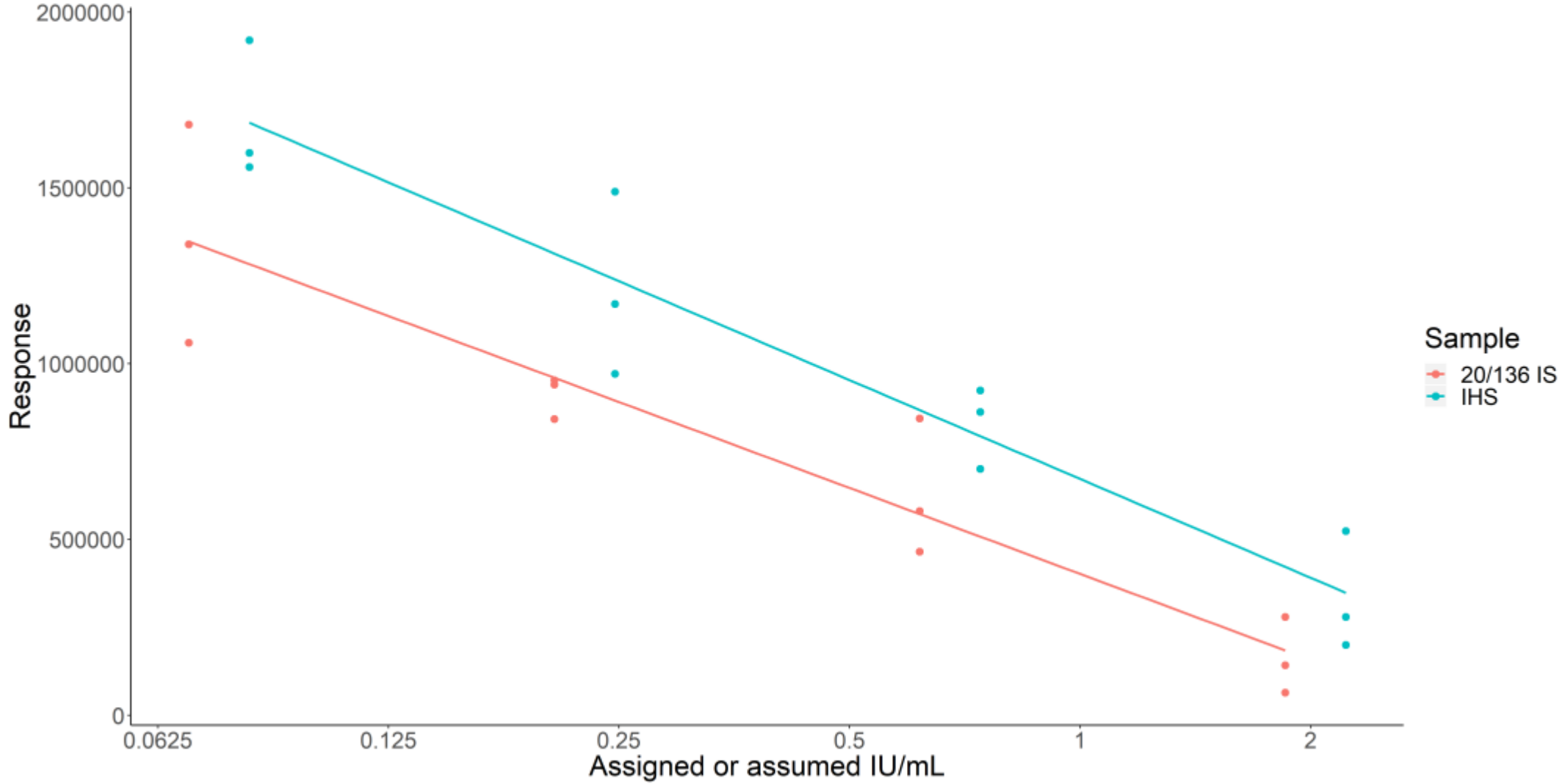
IS 20/136				IHS			
1000 IU/mL				20 IU/mL (assumed)			
Dilution	Responses			Dilution	Responses		
1/60	30	80	360	1/1	40	30	30
1/180	70800	92400	2510	1/3	580	2730	71600
1/540	142000	280000	64700	1/9	280000	200000	524000
1/1620	465000	844000	581000	1/27	863000	701000	924000
1/4860	953000	941000	843000	1/81	1170000	1490000	971000
1/14580	1060000	1680000	1340000	1/243	1560000	1920000	1600000
1/43740	870000	1480000	1290000	1/729	1210000	1410000	1310000

Data analysis

- Objectives of dose-response data analysis:
  - Assess assay validity e.g. linearity/parallelism for linear model
  - Estimate potency of IHS relative to WHO IS
- Various possible analysis methods, including:
  - Parallel line (parallel curve) analysis [recommended]
  - Interpolation from fitted dose-response curve for WHO IS
- Software options will depend on analysis method:
  - Specialised software for bioassay analysis
  - General statistical software packages, Excel



Calibration example – assay 1



Calibration example – assay 1 analysis

- Analysis output for assay 1:

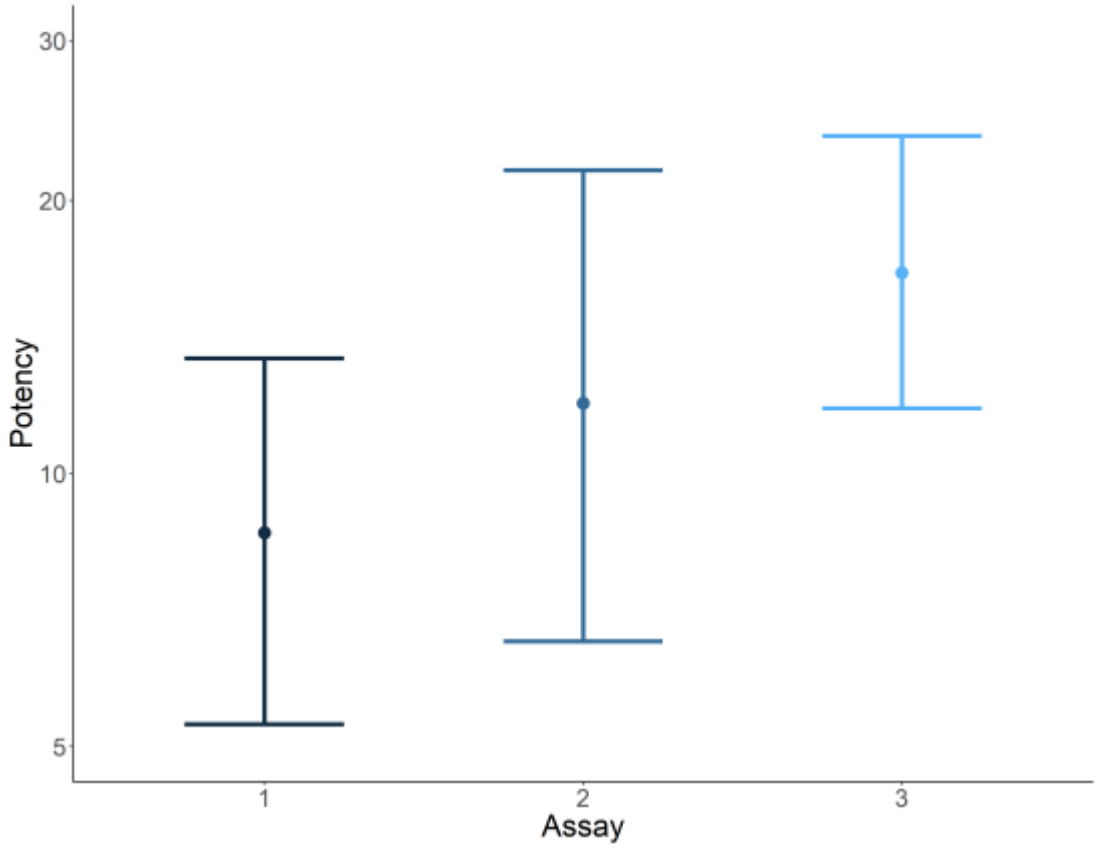
Source	Df	Sum of Sq	Mean Sq	F-ratio	P-Value
Treatments	7	5.63597E+12	8.05139E+11	21.549	<0.001 ***
Preparations	1	3.77329E+11	3.77329E+11	10.099	0.006 **
Regression	1	5.20746E+12	5.20746E+12	139.372	<0.001 ***
Non-parallelism	1	2.53490E+10	2.53490E+10	0.678	0.422
Non-linearity	4	2.58375E+10	6.45937E+09	0.173	0.949
Residual error	16	5.97818E+11	3.73637E+10		
Total	23	6.23379E+12	2.71034E+11		

IHS Potency (IU/mL)		
Estimate	95% LCL	95% UCL
8.60	5.29	13.40

Calibration example – combined estimate

- Combined estimate (IU/mL) from assays 1-3:

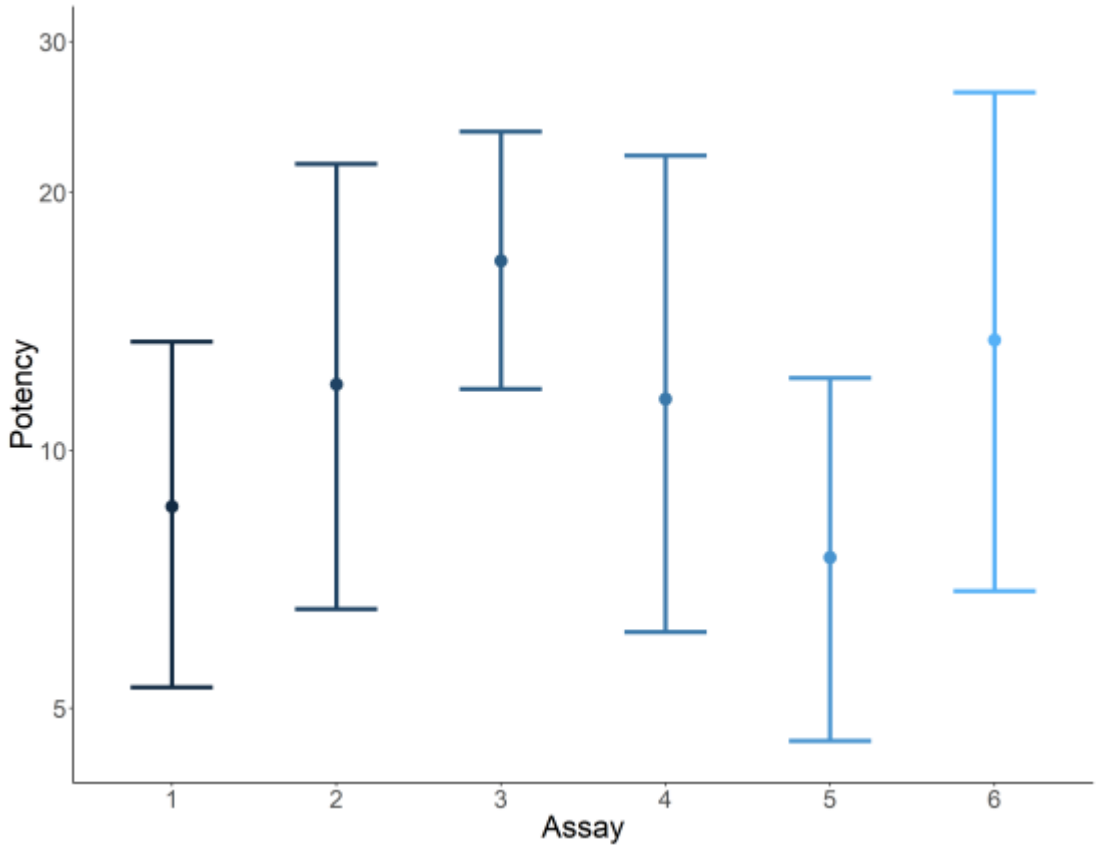
Estimate	95% LCL	95% UCL	LCL as %	UCL as %
12.3	8.5	17.9	68.9%	145.2%



Calibration example – after additional assays

- Combined estimate (IU/mL) from assays 1-6:

Estimate	95% LCL	95% UCL	LCL as %	UCL as %
11.5	9.2	14.4	79.9%	125.1%



Calibration example – conclusions

- Assigned value to internal standard = 11.5 IU/mL

- Assay currently states µg/mL unitage for internal standard

➢ Neat concentration used is 5 µg/mL

➢ Calibration exercise estimated neat concentration as 11.5 IU/mL

➢ Any existing results reported in µg/mL (from this assay platform, using this internal standard) can now be converted to IU/mL

Summary

- Expression of assay results in International Units (IU) requires use of the WHO International Standard (IS) directly, or the use of a secondary standard calibrated using the IS
- A calibration exercise can be performed to assign an IU value to the secondary standard
- Existing assay results already reported relative to a secondary standard (in µg, EU, relative titre etc.), can then be reported as IU
  - In most cases, existing assay analysis methods are unaffected

WHO standards for COVID-19: update from WHO ECBS

Dr Ivana Knezevic, Norms and Standards for Biologicals (WHO/MHP/HPS)

20 Jan 2021, WG on COVID-19 assays

WHO norms and standards for biologicals

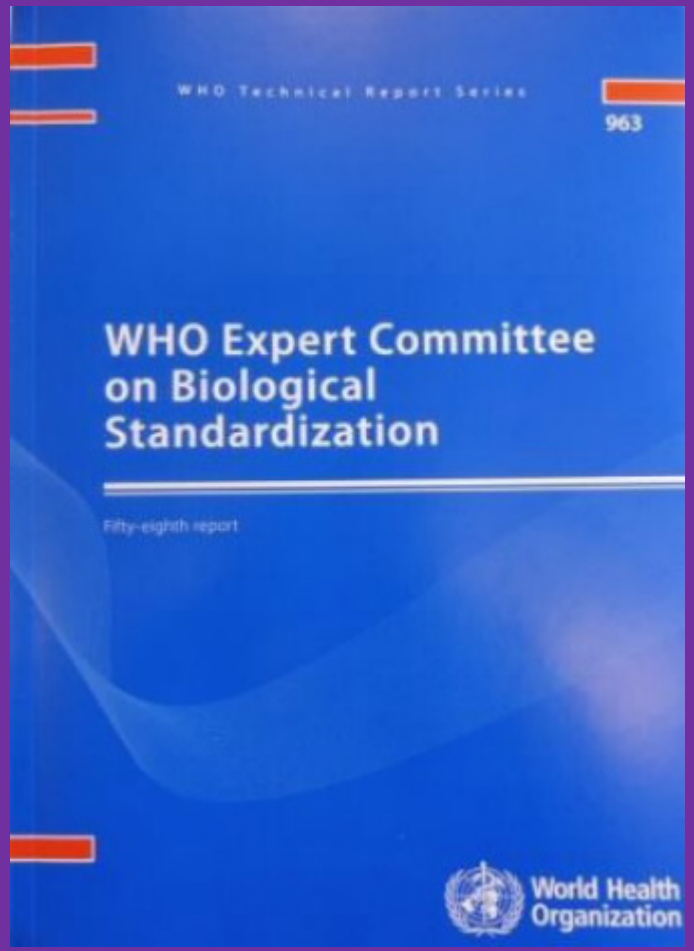
https://www.who.int/groups/expert-committee-on-biological-standardization

Global written standards

Total 103 docs (Recommendations/ Guidelines)
General docs that apply to vaccines & biologicals: 10
General documents that apply to all vaccines: 12
Vaccine specific: 71
BTP specific: 9

Scientific evidence

1) Standardization of assays
2) Further development and refinement of QC tests
3) Scientific basis for setting specifications



Global measurement standards

Measurement standards:
essential elements for development, licensing and lot release

Main outcomes of 73rd ECBS meeting in 2020

World Health Organization

1. ECBS meeting on 9-10 Dec 2020 (focused on COVID-19): published on WHO web site: https://www.who.int/groups/expert-committee-on-biological-standardization:

- Executive Summary posted on WHO web site on 16 Dec 2020:

https://www.who.int/groups/expert-committee-on-biological-standardization

3 new WHO International reference preparations established

Standards for use in public health emergencies		
SARS-CoV-2 RNA for NAT-based assays	7.40 $\log_{10}$ IU/ampoule	First WHO International Standard
Anti-SARS-CoV-2 immunoglobulin	250 IU/ampoule (neutralizing antibody activity)	First WHO International Standard
Anti-SARS-CoV-2 immunoglobulin panel	[no assigned units]	First WHO International Reference Panel

- Proposal to develop a standard for SARS-CoV-2 antigens to support the development, assessment and comparability of antigen-based rapid diagnostic tests.

- Update on written standards provided

Measurement standards for COVID-19

World Health Organization

Aim: to facilitate the development, validation and assessment of molecular and antibody assays. This will facilitate the comparability of results from different assays/labs and help harmonize the evaluation of diagnostics, vaccines and other products. Expression of the results of neutralization assays in the International Units would also contribute to the establishment of the correlates of protection.

Timeline: Feb to Dec 2020 is the absolute record for developing and establishing WHO International Standards in 9 months instead of 2-3 years.

Antibody standards during the COVID-19 pandemic: research reagents and WHO International Standards: joint effort by CEPI, NIBSC and WHO

WHO International Antibody Standards:

- **help interpreting results from vaccine CTs by providing the basis for the expression of the antibody titers in the International Units, particularly results from efficacy trials for various vaccine candidates. For instance, correlate of protection can be defined as IU/mL**
- **IS permits datasets across a range of assays to be compared by reference to the IU. This is especially important with the large number of vaccine candidates**

Uptake of WHO Ab standards – way forward

World Health Organization

1. **Why WHO Ab standards have not been used as expected?**

- Lack of information on the existence of these standards
- Misunderstandings regarding the intended use
- Labour intense calibration of secondary standards
- Lack of expertise for the use of standards and for the assays in CTs
- Other reasons?

2. **Need for secondary standards calibrated against WHO IS**

- Several initiatives ongoing
- How many vaccine developers plan to express results of neutralization assays in IU?

How can we help users of standards to express CT results in the International Units?

World Health Organization

1. ECBS – Executive Summary and report in WHO TRS

2. Collaborative study report provides lots of information about the standards but also about the assays

3. Instruction for use

4. WHO EUL: "Considerations for the Assessment of COVID-19 Vaccines for Listing by WHO" (https://www.who.int/teams/regulation-prequalification/eul/covid-19):

4.1. The assays used for immunogenicity evaluation should be validated for their intended purpose and calibrated against WHO international standards, where available (section 3.2)

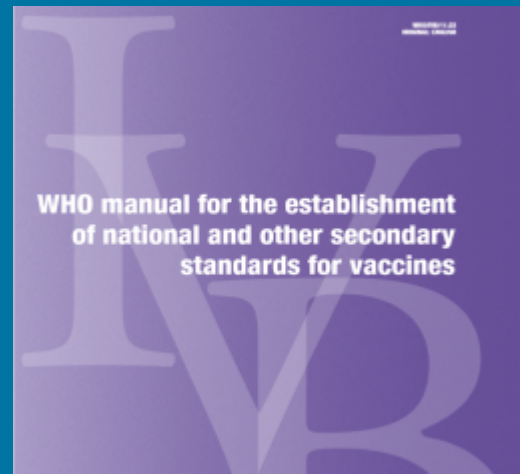
4.2. Assay results should be reported in international units wherever possible (section 3.3.8)

5. Webinars

6. Case studies with the examples

7. Manual for secondary standards for vaccines – Update and/or specific advice for COVID-19 standards

8. Other opportunities?



Medicines & Healthcare products Regulatory Agency

NIBSC

Roles and Importance of Standards in Assays

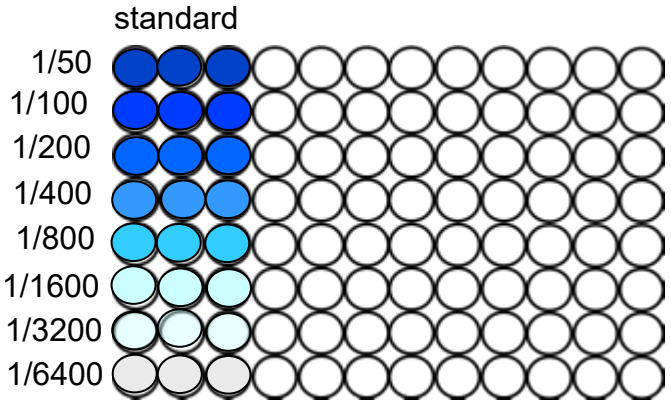
Giada Mattiuzzo

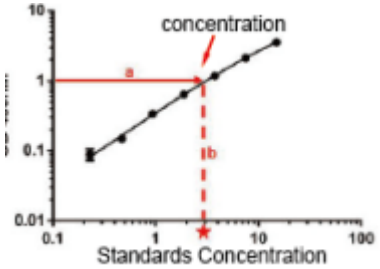
20th January 2021

Standards in assays

- 44 laboratories participated to the CS establishment of WHO IS for anti-SARS-CoV-2 immunoglobulin;
- 8 laboratories did not use an assay internal standard – results reported based on dilution factor
- Difficult to compare with other assays

- Internal calibrator/standard;
- run control;
- working reagent
- Secondary or tertiary reagent





Slide 2 Notes

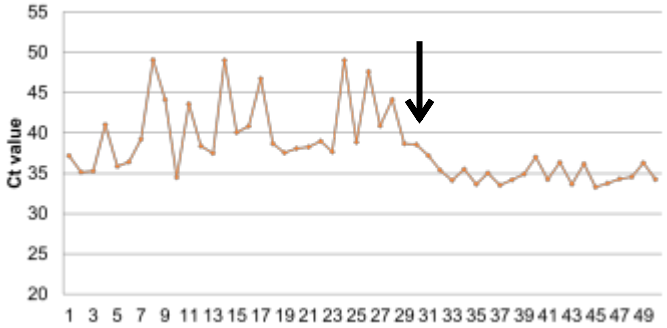
Most commercial assays have a calibrant

External controls

Allow to check for performance drifting of the assay over time/between operators

Monitoring data from diagnostic lab starting using NIBSC Working Reagent for Norovirus GII (⇩)

Kindly provided by NIBSC Diagnostics Group, IDD



Examples are: national standards, commercially available secondary reagents

Can be used to compare assay between laboratory

Primary calibrant

- WHO International Standard – established by the WHO ECBS
- Define the common language to expressed potency of the samples

Slide 4 Notes

Common language already reduces the difference between labs

Why International units? A bit of history

- 1890's First Biological: Diphtheria antitoxin
  No reliably produced. Lot to lot variation as biological activity differed from physical property
- Paul Ehrlich: STANDARD ANTITOXIN PREPARATION – calibrated in Units and used to calibrate future batches
- Henry Dale applied concept to insulin and other biologics (1920s) and on International level – appearance of International Units (IUs) as measure of strength or activity of a product

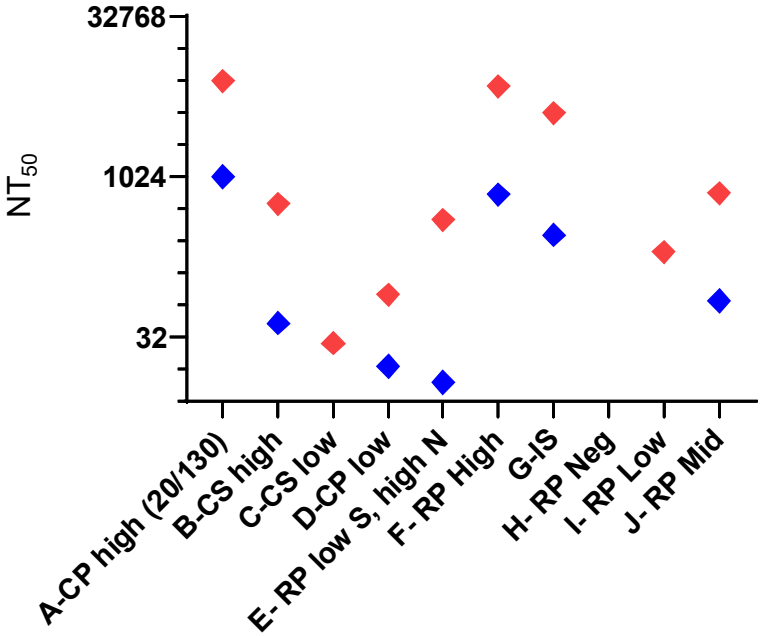
https://www.who.int/bloodproducts/ref_materials/en/

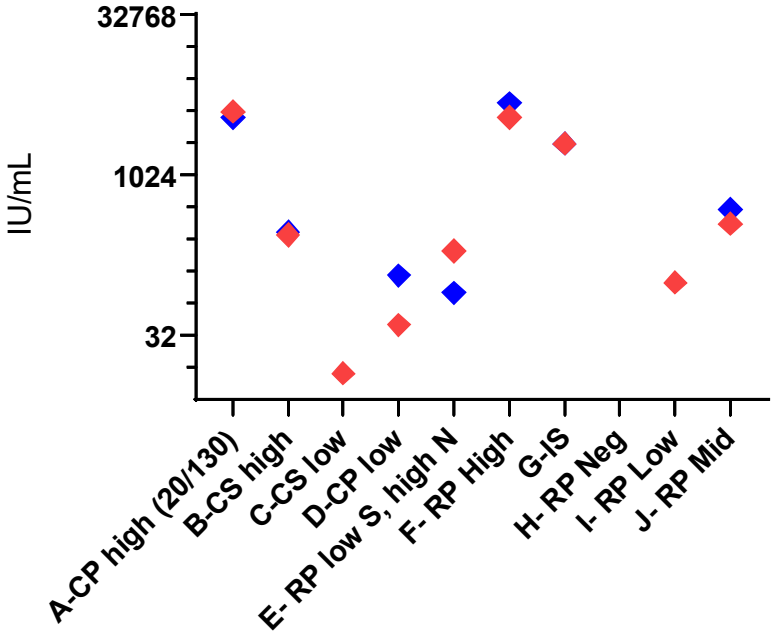
Slide 5 Notes

This is to answer the popular question on what’s the conversion factor to ng/mL

A recent example: SARS-CoV-2 antibody

Foci reduction neutralisation assay





Binding antibody assay- Spike ELISA

output	lab	A-20/130	B-CS High	C- CS Low	D- CP low	E-low S,high N	F-High	G-IS	H- neg	I - low	J- mid
Dil fact	22b	10159	5081	-	100	1270	12800.0	18102.0	-	635	2851
AU/mL	37a	3464	1764	-	42	510	5284	7215	-	340	1812
ug/mL	9a	25.2	13.3	0.4	0.8	4.5	47.4	55.4	-	2.6	13.3
ratio S/CO	38	18.7	13.9	-	-	4.1	21.4	23.7	-	3.1	11.9

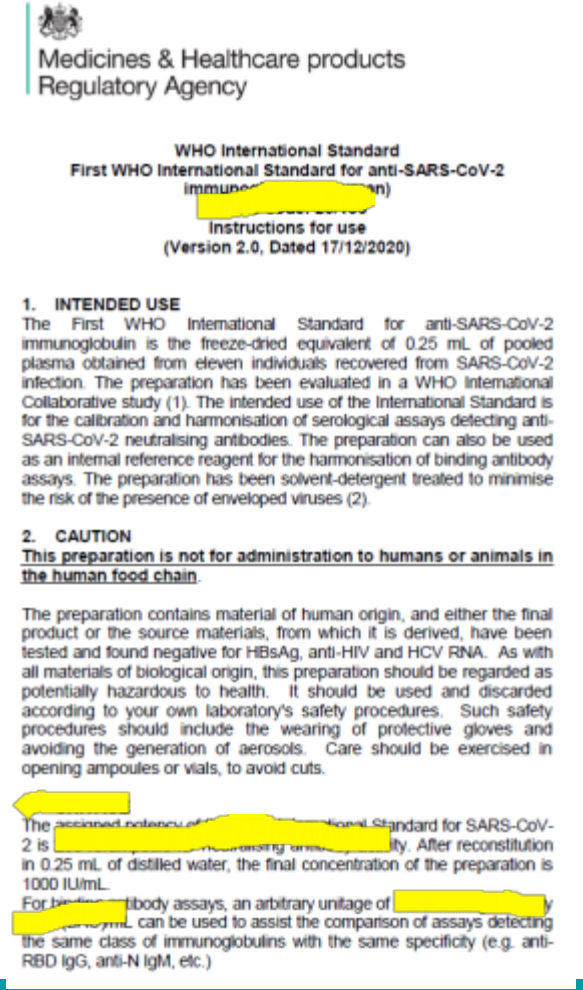
output	lab	A-20/130	B-CS High	C- CS Low	D- CP low	E-low S,high N	F-High	G-IS	H- neg	I - low	J- mid
IU/mL	22b	457	284	-	8	114	949	1000	-	47	154
	37a	480	244	-	6	71	732	1000	-	47	251
	9a	463	246	8	13	85	836	1000	-	47	230
	38	787	585	-	-	175	900	1000	-	131	503

Slide 7 Notes

2000 fold difference when expressed with different unitage vs less than 5 fold when expressed relative to IU

Unitage assign to the International Standard

- WHO IS established by WHO ECBS on 10th December 2020;
- Available in NIBSC catalogue on 18th December 2020;
- On 18th January 2021, approximately 50 end users have acquired the IS (tot shipped ampoules 162)



Medicines & Healthcare products Regulatory Agency

WHO International Standard
First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (human)
Instructions for use
(Version 2.0, Dated 17/12/2020)

1. INTENDED USE

The First WHO International Standard for anti-SARS-CoV-2 immunoglobulin is the freeze-dried equivalent of 0.25 mL of pooled plasma obtained from eleven individuals recovered from SARS-CoV-2 infection. The preparation has been evaluated in a WHO International Collaborative study (1). The intended use of the International Standard is for the calibration and harmonisation of serological assays detecting anti-SARS-CoV-2 neutralising antibodies. The preparation can also be used as an internal reference reagent for the harmonisation of binding antibody assays. The preparation has been solvent-detergent treated to minimise the risk of the presence of enveloped viruses (2).

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

The assigned potency of [redacted] International Standard for SARS-CoV-2 is [redacted] ity. After reconstitution in 0.25 mL of distilled water, the final concentration of the preparation is 1000 IU/mL.

For binding antibody assays, an arbitrary unitage of [redacted] /mL can be used to assist the comparison of assays detecting the same class of immunoglobulins with the same specificity (e.g. anti-RBD IgG, anti-N IgM, etc.)

First WHO International Reference Panel for anti-SARS-CoV-2 immunoglobulin (20/268)

Reference Panel will comprise 4 pools of COVID-19 convalescent plasma and a negative; freeze-dried equivalent of 0.25 mL

High (NIBSC code 20/150)

Mid (NIBSC code 20/148)

Low S, high N (NIBSC code 20/144)

Low (NIBSC code 20/140)

Negative (NIBSC code 20/142)

Representative data

	High 20/150	Mid 20/148	low S, high N 20/144	low 20/140	
Neut Ab	1473	210	95	44	**IU/mL**
anti-RBD IgG	817	205	66	45	**BAU/mL**
anti-S1 IgG	766	246	50	46	**BAU/mL**
anti-Spike IgG	832	241	86	53	**BAU/mL**
anti-N IgG	713	295	146	12	**BAU/mL**

The candidate Reference Panel samples were ranked similarly in almost all the assays used with very few exceptions

No unitage will be assigned for the Reference Panel, but representative data from CS include in IFU

Reseach Reagent 20/130

- Made available end April 2020 for the development of assay for the detection of SARS-CoV-2 antibody
- Secondary standard which allows assay to report data in IU/mL, retrospectively
- Calibrated to the WHO IS as part of the CS

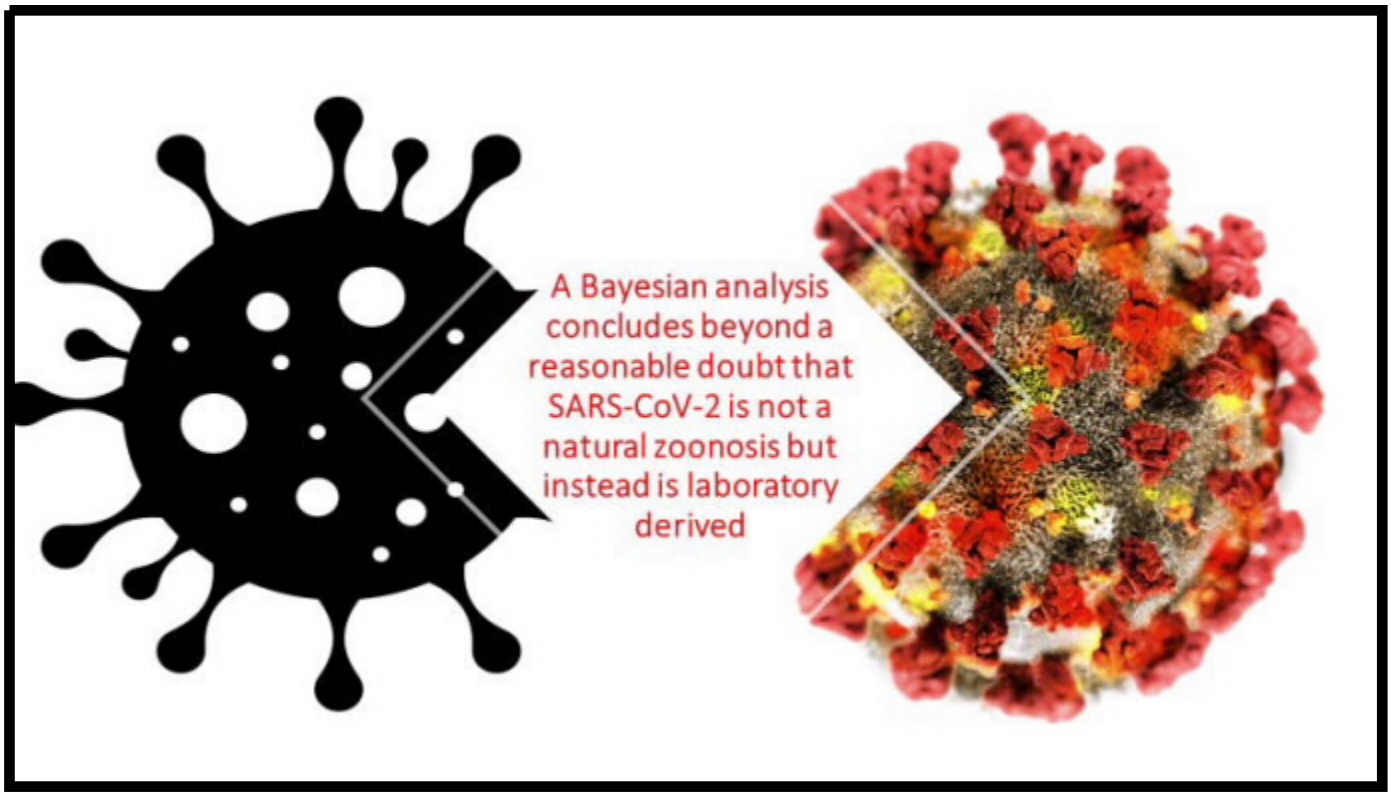
	GM	95% CI	
Neut Ab	1300	981-1719	**IU/mL**
anti-RBD IgG	502	382-660	**BAU/mL**
anti-S1 IgG	588	398-870	**BAU/mL**
anti-Spike IgG	476	418-542	**BAU/mL**
anti-N IgG	747	214-2606	**BAU/mL**

Slide 10 Notes

Slides available, however this data are also included in the revised Datasheet available in our website; less than 130 vials left- cannot be used as run control

Intended use the standards

- WHO International Standard 20/136 is primary calibrant; distribution restricted to 5 ampoules per end user per year
- Availability of secondary reagents- 20/130 is almost depleted; few example of national standards;
- other secondary standard (?)
- Internal Standard/run control can be calibrated to the IS or to secondary reagents- no need for a CS
- How?



By

Steven C. Quay, MD, PhD

www.DrQuay.com

A Bayesian analysis concludes beyond a reasonable doubt that SARS-CoV-2 is not a natural zoonosis but instead is laboratory derived

Executive Summary. The one-year anniversary of the COVID-19 pandemic records 2.1 million deaths, 99.7 million confirmed cases,[1] and trillions of dollars of economic damage. Although there is universal agreement that a coronavirus identified as Severe Acute Respiratory Syndrome Coronavirus 2 or SARS-CoV-2 (abbreviated CoV-2 henceforth) causes the disease COVID-19, there is no understanding or consensus on the origin of the disease.

The Chinese government, WHO, media, and many academic virologists have stated with strong conviction that the coronavirus came from nature, either directly from bats or indirectly from bats through another species. Transmission of a virus from animals to humans is called a zoonosis.

A small but growing number of scientists have considered another hypothesis: that an ancestral bat coronavirus was collected in the wild, genetically manipulated in a laboratory to make it more infectious, training it to infect human cells, and ultimately released, probably by accident, in Wuhan, China. For most of 2019 this theory was considered a crackpot idea, but in the last few weeks, more media attention has been given to the possibility that the Wuhan Institute of Virology, located in central Wuhan, may have been the source of the field specimen collection effort, laboratory genetic manipulation, and subsequent leak. On January 15, 2021, the U.S. Department of State issued a statement requesting the WHO investigation of the origin of COVID-19 include specific assertions related to a laboratory origin of the pandemic.[2]

Given the strong sentiment in the scientific community in favor of a zoonosis and the massive effort undertaken by China to find the natural animal source, one can assume that any evidence in favor of a natural origin, no matter how trivial, would become widely disseminated and known. This provides a potential evidence bias within the scientific community in favor of a natural origin which isn't quantifiable but should be kept in mind.

This becomes especially important background when evidence that could support a laboratory origin has been directly provided by leading Chinese scientists themselves, like Dr. Zhengli Shi, head of coronavirus research at the Wuhan Institute of Virology and Gao Fu (George Fu Gao), Director of Chinese CDC; by the Chinese government, as well as by powerful and vocal, pro-natural origin scientists, like Dr. Peter Daszak, of the NYC-based NGO, EcoHealth Alliance.

This report uses Bayesian inference, a common statistical tool in which Bayes' theorem, a well-known statistical equation, is used to update the likelihood for a particular hypothesis as more evidence or information becomes available. It is widely used in the sciences and medicine and has begun to be used in the law.

[1] https://www.worldometers.info/coronavirus/?
[2] https://www.state.gov/ensuring-a-transparent-thorough-investigation-of-covid-19s-origin/

The starting probability for origin of SARS-CoV-2 was set with the zoonotic or natural hypothesis at 98.8% likelihood with the laboratory origin hypothesis set at 1.2%. The initial state was biased as much as possible towards a zoonotic origin, with the starting point selected as the upper bounds of the 95% confidence interval for the mean and standard deviation of three independent estimates, including one by Daszak and colleagues. Each piece of new evidence for or against each hypothesis was then used to adjust the probabilities. If evidence favored a natural origin the math adjusts upward the probability of a natural origin, and so on.

The outcome of this report is the conclusion that the probability of a laboratory origin for CoV-2 is 99.8% with a corresponding probability of a zoonotic origin of 0.2%. This exceeds most academic law school discussions of how to quantify 'beyond a reasonable doubt,' the threshold for finding guilt in a criminal case. The report contains the detailed analysis and quantitative basis for the statistics and conclusion. It should be noted that because of the commutative property of the collected adjustments to the probabilities, the order in which they are used in the overall calculation is immaterial and the same end likelihoods will be reached regardless of the order of input.

The following Text-Table summarizes the evidence examined and the changes in probabilities:

Evidence	Zoonotic Origin	Laboratory Origin
Initial State	98.8%	1.2%
International committees to determine CoV-2 origin may not be impartial	98.8%	1.2%
Three key zoonotic papers: pros and cons	98.8%	1.2%
SARS-like infections among employees of the Wuhan Institute of Virology in the fall of 2019 reported by US Government	98.8%	1.2%
Location of first cases near Wuhan Institute of Virology	95.1%	4.9%
Lack of evidence of seroconversion in Wuhan and Shanghai	80.9%	19.1%
Lack of posterior diversity	30.8%	69.2%
Opportunity: The Wuhan Institute of Virology has publicly disclosed that by 2017 it had developed the techniques to collect novel coronaviruses, systematically modify the receptor binding domain to improve binding or alter zoonotic tropism and transmission, insert a furin site to permit human cell infection, make chimera and synthetic viruses, perform experiments in humanized mice, and optimize the ORF8 gene to increase human cell death.	30.8%	69.2%
Lack of furin cleavage sites in any other sarbecovirus	4.7%	95.3%
Rare usage of -CGG- single codons & no CGG-CGG pairs	0.5%	99.5%
Routine use of CGG in laboratory codon optimization, including Daszak & Shi	0.2%	99.8%
Spike Protein receptor binding region (200 amino acids) optimized for humans	0.2%	99.8%
Whole genome analysis shows pre-adaption of CoV-2	0.2%	99.8%
The finding of CoV-2 in Barcelona wastewater in early 2019 was an artifact	0.2%	99.8%
Shi and the WHO comment early on that CoV-2 seemed to begin with a single patient	0.2%	99.8%
Mammalian biodiversity between Yunnan and Hubei is significantly different, limiting a potential common intermediate host	0.2%	99.8%
The ancestor of CoV-2 can only obtain a furin site from other subgenera viruses but recombination is limited/non-existent between subgenera	0.2%	99.8%
Canvas of 410 animals shows humans and primates are the best, bats are the worst, for ACE2-Spike Protein interaction	0.2%	99.8%
A government requested review of samples collected from a mineshaft may have caused the COVID-19 pandemic	0.2%	99.8%
The Hunan Seafood Market and farmed animals in Hubei province are not the source of CoV-2	0.2%	99.8%
Line 2 of the Wuhan Metro System is the likely conduit of the pandemic and is the closest subway line to the WIV	0.2%	99.8%
Feral and domestic cats are not the intermediate host	0.2%	99.8%
Extraodinary pre-adaption for the use of human tRNA is observed	0.2%	99.8%
Evidence of lax operations and disregard of laboratory safety protocols and regulations in China	0.2%	99.8%
Previous SARS-CoV-1 laboratory accidents	0.2%	99.8%
Shi and Daszak use Wuhan residents as negative control for zoonotic coronavirus exposure	0.2%	99.8%
RaTG13 could be CoV-2 precursor using the synthetic biology 'No See 'Em' technique	0.2%	99.8%
Location, location, location: Based on the distance between known SARS-CoV-1 laboratory-acquired infections and the hospital of admission of the infected personnel, the WIV is within the expected hospital catchment for a CoV-2 LAI	0.2%	99.8%

The summary which follows will simply be a review and discussion of the evidence in the context of the two hypotheses.

Zoonosis Hypothesis

A viral zoonosis has at least three elements, a host, a virus, and the human population. With some viruses there are often two hosts. One is a 'reservoir host' where the virus can live for years or even decades in a relatively stable relationship. The reservoir host is never decimated by the virus, and the virus is never burned out by the reservoir host, disappearing completely. For coronaviruses the reservoir host is always one or more bat species. If there is a reservoir host that some viruses that cannot jump directly into the human population, there is a need for an second host, an intermediate host. In this case the virus spends time jumping into the intermediate host, 'practicing' adaption through random mutation and Darwinian selection for fitness to reproduce, infect, and transmit in the intermediate host. This process is then repeated between the intermediate host and the human population. Alternatively, the virus can jump directly between the bat reservoir and humans, without the need for an intermediate host.

For two prior human coronavirus epidemics, an intermediate or proximate host was identified. For SARS-CoV-1 in 2003-4 it was the civet cat while for Middle Eastern Respiratory Syndrome (MERS) in 2012-4 it was the camel. In both of these human epidemics, the intermediate host was identified within four to ten months of the first clinically identified human infection. With CoV-2 we are at 12 months since the pandemic began and still waiting for evidence of, despite a much larger effort inside China to find an intermediate host. For both of these previous pandemics, a bat species reservoir host was also identified, but not in the case of SARS-CoV-2.[3]

Based on the genome sequence of CoV-2, Drs. Shi and Daszak have proposed that the reservoir host for CoV-2 is the intermediate horseshoe bat (*Rhinolophus affinis*), which is found in Yunnan Province. Yunnan Province is in southern, rural China and about 1900 km from the north central province of Hubei, where the 11 million people of Wuhan live. In the US this would be equivalent in distance, climate change, and human population density difference to going from the Everglades in Florida to Manhattan, in New York City. The intermediate horseshow bat isn't found at all in Hubei province, making a direct bat-to-human transmission improbable.[4] Experiments in three independent laboratories also demonstrate that CoV-2 has changed genetically so much that it can no longer infect any bat species cell culture tested. So, while the leading US coronavirus expert, Dr. Ralph Baric of The University of North Carolina suggested in early 2020 that CoV-2 may have jumped into the human population directly from bats without an intermediate host, this hypothesis seems to no longer be viable.

For the zoonosis hypothesis to be advanced, it is now necessary to find an intermediate host. In January 2020 a theory was proposed that CoV-2 arose in the Huanan Seafood Market, a

[3] I am distinguishing here the difference between SARS-CoV-2 being a descendent of a bat coronavirus (with 3.8% or 1100 nucleotide (nt) differences between them) and the finding of the immediate precursor of SARS-CoV-2 in a bat colony population somewhere in the wild, which usually is <100 nt differences.

[4] "We have done bat virus surveillance in Hubei Province for many years but have not found that bats in Wuhan or even the wider Hubei Province carry any coronaviruses that are closely related to SARS-CoV-2. I don't think the spillover from bats to humans occurred in Wuhan or in Hubei Province," said Dr. Shi. Science, July 2020

traditional Chinese "wet market" where live animals are butchered and sold for food. The market theory was based on the observation that about 40% of early patients worked or shopped there. This was reminiscent of the wet market sources for civet cats infected with SARS-CoV-1 or the camel markets for the MERS coronavirus. The Chinese authorities closed the market on December 31, 2019 after performing extensive environmental sampling and sanitation.

But by May 2020 Dr. Gao Fu, Director of the Chinese CDC, announced that the market was not the source of CoV-2, as all of the animal specimens tested negative for CoV-2. And while SARS-CoV-1 was found in 100% of local farmed civets when tested, CoV-2 was different. In July 2020 Dr. Shi reported that extensive testing of farmed animals throughout Hubei Province failed to find CoV-2 in any animals.

For about six months, the pangolin, a scaly anteater, was suspected to be the intermediate host but finally Dr. Daszak reported that CoV-2 was not found in pangolins in the wild or from the (illegal) market trade.[5] Domestic and feral cats also were ruled out as a possible source. A comprehensive computer-based screen of 410 different animals reported the remarkable finding that the best ACE2 receptor matches to CoV-2 were human and other primates (or primate cells in the laboratory), including the favorite laboratory coronavirus host, the VERO monkey cell culture, and that all bat species were the worst host. At the time of this writing, there is not even a working hypothesis for the species of an intermediate host.

A typical zoonosis has a number of characteristic properties that can allow identification of a zoonotic infection, even in the absence of identifying an intermediate host. None of these properties are found for CoV-2.

All zoonotic infections have in common the principle that when a virus in nature uses evolution to move from, for example, a bat host to a camel host and then to a human host, it is a hit and miss, slow process. After all, evolution is the result of random genetic changes, mutations, and then enrichment of the ones that are helpful by amplification during reproduction. With both SARS-CoV-1 and MERS, the coronavirus spent months and years jumping from the intermediate host into humans, not having all of the necessary mutations needed to be aggressive, grow, and then spread, but spending enough time in humans to cause an infection and leaving behind a corresponding immune response.

The hallmark evidence of this 'practice' in abortive host jumping is in stored, archived human blood specimens taken from before the epidemic, where one can find evidence of pre-epidemic, usually sub-clinical, community spread from the antibodies to the eventual epidemic virus. For SARS-CoV-1 and MERS, about 0.6% of people in the region where the epidemic began showed signs of an infection in archived blood. With CoV-2, this seroconversion, as it is called, has never been observed, including in 540 specimens collected from 'fever clinics' in Wuhan between October 2019 and January 2020, reported by the WHO. Because this is such a potent signal of a zoonosis, and because I believe that China has over 100,000 stored specimens from

[5] https://link.springer.com/article/10.1007/s10393-020-01503-x

Wuhan taken in the fall of 2019, the lack of reports of seroconversion, the silence from China on this evidence, speaks volumes.

Another hallmark of a slow, natural zoonosis can be found in the virus. In SARS-CoV-1 and MERS, the coronavirus spent years in the intermediate host, passing back and forth among populations of hosts, the civets or camels, that were living in close proximity. During this time, they would accumulate a background of genetic mistakes, i.e., mutations- usually about one mistake every two weeks. When the final chip falls, and a mutation(s) happens allowing the jump into humans, the virus with that new mutation(s) also jumps around within the intermediate host population. The consequence of this latter behavior for a true zoonosis is that the genome sequences found in humans don't all descend from a single jump into a single human but show jumps from viruses that are only cousins of each other, not direct lineal descendants.

In a true zoonosis, the family tree of virus genome sequences doesn't pass back through the first patient but instead tracks all the way back to an ancestor months or years earlier. This is called posterior diversity, and it is an easy genetic test to perform. With CoV-2, every one of the more than 294,000 virus genomes sequenced can be traced back to the first genomic cluster and in the first patient in that cluster, a 39-year-old man who was seen at the People's Liberation Army (PLA) Hospital about one mile from the Wuhan Institute of Virology. The CoV-2 pandemic has the phylogenetic signature of one pure virus sequence infecting one human, with human-to-human spread thereafter; there is just the one and only jump into the human population ever seen. This lack of posterior diversity has been alluded to by Dr. Shi, by the WHO, and by other prominent virologists; they just never take that critical piece of the evidence to the next the proper inference.

The virus in a true zoonosis also contains the signature record of the gradual changes and adaptions it made in the protein key, the Spike Protein, it uses to unlock human cells and cause infection. With SARS-CoV-1 the Spike Protein had fewer than one-third of all the changes it would later develop by the time it became an epidemic. With CoV-2 the Spike Protein was almost perfectly adapted to the human lock, using 99.5% of the best amino acids possible.

Since with CoV-2 we have no evidence from stored blood that it was quietly practicing on humans in the community of Wuhan, it is surprising that when it finds its first patient, it has perfected to 99.5% the spike protein amino acid sequence, its ability to attack and infect humans. If this adaption couldn't have happened in the community, the only place it could have happened is in a laboratory, by what is called serial passage, a common laboratory process that repeatedly gives the virus a chance to practice on humanized mice or VERO monkey cells.[6] A related study showing human adaption right from the start of the pandemic looked at which of the dozens of protein manufacturing tools that CoV-2 uses (called tRNAs). It showed the same uncanny adaptation to the human tools with no evidence that the tools from other potential intermediate hosts would be suitable.

[6] It is noteworthy that the furin cleavage site is actually unstable in passage in VERO cells and is often deleted within a few passages. A laboratory origin theory needs to account for this observation. On the other hand, mutations in the furin site among the human CoV-2 genomes are exceedingly rare.

This evidence presented makes a strong case that CoV-2 did not come from nature. But is there affirmative evidence that it could have come from a laboratory? The answer is yes.

Laboratory Origin Hypothesis

The spike protein that gives the coronavirus its name, corona or crown, is the key to match with the lock found in host cells. But before it can inject its genetic material in the host cell, the spike protein needs to be cut, to loosen it in preparation for infection. The host cell has the scissors or enzymes that do the cutting. The singular, unique feature of CoV-2 is that it requires a host enzyme called furin to activate it at a spot called the S1/S2 junction. No other coronavirus in the same subgenera has a furin cleavage site, as it is called. The other coronaviruses are cleaved at a site downstream from the S1/S2 site, called the S' site.

This is of course a major problem for the zoonosis theory, but it gets worse.

Since 1992 the virology community has known that the one sure way to make a virus deadlier is to give it a furin cleavage site at the S1/S2 junction in the laboratory. At least eleven gain-of-function experiments, adding a furin site to make a virus more infective, are published in the open literature, including Dr. Zhengli Shi, head of coronavirus research at the WIV. This has caused a flurry of Chinese papers since the pandemic began trying to show a natural furin site in a related virus (this one example was later shown to be an error in interpretation) or to show that furin sites from distant cousins of CoV-2 might be the source through a process called recombination, where two different viruses infect the same host and then make a mistake in copying their genetic material, and swap sequences.

These convoluted, hypothetical methods each fail, however. It turns out that it is Daszak himself who has shown that the subgenera of coronaviruses that have furin sites are found in different bat hosts, which live in different regions of China, than the sarbecovirus subgenera of which CoV-2 is a member. And even with these barriers, they apparently are too far apart to recombine. "For the three focal subgenera, *Sarbecoviruses, Merbecoviruses and Embevoviruses*…none of the three focal subgenera recombines with one another."[7] As noted previously[2] Dr. Shi also does not believe the bats of Hubei province are capable of being a host for CoV-2-related coronaviruses.

But it gets worse still for the zoonosis theory. The gene sequence for the amino acids in the furin site in CoV-2 uses a very rare set of two codons, three letter words so six letters in a row, that are rarely used individually and have never been seen together in tandem in any coronaviruses in nature. But these same 'rare in nature' codons turn out to be the very ones that are always used by scientists in the laboratory when researchers want to add the amino acid arginine, the ones that are found in the furin site. When scientists add a dimer of arginine codons to a coronavirus, they invariably use the word, CGG-CGG, but coronaviruses in nature rarely (<1%) use this codon pair. For example, in the 580,000 codons of 58 Sarbecoviruses the only CGG pair is CoV-2; none of the other 57 sarbecoviruses have such a pair.[8]

[7] CoV-2 is in the subgenera Sarbecoviruses.
https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1009272
[8] https://virological.org/t/alignment-of-58-sarbecovirus-genomes-for-conservation-analysis-of-sars-cov-2/430

So, there is no natural example of a furin protein site in nature that could be introduced into CoV-2 by recombination, there is no natural example of the particular gene sequence for the furin protein site contained in CoV-2 being used to code for anything in nature, but this particular coding is exactly what Dr. Shi, Baric, and others have used previously in published experiments to insert or optimize arginine codons.

It is telling that when Dr. Shi introduced the world to CoV-2 for the first time in January 2020 she showed hundreds of gene sequences of this novel virus but stopped just short of showing the furin site, the one she is purported to have introduced, seemingly not wanting to call attention to her handywork. She apparently failed to realize that an accomplished but innocent virologist, finding the first furin site ever seen in this class of viruses apparently coming from nature, would have featured the presence of the furin site prominently, and also would have used its presence and her experience with furin sites in other viruses to predict what it would foretell for the world due to its aggressive nature.

She could have perhaps saved many lives just by telling the world that she saw a furin site in the virus sequence. It would be left to a French and Canadian team to later identify the furin site in a paper.[9] They would write: "This furin-like cleavage site…may provide a **gain-of-function** to the 2019-nCoV for efficient spreading in the human population compared to other lineage b betacoronaviruses." [Emphasis added.]

Dr. Shi has denied the virus came from her lab, but she has created such a record of multiple examples of obfuscation, half-truths, contrived specimens, genetic sequences taken from thin air but published in premier journals and US NIH databases, etc. that her veracity is deeply damaged. Perhaps her words and actions on December 30, 2019 show the truth. Her very first response when told there was an unknown outbreak in Wuhan and to return back quickly from a meeting she was attending in Shanghai was to say, "Could this have come from our lab?"[10]

"I wondered if [the municipal health authority] got it wrong," she says. "I had never expected this kind of thing to happen in Wuhan, in central China." Her studies had shown that the southern, subtropical provinces of Guangdong, Guangxi and Yunnan have the greatest risk of coronaviruses jumping to humans from animals—particularly bats, a known reservoir. After all, the US equivalent of the distance, climate change, and human population density change between Yunnan and Wuhan is comparing the Everglades National Park in Florida and New York City.

Her other action on December 30 was to alter WIV computer databases of novel coronaviruses used by the world's virologists for research to make it more difficult to search for which coronaviruses she had in her building. In short, the day she was asked to address the pandemic in Wuhan, she chose to spend time to make unavailable to her fellow scientists of the world her decades of coronavirus work.

[9] https://www.sciencedirect.com/science/article/pii/S0166354220300528?via%3Dihub
[10] https://www.scientificamerican.com/index.cfm/_api/render/file/?method=inline&fileID=E1FDF8DE-9E22-4CE5-AD8B2E4682F52A86

The notion that CoV-2 was a laboratory creation, designed for maximum virulence, that escaped the laboratory accidentally has additional rings of evidence. From President Xi announcing in February new laws about laboratory security, to abundant evidence that the WIV was closed in October with few personnel inside, to the top military medical research doctor, General Chen Wei, being placed in charge of the WIV, to many more clues, it is clear an event occurred in Wuhan sometime in late 2019 that is most consistent with a laboratory escape.

The Asian region has a two-decade record of a little less than one laboratory-acquired infection per year. After the first SARS-CoV-1 epidemic was ended, SARS-CoV-2 jumped four more times into the human population, all from laboratories, with two in China. The last smallpox death in the entire world was a secretary who worked two floors above a research lab in England and contracted it through the ventilation system. The head of that laboratory committed suicide over his anguish for causing her death.

Over and over again. there is a long history and record of laboratory acquired infections that provides the background for considering what happened here.

Lab-made Bio-Weapon Hypothesis

But was SARS-CoV-2 more than just a gain-of-function experiment that escaped a laboratory? Could it have been one part of a two-part novel virus-vaccine bioweapons program?

General Chen Wei has been involved in vaccine research since joining the People's Liberation Army after college. In a 2017 internal speech at the AMMS (Academy of Military Medical Sciences) she said: "只要有矛. 才能研究盾." which translates roughly as, "you need to have an arrow to study a shield."I believe a Rubicon has been crossed by the world with this pandemic and framing the proper understanding of how we got here, and the proper response will be the critical next steps.

When Oppenheimer saw the application of Einstein's physics in the embodiment of the atomic bomb, he is said to have quoted a line from the Hindu scripture, the Bhagavad Gita, which reads: 'Now I am become Death, the destroyer of worlds.' The contribution of physics' research to human killing would total less than 300,000 people in two ten-square mile zones in Japan, and the horrors of those events led the world to regulate the raw materials of such bombs and to sanction sovereign nations who attempted to violate the rules.

This had followed the contribution of chemistry to human killing in the form of chemical warfare during World War I, in which 100,000 were killed, and led the nations of the world to an historic agreement to never use chemical warfare again. It is now only 'rogue' operators who violate the norms civilized nations have agreed to.

It seems to be biology's turn to show its dark arts. If it is generally understood that biology/biotechnology has been harnessed to create a pandemic that has killed more people than physics and chemistry research combined, and to be a weapon where no place on earth is safe from its effects (SARS-CoV-2 has been detected in the deepest Amazon jungles and at research stations in Antarctica), there needs to be developed a new set of regulations, rules, etc. to both honor the 1.8 million innocent people who died from COVID-19 and to protect the world so this

never happens again. It is also urgent to gather further data to support or refute if this was a Chinese bioweapons program, as the consequences of that would be significant.

Pre-publication input has been sought. The following individuals were sent a copy of this unpublished manuscript by email and asked to provide comments, criticisms, or improvements.

No response has been received at the time of this publication. An update will be made at such time as useful comments are received.

First Name	Last Name
John	Amuasi
Kristian	Andersen
Danielle	Anderson
Ralph	Baric
Francis	Collins
Carlos	das Neves
Peter	Daszak
Vladimir	Dedkov
Dominic	Dwyer
Farag	El Moubasher
Anthony	Fauci
Hume	Field
Thea	Fisher
Robert	Garry
Eddie	Holmes
Gerald	Keusch
Marion	Koopmans
Dato' Sai Kit (Ken)	Lam
Fabian	Lendertz
W. Ian	Lipkin
Ken	Maeda
Hung	Nguyen
Stanley	Perlman
Andrew	Rambaut
Angelie	Rassmussen
Linda	Saif
Zhengli	Shi
Supaporn	Wacharapluesadde
John	Watson

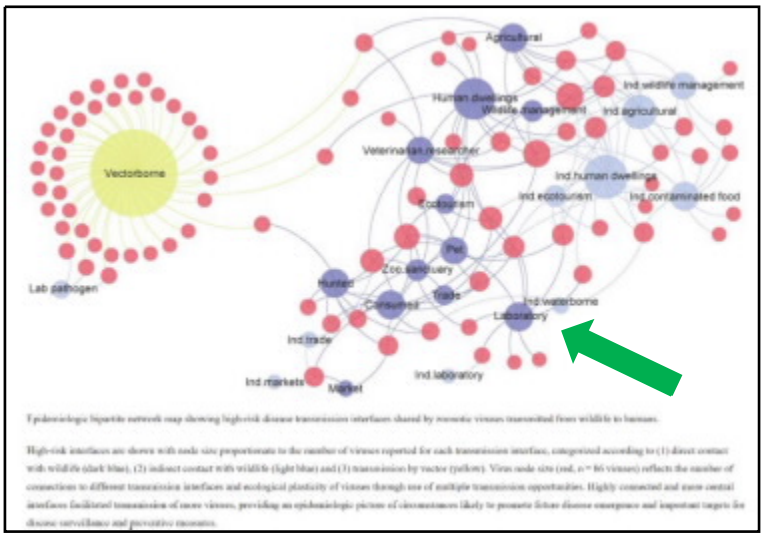
A Bayesian analysis concludes beyond a reasonable doubt that SARS-CoV-2 is not a natural zoonosis but instead is laboratory derived

Introduction. A two-hypothesis, Bayesian analysis was conducted to determine the origin of the SARS-CoV-2 pandemic. The conclusion was that it was created in a laboratory with synthetic biology tools from a bat beta coronavirus, subgenera sarbecovirus backbone (98.9% probability) and not from a natural, zoonotic transmission (1.1%).

There is no direct evidence of whether the release was accidental, or deliberate but circumstantial evidence makes it is highly likely it was accidental.

At the one-year anniversary of the first cases of COVID-19, the coronavirus pandemic caused by the SARS-CoV-2 virus, the origin of the virus remains unknown. While leading institutions and experts have been consistently adamant that it is a zoonotic disease which jumped from a bat reservoir host to humans directly or through an intermediate host the alternative possibility that it escaped from a laboratory conducting research remains a viable option.

In fact, in 2015 Peter Daszak, a leading zoonotic proponent of CoV-2 origin, wrote in, "Spillover and pandemic properties of zoonotic viruses with high host plasticity,"[11] that transmission from laboratories was a major source of zoonotic disease. The Figure below from the Daszak paper shows this important relationship (green arrow):

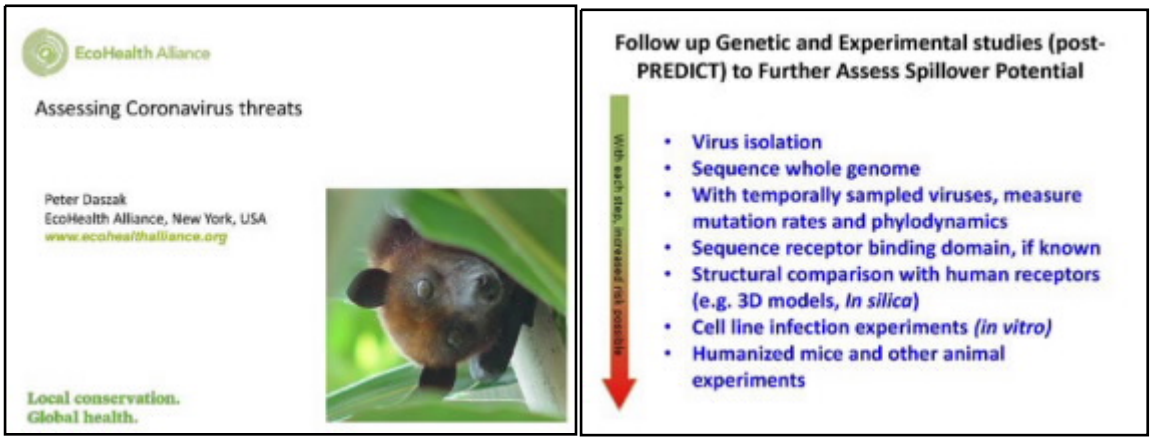


Daszak et al. also writes: "**Zoonotic virus spillover** from wildlife was most frequent in and around human dwellings and in agricultural fields, as well as **at interfaces with occupational exposure to animals** (hunters, **laboratory workers**, veterinarians, **researchers**, wildlife management, zoo and sanctuary staff). **Primate hosts were most frequently cited as the source of viruses transmitted by direct contact** during hunting (exact P = 0.051) and **in laboratories**

[11] https://www.nature.com/articles/srep14830

(exact P = 0.009).” [Emphasis added]. Primate “hosts” can presumably include monkey cell culture, such as the ubiquitous VERO cell used in all virology laboratories, including the WIV.

In 2015 Dr. Daszak spoke of the spillover danger of certain types of laboratory research:



He writes: “with each step, increased risk possible” with “Humanized mice and other animal experiments” the highest risk work.

In a prescient Twitter post in November 2019, he highlights the work he is doing using recombinant viruses with humanized mice and making viruses that “**don’t respond to MAbs, vaccines…**” in response to criticism his work is of limited value:

Clearly, before the beginning of the pandemic, Daszak, now a member of both the WHO and Lancet teams being sent to China to explore the origin of CoV-2, could entertain the eal possibility of a laboratory created virus escaping into the human population/community.

The purpose of this analysis is to use a Bayesian Inference Network approach to the collected circumstantial evidence that is available to provide likelihoods of the alternative hypotheses as to the origin of SARS-CoV-2. The analysis also will include certain prior probabilistic conclusions to help set the initial state before the proprietary evidence is used.

Origin hypotheses: Initial States to establish the posterior probabilities.

Two published Bayesian analyses and two independent studies of zoonotic spillover from nature and laboratory-acquired infections in Asia will be used to establish the posterior probabilities for this analysis.

Zoonotic spillover frequency versus laboratory acquired infection frequency based on two published papers, one by Daszak et al.

In 2015 Daszak et al. published a paper entitled, "Spillover and pandemic properties of zoonotic viruses with high host plasticity,"[1] in which they identified 162 zoonotic viruses with naturally occurring animal-to-human transmission from 1990-2010. This is a frequency of 162/20 = 8.1 events per year.

They also note: "The majority (94%) of zoonotic viruses described to date (n = 162) are RNA viruses, which is 28 times higher (95% CI 13.9–62.5, exact P < 0.001) than the proportion of RNA viruses among all vertebrate viruses recognized, indicating that RNA viruses are far more likely to be zoonotic than DNA viruses." CoV-2 is an RNA virus.

Finally, they note that: "In general, wild animals were suggested as the source of zoonotic transmission for 91% (86/95) of zoonotic viruses compared to 34% (32/95) of viruses transmitted from domestic animals and 25% (24/95) with transmission described from both wild and domestic animals."

One of the caveats of the Daszak data is that it categorizes a laboratory-acquired infection (LAI) from an animal collected from the wild as a zoonotic spillover. There is no data in the paper to assess this issue and leaving it uncorrected is a conservative approach since it only inflates the natural zoonotic frequency.

In 2018 a paper by Siengsanan-Lamont entitled, "A Review of Laboratory-Acquired Infections in the Asia-Pacific: Understanding Risk and the Need for Improved Biosafety for Veterinary and Zoonotic Diseases," was published.[12] They reported 27 LAIs between 1982 and 2016, a frequency of 27/(2016 – 1982) = 0.8 events per year.

Using these historical frequencies of zoonotic spillover versus LAI to predict a future event can be calculated in the following manner:

Evidence	ZoonoticOrigin	LaboratoryOrigin
Frequency per year from Daszak paper	8.1	NA
Frequency per year from Siengsanan-Lamont paper	NA	0.8
Total events per year	8.1 + 0.8 = 8.9	8.1 + 0.8 = 8.9
Likelihood of future event based on historical frequency	8.1/8.9 X 100 = 0.91	0.8/8.9 X 100 = 0.9

Daszak's initial state analysis. This evidence sets the likelihood that CoV-2 was a zoonotic origin event at 91% and a laboratory origin event at 9%.

[12] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6073996/

Independent prior analyses: Rootclaim.

The next data that will be used is a recent analysis published on the Rootclaim website.[13] Three hypotheses below were analyzed through a series of evidence statements and the probabilities that each was the origin of SARS-CoV-2 determined:

Hypothesis	Calculated Probability
Lab escape: The virus was the subject of genetic research, including gain-of-function, and was released by accident	81%
Zoonotic: The virus evolved in nature and was transmitted to humans from a non-human vertebrate animal	16%
Bioweapon: The virus was genetically engineered as a bioweapon and was deliberately released	3%

As can be seen, the highest likelihood probability is an accidental lab escape, the lowest a bioweapon. The details of the evidence used to arrive at this conclusion is contained in Appendix 1. A summary of the changes in probability at each level of evidence analysis is shown in this table:

Evidence	Laboratory	Zoonosis	Bioweapon
Starting point	1.2%	82%	16%
Contagion and mortality	1.4%	97%	1.9%
Outbreak location: Wuhan	42%	56%	2.8%
Virus sources near Wuhan	16%	83%	1.0%
Chimera	37%	60%	2.5%
Furin cleavage	72%	23%	4.8%
WIV lab procedures	80%	17%	3.5%
WIV disassociation	89%	9%	2.0%
Chinese response	90%	8%	1.7%
No reported infections at WIV	86%	11%	2.4%
No whistleblowers	81%	16%	2.8%

As can be seen, the starting point assumed an 82% probability of a zoonotic origin. This starting point is a reasonable value and will be used here. Since some of the evidence in the above analysis will be used here, only the starting point will be used and not the probability changes from there.

For purposes of this analysis only the Rootclaim initial state will be used since much of their evidence is also covered in the analysis here.

[13] https://www.rootclaim.com/analysis/what-is-the-source-of-covid-19-sars-cov-2

In a paper by Daszak and colleagues it states: "In general, wild animals were suggested as the source of zoonotic transmission for 91% (86/95) of zoonotic viruses compared to 34% (32/95) of viruses transmitted from domestic animals and 25% (24/95) with transmission described from both wild and domestic animals."[1]

On the other hand, domestic animals seem to have been ruled out for SARS-CoV-2. In an interview for *Science* in July 2020, Dr. Zhengli Shi, head of coronavirus research at the Wuhan Institute of Virology, stated: "Under the deployment of the Hubei Provincial Government, our team and researchers from Huazhong Agricultural University collected samples of farmed animals and livestock from farms around Wuhan and in other places in Hubei Province. We did not detect any SARS-CoV-2 nucleic acids in these samples."[14]

Reanalysis of Rootclaim initial state to remove Bioweapons option.

The US government uses the following definitions:

"Gain-of-function (GOF) studies, or research that improves the ability of a pathogen to cause disease, help define the fundamental nature of human-pathogen interactions, thereby enabling assessment of the pandemic potential of emerging infectious agents, informing public health and preparedness efforts, and furthering medical countermeasure development.

Gain-of-function studies may entail biosafety and biosecurity risks; therefore, the risks and benefits of gain-of function research must be evaluated, both in the context of recent U.S. biosafety incidents and to keep pace with new technological developments, in order to determine which types of studies should go forward and under what conditions."[15]

"Dual use research of concern (DURC) is life sciences research that, based on current understanding, can be reasonably anticipated to provide knowledge, information, products, or technologies that could be directly misapplied to pose a significant threat with broad potential consequences to public health and safety, agricultural crops, and other plants, animals, the environment, materiel, or national security. "[16]

For this analysis, the assumption is made that GOF and DURC are largely the same processes and techniques in the laboratory and thus can only be distinguished by direct, documentary evidence of the intent of the research from administers in the facilities conducting the work.

In the absence of any such documentary evidence that bioweapon research was being conducted or that SARS-CoV-2 is a bioweapon and to take the least inflammatory posture, the initial state for the above prior analysis will be recalculated by eliminating the hypothesis, and its accompanying probability, that SARS-CoV-2 was created as a bioweapon. The revised initial state calculation is shown in this table:[17]

[14] https://www.sciencemag.org/sites/default/files/Shi%20Zhengli%20Q%26A.pdf
[15] https://www.phe.gov/s3/dualuse/Pages/GainOfFunction.aspx
[16] https://www.phe.gov/s3/dualuse/Pages/default.aspx
[17] For clarity, the 3% bioweapon probability was simply dropped and the remaining likelihoods, 81% and 16%, were normalized.

Evidence	ZoonoticOrigin	LaboratoryOrigin	BioweaponsOrigin
Rootclaiminitial state	0.86	0.012	0.16
Remove bioweapons	NA	NA	0
Normalizeremaining hypotheses	0.86/(0.86+0.012) = 0.986	0.012/(0.86+0.012) = 0.014	NA

Rootclaim Initial state analysis, adjusted. This evidence sets the likelihood that CoV-2 was a zoonotic origin event at 98.6% and a laboratory origin event at 1.4%.

Additional Prior Evidence by Demaneuf and De Maistre. A second prior Bayesian analysis was performed by professionally educated risk assessment personnel and Chinese-language speaking professionals[18] and is included herein in its entirety. For the sake of brevity, the zoonotic origin evidence was based primarily on population size, distribution, and geographic distribution of bat populations relative to Wuhan. With respect to a lab accident, they separately analyze probabilities of a virus escape during collection, transport, and direct lab accidents and then separately the probability of a community outbreak following a lab escape. They also use primary Mandarin-language sources for Chinese estimates of the same events, showing corroboration of the probabilities. Their conclusion is that the probability of a lab escape ranges from 6% to 55% with a zoonotic origin a zoonotic origin probability being 45% to 94%.

Second Bayesian analysis. Using the most conservative probabilities, this evidence sets the likelihood that CoV-2 was a zoonotic origin event at 94% and a laboratory origin event at 6%.

Selection of initial state for Bayesian analysis.

The Text-Table below summarizes the three approaches to an initial state as to the origin of CoV-2. While the Demaneuf and De Maistre analyses set a range for the zoonotic origin of 45% to 94%, I have used the top of the range of their probability of a zoonotic origin to be conservative.

Prior Analysis	Zoonotic Origin	Laboratory Origin
Daszak et al. paper	91%	9%
Rootclaim Bayesian analysis	98.6%	1.4%
Demaneuf and De Maistre Bayesian analysis	94%	6%

Using a simple online calculator[19] the mean of these three value sets is 94.5%, the standard deviation is ± 3.8%, and the 95% confidence interval is ± 4.3%. Using these data, the upper bound of the 95% confidence interval is 98.8% and, to be most conservative, this will be used as the starting probability of a zoonotic origin.

Initial state for this analysis. The likelihood that SARS-CoV-2 began as a zoonotic event is 98.8% and the likelihood it began as a laboratory event is 1.2%.

[18] https://zenodo.org/record/4067919#.X-qIm9gzbOj . For reference purposes, this paper comes with a spreadsheet listing 112 individual BSL-3 labs in China across 62 lab-complexes.
[19] https://www.calculator.net/standard-deviation-calculator.html?numberinputs=91%2C+94%2C+98.6&ctype=s&x=48&y=19

1. General approach of this analysis[20]

This analysis is intended to examine two competing and mutually exclusive theories of the origin of the coronavirus, SARS-CoV-2 (CoV-2), and the pandemic it has caused, COVID-19.

At the time of this writing there have been 83 million confirmed cases and 1.8 million deaths.[21] Some sources place the economic damage at $21 trillion USD.

Bayes Theorem

This brief description of the Bayes Theorem was taken from the work of Jon Seymour: [22]

"The eponymously named Bayes Theorem was discovered by the Reverend Thomas Bayes in the 1700's and saved for posteriority by an archivist of his papers who discovered the work posthumously. In common language, it provides a rational technique for revising a prior belief in light of new evidence. The equation for Bayes Theorem is given below:

$$P(H|E) = \frac{P(E|H).P(H)}{P(E)}$$

where:

- H is the statement of the hypothesis of interest
- P(H) is the prior probability that the hypothesis is true, independent of the evidence.
- E is the evidence being used to revise the belief in hypothesis
- P(E) is the marginal likelihood of the evidence, independent of the hypothesis
- P(E|H) is the likelihood the evidence, given that the hypothesis is true
- P(H|E) is the posterior probability of the hypothesis, given the evidence.

P(E) is sometimes difficult to estimate, but the following identity must hold:

$$P(E) = P(E|H).P(H) + P(E|\hat{H}).P(\hat{H})$$

Here P(E|^H) is the probability of the evidence, assuming the hypothesis is false and P(^H) is the probability the hypothesis is false which is the same as 1-P(H). Estimating the two conditional probabilities P(E|H) and P(E|^H) is generally easier than estimating the unconditional probability, P(E)."

---

[20] The statistical approach and many of the individual statistical analyses were performed by Dr. Martin Lee, PhD, Adjunct Professor of Biostatistics, UCLA. https://ph.ucla.edu/faculty/lee The likelihood adjustments to the Bayesian analysis, which you can see are routine math, were conducted by the author.
[21] https://www.worldometers.info/coronavirus/coronavirus-cases/
[22] https://jonseymour.medium.com/a-bayesian-analysis-of-one-aspect-of-the-sars-cov-2-origin-story-where-the-first-recorded-1fbdcbea0a2b

Theory One. The zoonotic theory is that a vertebrate animal was infected with CoV-2 or an ancestor (Index Host) and that a human was infected with contact to that Index Host in some manner. Human-to-human spread then followed.

Theory Two. The laboratory origin theory is that CoV-2 or an ancestor was being used in laboratory experiments and that it 'escaped' from the lab via an infected person, lab animal, experimental waste, etc.

I have found no evidence of a deliberate release and early firsthand accounts of local officials and scientists suggest surprise and consternation. If this was a deliberate release, such evidence would be extremely local, limited in distribution, and highly compartmentalized. It is beyond the scope of this analysis.

Weight of the evidence. For purposes of the calculation of posterior probabilities in the Bayesian analysis, evidence which has a statistical basis will be used directly to adjust the probabilities.

Statistically significant evidence. Since some of the probability calculations have astronomical values which would make a single such evidence statement, if inputted directly, swamp any further calculation and make their later contribution mute, a decision was made to simply treat quantitative probabilities as significant at the $p = 0.05$ level, no matter how much 'more significant' the calculation suggested.

So, for example, a probability of certain codon usage coming from nature may be one in 440 or $p = 0.002$, the contribution of this evidence to the input to the posterior probability adjustment would be set at a p-value of 0.05. In such cases the adjustment would be to change the 'winning' hypothesis by multiplying by 19, since a $p = 0.05$ is the same as a 19 out of 20 likelihood event. This is a conservative treatment of what would be highly significant data.

Other quantitative evidence. If a piece of evidence can be quantified but it does not reach a significance of $p = 0.05$ it will be used directly in the likelihood adjustment.

Non-quantitative evidence. For evidence that cannot be quantified, the decision was made to treat these as quantitative outcomes with a 51% to 49% likelihood value with respect to the 'winning' hypothesis. This has the effect of increasing the probability of that hypothesis for that step in the Bayesian analysis by 1.04. This 51%/49% concept is related to the legal standard of the 'preponderance of the evidence' used in civil litigation.

Independence. An important qualitative assessment that must be made is whether or not two pieces of evidence are independent of each other. If they are independent, they can each be used in determining a new likelihood calculation. If they are dependent on each other then they must be combined and only a single new likelihood analysis can be made. Where ever possible, evidence statements that could be considered as dependent are called out and this rule is followed on their contribution to the analysis.

Subjective Discount Factor. The impact of each piece of evidence was adjusted further by a subjective discount factor. This is a qualitative assessment of the overall veracity of a particular

piece of evidence when all factors, samples, methods, data sources, etc. are taken into context. It varies from 60% to 100% and is used as a fraction to reduce the impact of a single piece of evidence even further.

Hearsay. Just as in a court of law, evidence, usually attributed to a given person or persons, that is not directly available but instead relies on statements of others is usually not allowed in a court trial and will accordingly not be used here to adjust the Bayesian analysis. It may be recorded and preserved as a placeholder and reminder for further research. If new, direct evidence can be found than the bar of using it is lifted and it can be used for adjustment.

Significant figures. Because of the overall nature of the analyses here, all math calculations related to likelihoods are performed and carried forward at the 'one significant figure' level, with standard rounding rules applied. This has the effect, near the end of the cumulative evidence, of failing to change the relative probabilities as the small adjustments are reversed in the rounding process.

Evidence. International committees to investigate the origin of SARS-CoV-2 may not be impartial.

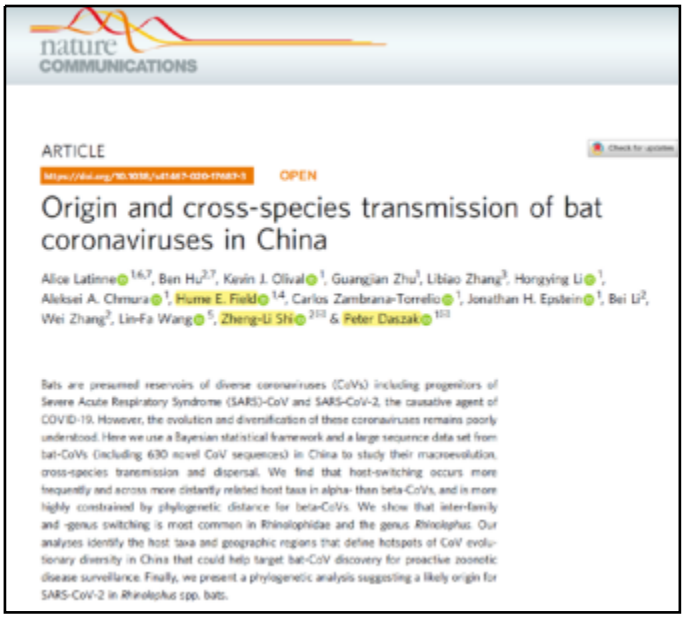
At the time of the writing of this manuscript there are two committees charged with examining the evidence and determining the origin of the SARS-CoV-2 virus. One committee is commissioned by the World Health Organization (WHO) and the other is an ad hoc committee established by the British medical journal, *The Lancet.*

The composition of the two committees is shown in the Text-Table below:

	Lancet Commission of CoV-2	WHO Commission on CoV-2 origin
	Dr. Peter Daszak, Chair	**Dr. Peter Daszak, Ph.D (EcoHealth Alliance, USA)**
	Dr. John Amuasi	Prof. John Watson (Public Health England, United Kingdom)
	Dr. Danielle Anderson	Prof. Dr. Marion Koopmans, DVM PhD (Erasmus MC, Netherlands)
	Dr. Isabella Eckerle	Prof. Dr. Dominic Dwyer, MD (Westmead Hospital, Australia)
Also co-author	Dr. Hume Field	Vladimir Dedkov, Ph.D (Institute Pasteur, Russia)
	Dr. Gerald Keusch	Dr. Hung Nguyen, PhD (International Livestock Research Institute (ILRI), Vietnam)
	Dr. Dato' Sai Kit (Ken) Lam	PD. Dr. med vet. Fabian Lendertz (Robert Koch-Institute, Germany)
	Dr. Carlos das Neves	Prof. Dr. Thea Fisher, MD, DMSc(PhD) (Nordsjællands Hospital, Denmark)
	Dr. Malik Peiris	Dr. Farag El Moubasher, Ph.D (Ministry of Public Health, Qatar)
	Dr. Stanley Perlman	Prof. Dr. Ken Maeda, PhD, DVM (National Institute of Infectious Diseases, Japan)
	Dr. Linda J. Saif	WHO Commission of CoV-2 origin
	Dr. Supaporn Wacharapluesadee	
	Lancet Commission on CoV-2	
	Signed Lancet letter	
	Co-author with Daszak	

There are a number of potential conflicts of interest:

Fully half of The Lancet's team had already suggested that any lab-leak hypothesis was a "conspiracy theory" in a January 2020 paper that has been shown elsewhere within to have been orchestrated behind the scenes to appear spontaneous.



nature COMMUNICATIONS

ARTICLE

OPEN

Origin and cross-species transmission of bat coronaviruses in China

Alice Latinne[1,6,7], Ben Hu[2,7], Kevin J. Olival[1], Guangjian Zhu[3], Libiao Zhang[3], Hongying Li[1], Aleksei A. Chmura[1], Hume E. Field[1,4], Carlos Zambrana-Torrelio[1], Jonathan H. Epstein[1], Bei Li[2], Wei Zhang[2], Lin-Fa Wang[5], Zheng-Li Shi[2✉] & Peter Daszak[1✉]

Bats are presumed reservoirs of diverse coronaviruses (CoVs) including progenitors of Severe Acute Respiratory Syndrome (SARS)-CoV and SARS-CoV-2, the causative agent of COVID-19. However, the evolution and diversification of these coronaviruses remains poorly understood. Here we use a Bayesian statistical framework and a large sequence data set from bat-CoVs (including 630 novel CoV sequences) in China to study their macroevolution, cross-species transmission and dispersal. We find that host-switching occurs more frequently and across more distantly related host taxa in alpha- than beta-CoVs, and is more highly constrained by phylogenetic distance for beta-CoVs. We show that inter-family and -genus switching is most common in Rhinolophidae and the genus *Rhinolophus*. Our analyses identify the host taxa and geographic regions that define hotspots of CoV evolutionary diversity in China that could help target bat-CoV discovery for proactive zoonotic disease surveillance. Finally, we present a phylogenetic analysis suggesting a likely origin for SARS-CoV-2 in *Rhinolophus* spp. bats.

The above paper published in August 2020 has as co-authors Drs. Hume, Daszak, and Shi. Having two of these scientists be asked to investigate a third co-author is a clear conflict of interest.

A newspaper piece about Peter Daszak entitled, "The doctor who denied COVID-19 was leaked from a lab had this major bias,"[23] questions his ability to be unbiased due to a deep, long history of work with Dr. Zhengli Shi of the WIV.

A lengthy piece in Wired was subtitled, "The two major investigations into the origins of the pandemic are compromised by potential conflicts of interest."[24]

Since the purpose of this manuscript is to evaluate the scientific evidence concerning the origin of SARS-CoV-2 no further effort will be put into these matters. If and when a report is prepared from either committee there will be time to analysis the work in the reports and compare it to prior publications and statements from the committee members to look for bias.

Likelihood from initial state is unchanged following this evidence analysis:

Zoonotic origin (98.8%) and laboratory origin (1.2%)

[23] https://nypost.com/2021/01/16/doctor-who-denied-covid-was-leaked-from-a-lab-had-this-major-bias/
[24] https://www.wired.com/story/if-covid-19-did-start-with-a-lab-leak-would-we-ever-know/?utm_source=twitter&utm_medium=social&utm_campaign=onsite-share&utm_brand=wired&utm_social-type=earned

Evidence. Three high visibility papers grounded the zoonotic origin hypothesis in the public conversation from February to May 2020: a pros and cons analysis.

Introduction. The two key data points from December 2019 concerning the origin of the SARS-CoV-2 coronavirus infection, the cause of COVID-19, are the observation that a large number of the earliest patients worked or had visited the Hunan Seafood Market in Wuhan, China and that the hospitals where the first patients were admitted were a short distance from the Wuhan Institute of Virology (WIV), the only high security, BSL-4 laboratory in all of China, and arguably the leading research institute in the world studying coronaviruses of the type causing COVID-19.

The first data point is reminiscent of the origin of SARS-CoV-1, a zoonosis with interspecies transmission from bats to civet cats and then to humans, identified in wet markets in southern China. The second data point is reminiscent of the four SARS-CoV-1 human spillovers that occurred after the 2003 epidemic ended and were each a laboratory-acquired infection (LAI) by a scientist working in a government research laboratory, much like the WIV, and then local human-to-human spread and nearby hospital admission.

To be clear in this paper, the term zoonosis will only be used to describe a interspecies transmission outside of a laboratory. This point seems important to clarify since Dr. Zhengli Shi, head of coronavirus research at the WIV, has previously reported: "An outbreak of hemorrhagic fever with renal syndrome occurred among students in a college (College A) in Kunming, Yunnan province, China in 2003. Subsequent investigations revealed the presence of hantavirus antibodies and antigens in laboratory rats at College A and two other institutions. Hantavirus antibodies were detected in 15 additional individuals other than the index case in these three locations. Epidemiologic data indicated that the human infections were a result of **zoonotic transmission** of the virus from laboratory rats."[25] [emphasis added.] The author has found no other support for the use of the term zoonotic transmission with respect to an LAI and its dual use could be confusing, and so will be avoided.

While the two initial data points would suggest that a balanced approach should be taken with respect to investigations of the origin of SARS-CoV-2, three high visibility publications that argued the laboratory origin idea was a "conspiracy theory" and strongly argued that it was of zoonotic origin foreclosed legitimate debate for much of 2019. The purpose of this evidence analysis is to examine these papers and weigh the strength of the evidence.

Paper 1: The February 3, 2020 paper by WIV scientist Dr. Shi et al. entitled: "A pneumonia outbreak associated with a new coronavirus of probable bat origin."

This seminal paper set the stage for the zoonotic origin of SARS-CoV-2 and has been accessed over one million times. According to *Nature*, this article is in the 99th percentile (ranked 24th) of the 326,159 tracked articles of a similar age in all journals and the 99th percentile (ranked 2nd) of the 783 tracked articles of a similar age in Nature.

[25] https://pubmed.ncbi.nlm.nih.gov/20380897/

However, a careful analysis of it shows serious issues which suggest it is unreliable. The following analysis is in the form of an independent manuscript:

The seminal paper from the Wuhan Institute of Virology claiming SARS-CoV-2 probably originated in bats appears to contain a contrived specimen, an incomplete and inaccurate genomic assembly, and the signature of laboratory-derived synthetic biology

The coronavirus RaTG13 was purportedly identified in a bat "fecal" specimen that is probably not feces, has significant unresolved method-dependent genome sequence errors and an incomplete assembly with significant gaps, and has an anomalous base substitution pattern that has never been seen in nature but is routinely used in codon-optimized synthetic genome constructions performed in the laboratory

Abstract. The species of origin for the SARS-CoV-2 coronavirus that has caused the COVID-19 pandemic remains unknown after over six months of intense research by investigators around the world. The current consensus theory among the scientific community is that it originated in bats and transferred to humans either directly or through an intermediate species; no credible intermediate species exists at this time. The suggested origin early on from a Wuhan "wet market" has been determined to be a red herring and the pangolin is no longer considered a likely intermediate by the virology community.

The basis for the hypothesis that SARS-CoV-2 probably evolved from bats initially came from a February 2020 paper[26] from Dr. Zheng-Li Shi's laboratory at the Wuhan Institute of Virology (WIV). In that paper the Wuhan laboratory made two claims: 1), "a bat fecal sample collected from Tongguan town, Mojiang county in Yunnan province in 2013" contained a coronavirus, originally designated "Rhinolophus bat coronavirus BtCoV/4991[27]" in 2016 but renamed in their paper, RaTG13; and 2), the genomes of RaTG13 and SARS-CoV-2 had an overall identity of 96.2%, making it the closest match to SARS-CoV-2 of any coronavirus identified at that time. RaTG13 remains the closest match to SARS-CoV-2 at the current time.

In this paper I document that:

1) The RaTG13 specimen was not a bat fecal specimen, based on a comparison of the relative bacterial and eukaryotic genetic material in the purported fecal specimen to nine authentic bat fecal specimens collected in the same field visits as RaTG13 was collected by the Wuhan laboratory, run on the same Illumina instrument (id ST-J00123), and published in a second paper in February 2020.[15] While the authentic bat fecal

[26] Zhou, P., Yang, X., Wang, X. *et al.* A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* **579,** 270–273 (2020). https://doi.org/10.1038/s41586-020-2012-7 .

[27] A Coronavirus BtCoV/4991 Genbank entry by Dr. Shi records: organism="Rhinolophus bat coronavirus BtCoV/4991." In July 2020 she wrote: "Ra4991 is the ID for a bat sample while RaTG13 is the ID for the coronavirus detected in the sample. We changed the name as we wanted it to reflect the time and location for the sample collection. 13 means it was collected in 2013, and TG is the abbreviation of Tongguan town, the location where the sample was collected."

samples were, as expected, largely bacterial (specifically, 65% bacteria and 12% eukaryotic genetic sequences), the purported RaTG13 specimen had a reversed composition, with mostly eukaryotic genes and almost no bacterial genetic material (0.7% bacteria and 68% eukaryotic). The RaTG13 specimen was also only 0.01% virus genes compared to an average of 1.4% for authentic bat fecal specimens. A Krona analysis identified 3% primate sequences consistent with VERO cell contamination, the standard monkey cell culture used for coronavirus research, including at the Wuhan laboratory. Based on using the mean and standard deviation of the nine authentic bat fecal specimens from the Wuhan laboratory, the probability that RaTG13 came from a true fecal sample but had the composition reported by the Wuhan laboratory is one in thirteen million;

2) According to multiple references, RaTG13 was identified via Sanger dideoxy sequencing before 2016, partially sequenced by amplicon sequencing in 2017 and 2018, and then complete sequencing and assembly by RNA-Seq in 2020, although some reports from WIV suggest the timing of the RNA-Seq experiments may have been performed earlier than 2020. In any case, a Blast analysis of sequences from the amplicon and RNA-Seq experiments indicates an approximate 5% nucleotide difference, 50-fold higher than the technical error rate for RNA-Seq of about 0.1%. At least two gaps of over 60 base-pairs, with no coverage in the RNA-Seq data, were easily identified. The incomplete assembly and anomalous, method-dependent sequence divergence for RaTG13 is troublesome;

3) The pattern of synonymous to non-synonymous (S/NS) sequence differences between RaTG13 and SARS-CoV-2 in a 2201 nucleotide region flanking the S1/S2 junction of the Spike Protein records 112 synonymous mutation differences with only three non-synonymous changes. Based on the S/NS mutational frequencies elsewhere in these two genomes and generally in other coronaviruses the probability that this mutation pattern arose naturally is approximately one in ten million. A similar pattern of unnatural S/SN substitutions was seen in a 10,818 nt region of the pp1ab gene. This pp1ab gene pattern has a probability of occurring naturally of less than one in 100 billion. A total of four regions of the RaTG13 genome, coding for 7,938 nt and about one-quarter of the entire genome, contain over 200 synonymous mutations without a single non-synonymous mutation. This has a probability of one in 10^{-17}. A possible explanation, the absolute criticality of the specific amino acid sequence in the regions which might make a non-synonymous change non-infective, is ruled out by the rapid appearance of an abundance of non-synonymous mutations in these very regions when examining the over 80,000 human SARS-CoV-2 specimens sequenced to date. An alternative hypothesis, that this arose by codon substitution is examined. It is demonstrated, by example from a published codon-optimized SARS-Cov-2 Spike Protein experiment, that the anomalous S/SN pattern is precisely the pattern which is produced, by design, when synthetic biology is used and represents a signature of laboratory construction.

Based on the findings concerning the RaTG13 data, including anomalies and inconsistent statements about RaTG13, its origin, renaming, and sequencing timing; the finding that the specimen it is purported to have come from is not bat feces and has a signature of cell culture contamination; the unexplained method-dependent 5% sequence difference for RaTG13; and the S/SN mutation pattern reported, which to my knowledge has never been seen in nature, it can be concluded that RaTG13 is not a pristine biological entity but shows evidence of genetic manipulation in the laboratory.

Until a satisfactory explanation of the findings in this paper have been offered by the Wuhan laboratory, all hypotheses of the proximal origin of the entry of SARS-CoV-2 into the human population should now include the likelihood that the seminal paper contains contrived data. For example, the hypothesis that SARS-CoV-2 was the subject of laboratory research and at some point escaped the laboratory should be included in the narrative of the origin of SARS-CoV-2 research.

Introduction. Since the first reported patient on December 1, 2019 with a SARS-CoV-2 infection, the virus has caused a pandemic that has led to twenty-five million cases worldwide and over 840,000 deaths as of August 30, 2020. To make progress on treating this disease and preventing the next viral outbreak, knowing the origin of the virus and how it entered the human population is critical.

On February 3, 2020 a paper was published from the Wuhan Institute of Virology that identified a bat coronavirus, RaTG13, as having a 96.2% identity to SARS-CoV-2, quickly providing support for a zoonotic origin, either from bats directly or from bats to humans through an unknown intermediary species. If true, this would replicate the model of SARS-CoV 2003 in which the transmission was from bats to civets to humans and for MERS in which the transmission was from bats to camels to humans. At the time of this paper and through August 30, 2020, no other virus has been identified with a closer sequence homology to SARS-CoV-2 than RaTG13. The publication containing the RaTG13 sequence has been cited over 1600 times in the six months since publication. None of these studies contain research on the isolated virus itself since the virus has never been isolated or cultured. It was apparently found in only one sample from 2013 and that sample has been exhausted.[28]

An examination of the raw data associated with RaTG13 immediately identified serious anomalies, bringing into question the existence of RaTG13 as a biological entity of completely nature origin.

[28] Dr. Shi Science interview July 2020

Materials and Methods.

GenBank accession URL table for sequences used in this paper.

The GenBank accession URLs for the specimens, raw reads, and sequences that are used in this paper are contained in the following Table, which can be used to reach the raw data.

Descriptor	URL Hyperlink
SARS-CoV-2 reference sequence in GenBank	SARS-CoV-2 complete genome
Bat coronavirus RaTG13, complete genome, Genbank	RaTG13 complete genome
RaTG13 purported bat fecal specimen	SRR11085797
Rhinolophus bat coronavirus BtCoV/4991 RNA-dependent RNA polymerase (RdRp) gene, partial cds	BtCoV/4991 RdRp gene
SRX8357956: amplicon_sequences of RaTG13	Specimen descriptor
RNA-Seq data for RaTG13	RNA-Seq data for RaTG13
Reference fecal bat specimens from WIV	SRR11085736
Reference fecal bat specimens from WIV	SRR11085734
Reference fecal bat specimens from WIV	SRR11085737
Reference fecal bat specimens from WIV	SRR11085733
Reference fecal bat specimens from WIV	SRR11085735
Reference fecal bat specimens from WIV	SRR11085738
Reference fecal bat specimens from WIV	SRR11085739
Reference fecal bat specimens from WIV	SRR11085740
Reference fecal bat specimens from WIV	SRR11085741

Below is a screen shot of the GenBank entry for the purported specimen from which RaTG13 was identified and upon which RNA-Seq was performed. While the title claims it is a "Rhinolophus affinis fecal swab" specimen it also records in the design of work entry that "(t)otal RNA was extracted from bronchoalveolar lavage fluid." These descriptions are clearly inconsistent.

SRX7724752: **RNA-Seq of Rhinolophus affinis:Fecal swab**
1 ILLUMINA (Illumina HiSeq 3000) run: 11.6M spots, 3.3G bases, 1.7Gb downloads

Design: Total RNA was extracted from bronchoalveolar lavage fluid using the QIAamp Viral RNA Mini Kit following the manufacturers instructions. An RNA library was then constructed using the TruSeq Stranded mRNA Library Preparation Kit (Illumina, USA). Paired-end (150 bp) sequencing of the RNA library was performed on the HiSeq 3000 platform (Illumina).

Submitted by: Wuhan Institute of Virology, Chinese Academy of Sciences

Study: Bat coronavirus RaTG13 Genome sequencing
PRJNA606165 • SRP249482 • All experiments • All runs
show Abstract

Sample:
SAMN14082201 • SRS6146537 • All experiments • All runs
Organism: unidentified coronavirus

Library:
Name: RaTG13
Instrument: Illumina HiSeq 3000
Strategy: RNA-Seq
Source: METAGENOMIC
Selection: RANDOM
Layout: PAIRED

Runs: 1 run, 11.6M spots, 3.3G bases, 1.7Gb

Run	# of Spots	# of Bases	Size	Published
SRR11085797	11,604,666	3.3G	1.7Gb	2020-02-13

Apparent missing amplicon reads for RaTG13 in GenBank.

There are 33 amplicon reads in GenBank for RaTG13 from experiments recorded as having been performed in 2017 and 2018. A file naming pattern was noticed among the data sets which suggests there may be amplicon runs that were not deposited in GenBank. These files, if related to RaTG13, may contain useful sequence data and an effort should be made to retrieve them and, if appropriate, upload them to GenBank. A Table with the apparently missing data (yellow) is shown here.

Date	Amplicon file name endings							
3-Jun-17	A07	A08						
17-Jun-17	A05	A06						
20-Jun-17						F03	G03	H03
27-Sep-18	A06	B06	C06		E05	F05	G05/G06	H05/H06
29-Sep-18				D05	E05		G04	H04
30-Sep-18	A02	B11						
8-Oct-18			C11				G10	H11
11-Oct-18	A12	B12						
14-Oct-18	A02	B02	C02	D02				

Relationship of *Rhinolophus* bat coronavirus BtCoV/4991 and Bat coronavirus RaTG13.

The Wuhan laboratory has reported on the bat coronaviruses, BtCoV/4991 and RaTG13, in two peer-reviewed publications, one in 2016 and one in February 2020.[29] They have submitted three entries to GenBank for these two viruses, in 2016, February 2020, and May 2020.[30] The GenBank entries confirm sequencing experiments using Sanger dideoxy sequencing in 2016, PCR-generated amplicon sequencing performed on an AB 310 Genetic Analyzer in 2017 and 2018, and RNA-seq performed on an Illumina HiSeq 3000 (instrument id ST-J00123) in 2020. A single GISAID entry records that the RNA-seq data was obtained from an original specimen without passage.[31] This is an important detail since evidence of primate sequences, consistent with VERO cell contamination, is found in this specimen, as reported below, which would suggest laboratory passage.

None of these disclosures report that BtCoV/4991 and RaTG13 are the same coronavirus, simply renamed. This information was only disclosed in a written Question and Answer publication from *Science* magazine by Dr. Shi on July 31, 2020.[4, 32] Given this disclosure months after the original publication concerning RaTG13 in *Nature* it is possible that the omission of the original publication and sequence data concerning BtCoV/4991 violated the "Reporting

[29] 2016 Virologica Sinica paper and February 2020 Nature paper
[30] RaTG13 complete genome Feb 2020, Raw sequence reads for RaTG13 published Feb 2020, Amplicon reads for RaTG13 from 2017 and 2018 published in May 2020.
[31] The GISAID entry is EPI_ISL_402131.
[32] Dr. Shi wrote: "Ra4991 is the ID for a bat sample while RaTG13 is the ID for the coronavirus detected in the sample. We changed the name as we wanted it to reflect the time and location for the sample collection. 13 means it was collected in 2013, and TG is the abbreviation of Tongguan town, the location where the sample was collected."

standards and availability of data, materials, code and protocols" required for *Nature* publications.[33]

The February 2020 papers uses the RNA-Seq data for RaTG13 genome determination but fails to disclose the previous data obtained by Sanger dideoxy sequencing in 2016 and by amplicon sequencing in 2017 and 2018. Since these unrecorded data establish method-dependent sequencing differences of up to 4% the failure to disclose this data or to reconcile these differences is troubling.

In addition, the raw assembly accession data for RaTG13 are not described or linked to the Genbank entry, MN669532, and also no assembly method is specified in the raw data SRX7724752 12 and the Illumina run. And the amplicon sequencing data has sequence gaps of approximately 20% of the genome. Therefore, no primary assembly data has been made available by the WIV for the RaTG13 genome. This is contrary to the *Nature* Reporting Standards[9] as they state: "When publishing reference genomes, the assembly must be made available in addition to the sequence reads."

Relationship of RaTG13 and SARS-CoV-2.

There have been two descriptions of the process by which the RaTG13 genome was identified as closely homologous to SARS-CoV-2. These seem to be inconsistent with each other.

In the February 2020 *Nature* paper[5] it states:

"We then found that a short region of RNA-dependent RNA polymerase (RdRp) from a bat coronavirus (BatCoV RaTG13)—which was previously detected in Rhinolophus affinis from Yunnan province—showed high sequence identity to 2019-nCoV. We carried out full-length sequencing on this RNA sample (GISAID accession number EPI_ISL_402131). Simplot analysis showed that 2019-nCoV was highly similar throughout the genome to RaTG13, with an overall genome sequence identity of 96.2%."

In a July 2020 interview the process was described:

"We detected the virus by pan-coronavirus RT-PCR in a bat fecal sample collected from Tongguan town, Mojiang county in Yunnan province in 2013, and obtained its partial RdRp sequence. Because the low similarity of this virus to SARS-CoV, we did not pay special attention to this sequence. In 2018, as the NGS sequencing technology and capability in our lab was improved, we did further sequencing of the virus using our remaining samples, and obtained the full-length genome sequence of RaTG13 except the 15 nucleotides at the 5' end. As the sample was used many times for the purpose of viral nucleic acid extraction, there was no more sample after we finished genome sequencing, and we did not do virus isolation and other studies on it. Among all the bat samples we collected, the RaTG13 virus was detected in only one single sample. In 2020, we compared the sequence of SARS-CoV-2 and our unpublished bat

[33] Nature research reporting standards for availability of data

coronavirus sequences and found it shared a 96.2% identity with RaTG13. RaTG13 has never been isolated or cultured."

If the full-length genome of RaTG13 was available by 2018 it is unclear why a database search within the WIV for coronaviruses that resembled SARS-CoV-2 would lead to identifying the 370-nt segment representing the RdRp gene (as stated in the February paper) but not the full length RaTG13 genome (which was stated to have been sequenced by 2018). In addition, an assembly of all available amplicon data for RaTG13 from 2017 and 2018 contains gaps of approximately 20% of the genome. If the sample was completely consumed during the 2017-8 sequencing it is unclear how RNA-Seq was conducted in 2020 to permit the full-length genome to be determined.

Analytical methods. Taxonomy of specimens was determined in the NCBI Sequence Read Archive and KRONA.[34] Blast was used for sequence alignment and comparisons.[35]

To evaluate the data from the bat species relative to the RaTG13 fecal sample analysis, the latter was treated as a fixed result with the comparison to the taxonomy results of the nine bat feces specimens. It also was noted that the data were clearly right skewed (and descriptively both mean/median and standard deviation/interquartile range were used). Therefore, a non-parametric procedure, the Wilcoxon signed-rank test was used with the p-value calculated by an exact procedure because of the small sample size. Considering the synonymous to non-synonymous mutation frequency and how to evaluate that for the various protein coding regions of the virus, it was noted that for all of the genes pooled, the ratio of the synonymous to non-synonymous regions was approximately 0.83. To analyze the corresponding distribution for each gene, we assumed that each mutation was an independent observation from a Bernoulli random variable and, therefore the number of synonymous mutations in the gene would have a binomial distribution (with probability 0.83). A probability was then computed for the actual number of synonymous mutations on this basis (the probability was determined on a one-sided basis, i.e. excess mutations, and was calculated as a strict inequality).

Results.

Original characterization of RaBtCoV/4991 (RaTG13) and related bat fecal specimen.

In 2016 Dr. Shi and colleagues published a paper entitled, "Coexistence of multiple coronaviruses in several bat colonies in an abandoned mineshaft[36]" in which a number of novel bat coronaviruses were isolated from bat fecal specimens collected during 2012 and 2013. The viruses were named, according to the paper, in the following fashion:

[34] NCBI Sequence Archive
[35] Blast alignment
[36] Xing-Yi Ge, et. al., Coexistence of multiple coronaviruses in several bat colonies in an abandoned mineshaft, Virologica Sinica, 2016, 31 (1): 31–40. DOI: 10.1007/s12250-016-3713-9

> "The positive samples detected in this study were named using the abbreviated bat species name plus the bat sample number abbreviation. For example, a virus detected from *Rhinolophus sinicus* in sample number 4017 was named RsBtCoV/4017. If the bat was co-infected by two different coronaviruses, numbers were appended to the sample names, such as RsBtCoV/4017-1 and RsBtCoV/4017-2."

In the July 2020 interview Dr. Shi wrote:

> "Ra4991 is the ID for a bat sample while RaTG13 is the ID for the coronavirus detected in the sample. We changed the name as we wanted it to reflect the time and location for the sample collection. 13 means it was collected in 2013, and TG is the abbreviation of Tongguan town, the location where the sample was collected."

The 2016 and 2020 statements about the naming of virus RsBtCoV/4991 appear inconsistent with each other.

Of the 152 coronaviruses identified, 150 were classified as alphacoronaviruses while only two were classified as betacoronaviruses, HiBtCoV/3740-2 and RaBtCoV/4991. The naming convention from the paper means this latter coronavirus was identified in a fecal specimen from a *Rhinolophus affinis* bat and was sample number 4991.

The latter virus was described in the paper as follows:

> "Virus RaBtCoV/4991 was detected in a R. affinis sample and was related to SL-CoV. The conserved 440-bp RdRp fragment of RaBtCoV/4991 had 89% nt identity and 95% aa identity with SL-CoV Rs672. In the phylogenetic tree, RaBtCoV/4991 showed more divergence from human SARS-CoV than other bat SL-CoVs and could be considered as a new strain of this virus lineage."

The Genbank accession number for RaBtCoV/4991 is MN KP876546.1 and in Genbank it is identified as having been collected in July 2013 as a "feces/swabs" specimen.

The RATG13 genome sequence was assembled from low coverage RNA-Seq data.

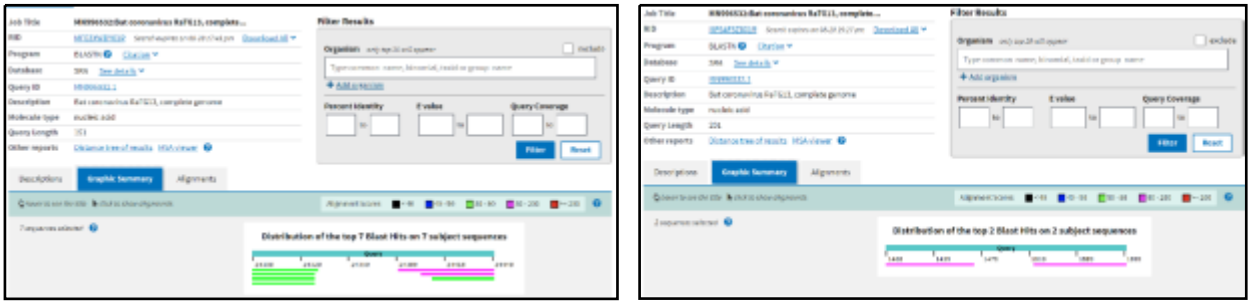
A Blast analysis of the RaTG13 genome against SRR11085797 retrieved about 1700 reads which covers only about 252,000 nt of the total reads of 3.3 Gb. Since the genome size of RaTG13 is known to be about 30,000 nt this represents an 8-fold coverage, typically insufficient for a definitive assembly. For example, some have suggested a 30-fold coverage is necessary to create high quality assemblies.[37]

[37] Sims, D. *et al.* Sequencing depth and coverage: key considerations in genomic analyses. Nature Reviews – Genetics. (2014) 15: 121-132. doi:10.1038/nrg3642.

At an eight-fold coverage and based on the typical practice of having four or more reads to call a SNP,[38] the 8-fold coverage of RaTG13 would have 4.2% bases or about 1260 calls of less than 4 reads and about 10 bases would be missed completely, with no calls at all.

A Blast of the RaTG13 published genome onto the RNA-Seq data documents at least two 60 base-pair gaps with no coverage, precluding a complete assembly.

Given the low coverage in the RNA-Seq data, an exploratory, non-exhaustive Blast search was conducted against the published RaTG13 sequence. Two gaps of over 60 nt, shown below, were easily found:

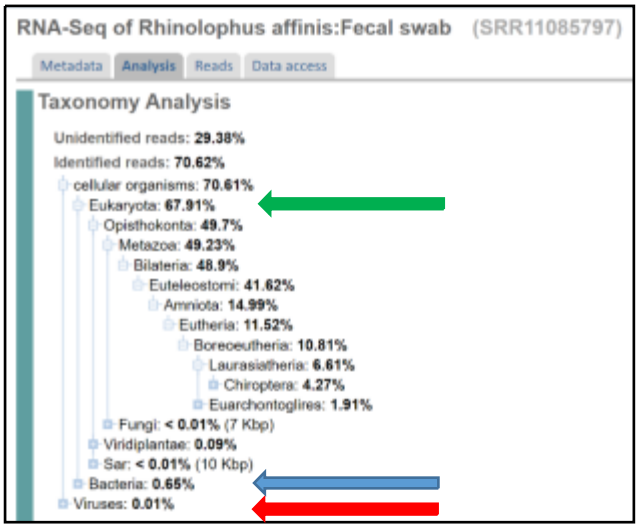


It is conceivable there are additional gaps but the above two are sufficient to document that the complete RaTG13 genome sequence could not have been assembled solely from the RNA-Seq data, as stated.[2]

Taxonomy analysis of the RaTG13 specimen is inconsistent with being from bat feces and shows evidence of laboratory cell culture contamination.

According to the Wuhan laboratory, the RaTG13 coronavirus was a fecal swab specimen collected from a *Rhinolophus affinis* bat in 2013. Unexpectedly, (Text-Figure below) the taxonomy analysis is primarily eukaryotic (green arrow; 67.91%) with only traces of bacteria (blue arrow; 0.65%). The viral genomes also make only a trace contribution (red arrow; 0.01%):

[38] Illumina Technical Bulletin Call Coverage



Taxonomy analysis for RaTG13 data SRR11085797

To compare this specimen composition to bat fecal specimens collected by Dr. Shi and her WIV colleagues and analyzed in other studies, a paper from Dr. Shi's laboratory, also published in February 2020, was identified. In this paper, entitled, "Discovery of Bat Coronaviruses through Surveillance and Probe Capture-Based Next-Generation Sequencing,"[39] a total of nine specimens "collected during previous bat CoV surveillance projects, (were) extracted from bat rectal swabs." According to the Methods section in this paper, the "previous bat CoV surveillance projects" include the field work in 2013 when the RaTG13 was said to have been collected. The comparison below is thus the same specimens collected on the same field surveillance projects by the same investigators from the Wuhan laboratory and sequenced on the same Illumina instrument. These nine specimens will be referred to as "reference fecal specimens" henceforth.

The following Text-Table compares the taxonomical analysis of the RaTG13 and reference fecal specimens. The reference fecal specimens have an average eukaryotic genome content of about 12% while RaTG13's eukaryotic content was 68%. On the other hand, the most abundant genes in the reference fecal specimens were bacterial, with an average of 65%; RaTG13 had less than 1% bacterial genes. And finally, the reference fecal specimens had 1.57% virus genes compared to the 0.01% virus genes of RaTG13.

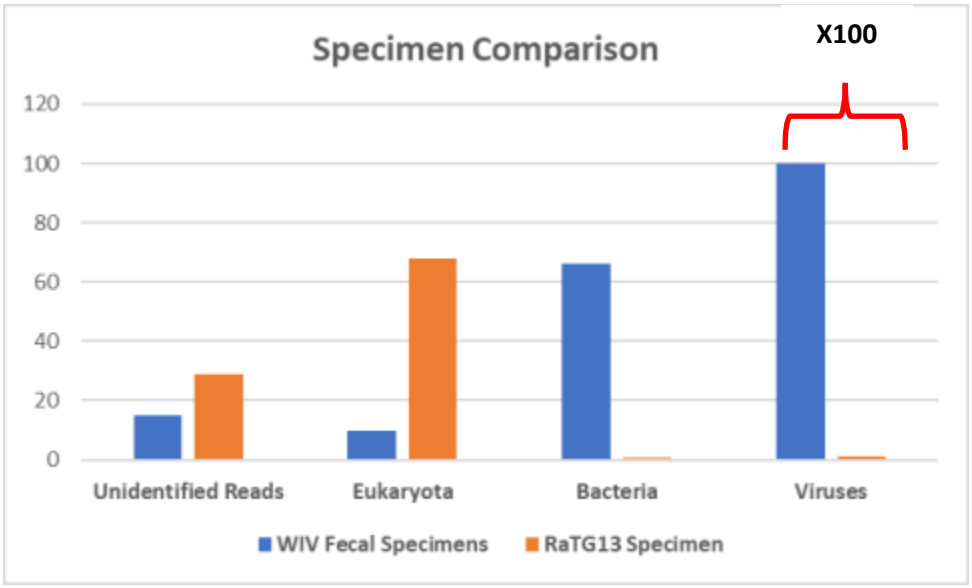
[39] Discovery of bat coronaviruses through surveillance and probe capture-based next-generation sequencing

Specimen ID	Specimen Type	Unidentified Reads	Eukaryota	Bacteria	Viruses	Sum
SRR11085736	*Rhinolophus affinis*	0.86	4.36	91.07	0.03	96.32
SRR11085734	*Miniopterus schreibersii*	3.81	16.03	76.15	0.11	96.1
SRR11085737	*Scotophilus kuhlii*	17.98	8.59	67.81	2.19	96.6
SRR11085733	*Hipposideros larvatus*	13.27	27.99	42.96	4.1	88.32
SRR11085735	*Hipposideros pomona*	34.33	7.96	54.78	0.71	97.78
SRR11085738	*Pipistrellus abramus*	20.33	21.44	47.3	6.45	95.52
SRR11085739	*Tylonycteris pachypus*	61.75	14.34	20.06	0.06	96.21
SRR11085740	*Miniopterus pusillus*	0.78	1.46	99.22	0.05	101.51
SRR11085741	*Rousettus aegyptiacus*	6.44	2.59	88.36	0.45	97.84
Mean +/- SD	Nine bat feces specimens	17.73+/-19.79	11.64+/-9.02	65.30+/-26.10	1.57+/-2.28	96.24+/-3.45
Median +/- IQR	Nine bat feces specimens	13.27+/-24.995	8.59+/-15.26	67.81+/-41.58	0.45+/-3.09	96.32+/-2.00
SRR11085797	**RaTG13 fecal specimen**	**29.38**	**67.91**	**0.65**	**0.01**	**97.95**
	P-value (exact Wilcoxon signed-rank test)	0.16	**0.0039**	**0.0048**	**0.0039**	0.098

As shown in the Text-Table above the RaTG13 specimen is significantly different from the reference fecal specimens in composition. The probabilities for each category, eukaryote, bacteria, and virus, are individually highly statistically significant. They are also independent of each other and therefore the overall probability that RaTG13 has the composition of eukaryote, bacteria, and virus genes that was reported by the Wuhan laboratory but is actually from an authentic bat fecal specimen is less than one in 13 million.

The alternative conclusion is that this sample was not a fecal specimen but was contrived. The data cannot, however, distinguish between a non-fecal specimen that came from true field work on the one hand and a specimen created *de novo* in the laboratory on the other hand.

A graphical comparison of the above data is shown below and visually shows the significant differences between the WIV fecal specimens and the RaTG13 specimen, despite the claim they were collected in the same field surveillance trips:



Another comparison can be made between the reference fecal specimens and the RaTG13 specimen by looking at the taxonomy of the nine to twelve "strong signals" identified on the NCBI Sequence Read Archive. The following Text-Table is a summary of these findings.

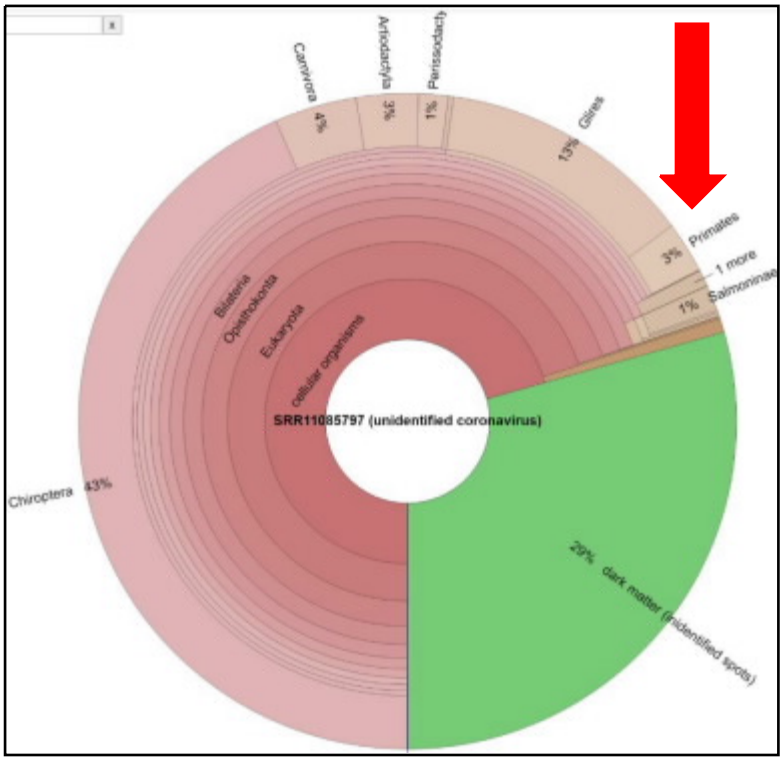
Specimen	The identity of the Strong Signals in the Specimens		
	Bacteria	Eukaryotes	Viruses
Rhinolophus affinis anal swab (SRR11085736)	92%	One magnaorder of placental mammals, includes bat	None
Miniopterus schreibersii anal swab (SRR11085734)	88%	**One bat**, the host bat, Miniopterus sp.	None
Scotophilus kuhlii anal swab (SRR11085737)	56%	**Two bats**, mouse-eared and big brown bats.	Two viruses, kobuvirus (host includes bats) and a Scotophilus kuhlii coronavirus
Hipposideros larvatus anal swab (SRR11085733)	56%	**One bat,** the host bat, Hipposideros sp. and one rodent.	Hipposideros pomona bat coronavirus
Hipposideros pomona: Anal swab (SRR11085735)	78%	**One bat**, the host bat, Hipposideros sp.	None
Pipistrellus abramus: Anal swab (SRR11085738)	73%	**Two bats**, the big brown bat and the mouse-eared bat.	Pipistrellus abramus bat coronavirus
Tylonycteris pachypus: Anal swab (SRR11085739)	67%	**Three bat**s, the microbat, the great roundleaf bat, and a superorder of mammals, which includes bats.	None
Miniopterus pusillus: Anal swab (SRR11085740)	89%	**One bat**, the Natal long-fingered bat.	None
Rousettus aegyptiacus: Anal swab (SRR11085741)	91%	One magnaorder of placental mammals, includes bats.	None
Average	77%		
RaTG13 Rhinolophus affinis:Fecal swab (SRR11085797)	None	All nine strong signals are eukaryotes. **Five bats**, the Great Roundleaf bat, resident of China, the Egyptian fruit bat, which is not found in China, a megabat, mouse-eared bat, and bent-winged bat. Two marmots, the Alpine marmot from Europe and the Yellow-bellied marmot of North America.The paraorder of whales. The red fox.	None

As can be seen, while the strong signals in the authentic specimens contain 56% to 92% (average 77%) bacterial signals, the RaTG13 specimen has no bacteria among the nine strong signals. Most specimens do not have virus strong signals but the three that do are host-related coronaviruses (four) or one host-related kobuvirus.

RaTG13 has no viral strong signals. Among the reference specimens with eukaryotic strong signals, they are either bat-related genes (eleven) or higher order taxonomy signals that include bats (three). There is one anomalous rodent-related signal among the reference specimens.

The RaTG13 specimen is again an outlier with all nine strong signals arising from eukaryotic genes. Five of the nine signals are bats, some resident to China and some with non-Chinese host ranges. Surprisingly, unlike three of the reference bat signals which are identified as host-related, the RaTG13 specimen did not contain *Rhinolophus* sp. host-related strong signals. The remaining four strong signals are marmot-related genes (two), whale-related gene (one), and red fox-related gene (one).

Finally, a Krona analysis (below) identifies 3% primate sequences (red arrow) in the RaTG13 sequence data. This is consistent with contamination by the standard laboratory coronavirus cell culture system, the VERO monkey kidney cell line.



Source: [Krona analysis of RaTG13 specimen]

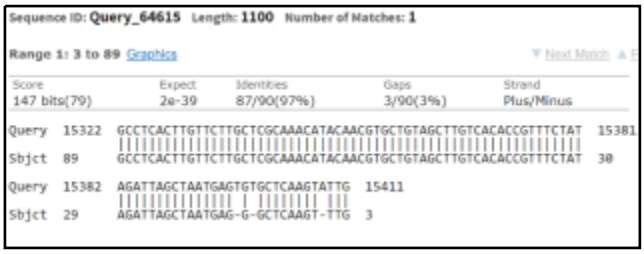
It is unclear why these obviously anomalous findings were not detected during the peer-review process prior to publication of this important work. At this point, an explanation is needed from the WIV to refute the conclusion that the specimen identified as the source of RaTG13 is **not** a bat fecal/anal specimen and that the primate genetic material is consistent with a VERO cell contaminated specimen.

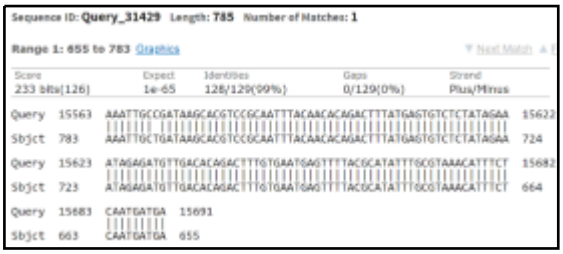
Method-related nt base substitutions in RaTG13.

The original Sanger dideoxy RdRp sequence reported in 2016 is homologous to RNA-seq data from 2020 but is non-homologous to amplicon sequencing data from 2017 and 2018.

As expected, a comparison of the 2016 RdRp GenBank sequence for BtCoV/4991 obtained by Sanger dideoxy sequencing with the RNA-seq sequencing of RaTG13 reported in *Nature* shows 100% identity over the 370 nt segment.

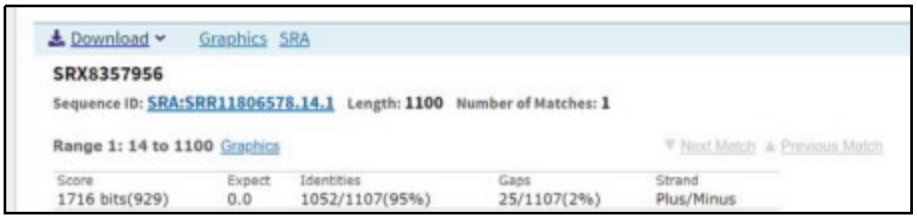
Surprisingly, the two amplicon sequences from 2017 that partially cover the 370 nt RdRp region have four base substitutions or gaps over a total segment of 219 nt (2% divergence).





RaTG13 Spike Protein gene has 5% substitutions when comparing 2020 RNA-Seq and 2017 amplicon sequencing data.

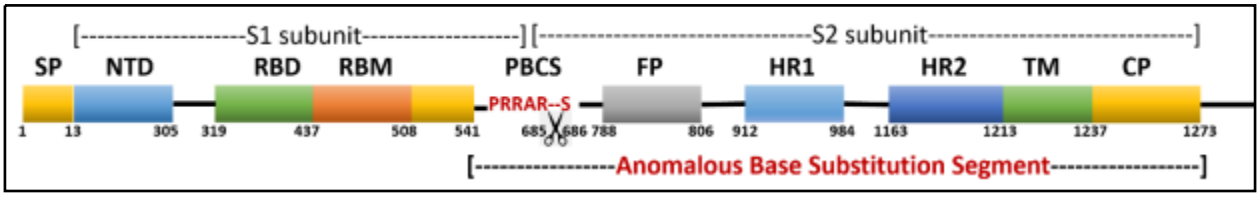
The segment of RaTG13 which shows the greatest sequence divergence between the RNA-seq and amplicon sequencing methods spans from A8886 to A9987 and is shown here below. It contains 80 base substitutions/indels in a 1107 nt sequence (5% substitution and 2% gaps).



No explanation has been offered in publications from the WIV for the method-dependent sequencing differences identified here, which are twenty- to 50-fold higher than the 0.1% technical error rate sometimes attributed to RNA-Seq data.

The Spike Protein gene sequence substitution divergence between RaTG13 and SARS-CoV-2 contains an improbable synonymous/non-synonymous pattern.

The functional structure of the SARS-CoV-2 Spike Protein is shown here:



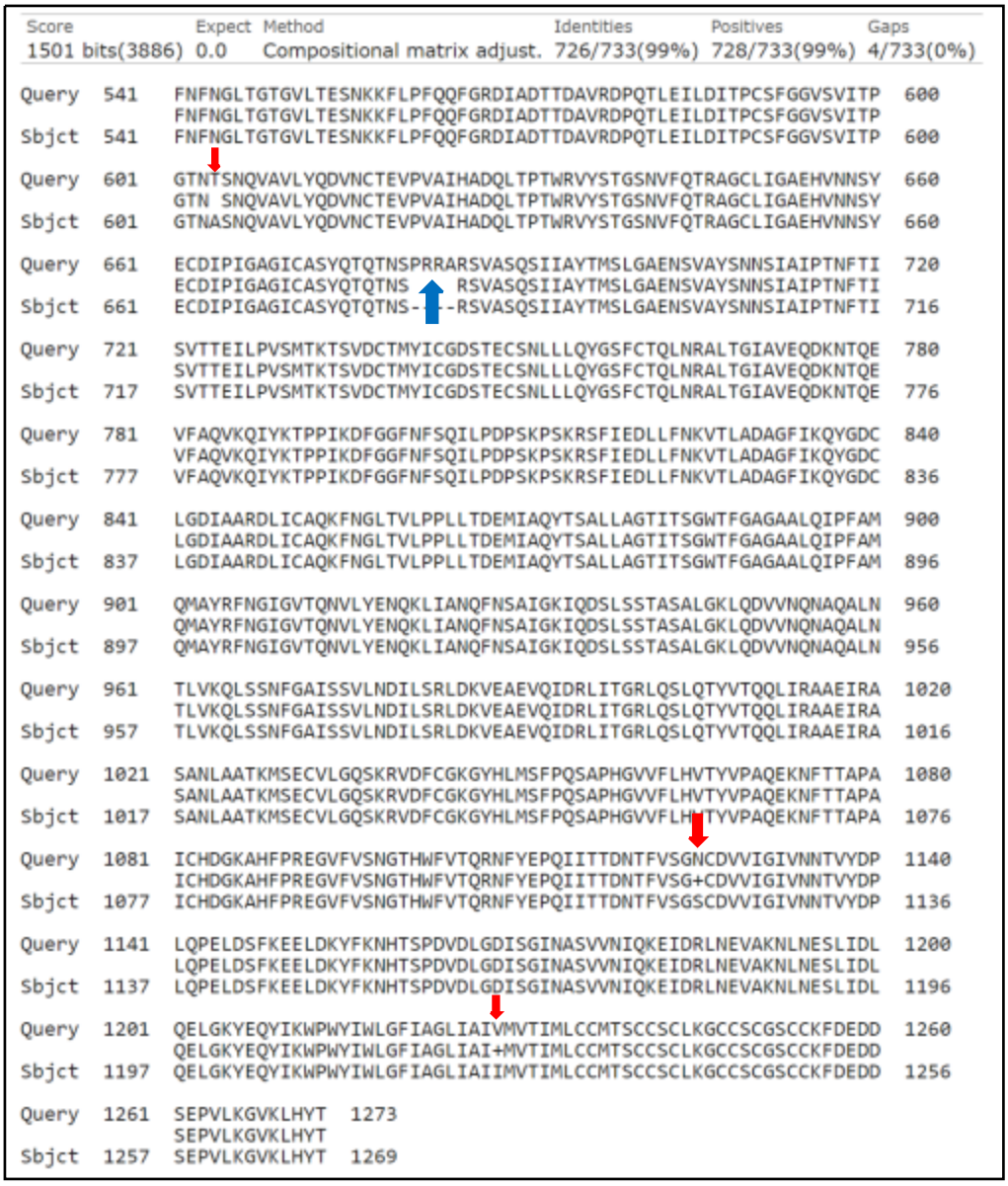
The SARS-CoV-2 Spike protein (above) contains an S1 subunit and S2 subunit with the Polybasic Cleavage Site (PBCS) between R685 and S686. This cleavage is performed by a host cell surface protease, furin, and is an important attribute in explaining the virulence of SARS-CoV-2 compared to other human coronaviruses, which do not have a furin cleavage site. The PBCS also contains the unusual PRRA insertion that has not been previously seen in Clade B coronaviruses and for which no natural mechanism for its appearance has been offered.[40]

The S1 subunit is located within the N-terminal 14–685 amino acids of S protein, containing N-terminal domain (NTD), receptor binding domain (RBD), and receptor binding motif (RBM). The S2 subunit contains a fusion peptide (FP), heptad repeat 1 (HR1), heptad repeat 2 (HR2), transmembrane domain (TM) and cytoplasmic domain (CP).

The base substitution pattern of synonymous and non-synonymous substitutions when comparing RaTG13 and the reference sequence of SARS-CoV-2 demonstrated an anomalous pattern for the coding region for aa 541 to 1273, a 733 aa protein segment representing over 60% of the SP gene.

As shown in the Text-Figure below, there are only three substitutions (red arrow) and the PBCS insertion (blue arrow) when comparing this segment of the RaTG13 and SARS-CoV-2 SP. Excluding the PBCS, the amino acid sequences are 99.6% identical.

[40] The proximal origin of SARS-CoV-2.



Score	Expect	Method	Identities	Positives	Gaps
1501 bits(3886)	0.0	Compositional matrix adjust.	726/733(99%)	728/733(99%)	4/733(0%)

```
Query  541   FNFNGLTGTGVLTESNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITP  600
             FNFNGLTGTGVLTESNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITP
Sbjct  541   FNFNGLTGTGVLTESNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITP  600

Query  601   GTNTSNQVAVLYQDVNCTEVPVAIHADQLTPTWRVYSTGSNVFQTRAGCLIGAEHVNNSY  660
             GTN SNQVAVLYQDVNCTEVPVAIHADQLTPTWRVYSTGSNVFQTRAGCLIGAEHVNNSY
Sbjct  601   GTNASNQVAVLYQDVNCTEVPVAIHADQLTPTWRVYSTGSNVFQTRAGCLIGAEHVNNSY  660

Query  661   ECDIPIGAGICASYQTQTNSPRRARSVASQSIIAYTMSLGAENSVAYSNNSIAIPTNFTI  720
             ECDIPIGAGICASYQTQTNS   RSVASQSIIAYTMSLGAENSVAYSNNSIAIPTNFTI
Sbjct  661   ECDIPIGAGICASYQTQTNS-   -RSVASQSIIAYTMSLGAENSVAYSNNSIAIPTNFTI  716

Query  721   SVTTEILPVSMTKTSVDCTMYICGDSTECSNLLLQYGSFCTQLNRALTGIAVEQDKNTQE  780
             SVTTEILPVSMTKTSVDCTMYICGDSTECSNLLLQYGSFCTQLNRALTGIAVEQDKNTQE
Sbjct  717   SVTTEILPVSMTKTSVDCTMYICGDSTECSNLLLQYGSFCTQLNRALTGIAVEQDKNTQE  776

Query  781   VFAQVKQIYKTPPIKDFGGFNFSQILPDPSKPSKRSFIEDLLFNKVTLADAGFIKQYGDC  840
             VFAQVKQIYKTPPIKDFGGFNFSQILPDPSKPSKRSFIEDLLFNKVTLADAGFIKQYGDC
Sbjct  777   VFAQVKQIYKTPPIKDFGGFNFSQILPDPSKPSKRSFIEDLLFNKVTLADAGFIKQYGDC  836

Query  841   LGDIAARDLICAQKFNGLTVLPPLLTDEMIAQYTSALLAGTITSGWTFGAGAALQIPFAM  900
             LGDIAARDLICAQKFNGLTVLPPLLTDEMIAQYTSALLAGTITSGWTFGAGAALQIPFAM
Sbjct  837   LGDIAARDLICAQKFNGLTVLPPLLTDEMIAQYTSALLAGTITSGWTFGAGAALQIPFAM  896

Query  901   QMAYRFNGIGVTQNVLYENQKLIANQFNSAIGKIQDSLSSTASALGKLQDVVNQNAQALN  960
             QMAYRFNGIGVTQNVLYENQKLIANQFNSAIGKIQDSLSSTASALGKLQDVVNQNAQALN
Sbjct  897   QMAYRFNGIGVTQNVLYENQKLIANQFNSAIGKIQDSLSSTASALGKLQDVVNQNAQALN  956

Query  961   TLVKQLSSNFGAISSVLNDILSRLDKVEAEVQIDRLITGRLQSLQTYVTQQLIRAAEIRA  1020
             TLVKQLSSNFGAISSVLNDILSRLDKVEAEVQIDRLITGRLQSLQTYVTQQLIRAAEIRA
Sbjct  957   TLVKQLSSNFGAISSVLNDILSRLDKVEAEVQIDRLITGRLQSLQTYVTQQLIRAAEIRA  1016

Query  1021  SANLAATKMSECVLGQSKRVDFCGKGYHLMSFPQSAPHGVVFLHVTYVPAQEKNFTTAPA  1080
             SANLAATKMSECVLGQSKRVDFCGKGYHLMSFPQSAPHGVVFLHVTYVPAQEKNFTTAPA
Sbjct  1017  SANLAATKMSECVLGQSKRVDFCGKGYHLMSFPQSAPHGVVFLHVTYVPAQEKNFTTAPA  1076

Query  1081  ICHDGKAHFPREGVFVSNGTHWFVTQRNFYEPQIITTDNTFVSGNCDVVIGIVNNTVYDP  1140
             ICHDGKAHFPREGVFVSNGTHWFVTQRNFYEPQIITTDNTFVSG+CDVVIGIVNNTVYDP
Sbjct  1077  ICHDGKAHFPREGVFVSNGTHWFVTQRNFYEPQIITTDNTFVSGSCDVVIGIVNNTVYDP  1136

Query  1141  LQPELDSFKEELDKYFKNHTSPDVDLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDL  1200
             LQPELDSFKEELDKYFKNHTSPDVDLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDL
Sbjct  1137  LQPELDSFKEELDKYFKNHTSPDVDLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDL  1196

Query  1201  QELGKYEQYIKWPWYIWLGFIAGLIAIVMVTIMLCCMTSCCSCLKGCCSCGSCCKFDEDD  1260
             QELGKYEQYIKWPWYIWLGFIAGLIAI+MVTIMLCCMTSCCSCLKGCCSCGSCCKFDEDD
Sbjct  1197  QELGKYEQYIKWPWYIWLGFIAGLIAIIMVTIMLCCMTSCCSCLKGCCSCGSCCKFDEDD  1256

Query  1261  SEPVLKGVKLHYT  1273
             SEPVLKGVKLHYT
Sbjct  1257  SEPVLKGVKLHYT  1269
```

Given the high amino acid identity of this 733 amino acid sequence (except for the PBCS insertion) and the typical coronavirus synonymous to non-synonymous mutation frequency of between three and five synonymous mutations for each non-synonymous mutation,[41] it was expected that a comparison of the nucleotide sequence for this region between SARS-CoV-2 and RaTG13 would show an almost identical sequence as well.

In fact, when the SARS-CoV-2 nt sequence 23,183-25,384 was compared to the RaTG13 nt sequence 23,165-25,354, the corresponding genome sequence to the 99.6% identical protein sequence above, the nucleotide identity was only 94.2% identical, with 122 synonymous substitutions and only the three non-synonymous substitutions.

[41] Comparative genomic analysis

To put this in context a comparison of thirteen other protein coding regions of SARS-CoV-2 and RaTG13 (Text-Table below) shows that the overall synonymous to non-synonymous mutation frequency is 549 synonymous to 109 non-synonymous or a ratio of about 5.0.

Gene	Region of Genome	Total Nucleotides	Synonymous mutations	Non-Synonymous mutations	S/NS	Probability of more than the number of synonymous mutations given the probability of a synonymous mutation is 0.83 (based on all genes pooled)
pp1ab	1-21,239	21,239	659	102	6.5	0.003
pp1ab ABSS	**7448-18266**	**10,818**	**283**	**13**	**21.8**	**5.73 x 10^-12**
Spike Protein RBD	1-1814	1814	131	27	4.9	0.48
Anomalous Base Substitution Segment	**23,183-25,384**	**2201**	**112**	**3**	**37.3**	**< 1.0 x 10^-7**
Entire Spike Protein	1-3810	3808	231	41	5.6	0.18
ORF1a polyprotein	1-13,215	13215	440	86	5.2	0.33
ORF3a protein	1-828	828	25	6	4.2	0.56
E Protein	1-228	228	1	0	Infinite	0.83
M Protein	1-669	669	27	3	9.0	0.1
ORF6 Protein	1-186	186	3	0	Infinite	0.17
ORF7a Protein	1-366	366	13	3	4.3	0.47
ORF7b Protein	1-132	132	0	1	0	0.83
ORF8 Protein	1-366	366	5	6	0.8	0.99
Nucleocapsid Phosphoprotein	1-1260	1260	35	4	8.75	0.083

With the exception of the anomalous base substitution segment (ABSS) in the Spike Protein gene and the pp1ab gene, the remainder of the S/SN substitution ratios are consistent with the literature values for coronaviruses. Only two genes or gene regions have a higher S/SN ratio than the ABSS because they have no non-synonymous mutations: the E protein gene with 228 nucleotides and the ORF6 protein gene with 186 nucleotides. Because of the short length of these two genes, the probabilities of the results for the E and ORF6 genes were not significant, with p-values of 0.86 and 0.17, respectively.

The p-value for the ABSS, on the other hand, was highly significant, with a p-value of <0.0000001. This strongly suggests a non-natural cause for this base substitution pattern, barring some unknown biological mechanism for such a result.

A second highly anomalous sequence was found in the pp1ab gene. This is about five-times larger than the Spike Protein region and is even more unlikely to have happened naturally, a chance of about one in 100 billion times.

Are there only synonymous mutations in these regions because non-synonymous mutations lead to non-replicative viruses?

A simple explanation for these results would be an extreme criticality for the specific sequences of these regions with respect to infectivity. If a single amino acid change yielded a non-transmissible viral particle that strong negative purification process could explain the above results.

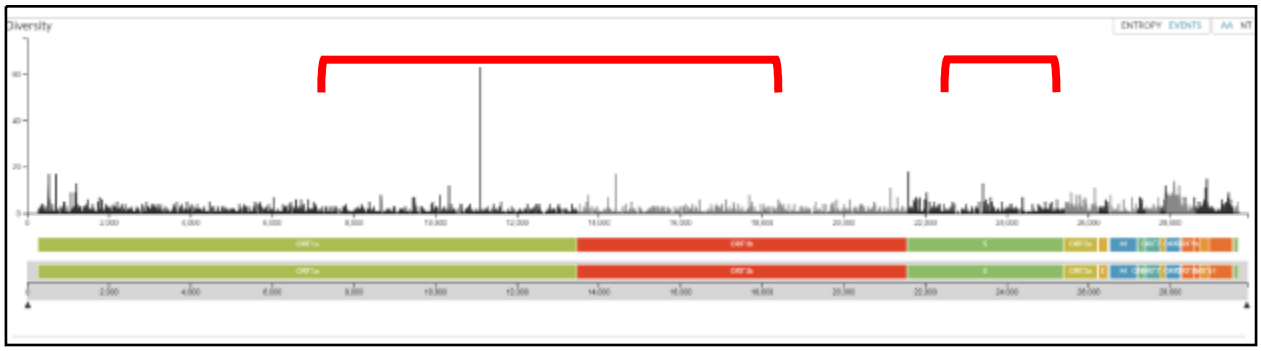
@2021. Steven C. Quay, MD, PhD

This hypothesis can be immediately rejected based on two observations.

In an examination of over 80,000 SARS-CoV-2 genome sequences, the most common Spike Protein non-synonymous mutation is within the ABSS (D614G) which was identified within weeks of the outbreak in January 2020 and which has become "the dominant virus…in every geographical region."[42] Specifically, as of August 28, 2020, GISAID reports that 65,738 full length SARS-CoV-2 genomes of a total of 83,387, or 79%, and comprising the G, GH, and GR clades, contain the D614G SNV. Under real world biological conditions, the ABSSN region has in fact, not a strong negative purification process in operation but in fact a strong positive selection process ongoing.

Secondly, in an analysis of mutations in 63,421 SARS-CoV-2 genomes the Spike Protein amino acid 605 to 1120 region had a total of 7,149 mutations. Fully 5,936 of these mutations (83%) are the above noted D614G non-synonymous change. Of the remaining 1213 mutations, 452 were non-synonymous while 755 were synonymous, a ratio of 1.7. There were also four indels and two stop codon mutations.

The following Text-Figure contains a map of the SARS-CoV-2 genome with the location of amino acid changes that have been found during the worldwide spread noted, with the frequency related to the height of the mark. The two ABSS in pp1ab and SP are marked with red brackets and clearly demonstrate an abundance of non-synonymous mutations in these regions during the human-to-human spread.



Nextstrain SARS-CoV-2 amino acid change events

Clearly, these regions can tolerate many non-synonymous mutations, rejecting the theory of a criticality for the amino acid sequence of this region. No other natural biological mechanism to explain these results has been identified.

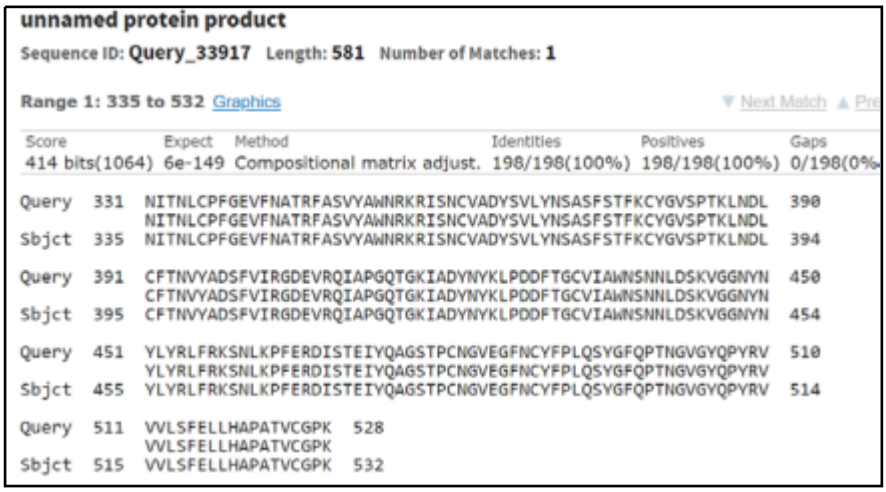
Codon modification, enhancement, or optimization is an example from synthetic biology in which the S/SN ratio is, by design, an anomaly when looked at through the lens of nature

[42] Biswas NK, Majumder PP. Analysis of RNA sequences of 3636 SARS-CoV-2 collected from 55 countries reveals selective sweep of one virus type. Indian J Med Res. 2020;151(5):450-458. doi:10.4103/ijmr.IJMR_1125_20.

Synonymous codon substitution is a decades old, well known method of enhancing gene expression when cloning exogenous genes in a laboratory experiment. In a paper on the immunogenicity of the SARS-CoV-2 Spike Protein[43] the following synthetic biology methods were used:

"We used the following structure coordinates of the coronavirus spike proteins from the PDB to define the boundaries for the design of RBD expression constructs: SARS-CoV-2 (6VSB), SARS-CoV-1 (6CRV), HKU-1 (5I08), OC43 (6NZK), 229E (6U7H) NL63 (6SZS). Accordingly, a codon-optimized gene encoding for S1-RBD [SARS-CoV-1 (318 – 514 aa, P59594), SARS-CoV-2 (331 – 528 aa, QIS60558.1), OC43 (329 – 613 aa, P36334.1), HKU-1 (310 – 611 aa, Q0ZME7.1), 229E (295 – 433 aa, P15423.1) and NL63 (480 – 617 aa, Q6Q1S2.1)] containing human serum albumin secretion signal sequence, three purification tags (6xHistidine tag, Halo tag, and TwinStrep tag) and two TEV protease cleavage sites was cloned into the mammalian expression vector pαH. S1 RBDs were expressed in Expi293 cells (ThermoFisher) and purified from the culture supernatant by nickel-nitrilotriacetic acid agarose (Qiagen)."

The Genbank alignment (below) confirms that the authentic SARS-CoV-2 Spike Protein sequence (https://www.ncbi.nlm.nih.gov/nuccore/1798174254) and the Synthetic construct SARS CoV-2 spike protein receptor binding domain gene, complete cds are 100% homologous at the protein level:



unnamed protein product

Sequence ID: **Query_33917** Length: **581** Number of Matches: **1**

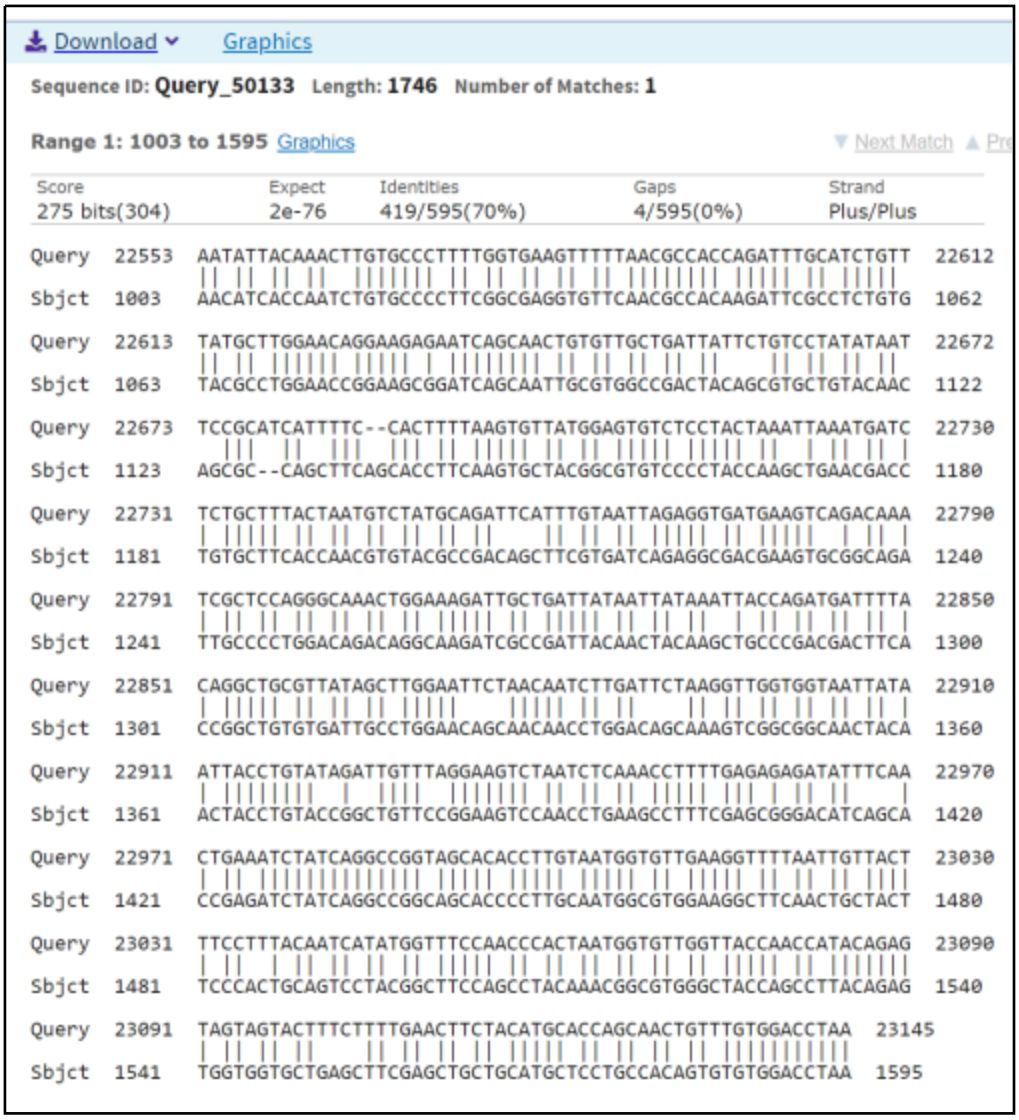
Range 1: 335 to 532 Graphics ▼ Next Match ▲ Pre

Score	Expect	Method	Identities	Positives	Gaps
414 bits(1064)	6e-149	Compositional matrix adjust.	198/198(100%)	198/198(100%)	0/198(0%

```
Query  331  NITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLYNSASFSTFKCYGVSPTKLNDL  390
            NITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLYNSASFSTFKCYGVSPTKLNDL
Sbjct  335  NITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLYNSASFSTFKCYGVSPTKLNDL  394

Query  391  CFTNVYADSFVIRGDEVRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYN  450
            CFTNVYADSFVIRGDEVRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYN
Sbjct  395  CFTNVYADSFVIRGDEVRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYN  454

Query  451  YLYRLFRKSNLKPFERDISTEIYQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRV  510
            YLYRLFRKSNLKPFERDISTEIYQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRV
Sbjct  455  YLYRLFRKSNLKPFERDISTEIYQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRV  514

Query  511  VVLSFELLHAPATVCGPK  528
            VVLSFELLHAPATVCGPK
Sbjct  515  VVLSFELLHAPATVCGPK  532
```

But a comparison of the authentic nucleotide sequence of SARS-CoV-2 to the codon-optimized synthetic construct shows no match using the "highly similar Megablast" algorithm setting. When the alignment algorithm is run in a more relaxed mode the impact of codon optimization in this case can be seen, a 70% homology:

[43] https://immunology.sciencemag.org/content/5/48/eabc8413/tab-pdf



Download Graphics

Sequence ID: **Query_50133** Length: **1746** Number of Matches: **1**

Range 1: 1003 to 1595 Graphics Next Match

Score	Expect	Identities	Gaps	Strand
275 bits(304)	2e-76	419/595(70%)	4/595(0%)	Plus/Plus

```
Query  22553  AATATTACAAACTTGTGCCCTTTTGGTGAAGTTTTTAACGCCACCAGATTTGCATCTGTT  22612
              || || || ||  ||||||| || || || || || |||||||| |||||| || |||||
Sbjct  1003   AACATCACCAATCTGTGCCCCTTCGGCGAGGTGTTCAACGCCACAAGATTCGCCTCTGTG  1062

Query  22613  TATGCTTGGAACAGGAAGAGAATCAGCAACTGTGTTGCTGATTATTCTGTCCTATATAAT  22672
              || || |||||| ||||| | |||||||| || || || || ||    || || || ||
Sbjct  1063   TACGCCTGGAACCGGAAGCGGATCAGCAATTGCGTGGCCGACTACAGCGTGCTGTACAAC  1122

Query  22673  TCCGCATCATTTTC--CACTTTTAAGTGTTATGGAGTGTCTCCTACTAAATTAAATGATC  22730
                |||  ||  |||  ||| || ||||| || || ||||| ||||| ||  | || || |
Sbjct  1123   AGCGC--CAGCTTCAGCACCTTCAAGTGCTACGGCGTGTCCCCTACCAAGCTGAACGACC  1180

Query  22731  TCTGCTTTACTAATGTCTATGCAGATTCATTTGTAATTAGAGGTGATGAAGTCAGACAAA  22790
              | ||||| || || || || || ||    || || || ||||| || |||||  | || |
Sbjct  1181   TGTGCTTCACCAACGTGTACGCCGACAGCTTCGTGATCAGAGGCGACGAAGTGCGGCAGA  1240

Query  22791  TCGCTCCAGGGCAAACTGGAAAGATTGCTGATTATAATTATAAATTACCAGATGATTTTA  22850
              | || || || || || || |||||| || ||||| || || ||  | || || || || |
Sbjct  1241   TTGCCCCTGGACAGACAGGCAAGATCGCCGATTACAACTACAAGCTGCCCGACGACTTCA  1300

Query  22851  CAGGCTGCGTTATAGCTTGGAATTCTAACAATCTTGATTCTAAGGTTGGTGGTAATTATA  22910
              | ||||| || || || |||||    ||||| || ||    || || || || || || |
Sbjct  1301   CCGGCTGTGTGATTGCCTGGAACAGCAACAACCTGGACAGCAAAGTCGGCGGCAACTACA  1360

Query  22911  ATTACCTGTATAGATTGTTTAGGAAGTCTAATCTCAAACCTTTTGAGAGAGATATTTCAA  22970
              | |||||||  |  |||| ||||||| || || || ||||| ||| | || ||    |
Sbjct  1361   ACTACCTGTACCGGCTGTTCCGGAAGTCCAACCTGAAGCCTTTCGAGCGGGACATCAGCA  1420

Query  22971  CTGAAATCTATCAGGCCGGTAGCACACCTTGTAATGGTGTTGAAGGTTTTAATTGTTACT  23030
              | || |||||||||||||| ||||| ||||| || ||||| || || || ||||
Sbjct  1421   CCGAGATCTATCAGGCCGGCAGCACCCCTTGCAATGGCGTGGAAGGCTTCAACTGCTACT  1480

Query  23031  TTCCTTTACAATCATATGGTTTCCAACCCACTAATGGTGTTGGTTACCAACCATACAGAG  23090
              | ||  | || || || || ||||| || || || || || || ||||| || |||||||
Sbjct  1481   TCCCACTGCAGTCCTACGGCTTCCAGCCTACAAACGGCGTGGGCTACCAGCCTTACAGAG  1540

Query  23091  TAGTAGTACTTTCTTTTGAACTTCTACATGCACCAGCAACTGTTTGTGGACCTAA  23145
              | || || ||    || || || || |||||| || || || || ||||||||||
Sbjct  1541   TGGTGGTGCTGAGCTTCGAGCTGCTGCATGCTCCTGCCACAGTGTGTGGACCTAA  1595
```

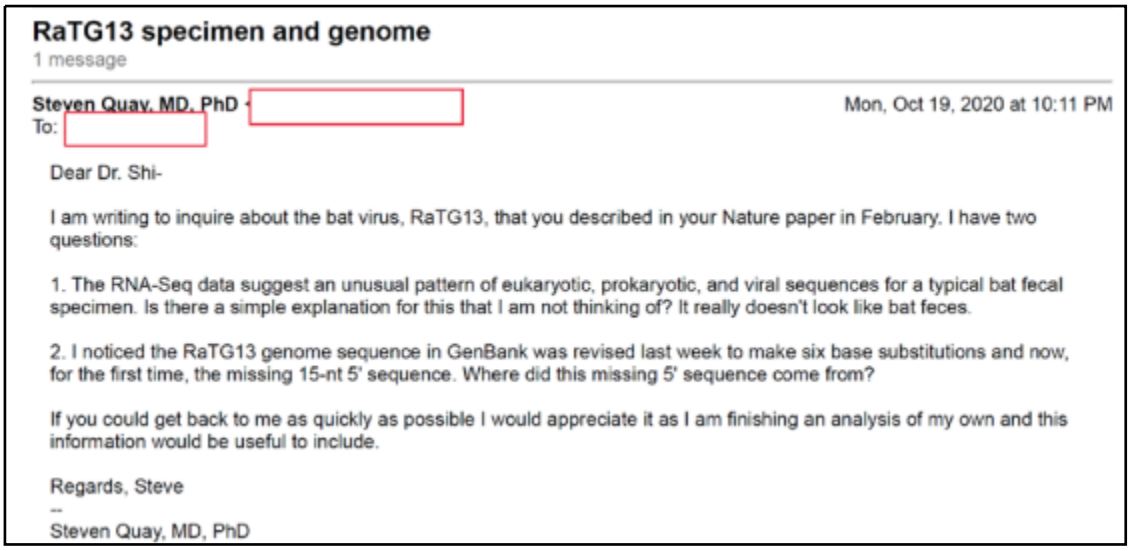
This is a situation in which there are 176 synonymous changes without a single non-synonymous change and is the genome signature of laboratory-derived synthetic biology. If these sequences were compared for phylogenetic divergence without the knowledge of their artificial construction, this synthetic laboratory experiment would create the impression that these two sequences had diverged in the wild from a common ancestor decades earlier.

The following Table identifies four regions of the RaTG13 and SARS-CoV-2 genomes in which there were a total of 220 synonymous mutations without a single non-synonymous change.

Protein/Gene	Protein Region	Total Nucleotides	Synonymous mutations	NS Mutations
S Protein	605-1124	1557	91	0
pp1ab	3607-4534	2781	66	0
pp1ab	4626-5111	1455	26	0
pp1ab	5113-5828	2145	37	0
	Total	7938	220	0

These regions represent over 26% of the entire genome and appear analogous to the outcome expected from the application of a synonymous codon modified, laboratory-derived synthetic biology project. They also represent about one-sixth of the 4% apparent phylogenetic divergence between RaTG13 and SARS-CoV-2.

October GenBank update. On October 13, 2020 the sequence for RaTG13 was updated. For the first time the first 15 nucleotides at the 5' end were present. However, these were not found in a blast of either the RNA-Seq raw reads or the Amplicons. The following email was sent to Dr. Shi asking for an explanation of the fecal specimen composition and the source for the 5' nt data.



RaTG13 specimen and genome
1 message

Steven Quay, MD, PhD [redacted] Mon, Oct 19, 2020 at 10:11 PM
To: [redacted]

Dear Dr. Shi-

I am writing to inquire about the bat virus, RaTG13, that you described in your Nature paper in February. I have two questions:

1. The RNA-Seq data suggest an unusual pattern of eukaryotic, prokaryotic, and viral sequences for a typical bat fecal specimen. Is there a simple explanation for this that I am not thinking of? It really doesn't look like bat feces.

2. I noticed the RaTG13 genome sequence in GenBank was revised last week to make six base substitutions and now, for the first time, the missing 15-nt 5' sequence. Where did this missing 5' sequence come from?

If you could get back to me as quickly as possible I would appreciate it as I am finishing an analysis of my own and this information would be useful to include.

Regards, Steve
--
Steven Quay, MD, PhD

At the time of this writing a response has not been received.

Discussion. The foundation of the working hypothesis that the COVID-19 pandemic arose via a natural zoonotic transfer from a non-human vertebrate host to man has been built on two publications: the February 3, 2020 *Nature* paper by Dr. Zheng-Li Shi and colleagues, in which the bat coronavirus RaTG13 is first identified as the closest sequence identity to SARS-CoV-2 at 96.2% and the March 17, 2020 *Nature Medicine* paper entitled, "The proximal origin of SARS-CoV-2," by Andersen *et al.,* in which the Shi *et al.* paper is cited as evidence for a bat origin for the pandemic. In the approximately six months since they were published, these two papers have been cited over 1600- and 200-times on PubMed, respectively.

However, research is beginning to question whether a bat species can be considered a natural reservoir for SARS-CoV-2. A recent paper performed an *in silico* simulation of the SARS-CoV-2 Spike Protein interaction with the cell surface receptor, ACE2, from 410 unique vertebrate species, including 252 mammals.[44] Among primates, 18/19 have an ACE2 receptor which is 100% homologous to the human protein in the 25 residues identified to be critical to infection,

[44] Broad host range of SARS-CoV-2 predicted by comparative and structural analysis of ACE2 in vertebrates Joana Damas, et al. Proc. of the Nat. Acad. of Sci. Aug 2020, 202010146; DOI: 10.1073/pnas.2010146117

including the *Chlorocebus sabaeus* (the Old World African Green monkey) and the rhesus macaques.

It is noteworthy that the laboratory workhorse of coronavirus research is the VERO cell, isolated from a female African Green monkey in 1962, and containing an ACE2 receptor that is 100% homologous to the human ACE2 in the 25 critical amino acids for infectivity.

This *in silico* work was confirmed in the laboratory with respect to rhesus macaques. Within weeks of the identification of SARS-CoV-2, the Wuhan laboratory had demonstrated that the pandemic virus would infect and produce a pneumonia in rhesus macaques.[45]

A surprising finding from the ACE2 *in silico* surveillance work was the very poor predicted affinity of the ACE2 receptors in both bats and pangolins. Of 37 bat species studied, 8 scored low and 29 scored very low. As expected by these predictions, cell lines derived from big brown bat (*Eptesicus fuscus*),[46] Lander's horseshoe bat (*Rhinolophus landeri*), and Daubenton's bat (*Myotis daubentonii*) could not be infected with SARS-CoV-2.[47]

It is unfortunate that growth of the RaTG13 specimen could not have been attempted in the *Rhinolophus sinicus* primary or immortalized cells generated and maintained in the Wuhan laboratory: kidney primary cells (RsKi9409), lung primary cells (RsLu4323), lung immortalized cells (RsLuT), brain immortalized cells (RsBrT) and heart immortalized cells (RsHeT).[48] However it should be noted that a synthetically created RaTG13 was reported not to infect human cells expressing *Rhinolophus sinicus* ACE2, providing evidence that RaTG13 may not be a viable coronavirus in a wild bat population.[49]

The other proposed intermediate host, the pangolin, also had predicted ACE-2 affinity that was either low or very low.

A recent paper that examined the high synonymous mutation difference between RaTG13 and SARS-CoV-2 used an *in silico* methodology to suggest that the difference could be largely attributed to the RNA modification system of hosts.[50] However, the authors do not "(t)he limitation of our study is that we were currently unable to provide experimental evidence for

---

[45] Infection with Novel Coronavirus (SARS-CoV-2) Causes Pneumonia in the *Rhesus Macaques.* C. Shan et al., Research Square, **DOI:** 10.21203/rs.2.25200/v1. Shan, C., Yao, Y., Yang, X. *et al.* Infection with novel coronavirus (SARS-CoV-2) causes pneumonia in *Rhesus macaques*. *Cell Res* **30,** 670–677 (2020). https://doi.org/10.1038/s41422-020-0364-z

[46] J. Harcourt et al., Severe acute respiratory syndrome coronavirus 2 from patient with coronavirus disease, United States. Emerg. Infect. Dis. 26, 1266–1273 (2020).

[47] M. Hoffmann et al., SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell 181, 271–280.e8 (2020).

[48] Zhou, P., Fan, H., Lan, T. et al. Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. Nature 556, 255–258 (2018). https://doi.org/10.1038/s41586-018-0010-9.

[49] Y. Li et al., Potential host range of multiple SARS-like coronaviruses and an improved ACE2-Fc variant that is potent against both SARS-CoV-2 and SARS-CoV-1. bioRxiv:10.1101/2020.04.10.032342 (18 May 2020).

[50] The divergence between SARS-CoV-2 and RaTG13 might be overestimated due to the extensive RNA modification

the modification on viral RNAs." The low S/SN ratio of 1.7 in the expansion of SARS-CoV-2 in the human population would argue against a robust host RNA modification mechanism.

In summary, the findings reported here are:

1. Inconsistences between published papers and interviews as to the source and sequencing history of the original specimen that was claimed to have been collected in 2013 (RaBtCoV/4991) and the specimen for the bat RaTG13 virus. For example, two explanations of the discovery of the close relationship between RaTG13 and SARS-Cov-2, a highly homologous match between the RdRp genes of the viruses noticed in 2020 followed by full genome sequencing, or identification in 2020 of a homologous match to full genome sequencing previously done in 2018. Current publicly available data for RaTG13 from 2017 and 2018 is a set of 33 amplicon sequencing runs but they cover only about 80% of the entire genome. In the *Science* interview Dr. Shi's says the specimen for RaTG was consumed during sequencing in 2018, but if this is true, the RNA-Seq referred to in the *Nature* paper could not have been performed in 2020. At this time, the Wuhan laboratory has not met the requirements of *Nature* with respect to the sharing of primary and sequence assembly data from their seminal paper[1] and this data should be provided immediately.
2. The specimen from which RaTG13 was reported to have been isolated and which has been repeatedly reported to have been a bat fecal specimen has a taxonomical composition of eukaryotes, bacteria, and viruses that is completely different from a set of nine bat fecal specimens collected in the same field visits by the same laboratory personnel from the Wuhan Institute of Virology. The probability that an authentic fecal specimen could have the composition reported is one in ten million, an impossibly low occurrence. Examination of the strong signals in the RaTG13 specimen identifies both a variety of bat genetic material, some that are not native to China, as well as unexpected species, such as marmots and a red fox. It also contains a telltale 3% primate sequence consistent with VERO cell contamination. I propose that this specimen is apparently either a mislabeled specimen (although I cannot conjure what the field source or specimen would be) or was artificially created in a laboratory.
3. The method-dependent sequence differences between the amplicon data and the RNA-Seq data are about 5% or about 50-times higher than expected as a technical error rate of 0.1%. This is an experimental quality issue that needs to be addressed; no explanation has been offered for this to date. In addition, no assembly methodology has been provided and at least two gaps, totaling over 60 nt, were easily identified.
4. The findings, reported here of a mutational drift of synonymous mutations only between SARS-CoV-2 and RaTG13 in the Spike Protein S1/S2 region and the pp1ab gene that has never been seen in nature before and which has a probability of having occurred by chance of less than one in ten million and one in one billion makes it more likely that, at least for these portions of the RaTG13 genome, comprising over one-quarter of the entire genome, another process is underway. With the demonstration

that codon-enhancement or optimization can produce this unnatural S/SN pattern, some form of laboratory-based synthetic biology was performed on RaTG13, SARS-CoV-2, or both.

Apparently, the entire specimen from which RaTG13 was purported to have been found has been consumed in previous sequencing experiments and the Principal Investigator has stated that no virus has ever been isolated or cultured from the specimen at any time in the past. Given the irregularities and anomalies identified in this paper it seems prudent to conclude that all data with respect to RaTG13 must be considered suspect. As such, reliance of the foundational papers of the origin of SARS-CoV-2 as having arisen from bats via a zoonotic mechanism must be reexamined and questioned.

Paper 2: The February 19, 2020 Lancet paper entitled: "Statement in support of the scientists, public health professionals, and medical professionals of China combatting COVID-19."

On February 19, 2020 *The Lancet* published a Correspondence entitled "Statement in support of the scientists, public health professionals, and medical professionals of China combatting COVID-19[51]" with 27 public health scientists from eight countries as authors. The statement seems to attempt to settle the question of the origin of SARS-CoV-2 and short circuit further debate, as the second sentence reads: "We stand together to strongly condemn conspiracy theories suggesting that COVID-19 does not have a natural origin." It goes on to state: "Conspiracy theories do nothing but create fear, rumors, and prejudice that jeopardize our global collaboration in the fight against this virus."

The letter provided an open solicitation for support and at this time has been signed by at over 20,300 people, as if to purport that science can be advanced through polling and the democratic process.[52] While it is a truism that conspiracy theories have no place in the academia, legitimate debate should not be foreclosed.

The statement itself provides a more nuanced discussion of the evidence for a zoonotic origin and contains 14 references, eight of which contain data about the COVID-19 pandemic and six of which are governmental policy statements without new data, background articles from 2003 and 2004 on zoonotic diseases, or a virus naming statement by the Coronavirus Study Group (CSG) of the International Committee on Taxonomy of Viruses, which is responsible for developing the official classification of viruses and taxa naming (taxonomy) of the Coronaviridae family. The eight articles with data were written at the end of January or early February, when there were fewer than 10,000 patients.

An analysis of the evidence for a zoonotic source given in support of the above Statement is contained in Text-Table here. The analysis shows there was very little actual data available at the

[51] https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(20)30418-9/fulltext#back-bib1

[52] This is reminiscent of the story attributed to Albert Einstein by Stephen Hawkins in his *Brief History of Time.* According to Hawkins, a book was published in 1930 in pre-war Germany entitled, "One Hundred Authors Against Einstein." When he was asked about the book Einstein is reported to have retorted, "If I were wrong, then one would have been enough!"

time to permit reaching such a definitive conclusion. There was also the absence of data or discussion that could support a laboratory origin.

Reference	Statements concerning origin of SARS-CoV-2	Response to statements
1.Gorbalenya AE Baker SC Baric RS et al. Severe acute respiratory syndrome-related coronavirus: the species and its viruses—a statement of the Coronavirus Study Group. bioRxiv. 2020; (published online Feb 11. DOI: 2020.02.07.937862 (preprint).)	A naming statement about SARS-CoV-2. The emergence of SARS-CoV-2 as a human pathogen in December 2019 may thus be perceived as completely independent from the SARS-CoV outbreak in 2002–2003. With respect to novelty, SARS-CoV-2 differs from the two other zoonotic coronaviruses, SARS-CoV and MERS-CoV, introduced to humans earlier in the twenty-first century.	Does not provide data on a potential zoonotic source.
2.Zhou P Yang X-L Wang X-G et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020; (published online Feb 3.)	The sequences of 2019-nCoV BetaCoV/Wuhan/WIV04/2019 among patient specimens are almost identical and share 79.6% sequence identity to SARS-CoV. Furthermore, we show that 2019-nCoV is 96% identical at the whole-genome level to a bat coronavirus. Pairwise protein sequence analysis of seven conserved non-structural proteins domains show that this virus belongs to the species of SARSr-CoV. The close phylogenetic relationship to RaTG13 provides evidence that 2019-nCoV may have originated in bats.	The bat genome identity of 96% described here, coupled with the known mutation rate of SARS-CoV-2 of about 26/year, implies a **lowest common ancestor about 44 years ago.**

3.Lu R Zhao X Li J et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. Lancet. 2020; (published online Jan 30.)	Genome sequences of 2019-nCoV sampled from nine patients who were among the early cases of this severe infection are almost genetically identical, which suggests very recent emergence of this virus in humans and that the outbreak was detected relatively rapidly. 2019-nCoV is most closely related to other betacoronaviruses of bat origin, indicating that these animals are the likely reservoir hosts for this emerging viral pathogen.	Figure 1A shows 8 sequences and the concensus sequence. These 8 sequences show 3 with 0 mutations, 2 with 1 mutation, 3 with 2 mutations, and none with more than 2 mutations. Based on current estimates of 1 mutation per human passage, these are at most two human-to-human transfers apart. Importantly, there is no background diversity as would be seen in two or more resevoir-to-human events. Fig 2 states strain Bat-SL-CoVZC45 is 87.6% sequence identity to the human virus, which means a difference of about 3700 mutations or over 70 years from lowest common ancestor.
4.Zhu N Zhang D Wang W et al. A novel coronavirus from patients with pneumonia in China, 2019. NEJM. 2020; (published online Jan 24.)	"more than 85% identity with a bat SARS-like CoV (bat-SL-CoVZC45, MG772933.1) genome published previously. Since the sequence identity in conserved replicase domains (ORF 1ab) is less than 90% between 2019-nCoV and other members of betacoronavirus, the 2019-nCoV — the likely causative agent of the viral pneumonia in Wuhan — is a novel betacoronavirus belonging to the	A >85% identity with a bat coronavirus means the **human and bat virus have over 70 years to LCA.**

	sarbecovirus subgenus of Coronaviridae family."	
5.Ren L Wang Y-M Wu Z-Q et al. Identification of a novel coronavirus causing severe pneumonia in humans: a descriptive study. Chin Med J. 2020; (published online Feb 11.)	All five patients have sequence homology of 99.8% to 99.9%. These isolates showed 79.0% nucleotide identity with the sequence of SARS-CoV (GenBank NC_004718) and 51.8% identity with the sequence of MERS-CoV (GenBank NC_019843). The virus is closest to a bat SARS-like CoV (SL-ZC45, GenBank MG772933) with 87.7% identity, but is in a separate clade. **Surprisingly, RNA-dependent RNA polymerase (RdRp), which is the most highly conserved sequence among different CoVs, only showed 86.3% to 86.5% nt identities with bat SL-CoV ZC45.**	Similar to reference 3 comments. Lack of conserved sequencing of the most highly conserved sequence with bat coronavirus would **suggest a non-bat source.**
6.Paraskevis D Kostaki EG Magiorkinis G Panayiotakopoulos G Tsiodras S Full-genome evolutionary analysis of the novel corona virus (2019-nCoV) rejects the hypothesis of emergence as a result of a recent recombination event. Infect Genet Evol. 2020; (published online Jan 29.)	A BLAST search of 2019-nCoV middle fragment revealed no considerable similarity with any of the previously characterized corona viruses. Bat_SARS-like coronavirus sequences cluster in different positions in the tree, suggesting that they are recombinants, and thus that the 2019-nCoV and RaTG13 are not recombinants. Codon usage analyses can resolve	**The middle segment with no similarity to other corona viruses is about 40% of the entire genome. I agree SARS-CoV-2 is not a recombinant of RaTG13. I agree, codon usage analysis here supports the furin binding site insertion as having been invented de novo. A recent recombination event is not necessary for a**

	the origin of proteins with deep ancestry and insufficient phylogenetic signal or **invented de novo**. Our study rejects the hypothesis of emergence as a result of a recent recombination event. Notably, the new coronavirus provides a new lineage for almost half of its genome, with **no close genetic relationships to other viruses** within the subgenus of sarbecovirus. This genomic part comprises half of the spike region encoding a multifunctional protein responsible also for virus entry into host cells	**laboratory derived theory of origin. Statements do not advance a zoonotic origin.**
7.Benvenuto D Giovanetti M Ciccozzi A Spoto S Angeletti S Ciccozzi M The 2019-new coronavirus epidemic: evidence for virus evolution. J Med Virol. 2020; (published online Jan 29.)	The epidemic originated in Wuhan, China. A phylogenetic tree has been built using the 15 available whole genome sequences of 2019-nCoV, 12 whole genome sequences of 2019-nCoV, and 12 highly similar whole genome sequences available in gene bank (five from the severe acute respiratory syndrome, two from Middle East respiratory syndrome, and five from bat SARS-like coronavirus). >97% maximum likelihood match to Bat SARS-like virus 2015 (Fig 1) is noted. The SARS and MERS viruses are excluded as a source of SARS-CoV-2. These results do not	A 3% genome **distance from the noted bat virus to human is about 34 years** at 26 mutations per year, the in-human mutation rate. Predicted a future mutation like the D614G mutation which is more infective.

	exclude the fact that **further mutation due to positive selective pressure, led by the epidemic evolution, could favor an enhancement of pathogenicity and transmission of this novel virus.**	
8.Wan Y Shang J Graham R Baric RS Li F Receptor recognition by novel coronavirus from Wuhan: an analysis based on decade-long structural studies of SARS. J Virol. 2020; (published online Jan 29.)	Based on predicted RBD-host ACE2 receptor affinities, civet, mice, and rats are fuled out as source species. Pigs, ferrets, cats, and **nonhuman primates** contain largely favorable 2019-nCoV-contacting residues in their ACE2. SARS-CoV was isolated in wild palm civets near Wuhan in 2005, and its RBD had already been well adapted to civet ACE2.	The **potential nonhuman primate ACE2 usage is noted. Consistent with a laboratory origin from VERO cells**, a monkey kidney cell line. It expresses an ACE2 that permits SARS-CoV-2 infection, making it a possible source for the virus. A common tissue culture cell line forSARS virus research.
9.US Center for Disease Control and Prevention Coronavirus disease 2019 (COVID-19) situation summary. https://www.cdc.gov/coronavirus/2019-nCoV/summary.html Date: Feb 16, 2020 Date accessed: February 8, 2020	Rarely, animal coronaviruses can infect people and then spread between people such as with MERS-CoV, SARS-CoV, and now with this new virus, named SARS-CoV-2. The SARS-CoV-2 virus is a betacoronavirus, like MERS-CoV and SARS-CoV. All three of these viruses have their origins in bats. The sequences from U.S. patients are similar to the one that China initially posted, suggesting a likely single, recent emergence of this virus from an animal reservoir.	There are no data to support these statements about bats as the source for SARS-CoV-2.

10.Andersen KG Rambaut A Lipkin WI Holmes EC Garry RF The proximal origin of SARS-CoV-2. http://virological.org/t/the-proximal-origin-of-sars-cov-2/398 Date: Feb 16, 2020 Date accessed: February 17, 2020	See Table 2.	See Table 2.
11.Bengis R Leighton F Fischer J Artois M Morner T Tate C The role of wildlife in emerging and re-emerging zoonoses. Rev Sci Tech. 2004; 23: 497-512	In one pattern, actual transmission of the pathogen to humans is a rare event but, once it has occurred, human-to-human transmission maintains the infection for some period of time or permanently. Some examples of pathogens with this pattern of transmission are human immunodeficiency virus/acquired immune deficiency syndrome, influenza A, Ebola virus and severe acute respiratory syndrome.	This 2004 paper describes the pattern of rare animal-to-human transmission followed by human-to-human spread as an example of the SARS virus. It does not address the origin of SARS-CoV-2.
12.Woolhouse ME Gowtage-Sequeria S Host range and emerging and reemerging pathogens. Emerg Infect Dis. 2005; 11: 1842-1847	Emerging and reemerging pathogens are disproportionately viruses, with 37% being RNA viruses. Emerging and reemerging pathogens more often are those with broad host ranges that often encompass several mammalian orders and even nonmammals. For pathogens that are minimally transmissible within human populations (R0 close to 0), outbreak size is determined largely by the number of introductions from the reservoir. For pathogens that are highly transmissible within human populations	This 2005 article has good general information about looking broadly for the reservoir species(s), identifies RNA viruses as a major source of human epidemics, predicts a large outbreak size for a high Ro virus, but does address the origin of SARS-CoV-2 origin.

	(R0>>1), outbreak size is determined largely by the size of the susceptible population.	
13.NASEM The National Academies of Science Engineering and Medicine of the USA. NAS, NAE, and NAM presidents' letter to the White House Office of Science and Technology Policy. https://www.nationalacademies.org/includes/NASEM%20Response%20to%20OSTP%20re%20Coronavirus_February%206,%202020.pdf Date: Feb 6, 2020 Date accessed: February 7, 2020	The closest known relative of 2019-nCoV appears to be a coronavirus identified from bat-derived samples collected in China.4 The experts informed us that additional genomic sequence data from geographically- and temporally-diverse viral samples are needed to determine the origin and evolution of the virus. Samples collected as early as possible in the outbreak in Wuhan and samples from wildlife would be particularly valuable. Understanding the driving forces behind viral evolution would help facilitate the development of more effective strategies for managing the 2019-nCoV outbreak and for preventing future outbreaks.	Agree. If additional genomic sequence data is available from geographically- and temporally-diverse viral samples are needed to determine the origin and evolution of the virus this should be made publicly available.
14.WHO Director-General's remarks at the media briefing on 2019 novel coronavirus on 8 February 2020. https://www.who.int/dg/speeches/detail/director-general-s-remarks-at-the-media-briefing-on-2019-novel-coronavirus---8-february-2020 Date: Feb 8, 2020 Date accessed: February 18, 2020	A general statement about the emerging pandemic without reference to the origin of SARS-CoV-2	There is no data about the origin of the pandemic.

In November 2020 the Watchdog group, US Right-to-Know, reported the following with respect to the *Lancet* article:[53]

"Emails obtained by U.S. Right to Know show that a statement in *The Lancet* authored by 27 prominent public health scientists condemning "conspiracy theories suggesting that COVID-19 does not have a natural origin" was organized by employees of EcoHealth Alliance, a non-profit group that has received millions of dollars of U.S. taxpayer funding to genetically manipulate coronaviruses with scientists at the Wuhan Institute of Virology."

"The emails obtained via public records requests show that EcoHealth Alliance President Peter Daszak drafted the Lancet statement, and that he intended it to "not be identifiable as coming from any one organization or person" but rather to be seen as "simply a letter from leading scientists". Daszak wrote that he wanted "to avoid the appearance of a political statement."

A separate, worrisome article entitled, "Peter Daszak's EcoHealth Alliance Has Hidden Almost $40 Million In Pentagon Funding And Militarized Pandemic Science,[54]" seems to indicate a serious conflict of interest with respect to Dr. Daszak's participation in any investigations on the origin of SARS-CoV-2.

Paper 3: The March 17, 2020 article in *Nature Medicine* entitled "The proximal origin of SARS-CoV-2" by Andersen et al.[55]

According to the journal, this article is in the 99th percentile (ranked 2nd) of the 312,683 tracked articles of a similar age in all journals and the 99th percentile (ranked 1st) of the 147 tracked articles of a similar age in *Nature Medicine.* The metrics also indicate it has been accessed over five million times. It is clearly the most cited paper and since its title and topic are the origin of the pandemic it clearly has an outsized influence on the topic.

The following statements form the evidence in the article of the natural origin of CoV-2:

- "While the analyses above suggest that SARS-CoV-2 may bind human ACE2 with high affinity, computational analyses **predict that the interaction is not ideal** and that the RBD sequence is different from those shown in SARS-CoV to be optimal for receptor binding. Thus, the high-affinity binding of the SARS-CoV-2 spike protein to human ACE2 is **most likely the result of natural selection on a human or human-like ACE2** that permits another optimal binding solution to arise. **This is strong evidence that SARS-CoV-2 is not the product of purposeful manipulation."** [emphasis added.]

    - A later analysis of over 3800 possible substitutions of amino acids in a 200 amino acid receptor binding region, much larger than the small, selective region referred to in this paper, shows that CoV-2 is 99.5% optimized for binding to the ACE-2

[53] https://usrtk.org/biohazards-blog/ecohealth-alliance-orchestrated-key-scientists-statement-on-natural-origin-of-sars-cov-2/
[54] https://www.independentsciencenews.org/news/peter-daszaks-ecohealth-alliance-has-hidden-almost-40-million-in-pentagon-funding/
[55] https://www.nature.com/articles/s41591-020-0820-9

receptor. This near perfect binding has never been seen before in a recent interspecies transmission jump.

- "Polybasic cleavage sites have not been observed in related 'lineage B' betacoronaviruses, although other human betacoronaviruses, including HKU1 (lineage A), have those sites and predicted O-linked glycans. Given the level of genetic variation in the spike, **it is likely that SARS-CoV-2-like viruses with partial or full polybasic cleavage sites will be discovered in other species.**" [emphasis added.]

    - As of the writing of this manuscript no other lineage B (sarbecovirus) has been found to have a furin site. In addition, the furin site of CoV-2 has the unusual -CGG-CGG- codon dimer, which has never been seen in an analysis of 58 other sarbecoviruses, that is, 580,000 codons. Since recombination between subgenera of beta coronaviruses is rare, or unknown, there is no source for the CGG-CGG dimer via a natural recombination event.

- "The acquisition of polybasic cleavage sites by HA has also been observed after repeated passage in cell culture or through animals."

    - It is curious why the above statement did not lead to a hypothesis somewhere in the article about a similar mechanism on CoV-2, a clear indication of a laboratory origin.

- "It is improbable that SARS-CoV-2 emerged through laboratory manipulation of a related SARS-CoV-like coronavirus."

    - This conclusory statement is unsupported my evidence.

- "Furthermore, if genetic manipulation had been performed, one of the several reverse-genetic systems available for betacoronaviruses would **probably have been used**. However, the genetic data irrefutably show that SARS-CoV-2 is not derived from any previously used virus backbone." [emphasis added.]

    - There is no explanation for why a prior backbone would necessarily be used. All synthetic biology chimera coronaviruses created in the past as published in prior papers have each used a unique backbone with no particular pattern in backbone selection. Each backbone was selected for the particular needs of those current experiments. This non-repeating prior pattern of reverse-genetic systems makes the above statement untenable. And with 16,000+ reported coronavirus specimens at the WIV it entirely reasonable a non-published virus could have been used.

- "Natural selection in an animal host before zoonotic transfer. For a precursor virus to acquire both the polybasic cleavage site and mutations in the spike protein suitable for

binding to human ACE2, **an animal host would probably have to have a high population density (to allow natural selection to proceed efficiently**) and an ACE2-encoding gene that is similar to the human ortholog." [emphasis added.]

  - The paragraph discusses the pangolin as the possible intermediate host but at the time of this manuscript the coronavirus data from pangolins has been discredited. This author agrees with statement that selection of the two unique features of CoV-2 require a high population density of the animal host. Of course, in the laboratory the animal hosts for either *in vitro* cell culture experiments or in animal experiments are a single species at high density.

- Natural selection in humans following zoonotic transfer. "It is possible that a progenitor of SARS-CoV-2 jumped into humans, acquiring the genomic features described above through adaptation during **undetected human-to-human transmission**. Once acquired, these adaptations would enable the pandemic to take off and produce a sufficiently large cluster of cases to trigger the surveillance system that detected it." [emphasis added.]

- "Studies of banked human samples could provide information on whether such cryptic spread has occurred. Further serological studies should be conducted to determine the extent of prior human exposure to SARS-CoV-2."

  - As will be shown in later sections, this prior undetected human-to-human transmission would be evident in archived specimens from before the fall of 2019. In both SARS-CoV-1 and MERS, this prior seroconversion averaged about 0.6% with almost 5% among workers exposed to the intermediate hosts. At the time of the writing of this manuscript, in limited sampling of archived specimens there has been no seroconversion detected. The author believes there are thousands of archived specimens from Wuhan taken in the fall of 2019 and these should be immediately examined for evidence of seroconversion. Since finding seroconversion among these specimens would be strong evidence for a zoonotic origin and not a laboratory accident, the absence of any information from China on this important evidence is hard to understand.

- Selection during passage. "Basic research involving passage of bat SARS-CoV-like coronaviruses in cell culture and/or animal models has been ongoing for many years in biosafety level 2 laboratories across the world, and there are documented instances of laboratory escapes of SARS-CoV. We must therefore examine the possibility of an inadvertent laboratory release of SARS-CoV-2."

- "In theory, it is possible that SARS-CoV-2 acquired RBD mutations during adaptation to passage in cell culture, as has been observed in studies of SARS-CoV."

- "New polybasic cleavage sites have been observed only after prolonged passage of low-pathogenicity avian influenza virus in vitro or in vivo. Furthermore, a hypothetical generation of SARS-CoV-2 by cell culture or animal passage would have required prior isolation of a progenitor virus with very high genetic similarity, **which has not been described.** Subsequent generation of a polybasic cleavage site would have then required repeated passage in cell culture or animals with ACE2 receptors similar to those of humans, **but such work has also not previously been described**." [emphasis added.]

    - The authors correctly describe a method for CoV-2 to have been generated in the laboratory and then dismiss it because the work has not been published previously. As active scientists themselves, the authors must know how disingenuous this sounds. Almost by definition elite scientists, like Dr. Shi of the WIV, work in secret until the publication of any given line of research. As the say, the absence of evidence cannot be used as evidence of its absence.

    - A peer-reviewed paper[56] entitled, "Might SARS-CoV-2 Have Arisen via Serial Passage through an Animal Host or Cell Culture? A potential explanation for much of the novel coronavirus' distinctive genome," provides a compelling argument that serial passage in the laboratory might indeed have been the manner in which CoV-2 acquired many of its devastating traits.

- "Although the **evidence shows that SARS-CoV-2 is not a purposefully manipulated virus**, it is currently impossible to prove or disprove the other theories of its origin described here. However, **since we observed all notable SARS-CoV-2 features, including the optimized RBD and polybasic cleavage site, in related coronaviruses in nature, we do not believe that any type of laboratory-based scenario is plausible**." [emphasis added.]

    - This author could identify no prior evidence in the paper to warrant saying it is not a purposefully manipulated virus. There is also no evidence that would point to a purposely manipulated virus.

    - The evidence in the paper shows that no prior zoonotic interspecies transmission has ever had an RBD as optimized as the CoV-2 RBD for the human ACE2. The evidence also shows that there is no natural source for the polybasic cleavage site (PCS). No other member of the subgenera to which CoV-2 belongs has a PCS. Since these are the only coronaviruses from which recombination could supply a polybasic cleavage site, the data in this paper refutes the natural origin.

[56] https://onlinelibrary.wiley.com/doi/full/10.1002/bies.202000091

- The belief statement concerning a laboratory-based scenario would be closer to the evidence if it was professed with, "despite evidence which is consistent with a laboratory-based scenario."

Based on the author's analysis of the paper, the following email was sent to the lead author:

Gmail Steven Quay, MD, PhD [redacted]

SARS-CoV-2 origin

[redacted]

Steven Quay, MD, PhD [redacted] Mon, May 25, 2020 at 7:14 PM
To: [redacted]

Dr. Andersen-

I read with interest your paper titled, The proximal origin of SARS-CoV-2, in which you conclude this is a natural, zoonotic-sourced infection. Three different approaches of analysis that I have done do not support this conclusion. Can you comment please:

1. The furin cleavage site insert has the unusual codon usage for the RR dimer of CGG-CGG. As you probably know, the frequency of this codon usage in the SARS-CoV-2 genome is 0.09. So having two next to each other is not likely as a random event. As support for the unlikeliness of these codons, using GISAID data, by March there is evidence of the third G being mutated out for either A or T at three-times the rate of the background mutation rate, 26/year from Nextstrain.org. Since codon usage in coronaviruses are not greatly influenced by the host it resides in, this means a jump to humans must have been in a host which did not have even a few months history with the virus, otherwise the terminal G would have been purified out. On the other hand, most laboratory use optimized codon primers and kits use CGG routinely; including in primers in published papers from the Wuhan Institute of Virology. So a laboratory source for a gain-of-function furin cleavage site would probably use these codons.

2. In the over 16,000 genomes in GISAID there is not one example of posterior diversity. With MERS, 93% of sequenced genomes did not pass through the index case but represented separate reservoir host to human jumps and this was apparent within 60 days of the index case. They collectively showed the most recent common ancestors among themselves was over 12 months before the index case. With SARS-CoV-2 it is acting like a 'pure culture' growth from the index case outward with no evidence of a reservoir host in the background. This would be the case for a laboratory acquired infection.

3. If you use a map of Wuhan and overlay the first four hospitals that saw cases with a map of the Metro system, you see that the hospitals straddle Line 2, which runs approximately east to west, carries 1,000,000 people a day, and is the Metro line with stops closest to both the Wuhan Institute of Virology and the original wet market that was considered an early source for the infection. There are 11 Metro lines in Wuhan, hundreds of stops on those lines, and over a dozen hospitals spread out over the city. I am working with a UCLA statistician to perform tests about the probability of this being simply an accident of statistics but the gestalt is, it does not look like a chance occurrence. But it is consistent with someone getting infected in the lab, riding Line 2 for a few days, and off you go.

It might be a truism to say that the six proven cases of laboratory derived SARS escapes occurred in big cities, Beijing, Singapore, or Taipei where the labs are located. But if you follow it with the fact that MERS and SARS, both proven as true zoonotic sources, on other hand began in rural settings in China and the Middle East, respectively. I am not sure why this obvious correlation was not at least pointed out in your paper and then addressed with a cogent argument.

I look forward to hearing your thoughts.

Regards, Steve

Soon after this email was written Dr. Andersen blocked the author from following his Twitter account. A reply to the above email was never received.

Conclusion. Three high visibility papers were published between January and May 202 which purported to settle the question of the origin of SARS-CoV-2 as a zoonotic transmission and not a laboratory accident. The analysis above concludes that these papers are not persuasive. The author has elected to not use evidence within these papers to change the prior likelihood of a

zoonotic versus laboratory origin. They are presented here as neutral evidence that supports neither theory.

Likelihood from initial state is unchanged following this evidence analysis:

Zoonotic origin (98.8%) and laboratory origin (1.2%)

Evidence. SARS-like infections among employees of the Wuhan Institute of Virology in the fall of 2019

The State Department of the United States issued the following statement on January 15, 2021[57]:

"1. Illnesses inside the Wuhan Institute of Virology (WIV):

- The U.S. government has reason to believe that several researchers inside the WIV became sick in autumn 2019, before the first identified case of the outbreak, with symptoms consistent with both COVID-19 and common seasonal illnesses. This raises questions about the credibility of WIV senior researcher Shi Zhengli's public claim that there was "zero infection" among the WIV's staff and students of SARS-CoV-2 or SARS-related viruses."

There is no additional evidence to support either parties position in the above statement. The U.S. Government statement would be considered hearsay in a court of law and probably not admissible. The veracity of Dr. Shi's statement above could be called into question due to other inconsistencies in some of her testimony, as reported elsewhere in this document.

At this time, the above evidence cannot be used to change the likelihood of either theory about the origin of SARS-CoV-2. The statement is kept within this analysis with the hope that in the future new information will come to light that could make this evidence a useful addition to the overall analysis.

Likelihood from initial state is unchanged following this evidence analysis:

Zoonotic origin (98.8%) and laboratory origin (1.2%)

[57] https://2017-2021.state.gov/fact-sheet-activity-at-the-wuhan-institute-of-virology//index.html

Evidence. A Bayesian Analysis of one aspect of the SARS-CoV-2 origin, where the first recorded outbreak occurred, increases the probability of a laboratory origin.

Introduction. The two competing hypotheses of the origin of SARS-CoV-2 as a natural, zoonotic spillover event versus a laboratory-acquired infection (LAI) or other laboratory accident each had supporting evidence from the very beginning of the pandemic.

On the one hand, about 40% of early patients with COVID-19 had an association with the Hunan Seafood Market in Wuhan. Since this mirrored SARS-CoV-1, where markets selling civet cats were determined to be the origin of that human epidemic, the natural origin hypothesis seemed logical. The Chinese CDC have now ruled out the market as a source for the outbreak.

On the other hand, the laboratory origin hypothesis also had an early beginning with the fact that the outbreak began adjacent to the only high security, BSL-4 laboratory in all of China, and one of the top coronavirus research centers in the world, was the Wuhan Institute of Virology (WIV). The hospitals of the first COVID patients were very close to the WIV.

This evidence statement is taken from an article applying a Bayesian analysis to the hypothesis that the proximal origin of SARS-CoV-2 was an uncontrolled[58] release from a laboratory using, as evidence, one aspect of the SARS-CoV-2 origin story — where the first recorded outbreak occurred.[59]

Hypothesis: The first recorded outbreak of SARS-CoV-2 in the human population occurred in a city that is also home to a virology laboratory that actively performs research on closely related viruses.

In this case, the city is Wuhan, and the virology laboratory is run by the Wuhan Institute of Virology.

Analysis. This analysis set the likelihood of a laboratory escape (the prior probability the hypothesis was true) at three values, 0.01%, 0.1%, and 1.0%. The second term was the conditional probability of the evidence, given that the hypothesis is actually false. This was set at 0.01. Finally, the third term was the conditional probability of the evidence, given the hypothesis is true. This was set, biasing to the natural origin, at 0.71.

Results. The paper provides the three-by-three cube of results for the three parameters of interest.

The ardent sceptic's probability begins at 0.01% and the revised estimate is no more than 0.05% or 5/10000. It applies to someone who was initially very skeptical about a lab origin (0.01% probability), who believes there is no more than 51% chance that an uncontrolled release of a highly contagious disease would lead to a local outbreak, and who thinks there was at least a

[58] By using the term uncontrolled release, the author was specifically excluding from consideration the possibility that the pathogen was deliberately released from the laboratory.
[59] https://jonseymour.medium.com/a-bayesian-analysis-of-one-aspect-of-the-sars-cov-2-origin-story-where-the-first-recorded-1fbdcbea0a2b

10% chance that a natural outbreak of a virus native to Yunnan would have occurred in Wuhan before any place else.

On the other extreme, is the ardent believer who started with at least a 1% belief in a laboratory outbreak, is 100% certain that an uncontrolled laboratory release would result in a local outbreak and believes that the probability that a natural outbreak of a virus native to Yunnan would occur in Wuhan before any place else is less than 0.1%. The ardent believer's revised belief is that the probability that the Wuhan outbreak was caused by an uncontrolled laboratory release changes from 1% to at least 91%.

In the center, is the so-called "central" observer who accepts that the central values for each of the parameter ranges are reasonable estimates of the true values of the probability being estimated. The central observer started with an initially skeptical belief in the hypothesis of 0.1%, believes that average citizen in Wuhan was a likely as any other citizen of China to be the initial vector of the virus into the human population and believes that there is no more or less than a 71% chance that an uncontrolled release from a laboratory of a highly contagious pathogen such as SARS-CoV-2 would result in a local outbreak as opposed to an outbreak in some other location. The central observer's revised belief in the hypothesis is 6.8%. If the central observer began with a 1% belief in a laboratory origin, this analysis would change that to 41.8%.

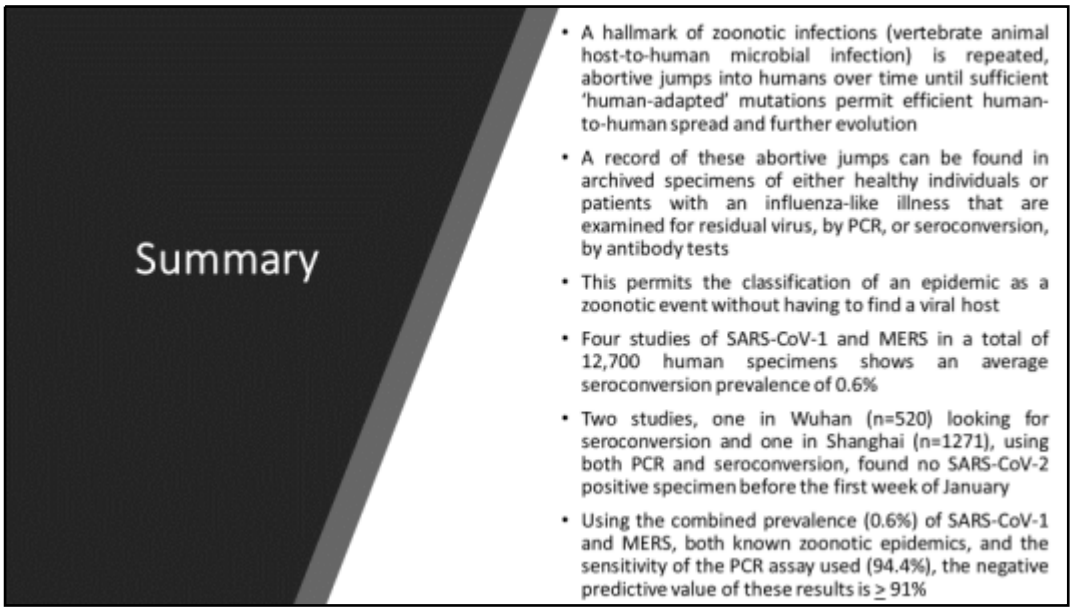
Conclusion. For purposes of this analysis and to be as conservative as possible, the assumptions will be that there is at least a 1% prior belief in a laboratory outbreak (because that was our starting probabilities), but there is no more than a 51% chance that an uncontrolled release of a highly contagious disease would lead to a local outbreak, and that there was at least a 10% chance that a natural outbreak of a virus native to Yunnan would have occurred in Wuhan before any place else. Using these assumptions, the initial likelihood of a 1% laboratory origin changes to 4.9%.

Starting likelihood from initial state: Zoonotic origin (98.8%) and laboratory origin (1.2%)

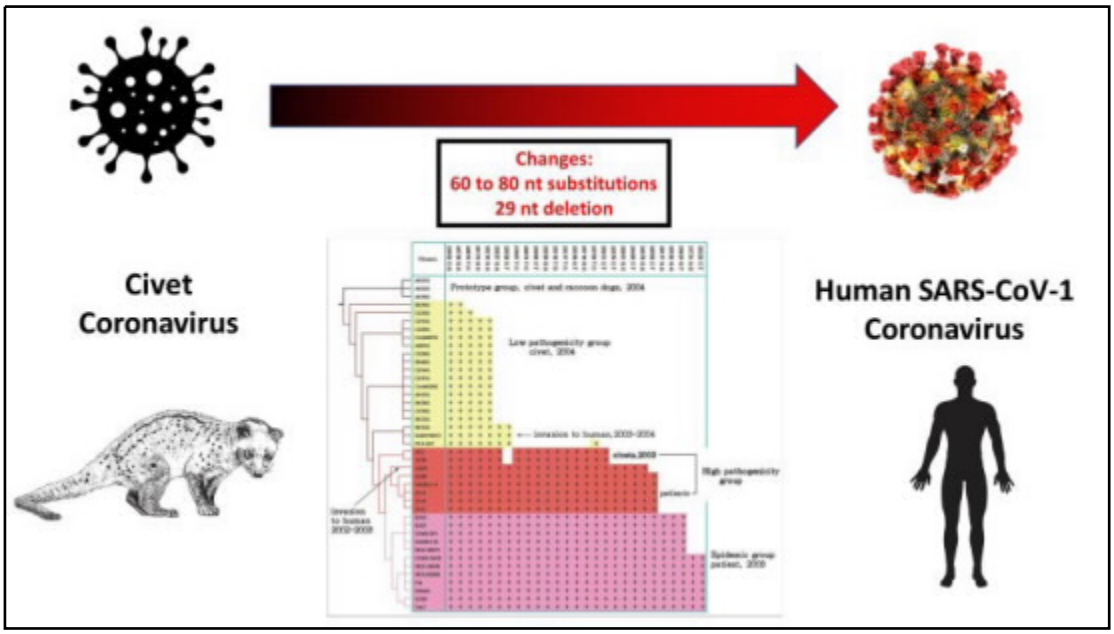
Adjusted likelihood: Zoonotic origin (95.1%) and laboratory origin (4.9%)

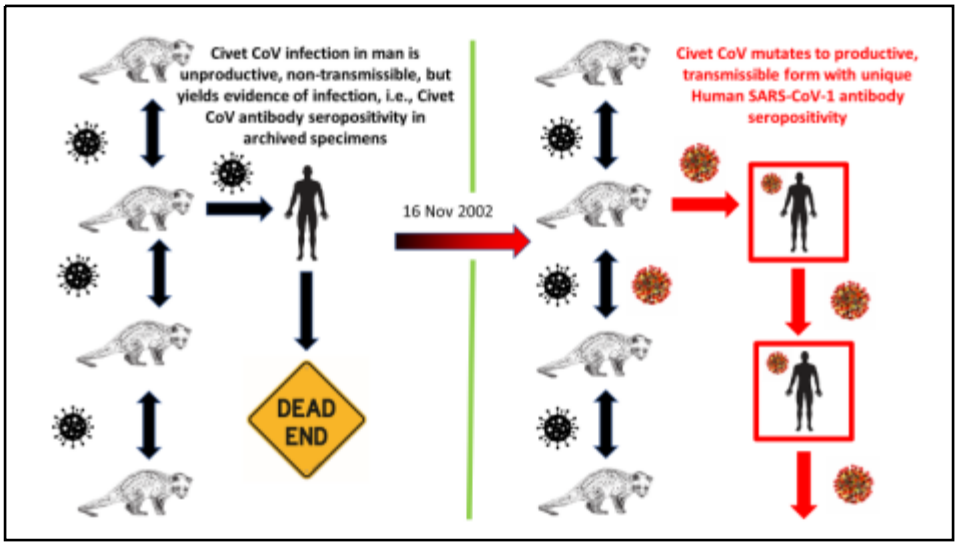
Evidence: Lack of seroconversion in Wuhan and Shanghai. Summary of evidence:

- A hallmark of zoonotic infections (vertebrate animal host-to-human microbial infection) is repeated, abortive jumps into humans over time until sufficient 'human-adapted' mutations permit efficient human-to-human spread and further evolution

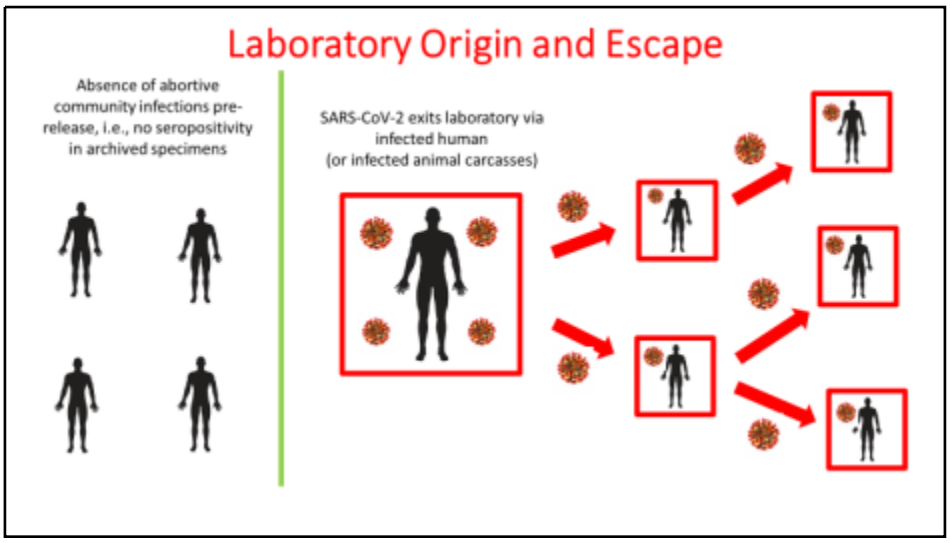


- A record of these abortive jumps can be found in archived specimens of either healthy individuals or patients with an influenza-like illness that are examined for residual virus, by PCR, or seroconversion, by antibody tests





- This permits the classification of an epidemic as a zoonotic event without having to find a viral host
- A laboratory accident is a situation in which there are no prior exposures within the human population as shown in the Figure below:



- Four studies of SARS-CoV-1 and MERS in a total of 12,700 human specimens shows an average seroconversion prevalence of 0.6%

SARS-related Virus Predating SARS Outbreak, Hong Kong

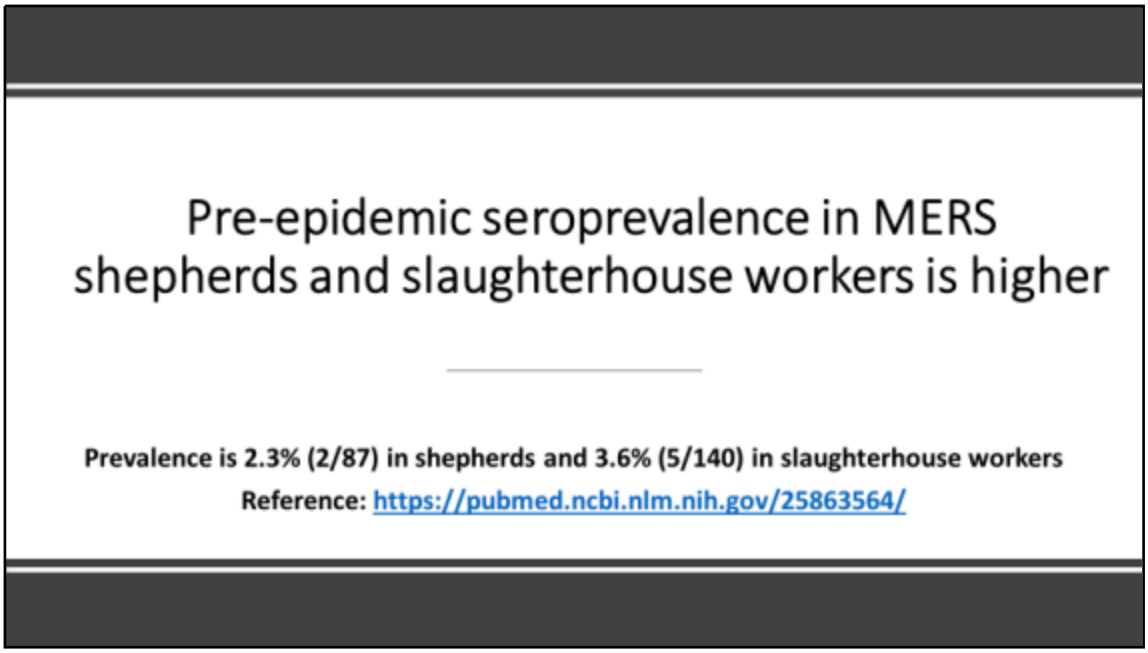
SARS-CoV-1 began in fall of 2002 in southern China

Patient Population	Serum samples collected in May 2001 from 938 healthy adults in Hong Kong	48 confirmed SARS patients diagnosed in February and March 2003 in Guangdong
Civet CoV > SARS-CoV-1 Seropositivity	13	0
SARS-CoV-1 > Civet CoV Seropositivity	4	48
Total	17 out of 938 = 1.8%	48 out of 48 = 100%

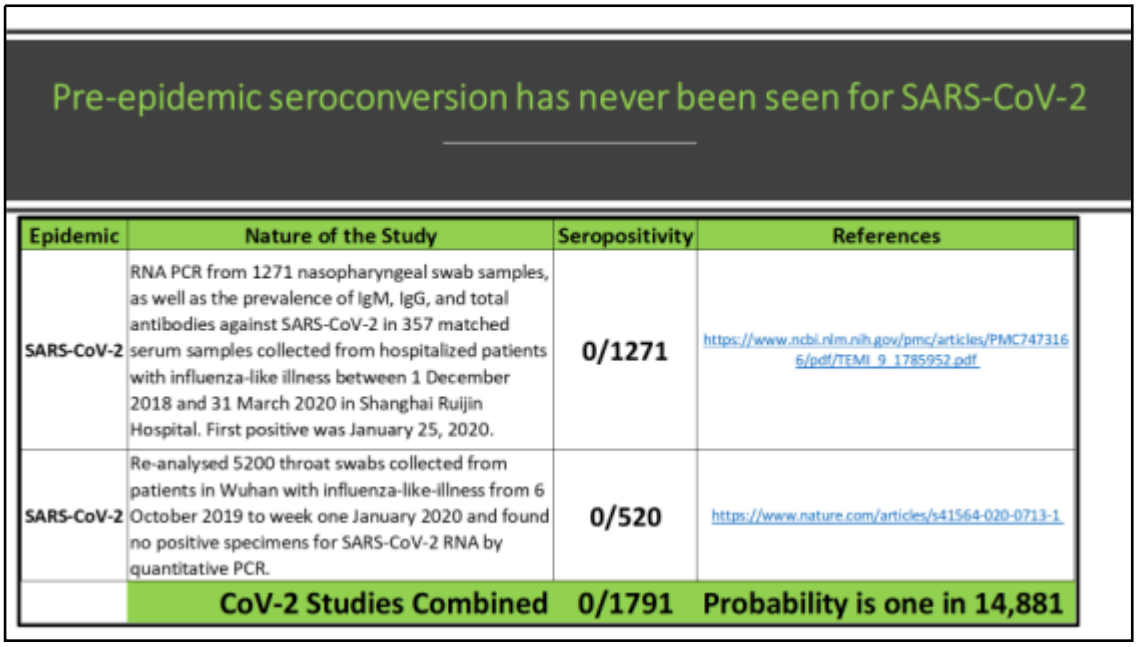
Pre-epidemic seroprevalence in the adult community

Prevalence is 0.6% for SARS-CoV-1 and MERS in 12,700 specimens

Epidemic	Nature of the Study	Seropositivity	Reference
SARS-CoV-1	Archived specimens from healthy adults in Hong Kong collected two years before CoV-1 were tested for Ab to civet or human CoV	17/938	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3322899/
MERS	Archived human sera collected in 2011 was tested for MERS-CoV S1-specific antibodies by ELISA	1/90	https://www.sciencedirect.com/science/article/pii/S1876034120300010#fig0010
SARS-CoV-1	Serum specimens collected from military recruits from the People's Republic of China in 2002 were tested for SARS-CoV-1 antibodies.	11/1621	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1074388/
MERS	Between Dec 1, 2012, and Dec 1, 2013, 10,009 individual serum samples were tested for anti-MERS-CoV antibodies in regions without cases.	15/10,009	https://pubmed.ncbi.nlm.nih.gov/25863564/
SARS-CoV-1	Serum samples that were collected from 42 individuals during 2001-2002, before the SARS outbreak, and tested for IgG antibody against SARS-CoV.	28/42	https://arxiv.org/ftp/arxiv/papers/1305/1305.2659.pdf

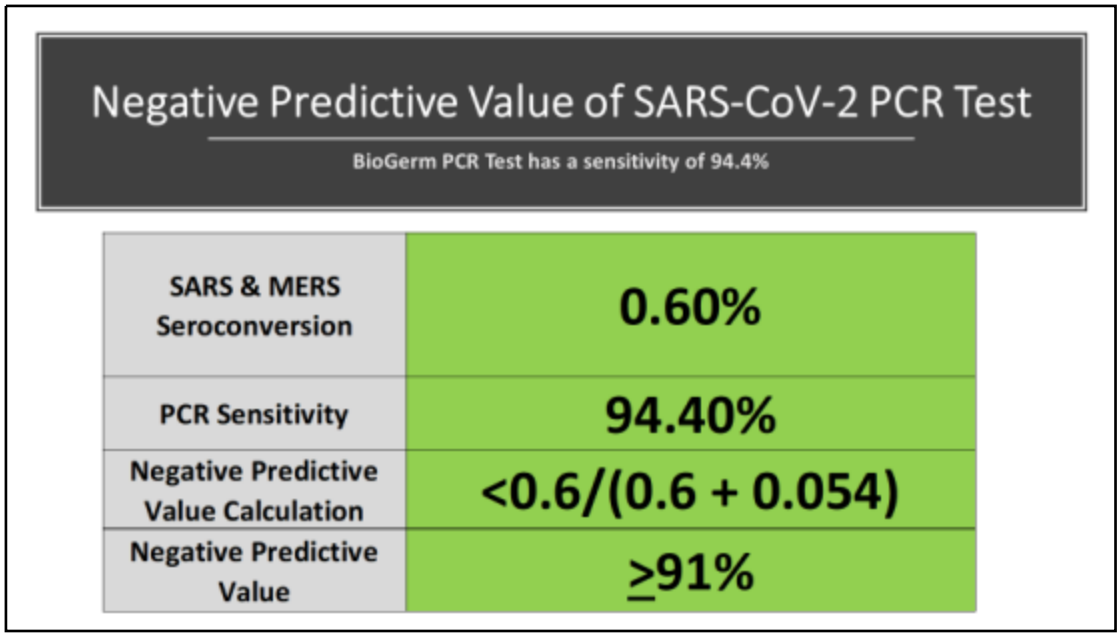


- Two studies, one in Wuhan (n=520) looking for seroconversion and one in Shanghai (n=1271), using both PCR and seroconversion, found no SARS-CoV-2 positive specimen before the first week of January



Epidemic	Nature of the Study	Seropositivity	References
SARS-CoV-2	RNA PCR from 1271 nasopharyngeal swab samples, as well as the prevalence of IgM, IgG, and total antibodies against SARS-CoV-2 in 357 matched serum samples collected from hospitalized patients with influenza-like illness between 1 December 2018 and 31 March 2020 in Shanghai Ruijin Hospital. First positive was January 25, 2020.	0/1271	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7473166/pdf/TEMI_9_1785952.pdf
SARS-CoV-2	Re-analysed 5200 throat swabs collected from patients in Wuhan with influenza-like-illness from 6 October 2019 to week one January 2020 and found no positive specimens for SARS-CoV-2 RNA by quantitative PCR.	0/520	https://www.nature.com/articles/s41564-020-0713-1
	CoV-2 Studies Combined	**0/1791**	**Probability is one in 14,881**

- Using the combined prevalence (0.6%) of SARS-CoV-1 and MERS, both known zoonotic epidemics, and the sensitivity of the PCR assay used (94.4%), the negative predictive value of these results is $\geq$ 91%



Here, the negative predictive value (NPV) represents the probability that a CoV-2 is not a zoonosis, given the negative seroconversion findings.

Subjective Discount Factor: 90% (a one in 10 chance this is wrong). This is a subjective value.

The change in origin likelihoods from this evidence and the calculations are shown in the Text-Table below.

Evidence or process	Zoonotic Origin (ZO)	Laboratory Origin
Starting likelihood	0.951	0.049
Negative predictive value of lack of seroconversion	0.91	
Reduced by 90% Subjective Discount Factor	0.91 x 0.9 = 0.82	
Impact of this evidence	Reduces the likelihood of ZO by 82/18 or 4.6-fold. For every 100 tests, a true ZO would be seen 18 times and a non-ZO would be seen 82 times	
Impact of evidence calculation	0.951/4.6 = 0.207	
Normalize this step of analysis	0.207/(0.207 + 0.049) = 0.809	0.049/(0.207 + 0.049) = 0.191

Adjusted likelihood: Zoonotic origin (80.9%) and laboratory origin (19.1%)

Evidence: Lack of posterior diversity for SARS-CoV-2 compared to MERS and SARS-CoV-1

- The earliest stages of human CoV-1 and MERS infections were characterized by viral genome base diversity as expected for multiple, independent jumps from a large and diverse intermediate host population into humans.
- Combining MERS and CoV-1 studies, out of the earliest 255 human infections in which virus genome sequences are available, 137 could not be rooted in a prior human-to-human infection and so are attributed to an independent intermediate host-to-human infection.[60]
- That is about 54% non-human-to-human transmission.
- On the other hand, Ralph Baric has written[61] that CoV-2 is different: "SARS-CoV-2 probably emerged from bats, and early strains identified in Wuhan, China, showed limited genetic diversity, which suggests that the virus **may have been introduced from a single source**." [emphasis added.]
- With CoV-2, there are 249 viral genomes in GISAID from Hubei province, where Wuhan is located, collected between Dec 24, 2019 and Mar 29, 2020.
- From Dec 24, 2019 to November 2020, there are 1001 genomes sequenced from all of China and 198,862 worldwide.
- For CoV-2, every single genome sequence is rooted in the first sequence from the PLA Hospital in Wuhan.
- Not one case of posterior diversity.
- Using the frequency of non-rooted genome diversity seen with MERS and CoV-1, about 50:50 or a coin toss, the probability that CoV-2 is a zoonotic pandemic with 0/249 genomes is the chance of tossing a coin 249 times and getting heads every time!
- Mathematically that is nonexistent; specifically, one in 10 with 84 zeros.
- Since Wuhan had approximately 500,000 cases during the time interval of this sampling, the potential sampling error of testing only 249/500,000 or 0.05% is significant. This sampling error, while large, is unable to obliterate the overwhelming odds that this did not arise from an intermediate host in Wuhan.
- Therefore, to permit continued evidence analysis, this finding will be set at the boundary of customary statistical significance, a p-value of 0.05 or a 1 in 20 likelihood that this is zoonotic.

Detailed explanation

[60] https://elifesciences.org/articles/31257#abstract ; https://www.researchgate.net/publication/225726653_Molecular_phylogeny_of_coronaviruses_including_human_SARS-CoV ; https://science.sciencemag.org/content/300/5624/1394/tab-pdf ; https://pubmed.ncbi.nlm.nih.gov/14585636/ ; https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.016378-0?crawler=true ; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7118731/

[61] https://www.nejm.org/doi/10.1056/NEJMcibr2032888

A fundamental difference between a laboratory and a non-laboratory acquired zoonotic disease, the imprint of phylogenetic diversity through pre-human spread within the source population, can be examined by the posterior diversity of human cases with no *a priori* knowledge of an intermediate host.

MERS. The MERS epidemic has been documented to have arisen from the initial jump from bats to camels, a three-to-five-year expansion within the camel population in which mutational diversity arose by random mistakes, and then a jump into humans. This model of spread predicts that there would, at some point, be additional jumps from other camels into other patients, and a pattern of "posterior diversity," would be found in the human specimens. If the COVID-19 pandemic arose by a similar mechanism the same pattern would be seen. The following Text-Table contains such data.

Phylogenetic Feature	MERS	SARS-CoV-2
Posteriority Diversity	28/30 (93%)	0
No Posteriority Diversity	2/30 (7%)	7666
Time from first patient to first example of posterior diversity	About 60 days	None at >120 days
Depth of posterior diversity to first patient	>365 days	None

The study of MERS noted above was published in 2013 in Lancet[62] in an article entitled, "Transmission and evolution of the Middle East respiratory syndrome coronavirus in Saudi Arabia: a descriptive genomic study." Thirty specimens were used in the analysis. The features of a camel-to-human zoonotic epidemic are easily identified. Specimens taken within sixty days of the first patient, "Patient Zero," began to show a background diversity that could not be traced back through Patient Zero. The analysis of all thirty, in fact, documented that 93% were transmitted directly from the camel intermediate reservoir. And looking only at the "background" diversity permitted a calculation of the last common ancestor for the spread within the camel population of over 365 days.

A study of SARS-CoV-2[63] available May 5, 2020 and entitled, "Emergence of genomic diversity and recurrent mutations in SARS-CoV-2," looked at 7666 patient specimens from around the world for phylogenetic diversity. The authors state: "There is a robust temporal signal in the data, captured by a statistically significant correlation between sampling dates and 'root-to-tip' distances for the 7666 SARS-CoV-2 ($R^2 = 0.20$, $p < .001$). Such positive association between sampling time and evolution is expected to arise in the presence of measurable evolution over the timeframe over which the genetic data was collected." This conclusion also argues against a MERS-like pattern of posterior diversity. In fact, the 95% upper bound for the probability of no posterior diversity being seen in SARS-CoV-2, given the data in MERS, is 3.9×10^{-4}.

[62] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3898949/
[63] https://www.sciencedirect.com/science/article/pii/S1567134820301829

The finding of posterior diversity in MERS was seen quickly, that is, within 60 days of the first patient and in only 30 specimens. In this study of COVID-19 the cutoff date of the 7666 specimens was April 19, 2020 or approximately 140 days after the first documented case. The lack of posterior diversity in COVID-19 at a much later date than what was seen with MERS also argues against a non-laboratory source for this pandemic.

A useful avenue of future research for those working to find an animal source for COVID-19 would be new mathematical models or statistical methods that might find a "hidden" signal of posterior diversity in the current data set which shows none. And given access to the unprecedented quantity of human data for COVID-19 which can be mined via bioinformatics, efforts to find the "missing link" in the wild through search and sample should be a second priority to mining the human specimen data set.

SARS-CoV-1. A similar pattern of clinical cases that do not show a common ancestor in the human population but instead is evidence of posterior diversity is shown in the Text-Table on the left for SARS-CoV-1[64] compared to CoV-2 on the right[65]. SARS-CoV-1 shows clusters of cases in humans that are connected only by phylogenetic branches that reach back in time (all of the branches inside the purple box. This is because of the extensive mutational background created while being in the intermediate host, the civet. With CoV-2 on the right, every clinical case descends from the first clinical case, in the 19A clade. There are no background mutations to account for. I will show elsewhere that the first Clade A patient was at the PLA Hospital about 3 km from the WIV.

[64] https://pubmed.ncbi.nlm.nih.gov/14585636/
[65] https://nextstrain.org/

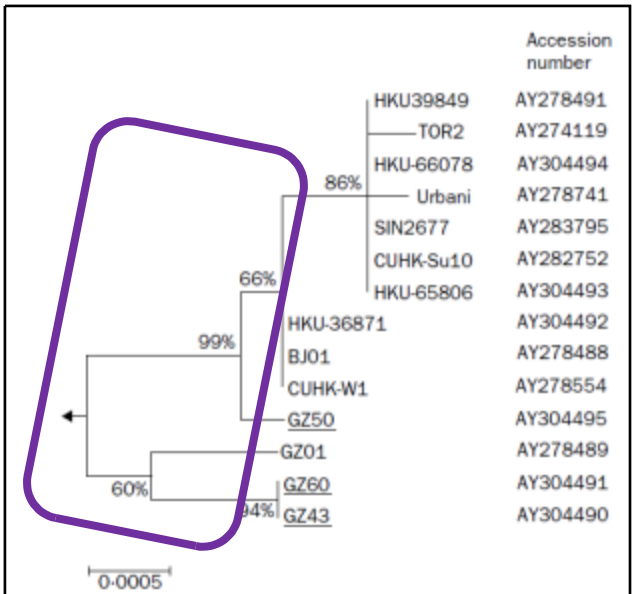
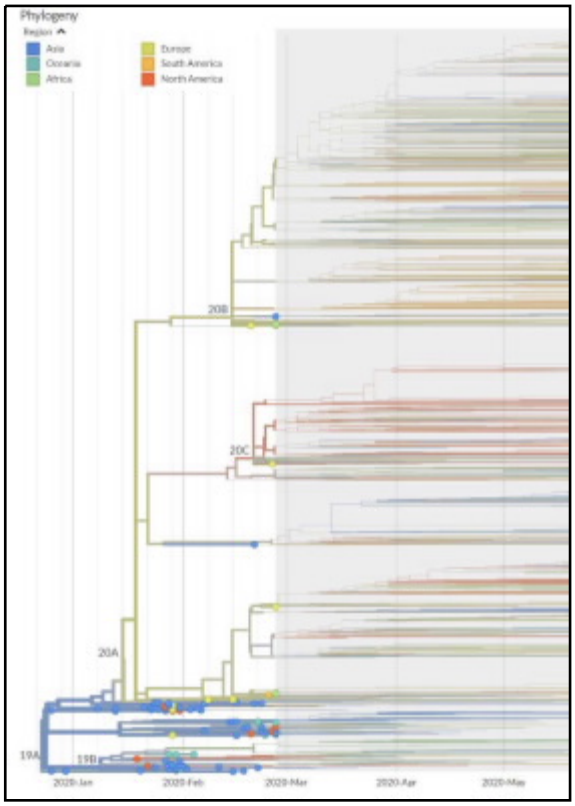
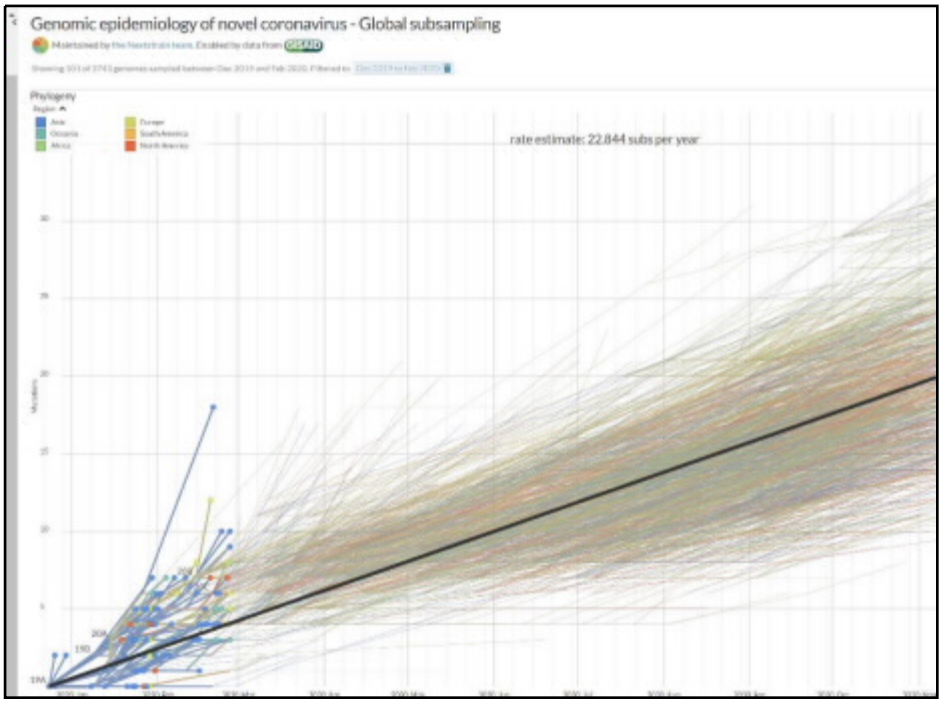


Figure 4: **Phylogenetic analysis of nucleotide acid sequence of spike gene of SARS CoV viruses**
Bootstrap values are shown as a percentage. The scale bar shows genetic distance estimated using Kimura's two parameter substitution model.[14] The nucleotide sequences of representative SARS CoV S genes (S gene coding region residue, 3765 bp) were analysed. Viruses sequenced in this study are underlined, and the other sequences used in the analysis can be accessed in GenBank with accession numbers as shown.



Given the rate of mutations of 22.8 per year for CoV-2 as shown in the Nextstrain graph below and a sequencing accuracy of about two calls per genome, CoV-2 could not have spent more than a few weeks in an intermediate host before a pattern of background mutations would be identified as posterior diversity. In the laboratory a pure culture on a single genome is used and the CoV-2 pattern is most consistent with a single pure culture infection a first human.

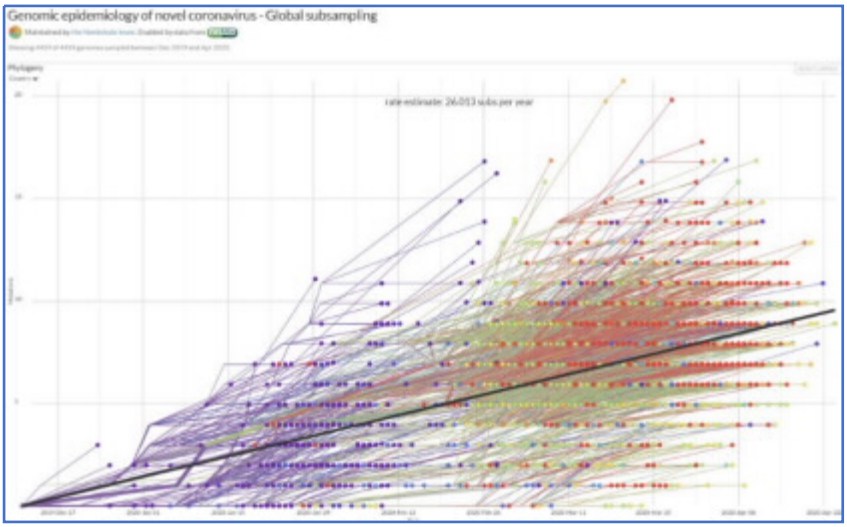


Non-zoonotic evolution. In a hypothetical in which there was a singular event in which one genetically pure virus infected one person and then the epidemic grow the development of the genetic diversity would have a clear, identifiable pattern: every new mutation would only appear on a background of the previous mutations.

The mutations in this virus are literally a personal tag. The general mutation rate leads to one mutation per patient. So, by definition, Patient Zero will have just one mutation. And then the 2-4 people that patient passes it to will have that mutation and then will add a new one, and so on. As time goes by two things happen: each patient gets a new mutation of their own and they pass on all the mutations of the past.

Since the virus has 29,900 nt and the mutation rate, as shown in this graph prepared by NextStrain is 26 mutations per year, there is very little chance a mutation will appear and then later get undone. By carefully going back in time, it is possible to literally name each person at each generation by the one (on average) new mutation they have and all of those that went before.

This graph of mutations on the Y-axis shows them gradually increasing and the color coding shows where they came from. In this infection, they only came from a previous patient and from the next previous patient and so on.



A NextStrain graphic.

How is that different from MERS, which was passed from camels to humans in a true zoonotic process?

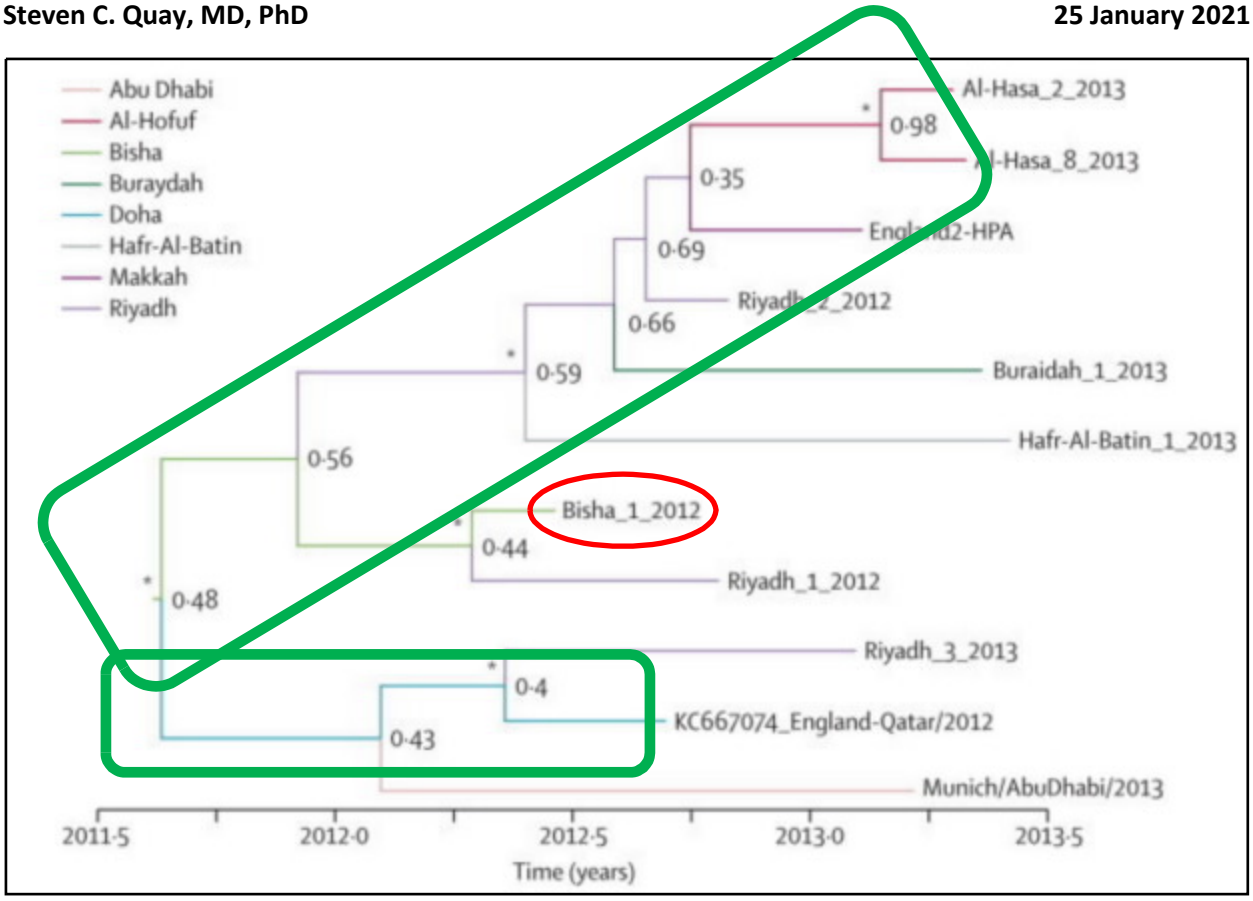
In a true zoonotic spread to humans there is usually an initiating species (in MERS it is bats), and then an intermediate species (in MERS it is camels), and then it moves to humans, either because of a new "enabling mutation" or for a non-domestic species, a chance encounter, and Source Zero and Patient Zero meet, and a cross species event occurs. But "Source Zero" doesn't stop there with one infection in one human; the virus also transmits itself vertically into the intermediate species. Source Zero also creates a vertical infection in the camels. Whether it is mild or not doesn't matter. The new human jumping gene is moving into a very diverse population of viruses, who have themselves been evolving since the first bat to camel transmission.

What is the outcome in terms of a test to show this is happening?

The diversity of the virus in humans becomes great, and the spots where the mutations occur don't match up to MERS Patient Zero like they do in COVID-19. In MERS, the virus in Patient Zero and the virus in a later infection are not direct descendants but cousins and only descended from an earlier virus that spent time in another camel population, collecting random mutations until it got the one it needed to infect humans, and then it begins again.

The chart below, from Lancet. 2013 Dec 14; 382(9909): 1993–2002, shows just how this works. The patient at Bisha is the earliest case in this chart (Patient Zero in the red circle). But notice, no other case comes from that patient. The viruses have such a diverse genetic background they appear to only be related to the Bisha virus with a posterior timeline of about one year. Their background is in the green boxes and it skips Patient Zero.

.



Even without knowing that camels are the zoonotic source for MERS, this data, from clinical sample only and without any field work in cave or camels, is all you need to know that this arose in the wild.

A paper just appeared with this analysis for a region of China and the posterior genomic diversity indicated a single starting point on December 1, 2019 for all cases. There was no posterior diversity. At this point with over 322,000 full genomes sequenced[66] and all showing an additive pattern of mutations and with none showing background diversity before the known appearance in Wuhan, the only conclusion is that there is no reservoir of genetic diversity.

On January 26, 2020 in an article in *Science* written by Jon Cohen, Kristian Andersen, an evolutionary biologist at the Scripps Research Institute who had analyzed sequences of CoV-2 to try to clarify its origin said: "The scenario of somebody being infected outside the market and then later bringing it to the market is one of the three scenarios we have considered that is still consistent with the data. It's entirely plausible given our current data and knowledge."

The negative predictive value of finding no posterior diversity in CoV-2 with 322,000 total infections sequenced, over 1000 in China, is 95%

Subjective Discount Factor: 95% (a one in 20 chance this is wrong)

[66] https://www.gisaid.org/

Below is the impact of the pack of posterior diversity on the likelihood of a zoonotic versus laboratory origin

Evidence or process	Zoonotic Origin (ZO)	Laboratory Origin
Starting likelihood	0.809	0.191
Negative predictive value of lack of posterior diversity	0.95	
Reduced by 95% Subjective Discount Factor	0.95 x 0.95 = 0.90	
Impact of this evidence	Reduces the likelihood of ZO by 90/10 or 9-fold. For every 100 tests, a true ZO would be seen 10 times and a non-ZO would be seen 90 times	
Impact of evidence calculation	0.809/9 = 0.085	
Normalize this step of analysis	0.085/(0.085 + 0.191) = 0.308	0.191/(0.085 + 0.191) = 0.692

Adjusted likelihood: Zoonotic origin (30.8%) and laboratory origin (69.2%)

Evidence: Opportunity.

The Wuhan Institute of Virology has publicly disclosed that by 2017 it had developed the techniques to collect novel coronaviruses, systematically modify the receptor binding domain to improve binding or alter zoonotic tropism and transmission, insert a furin site to permit human cell infection, make chimera and synthetic viruses, perform experiments in humanized mice, and optimize the ORF8 gene to increase human cell death (apoptosis).

Wuhan Institute of Virology scientists maps RBD and then takes a civet coronavirus that won't infect human cells, changes two amino acids in the receptor binding domain & it infects human cells.[67]

中国科技论文在线 http://www.paper.edu.cn

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 280, No. 33, Issue of August 19, pp. 29588–29595, 2005
© 2005 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

Identification of Two Critical Amino Acid Residues of the Severe Acute Respiratory Syndrome Coronavirus Spike Protein for Its Variation in Zoonotic Tropism Transition via a Double Substitution Strategy*

Received for publication, January 19, 2005, and in revised form, June 16, 2005
Published, JBC Papers in Press, June 24, 2005, DOI 10.1074/jbc.M500662200

Xiu-Xia Qu,[a,b] Pei Hao,[b,c] Xi-Jun Song,[a,b] Si-Ming Jiang,[a,b] Yan-Xia Liu,[a] Pei-Gang Wang,[a] Xi Rao,[a] Huai-Dong Song,[d] Sheng-Yue Wang,[e] Yu Zuo,[a] Ai-Hua Zheng,[a] Min Luo,[a] Hua-Lin Wang,[f] Fei Deng,[f] Han-Zhong Wang,[f] Zhi-Hong Hu,[f] Ming-Xiao Ding,[a] Guo-Ping Zhao,[c,e,g,h] and Hong-Kui Deng[a,i]

From the [a]Department of Cell Biology and Genetics, College of Life Sciences, Peking University, Beijing 100871, the [c]Bioinformation Center/Institute of Plant Physiology and Ecology/Health Science Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, the [d]State Key Laboratory for Medical Genomics/Pôle Sino-Francais de Recherche en Sciences du Vivant et Génomique, Ruijin Hospital Affiliated with the Shanghai Second Medical University, Shanghai 200025, the [e]Chinese National Human Genome Center, 250 Bi Bo Road, Zhang Jiang High Tech Park, Shanghai 201203, the [f]State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, 430071, and the [g]State Key Laboratory of Genetic Engineering/Department of Microbiology, School of Life Science, Fudan University, Shanghai 200433, China

Baric & Shi at WIV take bat coronavirus that won't infect human cells, change S746R to add an ARG at S1/S2 site to make furin-like cleavage site, & the new coronavirus infects human cells.[68]

Baric & Shi of WIV create completely synthetic coronavirus from bat spike & mouse adapted backbone that no treatment, monoclonal antibody, or vaccine will touch.[69]

- "Using the SARS-CoV reverse genetics system2, we generated and characterized a chimeric virus expressing the spike of bat coronavirus SHC014 in a mouse-adapted SARS-CoV backbone.

- The results indicate that group 2b viruses encoding the SHC014 spike in a wild-type backbone can efficiently use multiple orthologs of the SARS receptor human angiotensin

[67] http://www.paper.edu.cn/scholar/showpdf/NUT2kN0INTT0gxeQh
[68] https://jvi.asm.org/content/jvi/89/17/9119.full.pdf
[69] https://pubmed.ncbi.nlm.nih.gov/26552008/

converting enzyme II (ACE2), replicate efficiently in primary human airway cells and achieve in vitro titers equivalent to epidemic strains of SARS-CoV.

- Additionally, in vivo experiments demonstrate replication of the chimeric virus in **mouse lung with notable pathogenesis.**

- Evaluation of available SARS-based immune-therapeutic and prophylactic modalities revealed poor efficacy; both monoclonal antibody and vaccine approaches failed to neutralize and protect from infection with CoVs using the novel spike protein.

- On the basis of these findings, we **synthetically re-derived an infectious full-length** SHC014 recombinant virus and demonstrate robust viral replication both in vitro and in vivo."

This study was conducted, with permission, during the gain of function moratorium put in place by NIH in 2014:

"These studies were initiated before the US Government Deliberative Process Research Funding Pause on Selected Gain-of-Function Research Involving Influenza, MERS and SARS Viruses (http://www.phe.gov/s3/dualuse/Documents/gain-of-function.pdf). This paper has been reviewed by the funding agency, the NIH. Continuation of these studies was requested, and this has been approved by the NIH."

Drs. Daszak and Shi becomes world's expert on ORF8 induced apoptosis by CoVs in human cells (HeLa) & maximizing lethality.[70]

> The full-length ORF8 protein of SARS-CoV is a luminal endoplasmic reticulum (ER) membrane-associated protein that induces the activation of ATF6, an ER stress-regulated transcription factor that activates the transcription of ER chaperones involved in protein folding [35]. We amplified the ORF8 genes of Rf1, Rf4092 and WIV1, which represent three different genotypes of bat SARSr-CoV ORF8 (S3C Fig), and constructed the expression plasmids. All of the three ORF8 proteins transiently expressed in HeLa cells can stimulate the ATF6-dependent transcription. Among them, the WIV1 ORF8, which is highly divergent from the SARS-CoV ORF8, exhibited the strongest activation. The results indicate that the variants of bat SARSr-CoV ORF8 proteins may play a role in modulating ER stress by activating the ATF6 pathway. In addition, the ORF8a protein of SARS-CoV from the later phase has been demonstrated to induce apoptosis [28]. In this study, we have found that the ORF8a protein of the newly identified SARSr-CoV Rs4084, which contained an 8-aa insertion compared with the SARS-CoV ORF8a, significantly triggered apoptosis in 293T cells as well.

This paper also demonstrates the collection of 64 novel bat coronaviruses from caves in southern China, including Yunnan where Dr. Shi has said is the location of the bat ancestor of CoV-2.

This evidence is necessary for a laboratory origin hypothesis in which genetic manipulation to create CoV-2 is a precursor to a laboratory accident. However, it does not per se, provide

[70] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5708621/

increased weight in favor of a laboratory origin. It is however provided here to be a guide for the kinds of investigations to be conducted if access to the WIV records is ever provided.

Likelihood from prior state is unchanged following this evidence analysis:

Zoonotic origin (30.8%) and laboratory origin (69.2%)

Evidence and Motive for laboratory furin site insertion:

A key to infectivity of coronaviruses is the addition, in nature or the laboratory, of a furin cleavage site (FCS) at the S1/S2 junction of the Spike Protein.

Furin cleavage sites (FCS) have been widely understood to be important for many viral infections, including HIV, influenza, and others. It has also been widely understood before now that lineage B coronaviruses do not have FCS.

It was therefore surprising when an examination of SARS-CoV-2 Spike Protein found an insertion of a 12-nt, 4-AA sequence near the junction of the S1/S2 subunits which creates a furin site that is essential to human infectivity and transmission. As expected from previous work, no lineage B (sarbecovirus) coronavirus has this feature. This is the most difficult "molecular fingerprint" of SARS-CoV-2 to explain having been acquired in the wild and for that reason there are no even passingly feasible theories.

One database of whole genome sequences of 386 coronaviruses was devoid of furin cleavage sites.[71] Another database of 2956 genomes of sarbecovirus strains sequences shows that none have a furin site.[72] This is a highly significant finding with a probability that sarbecovirus has a furin site in the wild of one in about 985.[73]

It has been known since 1994 that viral glycoproteins can be cleaved by secreted proteases, including furin.[74] Even before that, in 1992, it was known the peptide sequence R-X-K/R-R in surface glycoproteins was required for avian influenza viruses of Serotype H7 pathogenesis.[75] The first paper using furin inhibitors to define a role for an FCS in coronavirus-cell fusion was published in 2004.[76]

Since that time, it has become common practice to insert FCS during laboratory gain-of-function experiments to increase infectivity. The following Text-Table illustrates the scope of just a few of the experiments conducted, with the hyperlink to the paper in column one.

URL for Paper	Title of Paper
One	Characterization of a panel of insertion mutants in human cytomegalovirus glycoprotein B.
Two	Insertion of the two cleavage sites of the respiratory syncytial virus fusion protein in Sendai virus fusion protein leads to enhanced cell-cell fusion and a decreased dependency on the HN attachment protein for activity.

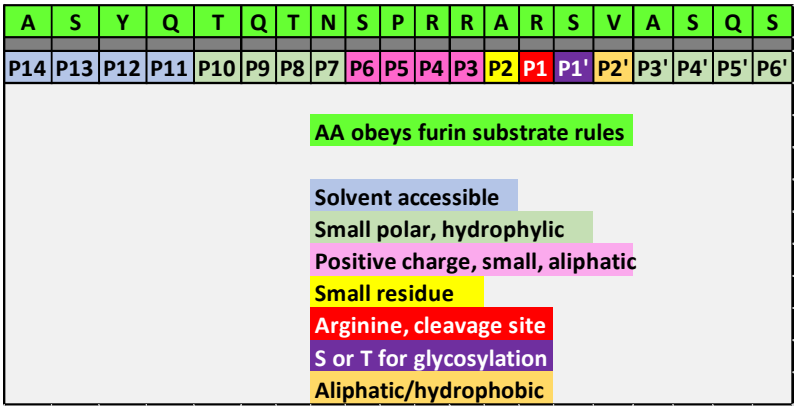
[71] https://academic.oup.com/bioinformatics/article/36/11/3552/5766118
[72] https://academic.oup.com/database/advance-article/doi/10.1093/database/baaa070/5909701
[73] When a series of samples are taken and none produce the result expected, the probability that this is a false negative finding can be estimated by taking the number of samples and dividing by three.Here, 2956 sarbecoviruses without a single furin site is a probability of one in 2956/3 or 985.
[74] https://www.ncbi.nlm.nih.gov/pubmed/8162439
[75] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7172898/pdf/main.pdf
[76] https://www.ncbi.nlm.nih.gov/pubmed/15141003

Three	Recombinant Sendai viruses expressing fusion proteins with two furin cleavage sites mimic the syncytial and receptor-independent infection properties of respiratory syncytial virus.
Four	Amino acid substitutions and an insertion in the spike glycoprotein extend the host range of the murine coronavirus MHV-A59
Five	Induction of IL-8 release in lung cells via activator protein-1 by recombinant baculovirus displaying severe acute respiratory syndrome-coronavirus spike proteins: identification of two functional regions.
Six	Coronaviruses as vectors: stability of foreign gene expression.
Seven	Experimental infection of a US spike-insertion deletion porcine epidemic diarrhea virus in conventional nursing piglets and cross-protection to the original US PEDV infection.
Eight	Minimum Determinants of Transmissible Gastroenteritis Virus Enteric Tropism Are Located in the N-Terminus of Spike Protein.
Nine	Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus.
Ten	Construction of a non-infectious SARS coronavirus replicon for application in drug screening and analysis of viral protein function
Eleven	A severe acute respiratory syndrome coronavirus that lacks the E gene is attenuated in vitro and in vivo.

The creation in the wild of a coronavirus FCS that is used as an example of what might have happened in SARS-CoV-2 is uninformative. In this case, a strain of influenza, in which a new polybasic site appears spontaneously leads to increased infectivity and lethality,[77] was reported by Tse *et al*. 2014. The mechanism of the FCS acquisition in this paper is an RNA polymerase dependent stuttering at a small, constrained loop in which one or more A nt were inserted, removing the strain in the loop and inserting an AAA codon which represents the basic amino acid lysine. No such method exists for the insertion of arginine, the amino acid in the CoV-2 furin site that needs to be created.

The insert generates a canonical 20 AA furin site sequence. In 2011 Tian et al.[78] published an analysis of 126 furin cleavage sites from three species: mammals, bacteria and viruses. The analysis showed that when the furin sites are recorded as a 20-residue motif, a canonical structure emerges. It includes one core cationic region (eight amino acids, P6–P2′) and two flanking solvent accessible regions (eight amino acids, P7–P14, and four amino acids, P3′–P6′).

[77] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3911587/
[78] https://www.nature.com/articles/srep00261



This figure above shows the 20-AA of the furin motif in SARS-CoV-2 (in green) with the P14 to P6' AA positions marked with the cleavage site being the amide bond between P1-R and the P1' residue. The motif is color coded with the requirements (in most cases, except for the positively charged AA requirements, most position requirements can be relaxed).

With the insertion, all 20 residues obey the rules as established by Tian. Since there are 20^4 different 4-AA peptides or 160,000 choices, it is remarkable that the 4 AA insert created a sequence that contained a small or cationic AA (8 AA/20 qualify), a cationic AA (3/20), another cationic AA (3/20), and a small AA (5/20) in that order. In fact, there are only 360 or the total or about 0.2% of all four amino acid inserts that would be expected to follow the exact rules for furin substrates. Of course, given the increase in infectivity SARS-CoV-2 has over other coronaviruses that do not have a well-designed furin cleavage site, selection pressure would drive this rare mutational event once it happened randomly. It would also be a likely choice for a laboratory designed furin cleavage site created *de novo*.

Based on the evidence that there are no furin cleavage sites in 2956 sarbecovirus (beta coronavirus) genome sequences[79], the likelihood that CoV-2 acquired the furin site from a wild sarbecovirus is one in 985 or 0.001. Because this is highly significant, we will use the conservative rule established in the beginning and use a likelihood of 0.05 for this evidence.

Subjective Discount Factor. 95% confidence (only a one in 20 chance this is wrong). Below is the calculation of the Bayesian adjustment.

Evidence or process	Zoonotic Origin (ZO)	Laboratory Origin
Starting likelihood	0.308	0.692
Negative predictive value of a lack of furin sites in sarbecovirus genomes	0.95	
Reduced by 95% Subjective Discount Factor	0.95 x 0.95 = 0.90	
Impact of this evidence	Reduces the likelihood of ZO by 90/10 or 9-fold. For every 100 tests, a true ZO would be seen 10 times and a non-ZO would be seen 90 times	
Impact of evidence calculation	0.308/9 = 0.034	
Normalize this step of analysis	0.034/(0.034 + 0.692) = 0.047	0.692/(0.692 + 0.034) = 0.953

Adjusted likelihood. Zoonotic origin (4.7%), laboratory origin (95.3%).

[79] https://academic.oup.com/database/advance-article/doi/10.1093/database/baaa070/5909701

Evidence: Codon usage can distinguish insertion events in the wild from those created in the laboratory.

Not only is the insertion of an FCS peptide unique among lineage B coronaviruses, the nt sequence used for the process is more broadly unique among coronaviruses in general, regardless of lineage:

-CCT-**CGG-CGG**-GCA-

I will now use synonymous codon bias methods to try to inform the question of the origin of SARS-CoV-2.

Because of the redundancy of the genetic code, more than one 3-nt sequence specifies any given amino acid. For example, there are six codons that specify arginine, R. The frequencies with which such synonymous codons are used are unequal and have coevolved with the cell's translation machinery to avoid excessive use of suboptimal codons that often correspond to rare or otherwise disadvantaged tRNAs. This results in a phenomenon termed "synonymous codon bias," which varies greatly between evolutionarily distant species and possibly even between different tissues in the same species.

Decades of research has identified that all life forms, viruses, bacteria, and humans alike, use the codons in a signature pattern of frequency which can be used to identify a particular sequence of RNA or DNA as human or non-human; viral or non-viral.

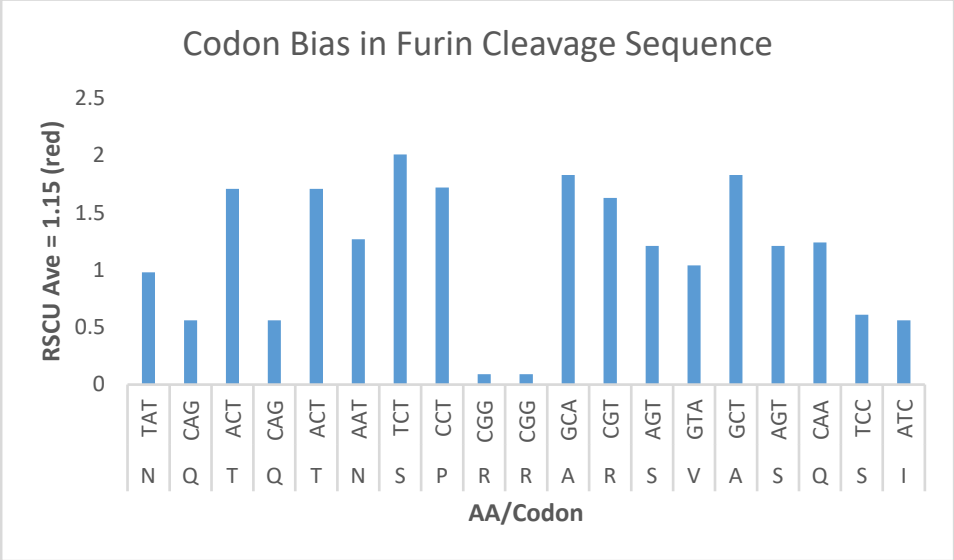
In this way, viruses in nature and scientists in the laboratory, with different goals and motivations, make distinguishing codon usage decisions which can sometimes provide a fingerprint of their source.

The Text-Table below contains the arginine codon usage for two populations, pooled data for SARS-CoV 2003 and related viruses and 13 Sars-CoV-2 human specimens from widely dispersed locations.

Codon	SARS-CoV 2003 and ten other evolutionary related viruses in the Nidovirales	SARS-CoV-2 from 13 Geo-locations
CGG	0.09	0.09
CGA	0.44	0.37
CGC	0.72	0.37
AGG	0.9	1.07
CGU	1.77	1.63
AGA	2.08	2.48

Since these values are of a type of multiplicative scale, they were fit using a log-normal distribution, which appears appropriate (although the sample size is small). Using the log mean and standard deviation and this distribution, the probability of finding a CGG codon is about 0.024. Assuming they are independent the probability of finding a CCG-CCG codon pair is effectively 0.024^2 or 0.00058. This is a likelihood of about one in 1700.

The following Figure shows the RSCU for the amino acids that comprise the new furin cleavage site in SARS-CoV-2. As one can see, the RSCU values are similar to each other with the exception of the RR dimer insert, which have a very low RSCU of 0.09.



The RSCU value for the CGG codon for R of 0.09 was taken from a 2004 paper of the RSCU for SARS-CoV 2003 and ten other evolutionary related viruses in the *Nidovirales* and is confirmed by 13 SARS-CoV-2 specimens obtained from diverse geographic locations. If one assumes that the RSCU observations are independent and that the probability distribution of these measurements is Gaussian (normal; a reasonable assumption), then one can calculate the probability of obtaining a result as small as 0.09. Removing the two 0.09 values, then the mean and standard deviation of the remaining values are 1.275 and 0.4992, respectively. Then the probability of a single 0.09 value is 0.0088. However, there are two 0.09 values. If we assume that these are independent findings, then the probability of both values being seen is 0.0088^2 or 7.7×10^{-5}. Using the RSCU of 0.2 from the Table above does not change the immense improbability of the usage of a CGGCGG codon pair in the wild.

Single Arginine CGG codon usage analysis suggests this will not be found in the wild.

The codon usage for SARS-CoV-2, like most coronaviruses studied, has a bias toward AT and away from GC nucleotides. The frequency of third position G use in CoV-2, for example, is 13%, 21%, 17%, and 16% for the spike protein, envelope, membrane, and nucleocapsid protein, respectively.

In that context, the scarcity of the CGG genome in SARS-CoV-2 and related coronaviruses, the relative synonymous codon usage, determined by the method of Behura and Severson,[80] was calculated and tabulated below. The color coding is blue for underutilized codons (RSCU < 1.0) and red for overutilized codons (RSCU > 1.0); light blue for RSCU values of 0.60 to 0.99 and

[80] https://www.ncbi.nlm.nih.gov/pubmed/22889422

light red for RSCU of 1.01 to 1.60. The highest RSCU usage of CGG is 1.21 in the membrane protein in the MERS virus but zero in SARS-CoV-2.

RSCU	SARS-CoV-2	Beta CoV Pangolin	SARS CoV	Bat SARS CoV	MERS CoV
Spike	0.29	0	0.19	0.08	0.25
Envelope	0	0	0	0	0
Membrane	0	0.35	0.74	0.24	1.21
Nucleocapsid	0.41	0.16	0.03	0.04	0.8

Looking at these five coronaviruses:

The largest structural protein of the coronaviruses is the spike protein, with 1273 amino acids. In SARS-CoV-2 there are 42 R residues, with only one RR dimer, the one in the insert that created SARS-CoV-2.

As a reminder none of these related coronaviruses have the 12-nucleotide insertion that forms the putative furin site in CoV-2. Interestingly, the pangolin coronavirus has no CGG residues in the spike protein. The significance of this is it makes the acquisition of this insert from pangolin by recombination impossible.

The smallest structural protein, the envelope protein, has 75 amino acids, including three R residues, but has no CGG codons in any of the related coronaviruses examined.

The SARS-CoV-2 membrane protein has 441 amino acids, 14 R residues and no CGG codons. Among related coronaviruses, this is the most unique finding of the four proteins for SARS-CoV-2 since the other four coronaviruses all utilize CGG to some extent in this protein. In the case of the MERS virus, this protein is the only occurrence in which this codon is overutilized.

The nucleocapsid protein has 418 amino acids and is responsible for packing the RNA genome. As expected for the role of R in protein-RNA interactions, it has 29 R residues and four RR dimers. None of the dimers use the CGGCGG sequence.

The nt usage of the 12-nt insert which forms the FCS cleavage site has a probability this sequence was selected for in the wild of one in 129,870.

A blast search was performed for the 12-nt inserted sequence and adjacent extensions and only the SARS-CoV-2 sequences were identified.

Shortening the search to just the two CGG-CGG codons was only slightly more fruitful. The Text-Table below shows the frequency of the middle half of the insert, CGGCGG, across the genomes of all seven known human coronaviruses, as well as a specimen bovine coronavirus and the bat and pangolin coronaviruses with greatest homology to SARS-CoV-2. Only a single example, outside of the Spike Protein gene, has been found.

Furin PBCS sequence	Beta Coronavirus	Total Arginine Dimers Anywhere	CGGCGG in Spike Protein *	CGGCGG Anywhere in genome *	CCGCCG Anywhere in genome
S**RR**K**RR**S	Human CoV-HKU1 GenBank: KF686346.1	12	0	0	0
K**RR**S**RR**A	Bovine CoV-Quebec GenBank: AF220295.1	12	0	0	0
PRRARSV	**SARS-CoV-2 Wuhan reference sequence GenBank: NC_045512.2**	**16**	**1; nt 23,606**	0	0
P**R**SV**R**S	MERS-CoV NCBI Reference Sequence: NC_019843.3	21	0	0	0
N**RR**S**R**GA	Human CoV-OC43 London/2011 GenBank: KU131570.1	16	0	0	0
None	Human CoV-229E GeneBank: KF514433.1	15	0	0	0
None	Human CoV NL63 NCBI Reference Sequence: NC_005831.2	9	0	0	0
None	SARS-CoV 2003 ZJ0301 from China GenBank: DQ182595.1	17	0	0	0
None	Bat coronavirus RaTG13 GeneBank: MN996532.1	11	0	1; nt 9394	0
None	Pangolin PCoV_GX-P4L GenBank: MT040333.1	10	0	0	0
	Total	139	1	0	0
* - Includes both in phase codons as well as out of phase, frameshift codons.					

To understand what this means for the search for the zoonotic source for SARS-CoV-2, a statistical approach was taken. Using the data from the nine viruses other than SARS-COV-2 there was a single incidence of the CGGCGG found in the bat coronavirus. Assuming 10,000 codons per genome, the frequency of CGGCGG in coronaviruses can be estimated at 2 per 45,000 codons or 4×10^{-5}. Therefore, the frequency of finding the center half of the SARS-CoV-2 insert is very small. This is consistent with the strong bias in all coronaviruses to place an A/U nt in the third codon position.

The last column above, the presence of -CCG-CCG- in these coronaviruses was included because it is the hybridization sequence partner for the negative strand sequence, which arises during genome replication. This eliminates the possibility of a strand jumping event to generate a CGGCGG codon dimer.

A similar analysis for the spike protein gene can be done. Since there are no instances of CGGCGG in the spike protein genome, and the gene is 3819 nucleotides long, there are 636 pairs of codons Thus, over the 9 other viruses, there are 5724 pairs of codons and no cases of the CGGCGG pair. To calculate the upper bound on the probability of such a pair from these data, one can use the Poisson "Rule of Three", which yields a value of 3/5724 or 0.00052 with 95% confidence. Now examining the SARS-COV-2 genome, there was one instance of the pair in question out of 636 pairs. The probability of this happening if the true rate of this occurrence for a beta coronavirus is 0.00052 is 0.044. Obviously for smaller assumed rates of this occurrence, this would result in probabilities less than 0.044.

Since the 12-nt insert has been found nowhere in the coronavirus genomic universe, examining over 300,000 sequences and using the Poisson "Rule of Three" again, the upper bound on the frequency that it exists in nature is less than one in 100,000 with 95% confidence.

This observation in conjunction with the lack of finding the 12-nt sequence in any candidate zoonotic species makes unlikely a natural source for the virus. One line of investigation to establish a wild source for this infection would be to find a coronavirus strain with the 12-nt sequence somewhere in nature. The fact that 10 of the 12 nts are either G or C coupled, the documented bias against GC suggests this search would be futile.

Based on these analyses that demonstrate that the finding of a -CGG-CGG- codon pair in the furin site of CoV-2 is a highly improbable event, and using the conservative value of a one in 20 chance (the value for a p-value of 0.05), one can recalculate the likelihood of the choice between a zoonotic origin and a laboratory origin.

Subjective Discount Factor. 95% confidence (only a one in 20 chance this is wrong). Below is the calculation of the Bayesian adjustment.

Evidence or process	Zoonotic Origin (ZO)	Laboratory Origin
Starting likelihood	0.047	0.953
Negative predictive value of the absence of the -CGG-CGG- pair in any coronavirus in nature	0.95	
Reduced by 95% Subjective Discount Factor	0.95 x 0.95 = 0.90	
Impact of this evidence	Reduces the likelihood of ZO by 90/10 or 9-fold. For every 100 tests, a true ZO would be seen 10 times and a non-ZO would be seen 90 times	
Impact of evidence calculation	0.047/9 = 0.005	
Normalize this step of analysis	0.005/(0.005 + 0.953) = 0.005	0.953/(0.953 + 0.005) = 0.995

Adjusted likelihood. Zoonotic origin (0.5%), laboratory origin (99.5%).

Evidence. Laboratory codon optimization uses CGG for laboratory insertions of arginine residues 50% of the time.

Codon optimization by recombinant methods (that is, to bring a gene's synonymous codon use into correspondence with the host cell's codon bias) has been widely used to improve cross-species expression of protein.

Though the opposite objective of reducing expression by intentional introduction of suboptimal synonymous codons has not been extensively investigated, isolated reports indicate that replacement of natural codons by rare codons can reduce the level of gene expression in different organisms. For example, one approach to vaccine development is to create an attenuated virus which comprises a modified viral genome containing nucleotide substitutions engineered in multiple locations in the genome, wherein the substitutions introduce synonymous de-optimized codons.

In US Patent 9,476,032[81] titled, "Attenuated viruses useful for vaccines," they state: "In one high-priority redesigned virus, most or all Arg codons are changed to CGC or **CGG** (the top two frequent human codons). This does not negatively affect translation." The patent contains numerous codon usages optimized for vaccine production, including the SARS-CoV virus, and in fact they use the CGG-CGG codon pair 45 times.

Beginning with a paper in 2004,[82] one motivation for codon-optimized SARS genomes is stated here: "The gene encoding the S protein of SARS-CoV contains many codons used infrequently in mammalian genes for efficiently expressed proteins. We therefore generated a codon-optimized form of the S-protein gene and compared its expression with the S-protein gene of the native viral sequence. S protein was readily detected in HEK293T cells transfected with a plasmid encoding the codon-optimized S protein."

Since that time, human optimized codons have been frequently used for coronavirus research, mostly in gain-of-function experiments. In that context the "molecular fingerprint" of CGG for R is one of those common laboratory reagent gene manipulators.

Other examples:

Examples of the use of CGG codon for arginine in coronavirus research	**Reference**
SARS was genetically modified to improve ACE2 binding using "human optimized" codons, like CGG for arginine, to grow better in the laboratory. The strains were more infective.Preparation of SARS-CoV S protein pseudotyped virus. "The full-length cDNA of	Wu, K. et al. Mechanisms of Host Receptor Adaptation by Severe Acute Respiratory Syndrome

[81] http://patft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&d=PALL&p=1&u=%2Fnetahtml%2FPTO%2Fsrchnum.htm&r=1&f=G&l=50&s1=9476032.PN.&OS=PN/9476032&RS=PN/9476032

[82] https://www.ncbi.nlm.nih.gov/pubmed/15367630

the SARS-CoV S gene was optimized according to human codon usage and cloned into the pCDNA3.1(+) vector (Invitrogen). The resulting "humanized" S sequence was identical with that of strain BJ01 at the amino acid level."	Coronavirus. J Biol Chem. 2012 Mar 16; 287(12): 8904–8911.
Predictions of future evolution of a virus are a difficult, if not completely impossible, task. However, our detailed structural analysis of the host receptor adaptation mutations in SARS-CoV RBD has allowed us to predict, design, and test optimized SARS-CoV RBDs that may resemble future evolved forms of the virus. "RBD might evolve into the human-optimized form by acquiring two mutations at the 442 and 472 position." SARS-CoV-2 acquired the mutation at position 472.	Fang Li. Receptor recognition and cross-species infections of SARS coronavirus. Antiviral Res. 2013 Oct; 100(1): 246–254.
Plasmid encoding a codon-optimized form of the SARS-CoV S protein of the TOR2 i	Wenhui Li, Chengsheng Z, et al., Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2. EMBO J. 2005 Apr 20; 24(8): 1634–1643.
The gene encoding the S protein of SARS-CoV contains many codons used infrequently in mammalian genes for efficiently expressed proteins. We therefore generated a codon-optimized form of the S-protein gene and compared its expression with the S-protein gene of the native viral sequence. S protein was readily detected in HEK293T cells transfected with a plasmid encoding the codon-optimized S protein (Fig. (Fig.1).1). No S protein was detected in cells transfected with a plasmid encoding the native S-protein gene.	Moore, MJ, Dorfman, T. Retroviruses Pseudotyped with the Severe Acute Respiratory Syndrome Coronavirus Spike Protein Efficiently Infect Cells Expressing Angiotensin-Converting Enzyme 2. J Virol. 2004 Oct; 78(19): 10628–10635.
Published in 2019 by **Dr. Zhengl-Li Shi,** entitled "Origin and evolution of pathogenic coronaviruses," reviews genetic optimized SARS viruses using human codons.	Cui, J, Fang, L. Origin and evolution of pathogenic coronaviruses. Nat Rev Microbiol. 2019; 17(3): 181–192.
In 2006, Montana scientists put a synthetic furin cleavage site into a SARS coronavirus by adding an R residue at position R667. They write: "We show that furin cleavage at the modified R667 position generates discrete S1 and S2 subunits and potentiates membrane fusion activity." Mutations were introduced by using	Follis, KE, York, J, Nunberg, JH. Furin cleavage of the SARS coronavirus spike glycoprotein enhances cell–cell fusion but does not affect virion entry. Virology 350 (2006) 358–369

QuikChange mutagenesis (Stratagene)[83]	
Identification of murine CD8 T cell epitopes in codon-optimized SARS-associated coronavirus spike protein is the title of a paper that shows that the expression of spike protein in vitro was greatly increased by expression cassette optimization.	Zhia, Y, Kobinger, GP, Jordan, H, et al. Identification of murine CD8 T cell epitopes in codon-optimized SARS-associated coronavirus spike protein
As for the human clec4C_1 and mouse clec14A, they showed very similar profiles with spike genes, especially with bat SARS-CoV, in the arginine coding groups, showing the high RSCU values over 2.50 in AGA.	Ahn,I, Jeong, B-J, Son, HS. Comparative study of synonymous codon usage variations between the nucleocapsid and spike genes of coronavirus, and C-type lectin domain genes of human and mouse. Experimental & Molecular Medicine volume 41, pages746–756, 2009.

One relevant paper,[84] in which arginine residues were being inserted into bovine herpesvirus-1, used primers to create RR dimers with nine separate -CGG-CGG- codon pairs. as testament to their broad use in the Wuhan Institute of Virology laboratory.

Scientists from the Wuhan Institute of Virology provided the scientific community with a technical bulletin on how to make genetic inserts in coronaviruses and proposed using the very tool that would insert this CGGCGG codon.

A Technical Appendix[85] entitled, "Detailed methods and primer sequences used in a study of genetically diverse filoviruses in Rousettus and Eonycteris spp. bats, China, 2009 and 2015, by Yang, Xinglou & Zhang, Yunzhi & Jiang, Ren-Di & Guo, Hua & Zhang, Wei & Li, Bei & Wang, Ning & Wang, Li & Rumberia, Cecilia & Zhou, Ji-Hua & Li, Shi-Yue & **Daszak, Peter** & Wang, Lin-Fa & **Shi, Zheng-Li.** (2017), from the Wuhan Institute of Virology identifies primer sequences for doing genetic experiments in coronaviruses and identifies CGG containing primers when a R amino acid is being inserted.

[83] Since the codon usage here was not reported I contacted Professor Nunberg to inquire which arginine codons were used. He replied: "Unfortunately, those files have all been archived and access to the nt sequences would involve considerable digging. If it is useful to you, I typically choose codons that are more frequent in highly expressed human proteins."
[84] From the Wuhan Institute of Virology; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7125963/
[85] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5382765/

Given that there are two codons of six possibilities that are used in codon optimization, CGG and CGC, the finding of a CGG pair would have a likelihood of happening by chance of (2/6) times (2/6) or one in nine.

Subjective Discount Factor: 80% (this has a probability of being wrong one in five times). This is arbitrary. The calculation to make this adjustment in likelihood is shown here:

Evidence or process	Zoonotic Origin (ZO)	Laboratory Origin (LO)
Starting likelihood	0.005	0.995
This is the outcome expected 8 of 9 times if this is codon optimization		0.88
Reduced by 80% confidence		0.88 x 0.8 = 0.704
Impact of this evidence		Increases the likelihood of LO by 70.4 divided by 29.6 or 2.378.
Impact of evidence calculation		0.995 x 2.378 = 2.37
Normalize this step of analysis	0.005/(2.37 + 0.005) = 0.002	2.37/(0.005 + 2.37) = 0.998

Adjusted likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

Evidence: SARS-CoV-2 Spike Protein is Highly Optimized for ACE2 Binding and Human Cell Infectivity, a Finding that is Inconsistent with Natural Selection but is Consistent with Laboratory Creation

Summary:

- Andersen et al.[86] hypothesized that if the CoV-2 interaction with the human ACE2 was apparently "not ideal," it was evidence that CoV-2 arose by natural selection.

- The alternative hypothesis would be that a finding that CoV-2 was optimized for ACE2 binding and human infection from the initial infection would be evidence of laboratory creation.

- Andersen relied on a paper for the "not ideal" interaction that relied on a computer algorithm rather than laboratory data, was qualitative in nature, sampled only five amino acids or 0.45% of the interaction region, and was over-interpreted.

- The analysis of the Baric et al. paper cited by Andersen as evidence the interaction was not ideal was reexamined, and it was concluded that Andersen had over-interpreted the paper. The paper was a computer simulation study of only 5 of 201 amino acids in the CoV-2-ACE2 interaction region. Only one of the five amino acids discussed was said to be inferior to the equivalent amino acid in SARS-CoV-1; the remainder were either positive or neutral with respect to binding.

- More recently, Baric has clarified his thoughts concerning the CoV-2 ACE2 receptor binding interaction. In a December 31, 2020 *New England Journal of Medicine* paper[57] he wrote: "Early zoonotic variants in the novel coronavirus SARS-CoV that emerged in 2003 affected the receptor-binding domain (RBD) of the spike protein and thereby enhanced virus docking and entry through the human angiotensin-converting–enzyme 2 (hACE2) receptor. **In contrast, the spike-protein RBD of early SARS-CoV-2 strains was shown to interact efficiently with hACE2 receptors early on**." [emphasis added.]

- A comprehensive, laboratory-based, and quantitative paper by Starr et al. of all 201 amino acids in the receptor binding region, not just five amino acids, was examined. Fully 99.6% of all of the possible 3819[87] amino acid substitutions were tested for their effect on CoV-2 binding to ACE2. Only 21 substitutions of the 3819 improved ACE2 binding. Therefore, CoV-2 has been optimized for human ACE2 binding in 99.45% of the possible amino acids in its Spike Protein interaction region.

[86] https://www.nature.com/articles/s41591-020-0820-9

[87] There are 201 amino acids in the residue 331 to 531 interaction region and so 201 times the 19 possible alternative amino acids not found in CoV-2 equals 3819.

- To support this finding, Starr also made an examination of 31,570 CoV-2 sequences from human infections, looking for the 21 substitutions that had been shown to improve CoV-2 binding in the above in vitro laboratory experiments. Among the 31, 570 CoV-2 cases, they failed to find even a single case in which there was an amino acid substitution that improved binding at the time of writing this analysis.[88]

- Based on Andersen's hypothesis and its alternative, SARS-CoV-2 is fully optimized for interaction with the human ACE2 receptor and was at the time of the first patient. There is no evidence of an evolving SP binding region, as was seen with SARS-CoV-1. This is consistent with a laboratory optimized coronavirus which entered the human population fully evolved.

Analysis

Quote from Andersen: "While the analyses above suggest that SARS-CoV-2 may bind human ACE2 with high affinity, computational analyses predict that the interaction is not ideal (reference 7) and that the RBD sequence is different from those shown in SARS-CoV to be optimal for receptor binding (references 7,11).

Thus, the high-affinity binding of the SARS-CoV-2 spike protein to human ACE2 is most likely the result of natural selection on a human or human-like ACE2 that permits another optimal binding solution to arise. This is strong evidence that SARS-CoV-2 is not the product of purposeful manipulation."

The apparent **hypothesis** for the above conclusion is:

"If the SARS-CoV-2 (CoV-2) Spike Protein interaction with the ACE2 receptor is not maximized, then it is evidence that the interaction is the product of natural selection and not purposeful (laboratory) manipulation."

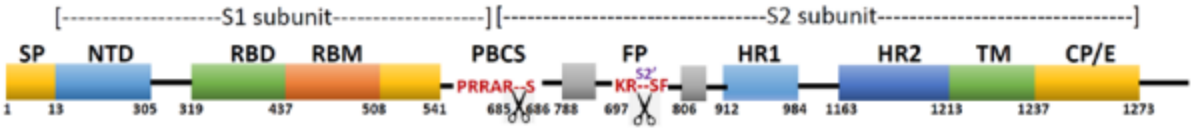
This would lead to an **alternative hypothesis**:

"If the CoV-2 Spike Protein interaction with the ACE2 receptor is maximized, then it is evidence that the interaction *was* the product of purposeful (laboratory) manipulation."

Background.

The Spike Protein (SP) structure and its functional domains are shown in this Figure. The S1 subunit is the initial host interaction portion while the S2 is the post-binding portion responsible for initiating host cell entry, with HR1, HR2, and TM being responsible for breaching the host cell membrane. Allowing viral RNA to enter the cell.

[88] The recent finding of the N501Y variant, first in the UK, and now spreading globally, is evidence of the power of this analysis. N501Y is one of only five potential substitutions in the Starr analysis that had a major effect in improving ACE2 binding.



The interaction of the SP portions which interact with the ACE2 of the host cell, which begins the internalization, infectious process, are contained in the Receptor Binding Domain (RBD) and to a lesser extent the Receptor Binding Motif (RBM), specifically residues 331 to 531. Herein, residues 331 to 531 are called the "interaction region."

Evidence given by Andersen:

Reference 7 in the Andersen paper above is a Ralph Baric paper[89] from early in the pandemic (submitted January 22, 2020) and examines five key residues in the receptor binding domain of the Spike Protein (SP) and whether they are "ideal" for interacting with the ACE2 of human cells. The entire paper is based on computer calculations or prior laboratory work but importantly does not do any new "wet" lab work with CoV-2.

Baric et al. had previously identified five amino acid residues that are important for SP-ACE2 interaction. Using the amino acid numbers of CoV-2, these amino acids are: 455, 486, 493, 494, and 501. Baric opines that the most critical residues are 493 and 501 and the next most important residues are 455, 486, and 494. The authors then discuss each amino acid in turn:

Residue 493: "Gln493 in 2019-nCoV RBD is compatible with hot spot 31, suggesting that 2019-nCoV is capable of recognizing human ACE2 and infecting human cells." In this analysis, 4 of the 20 amino acids are probed.

Residue 501: "This analysis suggests that 2019-nCoV recognizes human ACE2 less efficiently than human SARS-CoV (year 2002) but more efficiently than human SARS-CoV (year 2003). Hence, at least when considering the ACE2-RBD interactions, 2019-nCoV has gained some capability to transmit from human to human."

Direct binding evidence has shown that this statement is misleading, and CoV-2 binds the ACE2 receptor about ten-times better than SARS-CoV (year 2002).[90] In this analysis 3 of the 20 amino acids are probed.

Residues 455, 486, and 494: First, Baric et al. state: "Leu455 of 2019-nCoV RBD provides favorable interactions with hot spot 31, hence enhancing viral binding to human ACE2."

Next, they state: "Phe486 of 2019-nCoV RBD provides even more support for hot spot 31, hence also enhancing viral binding to human ACE2." Importantly, they also talk about their own laboratory work on an "optimized" receptor binding domain and state: "Leu472 of human and

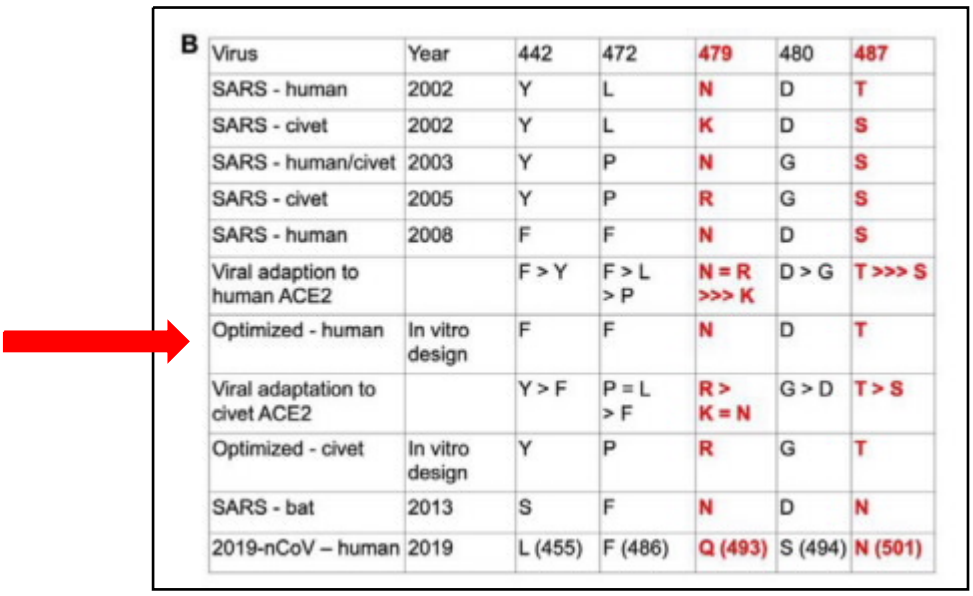
[89] https://jvi.asm.org/content/94/7/e00127-20
[90] https://www.cell.com/action/showPdf?pii=S0092-8674%2820%2931003-5 ; https://www.nature.com/articles/s41586-020-2179-y ; https://www.sciencedirect.com/science/article/pii/S0092867420302622 ; https://science.sciencemag.org/content/367/6483/1260

civet SARS-CoV RBDs provides favorable support for hot spot 31 on human ACE2 through hydrophobic interactions with ACE2 residue Met82 and several other hydrophobic residues **(this residue has been mutated to Phe472 in the optimized RBD)**." [emphasis added.]

Finally, they state: Ser494 in 2019-nCoV RBD still provides positive support for hot spot 353, but the support is not as favorable as that provided by Asp480. Overall, Leu455, Phe486, and Ser494 of 2019-nCoV RBD support the idea that 2019-nCoV recognizes human ACE2 and infects human cells."

In this analysis they probe 3 of 20 amino acid residues for position 480, 4 of 20 for position 486, and 4 of 20 for position 442.

As shown in the Figure below from the Baric paper, the in vitro designed, optimized human SP (red arrow) had the amino acid residues F, F, N, D, and T at these five key residues. Since CoV-2 was identical in only one of these five it was not "optimal" and, according to Andersen, it therefore was not laboratory derived.



B

Virus	Year	442	472	479	480	487
SARS - human	2002	Y	L	N	D	T
SARS - civet	2002	Y	L	K	D	S
SARS - human/civet	2003	Y	P	N	G	S
SARS - civet	2005	Y	P	R	G	S
SARS - human	2008	F	F	N	D	S
Viral adaption to human ACE2		F > Y	F > L > P	N = R >>> K	D > G	T >>> S
Optimized - human	In vitro design	F	F	N	D	T
Viral adaptation to civet ACE2		Y > F	P = L > F	R > K = N	G > D	T > S
Optimized - civet	In vitro design	Y	P	R	G	T
SARS - bat	2013	S	F	N	D	N
2019-nCoV – human	2019	L (455)	F (486)	Q (493)	S (494)	N (501)

Conclusion from the above paper: by examining five amino acid residues of the 200 residues encompassing the interaction region, and calculating the expected interaction of a total of 18 of the 4000 possible residues or 0.45% of all possibilities, they conclude CoV-2 can infect human cells, but is not optimized to do so. This data was twisted by Andersen to show 'strong evidence' of natural selection.

An alternative and comprehensive analysis in another paper:[91]

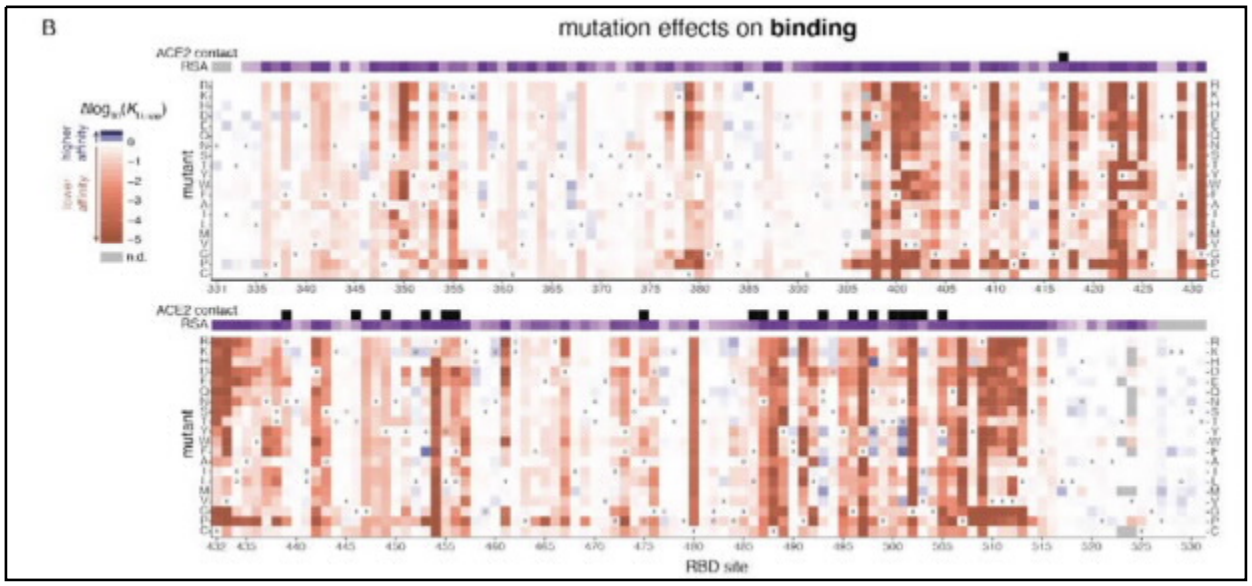
The receptor binding domain (RBD) of the CoV-2 SP is included in residues 331 to 531, a 201 amino acid sequence, of the SP. To examine the effect of each and every amino acid in each and every position, all 19 different amino acids were changed into all 201 positions of the RBD to the extent possible. Out of a total potential of 3819 different single amino acid variants, the scientists

[91] https://www.cell.com/action/showPdf?pii=S0092-8674%2820%2931003-5

were able to create 3804 of the potential variants or 99.6% of the possible variants. It is probable that the variants with the 0.4% amino acid substitutions could not be made for one reason or another. These 3804 were then tested for binding to the human ACE2. Finally, the RBD from SARS-CoV-1 also was tested.

The Figure below is the result of the experiment. Starting with amino acid 331 and ending with amino acid 531, the amino acids that were changed are in vertical columns and are color coded. Shades of brown are amino acid substitutions that reduce ACE2 binding affinity and blue are amino acid substitutions that improve binding, in all cases compared to the 'native' CoV-2 SP sequence. White is the color of a neutral substitution which neither enhances nor diminishes binding. Only the dark blue substitutions provide a strong improvement in ACE2 binding. There is a black square along the top row that denotes amino acids in the SP that interact with the ACE2 protein. Unlike in the Baric analysis above, in which only five amino acids were considered, this group of 19 amino acids provide a more complete interaction picture.

The first overarching observation is that most amino acid substitutions among the 201 amino acids are negative; while a large number are neutral. The fact that the vast majority of amino acid substitutions do not provide an improved ACE2 interaction is clear evidence that the CoV-2 SP interaction region is not newly evolved to the human ACE2 but arrived in the first patient having been "trained" to invade and kill human cells.

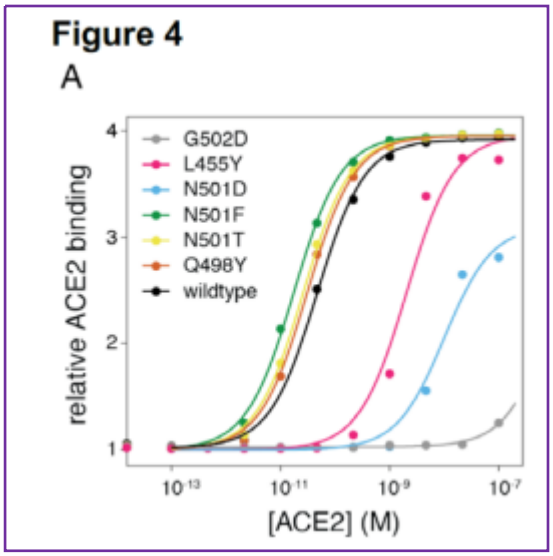


There are three levels of improved binding as designated by dark blue, medium blue, and pale blue. Out of the 3804 variants tested, there are 4 dark blue substitutions or 0.11% and 17 medium blue or 0.45%. According to the paper, the binding effect of the light blue could not be measured as different from the native sequence.

The conclusion of this comprehensive work is the demonstration that for 99.45% of the amino acids in the 201 amino acid interaction region, the CoV-2 choice is optimized, where any substitution is either detrimental or, at best, neutral with respect to the first step of CoV-2 entry to human cells, the binding step to the ACE2 receptor.

How much could CoV-2 binding be improved or made worse by substitutions during the human-to-human transmission of the pandemic?

The Figure 4 below, taken from the paper, shows that the three best amino acid substitutions have only a slight effect on the binding curve (Black is wildtype; curves to the left are better binding; curves to the right are worse binding). This is further evidence that CoV-2 is an optimized form of the original virus.



The authors also concluded that Anderson et al. was wrong: "An initially surprising feature of SARS-CoV-2 was that its RBD tightly binds ACE2 despite differing in sequence from SARS-CoV-1 at many residues that had been defined as important for ACE2 binding by that virus (Andersen et al., 2020; Wan et al., 2020)."

In fact, multiple studies have shown that CoV-2 binds ACE2 better than SARS-CoV-1, contradicting Andersen.

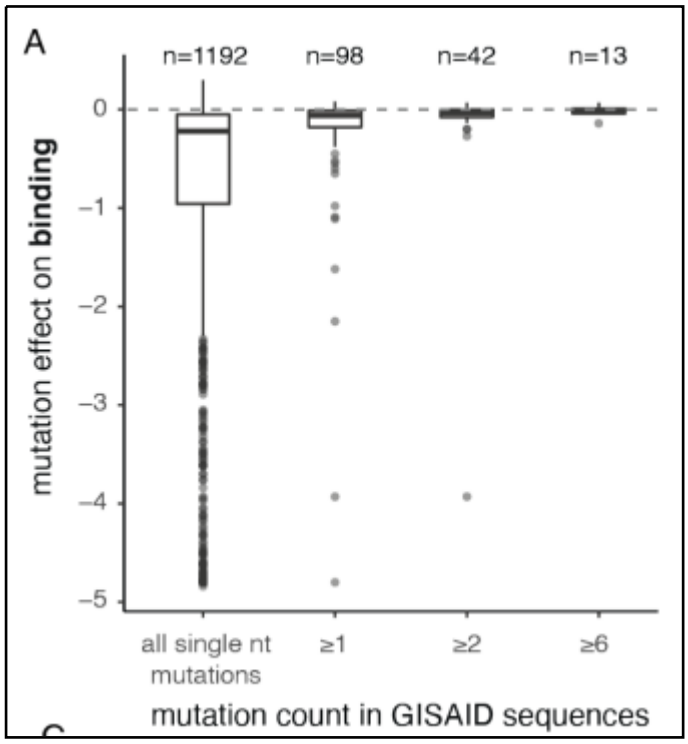
Is there evidence that CoV-2 in human circulation has mutations that enhance ACE2 binding?

Another measure of whether CoV-2 is optimized for human infection is to see if Spike Protein mutations have arisen during the pandemic that improve binding of the virus to the ACE2 receptor or if the SP amino acids are ideal from the very first human patient.

The Starr paper addressed this issue as well. A total of 31,570 human sequences were analyzed to see if any of the 21 amino acid substitutions from the binding experiments (or any other for that matter) were being selected for. That is, if there is any evidence of evolutionary pressure to improve SARS-CoV-2 infectivity.

Below is Figure 8 of the Starr paper. Of the 31,570 sequences, all mutations in the receptor interaction region were analyzed for their effect on ACE2 binding. The data below are for all examples of a single nt mutation (1192), two mutations (98), 3-5 mutations (42), and six or more (13) and the effect the mutation would have on ACE2 binding. The logarithmic scale has the

wildtype CoV-2 as 0 and each negative integer is a 10-fold reduction in affinity. Shockingly, there is not a single mutation that is above the 0 line, which would be an improved affinity for the ACE2 receptor. All of the mutations lower the receptor affinity.



Here are the results, in the words of Starr:

"Our discovery of multiple strong affinity-enhancing mutations to the SARS-CoV-2 RBD raises the question of whether positive selection will favor such mutations, since the relationship between receptor affinity and fitness can be complex for viruses that are well-adapted to their hosts (Callaway et al., 2018; Hensley et al., 2009; Lang et al., 2020). Strong affinity-enhancing mutations are accessible via single-nucleotide mutation from SARS-CoV-2 (Figure S8C), but **none are observed among circulating viral sequences in GISAID** (Figure 8A), and **there is no significant trend for actual observed mutations to enhance ACE2 affinity more than randomly drawn samples of all single nucleotide mutations** (see permutation tests in Figure S8D). **Taken together, we see no clear evidence of selection for stronger ACE2 binding, consistent with SARS-CoV-2 already possessing adequate ACE2 affinity at the beginning of the pandemic."** [emphasis added.]

It is striking that the authors, in observing the complete absence of any evidence for stronger ACE2 binding in over thirty thousand cases, would describe this as evidence of "adequate ACE2 affinity" and not as an exceptional finding of "optimized ACE2 affinity." Of course, calling the SP affinity exceptional from the beginning of the pandemic would beg the question of a laboratory derived virus.

Returning to the initial hypotheses, since the 3804 possible amino acids at the receptor interaction region of CoV-2 are 99.45% optimized for ACE2 binding, and there is not a single

example in 31,570 human CoV-2 genomes of a substitution that enhances ACE2 binding, the CoV-2 interaction with ACE-2 was maximized from the get-go.

Therefore, the hypothesis, "If the SARS-CoV-2 (CoV-2) Spike Protein interaction with the ACE2 receptor is not maximized, then it is evidence that the interaction is the product of natural selection and not purposeful (laboratory) manipulation," is **rejected**.

The alternative hypothesis, "If the CoV-2 Spike Protein interaction with the ACE2 receptor is maximized, then it is evidence that the interaction was the product of purposeful (laboratory) manipulation," is thus **accepted**.

At the time of this writing, a new RBD mutant N501Y has been observed. It is one of the five potential mutations that could be expected to increase RBD-ACE2 affinity.

This is the first example of evidence that will not be statistically quantified but treated as a 51%.49% preponderance of the evidence adjustment. The evidence is more consistent with having been optimized by various methods used in the laboratory than with the slow natural process as seen with SARS-CoV-1, and so the conservative rule that this is consistent with a laboratory origin (51%) versus zoonotic origin (49%) will be used. There will be no confidence adjustment.

The adjusted likelihoods are shown in the following table.

Evidence or process	Zoonotic Origin (ZO)	Laboratory Origin (LO)
Starting likelihood	0.002	0.998
This is the outcome favors LO over ZO at 51% versus 49%		0.51
Impact of this evidence		Increases the likelihood of LO by 51/49 = 1.041
Impact of evidence calculation		1.041 x 0.998 = 1.039
Normalize this step of analysis	0.002/(0.002 + 1.039) = 0.002	1.039/(0.002 + 1.039) = 0.998

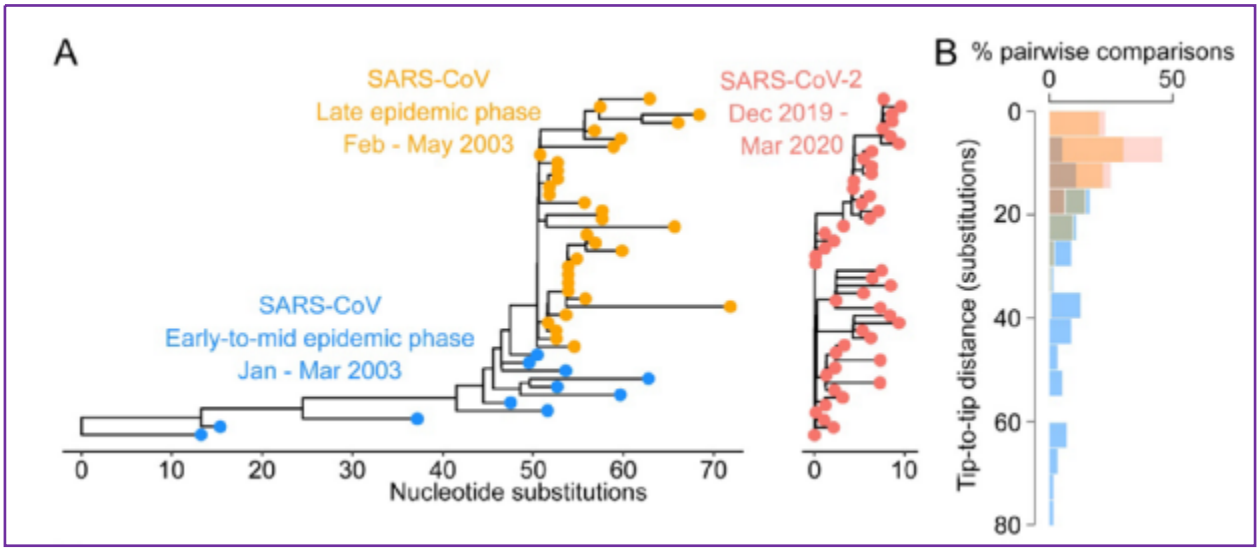
Adjusted likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

Evidence. Whole genome comparison of human adaption of CoV-2 compared to SARS-CoV-1 is consistent with a "pre-adaption" of CoV-2 to the human host

A paper[92] entitled, "SARS-CoV-2 is well adapted for humans. What does this mean for re-emergence?" by Shing Hei Zhan, Benjamin E. Deverman, and Yujia Alina Chan states in the abstract:

"In a side-by-side comparison of evolutionary dynamics between the 2019/2020 SARS-CoV-2 and the 2003 SARS-CoV, we were surprised to find that SARS-CoV-2 resembles SARS-CoV in the late phase of the 2003 epidemic, after SARS-CoV had developed several advantageous adaptations for human transmission. Our observations suggest that **by the time SARS-CoV-2 was first detected in late 2019, it was already pre-adapted to human transmission to an extent similar to late epidemic SARS-CoV**. **However, no precursors or branches of evolution stemming from a less human-adapted SARS-CoV-2-like virus have been detected.** The sudden appearance of a highly infectious SARS-CoV-2 presents a major cause for concern that should motivate stronger international efforts to identify the source and prevent re-emergence in the near future. [Emphasis added.]

The following Figure from the paper best illustrates the relative SNV adaption for SARS-CoV-1 versus CoV-2.



The paper also makes a tangential comment about posterior diversity: "It would be curious if no precursors or branches of SARS-CoV-2 evolution are discovered in humans or animals."

This is another example of evidence that will not be statistically quantified. The evidence is more consistent with having been adapted by various known methods used in a laboratory than with the slow natural process as seen with SARS-CoV-1, and so the conservative rule that this is consistent with a laboratory origin (51%) versus zoonotic origin (49%) will be used. There will be no confidence adjustment.

[92] https://www.biorxiv.org/content/10.1101/2020.05.01.073262v1

The adjusted likelihoods are shown in the following table.

Evidence or process	Zoonotic Origin (ZO)	Laboratory Origin (LO)
Starting likelihood	0.002	0.998
This is the outcome favors LO over ZO at 51% versus 49%		0.51
Impact of this evidence		Increases the likelihood of LO by 51/49 = 1.041
Impact of evidence calculation		1.041 x 0.998 = 1.039
Normalize this step of analysis	0.002/(0.002 + 1.039) = 0.002	1.039/(0.002 + 1.039) = 0.998

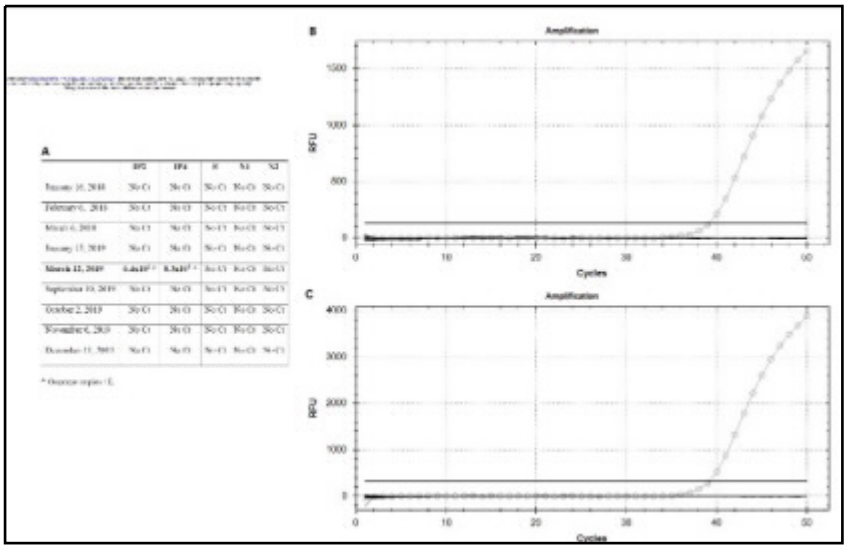
Adjusted likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

Evidence: Evidence of CoV-2 during early 2019 in wastewater from Barcelona, Spain is a false positive artifact

A paper entitled "Sentinel surveillance of SARS-CoV-2 in wastewater anticipates the occurrence of COVID-19 cases"[93] claims CoV-2 was present in Barcelona, Spain in March 2019. Specifically, they state:

"This possibility prompted us to analyze some archival WWTP samples from January 2018 to December 2019 (Figure 2). All samples came out to be negative for the presence of SARS-CoV-2 genomes with the exception of March 12, 2019, in which both IP2 and IP4 target assays were positive. This striking finding indicates circulation of the virus in Barcelona long before the report of any COVID-19 case worldwide."

This is a false positive



As shown above from the paper, they found 43/45 runs with zero and two runs had only 600-800 CoV-2 copies/L

But the limit of detection (LoD) of their assay is 1,000,000 CoV-2/L.

According to the Promega PCR assay FDA clearance package, the Ct at the LoD is 33-34 for the N1 and N2, respectively (Table 17, page 51).[94] Here the LoD is listed as 1 RNA/µL.

In the paper the Ct is 40 or 6-7 above the LoD.

This evidence is neutral as to origin and will not be used to adjust the likelihoods. It does reduce the credibility of some of the new origin theories coming out of China.

[93] https://www.medrxiv.org/content/10.1101/2020.06.13.20129627v1.full.pdf
[94] https://twitter.com/quay_dr/status/1340572543548227585/photo/1

Evidence: WHO and Dr. Shi have spoken of the singular nature of the beginning of COVID-19

On January 23, 2020 Dr. Shi wrote in the draft of her paper: "The almost identical sequences of this virus in different patients imply a probably recent introduction in humans…"[95] By February 3, 2020, when the final version of this paper was published, this sentence had been **deleted**.[96]

On April 23, 2020 the WHO stated: "All the published genetic sequences of SARS-CoV-2 isolated from human cases are very similar. This suggests that the start of the outbreak resulted from a single point introduction in the human population around the time that the virus was first reported in humans in Wuhan, China in December 2019."[97]

The evidence, like the lack of posterior diversity and seroconversion reported earlier, is more consistent with a single introduction in a laboratory accident. This evidence will not be used to adjust probabilities but is included because it could be a form of party admissions of unfavorable facts.

[95] RaTG13 paper as a preprint
[96] RaTG13 final Nature paper
[97] WHO document page 2 of 12

Evidence. As documented by Drs. Daszak, Humes, and Shi, mammalian biodiversity and bat species differences between Yunnan and Hubei Provence are significant and do not support a zoonotic origin

Summary. SARS-CoV-2 is most closely related to bat coronaviruses from Yunnan, a rural province in South West China. Wuhan, where the pandemic began, is a large urban city of 11 million inhabitants in north central China. These two areas are approximately 1900 km apart.

This is the US equivalent of the difference between New York City (population 8.4 million) and the Everglades in Florida, 2000 km away. The incongruent image of a bat or intermediate host in the Everglades somehow finding its way to New York City is a clear demonstration of the difficulty in this hypothetical transmission process. Nonetheless, a strict literature-based analysis will be conducted.

If COVID-19 is a zoonotic disease it must have travelled from bats to humans or from bats to an intermediate species to humans. Therefore, an examination of mammalian biodiversity differences and commonalities between Yunnan and Wuhan might provide useful information about the intermediate host or the particular bat species.

Peter Daszak, Zhengli-li Shi and colleagues published an August 2020 paper entitled, "Origin and cross-species transmission of bat coronaviruses in China,"[98] in which they make a number of observations that are relevant to this analysis. It should be remembered that both lead authors have made multiple, strong, public statements over many months where they assert that SARS-CoV-2 is a natural virus of zoonotic origin.

Yunnan and Hubei Provinces have very dissimilar mammalian diversity

Quoting from the Methods section of the Daszak, Shi paper:

"Defining zoogeographic regions in China:

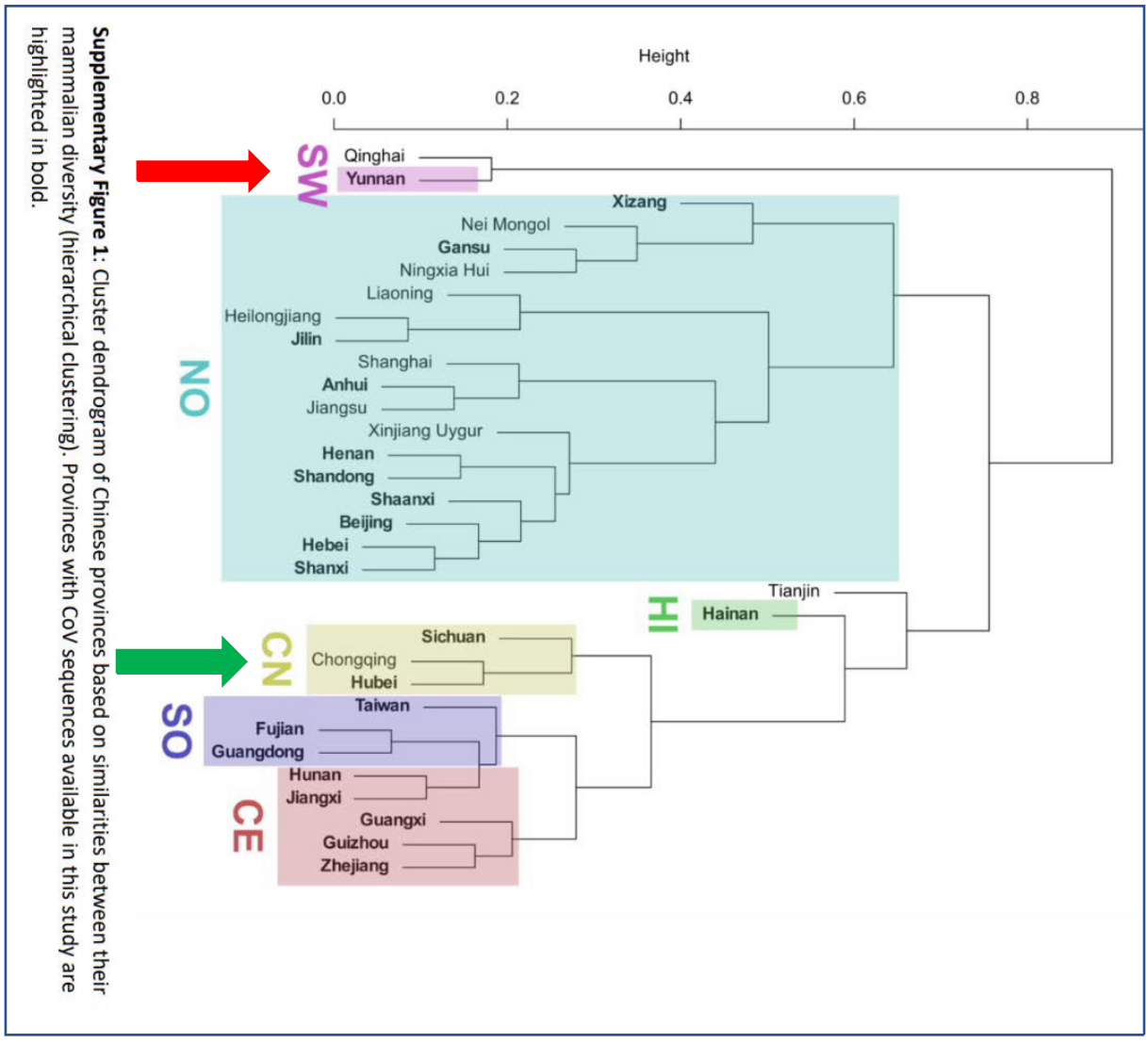
Hierarchical clustering was used to define zoogeographic regions within China by clustering provinces with similar mammalian diversity. Hierarchical cluster analysis classifies several objects into small groups based on similarities between them. To do this, we created a presence/absence matrix of all extant terrestrial mammals present in China using data from the IUCN spatial database and generated a cluster dendrogram using the function *hclust* with average method of the R package stats. Hong Kong and Macau were included within the neighboring Guangdong province. We then visually identified geographically contiguous clusters of provinces for which CoV sequences are available (Fig. 1 and Supplementary Fig. 1).

We identified six zoogeographic regions within China based on the similarity of the mammal community in these provinces: **SW (Yunnan province)**, NO (Xizang, Gansu, Jilin, Anhui, Henan, Shandong, Shaanxi, Hebei, and Shanxi provinces and Beijing municipality), **CN (Sichuan and Hubei provinces)**, CE (Guangxi, Guizhou, Hunan, Jiangxi, and Zhejiang provinces), SO (Guangdong and Fujian provinces, Hong Kong, Macau, and Taiwan), and HI.

[98] https://www.nature.com/articles/s41467-020-17687-3#Sec19

Hunan and Jiangxi, clustering with the SO provinces in our dendrogram, were included within the central region to create a geographically contiguous Central cluster (Supplementary Fig. 1). These six zoogeographic regions are very similar to the biogeographic regions traditionally recognized in China. The three β-CoV sequences from HI were included in the SO region to avoid creating a cluster with a very small number of sequences."

Below is a cluster dendrogram of Chinese provinces based on similarities between their mammalian diversity (hierarchical clustering). Provinces with CoV sequences available in this study are highlighted in bold.



Supplementary Figure 1: Cluster dendrogram of Chinese provinces based on similarities between their mammalian diversity (hierarchical clustering). Provinces with CoV sequences available in this study are highlighted in bold.

The y-axis height is a measure of the biodiversity with 1.0 being complete similarity and 0.0 being no similarity. As expected for the geography and location of the two provinces, Yunnan (red arrow above) and Hubei (green arrow above) have a height score of about 0.1, with seven branches and six nodes separating them. This is close to the biggest different in mammalian biodiversity of any two locations in all of China.

In conclusion, Daszak and Shi et al. demonstrate that the mammalian biodiversity between Yunnan and Hubei is very significant, reducing the options for a common intermediate host to be the natural conduit between bats and humans.

Shi, Humes, and Daszak statement: "SARS-CoV-2 is likely derived from a clade of viruses originating in horseshoe bats (*Rhinolophus* spp.). The geographic location of this origin appears to be Yunnan province."

This evidence will not be statistically quantified. The evidence reduces the biodiversity overlap needed to create a common intermediate species between the two provinces, and so the conservative rule that this is consistent with a laboratory origin (51%) versus zoonotic origin (49%) will be used. There will be no subjective discount factor adjustment.

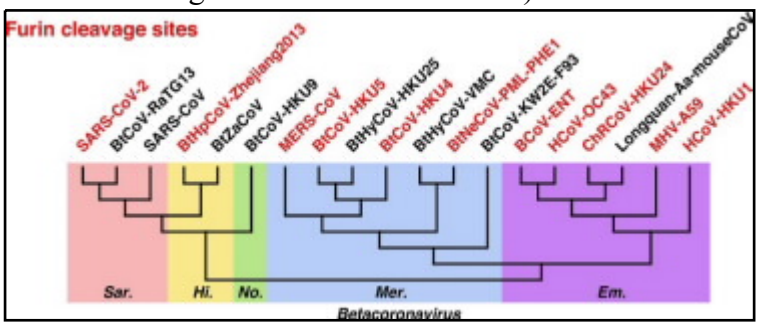
Evidence or process	Zoonotic Origin (ZO)	Laboratory Origin (LO)
Starting likelihood	0.002	0.998
This is the outcome favors LO over ZO at 51% versus 49%		0.51
Impact of this evidence		Increases the likelihood of LO by 51/49 = 1.041
Impact of evidence calculation		1.041 x 0.998 = 1.039
Normalize this step of analysis	0.002/(0.002 + 1.039) = 0.002	1.039/(0.002 + 1.039) = 0.998

Because of the rule on the use of significant figures, the likelihood does not change.

Adjusted likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

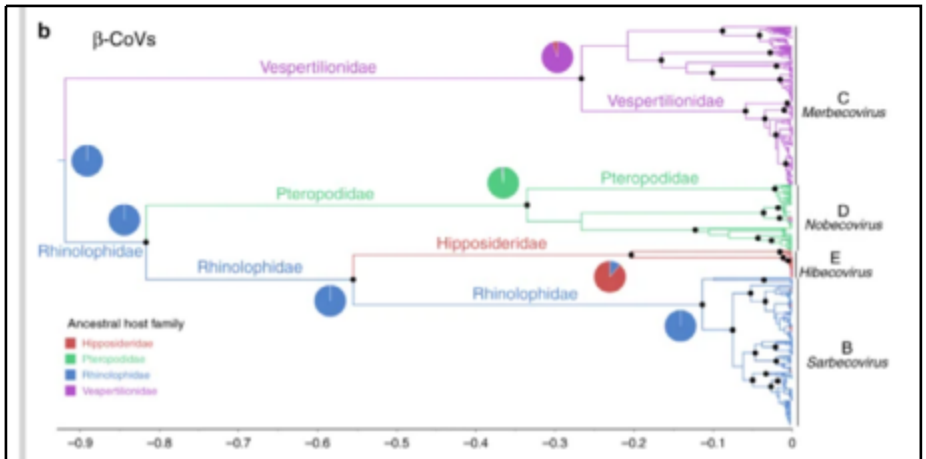
Evidence: The ancestor of SARS-CoV-2 can hypothetically only obtain a furin site by recombination outside of the sarbecovirus subgenera but there is strong evidence that coronavirus recombination is largely limited to the clade level, with limited evidence of sub-genera or genera recombination

- SARS-CoV-2 is a beta coronavirus, subgenera sarbecovirus and is the only sarbecovirus with a furin site.[99]
- Furin sites can be found in either alpha or gamma coronaviruses or the other beta coronavirus subgenera. The following Figure from reference 66 shows examples of such coronaviruses (furin containing viruses are shown in red):



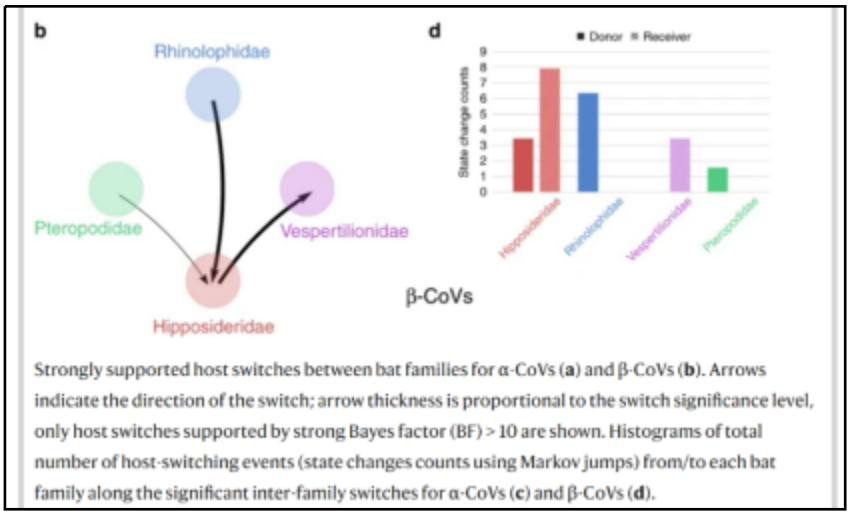
- To acquire a furin site in nature would require a co-infection between the CoV-2 sarbecovirus ancestor and a furin-containing non-sarbecovirus as shown above.
- However, there is no evidence of recombination in coronaviruses at either the genus level or the subgenus level; only at the clade level.[100][101]
- There is also evidence from Daszak and Shi that within the subgenera of the beta coronaviruses, there is bat host specificity. So, each subgenera of coronaviruses has a preferred bat host species. This reduces the opportunities for a co-host event to permit recombination.[102] The phylogeny below shows the problem of host incompatibility for beta coronaviruses:

[99] https://www.sciencedirect.com/science/article/pii/S1873506120304165#f0015
[100] file:///C:/Users/Steven%20Quay/Desktop/journal.pgen.1009272.pdf
[101] https://academic.oup.com/mbe/advance-article/doi/10.1093/molbev/msaa281/5955840
[102] https://www.nature.com/articles/s41467-020-17687-3#Sec2



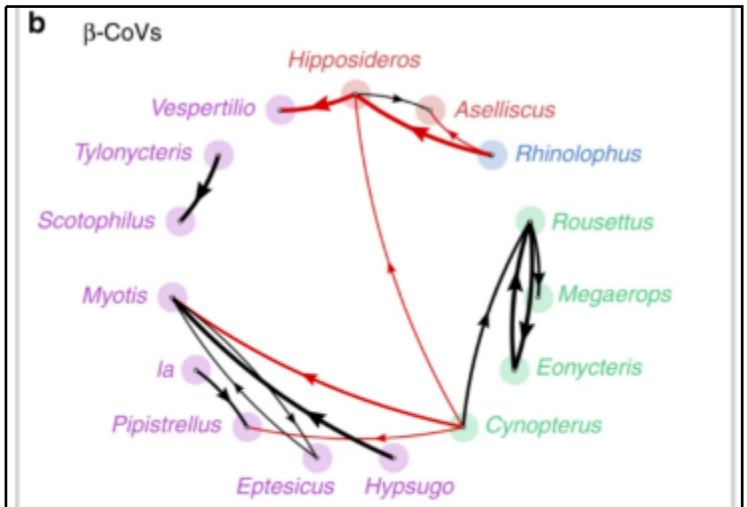
α-CoV (**a**) and β-CoV (**b**) maximum clade credibility annotated trees using complete datasets of *RdRp* sequences and bat host family as discrete character state. Pie charts located at the root and close to the deepest nodes show the state posterior probabilities for each bat family. Branch color correspond to the inferred ancestral family with the highest probability. Branch lengths are scaled according to relative time units (clock rate = 1.0). Well-supported nodes (posterior probability > 0.95) are indicated with a black dot. The ICTV approved CoV subgenera were highlighted: *Rhinacovirus* (L1), *Decacovirus* (L2), *Myotacovirus* (L3), *Pedacovirus* (L5), *Nyctacovirus* (L6), *Minunacovirus* (L7), and an unidentified lineage (L4) for α-CoVs; and *Merbecovirus* (Lineage C), *Nobecovirus* (lineage D), *Hibecovirus* (lineage E), and *Sarbecovirus* (Lineage B) for β-CoVs.

- Daszak and Shi also identified preferred directions of host switching. Since RaTG13, the closest coronavirus to SARS-CoV-2, is most closely related to viruses with bat hosts from the family, Rhinolophidae, it would be reasonable to expect furin-containing viruses from other bat hosts to migrate into Rhinolophidae, recombine by methods which have not been identified, and then the furin-containing sarbecovirus could evolve into the ancestor of SARS-CoV-2. Unexpectedly, Daszak et al. found host migration for the Rhinolophidae bats only outward and not inward, as required by the above, admittedly, convoluted process. The data Figure is shown here:



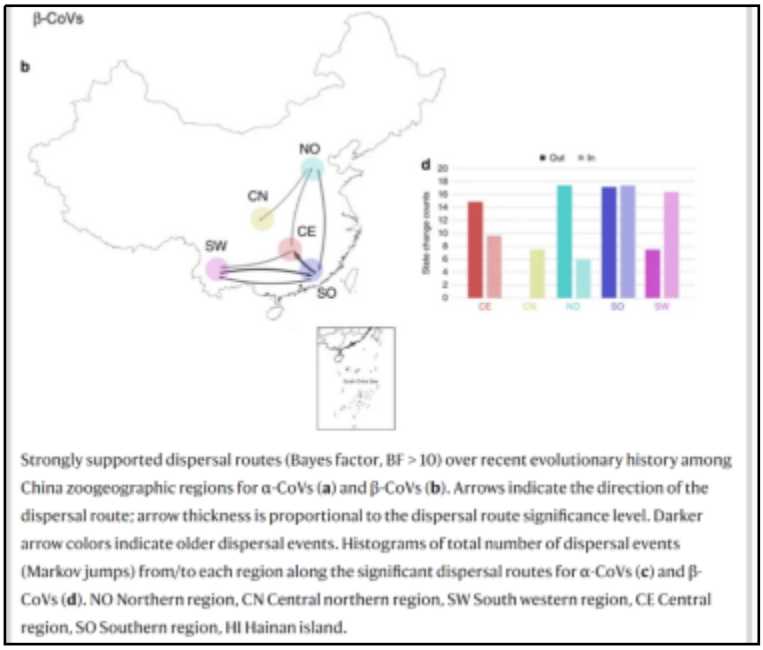
Strongly supported host switches between bat families for α-CoVs (**a**) and β-CoVs (**b**). Arrows indicate the direction of the switch; arrow thickness is proportional to the switch significance level, only host switches supported by strong Bayes factor (BF) > 10 are shown. Histograms of total number of host-switching events (state changes counts using Markov jumps) from/to each bat family along the significant inter-family switches for α-CoVs (**c**) and β-CoVs (**d**).

- Daszak and Shi also observed outward host switches from *Rhinolophus* at the genera level as well, also against a hypothesis for furin-site acquisition:



Strongly supported host switches between bat genera for α-CoVs (**a**) and β-CoVs (**b**) and their significance level (Bayes factor, BF). Only host switches supported by strong BF values >10 are shown. Line thickness is proportional to the switch significance level. Red lines correspond to host switches among bat genera belonging to different families, and black lines correspond to host switches among bat genera from the same family. Arrows indicate the direction of the switch. Genus names are colored according to the family they belong to using the same colors as in Figs. 2 and 3.

- Finally, this paper by Daszak and Shi states: "We used our Bayesian discrete phylogeographic model with zoogeographic regions as character states to reconstruct the spatiotemporal dynamics of CoV dispersal in China." If SARS-CoV-2 began in Yunnan and first crossed over into humans in Wuhan, this analysis should support a northernly spatiotemporal dispersal of beta coronaviruses. Unfortunately, Daszak and Shi cannot catch a break; their own data do not support the expected route of dispersion:



Strongly supported dispersal routes (Bayes factor, BF > 10) over recent evolutionary history among China zoogeographic regions for α-CoVs (**a**) and β-CoVs (**b**). Arrows indicate the direction of the dispersal route; arrow thickness is proportional to the dispersal route significance level. Darker arrow colors indicate older dispersal events. Histograms of total number of dispersal events (Markov jumps) from/to each region along the significant dispersal routes for α-CoVs (**c**) and β-CoVs (**d**). NO Northern region, CN Central northern region, SW South western region, CE Central region, SO Southern region, HI Hainan island.

As shown in the above Figure the only dispersal routes into Wuhan, which is in the CN region, are from the northern region. And the northern region has no inward dispersals from the SW, southwest region, where Yunnan and the origin of the ancestor of SARS-CoV-2, is located.

- Independent evidence documents that Hubei province does not have the bat species needed for SARS-CoV-2 reservoir host[103]

While statistical models of this data could be interesting and informative for general research about future spillovers, this is evidence will not be statistically quantified for this analysis. The evidence reduces the opportunities for subgenera co-infection and furin-site recombination into the CoV-2 ancestor and so the conservative rule that this is less consistent with a zoonotic origin (49%) versus laboratory origin (51%) will be used. There will be no subjective discount factor adjustment.

The results from the calculations are shown below.

Evidence or process	Zoonotic Origin (ZO)	Laboratory Origin (LO)
Starting likelihood	0.002	0.998
This is the outcome favors LO over ZO at 51% versus 49%		0.51
Impact of this evidence		Increases the likelihood of LO by 51/49 = 1.041
Impact of evidence calculation		1.041 x 0.998 = 1.039
Normalize this step of analysis	0.002/(0.002 + 1.039) = 0.002	1.039/(0.002 + 1.039) = 0.998

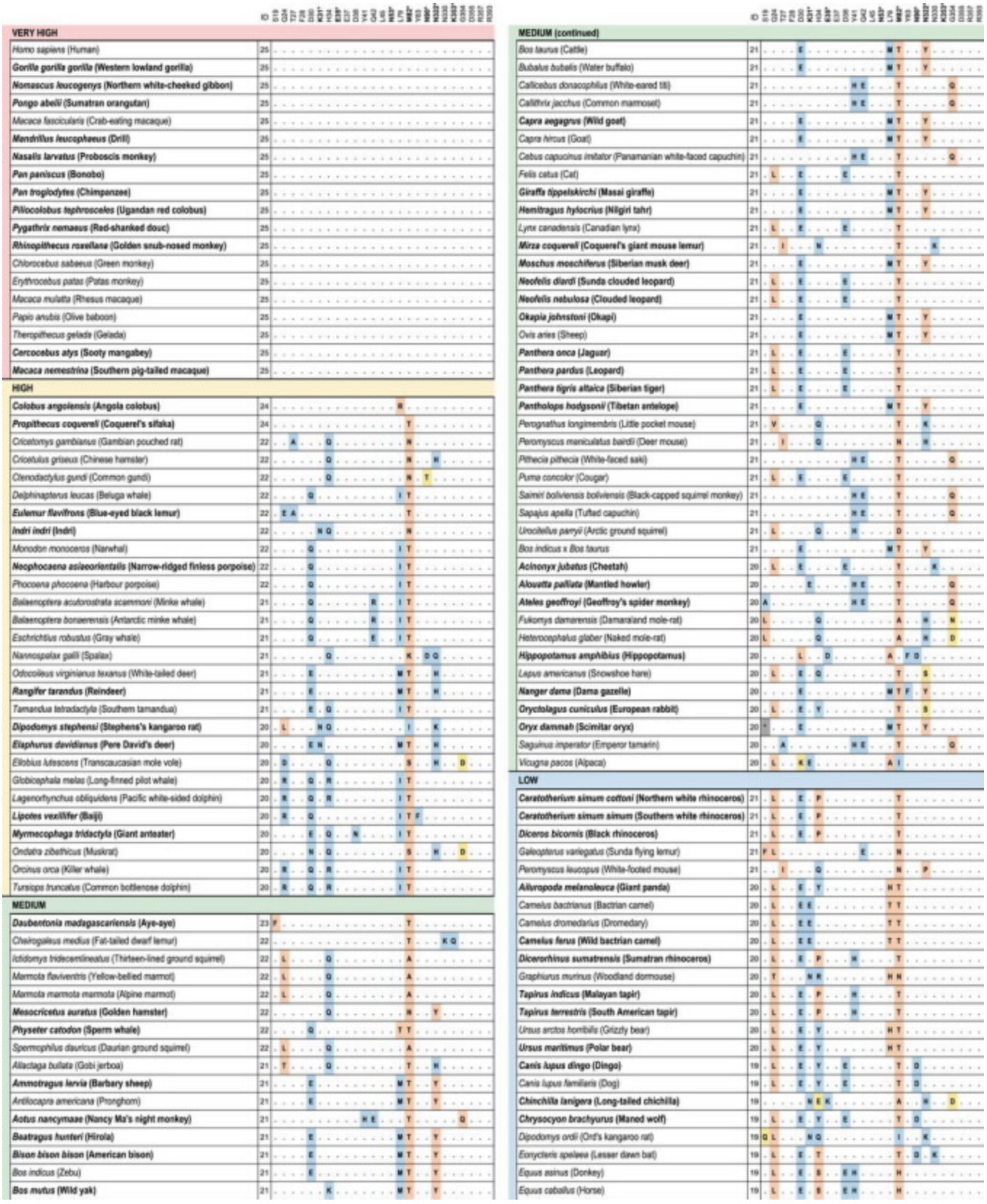
Adjusted likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

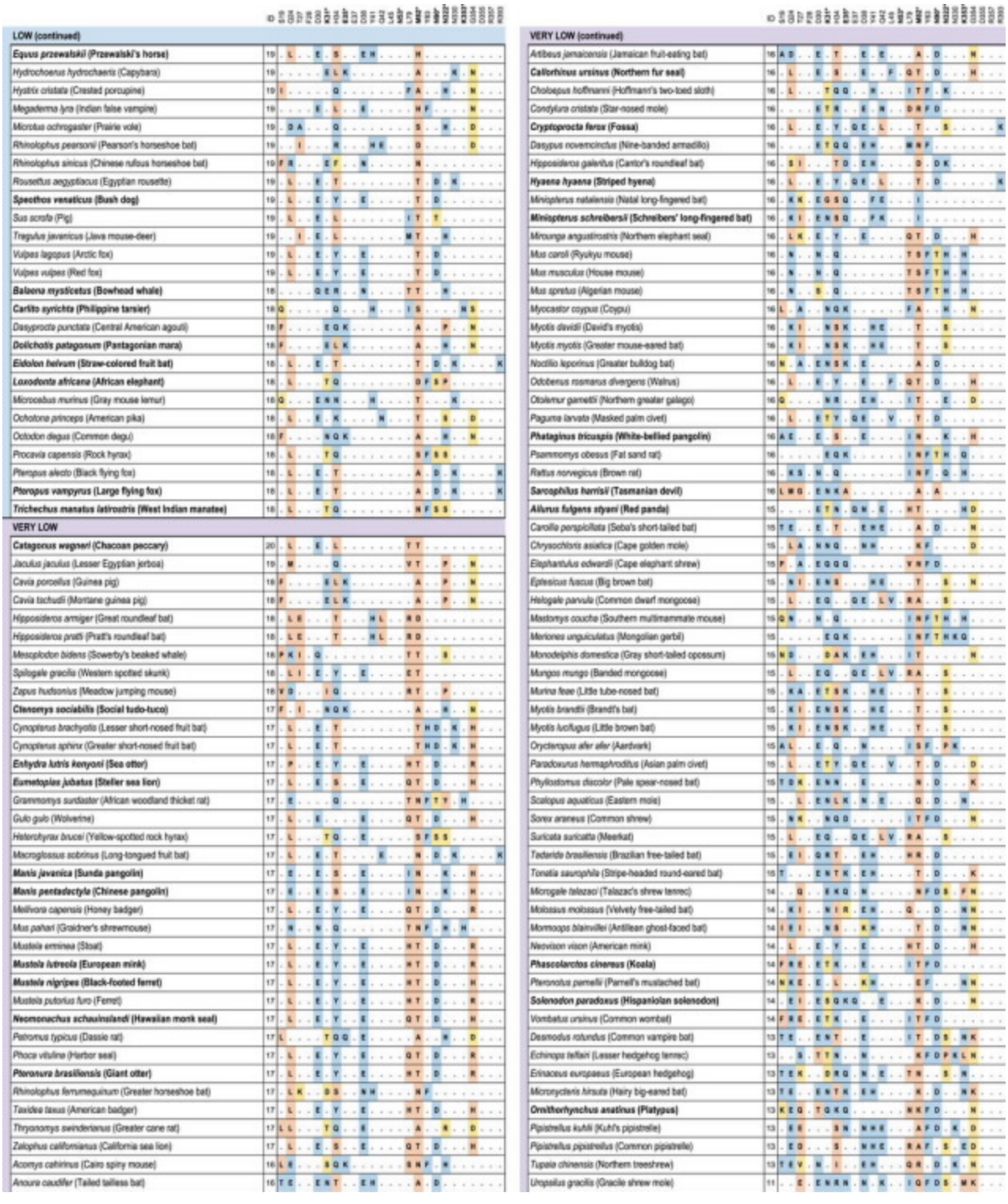
[103] file:///C:/Users/Steven%20Quay/Desktop/Zhangetal2009.pdf

Evidence: Of 410 vertebrate species tested for affinity to CoV-2 Spike Protein binding domain, primate ACE2 receptor, including human and VERO monkey cells, are the best at binding and bat species ACE2 are the worse, making direct bat-to-human host jumping extremely unlikely

- An examination of the ACE2 receptor binding domain amino acid sequences and their suitability for interacting with SARS-CoV-2 was performed in 410 vertebrates, including 252 mammals.[104]
- A five-category binding score was developed based on the conservation properties of 25 amino acids important for the binding between ACE2 and the SARS-CoV-2 spike protein.
- Only mammals fell into the medium to very high categories and only primates scored 25/25 for binding.
- This implies that SARS-CoV-2 is optimized for human ACE2-bearing cells from the first introduction into the human population, an observation that contradicts a zoonotic origin.
- It also suggests that other primates may be the proximate species from which SARS-CoV-2 entered the human population.
- Both VERO monkey kidney cells and ACE2 humanized mice would quality as an intermediate species by this criterion.
- Surprisingly, "all chiropterans (bats) scored low (n = 8) or very low (n = 29), including the Chinese rufous horseshoe bat, from which a coronavirus (SARSr-CoV ZC45) related to SARS-CoV-2 was identified."
- This is evidence that bats are probably not a reservoir host for SARS-CoV-2.
- A separate study observed: "Severe acute respiratory syndrome coronavirus 2 did not replicate efficiently in 13 bat cell lines."[105]
- The following two Tables are taken from the paper and are organized according to ACE2 SARS-CoV-2 affinity, from highest to lowest:

[104] https://www.pnas.org/content/117/36/22311
[105] https://wwwnc.cdc.gov/eid/article/26/12/20-2308_article


ID
S19
G24
T27
F28
D80
H241*
E264*
E37
D38
V41
G42
L45
L79*
M82*
Y83
N99*
N322S
K323R*
G324
D325
R327
VERY HIGH
Homo sapiens (Human) 25
Gorilla gorilla gorilla (Western lowland gorilla) 25
Nomascus leucogenys (Northern white-cheeked gibbon) 25
Pongo abelii (Sumatran orangutan) 25
Macaca fascicularis (Crab-eating macaque) 25
Mandrillus leucophaeus (Drill) 25
Nasalis larvatus (Proboscis monkey) 25
Pan paniscus (Bonobo) 25
Pan troglodytes (Chimpanzee) 25
Piliocolobus tephrosceles (Ugandan red colobus) 25
Pygathrix nemaeus (Red-shanked douc) 25
Rhinopithecus roxellana (Golden snub-nosed monkey) 25
Chlorocebus sabaeus (Green monkey) 25
Erythrocebus patas (Patas monkey) 25
Macaca mulatta (Rhesus macaque) 25
Papio anubis (Olive baboon) 25
Theropithecus gelada (Gelada) 25
Cercocebus atys (Sooty mangabey) 25
Macaca nemestrina (Southern pig-tailed macaque) 25
HIGH
Colobus angolensis (Angola colobus) 24
Propithecus coquereli (Coquerel's sifaka) 24
Cricetomys gambianus (Gambian pouched rat) 22
Cricetulus griseus (Chinese hamster) 22
Ctenodactylus gundi (Common gundi) 22
Delphinapterus leucas (Beluga whale) 22
Eulemur flavifrons (Blue-eyed black lemur) 22
Indri indri (Indri) 22
Monodon monoceros (Narwhal) 22
Neophocaena asiaeorientalis (Narrow-ridged finless porpoise) 22
Phocoena phocoena (Harbour porpoise) 22
Balaenoptera acutorostrata scammoni (Minke whale) 21
Balaenoptera bonaerensis (Antarctic minke whale) 21
Eschrichtius robustus (Gray whale) 21
Nannospalax galili (Spalax) 21
Odocoileus virginianus texanus (White-tailed deer) 21
Rangifer tarandus (Reindeer) 21
Tamandua tetradactyla (Southern tamandua) 21
Dipodomys stephensi (Stephens's kangaroo rat) 20
Elaphurus davidianus (Pere David's deer) 20
Ellobius lutescens (Transcaucasian mole vole) 20
Globicephala melas (Long-finned pilot whale) 20
Lagenorhynchus obliquidens (Pacific white-sided dolphin) 20
Lipotes vexillifer (Baiji) 20
Myrmecophaga tridactyla (Giant anteater) 20
Ondatra zibethicus (Muskrat) 20
Orcinus orca (Killer whale) 20
Tursiops truncatus (Common bottlenose dolphin) 20
MEDIUM
Daubentonia madagascariensis (Aye-aye) 23
Cheirogaleus medius (Fat-tailed dwarf lemur) 22
Ictidomys tridecemlineatus (Thirteen-lined ground squirrel) 22
Marmota flaviventris (Yellow-bellied marmot) 22
Marmota marmota marmota (Alpine marmot) 22
Mesocricetus auratus (Golden hamster) 22
Physeter catodon (Sperm whale) 22
Spermophilus dauricus (Daurian ground squirrel) 22
Allactaga bullata (Gobi jerboa) 21
Ammotragus lervia (Barbary sheep) 21
Antilocapra americana (Pronghorn) 21
Aotus nancymaae (Nancy Ma's night monkey) 21
Beatragus hunteri (Hirola) 21
Bison bison bison (American bison) 21
Bos indicus (Zebu) 21
Bos mutus (Wild yak) 21
MEDIUM (continued)
Bos taurus (Cattle) 21
Bubalus bubalis (Water buffalo) 21
Callicebus donacophilus (White-eared titi) 21
Callithrix jacchus (Common marmoset) 21
Capra aegagrus (Wild goat) 21
Capra hircus (Goat) 21
Cebus capucinus imitator (Panamanian white-faced capuchin) 21
Felis catus (Cat) 21
Giraffa tippelskirchi (Masai giraffe) 21
Hemitragus hylocrius (Nilgiri tahr) 21
Lynx canadensis (Canadian lynx) 21
Mirza coquereli (Coquerel's giant mouse lemur) 21
Moschus moschiferus (Siberian musk deer) 21
Neofelis diardi (Sunda clouded leopard) 21
Neofelis nebulosa (Clouded leopard) 21
Okapia johnstoni (Okapi) 21
Ovis aries (Sheep) 21
Panthera onca (Jaguar) 21
Panthera pardus (Leopard) 21
Panthera tigris altaica (Siberian tiger) 21
Pantholops hodgsonii (Tibetan antelope) 21
Perognathus longimembris (Little pocket mouse) 21
Peromyscus maniculatus bairdii (Deer mouse) 21
Pithecia pithecia (White-faced saki) 21
Puma concolor (Cougar) 21
Saimiri boliviensis boliviensis (Black-capped squirrel monkey) 21
Sapajus apella (Tufted capuchin) 21
Urocitellus parryii (Arctic ground squirrel) 21
Bos indicus x Bos taurus 21
Acinonyx jubatus (Cheetah) 20
Alouatta palliata (Mantled howler) 20
Ateles geoffroyi (Geoffroy's spider monkey) 20
Fukomys damarensis (Damaraland mole-rat) 20
Heterocephalus glaber (Naked mole-rat) 20
Hippopotamus amphibius (Hippopotamus) 20
Lepus americanus (Snowshoe hare) 20
Nanger dama (Dama gazelle) 20
Oryctolagus cuniculus (European rabbit) 20
Oryx dammah (Scimitar oryx) 20
Saguinus imperator (Emperor tamarin) 20
Vicugna pacos (Alpaca) 20
LOW
Ceratotherium simum cottoni (Northern white rhinoceros) 21
Ceratotherium simum simum (Southern white rhinoceros) 21
Diceros bicornis (Black rhinoceros) 21
Galeopterus variegatus (Sunda flying lemur) 21
Peromyscus leucopus (White-footed mouse) 21
Ailuropoda melanoleuca (Giant panda) 20
Camelus bactrianus (Bactrian camel) 20
Camelus dromedarius (Dromedary) 20
Camelus ferus (Wild bactrian camel) 20
Dicerorhinus sumatrensis (Sumatran rhinoceros) 20
Graphiurus murinus (Woodland dormouse) 20
Tapirus indicus (Malayan tapir) 20
Tapirus terrestris (South American tapir) 20
Ursus arctos horribilis (Grizzly bear) 20
Ursus maritimus (Polar bear) 20
Canis lupus dingo (Dingo) 19
Canis lupus familiaris (Dog) 19
Chinchilla lanigera (Long-tailed chichilla) 19
Chrysocyon brachyurus (Maned wolf) 19
Dipodomys ordii (Ord's kangaroo rat) 19
Eonycteris spelaea (Lesser dawn bat) 19
Equus asinus (Donkey) 19
Equus caballus (Horse) 19



While statistical models of this data could be interesting and informative, this is evidence will not be statistically quantified for this analysis. The evidence is another way of looking at the pre-adapted state of the CoV-2 for humans and suggests that primate animals, monkey cell cultures like the VERO cell, and humanized mice could be likely laboratory models that were used by the

WIV in GoF research. This will contribute a 51%/49% contribution in favor of laboratory compared to zoonotic origin. There will be no subjective discount factor adjustment.

The results from the calculations are shown below.

Evidence or process	Zoonotic Origin (ZO)	Laboratory Origin (LO)
Starting likelihood	0.002	0.998
This is the outcome favors LO over ZO at 51% versus 49%		0.51
Impact of this evidence		Increases the likelihood of LO by 51/49 = 1.041
Impact of evidence calculation		1.041 x 0.998 = 1.039
Normalize this step of analysis	0.002/(0.002 + 1.039) = 0.002	1.039/(0.002 + 1.039) = 0.998

Adjusted likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

Evidence: Did a Review of Samples Collected from a Mineshaft Cause the COVID-19 Pandemic?[106]

Abstract. The origin of the COVID-19 pandemic caused by SARS-CoV-2 has been hotly debated. Proponents of the natural spillover theory allege that the virus jumped species, possibly via an intermediary host, to cross over to humans via the wildlife trade or by other means. Proponents of a rival theory claim that the virus escaped from a laboratory in Wuhan. This research presents circumstantial evidence of a transmission route via a late 2019 review of samples collected from a mineshaft in Mojiang, Yunnan Province, China. It examines the activity at the Wuhan Institute of Virology in late 2019, when samples from a mineshaft associated with a suspected SARS outbreak were being reviewed. It proposes that spillover occurred during this review of samples including of a virus (BtCoV/4991) only 1% different to SARS-CoV-2 in its RNA-dependent RNA polymerase (RdRp).

It is a meticulous sourced analysis. It purposely avoids the question of whether SARS-CoV-2 was being grown or manipulated in the laboratory, but only addresses the evidence that events in the fall of 2019 are consistent with a laboratory accident.

This will not be used to adjust the likelihoods.

Current likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

[106] https://zenodo.org/record/4029545#.X-x_f9gzbOg. Author anonymous. A meticulously documented analysis that concludes an accident occurred at the Wuhan Institute of Virology during the fall of 2019.Includes many primary documents from Mandarin. No direct evidence of 'what' was the nature of the accident or if it was SARS-CoV-2.

Evidence: The Hunan market was not the source of SARS-CoV-2

From the WHO Terms of Reference for the investigation of the origin of SARS-CoV-2:[107]

"The Huanan wholesale market is a large market (653 stalls and more than 1180 employees) mainly supplying seafood products but also fresh fruits and vegetables, meat, and live animals. In late December 2019, 10 stall operators were trading live wild animals including chipmunks, foxes, racoons, wild boar, giant salamanders, hedgehogs, sika deer, and many others. Farmed, wild and domestic animals were also traded at the market including snakes, frogs, quails, bamboo rats, rabbits, crocodiles, and badgers. The market was closed on 1 January 2020, and several investigations followed, including environmental sampling, as well as sampling of frozen animal carcasses at the market. **Of the 336 samples collected from animals, none were PCR positive for SARS-CoV-2**, whereas 69 out of 842 environmental samples were positive by PCR for SARS-CoV-2. Sixty- one of those (88%) were from the western wing of the market. Of these, 22 samples were from 8 different drains and sewage, and 3 viruses were isolated, sequenced and shared on GISAID. These were virtually identical to the patient samples collected at the same time (>99.9 % homology)."

For contrast, with SARS-CoV-1 91 civets & 15 raccoon dogs in wet markets were tested with 106/106, 100% positive.[108]

This will not be used to adjust the likelihoods.

Current likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

[107] https://drive.google.com/file/d/1rx0W2efbE0R1Aq-lALWTqD22VsWbTlO-/view
[108] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1212604/

Evidence: Analysis of the hospital of admission for COVID-19 patients during December 2019 places "ground zero" for the outbreak somewhere along Line 2 of the Wuhan Metro System.

Line 2 carries one million people per day and services the Wuhan Institute of Virology, the Hunan Seafood Market, the high-speed rail system, and the Wuhan International Airport

A preprint manuscript[109] reported that the earliest genomic cluster of SARS-CoV-2 patients is a group of four individuals associated with the General Hospital of Central Theater Command of People's Liberation Army (PLA) of China in Wuhan. This cluster contains the "Founder Patients" of both Clade A and Clade B, from which every SARS-CoV-2 coronavirus that has infected every patient with COVID-19 anywhere in the world has arisen.

The PLA Hospital is about one mile from the Wuhan Institute of Virology (WIV) and the closest hospital to WIV. Both the PLA Hospital and WIV are serviced by Line 2 of the Wuhan Metro System. The Hunan Seafood Market is also located adjacent to Line 2. All patients between December 1st, 2019 and early January 2020 were first seen at hospitals that also are serviced by Line 2 of the Metro system.

With 40 hospitals located near seven of the nine Metro Lines, the likelihood that all early patients were seen at hospitals only near Line 2 by chance is about 1 in 68,500 (p-value = 0.0000146). The inference then would be that the early spread of SARS-CoV-2 was through human-to human transmission on Line 2.

Line 2 carries one million passengers per day and assuming most are round trip business workers going to and from work in the morning and evening, represents 500,000 riders or about 5% of the Wuhan population. A very recent publication determined that, in fact, 500,000 residents of Wuhan contracted COVID-19, a ten-fold upper estimate.[110] The coincidence of my prediction that 500,000 riders on Line 2 were likely exposed to SARS-CoV-2 in late 2019 and the recent admission from Chinese CDC that Wuhan had 500,000 COVID-19 cases is duly noted!

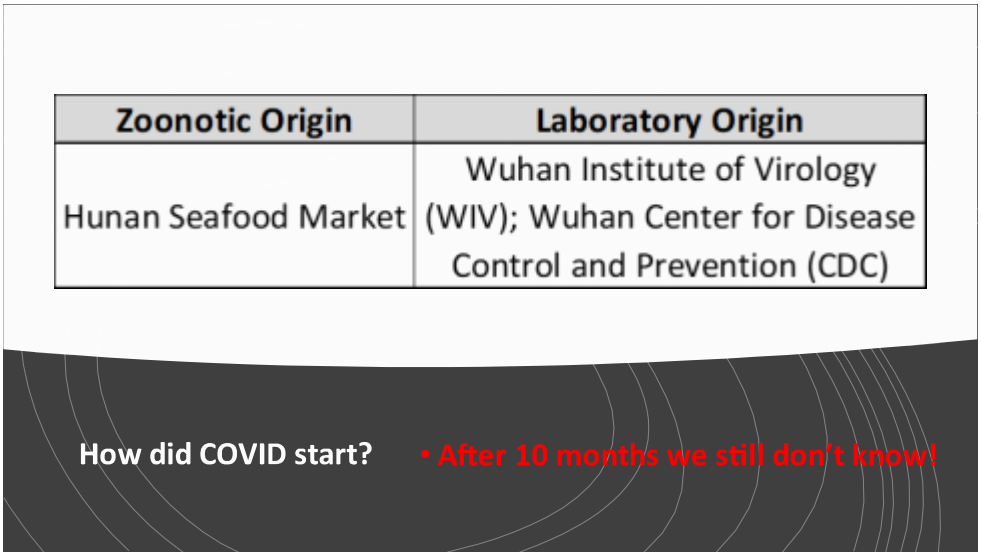
Line 2 connects to all eight other lines of the Wuhan Metro System (1, 3, 4, 6, 7, 8, 11, and Yanglu) facilitating rapid spread in Wuhan and Hubei Province, and also services both the high-speed rail station (Hankou Railway Station), facilitating rapid spread throughout China, and the Wuhan International Airport (Tianhe International Airport), facilitating rapid spread throughout Asia, Europe, and to the United States. In fact, direct human-to-human spread from the Reference Sequence patient to patients around the world is suggested by an unexpectedly reduced genome base substitution rate seen in patient specimens in cities with direct flights from Wuhan.

[109] https://zenodo.org/record/4119263#.X-rszNgzbOg
[110] https://mp.weixin.qq.com/s/LXTfDmsQLf3qZnu_S_MxcA ; https://thehill.com/policy/international/china/531935-study-shows-wuhan-coronavirus-cases-may-have-been-10-times-higher

In a separate paper by Quay and Dr. Martin Lee, Adjunct Professor of Statistics, UCLA, from May 2020, now accepted for publication in *Epidemics,*[111] the authors provide evidence that COVID-19 was appearing in California as early as the first week of 2020. This is likely due to direct flights connecting Line 2 to the Wuhan airport and then to San Francisco.

In conclusion, Line 2 of the Wuhan Metro System services the PLA Hospital with the first genomic cluster of patients with COVID-19, the hospitals where patients first went in December 2019 and early January 2020 and is the likely conduit for human-to-human spread throughout Wuhan, China, and the world.

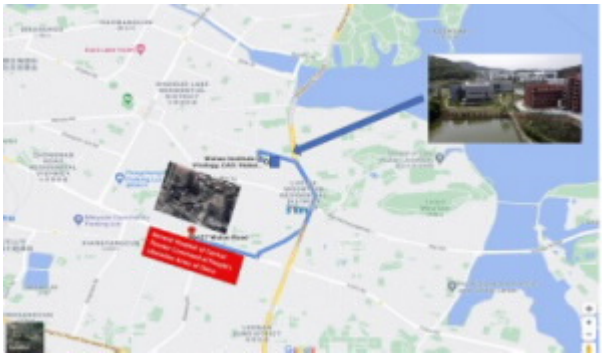
The following slide overview provides a visual analysis of this evidence:



[111] https://www.researchgate.net/publication/341742303_COVID-19_May_Have_Have_Reached_United_States_in_January_2020_05272020

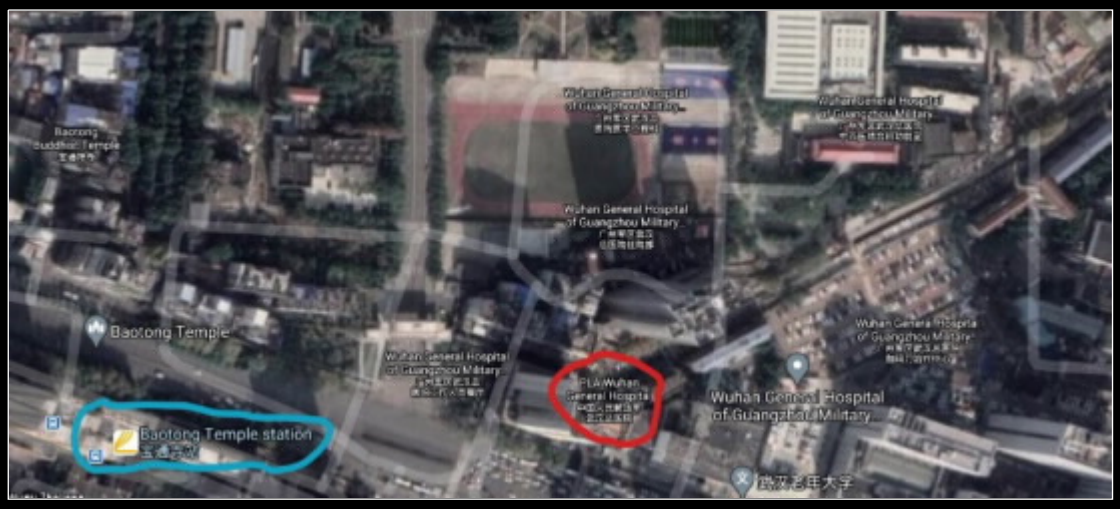
SARS 2003 and MERS 2015

- **Within four months (SARS 2003) and nine months (MERS 2015) we knew the zoonotic host**



GISAID Database

Earliest cases at the PLA Hospital



PLA Hospital is part of the Joint Logistic Support Force Complex

Position in RS	Bat-SL-CoVZC45	Bat-SL-CoVZXC21	RaTG13	PLA-4	PLA-3	PLA-2	Hu-1 Ref seq	PLA-1	GISAID #1
5' UTR	1-5 missing	1-5 missing	1-15 missing	1-16 missing	1-20 missing	1-36 missing	Intact	1-25 missing	Intact
3778	A	A	A	A	A	A	A	A	G
6968	T	T	C	C	C	C	C	A	C
8782	T	T	T	T	C	C	C	C	C
8987	T	T	T	T	T	T	T	T	A
11764	T	T	T	T	T	NA - Note 1	T	A	T
28144	C	C	C	C	T	T	T	T	T
3' UTR	last 4 poly-A missing	last 4 poly-A missing	last 13 poly-A missing	last 15 poly-A missing	last 15 poly-A missing	NA - Note 1	Intact	last 12 poly-A missing	last 4 poly-A missing
Genome length	29802	29732	29855	29872	29868	NA - Note 1	29903	29866	29899

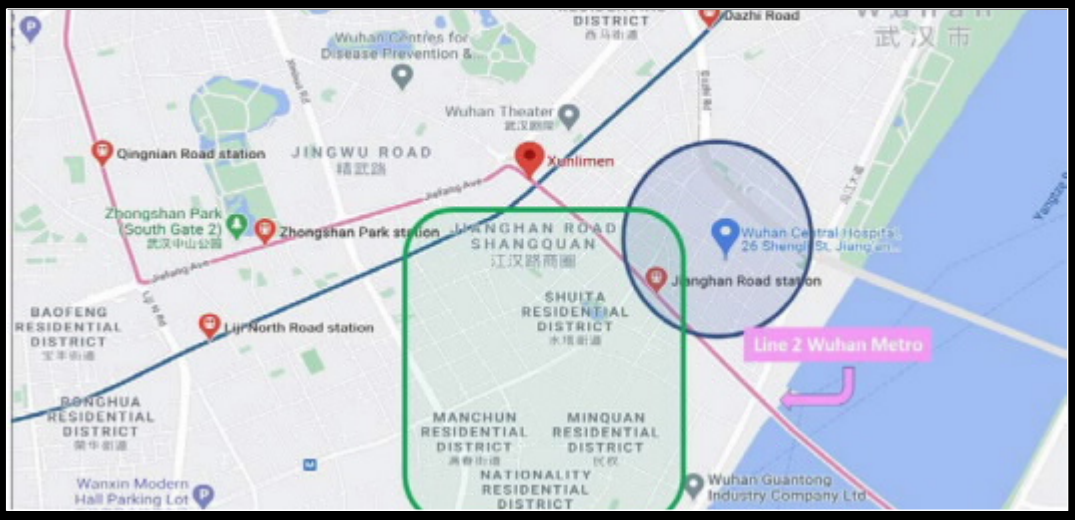
Clade A SNPs | Clade B SNPs | Non-RaTG13 DNPs

Note 1 - GISAID record: "Long stretches of NNNs (34.45% of overall sequence). Gap of 13 nucleotide(s) found at refpos 26171 (FRAMESHIFT). Gap of 13 nucleotides when compared to the reference sequence. 0.40% Unique Mutations."

The PLA patient cluster

- PLA-4 is genetically the closest human infection to the three closest bat viruses
- The four PLA patients have the close sequence pattern usually seen only in family transmissions

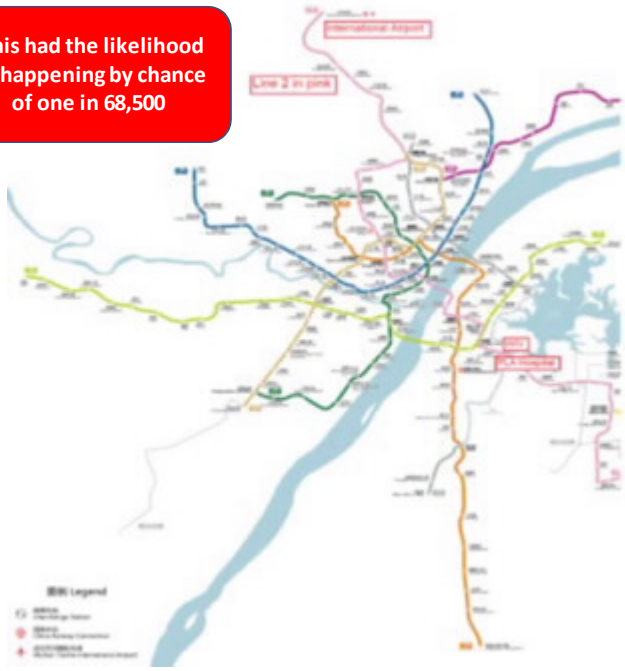
The Wuhan Metro System



Methodology: Metro station line for intersection of hospital catchment zone and residential living district zone was identified

All COVID patients from Dec 1 to early Jan were admitted to hospitals on Metro Line 2

This had the likelihood of happening by chance of one in 68,500



Feature	Relationship to Pandemic
Line 2 carried 1 MM passengers a day before COVID	Assuming 2 trips/d for commuters, about 5% of the Wuhan population uses this Line, making it an efficient transmission route for all of Wuhan as well as Hubei Provence. A single patient can leave a droplet/aerosol cloud for hours to infect others.
Line 2 shares stations with every other Metro Line	Permits human-to-human spread to every part of Wuhan at the stations shared with Line 2
Line 2, Hankou Railway Station	Connects Wuhan to all of China by high speed rail
Line 2, Tianhe International Airport	International destinations: New York City, San Francisco, London, Tokyo, Rome, Istanbul, Dubai, Paris, Sydney, Bali, Bangkok, Moscow, Osaka, Seoul, and Singapore.

The Line 2 COVID Conduit

The Line 2 COVID Conduit

- Line 2 Hankou High Speed Railway Station to all of China
- Line 2 Tianhe International Airport Station to the world

The Hunan Seafood Market, Wuhan Institute of Virology, and the Wuhan CDC, all locations suggested to be the possible source of SARS-CoV-2 in Wuhan, are also all serviced by Line 2 of the Metro system, suggesting this public transit line should become the focus for further investigations into the origin of this pandemic.

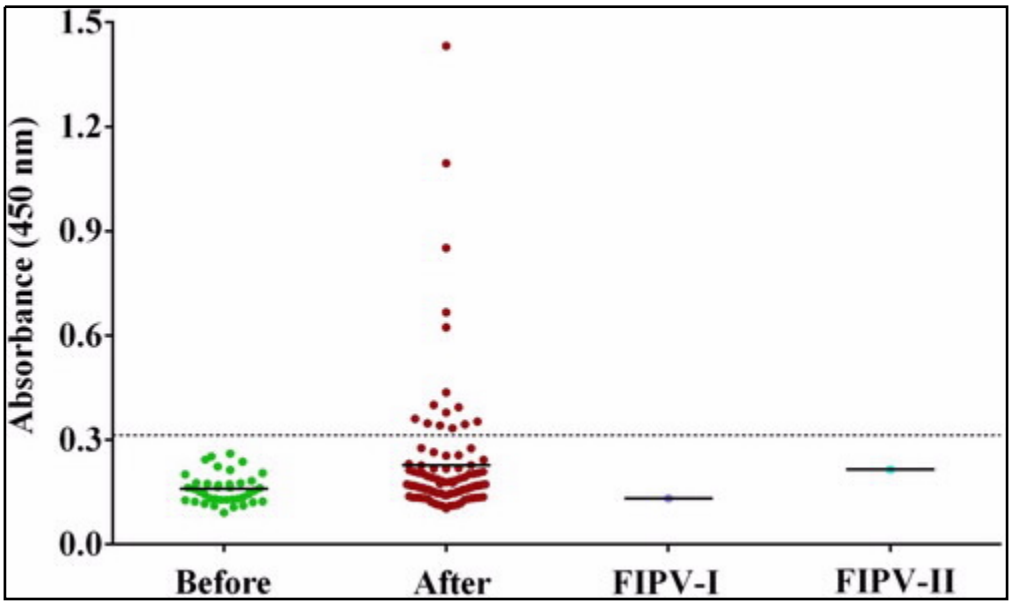
Given that the Hunan Seafood Market has been removed as a source for the origin of CoV-2, this evidence will contribute a 51%/49% contribution in favor of laboratory compared to zoonotic origin. There will be no Subjective Discount Factor adjustment.

The results from the calculations are shown below.

Evidence or process	Zoonotic Origin (ZO)	Laboratory Origin (LO)
Starting likelihood	0.002	0.998
This is the outcome favors LO over ZO at 51% versus 49%		0.51
Impact of this evidence		Increases the likelihood of LO by 51/49 = 1.041
Impact of evidence calculation		1.041 x 0.998 = 1.039
Normalize this step of analysis	0.002/(0.002 + 1.039) = 0.002	1.039/(0.002 + 1.039) = 0.998

Adjusted likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

Evidence: SARS-CoV-2 infection, based on antibody seroconversion, was not found in 39 archived specimens taken from cats (1/3 feral) between March and May 2019[112]



Based on these results, the prevalence of SARS-CoV-2 in domestic and feral cats prior to January 2020 is less than 8% with a 90% confidence interval.

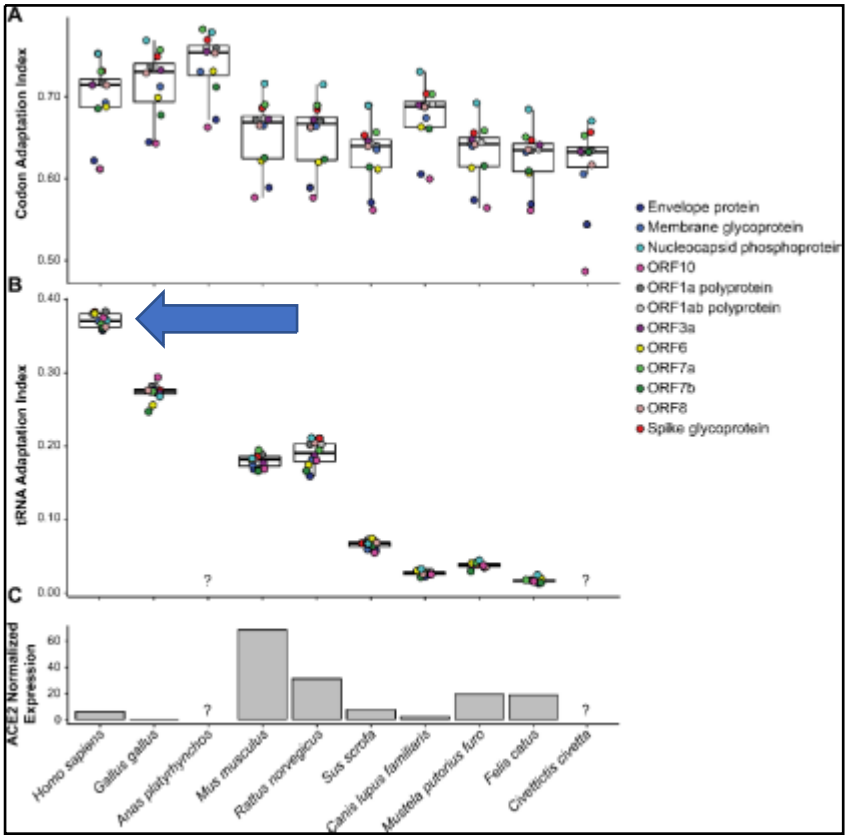
This will not be used to adjust the likelihoods.

Current likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

[112] https://www.tandfonline.com/doi/full/10.1080/22221751.2020.1817796

Evidence: The extraordinary pre-adaption of SARS-CoV-2 for human cells is demonstrated by a paper looking at a tRNA adaption index.[113]

"The proteome of SARS-CoV-2 is mainly composed of the replicase polyprotein (ORF1ab) and of structural proteins: the spike glycoprotein, the membrane and envelope proteins, and the nucleoprotein [41]. Based on the genomic codon usage of each of the possible host species, we compute the codon adaptation index (CAI) and the tRNA adaptation index (tAI) to estimate the translational efficiency of SARS-CoV-2 proteins in each host (Fig 3A and 3B and S2 Table). Humans are among the top three species whose CAIs are mostly over 0.70, together with ducks and chickens. In terms of the tAI, humans show the highest translational adaptation among all others, followed by chickens, and, to some extent, mice and rats. On the other hand, cats, ferrets, pigs, and dogs are less translationally adapted than humans both by CAI and tAI."



As shown in panel B above, the tRNA Adaption Index is highest, by far, for humans (blue arrow) followed by the red junglefowl. This is additional evidence of the extraordinary adaption of SARS-CoV-2 to humans from the very beginning. This also is the first evidence of a reasonable intermediate host but based only on these *in silico* data.

This will not be used to adjust the likelihoods.

Current likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

[113] https://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1008450#pcbi.1008450.s004

Evidence: Evidence of Lax procedures and disregard of laboratory safety protocols and regulations in China, including the Wuhan Institute of Virology

A collection[114] from the Chinese Q&A website, https://www.zhihu.com/, of first-hand documentation of laboratory safety breaches and incidents within a large number of laboratories with diverse research subjects and purposes in the People's Republic of China (PRC) is provided. The laboratories involved include Chemistry labs, Biolabs, Computer labs as well as Physics and Engineering labs.

From this first-hand documentation, we obtained evidence of relaxed safety regulations and frequent breaches of such regulations, with reasons ranging from poor training/education on lab safety and chronic ignorance of safety rules, to intentional breaches of protocols for purposes other than the research projects of the lab(s) of which the breach was documented in.

Such breaches often resulted in safety accidents ranging from physical injury, chemical burns, chemical leaks, and damage to property, to lab-acquired infection and escape of in-lab pathogens. With consequences ranging from personal-level to institution-wide impacts.

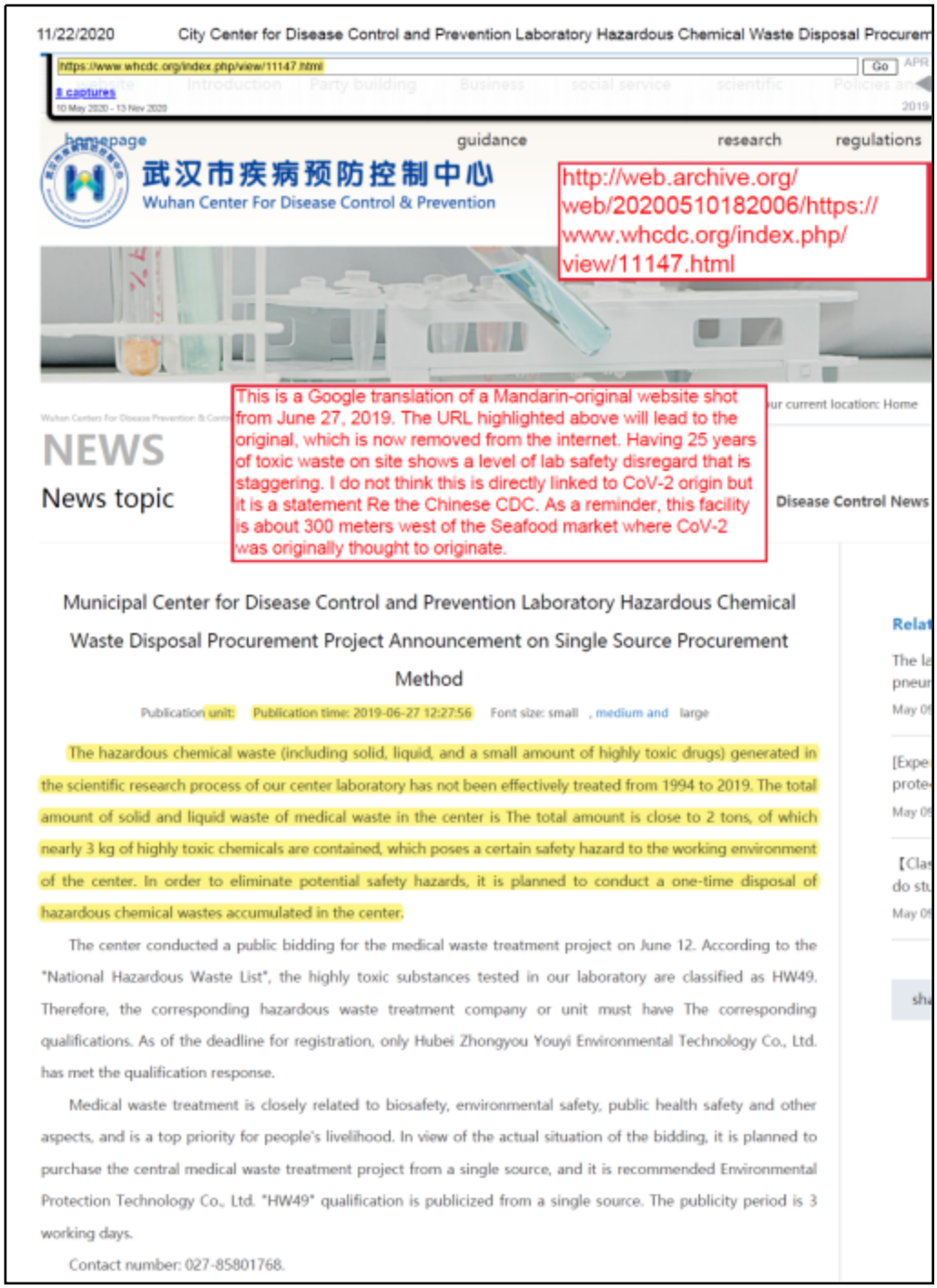
Here is the reference to the State Department cables concerning safety concerns at the WIV.[115]

The following document shows that in June 2019, the Chinese CDC was soliciting for the removal of 25-years-worth of solid and liquid medical waste. The total weight is close to two tons including three kg of highly toxic waste.

This is a Google translation of a Mandarin-original website shot from June 27, 2019. The URL highlighted above will lead to the original, which now has been removed from the internet. Having 25 years of toxic waste on site shows a staggering level of disregard for lab safety.

I do not think this is directly linked to CoV-2 origin, but it is a statement about the Chinese CDC. As a reminder, this facility is about 300 meters west of the Seafood market where CoV-2 was first thought to have originated.

[114] https://zenodo.org/record/4307879#.X-yUo9gzbOh
[115] https://foia.state.gov/Search/Results.aspx?caseNumber=F-2020-05255

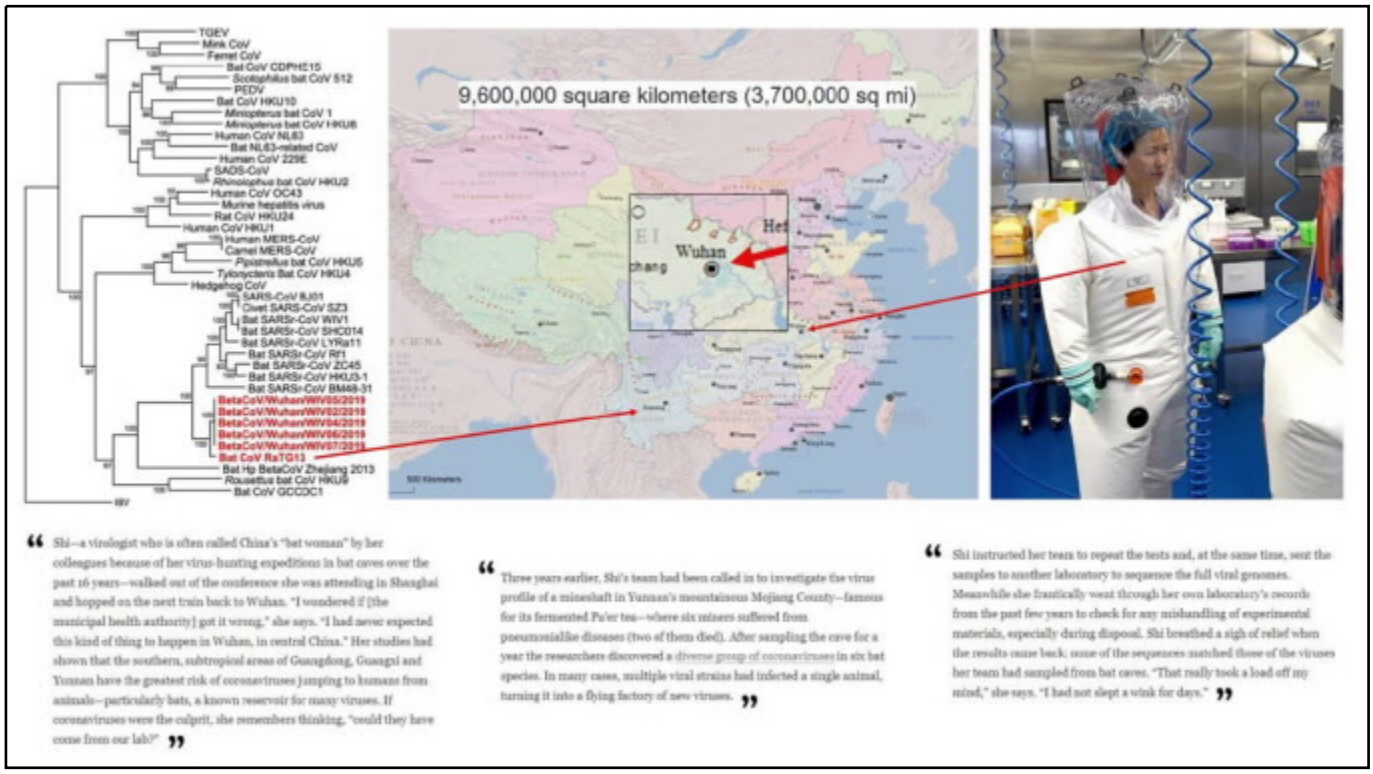


This will not be used to adjust the likelihoods.

Current likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

Evidence: The careful words of Dr. Shi do NOT say she did not have SARS-CoV-2 at the WIV.

This Figure contains quotes from an article about Dr. Shi and her reaction to the beginning of the COVID-19 pandemic.



Notice in the last frame Dr. Shi says two strange sentences:

Sentence 1: "…she frantically went through her own laboratory's records from the past few years to check for any mishandling of experimental materials, **especially during disposal**."

Why did she mention disposal? If you don't know what you are looking for this, "especially during disposal," is a bit of an odd qualifier. Other evidence from Wuhan suggests that, in fact, disposal may have been a likely source of the accidental lab release.

Sentence 2: "She breathed a sigh of relief when the results came back: none of the sequences matched those of the viruses her team had sampled from bat caves."

If Dr. Shi had created SARS-CoV-2 as a chimera, perhaps starting with one of those cave viruses, of course you would no longer have a sequence match. This is a probably truthful statement that leaves open the question of lab creation.

This will not be used to adjust the likelihoods.

Current likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

Evidence: The Good, the Bad, and the Ugly: a review of SARS Lab Escapes[116]

In 2003–04, in the wake of the SARS epidemics, there were multiple cases of laboratory acquired infection (LAI) with SARS within just a few months: first in a P3 in Singapore, then in a military P4 in Taipei and last a protracted case in a P3 in Beijing. The 'WHO SARS Risk Assessment and Preparedness Framework' has a good summary of these lab accidents:

Since July 2003, there have been four occasions when SARS has reappeared. Three of these incidents [note: Singapore, Taipei and Beijing] were attributed to breaches in laboratory biosafety and resulted in one or more cases of SARS. The most recent laboratory incident [note: in Beijing] resulted in 9 cases, 7 of which were associated with one chain of transmission and with hospital spread. Two additional cases at the same laboratory with a history of illness compatible with SARS in February 2004 were detected as part of a survey of contacts at the facility.[i.1]

This article reviews some of these cases and discusses briefly some of the insights that were gained from these at the time.

Another article along the same lines is, "10 incidents discovered at the nation's biolabs"[117] This included Dr. Baric's laboratory in which "(b)etween April 2013 and September 2014, eight individual mouse escapes were reported at the University of North Carolina-Chapel Hill. Several of the mice were infected with either SARS or the H1N1 flu virus."

Dozens of holes in BSL-4 'spacesuits'

As a key protection against the world's most deadly pathogens, including the Ebola virus, scientists in the BSL-4 labs at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) at Fort Detrick in Maryland wear pressurized, full-body spacesuit-like gear and breathe purified air. Yet those suits ruptured or developed holes in at least 37 incidents during a 20-month period in 2013 and 2014, according to lab incident reports obtained by USA TODAY under the federal Freedom of Information Act.

This will contribute a 51%/49% contribution in favor of laboratory compared to zoonotic origin. There will be no confidence adjustment. The results from the calculations are shown below.

Evidence or process	Zoonotic Origin (ZO)	Laboratory Origin (LO)
Starting likelihood	0.011	0.989
The history of SARS laboratory accidents is consistent with the laboratory origin hypothesis		0.51
Impact of this evidence		Increases the likelihood of LO by 51/49 = 1.041
Impact of evidence calculation		1.041 x 0.989 = 1.030
Normalize this step of analysis	0.011/(0.011 + 1.030) = 0.011	1.030/(0.011 + 1.030) = 0.989

Adjusted likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

[116] https://gillesdemaneuf.medium.com/the-good-the-bad-and-the-ugly-a-review-of-sars-lab-escapes-898d203d175d
[117] https://www.usatoday.com/story/news/2015/05/29/some-recent-us-lab-incidents/25258237/

Evidence: Drs. Shi and Daszak use Wuhan residents as negative controls for zoonotic coronavirus seroconversion[118]

"As a control, we collected 240 serum samples from random blood donors in **Wuhan >1000 km away from Jinning & where inhabitants have a much lower likelihood of contact with bats due to its urban setting**" [emphasis added]. As expected, 0/240 samples from the patients from Wuhan had a positive serological evidence of prior coronavirus infection.

"The 2.7% seropositivity for the high-risk group of residents living in close proximity to bat colonies suggests that spillover is a **relatively rare event**, however this depends on how long antibodies persist in people, since other individuals may have been exposed and antibodies waned."

In this paper from 2018, Drs. Shi and Daszak conclude that bat-to-human transfer is relatively rare for high-risk people living in close proximity to bat colonies and much less likely in Wuhan, a conclusion that does not support a hypothesis of bat-to-human transmission.

This will not be used to adjust the likelihoods.

Current likelihood: Zoonotic origin (0.2%), laboratory origin (99.8%).

[118] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6178078/

Evidence. The Bat Coronavirus RaTG13 has the Unique Genome Sequences Necessary to be the Precursor of SARS-CoV-2 Using the 'No See 'Em' Synthetic Biology Technology. ***The probability that RaTG13 acquired these 'No See 'Em' synthetic biology assembly sequences in nature is one in a billion.***

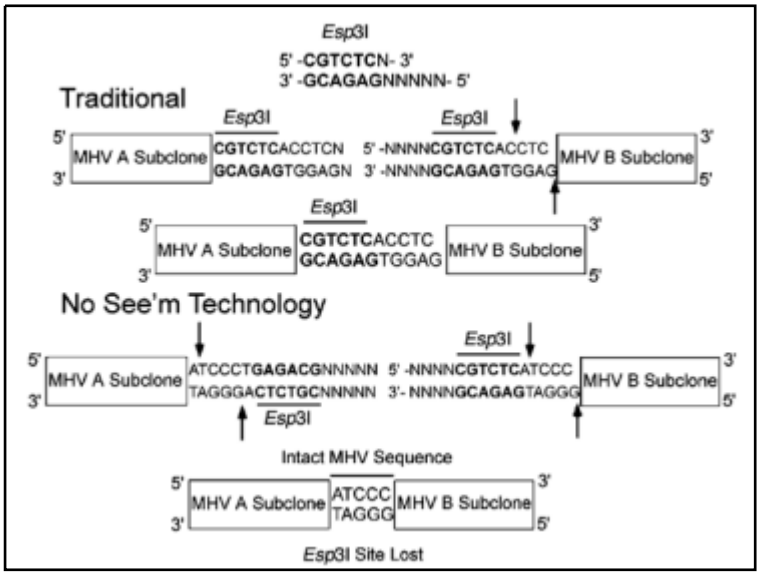
Summary.

- Synthetic biology techniques, like the engineered "No See 'Em'[119] restriction enzyme-enabled insertion method,[120] have been developed that, by design, extinguish the fingerprints of the insertion when only looking at the final genome.

- The use of these techniques is revealed however, if the precursor-product genome pair of such an insertion is available for inspection.

- **Hypothesis: the unique features of the SARS-CoV-2 Spike Protein, the receptor binding domain ACE2 contact amino acid residue region and the polybasic (furin) cleavage site, are the product of a genome insertion sequence into RaTG13 using engineered Esp3I restriction enzyme sites, the so-called, 'No See 'Em,' technology.**

- An example of the 'No See'm' Technology is shown below, taken from Baric and Sim.[1] By placing the restriction sites symmetrically on both strands of the cDNA, the resulting insertion no longer contains the identifying restriction site nts.



- According to Baric and Sims[1] "the type IIS restriction enzyme, Esp3I, recognizes an asymmetric sequence and makes a staggered cut 1 and 5 nucleotides downstream of the recognition sequence, leaving 256, mostly asymmetrical, 4-nucleotide overhangs

[119] Variably spelled 'No See 'Em,' 'No See 'um,' and 'No See'm.'
[120] https://www.researchgate.net/publication/8119695_Development_of_mouse_hepatitis_virus_and_SARS-CoV_infectious_cDNA_constructs

(GCTCTCN#NNNN). As identical Esp3I sites are generated every ~1,000,000 base pairs or so in a random DNA sequence, most restricted fragments usually do not self-assemble."

- Examination of RaTG13 identified two Esp3I cleavage sites in the Spike Protein gene, at nts 1366 and 2941 (positions 22,910 and 24,485 in the entire genome).

- As expected from the above rarity of such sites in an approximately 3800 nt gene, SARS-CoV-2 has no Esp3I sites in its SP gene. Neither do twelve other coronaviruses, including SARS-CoV-1, MERS, and other related human or bat coronaviruses.

- From all of the species other than bat RaTG13 gene source, the frequency of Esp3I sites **at any location** is 2 in 54,131 nucleotides or 0.000036947. If we assume the possibility of the occurrence of such a site at a given nucleotide is independent of any other nucleotide, then it is possible to use a binomial distribution calculation to determine the probability of 2 Esp3I sites in 3809 nucleotides for the bat RaTG13 gene. This calculation yields a probability of at least 2 sites anywhere in the Spike Protein gene of 0.009 or about one in a hundred. The probability of exactly 2 sites is 0.0086.[121]

- The 5' restriction site in RaTG13 begins at aa residue 455L, identified by Andersen et al, Nature, 2020. as the start of the "receptor-binding domain ACE2 contact residues." The downstream amino acids from this site are critical for why RaTG13 has such poor affinity for human ACE2 and the substitutions in CoV-2 are precisely why CoV-2 has such high affinity for human ACE2, why CoV-2 seems so 'preadapted' to human infections, etc. So this is the most important part of CoV-2 in explaining its ACE2 binding and infectivity. Further downstream is arguably the second most important site, the polybasic (furin) cleavage site.[122] Polybasic cleavage sites have not been observed in related 'lineage B' betacoronaviruses,' according to Andersen et al, Nature, 2020. and so there has been much speculation about how this site was acquired.

- The 3' restriction site in RaTG13 is at residue 980L. There is no protein-based rationale for this position.

- Comparing the nt sequences between RaTG13 and CoV-2, at the 5' restriction site, they are two codons in which only 2 of 6 nt bases are shared but, despite this low nt sequence homology, they are in fact synonymous base substitutions.

- Comparing the nt sequence between RaTG13 and CoV-2 at the 3' restriction site, this site has 5 of 6 identical nts with a single synonymous change in CoV-2 which destroys the restriction site. This is the only such five nt site in the RaTG13 spike protein gene and so

[121] Statistical analysis provided by Dr. Martin Lee, PhD, Adjunct Professor of Statistics, UCLA Fielding School of Public Health, UCLA, Los Angeles, CA.
[122] https://www.biorxiv.org/content/10.1101/2020.08.26.268854v1

is the easiest site in which a one nt substitution can create or destroy an Esp3I restriction site.

- The probability of having the restriction sites at **exactly these locations** can also be calculated.[2] Since there are 3809 nucleotides in the RaTG13 genome then, 3807 would not have a restriction site with probability (1-0.000036947), which was determined from the frequency of these restriction sites in other species. The other two sites would have this restriction site with probability 0.000036947. So the overall probability of this configuration has a probability of: $(1\text{-}0.00036947)^{3807} \times (0.000036947)^2 = 3.343 \times 10^{-10}$. This is a frequency of these site at their exact location being here from a natural process of approximately one in a billion.

- Dr. Zhengli-Li Shi, of the Wuhan Institute of Virology, collected the bat virus RaTG13 in 2013 and sequenced it between 2014 and 2018. In 2015, Dr. Shi and colleagues have also used the 'No See 'Em' technology' with a similar restriction enzyme, BgII, in the SARS-CoV reverse genetics system to generate chimeric coronaviruses. In that paper, they inserted a spike protein gene from a bat coronavirus into a mouse-adapted coronavirus, with a 'gain-of-function' phenotypic change.[123]

- **In conclusion:**

- **The bat coronavirus RaTG13 has two rare, Esp3I restriction sites strategically located to permit insertion of a genetic sequence that codes for the unique features of the SARS-CoV-2 Spike Protein, its receptor binding contact amino acids and its polybasic (furin) cleavage site, using the 'No See 'Em' synthetic biology techniques.**

- **This specific synthetic biology laboratory technique has been successfully performed previously by Wuhan Institute of Virology scientists to increase coronavirus infectivity.**

- **The probability these two sites are present and in their exact location in RaTG13 by an act of nature is one in a billion.**

[123] https://www.nature.com/articles/nm.3985

Text-Table. A record of the EspI restriction enzyme sites in the Spike Protein (SP) genes of fifteen coronaviruses, including RaTG13 and SARS-CoV-2. RaTG13 is unique in having two such sites, with SARS-CoV-2 and eleven other coronaviruses having no such site in the SP gene. The restriction sites were identified with the RestrictionMapper site algorithm: http://www.restrictionmapper.org/ .

Species	Spike Protein (SP) Gene Source	Nt Size of SP Gene	Esp3I Site Location in Spike Protein Gene	Reference
Bat	Bat Coronavirus RaTG13 from WIV	3809	1366, 2941 (22910, 24485 in genome)	
Human	SARS-CoV-2 Reference Sequence	3821	None	
Bat	Rhinolophus affinis coronavirus isolate LYRa11	3779	None	Daszak and Shi paper
Bat	Bat SARS coronavirus HKU3-1	3728	None	Daszak and Shi paper
Bat	SARS-like coronavirus isolate bat-SL-CoVZC45	3740	None	Third Military University publication
Bat	SARS-like coronavirus bat-SL-CoVZXC21	3737	None	Third Military University publication
Bat	hCoV-19/bat/Yunnan/RmYN02/2019	3873	None	Wild bat coronavirus with apparent furin-like insert
Bovine	Bovine coronavirus strain Quebec	4091	None	
Human	Human coronavirus HKU1 strain	4070	3208	
Human	MERS Reference Sequence	4061	None	
Human	Human coronavirus OC43 strain	4079	None	
Human	Human coronavirus 229E strain	3512	None	
Human	Human Coronavirus NL63 Reference Sequence	4070	None	
Human	SARS 2003 coronavirus ZJ0301	3767	None	
Pangolin	Pangolin coronavirus isolate PCoV_GX-P4L	3803	3351	
Human	SARS-CoV-1 Urbani	3767	None	

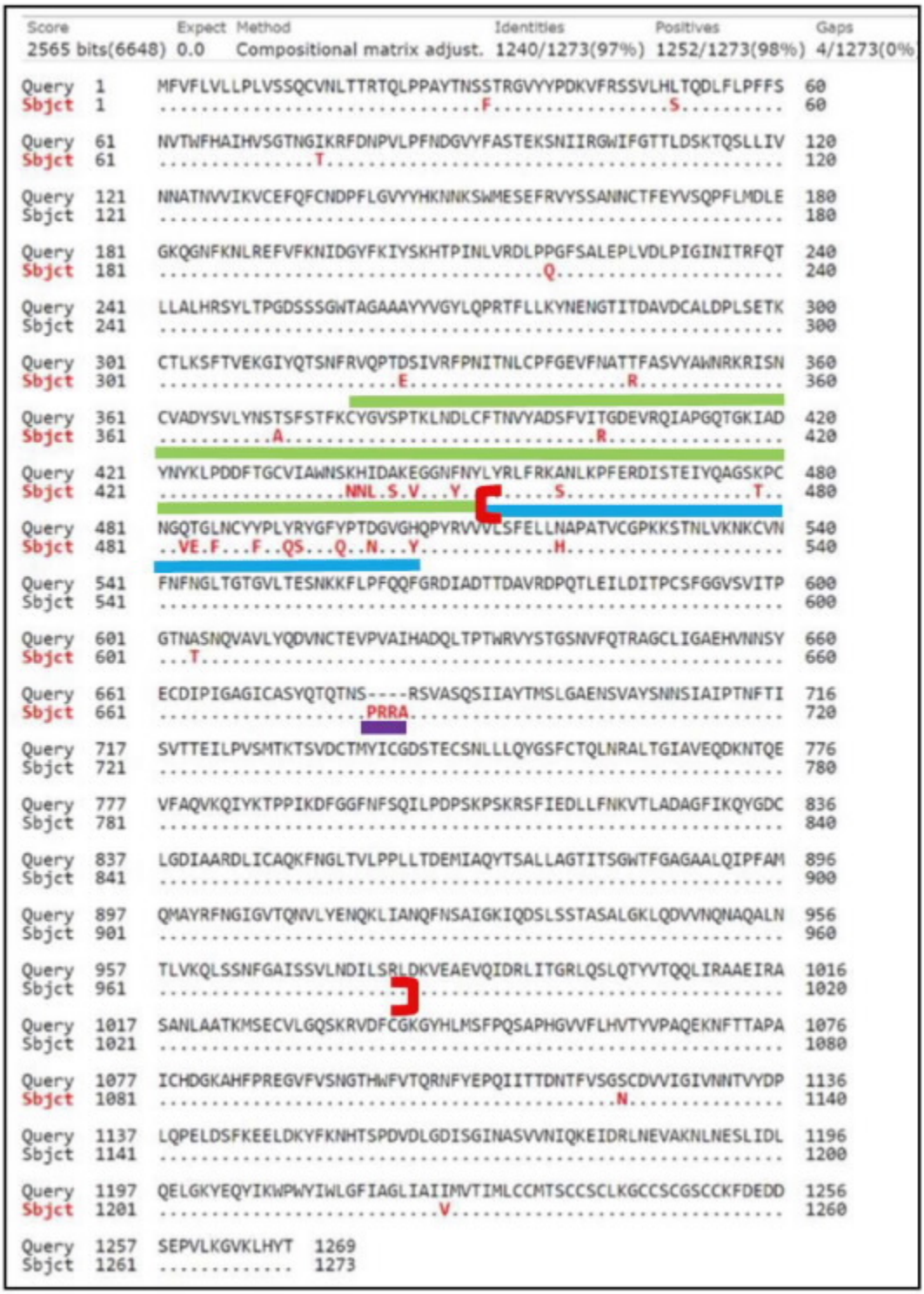
Figure. A comparison of the RaTG13 Spike Protein gene (Query) and the SARS-CoV-2 Reference Sequence (Sbjct) showing the only two Esp3I restriction enzyme cleavage site, both present in RaTG13 but absent in SARS-CoV-2. The restriction sites were identified with the RestrictionMapper site: http://www.restrictionmapper.org/ .The 5' cleavage site is strategically located at the beginning of the receptor binding domain ACE2 contact residues. Despite four of six nt are different these are synonymous changes.

```
Query  1321  ATTGATGCAAAAGAGGGCGGTAATTTTAACTATCTTTACCGTCTCTTTAGAAAAGCTAAT  1380
Sbjct  1321  CTTGATTCTAAGGTTGGTGGTAATTATAATTACCTGTATAGATTGTTTAGGAAGTCTAAT  1380
```

The 3' cleavage site is the only downstream -CGTCTN- sequence found in the CoV-2 Spike Protein, making it unique.

```
Query  2927  TCCTTTCACGTCTCGACAAAGTTGAGGCTGAAGTGCAGATTGACAGGTTGATCACAGGCA  2986
Sbjct  2939  TCCTTTCACGTCTTGACAAAGTTGAGGCTGAAGTGCAAATTGATAGGTTGATCACAGGCA  2998
```

Figure. Comparison of Spike Protein amino acid sequence between RaTG13 (Query) and SARS-CoV-2 (Sbjct). Amino acid substitutions in CoV-2 are shown in red, single letter abbreviation. Green band; receptor binding domain. Blue band; receptor binding domain ACE2 contact residues (Andersen et al, Nature, 2020.). Purple band; polybasic (furin) cleavage site. Red brackets; Esp3I cleavage sites in RaTG13.



Because it has not been established that RaTG13 was the precursor of CoV-2 this evidence statement will not be used at this time to adjust the likelihoods of the origin. If additional information is obtained at a later date this may be revisited.

Likelihood from prior state is unchanged following this evidence analysis:

Zoonotic origin (0.2%) and laboratory origin (99.8%)

Evidence. Location, location, location: Based on the distance between known SARS-CoV-1 laboratory-acquired infections and the hospital of admission of the infected personnel, the WIV is within the expected hospital catchment for a CoV-2 LAI

Hypothesis. Laboratory-acquired infections (LAI) have the property that the hospital of admission of the personnel from the laboratory with the acquired infection are close together, specifically they are within 24.64 km from the laboratory.

Prior data from SARS-CoV-1. There were four LAIs of SARS-CoV-1 that can be used to determine the distance between the laboratory where the infection occurred and the hospital of first admission. The data are here:

SARS-CoV-1 Laboratory Acquired Infection (LAI)	Hospital of admission	Distance (Google Maps)	
In September 2003, a 27-year-old student from the National University of Singapore (NUS) was infected with the SARS virus due to improper experimental procedures	Singapore General Hospital (SGH)	6.3 km	
Baiji Mountain, Sanxia, Taiwan	Taiwan Hoping Hospital, Taipei, Taiwan	27.8 km	
№100 Yingxin Street, Xicheng District, Bejing	Union Hospital, Beiijing, China	7.3 km	
№100 Yingxin Street, Xicheng District, Bejing	Friendship Hospital, Beijing, China	17.6 km	
		mean = 14.75	
		SD = 10.1	
		95% Confidence Interval	14.75 ±9.887

Based on these four cases, the 95% upper confidence limit for the distance from LAI patients to the hospitals of admission is 24.6 km of the laboratory where the infection was acquired.

SARS-CoV-2. Although it is not clear which hospital the first patient was admitted to the following Text-Table contains all likely candidates.

SARS-CoV-2 Potential LAI Source	Hospital of admission	Distance (Google Maps)	Probability of being closer than the average results for SARS-CoV-1	Probability of being farther than the average results for SARS-CoV-1
Wuhan Institute of Virology, Wuhan, China	PLA Hospital, NO. 627 Wuluo Road, Wuchang District, Wuhan, China	4.8 km	0.094	0.906
Wuhan Institute of Virology, Wuhan, China	Wuhan Central Hospital, Wuhan, China	9.1 km	0.338	0.662
Wuhan Institute of Virology, Wuhan, China	Zhongnan Hospital, Wuhan, China	2.8 km	0.019	0.981
Wuhan Institute of Virology, Wuhan, China	Tongji Hospital, Wuhan, China	5.1 km	0.109	0.891
Wuhan Institute of Virology, Wuhan, China	Hubei Maternity and Child Health Care Hospital, Wuhan, China	4.4 km	0.075	0.925
Hypothesis: Given the distance from the SARS-CoV-1 laboratory where an LAI occurred to the hospital of admission for the lab workers who became infected, what is the probability that CoV-2 is also an LAI, given the distance from the hospitals where the first patients were seen to the WIV, the hypothesized source.			Probability calculations based on the use of a log-normal distribution for distances	Probability calculations based on the use of a log-normal distribution for distances

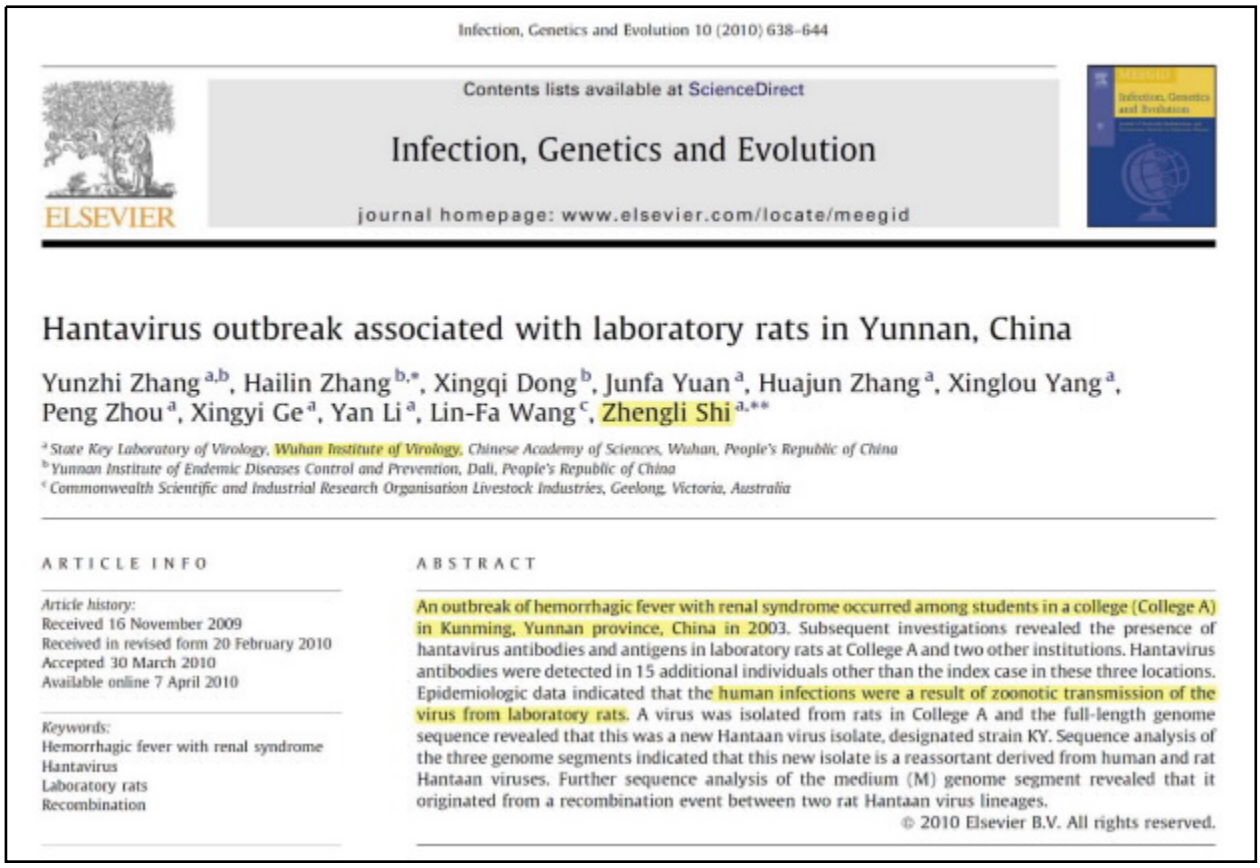
Based on the data for actual LAI for SARS-CoV-1 the distance between the WIV and the hospitals of admission for CoV-2 is consistent with the WIV being the origin for the LAI. There is no evidence the putative LAI for CoV-2 is any different than the known LAIs for CoV-1.

This evidence is not independent of other evidence that is based on location and so it cannot be used independently in the Bayesian analysis. It is included here for completeness.

Likelihood from prior state is unchanged following this evidence analysis:

Zoonotic origin (0.2%) and laboratory origin (99.8%)

Evidence. Dr. Shi successfully identifies a laboratory-acquired infection outbreak from Hanta virus in laboratory rodents.



Infection, Genetics and Evolution 10 (2010) 638–644

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Hantavirus outbreak associated with laboratory rats in Yunnan, China

Yunzhi Zhang [a,b], Hailin Zhang [b,*], Xingqi Dong [b], Junfa Yuan [a], Huajun Zhang [a], Xinglou Yang [a], Peng Zhou [a], Xingyi Ge [a], Yan Li [a], Lin-Fa Wang [c], Zhengli Shi [a,**]

[a] State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, People's Republic of China
[b] Yunnan Institute of Endemic Diseases Control and Prevention, Dali, People's Republic of China
[c] Commonwealth Scientific and Industrial Research Organisation Livestock Industries, Geelong, Victoria, Australia

ARTICLE INFO

Article history:
Received 16 November 2009
Received in revised form 20 February 2010
Accepted 30 March 2010
Available online 7 April 2010

Keywords:
Hemorrhagic fever with renal syndrome
Hantavirus
Laboratory rats
Recombination

ABSTRACT

An outbreak of hemorrhagic fever with renal syndrome occurred among students in a college (College A) in Kunming, Yunnan province, China in 2003. Subsequent investigations revealed the presence of hantavirus antibodies and antigens in laboratory rats at College A and two other institutions. Hantavirus antibodies were detected in 15 additional individuals other than the index case in these three locations. Epidemiologic data indicated that the human infections were a result of zoonotic transmission of the virus from laboratory rats. A virus was isolated from rats in College A and the full-length genome sequence revealed that this was a new Hantaan virus isolate, designated strain KY. Sequence analysis of the three genome segments indicated that this new isolate is a reassortant derived from human and rat Hantaan viruses. Further sequence analysis of the medium (M) genome segment revealed that it originated from a recombination event between two rat Hantaan virus lineages.

© 2010 Elsevier B.V. All rights reserved.

The significance of this evidence is that it demonstrates the methods used by Dr. Shi and the WIV to solve a laboratory-acquired infection outbreak. The methods described herein should be applied to the WIV in order to determine if CoV-2 was also a laboratory-acquired infection.

This will not be used to directly advance the Bayesian analysis.

Likelihood from prior state is unchanged following this evidence analysis:

Zoonotic origin (0.2%) and laboratory origin (99.8%)

Evidence. Bats hibernate when the temperature is below 10.5 C;[124] in Hubei province that begins in September and ends in May.

Average Hubei Temperature by Month

Month	Recommended Rate	Max Temp.	Min Temp.
Jan.	~	-17°C	-26°C
Feb.	√	-13°C	-23°C
Mar.	√√	-3°C	-14°C
Apr.	√√	7°C	-4°C
May.	√√	16°C	4°C
Jun.	√	23°C	11°C
Jul.	√	23°C	13°C
Aug.	√	21°C	11°C
Sep.	√√	15°C	4°C
Oct.	√√	6°C	-5°C
Nov.	√√	-5°C	-16°C
Dec.	√	-15°C	-23°C

Based on this evidence, they would have been hibernating at the time of the first human outbreak in the fall of 2019. Since this evidence is cumulative to the prior evidence from Dr. Shi that the bat host species for CoV-2 does not live in Hubei Province it will not be used to change the Bayesian analysis.

Likelihood from prior state is unchanged following this evidence analysis:

Zoonotic origin (0.2%) and laboratory origin (99.8%)

[124] https://zslpublications.onlinelibrary.wiley.com/doi/abs/10.1111/j.1469-7998.1971.tb01323.x

An official website of the United States government Here's how you know

Press

Business

Employees

Job Seekers

Students

Travelers

Menu

Searc

2017-2021 ARCHIVED CONTENT

You are viewing ARCHIVED CONTENT released online from January 20, 2017 to January 20, 2021.

Content in this archive site is NOT UPDATED, and links may not function.

For current information, go to www.state.gov.

★ ★ ★

Fact Sheet: Activity at the Wuhan Institute of Virology

FACT SHEET

OFFICE OF THE SPOKESPERSON

JANUARY 15, 2021

Share

For more than a year, the Chinese Communist Party (CCP) has systematically prevented a transparent and thorough investigation of the COVID-19 pandemic's origin, choosing instead to devote enormous resources to deceit and disinformation. Nearly two million

people have died. Their families deserve to know the truth. Only through transparency can we learn what caused this pandemic and how to prevent the next one.

The U.S. government does not know exactly where, when, or how the COVID-19 virus—known as SARS-CoV-2—was transmitted initially to humans. We have not determined whether the outbreak began through contact with infected animals or was the result of an accident at a laboratory in Wuhan, China.

The virus could have emerged naturally from human contact with infected animals, spreading in a pattern consistent with a natural epidemic. Alternatively, a laboratory accident could resemble a natural outbreak if the initial exposure included only a few individuals and was compounded by asymptomatic infection. Scientists in China have researched animal-derived coronaviruses under conditions that increased the risk for accidental and potentially unwitting exposure.

The CCP's deadly obsession with secrecy and control comes at the expense of public health in China and around the world. The previously undisclosed information in this fact sheet, combined with open-source reporting, highlights three elements about COVID-19's origin that deserve greater scrutiny:

1. Illnesses inside the Wuhan Institute of Virology (WIV):

- The U.S. government has reason to believe that several researchers inside the WIV became sick in autumn 2019, before the first identified case of the outbreak, with symptoms consistent with both COVID-19 and common seasonal illnesses. This raises questions about the credibility of WIV senior researcher Shi Zhengli's public claim that there was "zero infection" among the WIV's staff and students of SARS-CoV-2 or SARS-related viruses.
- Accidental infections in labs have caused several previous virus outbreaks in China and elsewhere, including a 2004 SARS outbreak in Beijing that infected nine people, killing one.

- The CCP has prevented independent journalists, investigators, and global health authorities from interviewing researchers at the WIV, including those who were ill in the fall of 2019. Any credible inquiry into the origin of the virus must include interviews with these researchers and a full accounting of their previously unreported illness.

2. Research at the WIV:

- Starting in at least 2016 – and with no indication of a stop prior to the COVID-19 outbreak – WIV researchers conducted experiments involving RaTG13, the bat coronavirus identified by the WIV in January 2020 as its closest sample to SARS-CoV-2 (96.2% similar). The WIV became a focal point for international coronavirus research after the 2003 SARS outbreak and has since studied animals including mice, bats, and pangolins.
- The WIV has a published record of conducting "gain-of-function" research to engineer chimeric viruses. But the WIV has not been transparent or consistent about its record of studying viruses most similar to the COVID-19 virus, including "RaTG13," which it sampled from a cave in Yunnan Province in 2013 after several miners died of SARS-like illness.
- WHO investigators must have access to the records of the WIV's work on bat and other coronaviruses before the COVID-19 outbreak. As part of a thorough inquiry, they must have a full accounting of why the WIV altered and then removed online records of its work with RaTG13 and other viruses.

3. Secret military activity at the WIV:

- Secrecy and non-disclosure are standard practice for Beijing. For many years the United States has publicly raised concerns about China's past biological weapons work, which Beijing has neither documented nor demonstrably eliminated, despite its clear obligations under the Biological Weapons Convention.
- Despite the WIV presenting itself as a civilian institution, the United States has determined that the WIV has collaborated on publications and secret projects with

China's military. The WIV has engaged in classified research, including laboratory animal experiments, on behalf of the Chinese military since at least 2017.

- The United States and other donors who funded or collaborated on civilian research at the WIV have a right and obligation to determine whether any of our research funding was diverted to secret Chinese military projects at the WIV.

Today's revelations just scratch the surface of what is still hidden about COVID-19's origin in China. Any credible investigation into the origin of COVID-19 demands complete, transparent access to the research labs in Wuhan, including their facilities, samples, personnel, and records.

As the world continues to battle this pandemic – and as WHO investigators begin their work, after more than a year of delays – the virus's origin remains uncertain. The United States will continue to do everything it can to support a credible and thorough investigation, including by continuing to demand transparency on the part of Chinese authorities.

TAGS

Bureau of East Asian and Pacific Affairs | China | Coronavirus | COVID-19 | Global Health | Office of the Spokesperson

---

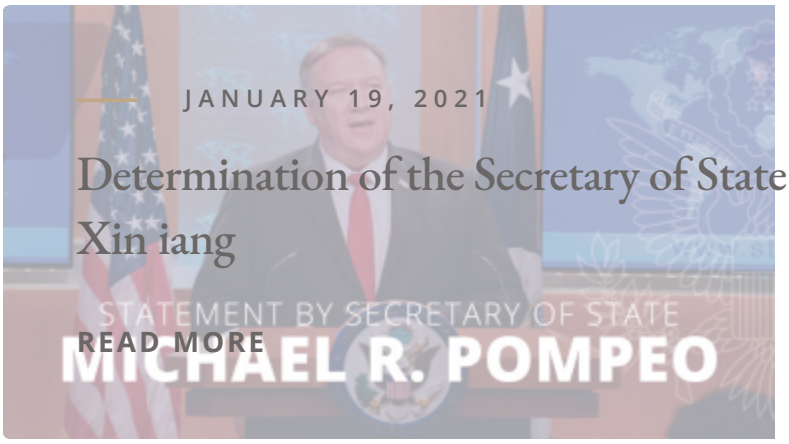
★ ★ ★

Related Articles

JANUARY 19, 2021

Secretary Michael R. Pompeo With John Roberts of Fox News America Reports

READ MORE



JANUARY 19, 2021

Determination of the Secretary of State on Atrocities in Xin iang

READ MORE

JANUARY 18, 2021

Secretary Pompeo's Call with Australian Prime Minister Morrison

READ MORE

JVI Accepts, published online ahead of print on 19 February 2014
J. Virol. doi:10.1128/JVI.00161-14
Copyright © 2014, American Society for Microbiology. All Rights Reserved.

Receptor variation and susceptibility to MERS coronavirus infection

Arlene Barlan[1], Jincun Zhao[2], Mayukh K. Sarkar[1], Kun Li[3],
Paul B. McCray, Jr.[3], Stanley Perlman[2], and Tom Gallagher[1]

[1]Department of Microbiology and Immunology, Loyola University Medical Center, Maywood, IL, 60153; Departments of [2]Microbiology and [3]Pediatrics, University of Iowa, Iowa City, IA, 52242

Running title: Utilization of animal receptors by MERS-CoV

*To whom correspondence should be addressed. E-mail: tgallag@lumc.edu

JVI Accepts published online ahead of print

JVI Accepts published online ahead of print

Abstract

The Middle East Respiratory Syndrome coronavirus (MERS-CoV) recently spread from an animal reservoir to infect humans, causing sporadic severe, and frequently fatal respiratory disease. Appropriate public health and control measures will require discovery of the zoonotic MERS coronavirus reservoirs. The relevant animal hosts are liable to be those that offer optimal MERS virus-cell entry. Cell entry begins with virus spike (S) protein binding to DPP4 receptors. We constructed chimeric DPP4 receptors that have the virus-binding domains of indigenous Middle Eastern animals, and assessed the activities of these receptors in supporting S protein binding and virus entry. Human, camel, and horse receptors were potent and nearly equally effective MERS virus receptors, while goat and bat receptors were considerably less effective. These patterns reflected S protein affinities for the receptors. However, even the low-affinity receptors could hyper-sensitize cells to infection when S-cleaving protease(s) were present, indicating that affinity thresholds for virus entry must be considered in the context of host-cell proteolytic environments. These findings suggest that virus receptors and S protein-cleaving proteases combine in a variety of animals to offer efficient virus entry, and that several Middle Eastern animals are potential reservoirs for transmitting MERS-CoV to humans.

Keywords: virus-receptor interaction / virus entry / zoonotic infection

JVI Accepts published online ahead of print

Importance

Middle East Respiratory Syndrome (MERS) is a frequently fatal disease that is caused by a zoonotic coronavirus (CoV). The animals transmitting MERS-CoV to humans are not yet known. Infection by MERS-CoV requires receptors and proteases on host cells. We compared the receptors of humans and Middle Eastern animals, and found that human, camel and horse receptors sensitized cells to MERS-CoV infection more robustly than goat and bat receptors. Infection susceptibility correlated with affinities of the receptors for viral spike proteins. We also found that the presence of a cell-surface lung protease will greatly increase susceptibility to MERS-CoV, particularly in conjunction with low-affinity receptors. This cataloguing of human and animal host cell factors allows one to make inferences on the distribution of MERS-CoV in nature.

Introduction

The Middle East Respiratory Syndrome coronavirus (MERS-CoV) emerged into the human population in April 2012 (1). This enveloped RNA virus is acquired by respiratory spread, either from infected humans or non-human animals. In individuals with underlying co-morbidities such as diabetes, respiratory or renal disease, infection by this virus can cause widespread pneumonia (2, 3), with case fatality rates of ~ 40%. (4, 5). While possible treatment options for this devastating infection may be forthcoming (6, 7), longer-term approaches to limiting human MERS-CoV infections will come after identifying and quarantining the animal source(s) of this virus.

Bats are implicated as virus reservoirs, as several known bat CoV genomes closely match with MERS-CoV (8-10), and bats harbor the progenitors to human SARS-CoV (11). Camels are also suspected virus reservoirs, because MERS-CoV neutralizing antibodies are prevalent in camel herds from neighboring countries as well as Egypt and the Canary Islands (12, 13). Further, MERS-CoV RNA was detected in a camel herd in Qatar, and two individuals who became infected with the virus were in contact with these animals (14).

Notably, there may be several animal reservoirs, as phylogenetic analyses suggest that there have been multiple, geographically distinct MERS-CoV transmissions from animals to humans (15). Indeed, given that current human-to-human transmissibility indices are low (16), and that animals might asymptomatically and abundantly shed virus, the animal-to-human transmission route may well account for the majority of MERS-CoV infected patients.

While widespread sampling of Saudi Arabian animals will help to identify sources of the virus, a complementary strategy is to carefully assess the infection sensitivities of cells from different animal species. Several laboratories have identified MERS-CoV susceptible cell types, which include human, bat, rabbit and pig (17). Thus these animals express MERS-CoV susceptibility factors, including the MERS-CoV receptor, dipeptidyl peptidase 4 (DPP4; also known as CD26) (17) and quite likely the proteases that cleave the MERS-CoV S proteins and allow them to refold into membrane fusion-active forms (18, 19). The widespread and relatively conserved nature of these susceptibility factors likely accounts for the remarkable polytropic character of MERS-CoV.

What is not known, however, are the relative extents with which each animal DPP4 sensitizes cells to MERS-CoV infection. Organizing animal DPP4s according to their affinity for MERS-CoV, and to their effectiveness at supporting virus entry, will add to our understanding of animal reservoirs and virus transmission pathways. Furthermore, comparative analyses of animal DPP4s and associated host proteases will address important questions concerning MERS-CoV adaptation toward a more narrow range of species, including humans. Our results reveal a hierarchy in animal DPP4 abilities to sensitize cells to MERS-CoV infection, and also demonstrate that the presence of a relevant host protease can potentially alter this hierarchy.

JVI Accepts published online ahead of print

JVI Accepts published online ahead of print

Materials and Methods

Cells. 293β5, 293T, and 293EBNA and Vero E6 cells were maintained in DMEM-10 [Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals), 100 IU/ml penicillin, 1 mg/ml streptomycin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, and 2 mM L-glutamine (Hyclone)]. 293″5 and 293T cells were obtained from Drs. Christopher Wiethoff and Edward Campbell at LUMC. Mouse astrocytoma DBT cells were maintained in MEM-7.5 [Minimal essential medium (MEM) supplemented with 7.5% FBS, 10% tryptose phosphate broth (Difco), 100 IU/ml penicillin, 1 mg/ml streptomycin, and 2mM L-glutamine. All cell lines were propagated as adherent monolayer cultures.

Plasmids and construction of chimeric receptors. Untagged mouse (Ms) DPP4 plasmid pCMV-Kan/Neo-msDPP4 (GenBank BC022183) and C-terminal Myc/FLAG tagged human (Hu) DPP4 plasmid pCMV6-Entry-huDPP4 (NCBI Reference Sequence NM_001935) were purchased from OriGene. pCMV-Kan/Neo-msDPP4 was PCR-amplified with the following primers: AsiSI-forward 5' –TTGGCGATCGCCATGAAGACACCGTGGAAG-3'; NotI-reverse 5' –ATAGTTTAGCGGCCGCAGTGTAAGGAGAAGCACTGCT-3'. Subsequently, MsDPP4 was then cloned into pCMV6-Entry vector between 5'AsiSI and 3'NotI sites. The MsDPP4 vector was then PCR-amplified with the following primers: forward 5'–AGTACCACGGGCTGG-3'; reverse 5'- CTCTTCATAAACCCAGTC-3'. Synthetic DNAs encoding DPP4 blades 4 and 5 of human (Hu), horse (Hs, NCBI Reference Sequence: NC_009161.2), Dubai camel (Cm), goat (Gt, GenBank: AJPT00000000), and pipistrelle bat (Bt, GenBank: KC249974.1) were purchased from GenScript. The camel sequence was obtained by RT-PCR and sequencing from a *Camelus dromedarius* cell line (Dubca; ATCC, CRL 2276). Gibson assembly techniques (20) with the In-Fusion HD Cloning System (Clontech) were then used to insert the various animal DPP4 blades 4 and 5 (b4-5) into the MsDPP4 vector. The chimeric DPP4 receptors were designated as human [Hu(b4-5)], horse [Hs(b4-5)], camel [Cm(b4-5)], goat [Gt(b4-5)] and bat [Bt(b4-5)]. pCAGGS-TMPRSS2-FLAG and pCAGGS-TMPRSS2(S441A)-FLAG were previously constructed (21). Codon optimized MERS S containing a C9 tag was

purchased from Genscript and subsequently cloned into pcDNA3.1+ between EcoRI and NotI restriction sites.

Transfections. Transfections were performed with polyethylenimine (PEI, Polysciences) at a ratio of 1ug DNA: 3ug PEI. DNA:PEI complexes were incubated in OptiMEM (Life Technologies) for 15 minutes before added to plated cells in 6-well or 12-well test plates. One day post transfection, cells were washed with 0.9% saline, treated with trypsin (Hyclone), and then replated into 96-well test plates for later inoculation with pseudoviruses or authentic MERS-CoV.

Pseudovirus production and transduction assays. To generate VSVluc-MERS S pseudovirus, 293T cells were transfected with pcDNA3.1-MERS-S-C9 via PEI transfection. One day post transfection, cells were then inoculated with G*ΔG-VSV-luc, as per recommendations in (22). One day later, media were collected, clarified for 10 minutes at 1,000 x g, labeled as VSVluc-MERS S, and stored at -80C. For transduction assays, 293β5 or DBT cells were first transfected via PEI with the various chimeric DPP4 receptor constructs. After 24 h, cells were then transduced with VSVluc-MERS S. 16 hours after pseudovirus transduction, cells were washed with serum-free DMEM (SFM) and then lysed with 1x passive lysis buffer (Promega). Luminescence was measured after addition of luciferase substrate (Promega) using a Veritas microplate luminometer (Turner BioSystems).

MERS CoV infections. DBT cells were transfected with chimeric DPP4 cDNAs and inoculated 24 h later with MERS-CoV at 1 pfu per cell. After 1h at 37°C, inocula were removed and cells incubated for 48h. Supernatants were collected and infectivities were determined by plaque assays on Vero 81 cells.

MERS S1-Fc production. The MERS S1 coding sequence (human codon-optimized) was PCR amplified with the following primers: NotI-forward 5'-CTAGCGGCCGCAGCCATGATCCATAGCG TCTTCCTCC-3'; ClaI-reverse 5'-ATCGATATACTTACCTGTGGGGGT CAGAGTGGAGGGGGT-3'. The resulting S1-encoding fragment was then recombined via Gibson assembly techniques with a vector fragment that

JVI Accepts published online ahead of print

was PCR amplified from pCEP4-sMHVR-Ig (23) using the following primers: ClaI-forward 5'-ACCCCCTCCACTCTGACCC CCACAGGTAAGTATATCGAT-3'; NotI-reverse 5' GGAGGAAGACGCTATGGATCATGGCTGCGGCCGCTAG-3'.

The resulting plasmid, designated as pCEP4-MERS-S1-Fc, was transfected using PEI into 293 EBNA cells, which contain the EBV EBNA-1 protein necessary for stable maintenance of transfected pCEP4 plasmid DNAs. After 36 h, cells were washed with pre-warmed SFM and incubated in SFM for ~16 h. Conditioned media were collected, cells were returned to DMEM-10 for ~8 h, then placed back with SFM for a subsequent round of S1-Fc secretion and collection. Pooled collections of S1-Fc – containing SFM were concentrated ~ 100 fold using Centricon-30 centrifugation devices (Millipore). Quantifications of MERS S1-Fc protein yields were determined by comparisons with serial dilutions of human IgG standards on western immunoblots, with band intensities quantified by CCD Alphaview instrumentation and software (Protein Simple).

MERS S1-Fc Blocking Assay. DBT cells were transfected with the various chimeric DPP4 receptor constructs in 12-well cell culture plates at seeding density of $3.8x10^5$ cells/well. One day post transfection, cells were trypsinized and re-plated in 96 well plates. MERS S1-Fc was added at indicated concentrations and incubated at 37°C for 1 hour, followed by addition of VSVluc-MERS S pseudoviruses. At 16 h post transduction, cells were lysed with 1X passive lysis buffer (Promega) and luminescence were measured as described above.

Flow Cytometry. DBT cells were transfected with the various chimeric DPP4 receptor constructs, at 1 ..g DNA per 10^6 cells. One day later, cells were detached from plates using Accutase (Millipore), suspended and washed in PBS-2% FBS. Cells were incubated with rabbit anti-FLAG antibody (1:500) (Sigma F7425) or MERS S1-Fc (~50nM) for 1 h on ice, followed by either Alexafluor488 goat anti-rabbit IgG (1:1000) (Lifetech) or Dylight549 goat anti-human IgG (1:1000) (Pierce), for 1h on ice. Cells were analyzed using an LSR Fortessa (BD Biosciences). Data were analyzed using FlowJo software.

JVI Accepts published online ahead of print

TMPRSS2 titration and transduction. DBT cells were PEI transfected in 12-well test plates with Ms DPP4, Hu(b4-5) or Bt(b4-5) with increasing TMPRSS2 or TMPRSS2(S441A). One day post transfection, cells were reseeded into 96-well plates. 6h later, the cells were transduced with VSVluc-MERS S and luminescence was measured 16 h post-transduction as described above.

Cell lysis and western blot analysis. Transfected cells were dissolved on ice for 15 min with TX100 buffer (1%Triton X-100/25mM TrisHCl [pH 7.4]/125 mM NaCl/0.1% protease inhibitors/ 1mM $MgCl_2$, 1mM $CaCl_2$), and nuclei removed by centrifugation. Post-nuclear supernatants were mixed 1:5 with SDS buffer (0.375 M TrisHCl [pH 6.8], 10% SDS, 6% 2-mercaptoethanol, 30% glycerol, 0.375 M TrisHCl [pH 6.8]) , and heated to 95C for 5 min. 30,000 cell equivalents were subjected to SDS-PAGE and proteins transferred to PVDF membranes (Millipore). DPP4 and TMPRSS2 proteins were detected with rabbit anti-FLAG (1:1000; Sigma F7425) and peroxidase-conjugated goat anti-rabbit IgG (1:5000; Pierce). Actin was detected with anti-″-actin peroxidase (1:20,000; Sigma). Membranes treated with ECL substrate (Pierce) were detected with FluorChem E and analyzed with Alphaview software (Protein Simple).

Statistical analyses. Statistical tests of significance were performed using the Holm-Sidak multiple Student's t-test procedure.

JVI Accepts published online ahead of print

JVI Accepts published online ahead of print

Results

Chimeric human – mouse DPP4 receptors. CD26/DPP4 is a type II integral membrane protein, organized as a homodimer, with each monomer comprising a membrane-proximal ±/″ hydrolase domain and a membrane-distal ″-propeller domain (24) (Fig. 1A). The receptor-binding domains (RBDs) of the MERS-CoV S proteins (25) bind to human blades 4 and 5 of the 8-blade ″-propeller (26, 27). Human blades 4 and 5 also bind extracellular adenosine deaminase (ADA) (28), tethering this enzyme onto DPP4+ T cells to provide local control of adenosine levels (29). Murine DPP4, however, does not bind ADA (28), and murine DPP4 is indeed divergent from human in the blade 4-5 region. This divergence suggests that murine DPP4 cannot bind MERS-CoV S proteins and provides an explanation for the observation that MERS-CoV does not infect murine cells (17) or mice (30, 31).

However, murine and human DPP4s are conserved at the flanking blade 3 and 6 regions. Therefore, we reasoned that murine blades 4 and 5 could be replaced with orthologous animal blades, without perturbing the seams between the murine and animal blades. We expected that, unlike the parental murine DPP4, some chimeric DPP4s would function as MERS-CoV receptors, with the receptor functions reflecting the complete, native, animal DPP4s. This expectation was based on the fact that the close contacts between MERS-CoV S and human DPP4 are within the blade 4-5 region (17, 27).

We obtained cDNA clones encoding C-terminal myc and FLAG-tagged mouse (Ms) and human (Hu) DPP4s, and synthetic DNAs encoding Ms and Hu blades 4 and 5. Gibson assembly techniques (20) were then used to replace the Ms blades with the synthetic human DNA, and to reciprocally replace Hu with Ms blades. This provided the construct designated Hu(b4-5), with humanized amino acids 194 – 350 in the Ms DPP4 background (Fig. 1A). The parental and chimeric DPP4s were evaluated by assessing their abilities to sensitize mouse DBT cells to MERS-CoV. Mouse DBT cells were chosen for these experiments because, unlike many human cell lines, they were completely refractory to MERS-CoV infection, and thus might be rendered susceptible by receptor expression. In a

JVI Accepts published online ahead of print

first test, we challenged DPP4-transfected DBT cells with VSV pseudoparticles (22) that encode firefly luciferase (luc) and also display MERS S on their surfaces (VSVluc-MERS S). These pseudoviruses serve as surrogates of authentic MERS-CoV, with Fluc activities after transduction being readouts of potential cell susceptibility to infection. By these assays, both Hu DPP4 and the chimeric Hu(b4-5) equally sensitized cells to transduction, while the Ms DPP4 did not (Fig. 1B). Notably, the reciprocal chimeric Hu DPP4 with Ms blades 4-5 also did not sensitize cells to transduction (data not shown). In a second test, we challenged the DBT-cell transfectants with authentic MERS-CoV, and measured virus output titers by plaque assays. The HuDPP4 and Hu (b4-5) DPP4-positive cells supported MERS-CoV infection equally well (Fig. 1C). Thus, in these assays, the readouts from VSVluc-MERS S pseudovirus transductions paralleled the readouts from authentic MERS-CoV infections. Additionally, the human DPP4 was indistinguishable from a mouse DPP4 that contained only human blades 4 and 5.

To further compare Hu DPP4 with the Hu(b4-5) DPP4, we measured affinities of these receptors for MERS S proteins. Coronavirus S1-Fc proteins are surrogates for authentic viral S (32) and MERS S1-Fc are known to block both MERS S-mediated transduction and MERS-CoV infection (33). To compare the affinities of the two DPP4s for viruses, we introduced graded doses of MERS S1-Fc proteins before and during VSVluc-MERS S transduction. While control N-CEACAM-Fc proteins (23) had no effect on transduction (data not shown), the S1-Fc proteins blocked transduction into Hu and Hu(b4-5) DPP4-positive cells, and with equal effectiveness (Fig. 1D). These analyses further demonstrated that S1-Fc 50% inhibitory concentrations (IC_{50}) were ~10 nM for both receptors. These ~10 nM affinity measurements were similar to the recently-reported Biacore determination of 17.6 nM affinity between human DPP4 and S1 (26).

Chimeric animal DPP4 receptors. These results from comparisons of Hu and Hu(b4-5) DPP4s engendered confidence that animal blade 4-5 regions, placed into the Ms DPP4 context, would reflect the complete animal DPP4s, at least with respect to MERS-CoV entry. Therefore, we proceeded with additional blade 4-5 replacements from other animals. For this study, we focused on DPP4s from indigenous Middle East animals that are considered

to be potential carriers of MERS-CoV. This included Dubai camels, from which we obtained the complete DPP4 cDNA sequence by RT-PCR and RACE. The camel DPP4 sequence was deposited in NCBI Gene Bank (accession # KJ002534) and was also compared with other animal DPP4s in a phylogenetic analysis (Fig. 2). The comparisons also included Pipistrelle bats, one of ~ 20 bat genera residing in Saudi Arabia. We note, however, that Pipistrellis kuhlii is the native Saudi Arabian species, while Pipistrellis pipistrellis is the bat DPP4 that we evaluated.

DPP4 blade 4-5 amino acid sequences from these animals diverge increasingly from humans in the order shown in Fig. 3: horses, camels, goats, bats, and then mice. With respect to the 14 residues contacting S proteins at < 3.6 angstroms ((27) and Fig. 3, arrows), horses have zero, camels two, goats and bats three, and mice five residues diverging from humans. Of note, K392 in blade 6 is close to MERS-CoV S1 in the co-complex structure (27). While blades 4-5 make the key contacts, it is possible that this residue, which also differs among species, could have an additional small effect on MERS-CoV binding.

All of the chimeric DPP4s sensitized DBT cells to VSVluc-MERS S transduction, but to varying levels (Fig. 4A). Human, horse and camel receptors were similarly effective, while goat and bat conferred significantly lower susceptibilities. These trends were also identified in results of authentic MERS-CoV infections (Fig. 4B). These trends did not arise from relative under-expression of the goat and bat DPP4 chimeras, as assessed by western immunoblotting (Fig. 4C). Notably, relative to the transduction assay results, the infection assays revealed far more prominent differences in the receptors' ability to sensitize cells (note that Fig. 4A and 4B y-axes are linear and logarithmic, respectively). This may reflect the fact that the transductions are necessarily single-cycle processes, while the 2-day infections involve multiple cycles of virus entry, release and re-entry, with each entry step being influenced by receptor abundance and affinity. Cumulative low-efficiency entry steps will multiply to create greater end-stage differences in authentic MERS-CoV yields.

JVI Accepts published online ahead of print

JVI Accepts published online ahead of print

S protein affinities for DPP4 receptors. Cell-surface receptor levels and receptor affinities for viruses both contribute to sensitize for infection (34). Cell-surface receptor levels were assessed by flow cytometry, using antibodies to the extracellular FLAG epitope tags. Consistent with the western blot analyses (Fig. 4C), the results (Fig. 5) demonstrated that all chimeric receptors were expressed similarly on surfaces, with minor differences perhaps reflecting slight variations in the ~ 50% efficient plasmid DNA transfections. These results indicated that the reduced susceptibilities of goat and bat DPP4$^+$ cells did not result from diminished surface expression but rather from reduced affinities for MERS-CoV. The same DPP4$^+$ transfectants, assessed in parallel for S1-Fc binding, revealed quite similar S1 associations for all of the receptors except for bat and mouse. Notably, the similar S1 associations were observed amongst fairly divergent receptors.

While flow cytometry results revealed some S-protein affinity differences between the animal DPP4s, they did not provide quantitative binding data, and therefore we adopted the more sensitive approach of measuring affinities via S1-Fc-mediated blockade of MERS S pseudotype transduction. To this end, animal DPP4$^+$ transfectants were incubated with serial dilutions of S1-Fc, then challenged with VSVluc-MERS S. These experiments revealed that the human DPP4 chimeras had the highest affinities of ~ 10 nM for S proteins, with affinities being modestly lower for horse and camel receptors (Fig. 6). In four of four separate experiments, the S1-Fc affinities for Hu(b4-5) exceeded that of Hs(b4-5) and Cm(b4-5) DPP4 chimeras, however, the modest differences between these S1-Fc affinities were not statistically significant. Gt(b4-5), however, had significantly lower affinities of ~ 40 nM, while Bt(b4-5) receptors bound so weakly with S1-Fc that an accurate affinity determination was not obtained by this method (Fig. 6). These quantitative data concurred with the flow cytometric determinations (Fig. 5), and, in part, with DPP4 phylogenetic relationships (Fig. 2).

Role of TMPRSS2 proteases. In addition to S-binding receptors, S-cleaving proteases are principal host factors determining the efficiency of CoV entry (35). Indeed, the cell-surface serine protease TMPRSS2 can augment MERS-CoV infection by up to 100-fold (18, 19). To evaluate the role of TMPRSS2 in relation to DPP4 affinities for MERS-CoV, target cells were

JVI Accepts published online ahead of print

transfected with DNAs encoding both DPP4 and TMPRSS2, or with DPP4 and the control, catalytically-inactive TMPRSS2 (S441A) (36). The catalytically-inactive S441A TMPRSS2 DNA was indistinguishable from empty vector DNA and had no effect on subsequent MERS S-mediated transductions (data not shown). However, relative to the S441A mutant control conditions, TMPRSS2 significantly promoted MERS S-mediated transduction into all of the DPP4$^+$ cells, except those expressing mouse DPP4 (Fig. 7A). We noted a trend in which transduction into cells with relatively low-affinity receptors (Bt(b4-5) and Gt(b4-5)) were augmented by TMPRSS2 more substantially than the corresponding transductions into cells with higher-affinity receptors. Specifically, transduction into Bt(b4-5) and Gt(b4-5) receptor-positive cells was augmented by WT TMPRSS2 ~ 3.6 times more than the corresponding transduction into Cm(b4-5), Hs(b4-5) and Hu(b4-5) DPP4$^+$ cells. These intriguing findings demanded further investigation by TMPRSS2 titration. In the context of the far less adhesive Bt(b4-5) DPP4, TMPRSS2 stimulated virus entry approximately 10-fold (from ~2 to ~20-fold above background levels), while the TMPRSS2 stimulation was a less pronounced ~ 5-fold in the human DPP4 context (Fig. 7B). These were statistically significant differences as assessed by Student's t-test. Notably, by western blotting, we detected relatively constant DPP4 levels with increasing TMPRSS2 (Fig. 7B, bottom panels). TMPRSS2 was not readily detectable by western blotting at the plasmid input doses used (data not shown). However, as it is known that TMPRSS2 proteolyzes the SARS-CoV receptor, angiotensin converting enzyme 2 (21), and augments SARS pseudovirus entry (37), we further investigated DPP4 proteolysis at higher TMPRSS2 levels, and in human 293 cells. In these experiments, human DPP4 was clearly diminished with increasing TMPRSS2, similar to the receptor for SARS-CoV, but only at supraphysiologic TMPRSS2 levels (Fig. 7C). In sum, the main conclusion from these studies involving TMPRSS2, one of the principal MERS-CoV activating proteases, is that cells bearing receptors with low affinities for MERS-CoV will be rendered far more susceptible to infection if the appropriate proteolytic environments are in place.

JVI Accepts published online ahead of print

Discussion

MERS-CoV preferentially infected cells expressing human DPP4, and its S1 domain bound to human DPP4 with higher affinity than to any of the other orthologous animal DPP4s. Thus it is possible that the MERS-CoVs (2012 and 2013 isolates), even with their limited residence in human hosts, are modestly "human-adapted". We suggest that the proximal source of the human-adapted MERS-CoV includes animals whose DPP4 proteins bind to MERS-CoV at near human DPP4 affinity. These animals are likely to maintain a MERS-CoV population that include variants with relatively high affinities for human receptors and thus become the founders that intrude into the human population. Our results suggest that camels and horses can harbor these human founder viruses. Goats and bats may be less prominent hosts for "human" MERS-CoVs, as their DPP4s are less adherent to the human MERS-CoV. However, a very recent report documenting susceptibility of two goat cell lines to MERS-CoV (38) makes it clear that goats cannot be excluded from animals potentially transmitting the virus to humans. Definitive exclusion from the MERS-CoV transmission processes applies to mice, and also ferrets, as a recent publication demonstrated that ferret DPP4 did not bind MERS S1 or support MERS-CoV infection (39). Quite likely there are several mammals entirely refractory to MERS-CoV binding and thus resistant to infection.

The affinity of the MERS-CoV S RBD for human DPP4 is ~ 17 nM (26). This value is ~ 10 times lower than SARS-CoV S RBD for human ACE2 (40). These findings suggest that the MERS-CoV could further adapt to the human DPP4, in the same ways that SARS-CoV adapted to humans. Indeed, the presumed animal precursors of human SARS-CoVs have affinities for ACE2 in the ~ 20-100 nM range (40), and the higher ~ 2 nM affinities of human-adapted SARS-CoV are considered to be central contributors to epidemic potential (41, 42). Thus careful monitoring of human and animal MERS-like CoV spike RBD changes is critical for assessing the likelihood of epidemic spread.

JVI Accepts published online ahead of print

421 It is notable, however, that there appears to be limited diversity in human MERS-CoVs.
422 Nearly identical MERS-CoVs have apparently transmitted from animal to human on more
423 than one occasion and in more than one geographic location (15). This observation is most
424 consistent with the notion that a single animal species, harboring MERS-CoV populations of
425 restricted genomic diversity, served as the reservoir for all human transmission thus far.
426 Furthermore, once in humans, while there is evidence of some genetic drift, there are no
427 changes in the MERS CoV spike RBDs (15). Even in virus isolates from patients with co-
428 morbidities that impaired anti-viral immune responses, and in whom virus might be
429 expected to persist, there is no evidence for changes in the MERS-CoV S RBD. While this
430 apparent genetic stability may simply reflect the small number of human infections and the
431 limited analysis of virus isolates, it is also possible that there is little selective force for
432 change at the level of virus-receptor binding.
433
434 A genetically stable MERS-CoV RBD suggests that binding affinity to the DPP4 receptor
435 may not be the most critical factor for adaptation to new species. MERS-CoV may have
436 stably adapted to an affinity range (~ 10 to ~ 20 nM) that allows for relatively similar
437 binding strength to many different animal DPP4s, i.e., human, camel and horse, and
438 consequent facile transfer between species. This affinity range, however, makes MERS-CoV
439 considerably less adherent than human SARS-CoV for its human ACE2 receptors. It is
440 possible that affinity requirements relate to virus entry events following receptor binding.
441 For SARS-CoV, endocytosis typically precedes S protein proteolysis and tethering of virus
442 to cells via S protein fusion machinery (43, 44). A durable virus:receptor connection may
443 therefore be necessary to maintain SARS-CoV on cells throughout endocytosis, or at least
444 until its S proteins refold into "fusion-intermediate" forms that directly connect the viral
445 and cellular membranes in a receptor-independent fashion. By contrast, the MERS-CoV,
446 appears to more rapidly adopt the fusion-intermediate, membrane-tethering forms at the
447 target cell plasma membrane, and thus may not require such long-lasting association with
448 receptors. This notion is supported by the fact that, unlike SARS-CoV (45), MERS-CoV
449 infection is quite sensitive to a membrane-impermeant inhibitory peptide that targets the
450 fusion intermediate forms (46). Additionally, MERS-CoV infection is remarkably increased
451 by TMPRSS2, a cell surface protease that quite likely cleaves and exposes S protein fusion

452 machinery immediately after receptor binding (19). Our results show that the weakly-
453 adhesive goat and bat DPP4s were especially assisted by TMPRSS2 (Fig. 7A and B),
454 suggesting that a low-affinity receptor can sensitize cells to infection as long as there are
455 protease co-factors available to cleave viral spikes and permit their transitions into
456 membrane fusion-active conformations.
457
458 While no MERS-CoV RBD mutations have been detected, adaptations in its membrane
459 fusion machinery have been repeatedly observed (15, 30). With the caveat of quite limited
460 data on MERS-CoV diversity, we suggest that the selective forces in MERS-CoV are at the
461 level past RBD interaction, and that the MERS-CoV has adapted to a more immediate cell
462 surface proteolysis, which does not demand this sort of stable association with receptors.
463 Indeed, high-affinity receptor interaction may not be a requisite feature of all CoV
464 infections. More relevant to infection may be the host cell proteases and the fusion
465 activation process. These views are consistent with previous hypotheses that enveloped
466 virus:receptor affinities adapt to the minimum levels sufficient to allow for the next
467 membrane fusion stage (34).
468
469
470
471
472
473
474
475
476
477
478
479
480
481

JVI Accepts published online ahead of print

Figure legends

Fig. 1: *Properties of Hu and Hu(b4-5) DPP4s.* (A) DPP4 includes a transmembrane anchor (residues 7-28), a propeller ectodomain (54-497) and a protease ectodomain (508-766). Exchangeable blades 4-5 comprise residues 194-350. Hu and Ms DPP4s are in black and grey, respectively. (B) Mouse DBT cells were transfected with pcDNA3.1 (empty vector, EV) Hu, Ms, or Hu(b4-5) DPP4s, then transduced one day later with VSVluc-MERS S. At 16 h post-transduction, luminescence values (RLU) were measured and plotted. (C) Mouse DBT cells were transfected and infected one day later with MERS-CoV (MOI = 1). Secreted viruses were collected after 2 days and infectivities (PFU/ml) were determined by titration on Vero cell indicators. The hatched horizontal line indicates the lower limit of assay sensitivity. (D) Mouse DBT cells were transfected, and after one day, exposed to the indicated doses of MERS S1-Fc. After 1h at 37°C, VSVluc-MERS S were inoculated, with MERS S1-Fc concentrations remaining the same. At 16 h post-transduction, luminescence values were measured and the relative transduction values were quantified. The concentration of MERS S1-Fc at the IC_{50} value reflects the affinity of MERS S for the DPP4 receptors. In all data sets, the error bars indicate the standard errors of means from triplicate values. Each experiment was repeated twice.

Fig. 2: *Camel DPP4 sequence and phylogenetic relationships with other animal DPP4s.* The complete camel DPP4 open reading frame is deposited in NCBI Gene Bank; accession number KJ002534. Camel DPP4 was compared with the indicated animal DPP4 protein sequences using Molecular Evolutionary Genetics Analysis (MEGA; www.megasoftware.net) to generate the neighbor-joined phylogenetic tree. Numbers at each node are bootstrap values calculated from 500 trees, and represent the percentage of trees that resolved clades at the indicated endpoints. The scale bar indicates the relationship between line lengths and sequence dissimilarities (0.05 = 5 / 100 amino acid divergence). Asterisks denote the animal DPP4s evaluated in this report.

JVI Accepts published online ahead of print

Fig. 3: *Alignment of DPP4 blade 4-5 regions.* The human and animal DPP4 sequences are arranged in order of increasing divergence from human blades 4-5. Light grey highlighting denotes divergent amino acids. Underlined regions denote the conserved terminal residues comprising the junctions between animal blades 4-5 and the mouse DPP4. The arrows and associated numbers at the top point to residues that, in human DPP4, interface closely with the MERS S protein.

Fig. 4: *Transduction and infection of animal DPP4 – positive cells.* (A) DPP4-transfected DBT cells were transfected with the indicated DPP4s, transduced one day later with VSVluc-MERS S, and luciferase accumulations determined 16 h later. Standard errors are shown; n = 6. Statistical significance tests were performed by Student t-test. Data shown are representative of five independent results. (B) DBT cells were infected with MERS-CoV and infectivity in media quantified by plaque assay on Vero cells. The hatched horizontal line indicates the lower limit of assay sensitivity. Standard errors are shown; n = 3. Statistical significance tests were performed by Student t-test. (C) DPP4-transfected DBT cells were lysed at one day post-transfection (the time of pseudovirus and virus inoculation). DPP4 and actin proteins were visualized by immunoblotting.

Fig. 5: *Relative levels of anti-FLAG antibody and MERS S1-Fc binding to cells.* DBT cells transfected with pcDNA3.1 (EV), or with pCMV6-DPP4 chimeras, were incubated with anti-FLAG antibodies (1:500), or with 50 nM S1-Fc, for 1h at 4°C, and analyzed by flow cytometry. . The bars in each panel indicate positive cell windows, and the numbers under the bars indicate the percentage of anti-FLAG antibody and MERS S1-Fc positive cells. Positive cell percentages, after background subtraction, are listed to the right of the figure.

Fig. 6: *Transduction blockade by MERS S1-Fc*. DBT cells were transfected with DPP4 chimeras, incubated with the indicated concentrations of MERS S1-Fc for 1h at 37°C, and transduced with VSVluc-MERS-S. Luminescence values were measured 16 h later. Data were normalized to transduction in the absence of S1-Fc. The concentrations of S1-Fc at each IC_{50} reflect the affinities of MERS spikes for the different DPP4 receptors. The experiment was repeated four times with similar results. Error bars indicate the standard

JVI Accepts published online ahead of print

errors of means from triplicate values. Student's t-tests were used to assess significance of differences between human and animal DPP4 data; n.s. = not significant.

Fig. 7: *Effect of TMPRSS2 on MERS S-mediated transduction*. (A) DBT cells were co-transfected with the indicated DPP4 chimeras and either wild type (WT) or catalytically-inactive (S441A) TMPRSS2 plasmids, at 1 : 0.1 DPP4 : TMPRSS2 DNA ratios. After one day, cell subsets were transduced with VSVluc-MERS-S and luciferase values determined 16 h later. (B) DBT cells were transfected with Hu(b4-5) or Bt(b4-5) DPP4 chimeras, in conjunction with increasing amounts of wild type (WT) or catalytically-inactive (S441A) TMPRSS2 plasmids. After one day, cell subsets were transduced with VSVluc-MERS-S and luciferase values determined 16 h later. The dotted line designates the basal transduction level into MsDPP4 transfected cells. Holm-Sidak Student's t-tests were used to reveal the significance of the relative differences between transduction levels in the presence of S441A and WT TMPRs; p-values are indicated. To obtain the images beneath the graph, the luciferase-containing lysates were subjected to electrophoresis and the DPP4 and actin proteins visualized by western blotting. (C) 293T cells were transfected with HuDPP4 (1 ug), in conjunction with the indicated TMPRSS2 doses. After one day, the HuDPP4, TMPR and actin proteins in cell lysates were visualized by western blotting. The uncleaved (inactive) TMPRSS2 zymogen has a molecular weight of ~ 60 kDa and the proteolytically-active (WT) TMPRSS2 has ~ 25 kDa molecular weight.

ACKNOWLEDGMENTS. This work was supported by a grant from the National Institutes of Health AI-P01 060699 (SP, PBM, TG) and by the Roy J. Carver Charitable Trust (PBM).

JVI Accepts published online ahead of print

JVI Accepts published online ahead of print

REFERENCES

1. **The WHO MERS-Cov Research Group.** 2013. State of Knowledge and Data Gaps of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) in Humans. PLoS currents **5**. DOI: 10.1371 / currents.outbreaks.
2. **Assiri A, Al-Tawfiq JA, Al-Rabeeah AA, Al-Rabiah FA, Al-Hajjar S, Al-Barrak A, Flemban H, Al-Nassir WN, Balkhy HH, Al-Hakeem RF, Makhdoom HQ, Zumla AI, Memish ZA.** 2013. Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory syndrome coronavirus disease from Saudi Arabia: a descriptive study. The Lancet infectious diseases **13:**752-761.
3. **Guery B, Poissy J, el Mansouf L, Sejourne C, Ettahar N, Lemaire X, Vuotto F, Goffard A, Behillil S, Enouf V, Caro V, Mailles A, Che D, Manuguerra JC, Mathieu D, Fontanet A, van der Werf S.** 2013. Clinical features and viral diagnosis of two cases of infection with Middle East Respiratory Syndrome coronavirus: a report of nosocomial transmission. Lancet **381:**2265-2272.
4. **Lim PL, Lee TH, Rowe EK.** 2013. Middle East Respiratory Syndrome coronavirus (MERS CoV): Update 2013. Current infectious disease reports **15:**295-298.
5. 2013. Updated Information on the Epidemiology of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) Infection and Guidance for the Public, Clinicians, and Public Health Authorities, 2012-2013. MMWR. Morbidity and mortality weekly report **62:**793-796.
6. **Falzarano D, de Wit E, Martellaro C, Callison J, Munster VJ, Feldmann H.** 2013. Inhibition of novel beta coronavirus replication by a combination of interferon-alpha2b and ribavirin. Scientific reports **3:**1686.
7. **Falzarano D, de Wit E, Rasmussen AL, Feldmann F, Okumura A, Scott DP, Brining D, Bushmaker T, Martellaro C, Baseler L, Benecke AG, Katze MG, Munster VJ, Feldmann H.** 2013. Treatment with interferon-alpha2b and ribavirin improves outcome in MERS-CoV-infected rhesus macaques. Nature medicine **19:**1313-1317.
8. **Memish ZA, Mishra N, Olival KJ, Fagbo SF, Kapoor V, Epstein JH, Alhakeem R, Durosinloun A, Al Asmari M, Islam A, Kapoor A, Briese T, Daszak P, Al Rabeeah AA, Lipkin WI.** 2013. Middle East Respiratory Syndrome Coronavirus in Bats, Saudi Arabia. Emerging infectious diseases **19**: 1819-1823.
9. **Ithete NL, Stoffberg S, Corman VM, Cottontail VM, Richards LR, Schoeman MC, Drosten C, Drexler JF, Preiser W.** 2013. Close relative of human middle East respiratory syndrome coronavirus in bat, South Africa. Emerging infectious diseases **19:**1697-1699.
10. **Lau SK, Li KS, Tsang AK, Lam CS, Ahmed S, Chen H, Chan KH, Woo PC, Yuen KY.** 2013. Genetic characterization of Betacoronavirus lineage C viruses in bats reveals marked sequence divergence in the spike protein of pipistrellus bat coronavirus HKU5 in Japanese pipistrelle: implications for the origin of the novel Middle East respiratory syndrome coronavirus. Journal of virology **87:**8638-8650.
11. **Ge XY, Li JL, Yang XL, Chmura AA, Zhu G, Epstein JH, Mazet JK, Hu B, Zhang W, Peng C, Zhang YJ, Luo CM, Tan B, Wang N, Zhu Y, Crameri G, Zhang SY, Wang LF, Daszak P, Shi ZL.** 2013. Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. Nature **503:** 535-538.

JVI Accepts published online ahead of print

12. **Reusken CB, Haagmans BL, Muller MA, Gutierrez C, Godeke GJ, Meyer B, Muth D, Raj VS, Vries LS, Corman VM, Drexler JF, Smits SL, El Tahir YE, De Sousa R, van Beek J, Nowotny N, van Maanen K, Hidalgo-Hermoso E, Bosch BJ, Rottier P, Osterhaus A, Gortazar-Schmidt C, Drosten C, Koopmans MP.** 2013. Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study. The Lancet infectious diseases **13:**859-866.
13. **Perera R, Wang P, Gomaa M, El-Shesheny R, Kandeil A, Bagato O, Siu L, Shehata M, Kayed A, Moatasim Y, Li M, Poon L, Guan Y, Webby R, Ali M, Peiris J, Kayali G.** 2013. Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013. Euro surveillance : European communicable disease bulletin **18:** (36):pii200574.
14. **Haagmans BL, Al Dhahiry SH, Reusken CB, Raj VS, Galiano M, Myers R, Godeke GJ, Jonges M, Farag E, Diab A, Ghobashy H, Alhajri F, Al-Thani M, Al-Marri SA, Al Romaihi HE, Al Khal A, Bermingham A, Osterhaus AD, Alhajri MM, Koopmans MP.** 2014. Middle East respiratory syndrome coronavirus in dromedary camels: an outbreak investigation. The Lancet infectious diseases **14**: 140-145.
15. **Cotten M, Watson SJ, Kellam P, Al-Rabeeah AA, Makhdoom HQ, Assiri A, Al-Tawfiq JA, Alhakeem RF, Madani H, Alrabiah FA, Hajjar SA, Al-Nassir WN, Albarrak A, Flemban H, Balkhy HH, Alsubaie S, Palser AL, Gall A, Bashford-Rogers R, Rambaut A, Zumla AI, Memish ZA.** 2013. Transmission and evolution of the Middle East respiratory syndrome coronavirus in Saudi Arabia: a descriptive genomic study. Lancet **382**: 1993-2002.
16. **Breban R, Riou J, Fontanet A.** 2013. Interhuman transmissibility of Middle East respiratory syndrome coronavirus: estimation of pandemic risk. Lancet **382:**694-699.
17. **Raj VS, Mou H, Smits SL, Dekkers DH, Muller MA, Dijkman R, Muth D, Demmers JA, Zaki A, Fouchier RA, Thiel V, Drosten C, Rottier PJ, Osterhaus AD, Bosch BJ, Haagmans BL.** 2013. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. Nature **495:**251-254.
18. **Gierer S, Bertram S, Kaup F, Wrensch F, Heurich A, Kramer-Kuhl A, Welsch K, Winkler M, Meyer B, Drosten C, Dittmer U, von Hahn T, Simmons G, Hofmann H, Pohlmann S.** 2013. The spike protein of the emerging betacoronavirus EMC uses a novel coronavirus receptor for entry, can be activated by TMPRSS2, and is targeted by neutralizing antibodies. Journal of virology **87:**5502-5511.
19. **Shirato K, Kawase M, Matsuyama S.** 2013. Middle East Respiratory Syndrome Coronavirus (MERS-CoV) Infection Mediated by the Transmembrane Serine Protease TMPRSS2. Journal of virology **87:** 12552-12561.
20. **Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, 3rd, Smith HO.** 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature methods **6:**343-345.
21. **Shulla A, Heald-Sargent T, Subramanya G, Zhao J, Perlman S, Gallagher T.** 2011. A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. Journal of virology **85:**873-882.

JVI Accepts published online ahead of print

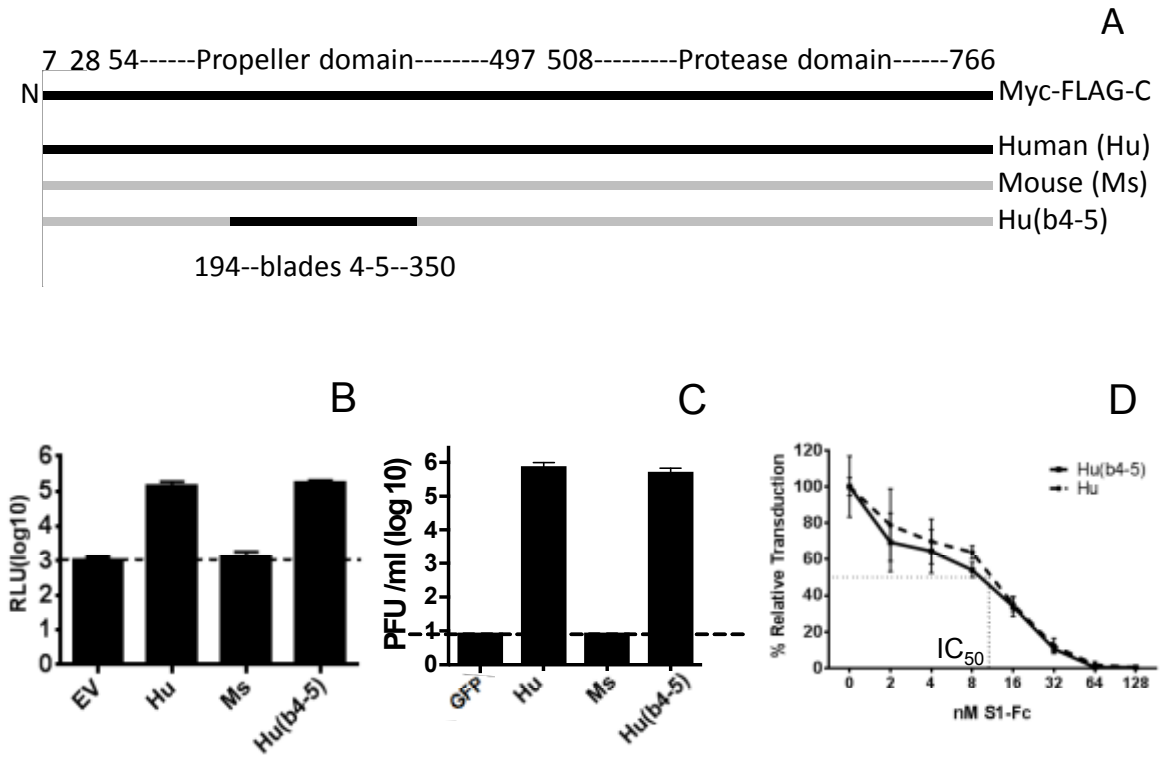
22. **Whitt MA.** 2010. Generation of VSV pseudotypes using recombinant DeltaG-VSV for studies on virus entry, identification of entry inhibitors, and immune responses to vaccines. Journal of virological methods **169:**365-374.
23. **Gallagher TM.** 1997. A role for naturally occurring variation of the murine coronavirus spike protein in stabilizing association with the cellular receptor. Journal of virology **71:**3129-3137.
24. **Engel M, Hoffmann T, Wagner L, Wermann M, Heiser U, Kiefersauer R, Huber R, Bode W, Demuth HU, Brandstetter H.** 2003. The crystal structure of dipeptidyl peptidase IV (CD26) reveals its functional regulation and enzymatic mechanism. Proceedings of the National Academy of Sciences of the United States of America **100:**5063-5068.
25. **Chen Y, Rajashankar KR, Yang Y, Agnihothram SS, Liu C, Lin YL, Baric RS, Li F.** 2013. Crystal structure of the receptor-binding domain from newly emerged Middle East respiratory syndrome coronavirus. Journal of virology **87:**10777-10783.
26. **Lu G, Hu Y, Wang Q, Qi J, Gao F, Li Y, Zhang Y, Zhang W, Yuan Y, Bao J, Zhang B, Shi Y, Yan J, Gao GF.** 2013. Molecular basis of binding between novel human coronavirus MERS-CoV and its receptor CD26. Nature **500:**227-231.
27. **Wang N, Shi X, Jiang L, Zhang S, Wang D, Tong P, Guo D, Fu L, Cui Y, Liu X, Arledge KC, Chen YH, Zhang L, Wang X.** 2013. Structure of MERS-CoV spike receptor-binding domain complexed with human receptor DPP4. Cell research **23:**986-993.
28. **Weihofen WA, Liu J, Reutter W, Saenger W, Fan H.** 2004. Crystal structure of CD26/dipeptidyl-peptidase IV in complex with adenosine deaminase reveals a highly amphiphilic interface. The Journal of biological chemistry **279:**43330-43335.
29. **Kameoka J, Tanaka T, Nojima Y, Schlossman SF, Morimoto C.** 1993. Direct association of adenosine deaminase with a T cell activation antigen, CD26. Science **261:**466-469.
30. **Scobey T, Yount BL, Sims AC, Donaldson EF, Agnihothram SS, Menachery VD, Graham RL, Swanstrom J, Bove PF, Kim JD, Grego S, Randell SH, Baric RS.** 2013. Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus. Proceedings of the National Academy of Sciences of the United States of America **110:**16157-16162.
31. **Coleman CM, Matthews KL, Goicochea L, Frieman MB.** 2013. Wild type and innate immune deficient mice are not susceptible to the Middle East Respiratory Syndrome Coronavirus. The Journal of general virology **95:** 408-412.
32. **Du L, Zhao G, Kou Z, Ma C, Sun S, Poon VK, Lu L, Wang L, Debnath AK, Zheng BJ, Zhou Y, Jiang S.** 2013. Identification of a receptor-binding domain in the s protein of the novel human coronavirus Middle East respiratory syndrome coronavirus as an essential target for vaccine development. Journal of virology **87:**9939-9942.
33. **Mou H, Raj VS, van Kuppeveld FJ, Rottier PJ, Haagmans BL, Bosch BJ.** 2013. The receptor binding domain of the new Middle East respiratory syndrome coronavirus maps to a 231-residue region in the spike protein that efficiently elicits neutralizing antibodies. Journal of virology **87:**9379-9383.
34. **Hasegawa K, Hu C, Nakamura T, Marks JD, Russell SJ, Peng KW.** 2007. Affinity thresholds for membrane fusion triggering by viral glycoproteins. Journal of virology **81:**13149-13157.

JVI Accepts published online ahead of print

35. **Simmons G, Zmora P, Gierer S, Heurich A, Pohlmann S.** 2013. Proteolytic activation of the SARS-coronavirus spike protein: Cutting enzymes at the cutting edge of antiviral research. Antiviral research **100**: 605-614.
36. **Hooper JD, Clements JA, Quigley JP, Antalis TM.** 2001. Type II transmembrane serine proteases. Insights into an emerging class of cell surface proteolytic enzymes. The Journal of biological chemistry **276:**857-860.
37. **Heurich A, Hofmann-Winkler H, Gierer S, Liepold T, Jahn O, Pohlmann S.** 2014. TMPRSS2 and ADAM17 Cleave ACE2 Differentially and Only Proteolysis by TMPRSS2 Augments Entry Driven by the Severe Acute Respiratory Syndrome Coronavirus Spike Protein. Journal of virology **88:**1293-1307.
38. **Eckerle I, Corman VM, Muller MA, Lenk M, Ulrich RG, Drosten C.** 2014. Replicative Capacity of MERS Coronavirus in Livestock Cell Lines. Emerging infectious diseases **20:** 276-279.
39. **Raj VS, Smits SL, Provacia LB, van den Brand JM, Wiersma L, Ouwendijk WJ, Bestebroer TM, Spronken MI, van Amerongen G, Rottier PJ, Fouchier RA, Bosch BJ, Osterhaus AD, Haagmans BL.** 2014. Adenosine deaminase acts as a natural antagonist for dipeptidyl peptidase 4-mediated entry of the middle East respiratory syndrome coronavirus. Journal of virology **88:**1834-1838.
40. **Wu K, Peng G, Wilken M, Geraghty RJ, Li F.** 2012. Mechanisms of host receptor adaptation by severe acute respiratory syndrome coronavirus. The Journal of biological chemistry **287:**8904-8911.
41. **Li W, Zhang C, Sui J, Kuhn JH, Moore MJ, Luo S, Wong SK, Huang IC, Xu K, Vasilieva N, Murakami A, He Y, Marasco WA, Guan Y, Choe H, Farzan M.** 2005. Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2. The EMBO journal **24:**1634-1643.
42. **Li W, Wong SK, Li F, Kuhn JH, Huang IC, Choe H, Farzan M.** 2006. Animal origins of the severe acute respiratory syndrome coronavirus: insight from ACE2-S-protein interactions. Journal of virology **80:**4211-4219.
43. **Wang H, Yang P, Liu K, Guo F, Zhang Y, Zhang G, Jiang C.** 2008. SARS coronavirus entry into host cells through a novel clathrin- and caveolae-independent endocytic pathway. Cell research **18:**290-301.
44. **Simmons G, Gosalia DN, Rennekamp AJ, Reeves JD, Diamond SL, Bates P.** 2005. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proceedings of the National Academy of Sciences of the United States of America **102:**11876-11881.
45. **Bosch BJ, Martina BE, Van Der Zee R, Lepault J, Haijema BJ, Versluis C, Heck AJ, De Groot R, Osterhaus AD, Rottier PJ.** 2004. Severe acute respiratory syndrome coronavirus (SARS-CoV) infection inhibition using spike protein heptad repeat-derived peptides. Proceedings of the National Academy of Sciences of the United States of America **101:**8455-8460.
46. **Gao J, Lu G, Qi J, Li Y, Wu Y, Deng Y, Geng H, Li H, Wang Q, Xiao H, Tan W, Yan J, Gao GF.** 2013. Structure of the fusion core and inhibition of fusion by a heptad-repeat peptide derived from the S protein of MERS-CoV. Journal of virology **87**: 13134-13140.

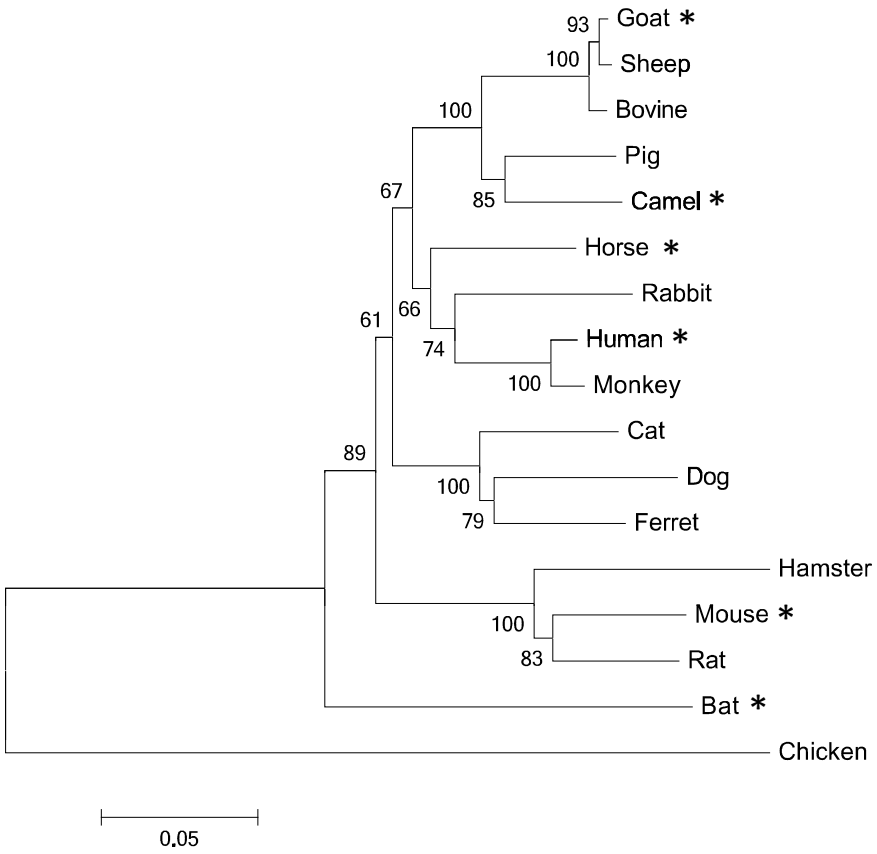
JVI Accepts published online ahead of print

Fig. 1



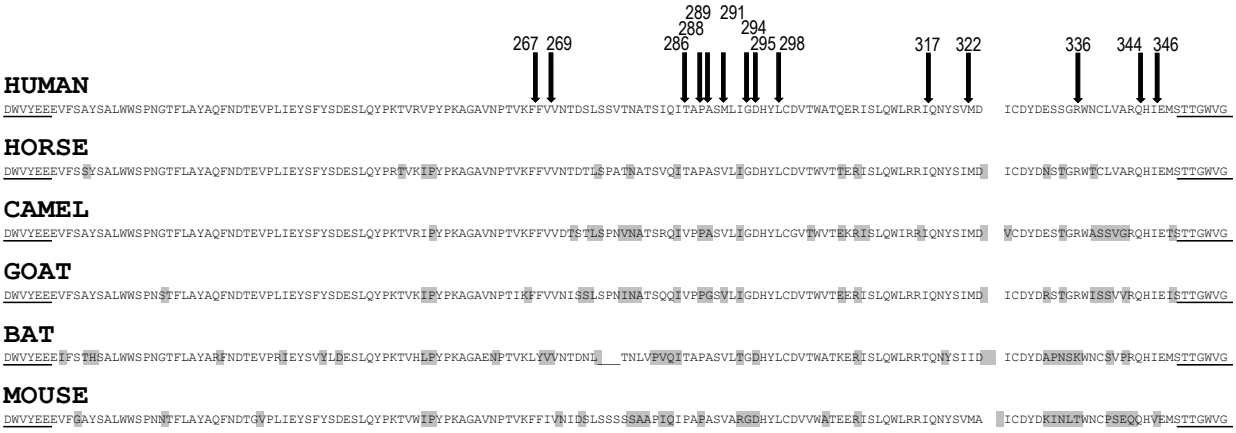
JVI Accepts published online ahead of print

Fig. 2



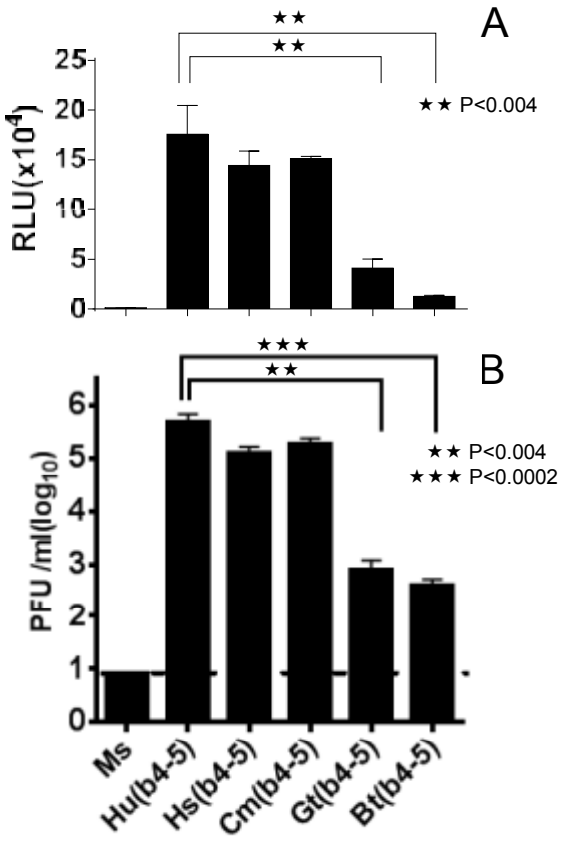
JVI Accepts published online ahead of print

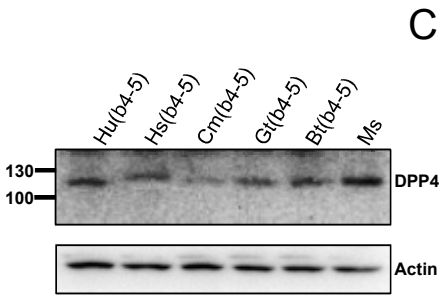
Fig. 3



JVI Accepts published online ahead of print

Fig. 4





JVI Accepts published online ahead of print

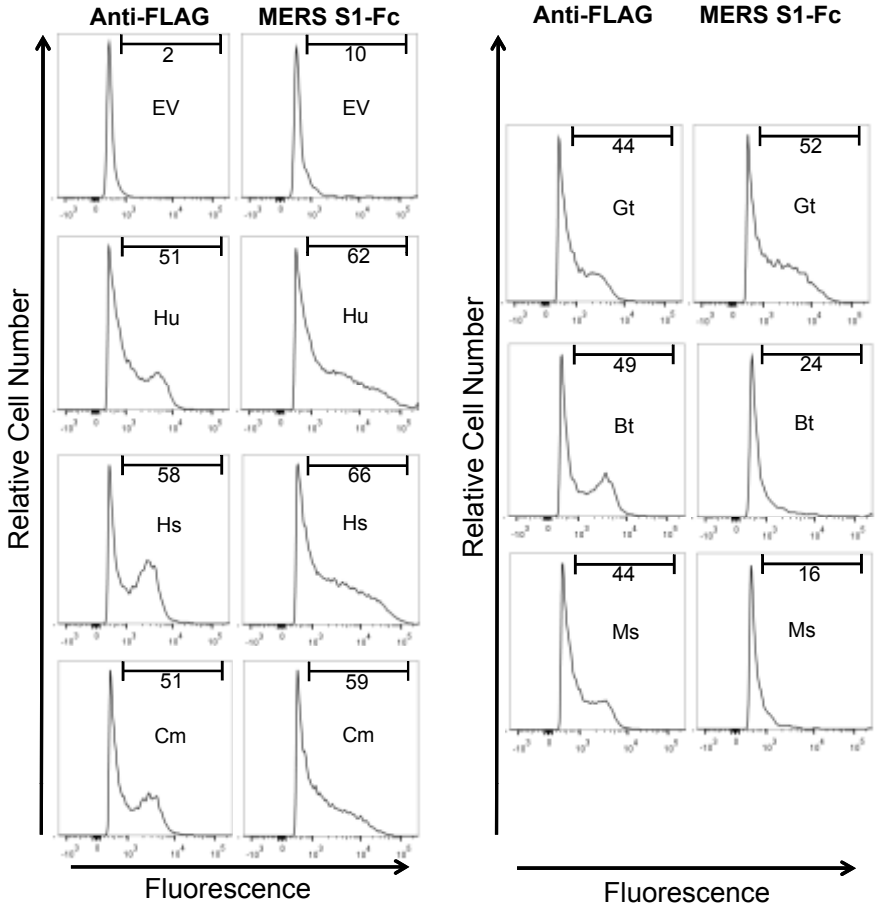
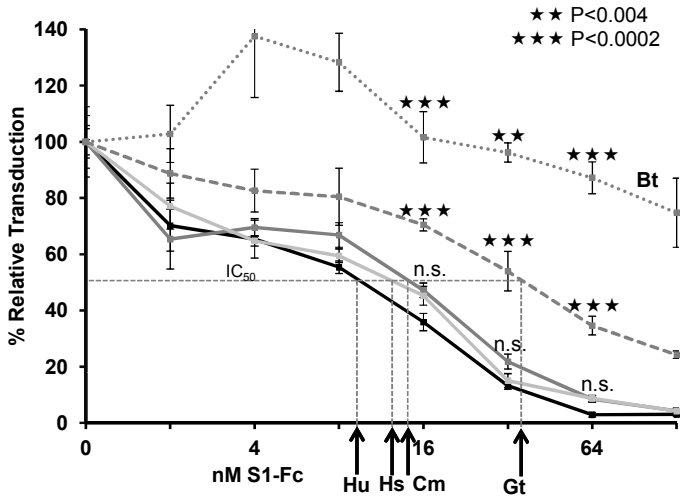


Fig. 5

	Anti-FLAG	S1-Fc
Hu	49	52
Hs	56	56
Cm	49	49
Gt	42	42
Bt	47	14
Ms	42	6

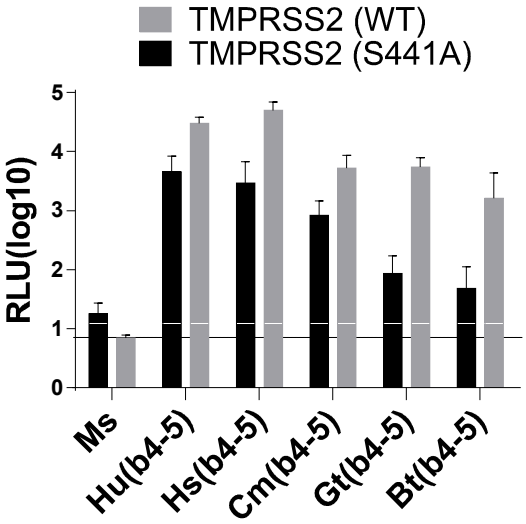
JVI Accepts published online ahead of print

Fig. 6



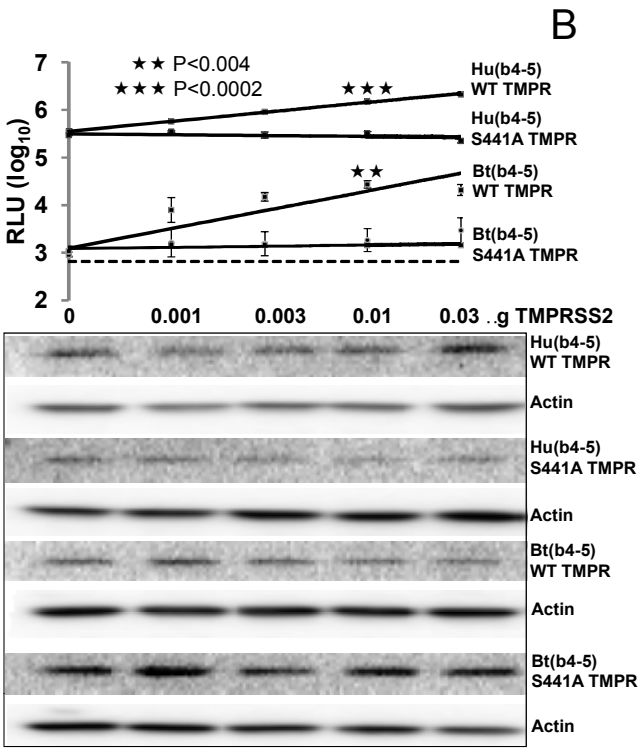
JVI Accepts published online ahead of print

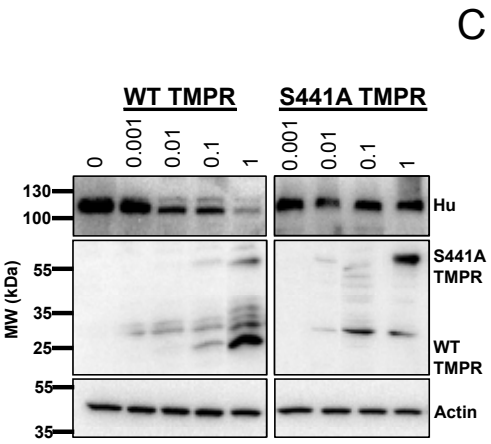
Fig. 7A



JVI Accepts published online ahead of print

Fig. 7BC





Bat Origins of MERS-CoV Supported by Bat Coronavirus HKU4 Usage of Human Receptor CD26

Qihui Wang,[1] Jianxun Qi,[1] Yuan Yuan,[1,2] Yifang Xuan,[3] Pengcheng Han,[4] Yuhua Wan,[1,5] Wei Ji,[6] Yan Li,[1] Ying Wu,[1] Jianwei Wang,[7] Aikichi Iwamoto,[8,9] Patrick C.Y. Woo,[10,11] Kwok-Yung Yuen,[10,11,12] Jinghua Yan,[1] Guangwen Lu,[1] and George F. Gao[1,2,3,6,12,13,*]

[1]CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
[2]School of Life Sciences, University of Science and Technology of China, Hefei 230027, Anhui Province, China
[3]Research Network of Immunity and Health, Beijing Institutes of Life Science, Chinese Academy of Sciences, Beijing 100101, China
[4]State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China
[5]School of Life Sciences, Anhui University, Hefei 230039, China
[6]National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (China CDC), Beijing102206, China
[7]MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, China
[8]China-Japan Joint Laboratory of Molecular Microbiology and Molecular Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
[9]Division of Infectious Diseases, Advanced Clinical Research Center, Department of Infectious Diseases and Applied Immunology, Research Hospital, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan
[10]State Key Laboratory for Emerging Infectious Diseases, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region 999077, China
[11]Department of Microbiology, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region 999077, China
[12]Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang University, Hangzhou 310003, China
[13]Office of Director-General, Chinese Center for Disease Control and Prevention (China CDC), Beijing 102206, China
*Correspondence: gaof@im.ac.cn
http://dx.doi.org/10.1016/j.chom.2014.08.009

SUMMARY

The recently reported Middle East respiratory syndrome coronavirus (MERS-CoV) is phylogenetically closely related to the bat coronaviruses (BatCoVs) HKU4 and HKU5. However, the evolutionary pathway of MERS-CoV is still unclear. A receptor binding domain (RBD) in the MERS-CoV envelope-embedded spike protein specifically engages human CD26 (hCD26)to initiate viral entry. The high sequence identity in the viral spike protein prompted us to investigate if HKU4 and HKU5 can recognize hCD26 for cell entry. We found that HKU4-RBD, but not HKU5-RBD, binds to hCD26, and pseudotyped viruses embedding HKU4 spike can infect cells via hCD26 recognition. The structure of the HKU4-RBD/hCD26 complex revealed a hCD26-binding mode similar overall to that observed for MERS-RBD. HKU4-RBD, however, is less adapted to hCD26 than MERS-RBD, explaining its lower affinity for receptor binding. Our findings support a bat origin for MERS-CoV and indicate the need for surveillance of HKU4-related viruses in bats.

INTRODUCTION

Coronaviruses (CoVs)are a group of enveloped, single-stranded RNA viruses taxonomically affiliated with the *Coronaviridae* family (Lai et al., 2007). Based on genotypic and serological characteristics, coronaviruses are further classified into four species: *Alpha*, *Beta*, *Gamma* (King et al., 2012), and *Deltacoronavirus* (Woo et al., 2009). Both alpha (alphaCoVs) and betacoronaviruses (betaCoVs) can cause human diseases (Lai et al., 2007). Most human infections related to alpha and betaCoVs, such as coronaviruses NL63, 229E, OC43, and HKU1 (reviewed in Wevers and van der Hoek, 2009), which commonly manifest as self-limiting common cold-like illnesses, though more severe diseases can develop in children, the elderly, and immunocompromised patients (Chiu et al., 2005; Gorse et al., 2009; Jean et al., 2013; Jevšnik et al., 2012). BetaCoVs, however, can also be life threatening and have pandemic potential (Ksiazek et al., 2003; Zaki et al., 2012). In 2003, the severe acute respiratory syndrome coronavirus (SARS-CoV), a lineage B betaCoV (Lu and Liu, 2012), caused >8,000 infections and >800 deaths worldwide (WHO, 2004). In 2012, another lineage C betaCoV named the Middle East respiratory syndrome coronavirus (MERS-CoV) (King et al., 2012) initially emerged in Saudi Arabia (Bermingham et al., 2012; Zaki et al., 2012) and then spread to other countries in the Middle East, Europe, Asia, and the US, leading to 701 confirmed cases as of June 16th, 2014 (WHO, 2014) with a fatality rate of approximately 35%. These unexpected outbreaks highlight the public health significance of betaCoVs, especially those in lineages B and C.

In addition to MERS-CoV, lineage C betaCoVs include two other important members: the bat coronaviruses (BatCoVs) HKU4 and HKU5 (Lu and Liu, 2012). These viruses were first identified as genomes in 2006 in lesser bamboo bats

(*Tylonycteris pachypus*) and Japanese pipistrelles (*Pipistrellus abramus*), respectively (Woo et al., 2006), and remain circulating in bats (Lau et al., 2013). Bats have been implicated as the largest reservoir of alpha and betaCoVs (Li et al., 2005b; Woo et al., 2012) and play pivotal roles in interspecies transmission of coronaviruses. This is best exemplified by SARS-CoV, which was recently shown to originate from Chinese horseshoe bats (Ge et al., 2013) and likely transmitted directly to humans (Ge et al., 2013) or through an intermediate host such as the palm civet (Guan et al., 2003). The emergent MERS-CoV might also be of bat origin, with gene fragments being identified in bats from both Saudi Arabia (Memish et al., 2013) and Africa (Ithete et al., 2013) that are nearly identical to those of MERS-CoV in sequence. Despite the fact that the BatCoVs HKU4 and HKU5 are bat derived, the potential of both viruses evolving for human adaptation cannot be excluded because of their close phylogenetic relationship with MERS-CoV (Lau et al., 2013; Lu and Liu, 2012; van Boheemen et al., 2012). Nevertheless, such presumptions are largely based on their genomic features and phylogenetic status (Lau et al., 2013; Woo et al., 2006, 2007).

Virus infections initiate with the binding of viral particles to host surface cellular receptors. Identification of the functional receptors represents a central issue in studying viral pathogenesis. Several host peptidases, including aminopeptidase N (Delmas et al., 1992; Yeager et al., 1992) and angiotensin converting enzyme 2 (hACE2)(Hofmann et al., 2005; Li et al., 2003), are utilized by coronaviruses for cell entry. A timely study on MERS-CoV identified a third peptidase recognized by coronaviruses: dipeptidyl peptidase 4 (DPP4 or CD26) (Raj et al., 2013). This enzyme is a type II transmembrane protein contributing to the regulation of various physiological processes (Gorrell et al., 2001) and is "hijacked" by MERS-CoV to infect humans. This work pioneered the study on lineage C betaCoVs for potential cellular receptors. However, it remains unknown which molecules can be recognized by BatCoVs HKU4 and HKU5.

The receptor binding of coronaviruses is mediated by the spike (S) protein embedded in the viral envelope (Lai et al., 2007). In most cases, S is further cleaved by host proteases into S1 and S2 subunits that function to engage receptors and mediate membrane fusion, respectively (Lai et al., 2007). High sequence identities (>50%) in S have been observed between BatCoV HKU4/HKU5 and MERS-CoV (Lau et al., 2013), raising the possibility that these two viruses may use human CD26 (hCD26) as a receptor.

We previously delineated the molecular basis of MERS-CoV binding to hCD26. The S1 domain responsible for hCD26 recognition is located in a C-terminal 240-residue receptor binding domain (RBD)that is composed of a core and an external subdomain (Lu et al., 2013). The latter engages the receptor and is therefore also designated as the receptor binding motif (RBM) (Lu et al., 2013). Despite the overall high sequence identities in S, amino acid variance in the RBD region, especially in the RBM region, is still obvious among BatCoV HKU4, HKU5, and MERS-CoV. Investigation of the hCD26-binding potential of BatCoVs HKU4 and HKU5 is therefore a key to understanding the biology of these bat-derived viruses, their potential threat to human health, and the evolutionary pathway of MERS-CoV.

In this study, we demonstrated that the RBD domain of BatCoV HKU4 (HKU4-RBD), but not the equivalent domain of BatCoV HKU5 (HKU5-RBD), can bind to hCD26. The binding affinity between HKU4-RBD and hCD26 was determined by surface plasmon resonance (SPR) to be within the micromolar range. Despite the observed lower affinity compared to that between the MERS-CoV RBD (MERS-RBD) and hCD26, we found that pseudoviruses containing BatCoV HKU4 S can infect human Huh7 cells via hCD26. The findings provided solid evidence that BatCoV HKU4 recognizes hCD26 as a cellular receptor, indicating its potential for adaptation to infect humans. We further solved the complex structure of HKU4-RBD bound to hCD26 to delineate the basis of receptor recognition. Our structural analysis and mutagenesis also demonstrated a similar hCD26-binding mode between BatCoV HKU4 and MERS-CoV, but the details of variant amino acid interactions responsible for the differences in binding affinity are distinct. Our work also supports the notion that MERS-CoV most likely originated from bats.

RESULTS

HKU4-RBD, but Not HKU5-RBD, Binds to hCD26

Our previous study determined the hCD26-interacting region as an RBD spanning amino acids 367–606 of the MERS-CoV S (Lu et al., 2013). The phylogenetic relatedness between BatCoV HKU4/HKU5 and MERS-CoV (Figure 1A) prompted us to further investigate the equivalent RBD regions (S amino acids 372–611 for BatCoV HKU4, HKU4-RBD; and 375–604 for BatCoV HKU5, HKU5-RBD) in these bat-derived viruses for their potential to bind hCD26. A recent phylogenetic analysis of 13 BatCoV HKU4 and 15 BatCoV HKU5 strains revealed that the S gene of MERS-CoV is more closely related to BatCoV HKU4 than to BatCoV HKU5 (Lau et al., 2013). Using only the RBD amino acids, a concise phylogenetic tree was constructed, and a sequence alignment comparison was performed among BatCoV HKU4, HKU5, MERS-CoV, and SARS-CoV. Consistently, BatCoV HKU4 clustered with MERS-CoV with high bootstrap support, together joining BatCoV HKU5 to form an evolutionary branch distant from SARS-CoV (Figure 1A). At the sequence level, MERS-RBD exhibits identity to HKU4-RBD slightly higher than HKU5-RBD does (54.4% versus 52.9%). Nevertheless, the pairwise alignment clearly showed two marked deletions located in a region corresponding to the external subdomain of MERS-RBD in HKU5-RBD, but not in HKU4-RBD (Figure 1B). According to our previous study (Lu et al., 2013), this subdomain is responsible for receptor recognition. Therefore, we hypothesized that BatCoV HKU4 stands a better chance than BatCoV HKU5 of engaging hCD26.

Thus, we prepared the individual Fc fusion RBD proteins and tested their binding avidities to human hepatoma Huh7 cells (CD26-expressing) via flow cytometry. Consistent with previous reports (Raj et al., 2013), Huh7 cells express abundant hCD26 protein on their membrane surface (Figure 2A). MERS-RBD, as a natural viral ligand of hCD26, avidly binds to Huh7 cells. HKU4-RBD, but not HKU5-RBD, also bound to Huh7 cells (Figure 2B). Compared to MERS-RBD, however, an evidently smaller fluorescence shift was observed for HKU4-RBD, indicating a lower binding potency than MERS-RBD. An hCD26-specific antibody rather than an isocontrol immunoglobulin G (IgG) blocked the Huh7 surface attachment by HKU4-RBD (Figure 2C). The observed Huh7 binding was also inhibited by the soluble

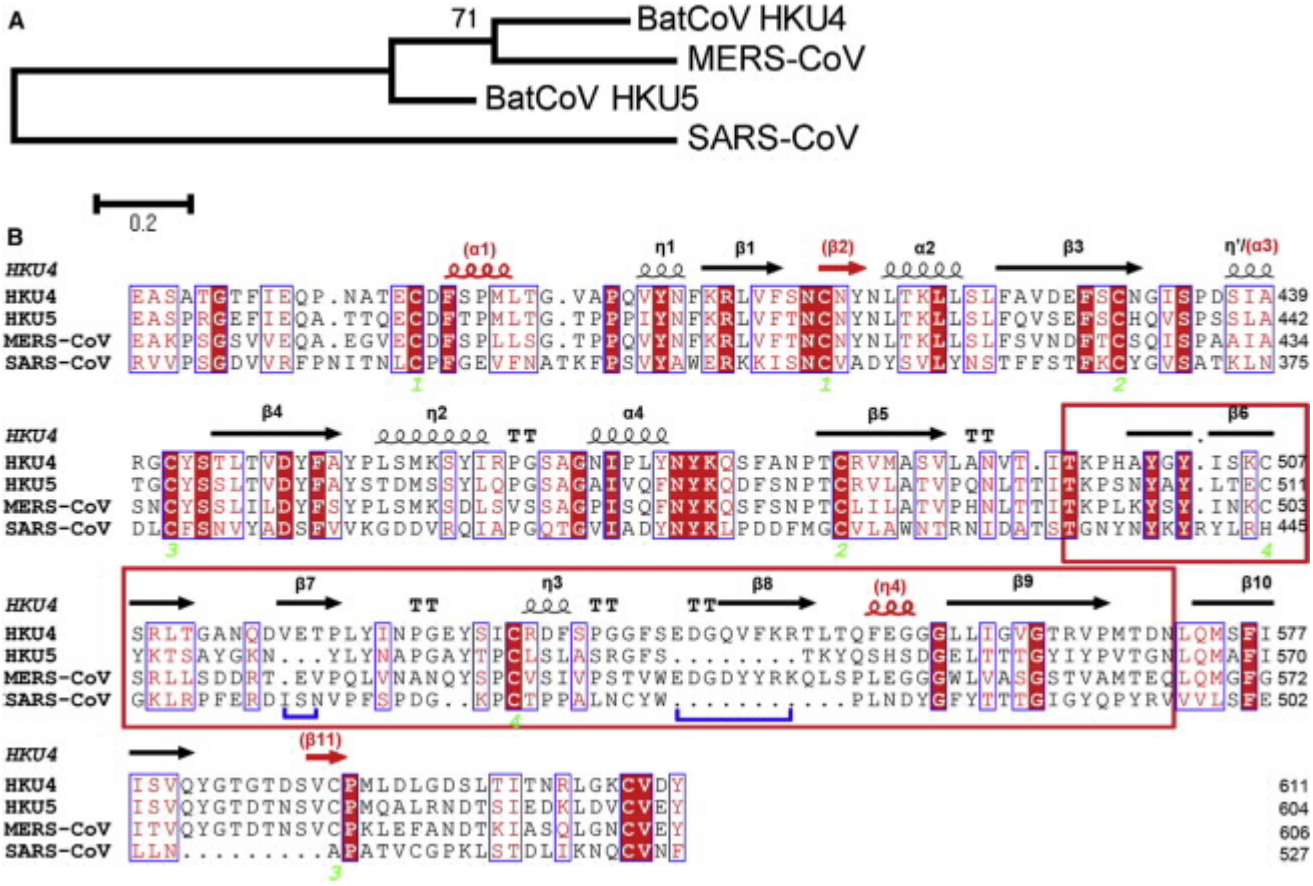


Figure 1. Comparison of the HKU4-RBD, HKU5-RBD, MERS-RBD, and SARS-RBD Sequences

(A) Phylogenetic tree generated using MEGA (Tamura et al., 2013) with the indicated RBD sequences.

(B) Structure-based sequence alignment. The secondary structure elements are defined based on an ESPript (Gouet et al., 1999) algorithm and are labeled as in a previous report on the MERS-RBD structure (Lu et al., 2013). Spiral lines indicate a or 3_{10} helices, while arrows represent b strands. Helices a1 and h4 and strands b2 and b11 are not preserved in the HKU4-RBD structure and are marked in red. The element equivalent to the MERS-RBD helix a3 exhibits characteristics of a 3_{10} helix in HKU4-RBD and is therefore labeled as h^0. The external subdomain is highlighted by enclosure with a red box. The two deletions in HKU5-RBD are marked with blue lines. The Arabic numerals 1–4 indicate cysteine residues that pair to form disulfide bonds. See also Figure S3.

ectodomain protein of hCD26, but not by the extracellular fragment of the SARS-CoV receptor hACE2 (Figure 2D). We also utilized the hCD26-negative BHK cell line in the binding assay. As expected, surface binding to BHK cells could only be observed for HKU4-RBD and MERS-RBD after transfection of the cells with an hCD26-expressing plasmid (Figures 2E–2H). Taken together, these results clearly demonstrated that BatCoV HKU4, unlike its close relative BatCoV HKU5, is capable of binding to hCD26 via the spike-RBD region.

The Interaction between HKU4-RBD and hCD26 Is Specific but of Low Affinity

We next set out to characterize the interaction between HKU4-RBD and hCD26 using real-time biophysical binding assays. The proteins prepared from insect cells were purified to homogeneity (Figure S1, available online) and subjected to SPR experiments. As assay controls, MERS-RBD and SARS-RBD were found to bind potently to their respective canonical receptors (Figures 3A and 3B). HKU4-RBD interacted with hCD26, but not with hACE2 (Figures 3C and 3D). However, in contrast to the control pairs showing slow on and off rates, the Biacore binding profile between HKU4-RBD and hCD26 revealed fast association/dissociation kinetics. The equilibrium dissociation constant (K_D) of HKU4-RBD binding to hCD26 was calculated to be 35.7 mM (Figures 3C and S2), which is approximately three orders of magnitude lower than that of MERS-RBD to hCD26 (Lu et al., 2013). Though HKU5-RBD behaved similarly to its HKU4 homolog protein by gel filtration (Figure S1), no hCD26 binding was observed (Figure 3E), which is consistent with our flow cytometric assays (Figures 2B and 2F).

Cell Infection of BatCoV HKU4 Pseudoviruses Is Mediated by hCD26

With evidence of binding between HKU4-RBD and hCD26, we then tested the potential of this human surface molecule to function as a receptor for BatCoV HKU4. Lentiviral particles pseudotyped with either the BatCoV HKU4 or the MERS-CoV S were individually prepared in 293T cells. The successful incorporation of the viral S protein into the pseudovirus envelope was ascertained by western blotting using a monoclonal antibody

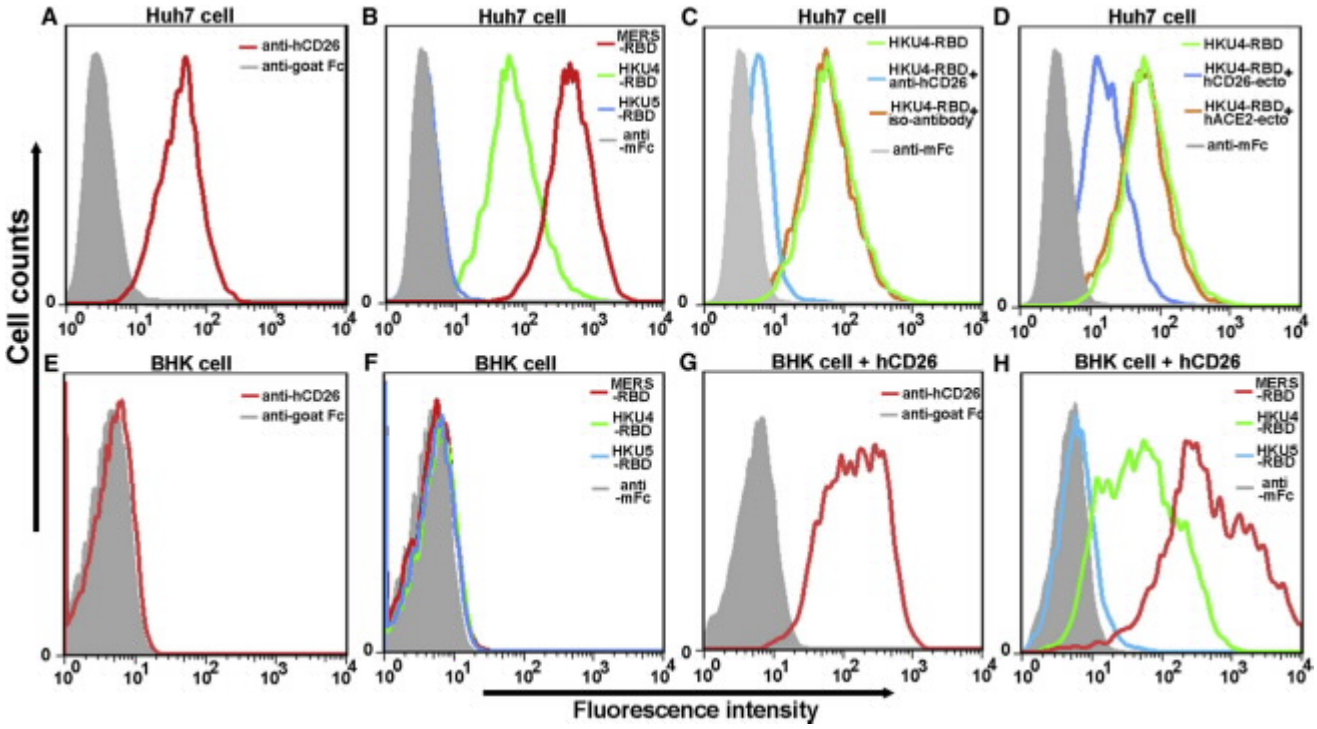


Figure 2. Characterization of Binding between HKU4-RBD and hCD26 by Flow Cytometry
(A) Huh7 cells stained with an anti-hCD26 antibody.
(B) Huh7 cells stained with MERS-RBD, HKU4-EBD, or HKU5-RBD.
(C) Huh7 cells stained with HKU4-RBD in the presence of an anti-hCD26 antibody or an isocontrol antibody.
(D) Huh7 cells stained with HKU4-RBD in the presence of hCD26 (hCD26-ecto) or hACE2 (hACE2-ecto) ectodomain protein.
(E) BHK cells stained with an anti-hCD26 antibody.
(F) BHK cells stained with MERS-RBD, HKU4-RBD, or HKU5-RBD.
(G) hCD26-transfected BHK cells stained with an anti-hCD26 antibody.
(H) hCD26-transfected BHK cells stained with MERS-RBD, HKU4-RBD, or HKU5-RBD.

recognizing a FLAG epitope engineered at the S C terminus (Figure 4A). In accordance with a previous report (Gierer et al., 2013), the majority of the MERS-CoV S protein was proteolytically cleaved (Figure 4A). In marked contrast, however, BatCoV HKU4 S remained largely intact (Figure 4A), displaying a pattern similar to that of SARS-CoV (Moore et al., 2004; Simmons et al., 2004).

The viral infection mediated by BatCoV HKU4 S was then tested in Huh7 cells. With a pseudotyped vector encoding luciferase, the infection efficiency was determined by quantifying luciferase activities in the cell lysates. As a positive control, pseudoparticles bearing the MERS-CoV S protein robustly infected Huh7 cells, showing an 400-fold increase in the luciferase signal as compared to particles bearing no S protein. However, no evident cell infection was observed for the BatCoV HKU4 pseudoviruses (Figure 4B).

According to previous studies on other coronaviruses (reviewed in Simmons et al., 2013), proteolytic separation of the S protein into functional S1 and S2 subunits by host cell proteases is a prerequisite to subsequent membrane fusion. Noting the inefficient S proteolysis in the BatCoV HKU4 pseudoviruses above, we hypothesized that in vitro treatment of the virus particles with proteases such as trypsin might activate the S protein, enabling viral entry into the cells upon receptor recognition. Therefore, the harvested BatCoV HKU4 pseudovirus particles were treated with various concentrations of trypsin (2.5–40 mg/ml), incubated with fetal bovine serum to inactivate the trypsin, and then used to infect Huh7 cells. Proteolysis of the S protein was observed in a concentration-dependent manner for the pseudoviruses (Figure 4A). Accordingly, significant increases (>150-fold) in the luciferase activity were recorded for pseudoviruses pretreated with 10, 20, and 40 mg/ml of trypsin, demonstrating successful entry into the cells (Figure 4B). Then, pseudoviruses digested with 10 mg/ml trypsin were used in the subsequent antibody blocking assays. As expected, a concentration-dependent inhibition of cell infection was observed using the hCD26 antibody that specifically blocked the Huh7 surface attachment of HKU4-RBD (Figure 4C). Therefore, we provided direct evidence that pseudoviruses bearing BatCoV HKU4 S infect human cells via hCD26 recognition.

Complex Structure between HKU4-RBD and hCD26

We further used cocrystallography to study the molecular basis of binding between HKU4-RBD and hCD26. The two purified proteins were mixed in vitro to allow the formation of heterocomplexes, and we managed to solve the structure at a resolution of 2.6 Å (Table S1). In the crystallographic asymmetric unit, HKU4-RBD and hCD26 were observed to form two 1:1 binding

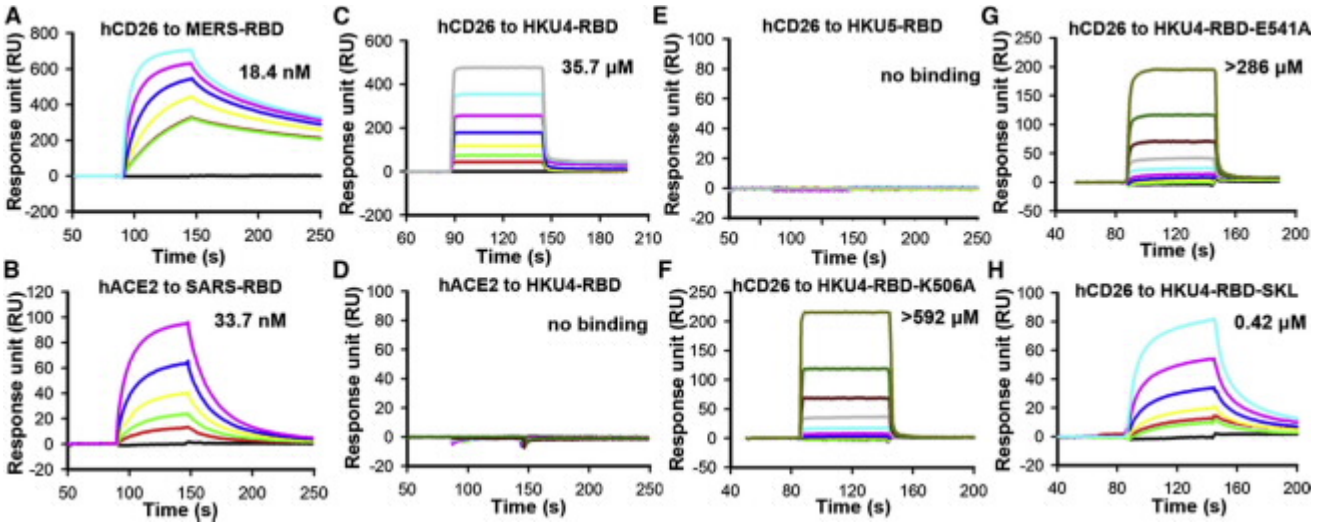


Figure 3. Specific Interaction between HKU4-RBD and hCD26 Characterized by SPR

(A–H)The indicated wild-type or mutant RBD proteins were immobilized on the chip and tested for binding with gradient concentrations of hCD26 or hACE2. The binding profiles are shown.

(A)hCD26 binding to MERS-RBD. (B)hACE2 binding to SARS-RBD. (C)hCD26 binding to HKU4-RBD. (D)hACE2 binding to HKU4-RBD. (E)hCD26 binding to HKU5-RBD. (F)hCD26 binding to HKU4-RBD-K506A. (G)hCD26 binding to HKU4-RBD-E541A. (H)hCD26 binding to HKU4-RBD-SKL, a triple mutant of S540W, K547R, and L558W. The dissociation constants were calculated to be 35.7 and 0.42 mM for the hCD26/HKU4-RBD and hCD26/HKU4-RBD-SKL pairs, respectively, and >400 mM for the hCD26/HKU4-RBD-K506A and hCD26/HKU4-RBD-E541A pairs. See also Figures S1 and S2.

complexes that are related by a two-fold axis (Figure 5A). These two complexes are of essentially the same structure, showing a root-mean-square deviation (rmsd) of 0.18 Å for all Ca pairs. For hCD26, clear electron densities could be traced for 727 amino acids from R40 to P766. As observed in previous reports (Engel et al., 2003), this human enzyme folds into two structural domains: an a/b hydrolase domain and an eight-bladed b-propeller domain. hCD26 utilizes its propeller blades IV and V to recognize HKU4-RBD (Figures 5A and 5B). The two hCD26 molecules in the asymmetric unit were assembled into a dimer in the structure, with each protomer engaging one HKU4-RBD via its propeller. This leads to an overall U-shaped structure very similar to that observed for the MERS-RBD/hCD26 complex (Lu et al., 2013).

In the complex structure, HKU4-RBD contains 208 consecutive density-traceable residues, spanning T386 to L593. The folded structure comprises a core subdomain located distally from the engaging hCD26 and an external subdomain recognizing blades IV and V of the receptor propeller (Figure 5B). The core subdomain involves a five-stranded b sheet (b1, b3, b4, b5, and b10) forming a "nucleus" in the center, several helices (a helices a2 and a4, and 3_{10} helices h1, h^0, and h2) decorating the center sheet on the exterior, and three disulfide bonds (C388/C412, C430/C483, and C442/C590) stabilizing the subdomain structure in the interior. The N and C termini are in close proximity, extending away from the bound receptor. The external subdomain is a strand-dominated structure with four anti-parallel b strands (b6–b9) and exposes a flat sheet-face for receptor engagement. An extra disulfide bond (C507/C531) located in this external region links helix h3 to strand b6 (Figures 1B and 5B).

As expected, this BatCoV HKU4 viral ligand exhibits significant structural homology to its MERS-CoV homolog, in agreement with the >50% sequence identity between the two molecules (Figure 1). Superimposition of the HKU4-RBD structure onto a previously reported MERS-RBD structure (Protein Data Bank [PDB]: 4KQZ) revealed an rmsd of 1.1 Å for 194 equivalent Ca atoms. In comparison to MERS-RBD, the majority of the secondary structure elements are well preserved in HKU4-RBD. The latter, however, lacks an a helix (a1) and two small strands (b2 and b11) in its core subdomain and is devoid of a 3_{10} helix (h4) in the external subdomain. In addition, the helix preceding strand b3 exhibits characteristics of a 3_{10} helix (h^0) in HKU4-RBD, rather than being a-helical (a3) as in MERS-RBD (Figures 1 and S3).

Atomic Details at the Binding Interface between HKU4-RBD and hCD26

In the complex structure, large surface areas (968.7 $Å^2$ in HKU4-RBD and 1,033.9 $Å^2$ in hCD26) are buried by the two binding entities. Therefore, we scrutinized this extended buried surface to identify key amino acids involved in complex formation. Residues located within the van der Waals (vdw) contact distance (4.5 Å resolution cutoff) between HKU4-RBD and hCD26 were selected (Table S2), and a series of hydrophilic amino acids located along the interface were found to form a solid network of H bond and salt bridge interactions (Figures 5B and 5C). These strong polar contacts include the HKU4-RBD residue K506 interacting with the receptor amino acids T288 and A289, N514 and Q515 with R317, D516 with Y322, E541 and D542 with K267, and K547 with I295 (Figure 5C). In addition, several residues (such as Y460 located in helix h2 of the RBD core subdomain) were further shown to contribute to the receptor binding by providing multiple vdw contacts (Table S2). It is notable that K506 and E541 of the viral ligand afforded both side-chain H bond and

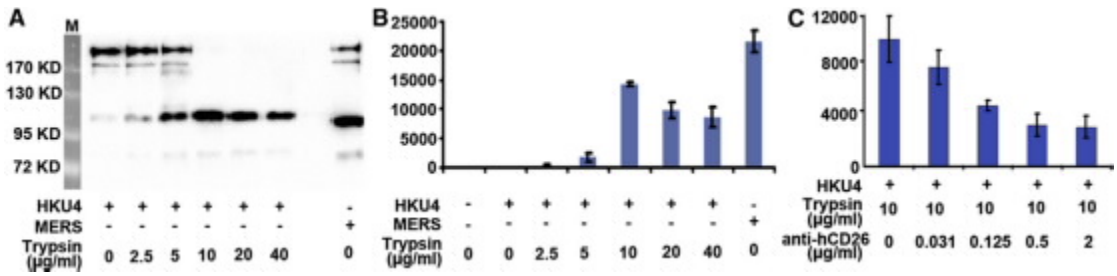


Figure 4. Huh7 Infection by Lentiviral Particles Pseudotyped with BatCoV HKU4 S

(A) Proteolytic processing of the embedded BatCoV HKU4 S by trypsin. The HKU4 pseudovirus was treated with trypsin at the indicated concentrations and characterized with an antibody recognizing a FLAG epitope engineered at the S C terminus. The untreated MERS pseudovirus was included as a reference control.

(B) A Huh7 cell infection assay with the indicated pseudoviruses.

(C) An antibody blocking assay using HKU4 pseudoviruses treated with 10 mg/ml trypsin and an anti-hCD26 antibody. The recorded fluorescence intensities were plotted as histograms, and the error bars represent ± SD for triplicate experiments.

multi-vdw interactions in this network (Table S2). Thus, these residues were individually mutated to alanine, and the resultant HKU4-RBD mutants were tested for receptor binding using SPR assays. The calculated K_Ds for the two mutants binding to hCD26 were >400 mM, which is an 10-fold decrease in binding affinity compared to that of the wild-type protein (Figures 2F, 2G, and S2), demonstrating their important roles in receptor recognition. Interestingly, these two amino acids are conserved between HKU4-RBD and MERS-RBD (Figure 1B) and are also involved in the binding of the MERS-CoV ligand to hCD26 (Lu et al., 2013).

In addition to the aforementioned contact network, extra HKU4-RBD/hCD26 interactions involve a small hydrophobic patch (Figure 5D) and a sugar-mediated engagement (Figure 5E). The former is located in the proximity to a bulged hCD26 helix, which packs amino acids A291, L294, and I295 against RBD residues L510 and I560; the latter consists of a carbohydrate moiety linked to hCD26 N229 and amino acids E541 and Q544 in HKU4-RBD, which together form two H bonds.

Variant Amino Acid Interaction Details with hCD26 between HKU4-RBD and MERS-RBD

Overall, the HKU4-RBD/hCD26 structure solved in this study is very similar to the previously reported structure of MERS-RBD bound to hCD26. The two complex structures can be superimposed onto each other well, except for two interstrand loops (the b6/b7 and b8/b9 loops) in the RBD, which are distinctly oriented between the two viral ligands (Figure 6A). Despite variance in sequence, HKU4-RBD and MERS-RBD engage the same IV and V blades of the hCD26 b-propeller, demonstrating an overall similar recognition mode for hCD26 between BatCoV HKU4 and MERS-CoV.

We next compared the receptor binding details between the two viruses by characterizing the vdw contacts for each RBD residue along the binding interface. In both HKU4-RBD and MERS-RBD, a limited number of intermolecule contacts are contributed by the core subdomain residues, such as those located in the h2 (e.g., HKU4-Y460 and MERS-D455) and a4 (e.g., HKU4-N468 and MERS-P463) helices. These amino acids are adapted for hCD26 in HKU4-RBD slightly better than for that in MERS-RBD. For example, both HKU4-Y460 and HKU4-N468 provide more vdw contacts than the equivalent residues in MERS-RBD (Figures 6B and 6C).

In contrast to the core subdomain, with only limited contributions to hCD26 engagement, the majority of the ligand/receptor intermolecule contacts are provided by the amino acids located in the RBD external subdomain. Most HKU4 residues in this region contact hCD26 less efficiently than the corresponding MERS-RBD amino acids. Among these, the S540/W535 and K547/R542 pairs in the b8 strand and the L558/W553 pair in the b9 strand are most evident, each showing 20–30 contact differences (Figures 6B and 6C). Reminiscent of the low binding affinity of HKU4-RBD to hCD26, these three residues in HKU4-RBD were mutated to the MERS-RBD amino acids, and the resultant mutant protein was tested for receptor binding by SPR. The calculated dissociation constant for this triple mutant was 0.42 mM, indicating that its binding affinity is 100-fold greater than that of the wild-type parental protein. In addition, the mutation also shifted the binding kinetics from a fast-on/fast-off mode, as observed for the wild-type HKU4-RBD, to a slow-on/slow-off mode, as observed for the MERS-RBD (Figures 2H and S2). We believe that the resultant introduction of extra vdw contacts and hydrophobic interactions by substituting these three residues in HKU4-RBD with more hydrophobic amino acids (e.g., tryptophan) led to the change in the binding kinetics. A similar phenomenon of altered K_{on} and/or K_{off} rates resulting from changes in hydrophobic interaction has also previously been observed in other protein interactions (Weihofen et al., 2004).

DISCUSSION

Entry into susceptible host cells is the first step in the virus life cycle, and each entry process starts with receptor recognition. Identification of the cellular receptors for a virus is therefore a key question in viral pathogenesis studies. The BatCoVs HKU4 and HKU5 represent two important bat-derived coronaviruses closely related to MERS-CoV (Lau et al., 2013; van Boheemen et al., 2012). In this study, we performed a functional assay to identify potential receptors for these viruses. We found that HKU4-RBD, a protein domain spanning residues 372–611 of the BatCoV HKU4 S, but not the equivalent S region of BatCoV

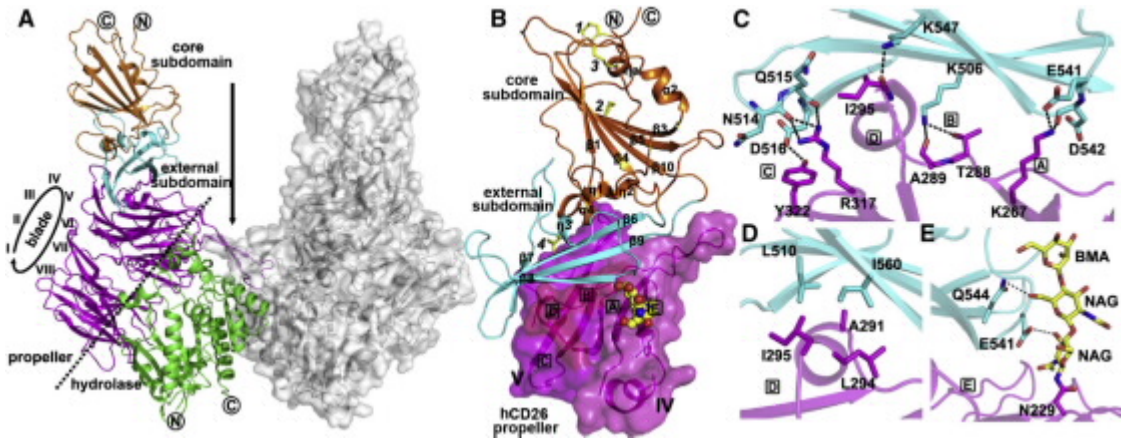


Figure 5. The Complex Structure of HKU4-RBD Bound to hCD26

(A) The overall structure. The two 1:1 complexes related by a two-fold axis (vertical arrow) are shown in cartoon and surface representations, respectively. The core and external subdomains of HKU4-RBD and the b-propeller and hydrolase domains of hCD26 are individually labeled and highlighted in orange, cyan, magenta, and green, respectively. The propeller blades (I–VIII)and the protein N/C termini are marked.

(B) A magnified view of the HKU4-RBD structure and the ligand/receptor interface. The secondary structure elements are specified by ESPript and labeled for the viral ligand. Yellow sticks marked with Arabic numbers indicate disulfide bonds. For the receptor, only propeller blades IV and V that engage HKU4-RBD are shown, using a surface representation.

(C–E) The important contact sites are marked with boxed letters A–E and are further delineated for interaction details as follows. (C) A solid network of H bond and salt bridge interactions. (D) A small patch of hydrophobic interactions. (E) Extra H bond contacts contributed by a carbohydrate moiety linked to hCD26 N229. The residues involved and the carbohydrates referred to are shown and labeled. See also Tables S1 and S2.

HKU5, binds to hCD26. In addition, the complex structure of HKU4-RBD bound to hCD26 was solved, demonstrating a receptor recognition mode similar to that observed for MERS-RBD, though with variant amino acid interaction details and a low binding affinity. We further showed that lentivirus particles pseudotyped with BatCoV HKU4 S could infect Huh7 cells via engagement of hCD26. Taken together, we provide the comprehensive data showing that hCD26 is also a functional receptor for the bat-derived HKU4 coronavirus. In support of this, a similar study of the functional reactivity of BatCoV HKU4 S for hCD26 was recently reported online (Yang et al., 2014) during the revision of our manuscript.

Coronavirus S proteins are among the typical class I membrane fusion proteins (Gao, 2007; Harrison, 2008; Xu et al., 2004a, 2004b). Activation of the subsequent membrane fusion process requires, in most cases, proteolysis of the fusion proteins by host cell proteases. This proteolytic process either occurs during or after maturation of the viruses, such as with murine hepatitis virus (MHV) (Frana et al., 1985) and MERS-CoV (Gierer et al., 2013), or occurs during viral entry, such as with SARS-CoV (Simmons et al., 2013). When we analyzed the lentivirus particles, the pseudotyped BatCoV HKU4 S was predominantly uncleaved. Accordingly, we initially were unable to observe any infection of Huh7 cells by the HKU4 pseudoviruses. However, by mimicking the maturation process of MERS-CoV, we showed that treatment of the pseudoviruses with trypsin enabled hCD26-mediated Huh7 infection. These results demonstrated that engagement of a receptor by, and proteolytic activation of, the envelope S protein remain two prerequisite factors for BatCoV HKU4 infection, as with other coronaviruses (Simmons et al., 2013). By identifying hCD26 as a functional receptor for BatCoV HKU4, the spatiotemporal processing of its S protein to allow subsequent membrane fusion remains an unresolved issue that should be further explored in the future.

Our results should also shed light on virus isolation. Since the first identification of the full-length HKU4 genome in bats in 2006 (Woo et al., 2006), culturing of the viruses has been unsuccessful. Further trials should consider cell lines expressing hCD26 but displaying variant proteases (e.g., cathepsin L, furin/furin-like proteases, HAT, and TMPRSS2) that are commonly involved in the processing of coronavirus spikes (Simmons et al., 2013). Alternatively, including small amounts of trypsin during viral culturing may facilitate the viral infection and thereby progeny virus production.

The previous SARS epidemic and the recent emergence of MERS-CoV in the Middle East serve as a constant reminder of the importance of identifying potential newly emerging coronaviruses in their natural animal reservoirs. Bats are natural reservoirs of many alphaCoVs and betaCoVs, which provide viral genes for the genesis of newly emerging coronaviruses with interspecies transmission potential. Phylogenetic dating suggests three suspected interspecies jumps of animal betaCoVs into humans, two of which (the SARS-CoV and MERS-CoV) are very likely of bat origin (Ge et al., 2013; Ithete et al., 2013; Li et al., 2005b; Memish et al., 2013) and were circulating in bats before they "jumped" to an intermediate host (e.g., civets for SARS-CoV and dromedary camels for MERS-CoV) and/or to humans (Ge et al., 2013; Guan et al., 2003; Haagmans et al., 2014; Reusken et al., 2013). A recent study demonstrates the circulation of BatCoV HKU4 in bats in the past years (Lau et al., 2013). With the data in this study, we found that BatCoV HKU4 has evolved to utilize hCD26 as a functional receptor and therefore gained one of the key factors sufficing for interspecies transmission and human infection. This highlights the necessity for a

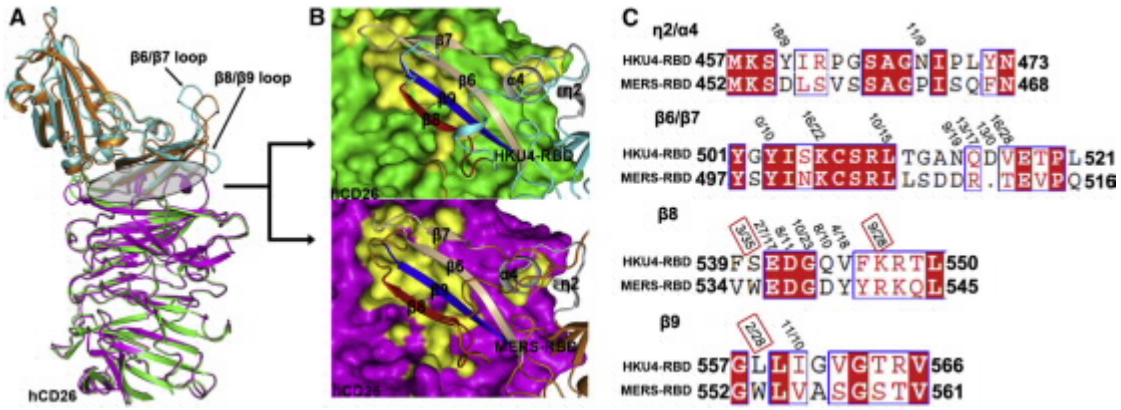


Figure 6. Comparison of the HKU4-RBD/hCD26 and MERS-RBD/hCD26 Pairs for Their Binding Modes and the Interaction Details

(A) Overall similar receptor binding mode between HKU4-RBD and MERS-RBD. Superimposition of the structure of HKU4-RBD (cyan) bound to hCD26 (green) and a complex structure of MERS-RBD (orange) with hCD26 (magenta). The loops exhibiting variant conformations are highlighted.

(B) A magnified view of the ligand/receptor (RBD in cartoon and hCD26 in surface) interface in the two binding pairs. The elements located within the vdw contact distance from the receptor are highlighted for the h2-a4 region (gray) in the core subdomain, and the b6-b7 (tint), b8 (red), and b9 (blue) strands in the external subdomain. Top: the HKU4-RBD/hCD26 structure. Bottom: the MERS-RBD/hCD26 structure.

(C) Better hCD26 adaptation in MERS-RBD than in HKU4-RBD. For each element specified in (B), the amino acid sequences were aligned between HKU4-RBD and MERS-RBD. The number pairs listed above the sequence highlight the differences in vdw contacts. For clarity, only those providing R 10 intermolecule contacts are labeled. The S540/W535, K547/R542, and L558/W553 pairs showing the most contact differences are marked with red boxes.

surveillance program to monitor HKU4 circulation in bats. Special attention should be paid to virus variants incorporating mutations in S that would increase their affinity for hCD26 and/or confer susceptibility to host protease cleavage.

The complex structure presented in this study makes BatCoV HKU4 the sixth coronavirus in the *Coronaviridae* family for which the receptor-recognition mode has been elucidated with complex structures, in addition to SARS-CoV (Li et al., 2005a), MERS-CoV (Lu et al., 2013; Wang et al., 2013), MHV (Peng et al., 2011), NL63 (Wu et al., 2009), and porcine respiratory coronavirus (Reguera et al., 2012). Among these, BatCoV HKU4 likely represents a coronavirus suboptimized for receptor adaptation, displaying micromolar affinity for hCD26. Compared to MERS-CoV, which recognizes the same hCD26 molecule as a receptor (Raj et al., 2013), HKU4-RBD and MERS-RBD form similar structures and engage hCD26 via similar binding modes. Nevertheless, the two viral ligands exhibit a difference in receptor binding affinity of three orders of magnitude. We noted that in the RBM region predominating the ligand binding interface with the receptor, the majority of the interface residues in MERS-RBD contribute more vdw contacts than the equivalent amino acids in HKU4-RBD. This demonstrates a much better adaptation to hCD26 for MERS-CoV than for BatCoV HKU4, which was further supported by our mutagenesis data. We therefore presented the molecular basis for the observed affinity variance and a structural explanation for the suboptimized hCD26 binding by BatCoV HKU4. This weak binding also raises the possibility for the presence of other high-affinity receptors for BatCoV HKU4. Nevertheless, the physical interaction between HKU4-RBD and hCD26 seems to favor a scenario in which BatCoV HKU4 has evolved to adapt to the human receptor. In this sense, we cannot rule out the possibility that this bat-derived virus has been exposed, at some stage, to humans or human tissues.

Though with slight differences, the BatCoVs HKU4 and HKU5 exhibit similar sequence identities to MERS-CoV in the RBD region (54.4% versus 52.9%). The marked difference between the HKU4-RBD and HKU5-RBD relative to MERS-RBD, however, lies in two sequence deletions in the HKU5-RBD. Both deletions are located in the external subdomain and in two regions corresponding to the scaffold strands b7 and b8 in the MERS/HKU4-RBD structure. The deletions would therefore alter the fold of HKU5-RBM and abolish its interaction with hCD26. Similar amino acid deletions that exclude the viral ligand from interacting with hACE2 have also been recorded for diverse SARS-like coronaviruses identified in bats (Ren et al., 2008). An intact full-length external subdomain is likely a prerequisite to maintain binding capacity for hCD26 or hACE2, in addition to preserving key interacting residues, such as K506 and E541 in HKU4-RBD.

EXPERIMENTAL PROCEDURES

Gene Construction and Protein Expression

The coding sequences for target proteins were separately cloned into the EcoRI and *Xho*I restriction sites of pFastBac1 vector for baculovirus expression (Bac-to-Bac baculovirus expression system, Invitrogen). For each protein, an N-terminal gp67 signal peptide and a C-terminal hexa-His were added to facilitate protein secretion and purification. Sf9 cells were used to package and amplify the baculovirus, and High5 cells were used to express the proteins, which were purified by nickel affinity chromatography and gel filtration. The proteins were then used for crystallization and SPR experiments.

To prepare the Fc chimeric proteins, the fragments were fused 5^{0}-terminally to a fragment coding for mouse Fc domain and ligated into the pCAGGS expression vector via the EcoRI and *Xho*I restriction sites. All fusion proteins were linked to the signal peptide of the MERS-CoV S protein. The proteins were transiently expressed in human embryonic kidney 293T (HEK293T)cells and purified by Protein A affinity chromatography and gel filtration. The purified proteins were then used for fluorescence-activated cell sorting (FACS)experiments (see Supplemental Experimental Procedures for detailed information).

Crystallization, Data Collection, and Structure Determination

For protein crystallization, monomeric HKU4-RBD was mixed with hCD26 at a 1:1 stoichiometry and crystallized by the sitting-drop vapor diffusion method at 4 C at 15 mg/ml in a buffer consisting of 0.1 M sodium citrate (pH 5.5) and 15%

PEG 6000. Diffraction data were collected with cryoprotected (in a reservoir solution containing 20% [v/v] glycerol) crystals at the Shanghai Synchrotron Radiation Facility (SSRF) BL17U. The complex structure was solved by molecular replacement using the structure of MERS-RBD/hCD26 (PDB: 4KR0) as the search model. For details, see Supplemental Experimental Procedures).

Binding Assays

Protein interactions were tested using both SPR analysis and FACS experiments. For the SPR assays, all proteins were exchanged into a buffer consisting of 10 mM HEPES (pH 7.4), 150 mM NaCl, and 0.005% v/v Tween 20. The indicated RBD proteins were immobilized onto CM5 chips and analyzed for real-time binding by flowing through gradient concentrations of hCD26 or hACE2.

For the cell sorting analysis, Huh7 or hCD26-transfected BHK cells were stained with different RBD-Fc fusion proteins and analyzed by flow cytometry. For the binding-block assay, Huh7 cells were incubated with an antibody (anti-hCD26 or anti-Flag; Sigma) or with the purified ectodomain protein (hCD26 or hACE2) before the addition of HKU4-RBD-mFc, then the cells were analyzed by cell sorting (for details, see Supplemental Experimental Procedures).

Pseudovirus Infection

BatCoV HKU4 pseudovirus particles were produced in HEK293T cells using a previously described method (Gao et al., 2013). For the pseudovirus infection, the virus particles were first treated with trypsin and then used to infect Huh7 cells in the presence or absence of the anti-hCD26 antibody. The luciferase activity was determined 48 hr postinfection using a GloMax 96 Microplate luminometer (Promega) (for details, see Supplemental Experimental Procedures).

ACCESSION NUMBERS

The atomic coordinate of the HKU4-RBD/hCD26 complex has been deposited in the Protein Data Bank with the PDB code 4QZV.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2014.08.009 .

AUTHOR CONTRIBUTIONS

G.F.G. and G.L. initiated and coordinated the project. G.F.G., G.L., Q.W., and J.Y. designed the experiments. Q.W. conducted the experiments with help from Y.Y., Y.X., P.H., Y.W., W.J., and Y.L. J.Q. solved the structure. J.W. and A.I. provided the reagents and helped with manuscript writing. P.C.Y.W. and K.-Y.Y. helped with the data analysis and manuscript writing. Q.W., G.L., J.Y., and G.F.G. analyzed the data. G.L., Q.W., and G.F.G. wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (NSFC, Grant No. 81290342), the China National Grand S&T Special Project (No. 2014ZX10004-001-006), and the Strategic Priority Research Program of the Chinese Academy of Sciences (Grant No. XDB08020100). We thank the staff at the Shanghai Synchrotron Radiation Facility (SSRF) and Tong Zhao at the Institute of Microbiology, Chinese Academy of Sciences for support. G.F.G. is a leading principal investigator of the NSFC Innovative Research Group (Grant No. 81321063).

Received: May 14, 2014
Revised: July 30, 2014
Accepted: August 22, 2014
Published: September 10, 2014

REFERENCES

Bermingham, A., Chand, M.A., Brown, C.S., Aarons, E., Tong, C., Langrish, C., Hoschler, K., Brown, K., Galiano, M., Myers, R., et al. (2012). Severe respiratory illness caused by a novel coronavirus, in a patient transferred to the United Kingdom from the Middle East, September 2012. Euro Surveill. *17*, 20290.

Chiu, S.S., Chan, K.H., Chu, K.W., Kwan, S.W., Guan, Y., Poon, L.L., and Peiris, J.S. (2005). Human coronavirus NL63 infection and other coronavirus infections in children hospitalized with acute respiratory disease in Hong Kong, China. Clin. Infect. Dis. *40*, 1721–1729.

Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L.K., Sjöström, H., Norén, O., and Laude, H. (1992). Aminopeptidase N is a major receptor for the entero-pathogenic coronavirus TGEV. Nature *357*, 417–420.

Engel, M., Hoffmann, T., Wagner, L., Wermann, M., Heiser, U., Kiefersauer, R., Huber, R., Bode, W., Demuth, H.U., and Brandstetter, H. (2003). The crystal structure of dipeptidyl peptidase IV (CD26) reveals its functional regulation and enzymatic mechanism. Proc. Natl. Acad. Sci. USA *100*, 5063–5068.

Frana, M.F., Behnke, J.N., Sturman, L.S., and Holmes, K.V. (1985). Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: host-dependent differences in proteolytic cleavage and cell fusion. J. Virol. *56*, 912–920.

Gao, G.F. (2007). Peptide Inhibitors Targeting Virus-Cell Fusion in Class I Enveloped Viruses. In Combating the Threat of Pandemic Influenza: Drug Discovery Approaches, P.F. Torrence, ed. (New York: John Wiley & Sons), pp. 226–246.

Gao, J., Lu, G., Qi, J., Li, Y., Wu, Y., Deng, Y., Geng, H., Li, H., Wang, Q., Xiao, H., et al. (2013). Structure of the fusion core and inhibition of fusion by a heptad repeat peptide derived from the S protein of Middle East respiratory syndrome coronavirus. J. Virol. *87*, 13134–13140.

Ge, X.-Y., Li, J.-L., Yang, X.-L., Chmura, A.A., Zhu, G., Epstein, J.H., Mazet, J.K., Hu, B., Zhang, W., Peng, C., et al. (2013). Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. Nature *503*, 535–538.

Gierer, S., Bertram, S., Kaup, F., Wrensch, F., Heurich, A., Krämer-Kühl, A., Welsch, K., Winkler, M., Meyer, B., Drosten, C., et al. (2013). The spike protein of the emerging betacoronavirus EMC uses a novel coronavirus receptor for entry, can be activated by TMPRSS2, and is targeted by neutralizing antibodies. J. Virol. *87*, 5502–5511.

Gorrell, M.D., Gysbers, V., and McCaughan, G.W. (2001). CD26: a multifunctional integral membrane and secreted protein of activated lymphocytes. Scand. J. Immunol. *54*, 249–264.

Gorse, G.J., O'Connor, T.Z., Hall, S.L., Vitale, J.N., and Nichol, K.L. (2009). Human coronavirus and acute respiratory illness in older adults with chronic obstructive pulmonary disease. J. Infect. Dis. *199*, 847–857.

Gouet, P., Courcelle, E., Stuart, D.I., and Métoz, F. (1999). ESPript: analysis of multiple sequence alignments in PostScript. Bioinformatics *15*, 305–308.

Guan, Y., Zheng, B.J., He, Y.Q., Liu, X.L., Zhuang, Z.X., Cheung, C.L., Luo, S.W., Li, P.H., Zhang, L.J., Guan, Y.J., et al. (2003). Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. Science *302*, 276–278.

Haagmans, B.L., Al Dhahiry, S.H., Reusken, C.B., Raj, V.S., Galiano, M., Myers, R., Godeke, G.J., Jonges, M., Farag, E., Diab, A., et al. (2014). Middle East respiratory syndrome coronavirus in dromedary camels: an outbreak investigation. Lancet Infect. Dis. *14*, 140–145.

Harrison, S.C. (2008). Viral membrane fusion. Nat. Struct. Mol. Biol. *15*, 690–698.

Hofmann, H., Pyrc, K., van der Hoek, L., Geier, M., Berkhout, B., and Pöhlmann, S. (2005). Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. Proc. Natl. Acad. Sci. USA *102*, 7988–7993.

Ithete, N.L., Stoffberg, S., Corman, V.M., Cottontail, V.M., Richards, L.R., Schoeman, M.C., Drosten, C., Drexler, J.F., and Preiser, W. (2013). Close relative of human Middle East respiratory syndrome coronavirus in bat, South Africa. Emerg. Infect. Dis. *19*, 1697–1699.

Jean, A., Quach, C., Yung, A., and Semret, M. (2013). Severity and outcome associated with human coronavirus OC43 infections among children. Pediatr. Infect. Dis. J. *32*, 325–329.

Jevšnik, M., Uršič, T., Zigon, N., Lusa, L., Krivec, U., and Petrovec, M. (2012). Coronavirus infections in hospitalized pediatric patients with acute respiratory tract disease. BMC Infect. Dis. *12*, 365.

King, A.M.Q., Adams, M.J., Carstens, E.B., and Lefkowitz, E.J. (2012). Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. (London: Elsevier Academic Press).

Ksiazek, T.G., Erdman, D., Goldsmith, C.S., Zaki, S.R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J.A., Lim, W., et al.; SARS Working Group (2003). A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. *348*, 1953–1966.

Lai, M.M.C., Perlman, S., and Anderson, L.J. (2007). Coronaviridae. In Fields Virology, D.M. Knipe and P.M. Howley, eds. (Philadelphia, PA: Lippincott Williams & Wilkins), pp. 1305–1335.

Lau, S.K., Li, K.S., Tsang, A.K., Lam, C.S., Ahmed, S., Chen, H., Chan, K.H., Woo, P.C., and Yuen, K.Y. (2013). Genetic characterization of Betacoronavirus lineage C viruses in bats reveals marked sequence divergence in the spike protein of pipistrellus bat coronavirus HKU5 in Japanese pipistrelle: implications for the origin of the novel Middle East respiratory syndrome coronavirus. J. Virol. *87*, 8638–8650.

Li, W., Moore, M.J., Vasilieva, N., Sui, J., Wong, S.K., Berne, M.A., Somasundaran, M., Sullivan, J.L., Luzuriaga, K., Greenough, T.C., et al. (2003). Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature *426*, 450–454.

Li, F., Li, W., Farzan, M., and Harrison, S.C. (2005a). Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. Science *309*, 1864–1868.

Li, W., Shi, Z., Yu, M., Ren, W., Smith, C., Epstein, J.H., Wang, H., Crameri, G., Hu, Z., Zhang, H., et al. (2005b). Bats are natural reservoirs of SARS-like coronaviruses. Science *310*, 676–679.

Lu, G., and Liu, D. (2012). SARS-like virus in the Middle East: a truly bat-related coronavirus causing human diseases. Protein Cell *3*, 803–805.

Lu, G., Hu, Y., Wang, Q., Qi, J., Gao, F., Li, Y., Zhang, Y., Zhang, W., Yuan, Y., Bao, J., et al. (2013). Molecular basis of binding between novel human coronavirus MERS-CoV and its receptor CD26. Nature *500*, 227–231.

Memish, Z.A., Mishra, N., Olival, K.J., Fagbo, S.F., Kapoor, V., Epstein, J.H., Alhakeem, R., Durosinloun, A., Al Asmari, M., Islam, A., et al. (2013). Middle East respiratory syndrome coronavirus in bats, Saudi Arabia. Emerg. Infect. Dis. *19*, 1819–1823.

Moore, M.J., Dorfman, T., Li, W., Wong, S.K., Li, Y., Kuhn, J.H., Coderre, J., Vasilieva, N., Han, Z., Greenough, T.C., et al. (2004). Retroviruses pseudotyped with the severe acute respiratory syndrome coronavirus spike protein efficiently infect cells expressing angiotensin-converting enzyme 2. J. Virol. *78*, 10628–10635.

Peng, G., Sun, D., Rajashankar, K.R., Qian, Z., Holmes, K.V., and Li, F. (2011). Crystal structure of mouse coronavirus receptor-binding domain complexed with its murine receptor. Proc. Natl. Acad. Sci. USA *108*, 10696–10701.

Raj, V.S., Mou, H., Smits, S.L., Dekkers, D.H.W., Müller, M.A., Dijkman, R., Muth, D., Demmers, J.A.A., Zaki, A., Fouchier, R.A.M., et al. (2013). Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. Nature *495*, 251–254.

Reguera, J., Santiago, C., Mudgal, G., Ordoño, D., Enjuanes, L., and Casasnovas, J.M. (2012). Structural bases of coronavirus attachment to host aminopeptidase N and its inhibition by neutralizing antibodies. PLoS Pathog. *8*, e1002859.

Ren, W., Qu, X., Li, W., Han, Z., Yu, M., Zhou, P., Zhang, S.Y., Wang, L.F., Deng, H., and Shi, Z. (2008). Difference in receptor usage between severe acute respiratory syndrome (SARS) coronavirus and SARS-like coronavirus of bat origin. J. Virol. *82*, 1899–1907.

Reusken, C.B., Haagmans, B.L., Müller, M.A., Gutierrez, C., Godeke, G.J., Meyer, B., Muth, D., Raj, V.S., Smits-De Vries, L., Corman, V.M., et al. (2013). Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study. Lancet Infect. Dis. *13*, 859–866.

Simmons, G., Reeves, J.D., Rennekamp, A.J., Amberg, S.M., Piefer, A.J., and Bates, P. (2004). Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry. Proc. Natl. Acad. Sci. USA *101*, 4240–4245.

Simmons, G., Zmora, P., Gierer, S., Heurich, A., and Pöhlmann, S. (2013). Proteolytic activation of the SARS-coronavirus spike protein: cutting enzymes at the cutting edge of antiviral research. Antiviral Res. *100*, 605–614.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. *30*, 2725–2729.

van Boheemen, S., de Graaf, M., Lauber, C., Bestebroer, T.M., Raj, V.S., Zaki, A.M., Osterhaus, A.D., Haagmans, B.L., Gorbalenya, A.E., Snijder, E.J., and Fouchier, R.A. (2012). Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. MBio *3*, e00473–e12.

Wang, N., Shi, X., Jiang, L., Zhang, S., Wang, D., Tong, P., Guo, D., Fu, L., Cui, Y., Liu, X., et al. (2013). Structure of MERS-CoV spike receptor-binding domain complexed with human receptor DPP4. Cell Res. *23*, 986–993.

Weihofen, W.A., Liu, J., Reutter, W., Saenger, W., and Fan, H. (2004). Crystal structure of CD26/dipeptidyl-peptidase IV in complex with adenosine deaminase reveals a highly amphiphilic interface. J. Biol. Chem. *279*, 43330–43335.

Wevers, B.A., and van der Hoek, L. (2009). Recently discovered human coronaviruses. Clin. Lab. Med. *29*, 715–724.

WHO (2004). Cumulative Number of Reported Probable Cases of Severe Acute Respiratory Syndrome (SARS). http://www.who.int/csr/sars/country/en/ .

WHO (2014). Middle East respiratory syndrome coronavirus (MERS-CoV) – update. http://www.who.int/csr/don/2014_06_16_mers/en/ .

Woo, P.C., Lau, S.K., Li, K.S., Poon, R.W., Wong, B.H., Tsoi, H.W., Yip, B.C., Huang, Y., Chan, K.H., and Yuen, K.Y. (2006). Molecular diversity of coronaviruses in bats. Virology *351*, 180–187.

Woo, P.C., Wang, M., Lau, S.K., Xu, H., Poon, R.W., Guo, R., Wong, B.H., Gao, K., Tsoi, H.W., Huang, Y., et al. (2007). Comparative analysis of twelve genomes of three novel group 2c and group 2d coronaviruses reveals unique group and subgroup features. J. Virol. *81*, 1574–1585.

Woo, P.C., Lau, S.K., Lam, C.S., Lai, K.K., Huang, Y., Lee, P., Luk, G.S., Dyrting, K.C., Chan, K.H., and Yuen, K.Y. (2009). Comparative analysis of complete genome sequences of three avian coronaviruses reveals a novel group 3c coronavirus. J. Virol. *83*, 908–917.

Woo, P.C., Lau, S.K., Lam, C.S., Lau, C.C., Tsang, A.K., Lau, J.H., Bai, R., Teng, J.L., Tsang, C.C., Wang, M., et al. (2012). Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. J. Virol. *86*, 3995–4008.

Wu, K., Li, W., Peng, G., and Li, F. (2009). Crystal structure of NL63 respiratory coronavirus receptor-binding domain complexed with its human receptor. Proc. Natl. Acad. Sci. USA *106*, 19970–19974.

Xu, Y., Liu, Y., Lou, Z., Qin, L., Li, X., Bai, Z., Pang, H., Tien, P., Gao, G.F., and Rao, Z. (2004a). Structural basis for coronavirus-mediated membrane fusion. Crystal structure of mouse hepatitis virus spike protein fusion core. J. Biol. Chem. *279*, 30514–30522.

Xu, Y., Lou, Z., Liu, Y., Pang, H., Tien, P., Gao, G.F., and Rao, Z. (2004b). Crystal structure of severe acute respiratory syndrome coronavirus spike protein fusion core. J. Biol. Chem. *279*, 49414–49419.

Yang, Y., Du, L., Liu, C., Wang, L., Ma, C., Tang, J., Baric, R.S., Jiang, S., and Li, F. (2014). Receptor usage and cell entry of bat coronavirus HKU4 provide insight into bat-to-human transmission of MERS coronavirus. Proc. Natl. Acad. Sci. USA in press.

Yeager, C.L., Ashmun, R.A., Williams, R.K., Cardellichio, C.B., Shapiro, L.H., Look, A.T., and Holmes, K.V. (1992). Human aminopeptidase N is a receptor for human coronavirus 229E. Nature *357*, 420–422.

Zaki, A.M., van Boheemen, S., Bestebroer, T.M., Osterhaus, A.D., and Fouchier, R.A. (2012). Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N. Engl. J. Med. *367*, 1814–1820.

CRDD Translational Core Sample Request Form

Type of specimen (e.g. serum, plasma, tissue, nucleic acid):

Source (e.g. human, bat, tick):

Pathogen(s):

Quantity (# of samples):

Volume of each sample:

Date needed by:

Other information (e.g. geographical origin of sample, animal species, sample never thawed):

Sample location:

- ☐ CII
- ☐ NYCDOHMH
- ☐ NYSDOH

*** Requested by:**

*** Contact phone:**

*** Contact e-mail**

(Please continue to page 2)

*** Courier address for mailing sample:**

Sample Request Correspondence: Please send all sample requests to dcm2120@columbia.edu with the Subject listed as TC request - Last name (project PI). Your request will be forwarded to the appropriate Translational Core member(s). You may subsequently be contacted directly by a Translational Core member for further information regarding your needs or sample availability.

*** Please copy dcm2120@columbia.edu on correspondence regarding sample transfer for tracking purposes.**

*** Required**

National Institute of Allergy and Infectious Diseases
Leading research to understand, treat, and prevent infectious, immunologic, and allergic diseases.

Research Funding

Highlights

- *NIAID Funding Newsletter,* October 1, 2014
- All About Grants
- Strategy for NIH Funding
- Samples and Examples
- Sample Applications
- Top Policy Changes
- Resources for Researchers

Look It Up

- advisory Council
- competitive segment
- direct costs
- dual assignment
- financial management plan
- fiscal year (FY)
- funding opportunity announcement (FOA)
- human subjects
- initial peer review
- institutes and centers (IC)
- modular budget
- new investigator
- noncompeting
- Notice of Award
- patent
- payline
- PHS 2590 Non-Competing Continuation Progress Report
- program officer
- program project (P)
- R01
- receipt date
- renewal
- Research Plan
- review cycle
- scope
- secondary assignment
- SF 424 Application Guide
- Specific Aims

See the Glossary for more terms.

Renewal Application Questions and Answers

Table of Contents

- What are renewals and where can I find basic information about them?
- How do I decide whether to apply as a renewal or submit a new application?
- Should my renewal use my original Specific Aims or should I write new Specific Aims?
- Can a PI submit two renewal applications to follow-up on a single R01?
- How should I time my renewal application?
- What should I consider when deciding whether to apply early with a renewal?
- Should I submit my renewal early if I do not have enough data at the earlier time?
- Can NIH fund a renewal before the existing grant ends?
- What happens to a fundable renewal application for a grant that ends the next fiscal year?

- What is the funding cap for renewals?
- To which grants does the funding cap apply?
- If the cap is 20 percent, how does NIAID determine the budget limit?
- For modular grants, can I round up to the next level if the cap has not been reached?
- The budget for my first R01 was low, $130,000; how much can I request for my new budget?
- If I ask for the cap amount for my first year, can I make a bigger request for subsequent years?
- If I request an amount within the cap level, I won't have enough funds for a new animal model—what should I do?
- Are modular R01 renewals affected by the budget cap?
- What should I do during this era of lean budgets?
- Must I keep the title of the grant unchanged when applying for a renewal?
- Does a grant renewal have to go to the same study section as its original R01?
- Can my renewal application be assigned to a different NIH institute than the one that funded my prior grant? If so, how should I make that request?
- What format should I use for the Research Plan section of an R01 renewal?
- I am preparing to submit a renewal application for an R01 grant. Where in the application do I include the progress report and how should I format it?
- Is the Progress Report Publication List part of the 12-page limit for the Research Strategy?
- Will resetting anniversary dates affect the timing of my renewal?
- Can you tell me about renewing a training grant?
- Is there a window of time that a PI can submit an application as a renewal? Must the original grant still be active?
- If I change some of the collaborating projects in my P01, is my submission considered to be a renewal or a new grant application?
- Do I need to submit a final progress report for my grant if I am applying for a renewal?
- What if my question wasn't answered here, or I'd like to suggest a question?

What are renewals and where can I find basic information about them?

A renewal application is a request for funding to continue your NIH-funded research. Before your project ends, you must again apply for support and undergo initial peer review.

Alternatively, you may submit a new application. The best approach depends on your circumstances—see the next question.

For information on renewals, go to NIAID's Apply for Renewal or Start Anew in the Strategy for NIH Funding.

How do I decide whether to apply as a renewal or submit a new application?

It's your choice. When deciding, consider your situation and the science; then contact your program officer for advice.

For example, it is advantageous to apply with a renewal if you have made progress and accomplished most or all of your Specific Aims, and you plan a logical continuation using the same funding mechanism.

Going in a new direction or expanding the scope of the research calls for a new application. Read more inApply for Renewal or Start Anew in our Strategy for NIH Funding.

Should my renewal use my original Specific Aims or should I write new Specific Aims?

Your renewal should have new or mostly new Specific Aims, given the expectation that most or all of your original aims have been achieved. Peer reviewers will take into account what you have accomplished when assessing the merits of your renewal application.

Also, a renewal should clearly link back to your previous grant's Specific Aims, showing progress without duplicating the aims of the previous grant. It's important to show that you successfully conducted relevant research, got results, and then will use those results to pursue the next set of experiments.

We suggest reading Apply for Renewal or Start Anew in our Strategy for NIH Funding.

Can a PI submit two renewal applications to follow-up on a single R01?

No, but you can submit one as a renewal, and another as a new application that's significantly different.

Be aware that there are different submission dates for new and renewal applications. You may also want to read our advice on renewals—go to Apply for Renewal or Start Anew in the Strategy for NIH Funding.

How should I time my renewal application?
Good planning is critical. Some people wait until the last possible receipt date to send in their renewal applications without a break in funding, though often it's best to apply earlier.

If you have sufficient data, you may consider submitting your application one or more review cycles early. Applying early gives you time to revise and resubmit if you do not get a fundable score.

For more guidance, read the Strategy for NIH Funding. Go to NIH's Review and Award Cycles table for receipt dates and funding timeframes.

What should I consider when deciding whether to apply early with a renewal?
If you are submitting a renewal application, reviewers expect to see data showing progress no matter when your application arrives.

Your timing hinges on your comfort level with your data and progress, the length of the grant, and the complexity of the project. Read more in the Strategy for NIH Funding.

Should I submit my renewal early if I do not have enough data at the earlier time?
No. Send in your application one or more review cycles early only if you have enough data to show progress.

The early approach does not apply to short awards or work progressing slowly. For more advice, read the Strategy for NIH Funding.

Can NIH fund a renewal before the existing grant ends?
No. Though you can submit your renewal application at any time, your renewal cannot be funded until shortly before the final grant year is about to end.

What happens to a fundable renewal application for a grant that ends the next fiscal year?
We will keep track of the application, and if it is within the next fiscal year's payline, we will make the award. Of course, you may also choose to resubmit.

What is the funding cap for renewals?
For the past several years—which we expect to continue—NIAID has limited the amount of money you can request for your renewal R01 to 20 percent more than the direct costs of the last noncompeting award.

The cap is part of NIAID's financial management plan. Go to our Paylines and Funding page for the latest information, and read Planning a Budget in the Strategy for NIH Funding for how NIAID calculates that cap.

To which grants does the funding cap apply?
The renewal cap applies only to R01 grants. Although sometimes NIAID requests an exception to a cap from our advisory Council, this is highly unusual, and you shouldn't expect it.

As always, we recommend discussing your options with your program officer.

If the cap is 20 percent, how does NIAID determine the budget limit?
Go to Planning a Budget in the Strategy for NIH Funding to see how we compute the cap.

For modular grants, can I round up to the next level if the cap has not been reached?
Yes, if the cap results in a number between modular increments, you round up to the next module.

The budget for my first R01 was low, $130,000; how much can I request for my new budget?
Here's an example. If you send in your application as a renewal, you are limited to 20 percent more than $130,000. This is a reasonable approach if you are pretty much continuing the same research you started with.

However, if your research has evolved in scope, you may want to apply with a new grant. In that case, change the title and abstract to include your new Specific Aims, so the focus of the application reflects the new scale of your research.

Because new investigators typically receive less money than more experienced grantees, the cap is often a problem for them. For more advice, contact your program officer, and read Strategies for Dealing With a Budget Cap in the Strategy for NIH Funding.

If I ask for the cap amount for my first year, can I make a bigger request for subsequent years?

No. All budgets are based on the first year budget. Modular grants get the same number of modules (same funding level) each year. Nonmodular grants may get a small increase or a decrease, depending on NIAID's financial management plan for that year.

You can request funds in excess of the cap for one-time costs, such as equipment. Go to Planning a Budget in the Strategy for NIH Funding and the Renewal Funding SOP for more information.

If I request an amount within the cap level, I won't have enough funds for a new animal model—what should I do?

You have several options. Regardless of Institute policy, you should request the resources needed for your research. Learn more in Strategies for Dealing With a Budget Cap in the Strategy for NIH Funding.

Are modular R01 renewals affected by the budget cap?

The cap affects all applications, modular or nonmodular, regardless of funding level.

What should I do during this era of lean budgets?

Keep in mind that it's harder to get funded in a lean budget year and that you may not get funded at the study section-approved level. Read more in When Funding Is Tight in the Strategy for NIH Funding.

Must I keep the title of the grant unchanged when applying for a renewal?

No. While it is often best to keep the same title, use a different title if it's a better fit.

If you do change the title, check the box indicating that your application is a renewal on the checklist (the last page) of the grant application, and enter your grant number. That way, NIH will know you are submitting a renewal with a new title.

Does a grant renewal have to go to the same study section as its original R01?

No. You can request any study section in your cover letter if it's relevant to the science. However, keep in mind that there's no guarantee your application will go there.

Can my renewal application be assigned to a different NIH institute than the one that funded my prior grant? If so, how should I make that request?

Although it's rare, grants can be funded in situations where the previous competitive segment was funded by another institute or center (IC). Check with a program officer in the other IC to see whether there is interest in your application before you submit the application.

Request that IC as the primary institute in your cover letter. If your application is able to fit a different IC's mission, you can work with your program officer to request a dual assignment if you didn't request it when you initially submitted the renewal application.

Many ICs will accept a transfer before review but not afterwards, so it's best to inquire whether there is interest in accepting the application at another IC before review.

If the assigned IC doesn't wish to fund the application, then a secondary IC can determine that it's important to their mission and request that the application moves to their IC. The secondary IC will generally only fund the application if the score is within their payline.

For more information, see the Request for Primary IC Assignment SOP.

What format should I use for the Research Plan section of an R01 renewal?

A Research Plan follows the same format and page limits for a renewal as a new application, with a few differences.

Instead of preliminary studies, include a Progress Report. You also include a Progress Report Publication List, information on Inventions and Patents, and a Planned Enrollment Report and Cumulative Inclusion Enrollment Report if you're conducting human subjects research.

See How to Fill Out the Forms in the Strategy for NIH Funding, and check the SF 424 Application Guide of the R01 funding opportunity announcement for further details.

I am preparing to submit a renewal application for an R01 grant. Where in the application package do I include the progress report and how should I format it?

NIH doesn't have a progress reporting form for renewal applications. You'll report progress in your Research Strategy. See a sample application that is a renewal, if you want to see how another PI did his.

For additional information, see How to Fill Out the Forms and Preliminary Studies or Progress Report in our Strategy for NIH Funding.

Is the Progress Report Publication List part of the 12-page limit for the Research Strategy?

No, it's not. The SF 424 (R&R) Application Guides state that the Progress Report Publication List should not be included in the Approach part of the Research Strategy. Therefore, it's not part of the 12-page limit for that section.

Instead, the Progress Report Publication List is a separate attachment for the PHS 398 Research Plan. The application guides don't specify a page limit for that attachment.

Will resetting anniversary dates affect the timing of my renewal?

Yes. NIAID does not reset grant dates every year. If we do, we let investigators know through our *NIAID Funding Newsletter*. Sign up to receive an email each time it is published at Subscribe to Email Alerts.

Can you tell me about renewing a training grant?

Go to Does NIAID have instructions for renewals of training grants? in the Training Grants questions and answers. Read Does my renewal application need to be as detailed as my original application? on that page.

Is there a window of time that a PI can submit an application as a renewal? Must the original grant still be active?

No. The grant need not be active and there is no time limit for a renewal application. However, reviewers will probably be concerned by major gaps.

If a significant amount of time has elapsed, indicate what you have done in the interim. Highlight any preliminary data you may have obtained, and show that your planned research is current with the latest science.

If I change some of the collaborating projects in my P01, is my submission considered to be a renewal or a new grant application?

See If I change some of the collaborating projects in my P01, is my submission a renewal or a new grant application? in the NIAID Investigator-Initiated Program Project (P01) Applications questions and answers.

Do I need to submit a final progress report for my grant if I am applying for a renewal?

See Do I need to submit a final progress report for my grant if I am applying for a renewal? in the Progress Reports questions and answers.

What if my question wasn't answered here, or I'd like to suggest a question?

Email deaweb@niaid.nih.gov with the title of this page or its URL and your question or comment. We answer questions by email and post them here. Thanks for helping us clarify and expand our knowledge base.

Last Updated September 25, 2014
Last Reviewed June 10, 2013

Stay Connected:

U.S. Government Gain-of-Function Deliberative Process and Research Funding Pause on Selected Gain-of-Function Research Involving Influenza, MERS, and SARS Viruses

Frequently Asked Questions

November 2014

What is "gain-of-function" research?

"Gain of function" refers to any modification of a biological agent that confers new or enhanced activity. Typically, researchers mutate or alter genes and examine the impact of these modifications on a particular property or trait of the organism. For example, some investigators can modify influenza viruses in ways that enhance pathogenicity and/or transmissibility in order to better understand the origins and nature of these traits at the molecular level, as well as their pathogenesis in susceptible hosts. Since influenza viruses constantly evolve in nature, these gain-of-function studies may help predict whether these viruses could evolve naturally over time to acquire these new or enhanced traits, and if so, how the viruses might affect hosts and the kinds of medical countermeasures that might be most effective. Some gain-of-function studies may entail biosafety and biosecurity risks that require unique risk assessment and mitigation measures.

Why is gain-of-function research undertaken?

Gain-of-function studies involving infectious agents help us understand the fundamental nature of host-pathogen interactions and can shed light on the potential for, and mechanisms of, evolution of viruses and other infectious agents to acquire certain properties. Greater insight into the biological processes that contribute to pathogenicity or transmissibility can provide a foundation for identifying and developing effective approaches to prevent or treat human or animal infections. Gain-of-function research may also enable assessment of the pandemic potential of emerging infectious agents, which could inform public health and preparedness efforts, including the development of effective medical countermeasures.

Why is the U.S. government[1] pausing the funding of certain types of gain-of-function studies at this time?

The occurrence this year of laboratory biosafety incidents at U.S. government research facilities have caused the federal government to re-assess the risk/benefit calculus underpinning funding decisions for a certain subset of gain-of-function research involving agents that pose a significant risk to public and animal health. The pause will allow the U.S. government, in partnership with the life sciences community, to conduct a comprehensive assessment of gain-of-function research with the explicit goal of developing a new policy framework to guide future funding decisions.

What is the scope of research covered by the funding pause?

During the pause, the U.S. government will not release new funding for gain-of-function research projects that reasonably may be anticipated to confer attributes to influenza[2], Middle East Respiratory Syndrome (MERS), or Severe Acute Respiratory Syndrome (SARS)

[1] http://www.whitehouse.gov/blog/2014/10/17/doing-diligence-assess-risks-and-benefits-life-sciences-gain-function-research

[2] Any influenza virus, not only highly pathogenic strains

viruses such that the resulting virus has enhanced pathogenicity and/or transmissibility (via the respiratory route) in mammals. This means that the U.S. government will not release new funding for experiments that meet the gain-of-function funding pause criteria. For the small subset of ongoing work that is determined to meet the gain-of-function funding pause criteria, the contract-funded work has been stopped and the grant-funded work is subject to a voluntary pause. The funding pause will not apply to characterization or testing of naturally occurring influenza, MERS, and SARS viruses, unless these tests reasonably are anticipated to increase transmissibility or pathogenicity.

Why is the funding pause limited to only research involving influenza, SARS, and MERS viruses?

These three viruses not only share the characteristic of being human health threats - causing in some instances significant morbidity and mortality - but they also have the potential to cause pandemics because they are transmitted easily by the respiratory route. A pandemic with any of these pathogens could have serious repercussions for health, security, and economics, both nationally and globally. While the U.S. government recognizes that understanding the pandemic potential of these organisms is one of the reasons investigators conduct gain-of-function studies, such experiments entail certain risks to individual and public safety and security. This year's federal laboratory incidents led the government to reassess the risk/benefit calculus underpinning funding decisions for a certain subset of gain of function research, and consequently pause funding for those high-risk gain of function experiments until a comprehensive review of the risks and benefits of gain of function studies could be completed.

How many ongoing research projects involving influenza, SARS, and MERS viruses entail experiments that fit the characteristics of those subject to the funding pause?

The National Institutes of Health (NIH) initially identified 18 projects that might entail experiments subject to the funding pause. Letters were sent to principal investigators on those projects to notify them of the funding pause and to request that they assess the applicability of the pause to their projects. Discussions between NIH and these investigators are currently ongoing and regard specific experimental details and research results in order to determine the applicability of the funding pause to those experiments. Therefore, the number of projects actually affected by the pause may change from the 18 currently identified.

What if I am not sure whether an experiment falls under the funding pause?

In cases where it is not clear whether an experiment involving influenza, MERS, or SARS virus would be subject to the pause, determinations will be made on a case-by-case basis by the funding agency. Investigators who have questions about whether an experiment may be subject to the pause should contact their funding agency.

Are experiments involving isolated genes of influenza, MERS, or SARS virus included in the funding pause?

No. The pause applies to research that would create viruses with certain attributes. If the research does not include production of infectious, replication-competent virus, then it would not be subject to the pause.

Are in silico or modeling studies of influenza, MERS, or SARS virus included in the funding pause?

Experiments *in silico* or modeling studies are not covered under the pause unless they also involve work with the actual viruses and meet the criteria.

Will influenza surveillance activities be affected by the funding pause?

The pause is not intended to affect influenza surveillance work generally, and the U.S. government is committed to ensuring the nation is prepared for seasonal and pandemic influenza. Influenza surveillance projects supported by the U.S. government should continue, unless these projects involve the manipulation of naturally occurring isolates such that the resulting viruses have enhanced transmissibility and/or pathogenicity.

What will occur during the pause?

During the pause, a deliberative process will be undertaken in which the potential benefits and risks of gain-of-function research will be evaluated by the life sciences community and federal government. The deliberative process will involve two distinct, but complementary, entities, and is explicitly structured to facilitate robust engagement between the federal government and life sciences community. The National Science Advisory Board for Biosecurity (NSABB) will serve as the official federal advisory body for providing advice on oversight of this area of dual use research, in keeping with federal rules and regulations.

Early in the deliberative process, the National Research Council (NRC) of the National Academies will be asked to convene a scientific symposium focused on the issues associated with gain-of-function. The NRC will also hold a second symposium later in the deliberative process, which will include a discussion of the NSABB draft recommendations regarding gain-of-function research.

The NSABB, informed by discussion at the NRC public consultations, will provide recommendations to the heads of all federal entities that conduct, support, or have an interest in life sciences research. The final NSABB recommendations as well as the outcomes of the NRC symposia will inform the development and adoption of a new U.S. government policy regarding gain-of-function research. The life sciences community will be encouraged to provide input through both the NRC and NSABB deliberative processes.

Will members of the life science community and public have opportunities to provide input to the NSABB and NRC discussions?

Yes. The NSABB and NRC proceedings will employ mechanisms to engage the life sciences community and public at large, who will be encouraged to provide input.

When will the funding pause end?

The funding pause will end when the U.S. government has adopted a federal policy to guide future funding decisions regarding gain-of-function studies on the basis of the deliberative process described above. The objective is for the pause not to be any longer than necessary, and the federal government has launched a process that is intended to take 12 months.

Is there a provision to allow gain-of-function research subject to the funding pause to proceed, for example, if the research is required for public health or national security purposes?

Yes, an exception from the funding pause may be granted by the head of the federal funding department or agency if that official determines in writing that the research is urgently necessary to protect public health or national security.

I believe that my research is indeed urgently necessary to protect public health or national security and should not be subject to the funding pause. What is the process for requesting and receiving an exception?

Principal investigators should begin by contacting the program or contract officer at the agency funding the research in question. Agency staff will then consider whether particular experiments are subject to the funding pause, and if so, whether they meet the exception criterion of being "urgently necessary to protect the public health or national security." If program staff believe the research in question may meet the exception criterion, the matter will then be referred to the Director of the department or agency that funds the research for a final evaluation and determination. Once the director makes a determination, agency staff will notify the investigator about the outcome of this evaluation in a timely way. While individual experiments and specific evaluation processes will vary, the U.S. government is committed to evaluating potential exceptions and making determinations as expeditiously as possible.

Is the funding pause related to ongoing efforts to respond to the Ebola outbreak in West Africa?

No. The research pause only affects release of new funding for gain-of-function research experiments that reasonably are anticipated to confer attributes to influenza, MERS, or SARS virus such that the resulting virus has enhanced pathogenicity and/or transmissibility via the respiratory route in mammals.

Department of Health and Human Services

Part 1. Overview Information

Participating Organization(s)	National Institutes of Health (NIH)
Components of Participating Organizations	National Institute of Allergy and Infectious Diseases (NIAID)
Funding Opportunity Title	**Centers of Excellence for Translational Research (CETR) (U19)**
Activity Code	U19 Research Program – Cooperative Agreements
Announcement Type	New
Related Notices	None
Funding Opportunity Announcement (FOA) Number	**RFA-AI-12-044**
Companion Funding Opportunity	None
Number of Applications	See Section III. 3. Additional Information on Eligibility.
Catalog of Federal Domestic Assistance (CFDA) Number(s)	93.855; 93.856
Funding Opportunity Purpose	The National Institute of Allergy and Infectious Diseases (NIAID) invites new applications from single institutions and consortia of institutions to participate in the Centers for Excellence in Translational Research (CETR) program. The purpose of this FOA is to support multidisciplinary translational research centers focused on generating, validating and advancing medical countermeasures to NIAID Emerging and Re-emerging Infectious Diseases.

Key Dates

Posted Date	November 23, 2012
Letter of Intent Due Date(s)	February 26, 2013
Application Due Date(s)	March 26, 2013
AIDS Application Due Date(s)	Not Applicable
Scientific Merit	July, 2013

Review	
Advisory Council Review	October, 2013
Earliest Start Date	March, 2014
Expiration Date	March 27, 2013
Due Dates for E.O. 12372	Not Applicable

Required Application Instructions

It is critical that applicants follow the instructions in the PHS 398 Application Guide except where instructed to do otherwise (in this FOA or in a Notice from the *NIH Guide for Grants and Contracts*). Conformance to all requirements (both in the Application Guide and the FOA) is required and strictly enforced. While some links are provided, applicants must read and follow all application instructions in the Application Guide as well as any program-specific instructions noted in Section IV. When the program-specific instructions deviate from those in the Application Guide, follow the program-specific instructions. **Applications that do not comply with these instructions may be delayed or not accepted for review.**

Looking ahead: NIH is committed to transitioning all grant programs to electronic submission using the SF424 Research and Related (R&R) format and is currently investigating solutions that will accommodate NIH's multi-project programs. NIH will announce plans to transition the remaining programs in the *NIH Guide to Grants and Contracts* and on NIH's Applying Electronically website.

Table of Contents

Part 1. Overview Information
Part 2. Full Text of Announcement
- Section I. Funding Opportunity Description
- Section II. Award Information
- Section III. Eligibility Information
- Section IV. Application and Submission Information
- Section V. Application Review Information
- Section VI. Award Administration Information
- Section VII. Agency Contacts
- Section VIII. Other Information

Part 2. Full Text of Announcement

Section I. Funding Opportunity Description

Purpose

The National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), supports extramural research focused on understanding, controlling and preventing diseases caused by virtually all infectious agents. In response to threats presented by emerging infectious diseases and bioterrorism, the NIAID Division of Microbiology and Infectious Diseases (DMID) has established complementary research programs to facilitate development of medical countermeasures for certain pathogens and toxins.

With this FOA, NIAID invites applications to establish Centers of Excellence for Translational Research (CETR) focused on the development of medical countermeasures and associated platforms/technologies targeting NIAID Emerging and Re-emerging Infectious Diseases (http://www.niaid.nih.gov/topics/emerging/pages/list.aspx), which includes NIAID Category A, B and C Priority Pathogens. For the purposes of this FOA, "translational research" is defined as research and developmental activities focused on transforming basic science outcomes (knowledge, technologies, infrastructure, etc.) into

new and innovative approaches for prevention, diagnosis, and treatment of disease. Priority will be given to Centers that address the greatest clinical need. Emphasis will be placed on Centers that integrate current research knowledge and infrastructure with highly innovative and synergistic approaches to facilitate medical countermeasure development, and address related constraints, challenges or barriers to product development, licensure and usage. Clinical trials will not be supported under this program.

Background

The NIH and other agencies in the Department of Health and Human Services (DHHS) support development of medical countermeasures to protect the public from emerging infectious diseases and bioterrorist threats. In 2002, NIAID published the initial Strategic Plan for Biodefense Research (http://www3.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/PDF/strategic_plan.pdf) and related research agendas (http://www3.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/about/strategicplan.htm), outlining research and development objectives to address threats posed by Category A, B and C Priority Pathogens. A significant component of the Strategic Plan was the establishment of the Regional Centers of Excellence for Biodefense and Emerging Infectious Diseases Research (RCE) program (http://www.niaid.nih.gov/labsandresources/resources/rce/Pages/default.aspx). The RCE program was initiated in 2003, renewed in 2008, and currently supports 11 Centers nationwide (http://www.niaid.nih.gov/labsandresources/resources/rce/Pages/default.aspx). The goal of the RCE program was to establish and maintain appropriate infrastructure and multifaceted research and development activities to provide scientific information and translational research capacity to facilitate discovery and development of the next generation of therapeutics, vaccines, and diagnostics against bioterror agents and emerging infectious diseases.

In 2007, NIAID published an updated Strategic Plan for Biodefense Research (http://www.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/Documents/biosp2007.pdf). The updated Strategic Plan is consistent with the DHHS Public Health Emergency Medical Countermeasure Enterprise (PHEMCE) Implementation Plan (http://www.hhs.gov/aspr/barda/phemce/enterprise/strategy/index.html), which outlined strategies for identifying medical countermeasure requirements and establishing priorities for their research, development and acquisition. The updated NIAID Strategic Plan continues to focus on translation of basic research to countermeasures, but with an emphasis shift from the “one bug-one drug” approach towards a more flexible, broad-spectrum approach. This approach is centered on development of countermeasures that are effective against multiple pathogens or toxins, development of technologies that can be widely applied to improve classes of products, and development of platforms that can reduce the time and cost of creating new products. The broad-spectrum approach recognizes the expanding range of biological threats and the limited resources available to address each individual threat. As one mechanism to achieve the goals of the updated Strategic Plan, the NIAID will establish the CETR program to facilitate innovative, interdisciplinary translational research efforts focused on countermeasure and/or related platform development.

The primary goals of the RCE program have been accomplished and the program will end in 2014. The CETR program will build upon the research and development accomplishments of the RCE program and other NIAID-supported programs, and is intended to complement and enhance ongoing translational and product development activities.

Research Objectives and Scope

The objective of this FOA is to establish a program of multi-project translational research Centers focused on advancing discovery, preclinical development, production, licensure and/or use of new or improved countermeasures (therapeutics, immunotherapeutics, vaccines, vaccine technologies, and medical diagnostics) or related technologies specific for NIAID Emerging and Re-emerging Infectious Diseases. Each Center will be organized around a single theme that addresses development and/or use of a targeted countermeasure or technology. NIAID anticipates considerable variety in Center themes and objectives, which can range from development of single or multiple countermeasures targeting a specific group of listed pathogens/toxins to development of new technologies or platforms that target a wide array of pathogens/toxins. Translational activities are anticipated to range from very early discovery-based efforts to late-stage preclinical development. Additionally, each Center must identify and address anticipated regulatory

barriers for the countermeasure or technology and propose research and/or strategies to overcome these barriers. This is particularly important for new classes of medical countermeasures for which there are no precedents for FDA approval.

Examples of translational research themes include, but are not limited to, the following areas:

- New or improved therapeutics against antibiotic-resistant pathogens.
- Development of a broad-spectrum countermeasure or technology.
- Countermeasures targeting intracellular pathogens.
- Development of a multivalent ("universal") or therapeutic vaccine.
- Host-targeted interventions as therapeutics.
- Development of broad-spectrum countermeasures against taxonomically-related viruses.
- Improved vaccine technologies or production platforms.
- Co-development of a therapeutic and diagnostic that have broad-spectrum activity.

Each application must clearly define the proposed Center theme and the range of activities being pursued, and the significance of the Center theme in regard to countermeasure development, public health need, and benefit to public health of a successful effort. Additionally, each application must detail how each Research Project contributes to the theme and objectives as well as project interdependence. Applications must outline expected synergies provided by the proposed center structure (see CETR Structure below). NIAID encourages Centers focused on development of medical countermeasures that are effective against a variety of pathogens and toxins, technologies that can be widely applied to improve classes of products, and platforms that can reduce the time and cost of creating new products. For this FOA, applications must focus translational activities towards development of one or more specific countermeasures or technologies as described below.

Note: For the purpose of this FOA, broad-spectrum is defined as a countermeasure which is effective against multiple infectious disease agents where one or more is on the list of NIAID Emerging and Re-emerging Infectious Diseases.

Note: Translational research themes supported by this program must target listed NIAID Emerging and Re-emerging Infectious Diseases. Accordingly, all Center research and development activities must utilize the virulent form of a listed human pathogen under appropriate biosafety conditions, or a CDC-approved excluded strain that is an attenuated form of the human pathogen.

Note: While clinical development strategies and/or clinical studies (e.g. sample collection, strain isolation, etc.) may be included within an overall project, this FOA will NOT support clinical trials; applications requesting support for clinical trials are nonresponsive to this FOA and will not be reviewed. Utilization of human-derived material in pre-clinical studies is encouraged.

In order to be responsive, projects must focus on the following countermeasures and/or technologies:

Therapeutics

This FOA will support the development of therapeutics, including immune-based and host-targeted

therapeutics, with special interest in broad-spectrum therapeutics and those targeting antimicrobial resistant pathogens and/or pathogens for which no standard clinical treatment exists.

Immune-based therapeutics may include both broad-spectrum (innate immunity, for example) and pathogen- or toxin-specific immunotherapeutics (antibodies, for example) that target listed pathogens/toxins. Of particular interest are immunotherapeutics that would enable prevention of infection or intoxication in the face of an immediate threat, protection of immunocompromised individuals, or post-exposure treatment to suppress infection and disease. NIAID encourages discovery and/or development of immunotherapeutics that directly affect pathogens/toxins and/or therapeutic approaches to stimulate non-specific immunity. Passive treatments may be especially valuable during the acute emergence of infectious diseases and may complement the use of antimicrobial drugs or vaccination programs to optimize protection.

This program will support discovery and development of therapeutics that target host-encoded functions required for infection, replication, spread and/or pathogenesis by one or more listed pathogens and potentially, additional non-listed pathogens. The development of therapeutics that target host-encoded functions provides a potential solution to emergence of microbial resistance and high developmental costs associated with treatments effective against only one microbe. Of particular interest are therapeutics that target specific host functions/pathways that are required for infection and pathogenesis by unrelated pathogens. For host-targeted intervention projects, applicants must clearly define the specific required host-pathogen interaction(s) for development of a corresponding targeted therapeutic(s).

Vaccines

This FOA will support the development of multivalent/universal vaccines and therapeutic vaccines. The development of monovalent vaccines against many listed pathogens and toxins is currently well represented within the NIAID emerging infectious diseases research portfolio. Accordingly, vaccine research supported by this FOA aligns with current HHS priorities that emphasize development of the following broad-spectrum vaccine forms targeting emerging diseases:

Multivalent/Universal Vaccines

For this FOA, multivalent or universal vaccines are defined as broad-spectrum vaccines that provide protection against a group of taxonomically related pathogens, or two or more unrelated pathogens. Vaccines characterized by broad-spectrum activity in this class include cross-protective forms, which induce an immune response against constant components of two or more microbes, and multiple component forms, which include elements that protect against microbes that are different, and may or may not be related. Examples of a multivalent/universal vaccine include a universal influenza vaccine and a multivalent vaccine that protects against both X and Y.

Therapeutic Vaccines

For this FOA, therapeutic vaccines are defined as those vaccines that can be delivered after disease onset to simplify, reduce, and/or shorten, or eliminate complicated treatment regimens, or reduce development of antimicrobial resistance. Examples of therapeutic vaccines include forms used for combination treatment of infections caused by *Mycobacterium tuberculosis* or *Burkholderia pseudomallei*.

Note: Applications for centers focused on development of a vaccine that cannot be classified as a multivalent/universal or therapeutic form are nonresponsive and will not be reviewed.

Vaccine Technologies

This FOA will support Center activities focused on discovery and/or development of innovative vaccine technologies (including adjuvants), particularly those that would improve vaccine effectiveness and/or simplify vaccine delivery to patient populations during a natural outbreak of an infectious disease or following the intentional release of an agent. Proposed projects must pair the candidate technology with one or more appropriately mature, well-characterized vaccine(s)/antigen(s) (except those pertinent to HIV) for which models and assays exist to allow evaluation of the technology. Applications focused on development of technologies such as delivery platforms, antigen targeting, adjuvants, stability and cold-chain minimization, production characterization or formulation methodologies are encouraged.

Note: While this FOA will not support the development of a candidate monovalent vaccine, a project focused on development of a new vaccine technology or production platform may use a validated or licensed monovalent vaccine as the demonstration and/or development platform.

Diagnostic Technologies

This FOA will support development of new and innovative integrated, sample-to-answer, rapid, easy-to-use, sensitive, and specific diagnostic platforms and/or technologies that address clinical priorities/needs/challenges pertinent to emerging infectious diseases. These medical diagnostics are to be targeted for use in clinical settings, including hospital-based clinical microbiology laboratories, point-of-care, and/or public health laboratories and will enable clinicians to rapidly provide the appropriate therapy

to patients. Applications must address methods for sample preparation, collection, and processing for the novel platform. The integrated medical diagnostic must be able to accept and process clinical sample volumes required for detection of targeted pathogen(s). Proposed novel, emerging diagnostic platforms may focus on detection of pathogen or host-specific targets. Successful projects will demonstrate the capability of the proposed platform to detect the targeted pathogens in valid infectious disease animal models (for in vivo technologies) or human clinical samples (for other technologies).

While it is not expected that proof-of-concept stage technologies should be ready for validation studies to enable FDA approval at the end of the project period, FDA approval would be an eventual long-term goal of projects supported under this program.

The following technologies/platforms are strongly encouraged:

Multiplex diagnostic technologies;

Technologies capable of identifying engineered or otherwise acquired genetic traits, such as patterns of antimicrobial resistance or enhanced virulence; and/or

Technologies that could be operational in non-traditional health care settings including rural and urban community health care clinics and temporary health care clinics (e.g., those established in response to a natural or man-made disaster).

Note: Applications focused on development of environmental, agricultural, industrial and/or workplace surveillance/detection technologies are nonresponsive and will not be reviewed.

CETR STRUCTURE

Each Center in the CETR program will be organized around a chosen theme focused on development and/or use of one or more countermeasures and/or technologies that target NIAID Emerging and Re-emerging infectious Diseases with the objective of translating research results to product development. Each Center will include individual Research Projects, an Administrative Core, and if necessary, Scientific Cores (see component descriptions below). NIAID anticipates considerable variety in Center themes and objectives, which can range from development of single or multiple countermeasures targeting a specific group of Emerging and Re-emerging Infectious Diseases to development of new technologies or platforms that target a wide array of pathogens/toxins. Additionally, translational activities are anticipated to range from very early discovery-based efforts to late-stage preclinical development.

As an example, a Center focused on discovery of new therapeutics that target select intracellular bacterial pathogens might have the following structure:

Research Project 1: Identification of Novel Therapeutic Targets in Pathogens

Research Project 2: Bioinformatic/Proteomic Approaches to Identify Essential Host Activities as Therapeutic Targets

Research Project 3: Screening for Small Molecule Effectors against Pathogen and Host Targets

Administrative Core

As a second example, a Center focused on an innovative broad-spectrum platform for production of vaccines might have the following structure:

Research Project 1: Production and Stabilization of a Smallpox Vaccine

Research Project 2: Production and Stabilization of Influenza Vaccines

Research Project 3: Production and Stabilization of a MRSA Vaccine

Administrative Core

Animal Model Core (Vaccine Efficacy Evaluation)

As a third example, a Center focused on development of countermeasures targeting a group of taxonomically related bacterial pathogens might have the following structure:

Research Project 1: Optimization and Preclinical Evaluation of Therapeutic X

Research Project 2: Optimization and Preclinical Evaluation of Immunotherapeutic Y

Research Project 3: Clinical Diagnostics for Targeted Bacteria and Antibiotic Resistance

Administrative Core

CETR COMPONENTS

Research Projects

Each Center must include at least three but not more than six interdependent translational Research Projects organized around the chosen theme; projects must focus on development and advancement of a new or improved medical countermeasure and/or associated platform/technology targeting one or more NIAID Emerging and Re-emerging Infectious Diseases. Each Research Project must contribute directly to the Center theme and objective(s). The Center Program Director/Principal Investigator (PD/PI) will monitor all Research Projects and promote efforts that foster integration, collaboration and synergy. Research Projects may be proposed for up to five years and each project leader must commit at least 1.2 calendar months effort to their project per year. Research Projects are expected to incorporate state-of-the-art technology and approaches and may include consortium arrangements for required activities. Applicants are encouraged to carefully consider the scope and range of research proposed and develop a Center that is coherent overall and consistent with available resources and personnel.

All Research Projects must include detailed project performance and timeline objectives (see Section IV. Application and Submission Information). Additionally, projects must identify and address anticipated regulatory barriers and propose research and/or strategies to overcome these barriers (see below). This is particularly important for new classes of medical countermeasures for which there are no precedents for FDA approval.

All Research Projects at the stage of preclinical product development must include a Product Development Strategy section (see Section IV. Application and Submission Information; Product Development Strategy). The Product Development Strategy must include both a Milestones and Timelines section detailing project performance and timeline objectives and a Product Development Plan. This plan should include the participation of consultants with expertise in technology transfer and the development of regulated products. Only those projects for which the mechanism of action of the candidate product is reasonably well understood, possible routes of administration and formulations suitable for scalable production identified, and assays to assess product quality that are well developed should be considered for support beyond Technology Readiness Level (TRL) 5 (https://www.medicalcountermeasures.gov/federal-initiatives/guidance/about-the-trls.aspx). In addition, animal models that are adequate to assess the ability of the product to induce a certain response and endpoints that will satisfy the Animal Rule (http://www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/ProductSecurity/ucm110322.htm) should be identified and included for candidate products that may not be tested for efficacy in humans.

Administrative Core

Each Center must include an Administrative Core, under which all Center activities will be managed, coordinated, and supervised. A well-structured and run Administrative Core is integral to a Center's success, and should be clearly described in the application. The PD/PI must be the director of the Administrative Core and must commit at least 0.6 calendar months effort per year to these responsibilities, in addition to his/her Research Project activities. An Administrative Core Associate should be a research administrator responsible for the daily administration and fiscal management of the Center. It is recommended that the Administrative Core Associate devote a level of effort ranging from 3 to 6 person months to the execution of the administrative details of the Center projects and to managing the day-to-day operations within the Center. Any additional Administrative Core personnel must be clearly justified. The Administrative Core budget may not

exceed 5% of total direct costs requested.

The Administrative Core must include a Management Plan that identifies and discusses: the Administrative Core structure; the roles of Administrative Core personnel; the composition and duties of the Scientific Advisory Committee (see below); and the facilitation of communications throughout the Center and with NIAID staff. The plan should specifically address continual evaluation of research and development progress, communications, group meetings and teleconferences, presentation and publication of data, resource and model sharing, transmission of information and reagents, the identification and proposed resolution of problems and engagement of the NIAID Staff as appropriate. A description of how consortia (subcontracts) will be managed should be provided and should include how communications such as periodic meetings and conference calls will be organized, managed and documented. The plan should include a Core budget and should identify how research-related travel will be managed. The plan must also detail the process for periodic solicitation and review of CETR program-sponsored supplemental research applications (see Section IV. Application and Submission Information under Administrative Core: Supplemental Research Projects).

The PD/PI and Administrative Core staff are responsible for ensuring that all research activities are carried out in compliance with all federal and NIH regulations. A necessary component of a Center's success will be the availability of adequate access to BSL3/4 biocontainment facilities. Applicants must identify Research Projects that will require high level containment facilities and provide a description of facilities that are available currently or planned at consortium institutions. A table listing each activity that requires BSL3/4 access and the likely facilities to be used should be included. The PD/PI and Administrative Core staff are also responsible for ensuring that appropriate systems are in place to provide for biosafety and security of materials, data, facilities and resources, including compliance with regard to Select Agent Regulations, Biosafety in Microbiology and Biomedical Laboratories (BMBL) Guidelines, Centers for Disease Control and Prevention and the National Institutes of Health, fifth Edition (http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm); U.S. Code of Federal Regulations 42 C.F.R. Part 73, 7 C.F.R. Part 331, and 9 C.F.R. Part 121 (http://www.cdc.gov/od/sap/).

Each Administrative Core **must** include the following components:

Scientific Advisory Committee

Each Center must establish a Scientific Advisory Committee that includes the PD/PI, some or all Research Project leaders, and at least 3 non-conflicted external advisors. For Centers engaged in preclinical product development activities, at least one of the external advisors must have demonstrated and relevant industry-level expertise. The Scientific Advisory Committee will be responsible for the annual scientific evaluation of all center research projects and Core activities. Additionally, the Scientific Advisory Committee will be responsible for soliciting and reviewing CETR program-sponsored Supplemental Research applications (see below).

Regulatory Expertise

All CETR program-supported countermeasure development must address anticipated regulatory requirements and challenges pertinent to Emerging and Re-emerging Infectious Diseases. Accordingly, each Center must include regulatory expertise appropriate for associated developmental activities. Regulatory expertise may be retained as defined effort or periodic consultation.

Programmatic Meetings

NIAID anticipates supporting an annual National CETR Program Meeting to highlight Center progress/activities, review progress, share knowledge of innovative technologies and methodologies, and encourage collaborations. The annual program meetings are expected to be attended by the PD/PI, some or all Research Project Leaders, Scientific Core Leaders, other appropriate key personnel and NIAID staff. These meetings are anticipated to be held at a location at/near Bethesda, MD or at another NIAID-approved site and will last for 1-2 days. Each Center application budget should include funds to travel and attend the annual meeting in Years 2-5 of the project period.

Each Center must participate in an annual reverse site visit to evaluate progress. The annual reverse site

visit will be attended by the PD/PI, 1-2 appropriate Project Leaders, and NIAID staff. These reverse site visits are anticipated to be held at a location at/near Bethesda, MD or at another NIAID-approved site and will last 1 day. Each Center application budget should include funds to travel and attend the reverse site visits in Years 1-4 of the project period.

Supplemental Research Projects

In order to facilitate development and/or improvement of innovative translational platforms/technologies, NIAID anticipates having funds to support a limited number of short-term (2-3 years) Supplemental Research Projects throughout the award period. The Supplemental Research Projects will begin in the second year of the CETR program with approved projects phased in to the parent award period. Under programmatic guidance, Centers will solicit and review applications for research projects that complement or facilitate specific theme objectives. Total funding of a Supplemental Research Project may not exceed $200,000 in total costs in any year and the Supplemental Research Project Leader must commit at least 1.2 person months per year to the effort.

Applicants may not submit in their applications descriptions of the projects that would be supported by the Supplemental Research program; these will be solicited and reviewed post-award. Applicants must include in the Administrative Core section a plan that outlines procedures for soliciting, reviewing and identifying the most meritorious Supplemental Research Project applications. Funding of Supplemental Research Projects will be determined by NIAID Program leadership based on Center recommendations, project merit, programmatic priorities and available funds. Do not include funds for Supplemental Research Projects in the budget.

Scientific Cores

A Center may include development and maintenance of one or more core resources/facilities that are essential for the activities of two or more Research Projects. A scientific core must provide a unique service that cannot be obtained through institutional or commercial means. Cores are intended to serve the needs of Center project researchers and they may not conduct research independent of the served Research Projects. The role of each core in overall Center research activities must be clearly described and justified. Additionally, plans for staffing, managing, and prioritizing use of the cores must be provided, as well as plans for determining fees to users if charging fees is necessary.

Section II. Award Information

Funding Instrument	Cooperative Agreement: A support mechanism used when there will be substantial Federal scientific or programmatic involvement. Substantial involvement means that, after award, NIH staff will assist, guide, coordinate, or participate in project activities.
Application Types Allowed	New The OER Glossary and the PHS 398 Application Guide provide details on these application types.
Funds Available and Anticipated Number of Awards	NIAID intends to commit $75 million in FY2014 to fund 10 to 20 awards.
Award Budget	Application budgets are limited to $4 million for FY2014 directs costs and need to reflect actual needs of the proposed project.
Award Project Period	Scope of the proposed project should determine the project period. The maximum period is 5 years.

NIH grants policies as described in the *NIH Grants Policy Statement* will apply to the applications submitted and awards made in response to this FOA.

Section III. Eligibility Information

1. Eligible Applicants

Eligible Organizations

Higher Education Institutions

- Public/State Controlled Institutions of Higher Education
- Private Institutions of Higher Education

The following types of Higher Education Institutions are always encouraged to apply for NIH support as Public or Private Institutions of Higher Education:

- Hispanic-serving Institutions
- Historically Black Colleges and Universities (HBCUs)
- Tribally Controlled Colleges and Universities (TCCUs)
- Alaska Native and Native Hawaiian Serving Institutions
- Asian American Native American Pacific Islander Serving Institutions (AANAPISIs)

Nonprofits Other Than Institutions of Higher Education

- Nonprofits with 501(c)(3) IRS Status (Other than Institutions of Higher Education)
- Nonprofits without 501(c)(3) IRS Status (Other than Institutions of Higher Education)

For-Profit Organizations

- Small Businesses
- For-Profit Organizations (Other than Small Businesses)

Governments

- State Governments
- County Governments
- City or Township Governments
- Special District Governments
- Indian/Native American Tribal Governments (Federally Recognized)
- Indian/Native American Tribal Governments (Other than Federally Recognized)
- Eligible Agencies of the Federal Government
- U.S. Territory or Possession

Other

- Independent School Districts
- Public Housing Authorities/Indian Housing Authorities
- Native American Tribal Organizations (other than Federally recognized tribal governments)
- Faith-based or Community-based Organizations
- Regional Organizations
- Non-domestic (non-U.S.) Entities (Foreign Institutions)

Foreign Institutions

Non-domestic (non-U.S.) Entities (Foreign Institutions) **are** eligible to apply.
Non-domestic (non-U.S.) components of U.S. Organizations **are** eligible to apply.
Foreign components, as defined in the *NIH Grants Policy Statement*, **are** allowed.

Required Registrations

Applicant organizations must complete the following registrations as described in the PHS 398 Application Guide to be eligible to apply for or receive an award. Applicants must have a valid Dun and Bradstreet

Universal Numbering System (DUNS) number in order to begin each of the following registrations.

- System for Award Management (SAM)– must maintain an active entity registration (formerly CCR registration), to be renewed at least annually. Use the Sam.gov "Manage Entity" function to manage your entity registrations. See the Grants Registration User Guide at SAM.gov for additional information.
- eRA Commons

All Program Directors/Principal Investigators (PD(s)/PI(s)) must also work with their institutional officials to register with the eRA Commons or ensure their existing eRA Commons account is affiliated with the eRA Commons account of the applicant organization.

All registrations must be completed by the application due date. Applicant organizations are strongly encouraged to start the registration process at least 6 weeks prior to the application due date.

Eligible Individuals (Program Director/Principal Investigator)

Any individual(s) with the skills, knowledge, and resources necessary to carry out the proposed research as the Program Director(s)/Principal Investigator(s) (PD(s)/PI(s)) is invited to work with his/her organization to develop an application for support. Individuals from underrepresented racial and ethnic groups as well as individuals with disabilities are always encouraged to apply for NIH support.

For institutions/organizations proposing multiple PDs/PIs, visit the Multiple Program Director/Principal Investigator Policy and submission details in the Senior/Key Person Profile (Expanded) Component of the PHS 398 Application Guide.

2. Cost Sharing

This FOA does not require cost sharing as defined in the *NIH Grants Policy Statement.*

3. Additional Information on Eligibility

Number of Applications

Applicant organizations may submit more than one application, provided that each application is scientifically distinct.

NIH will not accept any application that is essentially the same as one already reviewed within the past thirty-seven months (as described in the *NIH Grants Policy Statement*), except for submission:

- To an RFA of an application that was submitted previously as an investigator-initiated application but not paid;
- Of an investigator-initiated application that was originally submitted to an RFA but not paid; or
- Of an application with a changed grant activity code.

Section IV. Application and Submission Information

1. Address to Request Application Package

Applicants are required to prepare applications according to the current PHS 398 application forms in accordance with the PHS 398 Application Guide.

2. Content and Form of Application Submission

It is critical that applicants follow the instructions in the PHS 398 Application Guide, except where instructed in this funding opportunity announcement to do otherwise. Conformance to the requirements in the Application Guide is required and strictly enforced. Applications that are out of compliance with these instructions may be delayed or not accepted for review.

Letter of Intent

Although a letter of intent is not required, is not binding, and does not enter into the review of a subsequent application, the information that it contains allows IC staff to estimate the potential review workload and plan the review.

By the date listed in Part 1. Overview Information, prospective applicants are asked to submit a letter of intent that includes the following information:

- Descriptive title of proposed research
- Name(s), address(es), and telephone number(s) of the PD(s)/PI(s)
- Names of other key personnel
- Participating institution(s)
- Number and title of this funding opportunity

The letter of intent should be sent to:

Lynn Rust, Ph.D.
Division of Extramural Activities
National Institute of Allergy and Infectious Diseases (NIAID)
Room 3120, MSC-7616
6700-B Rockledge Drive
Bethesda, MD 20817-7616 (express mail), 20892-7616 (US mail)
Telephone: 301-402-3938
Email: lrust@mail.nih.gov

Application Submission

Applications must be prepared using the PHS 398 research grant application forms and instructions for preparing a research grant application. Submit a signed, typewritten original of the application, including the checklist, and three signed photocopies in one package to:

Center for Scientific Review
National Institutes of Health
6701 Rockledge Drive, Room 1040, MSC 7710
Bethesda, MD 20892-7710 (U.S. Postal Service Express or regular mail)
Bethesda, MD 20817 (for express/courier service; non-USPS service)

At the time of submission, two additional paper copies of the application and all copies of the Appendix files must be sent to:

Lynn Rust, Ph.D.
Division of Extramural Activities
National Institute of Allergy and Infectious Diseases (NIAID)
Room 3120, MSC-7616
6700-B Rockledge Drive
Bethesda, MD 20817-7616 (express mail), 20892-7616 (US mail)
Telephone: 301-402-3938
Email: lrust@mail.nih.gov

Page Limitations

All page limitations described in the PHS 398 Application Guide and must be followed , with the following exceptions or additional requirements:

- Overall Research Strategy is limited to 12 pages
- Research Projects are limited to 12 pages
- Product Development Strategy (limited to 12 pages; not included in the Research Strategy section)
- Administrative Core is limited to 12 pages
- Scientific Cores are limited to 6 pages

Supplemental Instruction for the Preparation of Multi-Project Applications

All instructions in the PHS 398 Application Guide must be followed, with the following additional instructions.

> The following section supplements the instructions found in Form PHS 398 for preparing a multi-project grant application. Additional instructions are required because the Form PHS 398 is designed primarily for individual, free-standing research project grant applications, and has no specific instructions for multi-project applications consisting of research projects interrelated by a common theme. All applications must be submitted on Form PHS 398. The multi-project grant application should be assembled and paginated as one complete document. Instructions for the Overall Component are presented first, followed by instructions for Research Projects, followed by instructions for Cores.

Instructions for Overall Component

Form Page 1. Face Page (Overall)

Complete this page as instructed in the Application Guide. This should be the first page of the entire application and all succeeding pages should be numbered consecutively.

Form Page 2. Summary, Relevance, Project/Performance Site,Senior/Key Peronnel, Other Signficant Contrbutor, and Human Embroyonic Stem Cells (Overall)

Description: Using Page 2 of Form 398; provide a succinct but accurate description (abstract) of the OVERALL multi-project application addressing the major, common theme of the program. Do not exceed the space provided.

Project/Performance Sites

List the performance sites where the research will be conducted.

Senior/Key Personnel (Overall)

Under "Key Personnel", list the PD/PI of the multi-project application, followed by the Project and Core Leaders of the component research projects and cores, and other key personnel and then other significant contributors.

Form Page 3. Table of Contents (Overall)

Do not use Form Page 3 of the PHS 398; a more comprehensive table of contents is needed for a multi-project application.

Bearing in mind that the application will be scientifically reviewed project by project and core by core, prepare a detailed Table of Contents that will enable reviewers to readily locate specific information pertinent to the overall application as well as to each component research project and core. A page reference should be included for the budget for each project and each core. Further, each research project should be identified by number (e.g. Project 1), title, and responsible Project Leader, and each Core should be identified by letter (e.g. Core A), title, and responsible Core Leader. The page location of a COMPOSITE BUDGET should be indicated in the "Table of Contents."

Form Page 4. Composite Budget (Overall)

Do not use Form Page 4 of PHS Form 398. Instead, using the suggested format presented below, prepare a Composite Budget for All Proposed Years of Support. (Justification for budget elements should not be presented here but in the individual budgets of the projects and cores.)

SAMPLE: Consolidated Direct Cost Budget for All Proposed Years of Support

Component	Year 1	Year 2	Year 3	Year 4	Year 5	All Years
Project 1. Invest.	125,000	130,000	135,200	140,608	146,232	677,040
Project 2. Study	125,000	130,000	135,200	140,608	146,232	677,040
Project 3. Develop.	100,000	104,000	108,160	112,486	116,985	541,631
Core A. Admin. Core.	50,000	52,000	54,080	56,243	58,493	270,816
Core B. DNA	25,000	50,000	52,000	54,080	56,243	237,323
Totals	425,000	466,000	484,640	504,025	524,185	2,403,850

Form Page 5.

Total Direct Costs for the Entire Budget Period (Overall)
Complete the Total Direct Cost line entries for all requested budget periods (years) and the Total Direct Cost for Entire Period of Support entry. Detailed budgets are required within the descriptions of each project and core (see below).

Biographical Sketch Format Page

Biographical sketches of all professional personnel for all components should be placed at the end of the application with the PD/PI first, followed by those of other key personnel in alphabetical order.

Resources Format Page

Do not complete. Essential information is to be presented in the individual research project and core sections of the application.

Center Program Overview Reseach Plan (Research Objectives and Strategic Plan) Overall

Specific Aims

List in priority order, the broad, long-range objectives and goals of the proposed Program. Concisely and realistically describe the hypothesis or hypotheses to be tested.

Overall Research Strategy

This narrative section summarizes the overall research plan for the multi-project application and is limited to (12) pages. The multi-project application should be viewed as a confederation of interrelated research projects, each capable of standing on its own scientific merit, but complementary to one another. This is an important section for it provides the group of investigators an opportunity to give conceptual wholeness to the overall program – by giving a statement of the general problem area and by laying out a broad strategy for attacking the problems. Applicants should define the Center theme and the range of activities being pursued, the significance of the Center theme in regard to countermeasure development, public health need, and benefit to public health of a successful effort. Additionally, discussions of how each Research Project contributes to the theme and objectives as well as project interdependence should be included. Applications should outline expected synergies provided by the proposed center and summarize the special features in the environment and/or resources that make this application strong or unique.

Resource Sharing Plan

Individuals are required to comply with the instructions for the Resource Sharing Plans (Data Sharing Plan, Sharing Model Organisms, and Genome Wide Association Studies (GWAS)) as provided in the PHS 398 Application Guide, with the following modification:

- All applications, regardless of the amount of direct costs requested for any one year, should address a

Data Sharing Plan.

Checklist

One Checklist, placed at the end of the Overall component, is to be submitted for the entire application.

Appendix Materials

Do not use the Appendix to circumvent page limits. Follow all instructions for the Appendix (please note all format requirements) as described in the PHS 398 Application Guide.

Instructions for Research Projects

A minimum of three and not more than six individual Research Projects are required.

For each individual Research Project, include:

- Face page (see special instructions, below)
- Description & Key personnel (PHS 398 Form Page 2)
- Table of Contents (PHS 398 Form Page 3)
- Budget Pages (PHS 398 Form Pages 4 and 5); with budget justifications
- Research Plan
- Resources

Cover Page (Reseach Projects)

The Face Page of the 398 Form should not be used as a cover page for individual research projects within a multi-project application. Instead, use the PHS 398 continuation page to create a "Cover Page" containing selected data about each individual research project. This Cover Page will demarcate each individual research project and should contain the following information items (these are a subset of the information provided on a PHS 398 Face Page):

- Project Number and Title: (e.g., 1. Preclinical Evaluation of HIV Microbicides)
- Name of Project Leader: (e.g., Jones, Roberta A.)
- Human Subjects: (Yes or No)
- If Yes:
- Exemption number, -or-
- IRB Approval Date (e.g., 12/13/2006,or "Pending"), and Federalwide Assurance (FWA) number
- Vertebrate Animals: (Yes or No)
- If Yes:
- IACUC Approval Date (e.g., 11/17/2006, or Pending) and Animal welfare assurance number:
- Proposed Period of Support:
- From: (mmddyy - e.g., 07/01/2007)
- To: (mmddyy - e.g., 06/30/2112)
- Costs Requested for Initial Budget Period: (e.g. 07/01/2007-06/30/2008)
- Direct Costs: (e.g., $ 150,000)
- Total Costs: (e.g., $162,000)
- Costs Requested for the Entire Budget Period: (e.g., 07/01/2007-06/30/2112)
- Direct Costs: (e.g., $700,000)
- Applicant Organization (full address)

Form Page 2. (Research Projects)

Provide a Description (abstract) of the research proposed in the individual research project according to the instructions on Form Page 2 of PHS Form 398. In addition, the abstract should contain a brief description of how the individual research project will contribute towards attainment of the multi-project Center objectives.

Project/Performance Sites (Research Projects)

List the performance sites where the research will be conducted.

Key Personnel (Research Projects)

Under "Key Personnel", list the Project Leader, followed by other key project personnel, and then other significant contributors.

Form Page 3. Table of Contents (Research Projects)

Prepare a Table of Contents for the research project using Form Page 3 of the PHS 398.

Form Page 4. and 5. Detailed Budget for Initial Budget Period (Research Projects)

Prepare a detailed budget and justification for the research project using Form Pages 4 and 5 of the PHS 398.

Individual Research Project Research Plan (Research Projects)

Specific Aims

List, in priority order, the broad long-range objectives and goals of the proposed project. Concisely and realistically describe the hypothesis or hypotheses to be tested. In addition, state the individual research project's relationship to the Program's goals and how it relates to other projects or cores.

Research Strategy

Use this section to describe how the proposed research will contribute to meeting the Center's goals and objectives and explain the rationale for selecting the methods to accomplish the specific aims. In addition to stating the biological significance of the research, indicate the project's relevance to the primary theme of the application.

Organize the Research Strategy in the specified order as stated in the PHS 398 Instructions, Section 5.5. Make sure to start each section with the appropriate section heading in order, Significance, Innovation, Approach, and include the appropriate information. Experimental details should be cited using the Bibliography and Reference Cited section and need not be detailed in the Research Strategy. Preliminary Studies for new projects must be included as part of the approach section, and must be contained within the page limits of the Research Strategy section.

For all Research Projects (within the Research Strategy page limit), describe the research design conceptual procedures, and analyses to be used to accomplish the specific aims of the project. Describe any new methodology and its advantage over existing methodologies. Describe any novel concepts, approaches, tools, or technologies for the proposed studies. Discuss associations with clinical project(s). Discuss the potential difficulties and limitations of the proposed procedures and alternative approaches to achieve the aims. As part of this section, provide a tentative sequence or timetable for the project. Describe how the research strategy is informed by an understanding of the regulatory process. Describe anticipated regulatory barriers and propose research and/or strategies to overcome these barriers. Note: Each project leader must commit at least 1.2 person months effort to their project per year.

Product Development Strategy

In addition to the instructions above for all research projects, research projects directly involved in preclinical development of a candidate product **must** also include the following two components in a clearly labeled separate section immediately following the Research Strategy.

Milestones and Timeline. Applicants are required to provide detailed project performance and timeline objectives in a section entitled "Milestones and Timeline." This section must be no more than 5 pages and must include:

- A clear description of all interim objectives (research and/or developmental milestones) to be achieved

during the course of the project. Applicants also must identify any impediments that could require a revision in the work plan or milestones with a discussion of alternative approaches.
- Detailed quantitative criteria by which milestone achievement will be assessed.
- A detailed schedule or timeline for the anticipated attainment of each milestone and the overall goal(s).
- Titles, abstracts, and status of patents and patent applications that are relevant to the project and are owned or licensed by the applicant.

Product Development Plan. Applicants are required to provide detailed development plans in a section entitled "Product Development Plan". This section must be no more than 7 pages and must include:

- A statement of the intended use/indication of the proposed product and biodefense/public health gap the product is intended to fill.
- A statement of the value of the project, including lay description of key technology objectives, innovation, and advantages compared to competing products, technologies, or services.
- A clear description of the goal(s) of the project, including one (or more) intermediate products (tools), final product(s) or stage(s) of product development to be completed during the award period. A specific product profile that is defined by licensing indication is not requested.

Additionally, the Product Development Plan must include descriptions pertaining to preclinical product development activities pertaining to the product proposed. For the purpose of this FOA, "preclinical" is defined as all activities beyond lead candidate identification or diagnostic assay/platform/prototype development or vaccine technology proof-of-principle. Please see below for a list of points to be discussed as part of the Product Development Plan based on the type of product proposed.

Product Development Plans for therapeutic, vaccine, or adjuvant projects should summarize:

- The performance specifications and features the product should have in order to provide immunological and/or therapeutic benefit (vaccines) or clinical, manufacturing and/or economic benefit (vaccine technologies).
- A description of the candidate product or lead series as it is currently configured.
- A description and developmental status of the assays for product release and characterization, including activity and efficacy.
- Data that support the characterization and selection of the candidate product for further development. Specifically for vaccines, a summary of data that demonstrates efficacy in in vitro assays and/or in vivo models for one or more of the selected agent(s). This includes: a detailed description of the assays and animal models, the choice of pathogen challenge, strain and route, and a rationale for the choice of animal model, pathogen challenge, strain and route, as well as for the outcome/endpoints selected; documentation that the animal infection experiments were performed under well-controlled experimental conditions.
- Discussions with FDA, if any, which are relevant to development activities for the candidate product/technology.

Product Development Plans for diagnostics projects should summarize:

- The performance specifications the product (diagnostic assay, method, technology, etc.) should have to demonstrate it is equivalent or superior to the gold standard for identifying the proposed agent(s), including clinical sensitivity in appropriate human samples, clinical specificity in appropriate human samples, analytical sensitivity (limit of detection), analytical reactivity (pathogen strains detected for each pathogen), analytical specificity (cross-reactivity), and analytical reproducibility (test replicates at different agent concentrations, at different sites, etc.). If no FDA-cleared test is available for the agent(s), the specifications should be equivalent to or exceed performance specifications of FDA-cleared tests for similar types of agent(s).
- Data that support the selection of the candidate product for further development, including an assessment of the present capacity of the diagnostic method, technology or assay to meet the performance specifications.
- Discussions with FDA, if any, which are relevant to development activities for the candidate product.

When appropriate and as part of the Product Development Plan, applicants must document compliance with guidelines that govern GLP, as defined by 21 CRF (58), and cGMP, as defined by 21 CRF (211), manufacturing and/or IND/IDE enabling studies that will be performed under the project award as they would be applicable to eventual product licensure in the U.S. Applications for projects involving cGMP manufacture should ensure inclusion of appropriate personnel to provide regulatory guidance before, during and after manufacture.

Resource Sharing Plan

Individuals are required to comply with the instructions for the Resource Sharing Plans (Data Sharing Plan, Sharing Model Organisms, and Genome Wide Association Studies (GWAS)) as provided in the PHS 398 Application Guide, with the following modification:

- All applications, regardless of the amount of direct costs requested for any one year, should address a Data Sharing Plan

Resources Format Page (Research Projects)

Provide information on resources available for the project. Describe how the scientific environment in which the research will be done contributes to the probability of success (e.g., institutional support, physical resources, and intellectual rapport.) For Early Stage Investigators, describe institutional investment in the success of the investigator. If there are multiple performance sites, describe the resources available at each site. Describe any special facilities used for working with biohazards or other potentially dangerous substances.

Biographical Sketch Format Page (Research Projects)

Do not repeat the biographical sketches of participating investigators since this information will be included at the end of the overall application (and therefore will be referenced in the Overall Table of Contents).

Instructions for Core(s)

Each Center must include an Administrative Core. Scientific Cores are optional.

For each individual Core, include:

- Cover page
- Description & Key personnel (PHS 398 Form Page 2)
- Table of Contents (PHS 398 Form Page 3)
- Budget Pages (PHS 398 Form Pages 4 and 5); with budget justifications
- Aministrative and Scientific Core Services Plan
- Resources Format Page

Cover Page (Cores)

The Face Page of the 398 Form should not be used as a cover page for cores within a multi-project application. Instead, use the 398 continuation page to create a "Cover Page" containing selected data about each individual core. This Cover Page will demarcate each core and should contain the following information items (which are a subset of the information provided on a Face Page - see PHS 398):

- Core Letter and Core Title: (e.g., A. Monoclonal Antibody Production Core)
- Name of Core Leader: (e.g., Smith, Robert A.)
- Human Subjects (Yes or No)
- If Yes,
- Exemption Number, -or-
- IRB Approval Date (e.g., 5/14/06, or Pending), and Federalwide Assurance (FWA) number
- Vertebrate Animals (Yes or No)
- If Yes,
- IACUC Approval Date (e.g., 4/15/07, or Pending), and Animal welfare assurance number

- Proposed Period of Support
- From: (mmddyy, e.g., 07/01/2007)
- To: (mmddyy, e.g., 06/30/2012)
- Costs Requested for Initial Budget Period
- Direct Costs (e.g. $50,000)
- Total Costs (e.g. $70,000)
- Costs Requested for the Entire Budget Period
- Direct Costs (e.g. $212,323)
- Total Costs (e.g. $297,252)
- Applicant Organization (ABC University; 111 Main Street; Anywhere, Else 99999)

Format Page 2. (Cores)

Provide a Description (abstract) of the core activities and services according to the instructions on Form Page 2 of the PHS 398. In addition, the abstract should contain a brief description of how the core services will contribute towards attainment of the objectives.

Project/Performance Sites (Cores)

List the performance sites where the core activities and services will be conducted.

Key Personnel (Cores)

Under "Key Personnel", list the Core Leader, followed by other key core personnel, and then other significant contributors. The PD/PI must be the director of the Administrative Core and must commit at least 0.6 person months effort per year to these responsibilities. An Administrative Core Associate should be a research administrator responsible for the daily administration and fiscal management of the Center. It is recommended that the Administrative Core Associate devote a level of effort ranging from 3 to 6 person months to the execution of the administrative details of the Center projects and to managing the day-to-day operations within the Center. Any additional Administrative Core personnel must be clearly justified.

Format Page 3. Table of Contents (Cores)

Prepare a Table of Contents for the core using page 3 of Form PHS 398.

Format Page 4. and 5. Budget Pages (Cores)

Prepare a detailed budget and justification for the core using Form Pages 4 and 5 of the PHS 398.

The Administrative Core budget may not exceed 5% of total direct costs requested. Requests for funds to support required travel may be included here.

Research Plan - Administrative and Scientific Cores

Specific Aims (Cores)

List in priority order, the broad, long-range objectives and goals of the proposed Administrative, or Scientific Cores. In addition, state the core's relationship to the Program's goals and how it relates to the individual Research Projects or other cores in the application.

Research Strategy for Admin Core (required)

The Administrative Core must include a Management Plan that identifies and discusses: the Administrative Core structure; the roles of Administrative Core personnel; the composition and duties of the Scientific Advisory Committee; and the facilitation of communications throughout the Center and with NIAID staff; and assurance that all research activities are carried out in compliance with all federal and NIH regulations. The plan should specifically address continual evaluation of research and development progress, communications, group meetings and teleconferences, presentation and publication of data, resource and model sharing, transmission of information and reagents, the identification and proposed

resolution of problems and engagement of the NIAID Staff as appropriate. A description of how consortia (subcontracts) will be managed should be provided and should include how communications such as periodic meetings and conference calls will be organized, managed and documented. The plan should include a Core budget and should identify how research-related travel will be managed. The plan must also detail procedures for anticipated solicitation and review of Supplemental Research Projects applications. All CETR program-supported countermeasure development must address anticipated regulatory requirements and challenges pertinent to Emerging and Re-emerging Infectious Diseases. Accordingly, each Center must include regulatory expertise appropriate for associated developmental activities. Regulatory expertise may be retained as defined effort or periodic consultation.

In addition for Supplemental Research Projects, describe the internal institutional plans and procedures to ensure that all projects supported from this award will comply fully with all applicable Federal regulations, policies, and guidelines for research involving human subjects, including the evaluation of risks and protections in project proposals, appropriate ethical oversight and funded projects, and plans for data and safety monitoring for clinical trials, if applicable. Total funding of a Supplemental Research Project may not exceed $200,000 in total costs in any year and the Supplemental Research Project Leader must commit at least 1.2 person months per year to the effort.

Research Strategy for Scientific Cores

Use this section to describe how the proposed core activities will contribute to meeting the Center's goals and objectives and explain the rationale for selection of the general methods and approaches proposed to accomplish the specific aims. In addition, this section should indicate the relevance of the core to the primary theme of the application. Provide details of the services or resources provided by the optional cores to at least two Research Projects and clarify how the optional cores are not duplicative of other services or facilities. Each Scientific Core should be headed by a Core Leader with at least 1.2 person months of effort committed to the Core.

Organize the section in the specified order as stated in the PHS 398 Application Guide. Make sure to start each section with the appropriate section heading in order, Significance, Innovation, Approach, and include the appropriate information. Experimental details should be cited using the Bibliography and Reference Cited section and need not be detailed in the Research Strategy. Preliminary Studies for new cores and progress reports for renewals must be included as part of the approach section and must be contained within the page limits of the Core Services Plan section.

Resource Sharing Plan (Cores)

Individuals are required to comply with the instructions for the Resource Sharing Plans (Data Sharing Plan, Sharing Model Organisms, and Genome Wide Association Studies (GWAS)) as provided in the PHS 398 Application Guide, with the following modification:

- All applications, regardless of the amount of direct costs requested for any one year, should address a Data Sharing Plan.

Biographical Sketch Format Page (Cores)

Do not repeat the biographical sketches of participating investigators since this information will be included at the end of the overall application (and therefore will be referenced in the Overall Table of Contents).

Resources Format Page (Cores)

Provide information on resources available for the core. Describe how the scientific environment in which the research will be done contributes to the probability of success (e.g., institutional support, physical resources, and intellectual rapport.) For Early Stage Investigators, describe institutional investment in the success of the investigator. If there are multiple performance sites, describe the resources available at each site. Describe any special facilities used for working with biohazards or other potentially dangerous substances. A table listing each activity that requires BSL3/4 access and the likely facilities to be used should be included.

Foreign Institutions

Foreign (non-U.S.) Institutions must follow policies described in the *NIH Grants Policy Statement*, and procedures for foreign institutions described throughout the PHS 398 Application Guide.

3. Submission Dates and Times

Part I. Overview Information contains information about Key Dates.

Information on the process of receipt and determining if your application is considered "on-time" is described in detail in the PHS 398 Application Guide.

Applicants may track the status of the application in the eRA Commons, NIH's electronic system for grants administration.

4. Intergovernmental Review (E.O. 12372)

This initiative is not subject to intergovernmental review.

5. Funding Restrictions

All NIH awards are subject to the terms and conditions, cost principles, and other considerations described in the *NIH Grants Policy Statement*.

Pre-award costs are allowable only as described in the *NIH Grants Policy Statement*.

6. Other Submission Requirements and Information

Applications must be received on or before the due dates in Part I. Overview Information. If an application is received after that date, it will not be reviewed.

Upon receipt, applications will be evaluated for completeness by the Center for Scientific Review and responsiveness by components of participating organizations, NIH. Applications that are incomplete and/or nonresponsive will not be reviewed.

Post Submission Materials

Applicants are required to follow the instructions for post-submission materials, as described in NOT-OD-10-115.

Section V. Application Review Information

1. Criteria

Only the review criteria described below will be considered in the review process. As part of the NIH mission, all applications submitted to the NIH in support of biomedical and behavioral research are evaluated for scientific and technical merit through the NIH peer review system.

Overall Impact - Overall

Reviewers will provide an overall impact score to reflect their assessment of the likelihood for the Center to exert a sustained, powerful influence on the research field(s) involved, in consideration of the following review criteria and additional review criteria (as applicable for the Center proposed).

- Does the application have a single clearly defined and scientifically justified theme?
- Does the application adequately explain the following:
  - Scientific merit of each Research Project and core?
  - The program as a whole? Significance of the overall program goals?
  - Scientific gains and synergy achieved by combining the component projects into a multiproject program beyond the gains achievable if each project were pursued independently?

- Are the overall program goals significant and focused on studies that increase knowledge needed to advance the translational activities of the center?
- How well does the overall program incorporate innovative concepts and approaches?
- Is the program as a whole cohesive and do the Research Projects and cores relate to a single common theme demonstrating cohesion, multidisciplinary interactions, coordination, and synergy?

Scored Review Criteria - Overall Individual Research Projects

Reviewers will consider each of the review criteria below in the determination of scientific merit, and give a separate score for each. An application does not need to be strong in all categories to be judged likely to have major scientific impact. For example, a project that by its nature is not innovative may be essential to advance a field.

Significance

Does the project address an important problem or a critical barrier to progress in the field? If the aims of the project are achieved, how will scientific knowledge, technical capability, and/or clinical practice be improved? How will successful completion of the aims change the concepts, methods, technologies, treatments, services, or preventative interventions that drive this field?

Investigator(s)

Are the PD(s)/PI(s), collaborators, and other researchers well suited to the project ? If Early Stage Investigators or New Investigators, or in the early stages of independent careers, do they have appropriate experience and training? If established, have they demonstrated an ongoing record of accomplishments that have advanced their field(s)? If the project is collaborative or multi-PD/PI, do the investigators have complementary and integrated expertise; are their leadership approach, governance and organizational structure appropriate for the project?

Innovation

Does the application challenge and seek to shift current research or clinical practice paradigms by utilizing novel theoretical concepts, approaches or methodologies, instrumentation, or interventions? Are the concepts, approaches or methodologies, instrumentation, or interventions novel to one field of research or novel in a broad sense? Is a refinement, improvement, or new application of theoretical concepts, approaches or methodologies, instrumentation, or interventions proposed?

Approach

Are the overall strategy, methodology, and analyses well-reasoned and appropriate to accomplish the specific aims of the project ? Are potential problems, alternative strategies, and benchmarks for success presented? If the project is in the early stages of development, will the strategy establish feasibility and will particularly risky aspects be managed?

If the project involves clinical research, are the plans for 1) protection of human subjects from research risks, and 2) inclusion of minorities and members of both sexes/genders, as well as the inclusion of children, justified in terms of the scientific goals and research strategy proposed?

Environment

Will the scientific environment in which the work will be done contribute to the probability of success? Are the institutional support, equipment and other physical resources available to the investigators adequate for the project proposed? Will the project benefit from unique features of the scientific environment, subject populations, or collaborative arrangements?

Additional Review Criteria - Overall Application, Individual Research Projects, and Cores

As applicable for the project proposed, reviewers will evaluate the following additional items while determining scientific and technical merit, and in providing an overall impact score, but will not give separate scores for these items.

For All Research Projects:

- Does the approach take into consideration the anticipated regulatory process and any anticipated regulatory barriers?
- For Research Projects engaged in preclinical product development activities;
  - Are the Milestones appropriate and feasible?
  - Does the Product Development Plan adequately address the specific areas described in the FOA? Is the proposed Product Development Plan feasible and appropriate for proposed and future product development?
- Is the Plan consistent with achieving the goals of this program?

Protections for Human Subjects

For research that involves human subjects but does not involve one of the six categories of research that are exempt under 45 CFR Part 46, the committee will evaluate the justification for involvement of human subjects and the proposed protections from research risk relating to their participation according to the following five review criteria: 1) risk to subjects, 2) adequacy of protection against risks, 3) potential benefits to the subjects and others, 4) importance of the knowledge to be gained, and 5) data and safety monitoring for clinical trials.

For research that involves human subjects and meets the criteria for one or more of the six categories of research that are exempt under 45 CFR Part 46, the committee will evaluate: 1) the justification for the exemption, 2) human subjects involvement and characteristics, and 3) sources of materials. For additional information on review of the Human Subjects section, please refer to the Human Subjects Protection and Inclusion Guidelines.

Inclusion of Women, Minorities, and Children

When the proposed project involves clinical research, the committee will evaluate the proposed plans for inclusion of minorities and members of both genders, as well as the inclusion of children. For additional information on review of the Inclusion section, please refer to the Human Subjects Protection and Inclusion Guidelines.

Vertebrate Animals

The committee will evaluate the involvement of live vertebrate animals as part of the scientific assessment according to the following five points: 1) proposed use of the animals, and species, strains, ages, sex, and numbers to be used; 2) justifications for the use of animals and for the appropriateness of the species and numbers proposed; 3) adequacy of veterinary care; 4) procedures for limiting discomfort, distress, pain and injury to that which is unavoidable in the conduct of scientifically sound research including the use of analgesic, anesthetic, and tranquilizing drugs and/or comfortable restraining devices; and 5) methods of euthanasia and reason for selection if not consistent with the AVMA Guidelines on Euthanasia. For additional information on review of the Vertebrate Animals section, please refer to the Worksheet for Review of the Vertebrate Animal Section.

Biohazards

Reviewers will assess whether materials or procedures proposed are potentially hazardous to research personnel and/or the environment, and if needed, determine whether adequate protection is proposed.

Resubmissions

Not Applicable

Renewals

Not Applicable

Revisions

Not Applicable

Additional Review Considerations - Overall Application, Individual Research Projects, and Cores

As applicable for the project proposed, reviewers will consider each of the following items, but will not give scores for these items, and should not consider them in providing an overall impact score.

Applications from Foreign Organizations

Reviewers will assess whether the project presents special opportunities for furthering research programs through the use of unusual talent, resources, populations, or environmental conditions that exist in other countries and either are not readily available in the United States or augment existing U.S. resources.

Select Agent Research

Reviewers will assess the information provided in this section of the application, including 1) the Select Agent(s) to be used in the proposed research, 2) the registration status of all entities where Select Agent(s) will be used, 3) the procedures that will be used to monitor possession use and transfer of Select Agent(s), and 4) plans for appropriate biosafety, biocontainment, and security of the Select Agent(s).

Resource Sharing Plans

Reviewers will comment on whether the following Resource Sharing Plans, or the rationale for not sharing the following types of resources, are reasonable: 1) Data Sharing Plan; 2) Sharing Model Organisms; and 3) Genome Wide Association Studies (GWAS).

Budget and Period of Support

Reviewers will consider whether the budget and the requested period of support are fully justified and reasonable in relation to the proposed research.

Scored Review Criteria – Cores

Reviewers will consider each of the review criteria below in the determination of scientific merit. An application does not need to be strong in all categories to be judged likely to have major scientific impact. For example, a core that by its nature is not innovative may be essential to advance a field.

Administrative Core

For the Administrative Core, is the administrative and organizational structure appropriate and adequate to achieve the goals of the proposed program? Is the Management Plan for fiscal accountability and communication within the program appropriate? Are the plans for coordination, problem identification and resolution, and the establishment of a strong collaborative environment for the program appropriate? Are plans for communication among the Centers adequate to facilitate collaborative activities? Do the PD/PI and Key Personnel commit sufficient time and effort to adequately manage the Program? Is the proposed membership on the Scientific Advisory Committee appropriate for the experience and expertise required for the planned scientific goals of the FOA? Are there adequate institutional plans and procedures to assure compliance with applicable federal regulations and NIH policies for the protection of human

research participants, including the evaluation of risks and protections in project proposals, appropriate ethical oversight of funded projects, and plans for monitoring data and safety in clinical research projects?

Scientific Core

For the Scientific Core(s), is it sufficiently justified? Does it support at least two Research Projects? Is the core adequately connected to the central focus of the overall program? Are the facilities or services provided by the core (including procedures, techniques, and quality control) high quality? Will the services be used effectively? Are the core leader and key personnel well qualified and is there an adequate commitment of time?

2. Review and Selection Process

Applications will be evaluated for scientific and technical merit by (an) appropriate Scientific Review Group(s) convened by NIAID, in accordance with NIH peer review policy and procedures, using the stated review criteria. Assignment to a Scientific Review Group will be shown in the eRA Commons.

As part of the scientific peer review, all applications:

- May undergo a selection process in which only those applications deemed to have the highest scientific and technical merit (generally the top half of applications under review) will be discussed and assigned an overall impact score.
- Will receive a written critique.

Appeals of initial peer review will not be accepted for applications submitted response to this FOA .

Applications will be assigned to the appropriate NIH Institute or Center and will compete for available funds with all other recommended applications submitted in response to this FOA . Following initial peer review, recommended applications will receive a second level of review by the National Advisory Allergy and Infectious Diseases Council I. The following will be considered in making funding decisions:

- Scientific and technical merit of the proposed project as determined by scientific peer review.
- Availability of funds.
- Relevance of the proposed project to program priorities.

3. Anticipated Announcement and Award Dates

After the peer review of the application is completed, the PD/PI will be able to access his or her Summary Statement (written critique) via the eRA Commons.

Information regarding the disposition of applications is available in the *NIH Grants Policy Statement*.

Section VI. Award Administration Information

1. Award Notices

If the application is under consideration for funding, NIH will request "just-in-time" information from the applicant as described in the *NIH Grants Policy Statement*.

A formal notification in the form of a Notice of Award (NoA) will be provided to the applicant organization for successful applications. The NoA signed by the grants management officer is the authorizing document and will be sent via email to the grantee's business official.

Awardees must comply with any funding restrictions described in Section IV.5. Funding Restrictions. Selection of an application for award is not an authorization to begin performance. Any costs incurred before receipt of the NoA are at the recipient's risk. These costs may be reimbursed only to the extent considered allowable pre-award costs.

Any application awarded in response to this FOA will be subject to the DUNS, SAM Registration, and

Transparency Act requirements as noted on the Award Conditions and Information for NIH Grants website.

Prior Approval of Supplemental Research Projects

Awardee-selected projects that involveclinical trials or studies involving greater than minimal risk to human subjects require prior approval by NIH prior to initiation.

- The awardee institution will provide NIH with written study protocols that address risks and protections for human subjects in accordance with NIH's Instructions for Preparing the Human Subjects Section of the Research Plan.
- The awardee institution will provide NIH with specific plans for data and safety monitoring, and will notify the IRB and NIH of serious adverse events and unanticipated problems, consistent with NIH DSMP policies.

2. Administrative and National Policy Requirements

All NIH grant and cooperative agreement awards include the *NIH Grants Policy Statement* as part of the NoA. For these terms of award, see the *NIH Grants Policy Statement* Part II: Terms and Conditions of NIH Grant Awards, Subpart A: General and Part II: Terms and Conditions of NIH Grant Awards, Subpart B: Terms and Conditions for Specific Types of Grants, Grantees, and Activities. More information is provided at Award Conditions and Information for NIH Grants.

Cooperative Agreement Terms and Conditions of Award

The following special terms of award are in addition to, and not in lieu of, otherwise applicable U.S. Office of Management and Budget (OMB) administrative guidelines, U.S. Department of Health and Human Services (DHHS) grant administration regulations at 45 CFR Parts 74 and 92 (Part 92 is applicable when State and local Governments are eligible to apply), and other HHS, PHS, and NIH grant administration policies.

The administrative and funding instrument used for this program will be the cooperative agreement, an "assistance" mechanism (rather than an "acquisition" mechanism), in which substantial NIH programmatic involvement with the awardees is anticipated during the performance of the activities. Under the cooperative agreement, the NIH purpose is to support and stimulate the recipients' activities by involvement in and otherwise working jointly with the award recipients in a partnership role; it is not to assume direction, prime responsibility, or a dominant role in the activities. Consistent with this concept, the dominant role and prime responsibility resides with the awardees for the project as a whole, although specific tasks and activities may be shared among the awardees and the NIH as defined below.

The PD(s)/PI(s) will have the primary responsibility for:

PD(s)/PI(s), will have the primary responsibility for coordinating the Projects and Cores within the overall Program. Specifically, the PD(s)/PI(s) have primary responsibility as described below.

The PD(s)/PI(s) will be responsible for defining the research objectives, approaches and details of the projects within the guidelines of the FOA and retains primary responsibility for the planning, directing, and executing the proposed scientific activities.

In addition, the PD(s)/PI(s) will be responsible for:

- Organizing and chairing annual site visit activities
- Making sure that the NIAID Data Release Guidelines (see below) are properly implemented by the personnel of the Research Projects and Cores
- Advertising the availability of the Program generated resources through outreach activities.
- Ensuring that results obtained from the Program are analyzed and published in a timely manner.
- Provide semi-annual progress reports in a format to be defined by the NIAID after award.

The multi-disciplinary and collaborative nature of the Programs funded under this FOA creates an extraordinary opportunity for information exchange and scientific advancement of translational research applied to infectious diseases research. Programs' investigators are expected to take advantage of this

opportunity by participating in both formal events established expressly for this purpose and informal investigator-initiated dialogues.

Federally Mandated Regulatory Requirements for Clinical Research

Each institution participating in clinical research is required to meet DHHS regulations for the protection of human subjects. At a minimum, this includes:

- Methods for assuring that each institution at which Program investigators are conducting clinical studies has registered with the Office of Human Research Protections (OHRP; http://www.hhs.gov/ohrp) and has a Federalwide Assurance; that study protocols are reviewed and approved by the responsible Institutional Review Board (IRB) prior to participant entry; that active protocols are reviewed at least annually by the IRB, and that amendments are approved by the IRB.
- Methods for assuring or documenting that each participant, or participant's parent/legal guardian, gives fully informed consent to participation in a research protocol prior to the initiation of the intervention.

Intellectual Property

The awardee is solely responsible for the timely acquisition of all appropriate propriety rights, including intellectual property rights, and all materials needed for the awardee to perform the project.

Before, during, and subsequent to the award, the U.S. Government is not required to obtain for the awardee any propriety rights, including intellectual property rights, or any materials needed by the awardee to perform the project.

The awardee is required to report to the U.S. Government all inventions made in the performance of the project, as specified by 35 U.S.C. Sect. 202 (Bayh-Dole Act).

Program Generated Data, Software and Other Resources

Awardees will retain custody of and have primary rights to the data and software developed under these awards, subject to Government rights of access consistent with current HHS, PHS, and NIH policies.

The Programs funded under this FOA are expected to follow the requirements and timelines described in the NIAID Data and Reagents Sharing and Release Guidelines (http://www.niaid.nih.gov/LabsAndResources/resources/dmid/gsc/Pages/data.aspx) as well as those in the Data Sharing Guiding Principles for the NIAID/DMID Systems Biology Program (http://www.niaid.nih.gov/LABSANDRESOURCES/RESOURCES/DMID/SB/Pages/DataReleaseGuidelines.aspx) Program-generated data and software should be made available through publicly accessible web and database sites, including the Web Portal, the BRCs (http://www.niaid.nih.gov/dmid/genomes/brc/default.htm), the NCBI (http://www.ncbi.nlm.nih.gov/) and/or other public repositories, as identified by the Steering Committee in consultation with the NIAID.

Program-generated data and software include, for example:

- all research data (both experimentally and computationally generated) and associated metadata;
- database schema and specifications;
- experimental protocols and Standard Operating Procedures (SOPs); and
- data analysis tools, models and algorithms generated under this contract, including model parameters and source code, complete use documentation and tutorials.

Whenever possible, the awardee shall provide software certified by the Open Source Initiative (http://www.opensource.org/licenses/), to guarantee the right of others to read, redistribute, modify, and freely use the software.

Program-generated novel reagents (e.g., expression vectors, mutant strains, libraries, protein clones), should be made available through NIAID-supported repositories, such as the NIAID BEI Resources (http://www.beiresources.org/) or in other repositories as identified by the Steering Committee in consultation with the NIAID.

Publications

The PD(s)/PI(s) will be responsible for the timely submission to the NIAID of all abstracts, manuscripts and reviews (co)authored by members of the grant and supported in whole or in part under this Cooperative Agreement. The PD(s)/PI(s) are requested to provide manuscripts to the NIAID Program staff prior to the submission to the Journal so that an up-to-date summary of program accomplishments can be maintained. Publications and oral presentations of work conducted under this Cooperative Agreement are the responsibility of the PD(s)/PI(s) and appropriate Research Project and Core Leaders and will require appropriate acknowledgement of NIAID support. Awardees are expected to make new information and materials known to the research community in a timely manner through publications, web announcements, and reports to the NIAID, or other mechanisms.

NIH staff have substantial programmatic involvement that is above and beyond the normal stewardship role in awards, as described below:

The role of the NIAID/NIH Project Scientist in the cooperative agreement is to support and encourage the recipient's activities by substantial involvement as partners and facilitators in the process without assuming responsibilities that remain with the PDs/PIs. The NIAID Project Scientist will work closely with the PD(s)/PI(s) and other Program member scientists to facilitate collaborations and to leverage the resources available to the Program.

The NIAID Project Scientist will monitor the progress of the Program, help coordinate research approaches among all Programs funded through the FOA, and contribute to the shaping of research projects or approaches as warranted. The NIAID Project Scientist will support and facilitate this process but will not direct it.

The NIAID Project Scientist will keep the Program informed about other ongoing studies supported by NIAID to avoid duplication of effort and encourage sharing/collaboration in infectious diseases research. The NIAID Project Scientist will coordinate access for the Program to other NIAID resources, as well as assist the research efforts of the Program by facilitating access to fiscal and intellectual resources provided by industry, private foundations, NIH intramural scientists and other federal government agencies as appropriate.

The NIAID Project Scientist will review and make funding recommendations for Supplemental Research Project applications.

The NIAID Project Scientist will retain the option to recommend withholding or reduction of support from any cooperative agreement that substantially fails to achieve its goals according to the milestones agreed to at the time of the award or fails to comply with the Terms and Conditions of the award.

Additionally, a NIAID program director will be responsible for the normal scientific and programmatic stewardship of the award and will be named in the award notice. The assigned program official may also serve as the NIAID Project Scientist.

Areas of Joint Responsibility include:

The NIAID Project Scientist will provide overall coordination across all funded Programs, will coordinate with the PD(s)/PI(s) and hold regular program-wide discussions to facilitate the achievement of program goals. In the event that some members of the Programs develop common research interests working groups may be formed to pursue collaborative activities.

Dispute Resolution:

Any disagreements that may arise in scientific or programmatic matters (within the scope of the award) between award recipients and the NIH may be brought to Dispute Resolution. A Dispute Resolution Panel composed of three members will be convened. It will have three members: a designee of the Steering Committee chosen without NIH staff voting, one NIH designee, and a third designee with expertise in the relevant area who is chosen by the other two; in the case of individual disagreement, the first member may be chosen by the individual awardee. This special dispute resolution procedure does not alter the awardee's right to appeal an adverse action that is otherwise appealable in accordance with PHS regulation 42 CFR Part 50,

Subpart D and DHHS regulation 45 CFR Part 16.

3. Reporting

When multiple years are involved, awardees will be required to submit the Non-Competing Continuation Grant Progress Report (PHS 2590 or RPPR) annually and financial statements as required in the *NIH Grants Policy Statement.* Progress reports should briefly describe status of Supplemental Research Projects, including data and safety monitoring, and should notify NIH of serious adverse events and unanticipated problems.

A final progress report, invention statement, and the expenditure data portion of the Federal Financial Report are required for closeout of an award, as described in the *NIH Grants Policy Statement*.

The Federal Funding Accountability and Transparency Act of 2006 (Transparency Act), includes a requirement for awardees of Federal grants to report information about first-tier subawards and executive compensation under Federal assistance awards issued in FY2011 or later. All awardees of applicable NIH grants and cooperative agreements are required to report to the Federal Subaward Reporting System (FSRS) available at www.fsrs.gov on all subawards over $25,000. See the *NIH Grants Policy Statement* for additional information on this reporting requirement.

Section VII. Agency Contacts

We encourage inquiries concerning this funding opportunity and welcome the opportunity to answer questions from potential applicants.

Application Submission Contacts

GrantsInfo (Questions regarding application instructions and process, finding NIH grant resources)
Telephone 301-435-0714
TTY 301-451-5936
Email: GrantsInfo@nih.gov

eRA Commons Help Desk (Questions regarding eRA Commons registration, tracking application status, post submission issues)
Phone: 301-402-7469 or 866-504-9552 (Toll Free)
TTY: 301-451-5939
Email: commons@od.nih.gov

Scientific/Research Contact(s)

N. Kent Peters, Ph.D.
Division of Microbiology and Infectious Diseases
National Institute of Allergy and Infectious Diseases (NIAID)
Telephone: 301-402-8584
Email: petersn@niaid.nih.gov

Michael R. Schaefer, Ph.D.
Division of Microbiology and Infectious Diseases
National Institute of Allergy and Infectious Diseases (NIAID)
Telephone: 301-451-3758
Email: mschaefer@niaid.nih.gov

Peer Review Contact(s)

Lynn Rust, Ph.D.
Division of Extramural Activities
National Institute of Allergy and Infectious Diseases (NIAID)
Telephone: 301- 402-3938
Email: lrust@mail.nih.gov

Financial/Grants Management Contact(s)

Theresa Mercogliano
Division of Extramural Activities
National Institute of Allergy and Infectious Diseases (NIAID)
Telephone: 301- 402-5512
FAX: 301-493-0597
Email: tmercoglia@niaid.nih.gov

Section VIII. Other Information

Recently issued trans-NIH policy notices may affect your application submission. A full list of policy notices published by NIH is provided in the *NIH Guide for Grants and Contracts*. All awards are subject to the terms and conditions, cost principles, and other considerations described in the *NIH Grants Policy Statement*.

Authority and Regulations

Awards are made under the authorization of Sections 301 and 405 of the Public Health Service Act as amended (42 USC 241 and 284) and under Federal Regulations 42 CFR Part 52 and 45 CFR Parts 74 and 92.

Weekly TOC for this Announcement
NIH Funding Opportunities and Notices

NIH National Institutes of Health
Office of Extramural Research

Department of Health and Human Services (HHS)

USA.gov Government Made Easy

NIH... Turning Discovery Into Health®

Note: For help accessing PDF, RTF, MS Word, Excel, PowerPoint, Audio or Video files, see Help Downloading Files.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME:

eRA COMMONS USER NAME (credential, e.g., agency login):

POSITION TITLE:

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY

Please refer to the Biographical Sketch sample in order to complete sections A, B, C, and D of the Biographical Sketch.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. DO NOT EXCEED FIVE PAGES.

NAME:

eRA COMMONS USER NAME (credential, e.g., agency login):

POSITION TITLE:

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY

NOTE: The Biographical Sketch may not exceed five pages. Follow the formats and instructions below.

A. Personal Statement

Briefly describe why you are well-suited for your role in the project described in this application. The relevant factors may include aspects of your training; your previous experimental work on this specific topic or related topics; your technical expertise; your collaborators or scientific environment; and your past performance in this or related fields (you may mention specific contributions to science that are not included in Section C). Also, you may identify up to four peer reviewed publications that specifically highlight your experience and qualifications for this project. If you wish to explain impediments to your past productivity, you may include a description of factors such as family care responsibilities, illness, disability, and active duty military service.

B. Positions and Honors

List in chronological order previous positions, concluding with the present position. List any honors. Include present membership on any Federal Government public advisory committee.

C. Contribution to Science

Briefly describe up to five of your most significant contributions to science. For each contribution, indicate the historical background that frames the scientific problem; the central finding(s); the influence of the finding(s) on the progress of science or the application of those finding(s) to health or technology; and your specific role in the described work. For each of these contributions, reference up to four peer-reviewed publications or other non-publication research products (can include audio or video products; patents; data and research materials; databases; educational aids or curricula; instruments or equipment; models; protocols; and software or netware) that are relevant to the described contribution. The description of each contribution should be no longer than one half page including figures and citations. Also provide a URL to a full list of your published work as found in a publicly available digital database such as SciENcv or My Bibliography, which are maintained by the US National Library of Medicine.

SAMPLE

D. Research Support

List both selected ongoing and completed research projects for the past three years (Federal or non-Federally-supported). *Begin with the projects that are most relevant to the research proposed in the application.* Briefly indicate the overall goals of the projects and responsibilities of the key person identified on the Biographical Sketch. Do not include number of person months or direct costs.

SAMPLE

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES**.

NAME: Hunt, Morgan Casey

eRA COMMONS USER NAME (credential, e.g., agency login): huntmc

POSITION TITLE: Associate Professor of Psychology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of California, Berkeley	B.S	05/1990	Psychology
University of Vermont	Ph.D.	05/1996	Experimental Psychology
University of California, Berkeley	Postdoctoral	08/1998	Public Health and Epidemiology

A. Personal Statement

I have the expertise, leadership, training, expertise and motivation necessary to successfully carry out the proposed research project. I have a broad background in psychology, with specific training and expertise in ethnographic and survey research and secondary data analysis on psychological aspects of drug addiction. My research includes neuropsychological changes associated with addiction. As PI or co-Investigator on several university- and NIH-funded grants, I laid the groundwork for the proposed research by developing effective measures of disability, depression, and other psychosocial factors relevant to the aging substance abuser, and by establishing strong ties with community providers that will make it possible to recruit and track participants over time as documented in the following publications. In addition, I successfully administered the projects (e.g. staffing, research protections, budget), collaborated with other researchers, and produced several peer-reviewed publications from each project. As a result of these previous experiences, I am aware of the importance of frequent communication among project members and of constructing a realistic research plan, timeline, and budget. The current application builds logically on my prior work. During 2005-2006 my career was disrupted due to family obligations. However, upon returning to the field I immediately resumed my research projects and collaborations and successfully competed for NIH support.

1. Merryle, R.J. & Hunt, M.C. (2004). Independent living, physical disability and substance abuse among the elderly. Psychology and Aging, 23(4), 10-22.
2. Hunt, M.C., Jensen, J.L. & Crenshaw, W. (2007). Substance abuse and mental health among community-dwelling elderly. International Journal of Geriatric Psychiatry, 24(9), 1124-1135.
3. Hunt, M.C., Wiechelt, S.A. & Merryle, R. (2008). Predicting the substance-abuse treatment needs of an aging population. American Journal of Public Health, 45(2), 236-245. PMCID: PMC9162292 Hunt, M.C., Newlin, D.B. & Fishbein, D. (2009). Brain imaging in methamphetamine abusers across the life-span. Gerontology, 46(3), 122-145.

SAMPLE

B. Positions and Honors

Positions and Employment

1998-2000	Fellow, Division of Intramural Research, National Institute of Drug Abuse, Bethesda, MD
2000-2002	Lecturer, Department of Psychology, Middlebury College, Middlebury, VT
2001-	Consultant, Coastal Psychological Services, San Francisco, CA
2002-2005	Assistant Professor, Department of Psychology, Washington University, St. Louis, MO
2007-	Associate Professor, Department of Psychology, Washington University, St. Louis, MO

Other Experience and Professional Memberships

1995-	Member, American Psychological Association
1998-	Member, Gerontological Society of America
1998-	Member, American Geriatrics Society
2000-	Associate Editor, Psychology and Aging
2003-	Board of Advisors, Senior Services of Eastern Missouri
2003-05	NIH Peer Review Committee: Psychobiology of Aging, ad hoc reviewer
2007-11	NIH Risk, Adult Addictions Study Section, members

Honors

2003	Outstanding Young Faculty Award, Washington University, St. Louis, MO
2004	Excellence in Teaching, Washington University, St. Louis, MO
2009	Award for Best in Interdisciplinary Ethnography, International Ethnographic Society

C. Contribution to Science

1. My early publications directly addressed the fact that substance abuse is often overlooked in older adults. However, because many older adults were raised during an era of increased drug and alcohol use, there are reasons to believe that this will become an increasing issue as the population ages. These publications found that older adults appear in a variety of primary care settings or seek mental health providers to deal with emerging addiction problems. These publications document this emerging problem but guide primary care providers and geriatric mental health providers to recognize symptoms, assess the nature of the problem and apply the necessary interventions. By providing evidence and simple clinical approaches, this body of work has changed the standards of care for addicted older adults and will continue to provide assistance in relevant medical settings well into the future. I served as the primary investigator or co-investigator in all of these studies.
    a. Gryczynski, J., Shaft, B.M., Merryle, R., & Hunt, M.C. (2002). Community based participatory research with late-life addicts. American Journal of Alcohol and Drug Abuse, 15(3), 222-238.
    b. Shaft, B.M., Hunt, M.C., Merryle, R., & Venturi, R. (2003). Policy implications of genetic transmission of alcohol and drug abuse in female nonusers. International Journal of Drug Policy, 30(5), 46-58.
    c. Hunt, M.C., Marks, A.E., Shaft, B.M., Merryle, R., & Jensen, J.L. (2004). Early-life family and community characteristics and late-life substance abuse. Journal of Applied Gerontology, 28(2),26-37.
    d. Hunt, M.C., Marks, A.E., Venturi, R., Crenshaw, W. & Ratonian, A. (2007). Community-based intervention strategies for reducing alcohol and drug abuse in the elderly. Addiction, 104(9), 1436-1606. PMCID: PMC9000292

2. In addition to the contributions described above, with a team of collaborators, I directly documented the effectiveness of various intervention models for older substance abusers and demonstrated the importance of social support networks. These studies emphasized contextual factors in the etiology and maintenance of addictive disorders and the disruptive potential of networks in substance abuse treatment. This body of work also discusses the prevalence of alcohol, amphetamine, and opioid abuse in older adults and how networking approaches can be used to mitigate the effects of these disorders.
    a. Hunt, M.C., Merryle, R. & Jensen, J.L. (2005). The effect of social support networks on morbidity among elderly substance abusers. Journal of the American Geriatrics Society, 57(4), 15-23.
    b. Hunt, M.C., Pour, B., Marks, A.E., Merryle, R. & Jensen, J.L. (2005). Aging out of methadone treatment. American Journal of Alcohol and Drug Abuse, 15(6), 134-149.

SAMPLE

c. Merryle, R. & Hunt, M.C. (2007). Randomized clinical trial of cotinine in older nicotine addicts. Age and Ageing, 38(2), 9-23. PMCID: PMC9002364

3. Methadone maintenance has been used to treat narcotics addicts for many years but I led research that has shown that over the long-term, those in methadone treatment view themselves negatively and they gradually begin to view treatment as an intrusion into normal life. Elderly narcotics users were shown in carefully constructed ethnographic studies to be especially responsive to tailored social support networks that allow them to eventually reduce their maintenance doses and move into other forms of therapy. These studies also demonstrate the policy and commercial implications associated with these findings.

   a. Hunt, M.C. & Jensen, J.L. (2003). Morbidity among elderly substance abusers. Journal of the Geriatrics, 60(4), 45-61.
   b. Hunt, M.C. & Pour, B. (2004). Methadone treatment and personal assessment. Journal Drug Abuse, 45(5), 15-26.
   c. Merryle, R. & Hunt, M.C. (2005). The use of various nicotine delivery systems by older nicotine addicts. Journal of Ageing, 54(1), 24-41. PMCID: PMC9112304
   d. Hunt, M.C., Jensen, J.L. & Merryle, R. (2008). The aging addict: ethnographic profiles of the elderly drug user. NY, NY: W. W. Norton & Company.

Complete List of Published Work in MyBibliography:
http://www.ncbi.nlm.nih.gov/sites/myncbi/collections/public/1PgT7IEFIAJBtGMRDdWFmjWAO/?sort=date&direction=ascending

D. Research Support

Ongoing Research Support

R01 DA942367 Hunt (PI) 09/01/08-08/31/16
Health trajectories and behavioral interventions among older substance abusers
The goal of this study is to compare the effects of two substance abuse interventions on health outcomes in an urban population of older opiate addicts.
Role: PI

R01 MH922731 Merryle (PI) 12/15/07-11/30/15
Physical disability, depression and substance abuse in the elderly
The goal of this study is to identify disability and depression trajectories and demographic factors associated with substance abuse in an independently-living elderly population.
Role: Co-Investigator

Faculty Resources Grant, Washington University 08/15/09-08/14/15
Opiate Addiction Database
The goal of this project is to create an integrated database of demographic, social and biomedical information for homeless opiate abusers in two urban Missouri locations, using a number of state and local data sources.
Role: PI

Completed Research Support

R21 AA998075 Hunt (PI) 01/01/11-12/31/13
Community-based intervention for alcohol abuse
The goal of this project was to assess a community-based strategy for reducing alcohol abuse among older individuals.
Role: PI

SAMPLE

ALL PERSONNEL REPORT

Place this form at the end of the signed original copy of the application. Do not duplicate.

GRANT NUMBER

Always list the PD/PI(s). In addition, list all other personnel who participated in the project during the current budget period for at least one person month or more, regardless of the source of compensation (a person month equals approximately 160 hours or 8.3% of annualized effort). Use the following abbreviated categories for describing Role on Project:

- **PD/PI***
- **Co-Investigator**
- **Faculty**
- **Postdoctoral (scholar, fellow, or other postdoctoral position)***
- **Technician**
- **Staff Scientist (doctoral level)**
- **Statistician**
- **Graduate Student (research assistant)**
- **Non-student Research Assistant**
- **Undergraduate Student**
- **High School Student**
- **Consultant**
- **Other (please specify)**

If personnel are supported by a Reentry or Diversity Supplement please indicate such after the Role on Project, using the following abbreviations: RS - Reentry Supplement; DS - Diversity Supplement.

***Commons ID required for any personnel holding this Role on Project and for all individuals supported by a Reenty or Diversity Supplement. The Commons ID will be required in the future for all individuals with a graduate student, or undergraduate role. The Commons ID is strongly encouraged, but not required, for all other Project Personnel.**

Use Cal (calendar), Acad, or Summer to enter months devoted to project.

Commons ID*	Name	Degree(s)	SSN (last 4 digits)	Role on Project	DoB (MM /YY)	Cal	Acad	Summer

NIH Research Performance Progress Reports (RPPR)

Helpful Hints for the UW Research Community

This document is meant as a tool for research administrators to collect information from PIs for the new RPPR format and to give some tips/explanations for areas that look/feel different than eSNAP. It is not meant as a step-by-step tool for filling out the RPPR.

NIH has an RPPR Instruction Guide that explains every section in great detail as well as a very helpful Webcast which is posted on OSP website.

What makes RPPR different from eSNAP?

- ✓ RPPR data fields are specific to your grant type and grant number only. This means that all active sections are or can be relevant to your grant. Data fields not relevant to your grant will be grayed out or labeled "Not Applicable."
- ✓ After you have submitted the first RPPR, future applications will be pre-populated with information from other databases, proposals and previous progress reports. If you think that the pre-populated information is incorrect and the system does not allow you to change it, then you can either change it in the source database or contact the eRA Help Desk.
- ✓ Text boxes only accept rich text format. Do not enter any symbols. Scientific report on accomplishments is attached as a PDF document and can include symbols.
- ✓ If the question is not applicable to your grant or if you have no change or progress to report just select "noting to report."

A. Cover page

Approval for any PD/PI change will have to be received from the sponsor prior to recording it on RPPR.

A.4. Addresses, emails and phone numbers are pre-populated from the PI's eRA Commons profile. If you want to change this information please update your eRA Commons profile first and the information will automatically be updated on the RPPR.

Recipient ID: Please enter the eGC1 number (if any) for UW internal tracking.

B. Accomplishments

Important note - Each part of this section, B1-B6, has to be distinct and non-repetitive. All progress cannot be summarized in one text/word file.

B.1. What are the major goals of this project?

Major goals are the same as specific aims from the proposal document. These will only have to be entered the first year of submitting RPPR. In subsequent years this area will be pre-populated.

If there have been changes to the goals then written approval from the sponsor will have to be received prior to recording it on RPPR.

Even though maximum of 3 pages (or 8000 characters) of text is allowed in each text box, NIH strongly recommends not exceeding one page.

B.2. What was accomplished under these goals?

Publications are not reported in this section. Please do not add them in the text. They will not be recorded.

Uploadable PDF file. 6MB maximum limit, 2 pages preferred.

B.3. Are there any competitive revisions and administrative supplements related to this award? What are these? (ARRAs and other supplements belong here)

¼ page or 700 character limit.

B.4. What opportunities for training and professional development has the project provided?

Include participation in conferences, workshops, seminars, and/or mentoring not listed under major activities and accomplishments.

Response required for T, F, K, R25, R13, D43 and other training awards.

PDF attachment, no page limits.

B.5. Did your award include a requirement to disseminate information to any particular community? If so, please describe. "Routine" websites, press releases, scientific publications and the sharing of research resources do not apply.

3 page or 8000 character limit, 1 page preferred.

B.6. What do you plan to do for the next reporting period to accomplish the goals?

3 page or 8000 character limit, 1 page preferred.

C. Products

C.1. Are there publications or manuscripts accepted for publication in a journal or other publication (e.g., book, one-time publication, monograph) during the reporting period resulting directly from this award?

This is the only place to report on publications. Any publication information entered in other places will not be recorded as publications.

All publications reported in this section are drawn from MyNCBI site. The PIs MyNCBI account and eRA Commons accounts must be linked ("partnering accounts") otherwise the publications will not auto-populate into RPPR.

If your publication is not in compliance with the NIH Public Access Policy or in other words, does not have the PMCID number then you can still submit the RPPR and you have 10 business days to bring those publications into compliance in MyNCBI. It will automatically change their status on RPPR.

C.2. Website(s) or internet site(s) – list the URL for any site that disseminates the results of the research activities in the past reporting year and provide a short description.

Only for awards designed to create one or more websites.

3 page or 8000 character limit, 1 page preferred.

C.3. Technologies or techniques – identify technologies and techniques that have resulted from the research activities in the past reporting year.

Separate from accomplishments.

3 page or 8000 character limit, 1 page preferred.

C.4. Have inventions, patent applications and/or licenses resulted from the award during this reporting period?

If yes, has this information been previously provided to the PHS or to the official responsible for patent matters at the grantee organization?

C.5.a. Were there any other products developed under this project?

PDF attachment, no page limits. Do not repeat information provided earlier.

C.5.b. Resource Sharing plan – Does this award include a required resource sharing plan? If yes, please describe how data has been shared.

PDF attachment, no page limits.

D. Participants

D.1. Participant effort must be rounded to the nearest whole person month.

If a PD's/PI's effort is 0.4 or less then it should be marked as 0. NIH will compare it to the original proposal and will not consider the report non-compliant. Other participants should have at least one whole person month of effort.

If a person has an eRA Commons user ID then most of the information will populate automatically.

D.2.a. Will there be, in the next budget period, either (1) a reduction of 25% or more in the level of effort from what was approved by the agency for the PD/PI(s) or other senior/key personnel designated in the Notice of Award, or (2) a reduction in the level of effort below the minimum amount of effort required by the Notice of Award? If yes, please explain.

This is the only occasion in this report where significant change is allowed without separate prior approval from sponsor. Selecting "YES" is your request to the agency and the issuance of a subsequent year funding constitutes agency approval of the request.

¼ page, 700 characters limit.

D.2.b. Are there, or will there be, new senior/key personnel?

PDF attachment of biosketches.

D.2.c. Has there been a change in the active Other Support of senior/key personnel since the last reporting period?

Other Support will only be required if there has been a change.

PDF attachment, no page limits.

D.2.d. Are there, or will there be, new other significant contributors?

PDF attachment of biosketches.

D.2.e. Will there be a change in the MPI Leadership plan for the next budget period? If you had one.

Adding, removing or changing the makeup of the PIs requires prior approval. Any other changes to the Leadership Plan, do not require prior approval.

PDF attachment, no page limits.

E. Impact

E.1. What is the impact on the development of human resources?

Only applicable to education awards (D24, K30, R13, R25, RL5, T14, T36, U13, U2R)

E.2. Describe ways, if any, in which the project made an impact, or is likely to make an impact, on physical, institutional, and information resources that form infrastructure, including:

- physical resources (such as facilities, laboratories, or instruments);
- institutional resources (such as establishment or sustenance of societies or organizations); or
- information resources, electronic means for accessing such resources or for scientific communication, or the like.

3 page or 8000 character limit, 1 page preferred.

E.4. Has any money from this grant been spent in foreign countries?

Dollar amount and country.

F. Changes

F.1. Changes in approach and reasons for change

Only applicable to Education awards (D24, K30, R13, R25, RL5, T14, T36, U13, U2R)

F.2. Actual or anticipated challenges or delays and actions or plans to resolve them.

Only Significant challenges need to be reported.

3 page or 8000 character limit, 1 page preferred

F.3.a-F.3.d. Only significant deviations, unexpected outcomes, or changes in approved protocols to human subjects, vertebrate animals, biohazards and/or select agents need to be reported.

PDF attachment, no page limits.

G. Special reporting requirements

G.1. Special NoA and FOA reporting requirements

Link to the NoA Notice of Award link for easy reference.

G.2. Responsible Conduct of Research education received

Only applicable to K, F and other education awards

G.3. Mentor's report/sponsor comments

Only applicable to K, F awards

G.4.b. Inclusion enrollment data

If your grant supports human subjects and a clinical trial then the system will display one of two messages:

- *"The RPPR does not have any Inclusion Enrollment Reports or the report records have a status of Close"*
- *"You are required to complete Inclusion Enrollment Report for the following studies:[list of all protocols]"*

Follow their lead.

If you have questions about Inclusion Enrollment Report then contact your Program Official.

G.4.c. If it is a clinical trial it requires an NCT (ClinicalTrials.gov identifier) number.

G.5. Human subjects education requirement - Are there personnel on this project who are or will be newly involved in the design or conduct of human subjects research?

Details of their training

G.6. Human Embryonic Stem Cells

Requires hESC registration number from NIH registry. Link to the registry provided in the RPPR.

Has there been a change in the use of hESCs?

¼ page, 700 characters limit.

G.8. Is there a change in (primary) project performance sites?

If yes, provide addresses

G.9. Foreign component – NEW

Was significant scientific activity performed outside of the US, either by the grantee or by researcher employed by a foreign organization, whether or not grant funds were expended? The following grant related activities are considered significant and must be reported:

- involvement in human subjects or research with live vertebrate animals;
- extensive foreign travel by grantee project staff to collect data, or conduct surveys or sampling activities; or
- any grantee activity that may have an impact on U.S. foreign policy.

Provide organization name and country and short description

¼ page or 700 characters limit.

G.10. Estimated unobligated balance

G.10.a. It is anticipated that an estimated unobligated balance (including prior year carryover) will be greater than 25% of the current year's total approved budget? And total dollars.

G.10.b. Explanation for estimated unobligated balance

¼ page or 700 characters limit.

G.10.c If authorized to carry over balance describe how you are going to use it

½ page or 1300 character limit

G.11 Is program income anticipated during the next budget period?

Total amount in dollars and sources

G.12 Is there a change in performance sites that will affect F&A costs?

½ page or 1300 character limit

FOIA Request 44416

Redactable content (patentable or privileged, unpublished)

(1) File: 0114 email

Redactable:
(1) PDF pages 2-3: Spreadsheet of pOME constructs: remove rows containing the tag "Hypothetical" in the "Target type" column (applies to 3 Ebola and 2 KSHV constructs)
(2) Project 2, PDF page 11: References to sGP and ssGP and Lloviu in section C
(3) Project 2, PDF pages 12-13:
- (a) Paragraph referencing Ebola lacking sGP + ssGP
- (b) Paragraph referencing the testing of uncharacterized proteins from Lloviu virus
- (c) PDF page 13, Section G, point 4

(2) File: 0220 email

Redactable:
(1) Project 1: PDF pages 9-10: Text summarizing functional assay results (section C.2, spanning PDF pages 9-10), and Figure 1 on PDF page 10
(2) Project 2:
- (a) PDF page 11: Section C, points 3 and 4
- (b) All of section F, including figures, spanning PDF pages 13-15
- (c) PDF page 13: Section C, points beginning with "further investigated the induction of apoptosis..." and "generated a mutant Ebola virus..."
- (d) PDF page 13, Section D, reference to pOME103, hypothetical gene in the M segment of influenza virus
- (e) PDF page 15, Section G, text "no longer expressing sGP and ssGP"

(3) PDF pages 25-27: Spreadsheet of pOME constructs: remove rows containing the tag "Hypothetical" in the "Target type" column
- Applies to the following pOME constructs: 94-99, [129-132 misidentified as hypothetical], 137-156, 171-184

(4) PDF pages 28-29, Spreadsheet of pOME characterization data: remove entire chart (shows assay data)

(3) File: 1015 email

Redactable:
(1) PDF pages 2-22: Spreadsheet of pOME constructs: remove rows containing the tag "Hypothetical" in the "Type" column
- Applies to the following pOME constructs: 94-99, 137-156, 171-184, 203-204, 216-231

(2) Table on PDF pages 25-36: Contains functional assay information that has not yet been published; remove in its entirety

(3) Project 1: Text summarizing functional assay results (section C.2, spanning PDF pages 39-40), and all of the Figure page 40
(4) Project 2: Section 2, page 45, Text and table referencing hypothetical constructs (esp. PB2 100 references), Section 3, PDF pages 46-47, text and Figures 2-4

http://grants.nih.gov/grants/guide/pa-files/PAR-16-366.html

Department of Health and Human Services
Part 1. Overview Information

Participating Organization(s)

National Institutes of Health (NIH)

United States Department of Agriculture (USDA)

Components of Participating Organizations

Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD)

National Institute of Food and Agriculture (USDA-NIFA)

Funding Opportunity Title

Dual Purpose with Dual Benefit: Research in Biomedicine and Agriculture Using Agriculturally Important Domestic Animal Species (R01)

Activity Code

R01 Research Project Grant

Announcement Type

Reissue of PAR-13-204

Related Notices

None

Funding Opportunity Announcement (FOA) Number

PAR-16-366

Companion Funding Opportunity

None

Number of Applications

See Section III. 3. Additional Information on Eligibility.

Catalog of Federal Domestic Assistance (CFDA) Number(s)

93.865

Funding Opportunity Purpose

The purpose of this interagency Funding Opportunity Announcement (FOA) is to invite the submission of grant applications that utilize agriculturally important domestic animal species to improve human health through the advancement of basic and translational research deemed highly relevant to both agricultural

and biomedical research. This initiative is designed to facilitate and encourage comparative medicine research studies through the careful selection and refinement of farm animal models that mimic human developmental, physiological and etiological processes to better understand the biology of fertility and infertility, normal and abnormal metabolism, developmental origin of diseases, and improve prevention and treatment of infectious diseases in both human and agriculturally important domestic animals. The anticipated outcomes include both the elucidation of fundamental information relevant for the improvement of human health and an increase in food animal production and improvement in animal health and product quality. It is envisioned that each application will address mission-relevant areas of both agencies.

Key Dates

Posted Date
July 12, 2016

Open Date (Earliest Submission Date)
August 27, 2016

Letter of Intent Due Date(s)
30 days prior to the application due date

Application Due Date(s)
September 27, 2016; September 27, 2017; September 27, 2018, by 5:00 PM local time of applicant organization. Alltypes of non-AIDS applications allowed for this funding opportunity announcement are due on these dates.

Applicants are encouraged to apply early to allow adequate time to make any corrections to errors found in the application during the submission process by the due date.

AIDS Application Due Date(s)
Not Applicable

Scientific Merit Review
February/March 2017, February/March 2018, February/March 2019

Advisory Council Review
May 2017, May 2018, May 2019

Earliest Start Date
July 2017, July 2018, July 2019

Expiration Date
September 28, 2018

Due Dates for E.O. 12372
Not Applicable

Required Application Instructions

It is critical that applicants follow the instructions in the SF424 (R&R) Application Guide, except where instructed to do otherwise (in this FOA or in a Notice from the ***NIH Guide for Grants and Contracts***). Conformance to all requirements (both in the Application Guide and the FOA) is required and strictly enforced. Applicants must read and follow all application instructions in the Application Guide as well as any program-specific instructions noted in Section IV. When the program-specific instructions deviate from those in the Application Guide, follow the program-specific instructions. **Applications that do not comply with these instructions may be delayed or not accepted for review.**

There are several options to submit your application to the agency through Grants.gov. You can use the ASSIST system to prepare, submit and track your application online. You can download an application package from Grants.gov, complete the forms offline, submit the completed forms to Grants.gov and track your application in eRA Commons. Or, you can use other institutional system-to-system solutions to prepare and submit your application to Grants.gov and track your application in eRA Commons. Learn more.

Apply Online Using ASSIST

Apply Using Downloadable Forms

Problems accessing or using ASSIST should be directed to the eRA Service Desk.
Problems downloading forms should be directed to Grants.gov Customer Support.

Table of Contents

Part 1. Overview Information
Part 2. Full Text of the Announcement

Section I. Funding Opportunity Description
Section II. Award Information
Section III. Eligibility Information
Section IV. Application and Submission Information
Section V. Application Review Information
Section VI. Award Administration Information
Section VII. Agency Contacts
Section VIII. Other Information

Part 2. Full Text of Announcement

Section I. Funding Opportunity Description

Purpose

The purpose of this FOA is to facilitate new and innovative comparative medicine research that expands our knowledge of basic and translational strategies to ameliorate human and agricultural animal disease and developmental and metabolic disorders and improve reproductive efficiency. The focal point of the FOA will be to stimulate and strengthen the application of large domestic animal species for addressing and elucidating specific complex biological mechanisms important to both biomedicine and agriculture. New investigators (including Early-Stage Investigators), and PD(s)/PI(s) who received their first R01 under the previous program announcements (PAR-10-276; PAR-13-204) to the National Institutes of Health are highly encouraged to submit research grant applications projected to have a “dual benefit” for the improvement of health, nutrition, disease prevention, and reproduction in both humans and agriculturally important domestic animals.

Background

A look at the history of biomedical research reveals that agriculturally important domestic animal species such as the pig, sheep, cow, horse, and chicken have been central for the acquisition of basic fundamental knowledge regarding the physiology and pathophysiology of organ systems and the development of reproductive technologies. Despite these documented advances in biomedical research using domestic animal species, their utilization in this era of molecular biology, genomics, and proteomics has often been limited. However, with the recent elucidation of the genome of several of the domestic animal species (cow, horse, pig, chicken, and turkey) and their high gene conservation and similar chromosomal order to humans, these animals become superior genetic models for mapping, as well as the inference of genes associated with disease or physiological mechanisms. While research to curtail human disease is funded primarily by the NIH, the USDA-NIFA provides support for research aimed at improving human health by the provision of safe food with a high nutritive value.

During the past decade, two workshops, convened in 2004 and 2007, highlighted the need for greater interagency cooperation between the NIH and USDA-NIFA so as to better advocate for the utility of these animals as models to study high priority areas in both biomedical and agricultural research. Four priority topics (assisted reproduction/stem cell biology, metabolism with respect to obesity and nutrition, developmental origins of adult disease, and infectious disease) were chosen since it was envisioned that knowledge gained and advances in these areas could greatly benefit both biomedicine and animal agriculture.

In 2007, the American Medical Association and the American Veterinary Medical Association considered a “One Health” initiative (http://www.onehealthinitiative.com/taskForce.php) to strengthen ties and collaborative efforts between schools of medicine and veterinary science. The basis of this initiative was recognition of the mutualistic relationship humans, animals and ecosystems have in the maintenance of overall health. Recently in March 2015, the Division of Comparative Medicine, Office of Research Infrastructure Programs (ORIP) at NIH convened a workshop to assess the progress of the One Health initiative and suggest future steps. The meeting recognized the contribution of the two successful programs funded jointly by NIH and USDA-NIFA, i.e., "Ecology and Evolution of Infectious Diseases", and "Dual Purpose with Dual Benefit: Research in Biomedicine and Agriculture Using Agriculturally Important Domestic Animal Species". As such, “dual purpose for dual benefit” research utilizing domestic agricultural animals should not only further stimulate the progression of scientific knowledge towards multipurpose research endeavors, but it should also provide additional funding opportunities for investigators whose research has traditionally been in the domain of either NIH or USDA-NIFA.

Scientific Knowledge to be Achieved

Knowledge gained through comparative research using domestic agricultural animal models supported by this FOA will broaden the fundamental understanding of normal animal and human physiology but also the etiology of metabolic and infectious diseases in four main topic areas to advance the development of biomedical and agricultural interventions or therapies to improve both human health and farm animal health and production.

- ***Reproduction, Stem cell biology and Regenerative medicine***: Expand fundamental knowledge of processes that underlie human and animal reproduction by advancing our understanding of gametogenesis through the identification of molecular, physiological, and developmental mechanisms that regulate oogenesis and spermatogenesis; improve the efficiency of assisted reproductive technologies in human or cloning in farm animals; elucidate the molecular processes regulating reprogramming in early embryos, embryonic stem cells, induced pluripotent stem cells, and other lineage-specific stem cells, and in cloning (somatic cell nuclear transfer) to enhance tools and improve methods for cloning or the generation of transgenic animals; modify genetic traits of large animals using gene-editing technologies for selective breeding and generation of transgenic animals that mimic human diseases, or provide living, functional tissues to repair or replace tissue or organ functionality lost due to age, disease, damage or congenital defects in the human; characterize the core microbiome (including the metabolome of the microbiome) of the reproductive tract and study its impact on physiology and pathophysiology of reproduction.
- ***Metabolism***: Establish the contribution of specific cell types, such as the adipocyte, and central or peripheral factors in the metabolism of lipids and their role in modulating fat accretion in tissues and/or during specific developmental stages; identify genetic and/or exogenous factors, such as diet or hormones, and determine their role in predisposition to or the onset of obesity; characterize the role of gastrointestinal microbiome (including the metabolome of the microbiome) on host metabolism in the context of adipose deposition and obesity. An understanding of the metabolism and regulation of fat accretion will identify ways in which to improve meat quality, thus its nutritive value to humans, and concomitantly provide insight for the development of novel therapies to reduce the current global epidemic of obesity.
- ***Developmental origin of adult disease***: Investigate the role of placenta in health and disease of the mother and her fetus during pregnancy, postnatally in the offspring and during adulthood; Define in utero developmental programming events altered by maternal exposure to environment stressors (e.g. nutrition, drugs, temperature, pathogens, toxicants) or maternal health status (e.g. disease, obesity) that may be the origin of adult disease or may impair growth, fertility, meat quality, disease resistance, and other traits that are particularly important for agriculture; characterize the gestational and perinatal microbiomes (including the metabolomes of their microbiome) and study their relationship to growth, development, anti-microbial resistance, and to the origin of adult diseases. Quantifiable benefits to NIH and the USDA-NIFA will be the creation of innovative interventions that prevent or ameliorate disease and enhance desired traits to promote human and animal health, as well as economically important production traits for agriculture.
- ***Infectious diseases***: Elucidate the genes and physiological mechanisms that regulate resistance and/or susceptibility to infectious pathogens of human or agricultural animals which will allow for the selection and generation of pathogen-resistant animals; develop interventions that decrease or prevent the transmission of pathogens from animal reservoirs to humans; identify environmental

factors that contribute to the emergence of infectious pathogens to decrease or eradicate their occurrence; define organ or tissue-specific microbiome (including the metabolome of the microbiome) and its relationship to inflammation, immune response, infectious disease development, and antimicrobial resistance; develop genome editing strategies for treatment of infectious diseases. The prevention or eradication of infectious diseases substantially impacts human health and enables the generation of healthier, more superior farm animals.

Objectives

The main objective of this FOA is to stimulate and encourage investigations in the four areas identified as high priority issues to both biomedicine and agriculture through the use of pertinent domestic farm animal species that better mimic the specific human developmental, physiological or disease state. Knowledge gained will provide “dual benefit” to both human and agricultural animal species and advance applications for the improvement of health, productivity and reproductive efficiency. New Investigators, Early Stage Investigators, and PD(s)/PI(s) who received their first R01 under this program are strongly encouraged to apply.

Research and Experimental Approaches

This FOA has been established to encourage the use of domestic agricultural animal species to address four priority research areas of high impact to biomedicine and agriculture. Though research with rodent models has been useful, grasping a more complete fundamental understanding of specific biological questions and translating the knowledge gained to improve both animal and human health and reproduction require the use of models that more closely resemble each other developmentally and physiologically or that exhibit similar susceptibility to common infectious pathogens. The areas of investigation and experimental approaches may involve the characterization of animal models, elucidation of the etiology involved in disease or transmission of pathogens, a determination of environmental insults underlying developmental or epigenetic changes leading to pathophysiology, metabolic studies focused on obesity, or the identification of diagnostic tools (genetic markers, biomarkers, or antibodies). Genomic or proteomic approaches to pinpoint potential gene products or molecular mechanisms involved in the developmental, reproductive, or disease process being investigated are encouraged but findings and interpretations will require validation by cellular, systemic, or in vivo studies. Similarly, in vitro (cellular) studies with downstream functional in vivo (whole animal) studies will be crucial to demonstrate and verify the utility of the findings for applications that improve specific health or reproductive issues addressed. Efforts to address the identification of markers that will predict reproductive capacity of oocytes or embryos should involve non-invasive approaches. This research effort is expected to deepen the fundamental knowledge of biological mechanisms essential to both humans and farm animals, highlight the utility of domestic agricultural animals as more appropriate animal models for specific mechanistic studies, and encourage investigators with farm animal expertise to be involved in research benefiting both agriculture and biomedicine.

Research Scope

Examples of research topics considered appropriate to this FOA include, but are not limited to the following:

- Study ovarian function and dysfunction, including the mechanisms that regulate follicle endowment, follicular development, ovulation, development and function of the corpus luteum, and ovarian pathologies that affect fertility
- Establish or improve methods for the in vitro expansion of spermatogonial stem cells, their cryopreservation, and testicular repopulation that can be applied 1) as an alternative approach for male germplasm preservation, 2) towards regenerative medicine to alleviate detrimental male-inherited traits or gonadotoxic exposure, or 3) as an alternative transgenesis tool.
- Design studies to enhance the efficacy of assisted reproduction technology (ART) and reduce simultaneous multiple pregnancies in humans and thus, the risks associated with multiple births such as prematurity, small for gestational age, and improve the efficacy of in vitro conditions for the manipulation of oocytes by: Identifying molecular mechanisms that regulate oocyte and embryo competency; Pinpointing and validating biomarkers and other types of non-invasive hallmarks that may be used reliably to determine oocyte and embryo quality; Investigating the effect of current and novel in vitro oocyte maturation methods on embryo competency.
- Elucidate mechanisms that regulate nuclear reprogramming, including maintenance of genomic integrity and, the identification of factors in the in vitro milieu that trigger epigenetic changes to improve cloning or transgenesis methodologies.
- Establish the core microbiome (including the metabolome of the microbiome) of the reproductive tract, and design studies to understand the effect of altered microbiome on fertility and infertility.
- Improve or identify novel interventions to prevent or decrease the incidence of obesity in humans and to improve the carcass quality of farm animals by: Identifying central and peripheral factors involved in the regulation of energy balance and examining their role in nutrient utilization and/or feeding behavior with respect to obesity.
- Elucidate the biology of the adipocyte, including mechanisms that regulate lipid metabolism, generation of adipose tissue-derived cytokines (adipokines), and fat accretion
- Deciphering genetic, dietary or hormonal influences on the regulation of pre- or postnatal adiposity.
- Characterize the role of gastrointestinal microbiome (including the metabolome of the microbiome) on host metabolism in the context of obesity.
- Examine the impact of environmental factors, the mother's physical state, and placental structure and/or metabolism on in utero developmental programming of the embryo and fetus and their role in the etiology of subsequent pathogenesis in adulthood that 1) negatively impacts the ability to thrive or survive, 2) increases disease susceptibility and/or 3) decreases fertility and reproductive capacity in humans and farm animals through: the identification of fetal/placental developmental factor, placentation, or epigenetic changes influenced by exposure of the mother to specific environmental stressors (e.g. malnutrition, obesity, toxicants, drugs, temperature, disease) that alter development processes in the embryo and fetus and directly increase the incidence of disease susceptibility, pathogenesis, or decreased fertility and reproductive capacity in adulthood.
- Investigate the effects of specific nutrients on gene expression and the elucidation of genetic components that modulate unique responses (i.e. nutrigenomics) to determine its application for the prevention, alleviation or treatment of chronic disease or to enhance reproduction.
- Study the role of gestational and perinatal microbiomes (including the metabolomes of their microbiome) on growth, development, antimicrobial resistance, and to the origin of adult diseases.
- Identify interventions that halt the transmission of infectious diseases from animal reservoirs to abrogate disease in the hosts through: the elucidation of the factors and mechanisms that regulate

horizontal and vertical transmission of virulent infectious pathogens and the genetic factors that predispose susceptibility of a human or animal.
- The identification of determinants, such as human demographics and behavior, practices in technology, industry or agriculture and economic development that contribute to the emergence of eradicated and relatively new infectious pathogens.
- Define organ or tissue-specific microbiome (including the metabolome of the microbiome) and its relationship to inflammation, immune response, infectious development and antimicrobial resistance
- Develop genome-editing strategies for the treatment of infectious diseases
- The development of vaccines or novel therapies to block transmission and/or abrogate susceptibility.

See Section VIII. Other Information for award authorities and regulations.

Section II. Award Information

Funding Instrument

Grant: A support mechanism providing money, property, or both to an eligible entity to carry out an approved project or activity.

Application Types Allowed

New
Renewal
Resubmission
Revision
The OER Glossary and the SF424 (R&R) Application Guide provide details on these application types.

Funds Available and Anticipated Number of Awards

The number of awards is contingent upon NIH appropriations and the submission of a sufficient number of meritorious applications.

It is the intention of NIH and USDA-NIFA to independently fund grants, and funding in subsequent years will be the responsibility of the agency awarding the grant.

Award Budget

Application budgets are not limited but need to reflect the actual needs of the proposed project.

Award Project Period

The scope of the proposed project should determine the project period. The maximum project period is 5 years.

NIH grants policies as described in the ***NIH Grants Policy Statement*** will apply to the applications submitted and awards made in response to this FOA.

Section III. Eligibility Information

1. Eligible Applicants

Eligible Organizations

Higher Education Institutions

- Public/State Controlled Institutions of Higher Education
- Private Institutions of Higher Education

The following types of Higher Education Institutions are always encouraged to apply for NIH support as Public or Private Institutions of Higher Education:

  - Hispanic-serving Institutions
  - Historically Black Colleges and Universities (HBCUs)
  - Tribally Controlled Colleges and Universities (TCCUs)
  - Alaska Native and Native Hawaiian Serving Institutions
  - Asian American Native American Pacific Islander Serving Institutions (AANAPISIs)

Nonprofits Other Than Institutions of Higher Education

- Nonprofits with 501(c)(3) IRS Status (Other than Institutions of Higher Education)
- Nonprofits without 501(c)(3) IRS Status (Other than Institutions of Higher Education)

For-Profit Organizations

- Small Businesses
- For-Profit Organizations (Other than Small Businesses)

Governments

- State Governments
- County Governments
- City or Township Governments
- Special District Governments
- Indian/Native American Tribal Governments (Federally Recognized)
- Indian/Native American Tribal Governments (Other than Federally Recognized)
- Eligible Agencies of the Federal Government
- U.S. Territory or Possession

Other

- Independent School Districts
- Public Housing Authorities/Indian Housing Authorities
- Native American Tribal Organizations (other than Federally recognized tribal governments)
- Faith-based or Community-based Organizations
- Regional Organizations
- Non-domestic (non-U.S.) Entities (Foreign Institutions)

Foreign Institutions

Non-domestic (non-U.S.) Entities (Foreign Institutions) **are** eligible to apply.
Non-domestic (non-U.S.) components of U.S. Organizations **are** eligible to apply.
Foreign components, as defined in the ***NIH Grants Policy Statement***, **are** allowed.

Required Registrations

Applicant Organizations

Applicant organizations must complete and maintain the following registrations as described in the SF 424 (R&R) Application Guide to be eligible to apply for or receive an award. All registrations must be completed prior to the application being submitted. Registration can take 6 weeks or more, so applicants should begin the registration process as soon as possible. The NIH Policy on Late Submission of Grant Applications states that failure to complete registrations in advance of a due date is not a valid reason for a late submission.

- Dun and Bradstreet Universal Numbering System (DUNS) - All registrations require that applicants be issued a DUNS number. After obtaining a DUNS number, applicants can begin both SAM and eRA Commons registrations. The same DUNS number must be used for all registrations, as well as on the grant application.
- System for Award Management (SAM) (formerly CCR) – Applicants must complete and maintain an active registration, **which requires renewal at least annually**. The renewal process may require as much time as the initial registration. SAM registration includes the assignment of a Commercial and Government Entity (CAGE) Code for domestic organizations which have not already been assigned a CAGE Code.
    - NATO Commercial and Government Entity (NCAGE) Code – Foreign organizations must obtain an NCAGE code (in lieu of a CAGE code) in order to register in SAM.
- eRA Commons - Applicants must have an active DUNS number and SAM registration in order to complete the eRA Commons registration. Organizations can register with the eRA Commons as they are working through their SAM or Grants.gov registration. eRA Commons requires organizations to identify at least one Signing Official (SO) and at least one Program Director/Principal Investigator (PD/PI) account in order to submit an application.
- Grants.gov – Applicants must have an active DUNS number and SAM registration in order to complete the Grants.gov registration.

Program Directors/Principal Investigators (PD(s)/PI(s))

All PD(s)/PI(s) must have an eRA Commons account. PD(s)/PI(s) should work with their organizational officials to either create a new account or to affiliate their existing account with the applicant organization in eRA Commons. If the PD/PI is also the organizational Signing Official, they must have two distinct eRA Commons accounts, one for each role. Obtaining an eRA Commons account can take up to 2 weeks.

Eligible Individuals (Program Director/Principal Investigator)

Any individual(s) with the skills, knowledge, and resources necessary to carry out the proposed research as the Program Director(s)/Principal Investigator(s) (PD(s)/PI(s)) is invited to work with his/her organization to develop an application for support. Individuals from underrepresented racial and ethnic groups as well as individuals with disabilities are always encouraged to apply for NIH support.

For institutions/organizations proposing multiple PDs/PIs, visit the Multiple Program Director/Principal Investigator Policy and submission details in the Senior/Key Person Profile (Expanded) Component of the SF424 (R&R) Application Guide.

2. Cost Sharing

This FOA does not require cost sharing as defined in the ***NIH Grants Policy Statement***.

3. Additional Information on Eligibility

Number of Applications

Applicant organizations may submit more than one application, provided that each application is scientifically distinct.

The NIH will not accept duplicate or highly overlapping applications under review at the same time. This means that the NIH will not accept:

- A new (A0) application that is submitted before issuance of the summary statement from the review of an overlapping new (A0) or resubmission (A1) application.
- A resubmission (A1) application that is submitted before issuance of the summary statement from the review of the previous new (A0) application.
- An application that has substantial overlap with another application pending appeal of initial peer review (see NOT-OD-11-101).

Section IV. Application and Submission Information

1. Requesting an Application Package

Applicants must obtain the SF424 (R&R) application package associated with this funding opportunity using the "Apply for Grant Electronically" button in this FOA or following the directions provided at Grants.gov.

2. Content and Form of Application Submission

It is critical that applicants follow the instructions in the SF424 (R&R) Application Guide, including Supplemental Grant Application Instructions except where instructed in this funding opportunity announcement to do otherwise. Conformance to the requirements in the Application Guide is required and strictly enforced. Applications that are out of compliance with these instructions may be delayed or not accepted for review.

For information on Application Submission and Receipt, visit Frequently Asked Questions – Application Guide, Electronic Submission of Grant Applications.

Letter of Intent

Although a letter of intent is not required, is not binding, and does not enter into the review of a subsequent application, the information that it contains allows IC staff to estimate the potential review workload and plan the review.

By the date listed in Part 1. Overview Information, prospective applicants are asked to submit a letter of intent that includes the following information:

- Descriptive title of proposed activity
- Name(s), address(es), and telephone number(s) of the PD(s)/PI(s)
- Names of other key personnel
- Participating institution(s)
- Number and title of this funding opportunity

The letter of intent should be sent to:

Ravi (Neelakanta) Ravindranath, DVM, PhD
Fertility and Infertility Branch (FIB)
Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD)
6100 Executive Boulevard, Rm8B01G, MSC 7510
Bethesda, MD 20892
Rockville, MD 20852 (for express/courier service; non-USPS service)
Telephone: 301-435-6889
Fax: 301-480-2389
Email: ravindrn@mail.nih.gov

Page Limitations

All page limitations described in the SF424 Application Guide and the Table of Page Limits must be followed.

Instructions for Application Submission

The following section supplements the instructions found in the SF424 (R&R) Application Guide and should be used for preparing an application to this FOA.

SF424(R&R) Cover

All instructions in the SF424 (R&R) Application Guide must be followed.

SF424(R&R) Project/Performance Site Locations

All instructions in the SF424 (R&R) Application Guide must be followed.

SF424(R&R) Other Project Information

All instructions in the SF424 (R&R) Application Guide must be followed.

SF424(R&R) Senior/Key Person Profile

All instructions in the SF424 (R&R) Application Guide must be followed.

R&R or Modular Budget

All instructions in the SF424 (R&R) Application Guide must be followed.

R&R Subaward Budget

All instructions in the SF424 (R&R) Application Guide must be followed.

PHS 398 Cover Page Supplement

All instructions in the SF424 (R&R) Application Guide must be followed.

PHS 398 Research Plan

All instructions in the SF424 (R&R) Application Guide must be followed, with the following additional instructions:

Resource Sharing Plan: Individuals are required to comply with the instructions for the Resource Sharing Plans as provided in the SF424 (R&R) Application Guide, with the following modification:

- All applications, regardless of the amount of direct costs requested for any one year, should address a Data Sharing Plan.

Appendix: Do not use the Appendix to circumvent page limits. Follow all instructions for the Appendix as described in the SF424 (R&R) Application Guide.

PHS Inclusion Enrollment Report

When conducting clinical research, follow all instructions for completing PHS Inclusion Enrollment Report as described in the SF424 (R&R) Application Guide.

PHS Assignment Request Form

All instructions in the SF424 (R&R) Application Guide must be followed.

Foreign Institutions

Foreign (non-U.S.) institutions must follow policies described in the ***NIH Grants Policy Statement***, and procedures for foreign institutions described throughout the SF424 (R&R) Application Guide.

3. Unique Entity Identifier and System for Award Management (SAM)

See Part 1. Section III.1 for information regarding the requirement for obtaining a unique entity identifier and for completing and maintaining active registrations in System for Award Management (SAM), NATO Commercial and Government Entity (NCAGE) Code (if applicable), eRA Commons, and Grants.gov

4. Submission Dates and Times

Part I. Overview Information contains information about Key Dates and times. Applicants are encouraged to submit applications before the due date to ensure they have time to make any application corrections that might be necessary for successful submission. When a submission date falls on a weekend or Federal holiday, the application deadline is automatically extended to the next business day.

Organizations must submit applications to Grants.gov (the online portal to find and apply for grants across all Federal agencies). Applicants must then complete the submission process by tracking the status of the application in the eRA Commons, NIH's electronic system for grants administration. NIH and Grants.gov systems check the application against many of the application instructions upon submission. Errors must be corrected and a changed/corrected application must be submitted to Grants.gov on or before the application due date and time. If a Changed/Corrected application is submitted after the deadline, the application will be considered late. Applications that miss the due date and time are subjected to the NIH Policy on Late Application Submission.

Applicants are responsible for viewing their application before the due date in the eRA Commons to ensure accurate and successful submission.

Information on the submission process and a definition of on-time submission are provided in the SF424 (R&R) Application Guide.

5. Intergovernmental Review (E.O. 12372)

This initiative is not subject to intergovernmental review.

6. Funding Restrictions

All NIH awards are subject to the terms and conditions, cost principles, and other considerations described in the ***NIH Grants Policy Statement***.

Pre-award costs are allowable only as described in the ***NIH Grants Policy Statement***.

NIFA awards are subject to the terms and conditions, cost principles, and other considerations described in 7 CFR 3430, Competitive and Noncompetitive Non-formula Grant Programs – General Grant Administrative Provisions.

Section 7132 of the Food, Conservation, and Energy Act of 2008 (Pub. L. 110-246) amended section 1462(a) of the National Agricultural Research, Extension, and Teaching Policy Act of 1977 (7 U.S.C. 3310(a)) on recovery of indirect costs. The recovery of indirect costs on awards made by USDA-NIFA under this program may not exceed the lesser of the institution's official negotiated indirect cost rate or the equivalent of 22 percent of total Federal funds awarded. If an applicant is selected for an award by USDA-NIFA, they will be instructed to resubmit their budget forms in accordance with this limitation.

Funds made available for grants under this FOA shall not be used for the construction of a new building or facility or the acquisition, expansion, remodeling, or alteration of an existing building or facility (including site grading and improvement, and architect fees).

7. Other Submission Requirements and Information

Applications must be submitted electronically following the instructions described in the SF424 (R&R) Application Guide. Paper applications will not be accepted.

Applicants must complete all required registrations before the application due date. Section III. Eligibility Information contains information about registration.

For assistance with your electronic application or for more information on the electronic submission process, visit Applying Electronically. If you encounter a system issue beyond your control that threatens your ability to complete the submission process on-time, you must follow the Guidelines for Applicants Experiencing System Issues. For assistance with application submission, contact the Application Submission Contacts in Section VII.

Important reminders:

All PD(s)/PI(s) must include their eRA Commons ID in the Credential field of the Senior/Key Person Profile Component of the SF424(R&R) Application Package**.** Failure to register in the Commons and to include a valid PD/PI Commons ID in the credential field will prevent the successful submission of an electronic application to NIH. See Section IIIof this FOA for information on registration requirements.

The applicant organization must ensure that the DUNS number it provides on the application is the same number used in the organization’s profile in the eRA Commons and for the System for Award Management. Additional information may be found in the SF424 (R&R) Application Guide.

See more tips for avoiding common errors.

Upon receipt, applications will be evaluated for completeness and compliance with application instructions by the Center for Scientific Review, NIH. Applications that are incomplete or non-compliant will not be reviewed.

Requests of $500,000 or more for direct costs in any year

Applicants requesting $500,000 or more in direct costs in any year (excluding consortium F&A) must contact a Scientific/ Research Contact at least 6 weeks before submitting the application and follow the Policy on the Acceptance for Review of Unsolicited Applications that Request $500,000 or More in Direct Costs as described in the SF424 (R&R) Application Guide.

Post Submission Materials

Applicants are required to follow the instructions for post-submission materials, as described in NOT-OD-13-030.

Section V. Application Review Information

1. Criteria

Only the review criteria described below will be considered in the review process. As part of the NIH mission, all applications submitted to the NIH in support of biomedical and behavioral research are evaluated for scientific and technical merit through the NIH peer review system.

Overall Impact

Reviewers will provide an overall impact score to reflect their assessment of the likelihood for the project to exert a sustained, powerful influence on the research field(s) involved, in consideration of the following review criteria and additional review criteria (as applicable for the project proposed).

Scored Review Criteria

Reviewers will consider each of the review criteria below in the determination of scientific merit, and give a separate score for each. An application does not need to be strong in all categories to be judged likely to have major scientific impact. For example, a project that by its nature is not innovative may be essential to advance a field.

Significance

Does the project address an important problem or a critical barrier to progress in the field? Is there a strong scientific premise for the project? If the aims of the project are achieved, how will scientific knowledge, technical capability, and/or clinical practice be improved? How will successful completion of the aims change the concepts, methods, technologies, treatments, services, or preventative interventions that drive this field?

Investigator(s)

Are the PD(s)/PI(s), collaborators, and other researchers well suited to the project? If Early Stage Investigators or New Investigators, or in the early stages of independent careers, do they have appropriate experience and training? If established, have they demonstrated an ongoing record of accomplishments that have advanced their field(s)? If the project is collaborative or multi-PD/PI, do the investigators have complementary and integrated expertise; are their leadership approach, governance and organizational structure appropriate for the project?

Innovation

Does the application challenge and seek to shift current research or clinical practice paradigms by utilizing novel theoretical concepts, approaches or methodologies, instrumentation, or interventions? Are the concepts, approaches or methodologies, instrumentation, or interventions novel to one field of

research or novel in a broad sense? Is a refinement, improvement, or new application of theoretical concepts, approaches or methodologies, instrumentation, or interventions proposed?

Approach

Are the overall strategy, methodology, and analyses well-reasoned and appropriate to accomplish the specific aims of the project? Have the investigators presented strategies to ensure a robust and unbiased approach, as appropriate for the work proposed? Are potential problems, alternative strategies, and benchmarks for success presented? If the project is in the early stages of development, will the strategy establish feasibility and will particularly risky aspects be managed? Have the investigators presented adequate plans to address relevant biological variables, such as sex, for studies in vertebrate animals or human subjects?

If the project involves human subjects and/or NIH-defined clinical research, are the plans to address 1) the protection of human subjects from research risks, and 2) inclusion (or exclusion) of individuals on the basis of sex/gender, race, and ethnicity, as well as the inclusion or exclusion of children, justified in terms of the scientific goals and research strategy proposed?

Environment

Will the scientific environment in which the work will be done contribute to the probability of success? Are the institutional support, equipment and other physical resources available to the investigators adequate for the project proposed? Will the project benefit from unique features of the scientific environment, subject populations, or collaborative arrangements?

Additional Review Criteria

As applicable for the project proposed, reviewers will evaluate the following additional items while determining scientific and technical merit, and in providing an overall impact score, but will not give separate scores for these items.

Protections for Human Subjects

For research that involves human subjects but does not involve one of the six categories of research that are exempt under 45 CFR Part 46, the committee will evaluate the justification for involvement of human subjects and the proposed protections from research risk relating to their participation according to the following five review criteria: 1) risk to subjects, 2) adequacy of protection against risks, 3) potential benefits to the subjects and others, 4) importance of the knowledge to be gained, and 5) data and safety monitoring for clinical trials.

For research that involves human subjects and meets the criteria for one or more of the six categories of research that are exempt under 45 CFR Part 46, the committee will evaluate: 1) the justification for the exemption, 2) human subjects involvement and characteristics, and 3) sources of materials. For additional information on review of the Human Subjects section, please refer to the Guidelines for the Review of Human Subjects.

Inclusion of Women, Minorities, and Children

When the proposed project involves human subjects and/or NIH-defined clinical research, the committee will evaluate the proposed plans for the inclusion (or exclusion) of individuals on the basis of sex/gender, race, and ethnicity, as well as the inclusion (or exclusion) of children to determine if it is justified in terms of the scientific goals and research strategy proposed. For additional information on

review of the Inclusion section, please refer to the Guidelines for the Review of Inclusion in Clinical Research.

Vertebrate Animals

The committee will evaluate the involvement of live vertebrate animals as part of the scientific assessment according to the following criteria: (1) description of proposed procedures involving animals, including species, strains, ages, sex, and total number to be used; (2) justifications for the use of animals versus alternative models and for the appropriateness of the species proposed; (3) interventions to minimize discomfort, distress, pain and injury; and (4) justification for euthanasia method if NOT consistent with the AVMA Guidelines for the Euthanasia of Animals. Reviewers will assess the use of chimpanzees as they would any other application proposing the use of vertebrate animals. For additional information on review of the Vertebrate Animals section, please refer to the Worksheet for Review of the Vertebrate Animal Section.

Biohazards

Reviewers will assess whether materials or procedures proposed are potentially hazardous to research personnel and/or the environment, and if needed, determine whether adequate protection is proposed.

Resubmissions

For Resubmissions, the committee will evaluate the application as now presented, taking into consideration the responses to comments from the previous scientific review group and changes made to the project.

Renewals

For Renewals, the committee will consider the progress made in the last funding period.

Revisions

For Revisions, the committee will consider the appropriateness of the proposed expansion of the scope of the project. If the Revision application relates to a specific line of investigation presented in the original application that was not recommended for approval by the committee, then the committee will consider whether the responses to comments from the previous scientific review group are adequate and whether substantial changes are clearly evident.

Additional Review Considerations

As applicable for the project proposed, reviewers will consider each of the following items, but will not give scores for these items, and should not consider them in providing an overall impact score.

Applications from Foreign Organizations

Reviewers will assess whether the project presents special opportunities for furthering research programs through the use of unusual talent, resources, populations, or environmental conditions that exist in other countries and either are not readily available in the United States or augment existing U.S. resources.

Select Agent Research

Reviewers will assess the information provided in this section of the application, including 1) the Select Agent(s) to be used in the proposed research, 2) the registration status of all entities where Select Agent(s) will be used, 3) the procedures that will be used to monitor possession use and transfer of

Select Agent(s), and 4) plans for appropriate biosafety, biocontainment, and security of the Select Agent(s).

Resource Sharing Plans

Reviewers will comment on whether the following Resource Sharing Plans, or the rationale for not sharing the following types of resources, are reasonable: (1) Data Sharing Plan; (2) Sharing Model Organisms; and (3) Genomic Data Sharing Plan (GDS).

Authentication of Key Biological and/or Chemical Resources:

For projects involving key biological and/or chemical resources, reviewers will comment on the brief plans proposed for identifying and ensuring the validity of those resources.

Budget and Period of Support

Reviewers will consider whether the budget and the requested period of support are fully justified and reasonable in relation to the proposed research.

2. Review and Selection Process

Applications will be evaluated for scientific and technical merit by (an) appropriate Scientific Review Group(s) convened by the Center for Scientific Review, in accordance with NIH peer review policy and procedures, using the stated review criteria. Assignment to a Scientific Review Group will be shown in the eRA Commons.

As part of the scientific peer review, all applications:

- May undergo a selection process in which only those applications deemed to have the highest scientific and technical merit (generally the top half of applications under review) will be discussed and assigned an overall impact score.
- Will receive a written critique.

Applications will be assigned on the basis of established PHS referral guidelines to the appropriate NIH Institute or Center. Applications will compete for available funds with all other recommended applications . Following initial peer review, recommended applications will receive a second level of review by the appropriate national Advisory Council or Board. The following will be considered in making funding decisions:

- Scientific and technical merit of the proposed project as determined by scientific peer review.
- Availability of funds.
- Relevance of the proposed project to program priorities.

For applications that are not awarded by NICHD, the written critique (Summary Statement) may be shared with appropriate staff at USDA for their consideration.

3. Anticipated Announcement and Award Dates

After the peer review of the application is completed, the PD/PI will be able to access his or her Summary Statement (written critique) via the eRA Commons. Refer to Part 1 for dates for peer review, advisory council review, and earliest start date.

Information regarding the disposition of applications is available in the ***NIH Grants Policy Statement***.

Section VI. Award Administration Information

1. Award Notices

If the application is under consideration for funding, NIH will request "just-in-time" information from the applicant as described in the ***NIH Grants Policy Statement***.

A formal notification in the form of a Notice of Award (NoA) will be provided to the applicant organization for successful applications. The NoA signed by the grants management officer is the authorizing document and will be sent via email to the grantee's business official.

Awardees must comply with any funding restrictions described in Section IV.5. Funding Restrictions. Selection of an application for award is not an authorization to begin performance. Any costs incurred before receipt of the NoA are at the recipient's risk. These costs may be reimbursed only to the extent considered allowable pre-award costs.

Any application awarded in response to this FOA will be subject to terms and conditions found on the Award Conditions and Information for NIH Grants website. This includes any recent legislation and policy applicable to awards that is highlighted on this website.

USDA-NIFA Award Notices:

Applicants selected for USDA-NIFA funding will be required to submit additional forms and documents as detailed in "A Guide for Preparation and Submission of NIFA Applications via Grants.gov (PDF)" (http://www.csrees.usda.gov/funding/grant_forms/adobe_grants_gov_instructions.pdf). All awards made from the USDA will be limited to an indirect cost cap of 22% of the total Federal funds awarded. Revised budgets will be requested if these guidelines are not met by an application to be awarded by USDA/NIFA.

USDA-NIFA award documents will provide pertinent instructions and information and shall include at a minimum the following:

1. Legal name and address of performing organization or institution to which the Director has issued an award under the terms of this FOA;

2. Title of project;

3. Name(s) and institution(s) of PDs chosen to direct and control approved projects;

4. Identifying award number assigned by the Department;

5. Award type, specifying whether the grant is a standard or continuation award;

6. Project period, specifying the amount of time the Department intends to support the project without requiring re-competition for funds, and that no-cost extensions of time beyond the five year performance period will be granted only in extenuating circumstances, require prior approval and will be contingent on a satisfactory merit review conducted by NIFA;

7. Total amount of Departmental financial assistance approved by the Director during the project period;

8. Legal authority(ies) under which the award is issued;

9. Appropriate Catalog of Federal Domestic Assistance (CFDA) number;

10. Applicable award terms and conditions
(see http://www.nifa.usda.gov/business/awards/awardterms.html to view NIFA award terms and conditions);

11. Approved budget plan for categorizing allocable project funds to accomplish the stated purpose of the award; and

12. Other information or provisions deemed necessary by NIFA to carry out its respective awarding activities or to accomplish the purpose of a particular award.

2. Administrative and National Policy Requirements

All NIH grant and cooperative agreement awards include the ***NIH Grants Policy Statement*** as part of the NoA. For these terms of award, see the ***NIH Grants Policy Statement*** Part II: Terms and Conditions of NIH Grant Awards, Subpart A: General and Part II: Terms and Conditions of NIH Grant Awards, Subpart B: Terms and Conditions for Specific Types of Grants, Grantees, and Activities. More information is provided at Award Conditions and Information for NIH Grants.

Recipients of federal financial assistance (FFA) from HHS must administer their programs in compliance with federal civil rights law. This means that recipients of HHS funds must ensure equal access to their programs without regard to a person's race, color, national origin, disability, age and, in some circumstances, sex and religion. This includes ensuring your programs are accessible to persons with limited English proficiency. HHS recognizes that research projects are often limited in scope for many reasons that are nondiscriminatory, such as the principal investigator's scientific interest, funding limitations, recruitment requirements, and other considerations. Thus, criteria in research protocols that target or exclude certain populations are warranted where nondiscriminatory justifications establish that such criteria are appropriate with respect to the health or safety of the subjects, the scientific study design, or the purpose of the research.

For additional guidance regarding how the provisions apply to NIH grant programs, please contact the Scientific/Research Contact that is identified in Section VII under Agency Contacts of this FOA. HHS provides general guidance to recipients of FFA on meeting their legal obligation to take reasonable steps to provide meaningful access to their programs by persons with limited English proficiency. Please see http://www.hhs.gov/ocr/civilrights/resources/laws/revisedlep.html. The HHS Office for Civil Rights also provides guidance on complying with civil rights laws enforced by HHS. Please see http://www.hhs.gov/ocr/civilrights/understanding/section1557/index.html; andhttp://www.hhs.gov/ocr/civilrights/understanding/index.html. Recipients of FFA also have specific legal obligations for serving qualified individuals with disabilities. Please seehttp://www.hhs.gov/ocr/civilrights/understanding/disability/index.html. Please contact the HHS Office for Civil Rights for more information about obligations and prohibitions under federal civil rights laws at http://www.hhs.gov/ocr/office/about/rgn-hqaddresses.html or call 1-800-368-1019 or TDD 1-800-537-7697. Also note it is an HHS Departmental goal to ensure access to quality, culturally competent care, including long-term services and supports, for vulnerable populations. For further guidance on providing culturally and linguistically appropriate services, recipients should review the National Standards for Culturally and Linguistically Appropriate Services in Health and Health Care athttp://minorityhealth.hhs.gov/omh/browse.aspx?lvl=2&lvlid=53.

USDA-NIFA Administrative and National Policy Requirements:

Several Federal statutes and regulations apply to USDA-NIFA grant applications considered for review and to project grants awarded under this program.

These include, but are not limited to:

7 CFR Part 1, subpart A—USDA implementation of the Freedom of Information Act.

7 CFR Part 3-USDA implementation of OMB Circular No. A-129, regarding debt collection

7 CFR Part 15, subpart A—USDA implementation of Title VI of the Civil Rights Act of 1964, as amended.

7 CFR Part 331 and 9 CFR Part 121—USDA implementation of the Agricultural Bioterrorism Protection Act of 2002.

7 CFR Part 3015—USDA Uniform Federal Assistance Regulations, implementing OMB directives (i.e., OMB Circular Nos. A-21 and A-122 (2 CFR Parts 220 and 230) and incorporating provisions of 31 U.S.C. 6301-6308 (formerly the Federal Grant and Cooperative Agreement Act of 1977, Pub. L. No. 95-224), as well as general policy requirements applicable to recipients of Departmental financial assistance.

7 CFR Part 3017—USDA implementation of Governmentwide Debarment and Suspension (Nonprocurement).

7 CFR Part 3018—USDA implementation of Restrictions on Lobbying. Imposes prohibitions and requirements for disclosure and certification related to lobbying on recipients of Federal contracts, grants, cooperative agreements, and loans.

7 CFR Part 3019—USDA implementation of OMB Circular A-110, Uniform Administrative Requirements for Grants and Other Agreements With Institutions of Higher Education, Hospitals, and Other Nonprofit Organizations (2 CFR Part 215).

7 CFR Part 3021—Governmentwide Requirements for Drug-Free Workplace (Financial Assistance).

7 CFR Part 3052—USDA implementation of OMB Circular No. A-133, Audits of States, Local Governments, and Nonprofit Organizations.

7 CFR Part 3407—NIFA procedures to implement the National Environmental Policy Act of 1969, as amended.

7 CFR 3430—Competitive and Noncompetitive Non-formula Grant Programs--General Grant Administrative Provisions.

29 U.S.C. 794 (section 504, Rehabilitation Act of 1973) and 7 CFR Part 15b (USDA implementation of statute) —prohibiting discrimination based upon physical or mental handicap in Federally assisted programs.

35 U.S.C. 200 et seq. —Bayh Dole Act, controlling allocation of rights to inventions made by employees of small business firms and domestic nonprofit organizations, including universities, in Federally assisted programs (implementing regulations are contained in 37 CFR Part 401). For additional information on NIFA patents, inventions and copyrights requirements, visit

http://www.nifa.usda.gov/business/awards/intellprop.html.

Cooperative Agreement Terms and Conditions of Award

Not Applicable

3. Reporting

When multiple years are involved, awardees will be required to submit the Research Performance Progress Report (RPPR) annually and financial statements as required in the NIH Grants Policy Statement.

A final progress report, invention statement, and the expenditure data portion of the Federal Financial Report are required for closeout of an award, as described in the ***NIH Grants Policy Statement***.

The Federal Funding Accountability and Transparency Act of 2006 (Transparency Act), includes a requirement for awardees of Federal grants to report information about first-tier subawards and executive compensation under Federal assistance awards issued in FY2011 or later. All awardees of applicable NIH grants and cooperative agreements are required to report to the Federal Subaward Reporting System (FSRS) available at www.fsrs.gov on all subawards over $25,000. See the ***NIH Grants Policy Statement*** for additional information on this reporting requirement.

USDA-NIFA Awards:

NIFA reports its awards on the new REEport system. Information is made available at https://portal.nifa.usda.gov/portal

For informational purposes, the “Federal Financial Report,” Form SF-425, consolidates into a single report the former Financial Status Report (SF-269 and SF-269A) and the Federal Cash Transactions Report (SF-272 and SF-272A). The NIFA Agency specific Terms and Conditions include the requirement that Form SF-425 is due on a quarterly basis no later than 30 days following the end of each reporting period. A final “Federal Financial Report,” Form SF-425, is due 90 days after the expiration date of this award.

Section VII. Agency Contacts

We encourage inquiries concerning this funding opportunity and welcome the opportunity to answer questions from potential applicants.

Application Submission Contacts

eRA Service Desk (Questions regarding ASSIST, eRA Commons registration, submitting and tracking an application, documenting system problems that threaten submission by the due date, post submission issues)
Finding Help Online: http://grants.nih.gov/support/ (preferred method of contact)
Telephone: 301-402-7469 or 866-504-9552 (Toll Free)

Grants.gov Customer Support (Questions regarding Grants.gov registration and submission, downloading forms and application packages)
Contact CenterTelephone: 800-518-4726

Email: support@grants.gov

GrantsInfo (Questions regarding application instructions and process, finding NIH grant resources)
Email: GrantsInfo@nih.gov (preferred method of contact)
Telephone: 301-710-0267

Scientific/Research Contact(s)

Ravi (Neelakanta) Ravindranath, DVM, PhD
Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD)
Telephone: 301-435-6889
Email: ravindrn@mail.nih.gov

Mark Mirando, PhD
Institute of Food Production and Sustainability
USDA National Institute of Food and Agriculture
Telephone: 202-401-4336
Email: mmirando@nifa.usda.gov

Peer Review Contact(s)

Elaine Sierra-Rivera, Ph.D.
Center for Scientific Review (CSR)
Telephone: 301-435-1043
Email: riverase@csr.nih.gov

Financial/Grants Management Contact(s)

Bryan S. Clark, MBA
Grants Management Branch (NICHD)
Telephone: 301-435-6975
Email: clarkb1@mail.nih.gov

Section VIII. Other Information

Recently issued trans-NIH policy notices may affect your application submission. A full list of policy notices published by NIH is provided in the ***NIH Guide for Grants and Contracts***. All awards are subject to the terms and conditions, cost principles, and other considerations described in the NIH Grants Policy Statement.

Authority and Regulations

Awards are made under the authorization of Sections 301 and 405 of the Public Health Service Act as amended (42 USC 241 and 284) and under Federal Regulations 42 CFR Part 52 and 45 CFR Part 75.

Weekly TOC for this Announcement
NIH Funding Opportunities and Notices

MAJOR ARTICLE

Evaluation of Serological and Antigenic Relationships Between Middle Eastern Respiratory Syndrome Coronavirus and Other Coronaviruses to Develop Vaccine Platforms for the Rapid Response to Emerging Coronaviruses

Sudhakar Agnihothram,[1,2,a] Robin Gopal,[4,a] Boyd L. Yount Jr,[1,2] Eric F. Donaldson,[1,2] Vineet D. Menachery,[1,2] Rachel L. Graham,[1,2] Trevor D. Scobey,[1,2] Lisa E. Gralinski,[1,2] Mark R. Denison,[3] Maria Zambon,[4] and Ralph S. Baric[1,2]

[1]Department of Epidemiology and [2]Department of Microbiology and Immunology, University of North Carolina, Chapel Hill; [3]Department of Pediatrics and Microbiology and Immunology, Vanderbilt University, Nashville, Tennessee; and [4]Viral Zoonosis Unit, Public Health of England, London, United Kingdom

Background Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in 2012, causing severe acute respiratory disease and pneumonia, with 44% mortality among 136 cases to date. Design of vaccines to limit the virus spread or diagnostic tests to track newly emerging strains requires knowledge of antigenic and serologic relationships between MERS-CoV and other CoVs.

Methods Using synthetic genomics and Venezuelan equine encephalitis virus replicons (VRPs) expressing spike and nucleocapsid proteins from MERS-CoV and other human and bat CoVs, we characterized the antigenic responses (using Western blot and enzyme-linked immunosorbent assay) and serologic responses (using neutralization assays) against 2 MERS-CoV isolates in comparison with those of other human and bat CoVs.

Results Serologic and neutralization responses against the spike glycoprotein were primarily strain specific, with a very low level of cross-reactivity within or across subgroups. CoV N proteins within but not across subgroups share cross-reactive epitopes with MERS-CoV isolates. Our findings were validated using a convalescent-phase serum specimen from a patient infected with MERS-CoV (NA 01) and human antiserum against SARS-CoV, human CoV NL63, and human CoV OC43.

Conclusions Vaccine design for emerging CoVs should involve chimeric spike protein containing neutralizing epitopes from multiple virus strains across subgroups to reduce immune pathology, and a diagnostic platform should include a panel of nucleocapsid and spike proteins from phylogenetically distinct CoVs.

Keywords MERS-CoV; Vaccine Design; Diagnostics; Serology; Synthetic Genomics.

Novel approaches are needed to respond rapidly to new emerging diseases, especially early in the epidemic, when prompt public health interventions can limit mortality and epidemic spread. Coronaviruses (CoVs) constitute a group of phylogenetically diverse enveloped viruses that have the largest plus-strand RNA genomes and replicate efficiently in most mammals [1, 2]. Human CoV (HCoV-229E, -OC43, -NL63, and -HKU 1) infections typically result in mild-to-severe upper and lower respiratory tract disease [3, 4]. SARS-CoV emerged in 2002–2003, causing acute respiratory distress syndrome with 10% mortality overall and up to 50% mortality among aged individuals [5]. Most recently, Middle East respiratory syndrome CoV (MERS-CoV) emerged in the Middle East in April 2012, manifesting as severe pneumonia, acute respiratory distress syndrome, and acute renal failure. The virus is still circulating and has caused 136 human infections with 58 deaths (mortality rate, approximately 44%) [6, 7].

Received 16 August 2013; accepted 11 October 2013; electronically published 18 November 2013.

[a]S.A. and R.G. contributed equally to this work.

Presented in part: MERS-CoV study group meeting, June 20–22 2013, Cairo, Egypt.

Correspondence: Ralph S. Baric, PhD, Department of Microbiology and Immunology, 3304 Michael Hooker Research Bldg, Campus Box 7435, Chapel Hill, NC 27599-7435 (baric@email.unc.edu).

The Journal of Infectious Diseases 2014; 209: 995–1006
© The Author 2013. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com.
DOI: 10.1093/infdis/jit609

Downloaded from http://jid.oxfordjournals.org/ by guest on August 15, 2016

Phylogenetic analysis groups CoVs into 4 genera—Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus—and for many mammalian CoVs, bats are considered reservoirs [6, 8, 9]. SARS-CoVs closely related to bat CoV (BtCoV) HKU 3 [1, 10–12], whereas MERS-CoV is closely related to Pipistrellus BtCoV HKU 5 and Tylonycteris BtCoV HKU 4 [9]. However, the serologic and antigenic relationship between strains is unclear. Given the vast number of genetically distinct CoVs, well-defined serologic and virologic reagents are needed to rapidly track MERS-CoV and other CoV infections in natural populations and to optimize vaccine and therapeutic designs early in an outbreak setting, especially within and between phylogenetic subgroups.

The spike (S) and nucleocapsid (N) proteins are major immunogenic components of CoVs and are produced in abundant quantities during infection. The S protein is the principle determinant of protective immunity and cross-species transmission in CoV [11]. Antibodies against S protein protect from homologous and heterologous SARS-CoV challenge in vivo [13], whereas N protein–specific immune responses may offer limited protection especially against low-dose challenge [13]. Therefore, antibodies against S and N protein have diagnostic and therapeutic potential [14, 15].

In this article, we use alphavirus replicon vaccine vectors to express a panel of recombinant S and N proteins from distantly related alphacoronaviruses and betacoronaviruses, including MERS-CoV and other subgroup 2c CoVs. Using mouse polyclonal antisera and recombinant proteins, we compare the cross-reactivity and neutralization titers of these antisera between distantly related human and bat CoVs. Our results indicate that the S glycoprotein but not the N protein is the major determinant of the neutralizing antibody response to MERS-CoV; that the N proteins of CoVs only cross-react within but not between subgroups; that little if any cross-neutralization or cross-reactivity exists between the S proteins of CoVs within subgroup 2c or any other subgroup; and that cross-neutralization and cross-reactive patterns were validated with the convalescent-phase serum sample from a patient infected with MERS-CoV Hu/England-N1/2012 and a donor panel of human antisera against 3 different HCoVs. Our approach provides critical reagents, antisera, and recombinant virus vaccines that allow for rapid diagnosis of and intervention against MERS-CoV and other zoonotic CoVs that emerge in the future.

MATERIALS AND METHODS

Viruses, Cells, and Plaque Assays

MERS-CoV Hu/England-N1/2012 and MERS-CoV Hu/SA-N1/2012 were cultured on Vero 81 cells and grown in Opti-Mem (Gibco, Carlsbad, CA) with 5% fetal clone serum (Hyclone, South Logan, UT) and gentamicin/kanamycin (Gibco). Viral growth assays in Vero and Calu-3 cells were performed as previously described [16].

Generation of Polyclonal Mouse Antisera, Neutralization Assays, and Western and Northern Blot Analysis

Genes encoding the indicated S and N proteins were synthesized from Bio Basic (Ontario, Canada) and packaged into Venezuelan equine encephalitis virus replicon particles (VRPs). Following vaccination, mouse polyclonal sera were generated from BALB/c mice, and neutralization assays involving MERS-CoV strains and SARS-CoV were as described previously [17]. For Western blots, VRP or virus-infected cell lysates and controls were prepared as described before in detail [8], and these blots were probed using the indicated mouse polyclonal sera. Vero cells inoculated with MERS-CoV isolates were harvested 12 hours after infection by means of Trizol reagent (Invitrogen) and were used to perform Northern blots [18].

Enzyme-Linked Immunosorbent Assay (ELISA) and Blocking Assay

An ELISA using indicated virus-infected cell lysates or antigens expressed from VRPs was performed as described previously [19], and the reactivity of mouse or human serum was determined using a chemiluminescent substrate. Blocking ELISA was performed by sequentially reacting plate-bound MERS-CoV lysate antigen with convalescent-phase serum obtained from a patient with MERS, followed by mouse polyclonal serum raised against VRP-packaged MERS-CoV N or S proteins. Blocking was expressed as the percentage reduction in the reactivity of the mouse serum alone.

RESULTS

Molecular Characterization of MERS-CoV Hu/England-N1/2012

MERS-CoV Hu/England-N1/2012 (MERSEng 1) was isolated from a 49-year-old patient with severe respiratory illness and was transferred to London for treatment [7]. Twenty-nine mutations in MERSEng 1 at the amino acid level were identified and compared to the published sequence of MERS-CoV Hu/SA-N1/2012 (MERSSA 1; GenBank JX869059.2; Supplementary Figure S2). To identify whether these mutations altered virus growth, we analyzed the replication kinetics of the 2 isolates in Vero cells and a continuous epithelial cell line, Calu-3 (Figure 1A and 1B). Although the replication kinetics were slightly different between 2 isolates in Vero cells, peak viral titers were equivalent. In contrast, virus growth was markedly distinct in human Calu-3 cells and could have represented differences in strain-specific in vitro adaptation phenotypes or resulted from functional differences in the sensitivity to innate immune responses [20].

CoV replication and transcription involves production of a nested set of subgenomic messenger RNAs (mRNAs) [2], and

Downloaded from http://jid.oxfordjournals.org/ by guest on August 15, 2016

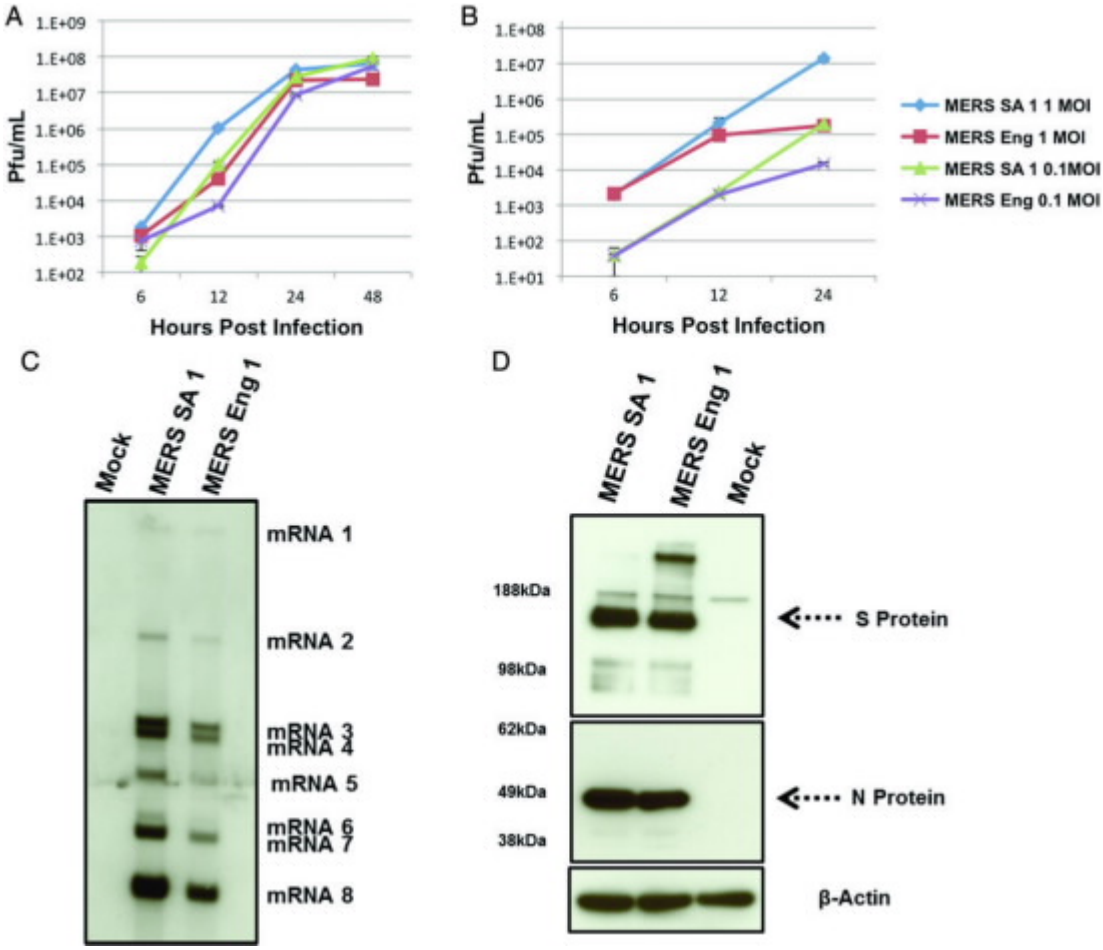


Figure 1. Growth of Middle East respiratory syndrome coronavirus (MERS-CoV) isolates at indicated multiplicity of infection (MOI) in Vero cells (A) and Calu-3 cells (B). Infected cultures were sampled in triplicate at times indicated, and viral titers (shown as plaque-forming units [PFU]/mL) were determined by plaque assay on Vero cells. Error bars indicate standard error of the mean. C, Northern blot analysis of RNA harvested 12 hours after infection from Vero cells infected with MERS-CoV isolates at a MOI of 5. D, Western blots of lysates harvested 12 hours after infection from Calu-3 2B4 cells infected with MERS-CoV isolates at a MOI of 5 that were probed with antisera to spike (S) and nucleocapsid (N) proteins. β-actin indicates loading control.

previous reports predicted 10 open reading frames in MERS SA 1 and MERS Eng 1 [9]. Northern blot analysis identified 8 subgenomic mRNAs after infection in both viruses (Figure 1C). The observed nested set of subgenomic mRNA expression is consistent with observations for other CoVs [2, 9]. The open reading frames encoded by each mRNA in these isolates are detailed in Supplementary Table 1.

Serologic Relationships Among MERS-CoV Strains

VRPs function as efficient expression and vaccine platforms for a variety of antigens [13, 21]. We generated VRPs expressing MERS SA 1 S and N proteins and then immunized mice. N protein–specific antiserum recognized a discrete 50-kDa band at the predicted molecular weight in lysates from Vero cells (Supplementary Figure 1A) and Calu-3 cells (Figure 1D) infected with VRP-N or with the 2 different MERS-CoV isolates. For the most part, similar expression patterns were evident between VRPs and viruses; however, the N protein of MERS Eng 1 had a slightly lower molecular weight, which was consistent with amino acid deletions at positions S391 and I392 (Supplementary Figure 2).

Mouse anti-S serum identified an approximately 180-kDa S protein in VRP-S– or MERS-CoV–infected Vero cells (Supplementary Figure 1A). The observed molecular weights were consistent with the sizes of the S proteins of other CoVs [2, 22]. We noted similar results in Calu-3 cells (Figure 1D), and interestingly, an increased amount of a higher-molecular-weight form of S protein (glycosylated dimer) was noted in MERS Eng 1 in both cell lines. Antiserum against MERS SA 1 S and N proteins also recognized MERS Eng 1 in ELISAs (data not shown), and N and S proteins recognized the MERS-CoV Jordan isolate (Supplementary Figure 1B).

Downloaded from http://jid.oxfordjournals.org/ by guest on August 15, 2016

Cross-neutralization Patterns Across Strains

Plaque reduction neutralization tests ($PRNT_{50}$) indicated complete neutralization of both MERS-CoV isolates ($PRNT_{50}$ titer, approximately 1:1400 for each; Figure 2A) and the MERS-CoV Jordan isolate (Supplementary Figure 1C) by VRP-S antiserum, whereas no neutralization was observed with N antiserum. Similar findings have been reported with SARS-CoV, as well as with other known human and animal CoVs [13, 23]. Interestingly, serum from aged mice vaccinated with VRP S showed a 6-fold reduction in $PRNT_{50}$ titers (approximately 1:200), indicating that immunosenescence attenuates vaccine responses to MERS-CoV antigens, as was noted with SARS-CoV vaccines [13, 17]. Using serum from NA 01 patient, ELISA demonstrated high reactivity of the patient's serum to N and S antigens of MERS SA 1 expressed from VRPs (Figure 2B and 2C). Titers of antibody against N protein in patient serum peaked 3–5 weeks after onset of illness (which occurred on 3 September 2012) and waned thereafter, but the antibodies were still detected up to 5 months after illness onset. Titers of antibody against S protein were consistent from 3 weeks to 5 weeks after illness onset, after which they remained detectable. Most importantly, patient serum collected on 16 November 2012 (which contained high titers against S protein) outcompeted the binding of mouse S antiserum to intact virus in a blocking assay (Figure 2D). These data suggest that different/overlapping epitopes are recognized by human and mouse antisera following virus or VRP-S infection.

Cross-reactive and Cross-neutralizing Antibody Responses Within and Across Alphacoronaviruses and Betacoronaviruses

MERS SA 1 and MERS Eng 1 are closely related to BtCoV HKU 5 and BtCoV HKU 4 (Figure 2E) [6, 9, 24]. To evaluate antigenic relationships with the subgroup 2c betacoronaviruses, VRPs expressing S and N proteins of BtCoV HKU 4.2 and BtCoV HKU 5.5 were inoculated into mice. Antisera against both HKU 4.2 and 5.5 N proteins recognized the N proteins of both MERS-CoV isolates, whereas MERS SA 1 N antisera also detected the VRP-expressed HKU 4.2 and 5.5 N proteins, as revealed by Western blot (Figure 3A and 3B). We obtained similar results using ELISA and immunofluorescence assays (data not shown). In contrast, there was little if any observable cross-reactivity observed between MERS SA 1 S antisera with the VRP-expressed S proteins of HKU 4.2 and HKU 5.5, whereas antisera to HKU 5.5 S protein but not HKU 4.2 S protein recognized the S proteins of both MERS-CoV isolates (Figure 3C and 3D). We also measured serologic relationships using ELISA, which captures cross-reactivity to conformational epitopes, and confirmed these antigenic relationships (Figure 6C). Consistent with results of serologic tests, antisera against HKU 4.2 and HKU 5.5 S proteins did not cross-neutralize the MERS-CoV isolates. These data indicate that the N protein but not the S glycoprotein are antigenically conserved within the subgroup 2c betacoronaviruses evaluated in this panel.

We then extended our analysis to the highly pathogenic SARS-CoV and related subgroup 2b betacoronaviruses. Polyclonal mouse sera to SARS-CoV or MERS SA 1 N or S proteins exhibited no cross-reactivity to the reciprocal strains (Figure 4A and 4B). We observed very low levels of cross-neutralization of MERS SA 1 by mouse antisera to SARS-CoV, using very high but not low concentrations of serum (Figure 4C), a finding that is consistent with a recent report [24]. Interestingly, ELISA results also showed very minimal cross-reactivity of the NA 01 patient sera obtained on 23 September 2012 to SARS-CoV S antigen (Figure 4D). Consistent with this observation, binding of mouse SARS-CoV S antiserum to SARS-CoV was not inhibited by NA01 patient sera in blocking assays (Figure 2D), indicating the absence of antibodies to SARS-CoV in the patient serum.

Consonant with these findings, no cross-reactivity was observed with antisera against the VRP-expressed N or S glycoproteins of BtCoV HKU 3 and 279 and the MERS-CoV isolates (Figure 5A–D). Furthermore, no cross-neutralization of MERS-CoV isolates by HKU 3 S antiserum was observed, although this serum has previously been shown to neutralize a synthetically resurrected HKU 3 variant encoding the SARS S glycoprotein receptor-binding domain [23]. Interestingly, we observed very low levels of cross-neutralization of SARS-CoV by BtCoV 279 S antiserum (Figure 4C).

To further elucidate the antigenic relationships between the S glycoproteins of alphacoronaviruses and the MERS-CoV isolates, we expressed and generated mouse antisera to BtCoV 1A and BtCoV HKU 2 (group 1b alphacoronaviruses), using the VRP platforms. Despite efficient recombinant S glycoprotein expression (Figure 7A and 7B), none of the recombinant S glycoproteins were recognized by MERS SA 1 S antisera. Antisera against BtCoV 1A and HKU 2 S glycoproteins had little if any cross-reactivity with and did not neutralize MERS-CoV (Figure 7A–C).

Antigenic Relationships Among the HCoVs

We next analyzed the antigenic relationships between VRP-derived mouse serum with the following representative HCoVs from each subgroup, using ELISA (Figure 6C): MERS Eng 1, from subgroup 2c; SARS-CoV, from subgroup 2b; HCoV-NL63, from subgroup 1b; and HCoV-OC43, from subgroup 2a. MERS Eng 1 was recognized by antisera targeting the N but not the S glycoprotein of viruses within the subgroup 2c betacoronaviruses. Likewise, SARS-CoV was only recognized by antisera to N but not S glycoproteins of viruses with the subgroup 2b betacoronaviruses. None of the antiserums screened reacted with HCoV-NL63 (subgroup 1b) or HCoV-OC43 (subgroup 2a). Although BtCoV HKU 2 is genetically close to HCoV-NL63, we did not observe any cross-reactivity within the S glycoprotein.

Serum from patients infected with SARS-CoV, HCoV-NL63, or HCoV-OC43 was screened against the N proteins from representative subgroup 2c and 2d betacoronaviruses. Consistent

Downloaded from http://jid.oxfordjournals.org/ by guest on August 15, 2016

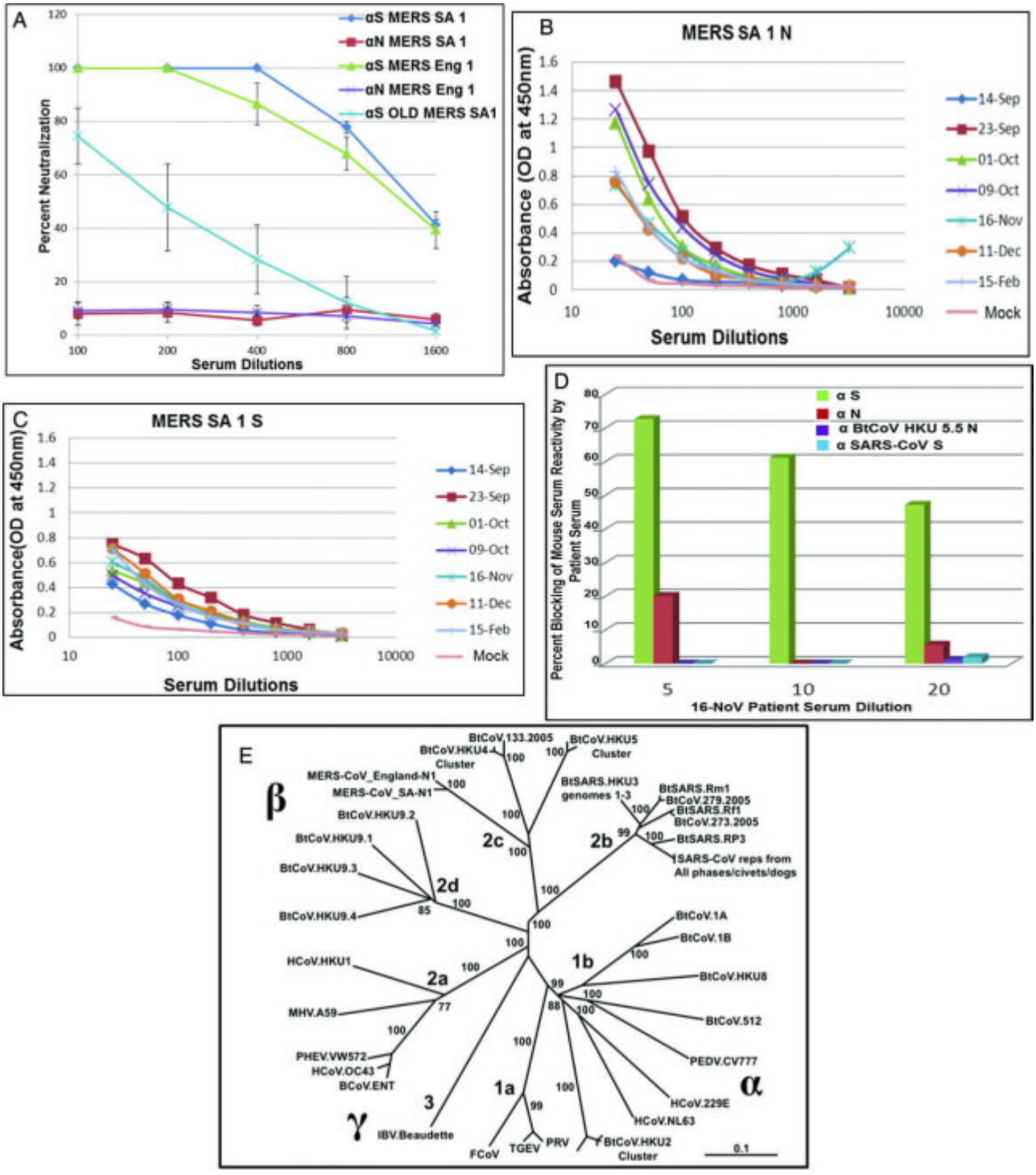


Figure 2. A, Serum from each of 4 young and aged mice immunized with Venezuelan equine encephalitis virus replicon particles (VRPs) expressing spike (S) or nucleocapsid (N) proteins were tested in a plaque reduction neutralization test to neutralize Middle East respiratory syndrome coronavirus (MERS-CoV) isolates. Error bars indicate standard error of the mean. B and C, NA01 patient sera collected at indicated dates after hospitalization were analyzed in an enzyme-linked immunosorbent assay using cell lysates expressing S and N antigens from VRPs. D, Indicated dilutions of NA01 patient sera collected on November 16, 2012 were screened with 1:800 dilutions of mouse antisera to S, N, bat CoV (BtCoV) HKU 5.5N, or SARS-CoV S in an in vitro competition assay for binding to MERS-CoV or SARS-CoV S. E. The full-length genome sequences of 51 CoVs were downloaded from GenBank or PATRIC, aligned with ClustalX, and phylogenetically compared by maximum likelihood estimation, using 100 bootstraps. The tree shows that CoVs are divided into 3 distinct phylogenetic groups, defined as α, β, and γ. This taxonomic nomenclature replaces the former group 1, 2, and 3 designations, respectively. Classical subgroup clusters are marked as 2a–2d for the β-CoVs and as 1a and 1b for the α-CoVs. The tree was generated using maximum likelihood estimation with the PhyML package. The scale bar represents nucleotide substitutions. Only nodes with bootstrap support of >70% are labeled. Accession numbers and definitions of various CoV strains will be provided upon request.

Downloaded from http://jid.oxfordjournals.org/ by guest on August 15, 2016

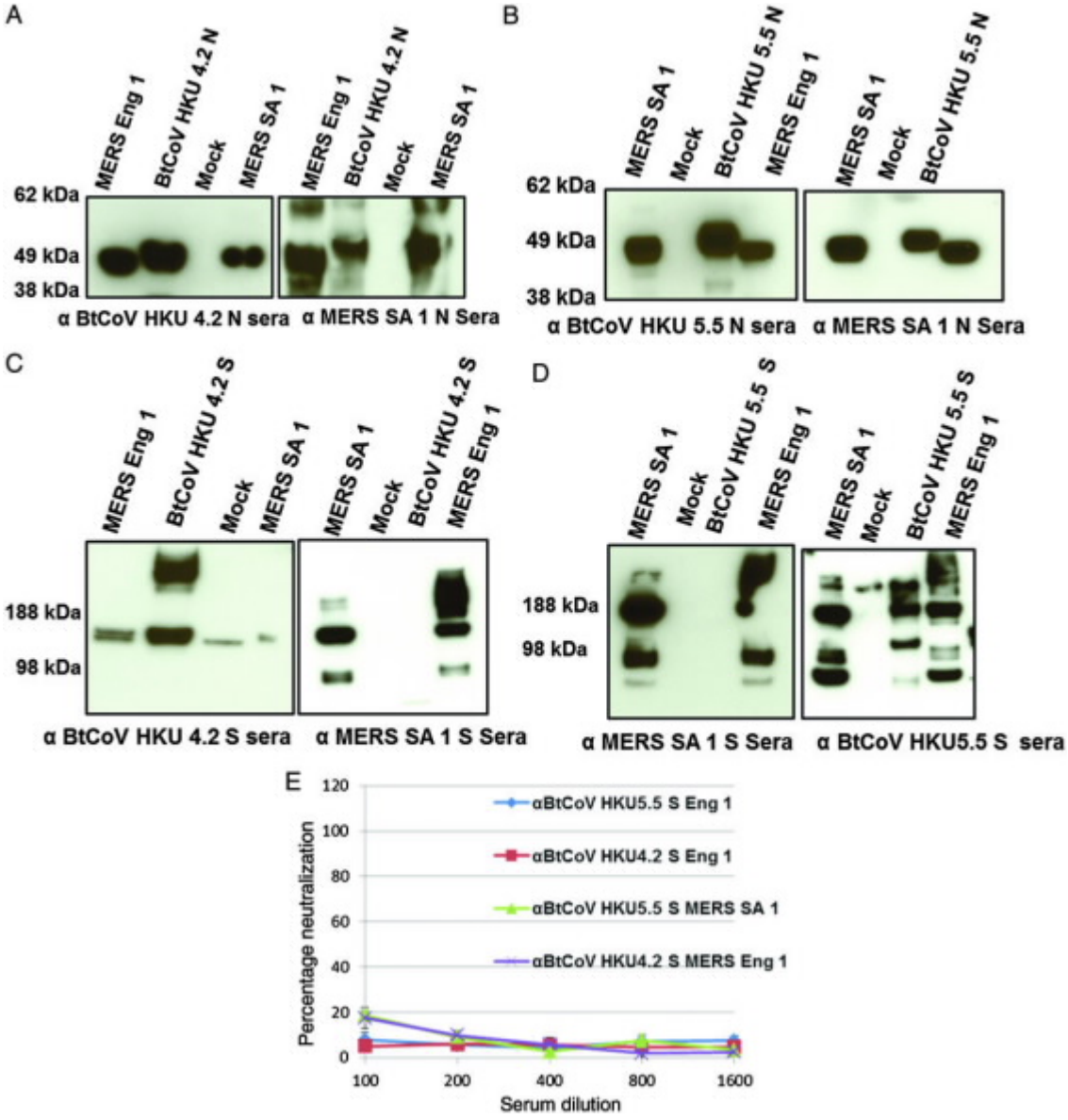


Figure 3. Western Blots showing cross-reactivity between nucleocapsid (N; A and B) and spike (S; C and D) proteins of Middle East respiratory syndrome coronavirus (MERS-CoV) isolates and N and S proteins of bat CoV (BtCoV) HKU 4.2 and HKU 5.5 (E). Plaque reduction neutralization tests showing absence of cross-neutralization of MERS-CoV isolates by antisera to BtCoV HKU 4.2 and 5.5 S proteins. Serum from groups of 4 mice immunized with Venezuelan equine encephalitis virus replicons was tested in this assay. Error bars indicate standard error of the mean. Note the cross-reactivity of antisera to BtCoV HKU 5.5 S protein to S proteins of MERS-CoV isolates (D) but the absence of cross-neutralization.

with our previous findings, human serum to SARS-CoV recognized BtCoV HKU 3N, BtCoV 279N, and SARS-CoV N (subgroup 2b) but did not recognize N proteins from other subgroups (Figure 6B). Similarly, there was no cross-reactivity of the human antisera from HCoV-NL63 (subgroup 1b) and HCoV-OC43 (subgroup 2a) infections with any of the viral antigens within the panel. Serum collected from the patient infected with MERS-CoV NA01 showed cross-reactive binding only to BtCoV HKU 5.5 N (subgroup 2c), and little if any cross-reactivity was noted outside the subgroup (Figure 6A), apart from very low, transient cross-detection of BtCoV 279N and from cross-reactivity to SARS-CoV S and N recombinant proteins on a single day (23 September 2012).

DISCUSSION

Emerging respiratory CoVs offer a considerable threat to the health of global populations and the economy. Platforms for generating well-characterized molecular reagents and recombinant viruses are needed to detect and control the emergence of

Downloaded from http://jid.oxfordjournals.org/ by guest on August 15, 2016

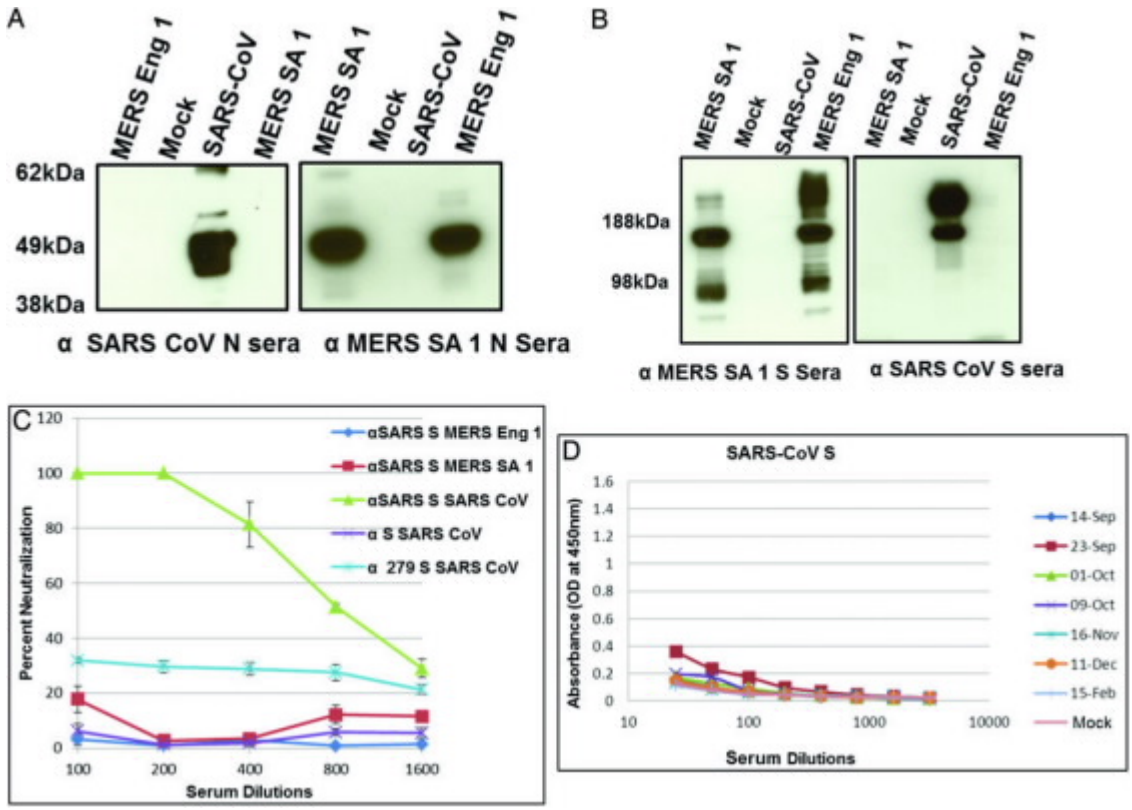


Figure 4. Western Blots showing no cross-reactivity between nucleocapsid (N; A) and spike (S; B) proteins of Middle East respiratory syndrome coronavirus (MERS-CoV) Vu isolates and SARS-CoV. C, Plaque reduction neutralization tests showing the absence of cross-neutralization of MERS-CoV Vu isolates by antisera to SARS-CoV S and of SARS-CoV by antisera to MERS-CoV/SA-1/2012 S protein and BtCoV 279 S protein. Note that antisera to SARS-CoV S neutralize SARS-CoV. Serum from groups of 4 mice immunized with Venezuelan equine encephalitis virus replicons was tested in this assay and error bars indicate standard error of the mean. D, Enzyme-linked immunosorbent assay results showing the absence of reactivity of NA01 patient sera to SARS-CoV S antigen.

new strains, especially early in an outbreak, before the development of type-specific serologic reagents and therapeutics. Here, we characterized the genome organization, subgenomic mRNA expression, and protein expression patterns of 2 isolates of MERS-CoV. Using alphavirus replicon particles and synthetic gene design, we assembled a panel of recombinant proteins from and donor antisera against phylogenetically distant alphacoronaviruses and betacoronaviruses to evaluate the antigenic relationships between strains and to inform vaccine design. MERS-CoV is a highly pathogenic respiratory CoV of humans, causing acute respiratory distress syndrome, with mortality rates approaching 44%. CoV primer sets were not successful in diagnosing the etiology of the Jordan outbreak in April 2012, demonstrating a critical need for paneled reagent sets of recombinant proteins and sera that allow for serologic evaluations of cases, contact cases, and asymptomatic infections, using Western blot or ELISA-based techniques [25]. This article is also the first report that describes the serologic characterization of MERS-CoV and CoV reagents. An advantage of the VRP platform is that it can also function as a vaccine vector, affording the rapid production of candidate vaccines against newly emerged strains [23]. Using SARS-CoV and MERS-CoV as models, we clearly demonstrated that S protein–based recombinant vaccines elicit robust neutralization responses in young and aged rodent models. Because VRP-S vectors against SARS-CoV protected young and aged animals [17], we have developed a recombinant S vectored vaccine that could likely prove successful in preventing heterologous MERS-CoV infection in aged individuals, but this remains to be tested.

MERS Eng 1 replicated to lower titers than SA1 in Calu-3 cells. As the 2 viruses have different passage histories in vitro, tissue culture adaptive mutations may account for these differences, as reported with many SARS-CoV isolates [26]. Alternatively, 29 amino acid differences have been described, most of which reside in the replicase polyprotein (Supplementary Figure 2) and may affect replication efficiency. In addition, the S glycoprotein of MERS Eng 1 differs from that of MERS SA 1 by 2 amino acids, L506F and Q1020H (Supplementary Figure 2), which may account for the increased amount of the higher-molecular-weight form of S protein in MERS Eng 1

Downloaded from http://jid.oxfordjournals.org/ by guest on August 15, 2016

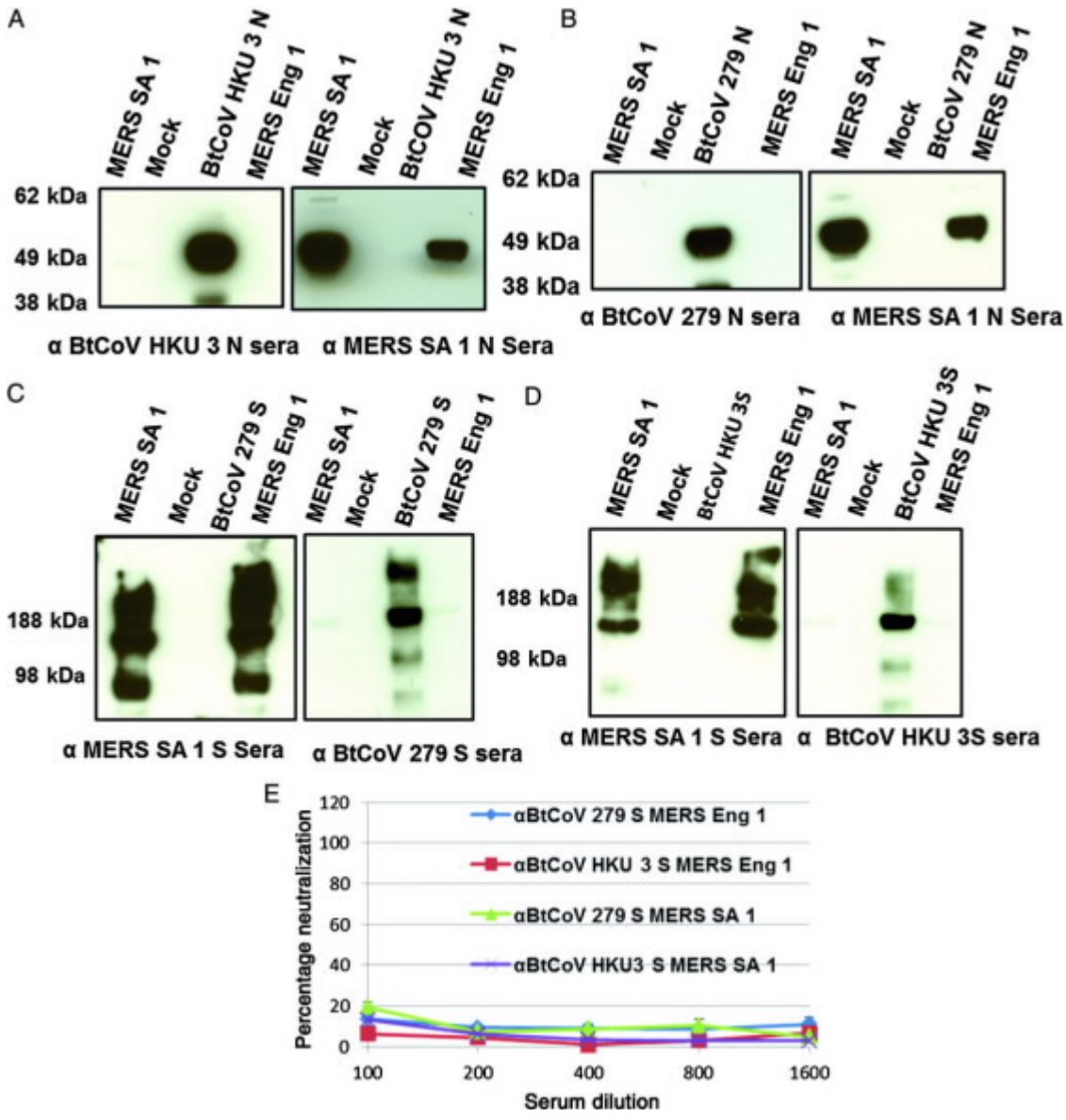


Figure 5. Western blots showing cross-reactivity between nucleocapsid (N; A and B) and spike (S; C and D) proteins of Middle East respiratory syndrome coronavirus (MERS-CoV) isolates and N and S proteins of BtCoV 279 and HKU3. E, Plaque reduction neutralization tests showing absence of cross-neutralization of MERS-CoV isolates by antisera to BtCoV 279 S and HKU3 S proteins. Serum from groups of 4 mice immunized with Venezuelan equine encephalitis virus replicons was tested in this assay. Error bars indicate standard error of the mean.

(Figure 1D and Supplementary Figure 1A). Recent studies indicate that TMPRSS2 likely plays important roles in viral entry by enhancing fusogenic potential through proteolytic processing of MERS-CoV S glycoprotein [22]. In addition, we identified a unique mutation, T1015N, in the MERS SA 1 isolate but not in the MERS Eng 1 isolate and showed that this mutation is responsible for increased in vitro fitness and for plaque morphology [20]. It is possible that the presence or absence of 1 or more of the S glycoprotein mutations in MERS Eng 1 may result in the slower growth phenotype in Calu3 cells.

Alphavirus VRPs have considerable potential as recombinant virus vaccine platforms in the absence of preexisting immunity [27–30]. We demonstrate efficient expression of several CoV S and N structural proteins both in vitro and in vivo, resulting in robust serologic responses in vaccinated mice. Antiserum to VRP-S glycoprotein but not to VRP-N protein neutralized both isolates of MERS-CoV. Furthermore, we and others have demonstrated that vaccine-induced immunopathology observed after challenge is minimized in VRP-S protein–based vaccines, partly because of the T-helper type 1–biased immune response

Downloaded from http://jid.oxfordjournals.org/ by guest on August 15, 2016

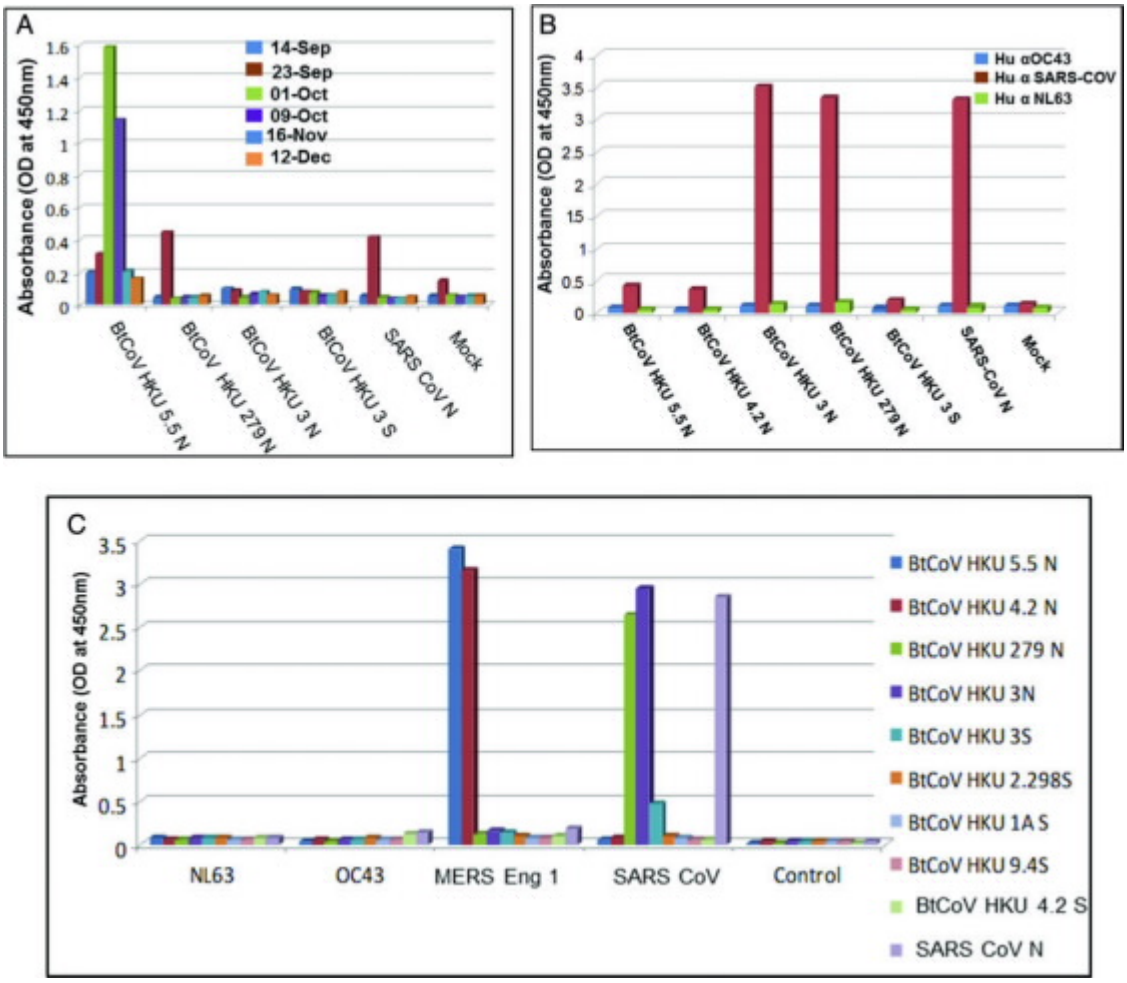


Figure 6. A, NA01 patient serum specimens collected at indicated dates were analyzed in an enzyme-linked immunosorbent assay (ELISA), using cell lysates expressing indicated antigens. B, Mouse antisera to the indicated antigens were screened in an ELISA. C, Human antisera to indicated CoVs were screened in an ELISA with cell lysates expressing indicated antigens.

and high neutralization titers elicited by VRP vectors [13, 31]. Importantly, vaccination of aged mice demonstrated that immunosenescence contributes to a reduction in the magnitude of the antibody response to MERS-CoV S glycoprotein, an important point to be considered in candidate vaccine designs. To date, wild-type and VRP 3526–coated VRP-S vaccines represent one of the few vaccine platforms that functioned well in aged animals, in addition to recombinant subunit–based vaccines and poxvirus-vectored vaccines [21, 32–34]. In SARS-CoV pathogenesis in aged animals, increased age-related susceptibility is linked to increased prostaglandin D2 expression; it remains uncertain whether increased prostaglandin D2 levels have contributed to reduced vaccine performance, as well [35]. Because we have not observed MERS-CoV replication in immunocompetent and immunocompromised mice, these vectors must be tested directly in primates [36]. The safety of the VRP platform has been demonstrated in high-risk human populations and immunosenescent nonhuman primates [27, 28, 37, 38], and we believe that these vectors will be efficacious in healthcare workers and target populations infected with MERS-CoV.

Our results indicate the presence of strongly cross-reactive epitopes in the N protein within a particular subgroup but not between subgroups. Under identical conditions, little cross-reactivity or conservation of cross-neutralizing epitopes was observed between S proteins within and across subgroups. Similar studies showing strong conservation of cross-reactive epitopes between N proteins, but to a lesser extent between S proteins of the subgroup 2a CoVs, has been reported [39, 40]. Importantly, the pattern of serological and antigenic relationship observed using the mouse antisera was recapitulated using the human antiserum to 4 different CoVs. Neutralization assays demonstrated little if any conservation of cross-neutralizing epitopes between S glycoproteins of CoVs within and across subgroups. In particular, the absence of cross-neutralization of MERS-CoV

Downloaded from http://jid.oxfordjournals.org/ by guest on August 15, 2016

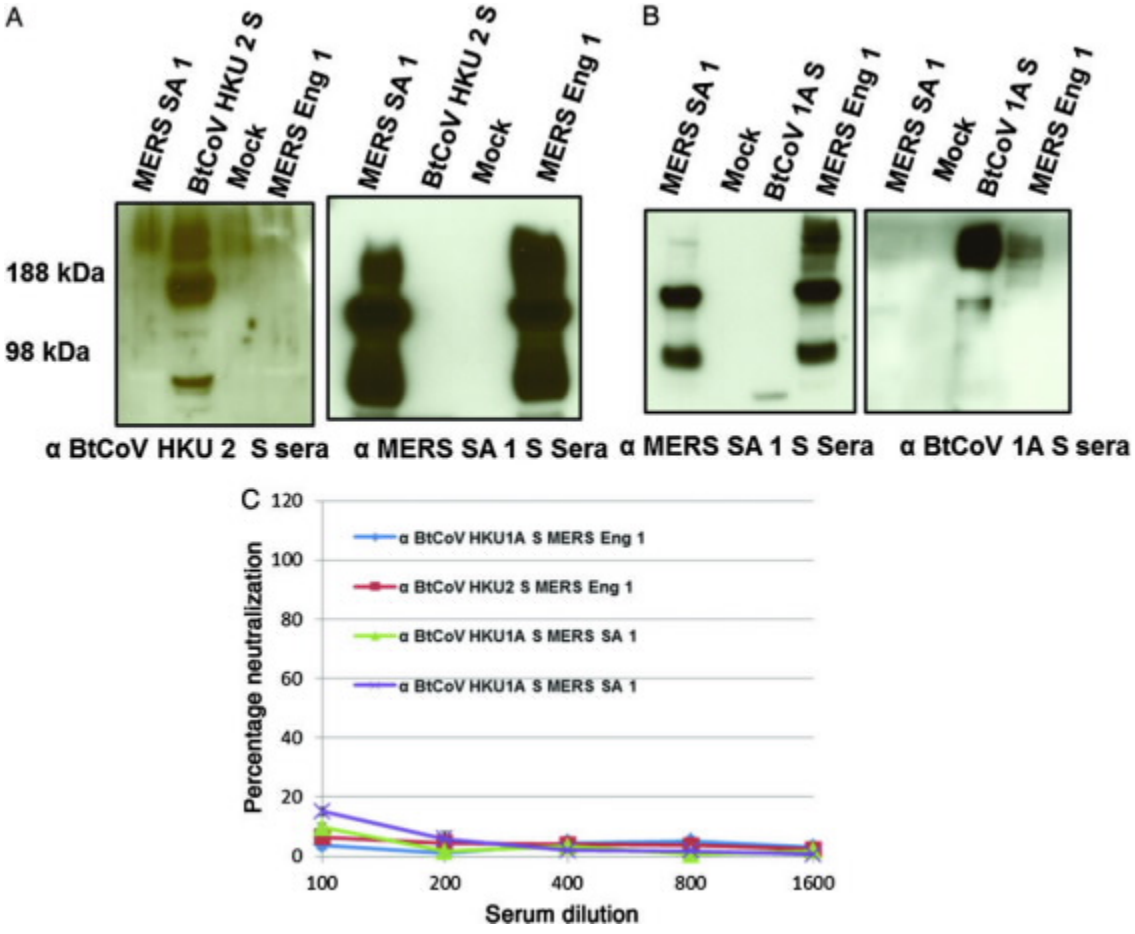


Figure 7. Western blots showing the cross-reactivity of spike (S) proteins of bat coronavirus (BtCoV) HKU2.298 (A) and BtCoV 1A (B) with Middle East respiratory syndrome coronavirus (MERS-CoV) HCoV isolates C, Plaque reduction neutralization tests showing the absence of cross-neutralization of MERS-CoV HCoV isolates by antisera. Serum from groups of 4 mice immunized with Venezuelan equine encephalitis virus replicons was tested in this assay. Error bars indicate standard error of the mean.

isolates by antiserum to HKU 4 or HKU 5 S glycoprotein and of SARS-CoV by antiserum to the HKU 3 or BtCoV 279 S glycoprotein suggests very limited conservation or, possibly, the deliberate masking of conserved cross-neutralizing sites within a subgroup. Although speculative, these cross-neutralization relationships suggest that at least 3 antigenically distinct CoVs could emerge from zoonotic viruses circulating within subgroup 1a/b, 2b, and 2c reservoirs and then simultaneously circulate in humans. These findings are evidence that vaccine design for any new emerging CoV should either focus on the development of chimeric S glycoproteins containing neutralizing epitopes from multiple strains within or across subgroups or on the development of new paradigms in structure-guided antigen design that improve the presentation of broadly neutralizing epitopes. Regions of S glycoprotein are interchangeable between CoVs within and across subgroups, rendering viable recombinant viruses [23]. Inclusion of N protein in such chimeric vaccines may broaden the protective response, although this remains to be tested using lethal challenge viruses. Such a vaccine might provide robust protection against several homologous and heterologous viruses within or across genoclusters.

After the SARS-CoV epidemic and in stark contrast to the situation with emerging influenza viruses such as influenza A (H7N9), the research and biomedical communities failed to develop broadly applicable biopreparedness platforms for rapid response against future emerging CoV threats. Because CoVs have demonstrated an accelerating pattern of zoonotic emergence since the 1980s [25, 41], our data indicate that an appropriate diagnostic platform should include a large panel of phylogenetically distinct CoV S and N structural proteins, which must be validated using larger panels of antisera against other HCoVs in the general population. While molecular-based platforms like polymerase chain reaction and deep sequencing offer clear advantages in early detection of active infections, public health response platforms would be

Downloaded from http://jid.oxfordjournals.org/ by guest on August 15, 2016

strengthened by the availability of recombinant proteins and subgroup- and type-specific antisera that can track subclinical infections, determine the prevalence of infection in populations, and identify hospital-acquired infections. A recent report identified subclinical cases of MERS-CoV infection through reverse-transcription polymerase chain reaction, and the screen using the panel of recombinant proteins described here would have provided more-specific information about the presence of other CoVs in these cases [42]. The VRP platform we describe not only yields high-level expression of key recombinant proteins across the alphacoronaviruses and betacoronaviruses tested, also provides the first candidate vaccine vectors with the potential to augment the T-helper type 1–based immune responses to MERS-CoV infection and to reduce associated immune pathology. The VRP 3526-associated approach is also applicable to improving the public health response to and the control of future outbreaks of other highly pathogenic emerging infectious diseases due to CoV in human populations.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank the University of North Carolina–Chapel Hill genome analysis facility and Dr Mark Heise at Carolina Vaccine Institute, for providing sequencing services and sharing laboratory space for animal experiments, respectively; Dr Michael Cooper at the Armed Forces Health Surveillance Center, Dr Emad Mohareb at Navy Medical Research Unit 3, and Dr Kanta Subbarao at the National Institute of Allergy and Infectious Diseases, for providing us with MERS-CoV-Hu/Jordan-N3/2012.

Financial support. This work was supported by the National Institutes of Allergy and Infectious Diseases, National Institute of Health (grants AI085524, AI057157, and U19 AI107810) and Public Health of England (formerly, Health Protection Agency, England).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Graham RL, Baric RS. Recombination, reservoirs, and the modular spike: mechanisms of coronavirus cross-species transmission. J Virol 2010; 84:3134–46.
2. Masters PS. The molecular biology of coronaviruses. Adv Virus Res 2006; 66:193–292.
3. Pyrc K, Sims AC, Dijkman R, et al. Culturing the unculturable: human coronavirus HKU1 infects, replicates, and produces progeny virions in human ciliated airway epithelial cell cultures. J Virol 2010; 84: 11255–63.
4. van der Hoek L, Pyrc K, Jebbink MF, et al. Identification of a new human coronavirus. Nat Med 2004; 10:368–73.
5. Rota PA, Oberste MS, Monroe SS, et al. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science 2003; 300:1394–9.
6. Lau SK, Li KS, Tsang AK, et al. Genetic characterization of Betacoronavirus lineage C viruses in bats reveals marked sequence divergence in the spike protein of Pipistrellus bat coronavirus HKU5 in Japanese pipistrelle: implications on the origin of the novel Middle East respiratory syndrome coronavirus. J Virol 2013; 87:8638–50.
7. Bermingham A, Chand MA, Brown CS, et al. Severe respiratory illness caused by a novel coronavirus, in a patient transferred to the United Kingdom from the Middle East, September 2012. Euro Surveill 2012; 17:20290.
8. Huynh J, Li S, Yount B, et al. Evidence supporting a zoonotic origin of human coronavirus strain NL63. J Virol 2012; 86:12816–25.
9. van Boheemen S, de Graaf M, Lauber C, et al. Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. MBio 2012; 3:e00473-12.
10. Müller MA, Raj VS, Muth D, et al. Human coronavirus EMC does not require the SARS-coronavirus receptor and maintains broad replicative capability in mammalian cell lines. MBio 2012; 3:e00515-12.
11. Bolles M, Donaldson E, Baric R. SARS-CoV and emergent coronaviruses: viral determinants of interspecies transmission. Curr Opin Virol 2011; 1:624–34.
12. Perlman S, Zhao J. Human coronavirus EMC is not the same as severe acute respiratory syndrome coronavirus. MBio 2013; 4:e00002-13.
13. Deming D, Sheahan T, Heise M, et al. Vaccine efficacy in senescent mice challenged with recombinant SARS-CoV bearing epidemic and zoonotic spike variants. PLoS Med 2006; 3:e525.
14. Rockx B, Corti D, Donaldson E, et al. Structural basis for potent cross-neutralizing human monoclonal antibody protection against lethal human and zoonotic severe acute respiratory syndrome coronavirus challenge. J Virol 2008; 82:3220–35.
15. Chan RW, Chan MC, Agnihothram S, et al. Tropism and innate immune responses of the novel human betacoronavirus lineage C virus in human ex vivo respiratory organ cultures. J Virol 2013; 87:6604–14.
16. Josset L, Menachery VD, Gralinski LE, et al. Cell host response to infection with novel human coronavirus EMC predicts potential antivirals and important differences with SARS coronavirus. MBio 2013; 4:e00165-13.
17. Sheahan T, Whitmore A, Long K, et al. Successful vaccination strategies that protect aged mice from lethal challenge from influenza virus and heterologous severe acute respiratory syndrome coronavirus. J Virol 2011; 85:217–30.
18. Donaldson EF, Sims AC, Graham RL, Denison MR, Baric RS. Murine hepatitis virus replicase protein nsp10 is a critical regulator of viral RNA synthesis. J Virol 2007; 81:6356–68.
19. Höschler K, Gopal R, Andrews N, et al. Cross-neutralisation of antibodies elicited by an inactivated split-virion influenza A/Vietnam/1194/2004(H5N1) vaccine in healthy adults against H5N1 clade 2 strains. Influenza Other Respir Viruses 2007; 1:199–206.
20. Scobey T, Yount BL, Sims AC, et al. Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus. Proc Natl Acad Sci U S A 2013; 110:16157–62.
21. Sheahan T, Whitmore A, Long K, et al. Successful vaccination strategies that protect aged mice from lethal challenge from influenza virus and heterologous severe acute respiratory syndrome coronavirus. J Virol 2011; 85:217–30.
22. Gierer S, Bertram S, Kaup F, et al. The spike protein of the emerging betacoronavirus EMC uses a novel coronavirus receptor for entry, can be activated by TMPRSS2, and is targeted by neutralizing antibodies. J Virol 2013; 87:5502–11.
23. Becker MM, Graham RL, Donaldson EF, et al. Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice. Proc Natl Acad Sci U S A 2008; 105:19944–9.
24. Chan KH, Chan JF, Tse H, et al. Cross-reactive antibodies in convalescent SARS patients' sera against the emerging novel human coronavirus EMC (2012) by both immunofluorescent and neutralizing antibody tests. J Infect 2013; 67:130–40.

Downloaded from http://jid.oxfordjournals.org/ by guest on August 15, 2016

25. Giménez LG, Rojas J, Rojas A, Mendoza J, Camacho AG. Development of an enzyme-linked immunosorbent assay-based test with a cocktail of nucleocapsid and spike proteins for detection of severe acute respiratory syndrome-associated coronavirus-specific antibody. Clin Vaccine Immunol 2009; 16:241–5.
26. Vega VB, Ruan Y, Liu J, et al. Mutational dynamics of the SARS coronavirus in cell culture and human populations isolated in 2003. BMC Infect Dis 2004; 4:32.
27. Slovin SF, Kehoe M, Durso R, et al. A phase I dose escalation trial of vaccine replicon particles (VRP) expressing prostate-specific membrane antigen (PSMA) in subjects with prostate cancer. Vaccine 2013; 31:943–9.
28. Wecker M, Gilbert P, Russell N, et al. Phase I safety and immunogenicity evaluations of an alphavirus replicon HIV-1 subtype C gag vaccine in healthy HIV-1-uninfected adults. Clin Vaccine Immunol 2012; 19:1651–60.
29. Fillis CA, Calisher CH. Neutralizing antibody responses of humans and mice to vaccination with Venezuelan encephalitis (TC-83) virus. J Clin Microbiol 1979; 10:544–9.
30. Tesh RB, Gajdusek DC, Garruto RM, Cross JH, Rosen L. The distribution and prevalence of group A arbovirus neutralizing antibodies among human populations in Southeast Asia and the Pacific islands. Am J Trop Med Hyg 1975; 24:664–75.
31. Tseng CT, Sbrana E, Iwata-Yoshikawa N, et al. Immunization with SARS coronavirus vaccines leads to pulmonary immunopathology on challenge with the SARS virus. PLoS One 2012; 7:e35421.
32. Bolles M, Deming D, Long K, et al. A double-inactivated severe acute respiratory syndrome coronavirus vaccine provides incomplete protection in mice and induces increased eosinophilic proinflammatory pulmonary response upon challenge. J Virol 2011; 85:12201–15.
33. Ben-Yehuda A, Ehleiter D, Hu AR, Weksler ME. Recombinant vaccinia virus expressing the PR/8 influenza hemagglutinin gene overcomes the impaired immune response and increased susceptibility of old mice to influenza infection. J Infect Dis 1993; 168:352–7.
34. Asanuma H, Hirokawa K, Uchiyama M, et al. Immune responses and protection in different strains of aged mice immunized intranasally with an adjuvant-combined influenza vaccine. Vaccine 2001; 19:3981–9.
35. Zhao J, Legge K, Perlman S. Age-related increases in PGD(2) expression impair respiratory DC migration, resulting in diminished T cell responses upon respiratory virus infection in mice. J Clin Invest 2011; 121:4921–30.
36. Munster VJ, de Wit E, Feldmann H. Pneumonia from human coronavirus in a macaque model. N Engl J Med 2013; 368:1560–2.
37. Fine DL, Roberts BA, Teehee ML, et al. Venezuelan equine encephalitis virus vaccine candidate (V3526) safety, immunogenicity and efficacy in horses. Vaccine 2007; 25:1868–76.
38. Fine DL, Roberts BA, Terpening SJ, Mott J, Vasconcelos D, House RV. Neurovirulence evaluation of Venezuelan equine encephalitis (VEE) vaccine candidate V3526 in nonhuman primates. Vaccine 2008; 26:3497–506.
39. Dea S, Verbeek AJ, Tijssen P. Antigenic and genomic relationships among turkey and bovine enteric coronaviruses. J Virol 1990; 64:3112–8.
40. Hogue BG, King B, Brian DA. Antigenic relationships among proteins of bovine coronavirus, human respiratory coronavirus OC43, and mouse hepatitis coronavirus A59. J Virol 1984; 51:384–8.
41. Cao Z, Liu L, Du L, et al. Potent and persistent antibody responses against the receptor-binding domain of SARS-CoV spike protein in recovered patients. Virol J 2010; 7:299.
42. Memish ZA, Zumla AI, Assiri A. Middle east respiratory syndrome coronavirus infections in health care workers. N Engl J Med 2013; 369:884–6.

Downloaded from http://jid.oxfordjournals.org/ by guest on August 15, 2016

Name

PROOF & WEB (0030713).joboptions

Comment	Size	Modified

ALL PERSONNEL REPORT

Place this form at the end of the signed original copy of the application. Do not duplicate.

GRANT NUMBER

Always list the PD/PI(s). In addition, list all other personnel who participated in the project during the current budget period for at least one person month or more, regardless of the source of compensation (a person month equals approximately 160 hours or 8.3% of annualized effort). Use the following abbreviated categories for describing Role on Project:

- **PD/PI***
- **Co-Investigator**
- **Faculty**
- **Postdoctoral (scholar, fellow, or other postdoctoral position)***
- **Technician**
- **Staff Scientist (doctoral level)**
- **Statistician**
- **Graduate Student (research assistant)**
- **Non-student Research Assistant**
- **Undergraduate Student**
- **High School Student**
- **Consultant**
- **Other (please specify)**

If personnel are supported by a Reentry or Diversity Supplement please indicate such after the Role on Project, using the following abbreviations: RS - Reentry Supplement; DS - Diversity Supplement.

***Commons ID required for any personnel holding this Role on Project and for all individuals supported by a Reenty or Diversity Supplement. The Commons ID will be required in the future for all individuals with a graduate student, or undergraduate role. The Commons ID is strongly encouraged, but not required, for all other Project Personnel.**

Use Cal (calendar), Acad, or Summer to enter months devoted to project.

Commons ID*	Name	Degree(s)	SSN (last 4 digits)	Role on Project	DoB (MM /YY)	Cal	Acad	Summer

Broad Agency Announcement

Pandemic Prevention Platform (P3)

BIOLOGICAL TECHNOLOGIES OFFICE

HR001117S0019

February 6, 2017

TABLE OF CONTENTS

PART I: OVERVIEW INFORMATION....3

PART II: FULL TEXT OF ANNOUNCEMENT....4

1. Funding Opportunity Description....4
1.1. PROGRAM OVERVIEW....4
1.2. PROGRAM STRUCTURE....6
1.3. TECHNICAL AREAS....9
1.4. PROGRAM METRICS....15
2. Award Information....16
2.1. GENERAL AWARD INFORMATION....16
2.2. FUNDAMENTAL RESEARCH....17
3. Eligibility Information....17
3.1. ELIGIBLE APPLICANTS....17
3.2. ORGANIZATIONAL CONFLICTS OF INTEREST....18
3.3. COST SHARING/MATCHING....19
3.4. COLLABORATIVE EFFORTS....20
4. Application and Submission Information....20
4.1. ADDRESS TO REQUEST APPLICATION PACKAGE....20
4.2. CONTENT AND FORM OF APPLICATION SUBMISSION....20
4.3. FUNDING RESTRICTIONS....33
4.4. OTHER SUBMISSION REQUIREMENTS....33
5. Application Review Information....33
5.1. EVALUATION CRITERIA....33
5.2. REVIEW OF PROPOSALS....34
6. Award Administration Information....35
6.1. SELECTION NOTICES....35
6.2. ADMINISTRATIVE AND NATIONAL POLICY REQUIREMENTS....35
6.3. REPORTING....36
6.4. ELECTRONIC SYSTEMS....36
7. Agency Contacts....36
8. Other Information....37
9. APPENDIX 1 – Volume II checklist....39

PART I: OVERVIEW INFORMATION

- **Federal Agency Name** – Defense Advanced Research Projects Agency (DARPA), Biological Technologies Office
- **Funding Opportunity Title –** Pandemic Prevention Platform (P3)
- **Announcement Type –** Initial Announcement
- **Funding Opportunity Number –** HR001117S0019
- **Catalog of Federal Domestic Assistance Numbers (CFDA) – 12.910 Research and Technology Development**
- **Dates**
    - Posting Date: February 6, 2017
    - Proposal Abstract Due Date and Time: March 13, 2017 by 12:00 pm ET
    - Proposal Due Date and Time: May 1, 2017 by 5:00 pm ET
    - Any other relevant date(s): Washington D.C. Proposer's Day, February 22, 2017. San Diego Proposer's Day, March 2, 2017.

- **Concise description of the funding opportunity:** The P3 program will create an integrated platform that delivers pandemic prevention medical countermeasures within 60 days after identification of the pathogen.
- **Anticipated individual awards** - Multiple awards are anticipated.
- **Types of instruments that may be awarded** - Procurement contract, cooperative agreement, or other transaction.
- **Agency contact**
    - Points of Contact
      The BAA Coordinator for this effort may be reached at:
      P3@darpa.mil.
      DARPA/BTO
      ATTN: HR001117S0019
      675 North Randolph Street
      Arlington, VA 22203-2114

PART II: FULL TEXT OF ANNOUNCEMENT

1. Funding Opportunity Description

This publication constitutes a Broad Agency Announcement (BAA) as contemplated in Federal Acquisition Regulation (FAR) 6.102(d)(2) and 35.016 and 2 CFR § 200.203. Any resultant award negotiations will follow all pertinent law and regulation, and any negotiations and/or awards for procurement contracts will use procedures under FAR 15.4, Contract Pricing, as specified in the BAA.

DARPA is soliciting innovative research proposals to develop an end-to-end platform capability for preventing a pandemic threat in <60 days. Recent advances in medical countermeasures have formed a strong foundation, enabling the creation of a true end-to-end pandemic prevention platform. However, experience gained from conventional responses to emerging infectious diseases (MERS, SARS, dengue, chikungunya, Ebola) has demonstrated that significant bottlenecks hinder the rapid response to an emerging infectious threat[1]. Current challenges include the ability to rapidly produce virus needed to test and evaluate therapies, to obtain high potency antibodies within the first weeks of an outbreak, or to scale delivery methods into humans to produce protective levels inside the patient.

1.1. PROGRAM OVERVIEW

Eliminating pandemic outbreaks and mitigating the impact of a potential high threat biological agent release are national security priorities. The U.S. military supports U.S. Government responses to public health emergencies such as Ebola, which can cause regional destabilization and spread through global travel. Warfighters must also operate in regions where diseases like chikungunya and dengue are endemic, and even seemingly mild challenges like seasonal influenza affect force readiness. In addition to these naturally occurring threats, terrorists and other potential adversaries have a growing palette of biological tools to engineer new biological threats. The threat of infectious agents on U.S. and global national security can be mitigated if the Department of Defense (DoD) has the capability to rapidly deploy and impart near-immediate immunity to military personnel and civilian populations for known and newly emerging pathogens.

Existing capabilities to respond to an outbreak and develop vaccines and therapeutics often take years or even decades to achieve results. The R&D process for identifying the protective antigens can take months for a simple variant of a known infectious agent, such as seasonal influenza, and years to decades for a newly discovered agent. In many cases, even decades of research have not led to the development of licensed vaccines for known infections. Further, manufacture of traditional vaccine products typically takes an additional six to nine months. Finally, even when effective vaccines are readily available, immunity in humans can take weeks

[1] Letter Report on Action Needed to Protect against Biological Attack; President's Council of Advisors on Science and Technology – Executive Office of the President. November 2016; https://www.whitehouse.gov/sites/default/files/microsites/ostp/PCAST/pcast_biodefense_letter_report_final.pdf

or months to establish. Recent examples of public health emergencies have demonstrated a national and global inability to develop effective preventive or therapeutic solutions in a relevant timescale when an infectious threat emerges. Reviews of recent outbreaks have repeatedly highlighted this capability gap; the significant delay in deployment of solutions during the West Africa Ebola outbreak is a recent example.

The goal of the Pandemic Prevention Platform (P3) program is to develop an integrated capability to deliver pandemic prevention countermeasures to humans in <60 days. The P3 program aims to revolutionize outbreak response capabilities to allow rapid discovery, characterization, production, and testing of efficacious medical countermeasures. **The P3 program aims to innovate in the following areas:**

1. Generation of virus stock (including viral unknowns).
2. Rapid evolution of antibody candidates.
3. Gene-encoded antibody delivery methods.

Foundational DARPA research investments in gene-encoded antibodies have demonstrated the feasibility and impact of this therapy for the rapid protection of a large population against emerging infectious disease threats. Gene-encoded antibodies have significant advantages over antibodies produced in mammalian cell lines because of their shortened timeline to manufacture and prolonged effect. The increased stability of gene-encoded products further strengthens readiness through improved robustness that enables stockpiling for extended periods of time and distribution in remote areas without the need for cold chain. Further, cutting-edge work in immune engineering can enable construction of critical human immune system components external to the body, thereby enabling higher throughput evolution and screening of antibody candidates. Collectively, these advances and others in the fields of molecular biology, immunology, and drug delivery strengthen the genesis of a functional pandemic prevention platform. DARPA investments that accelerate the early phases of medical countermeasure development will enable the US Government and DoD to effectively respond to public health emergencies due to infectious threats, thereby decreasing the likelihood that an outbreak becomes a pandemic.

Performer teams are required to functionally integrate the key innovations above, along with established technologies for finding antibodies and manufacturing medical countermeasures, into an end-to-end capability platform such that a product is available for human use in <60 days from the time of pathogen identification. The integrated platform must be adaptable to any virus, including unidentified agents. By functionally integrating innovative technologies, the response window will be reduced to an epidemiologically relevant timescale of <60 days. The proposed approach must be capable of delivering a prophylactic countermeasure to **prevent or halt** the spread of an infectious outbreak.

Due to significant advances in identifying antibody products for use in gene-encoded medical countermeasure, it is anticipated that the proposed approach will utilize an antibody format (bi- or multi-specific, single chain or full length, etc.). Alternatives to antibody approaches using biologics will be considered and may include, but are not limited to, proteins/peptides (natural

or non-natural) and oligonucleotides. While these approaches are appropriate, the time and performance metrics defined in Table 1 must be met, and additional metrics should be proposed to meet the unique features of the alternative technologies.

1.2. PROGRAM STRUCTURE

The P3 program will be a four-year effort aimed at developing an integrated platform that produces an effective medical countermeasure, ready for administration to patients, in <60 days after pathogen identification.

The platform will incorporate the following five objectives:

1. Grow virus.
2. Identify an antibody or biologic product that targets the virus for use in medical countermeasure.
3. Evolve the antibody or biologic product for increased potency.
4. Clinical grade product manufacturing.
5. Deliver the product to patients to induce protection in < 3 days following administration.

Previous DARPA investments through the Autonomous Diagnostics to Enable Prevention and Therapeutics (ADEPT) program have resulted in significant scientific and technical progress towards the ability to identify an antibody (Objective 2) and manufacturing a genetic construct (Objective 4) for the in-person production of an antibody. The three remaining objectives (Objectives 1, 3, and 5) will require significant technical innovation to make them suitable for a pandemic platform capable of a response in <60 days. These objectives represent the technical areas discussed in Section 1.3. Performer teams must propose an approach that addresses all three technical areas (TAs) while also integrating Objectives 2 and 4 into a functional response platform (antibody/biologic identification, development, and manufacturing). At the end of the four-year effort, the performer teams must maintain the platform for use in future pandemic response.

The proposed integrated platform must represent a prophylactic approach that will prevent or halt the spread of an outbreak as opposed to an approach that will primarily treat infected individuals, although countermeasures with both prophylactic and therapeutic functions are appropriate. The intent of the P3 program is not to fund significant investment in research into novel manufacturing technologies; therefore, proposers may want to consider pre-existing manufacturing technology. The proposing teams should have manufacturing capacity, or include subcontractors or contract manufacturing organizations capable of producing clinical (Good Manufacturing Practices) grade material for both first-in-human studies as well as the potential for scaled-up manufacture. To achieve all of the goals of the integrated platform, it is anticipated that proposer teams will consist of several members led by a prime contractor integrating team. The integrating prime contractor may choose to propose several solutions or teams for the initial phase of each technical area as a risk mitigation strategy. While this is

allowable within this program, the prime contractor must ensure that the effort still results in a functionally integrated platform.

The program timeline (Figure 1) is outlined below:

Platform development (months 1-30)

Platform development will focus on the technical and innovative development of each of the TAs. The performers will be required to meet specific metrics associated with each TA as outlined below in Section 1.3. Fulfillment of the metrics for each TA can occur independently of each other, but the performers will be required to test the entire end-to-end platform in capability demonstrations beginning in month 12.

Capability demonstrations on known pathogen and platform integration (months 12-40; overlapping with platform development)

As the P3 program requires functional platform integration, capability demonstrations (demos) will focus on demonstrating full end-to-end functionality and complete integration of the entire platform. To ensure the ability to produce a quality product with a regulatory path using this platform**, a clinical phase I safety trial is required during the course of the program.** The capability demonstration portion of the program will overlap with platform development to address the end-to-end capability throughout the TA innovation development phase. As part of the demos, the performers must demonstrate the end-to-end ability to complete the five objectives[2] and the ability to complete a Phase I clinical safety trial. Only one such trial will be funded per performer team, and while capability demonstrations are required before month 24 (Figure 1), clinical trial completion is not required until the end of the program.

A series of capability demonstrations of escalating difficulty will test platform functionality at months 12, 24, and 36 (Figure 1). Two of these capability demonstrations will utilize performer-defined pathogens. It is strongly encouraged that performer-selected pathogens in capability demonstrations 1 or 2 are of commercial interest to the performer group for use in the Phase I clinical safety trial. All proposals are required to make a compelling case for both pandemic potential and commercial interest of each pathogen selected for the capability demonstrations.

The first capability demonstration will occur at the end of month 12 of the program and will give performer groups the opportunity to test and validate an 'at-the-ready' pandemic prevention platform by integrating innovation areas (TA1-3) with existing technologies. Proposers should attempt to deliver a countermeasure tested in animals within 60 days. However, additional time (up to 90 days) will be allotted for the first demonstration to evaluate the progress of proposed approaches and gain an understanding of successes and bottlenecks.

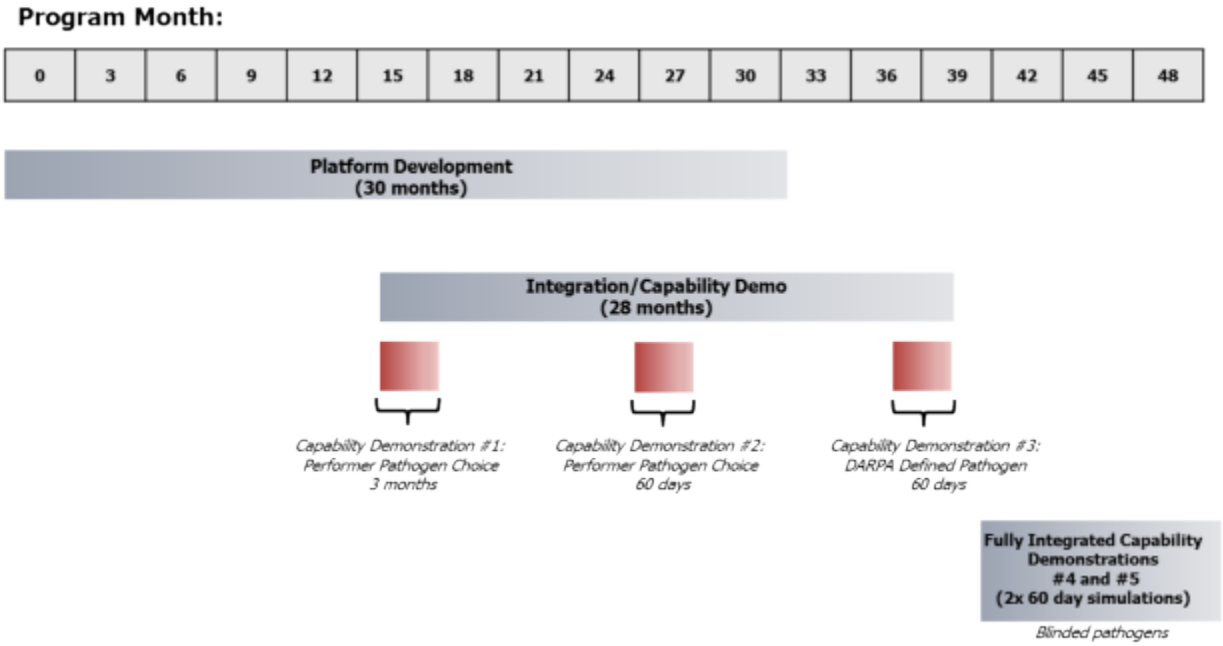
[2] 1. Grow virus
2. Identify an antibody or biologic product that targets the virus for use in medical countermeasure
3. Evolve the antibody or biologic product for increased potency
4. Clinical grade product manufacturing
5. Deliver the product to patients to induce protection in <3 days following administration

At approximately 24 months, performers will have 60 days to conduct a second capability demonstration with a second pathogen. Performer teams may elect to conduct the first two capability demonstrations using the same pathogen, or may choose two different pathogens. Finally, at 36 months, performers will conduct a third 60-day capability demonstration with a DARPA selected pathogen. The specific biosafety concerns for the third capability demonstration will be shared with performer teams at least 2 months in advance of test start date. Thus, performers will have at least a 60-day notice of the biosafety concerns of the pathogen to be tested, and 60 days to complete the capability demonstration. At least one of the capability demonstrations must demonstrate proof-of-concept GMP manufacturing capabilities of the product. The production of 20,000 doses will not be required during the course of the program but proposers must demonstrate the ability to scale proof-of-concept manufacture up to 20,000 doses during the platform development phase of the program (months 1-30).

Final capability demonstrations on blinded pathogen (months 40-48)

The final demonstrations of the P3 program will focus on demonstrating full end-to-end platform integration with blinded pathogens to simulate "real-world" events. There will be two capability demonstrations as part of the final demos. These capability demonstrations will each encompass 60 days and intend to demonstrate the end-to-end capability on blinded targets as defined by DARPA. Performers will be made aware of any biosafety concerns surrounding the pathogen greater than 12 months prior to the initiation of the demonstrations.

Figure 1. Program Timeline



1.3. TECHNICAL AREAS

Based on achievements and lessons learned in previous countermeasure development efforts (DARPA and across the scientific community), three Technical Areas (TA) have been identified in which specific investments may lead to breakthroughs for countermeasure development. These Technical Areas are:

1. On-demand platform to grow virus.
2. System to evolve antibodies: rapid creation of highly potent medical countermeasures.
3. Delivery of medical countermeasure(s): reproducible effects which lead to protective levels of product.

Functional integration of these technical areas along with antibody discovery and manufacturing is essential to achieving a medical countermeasure ready for first-in-human studies in <60 days.

TA1: On-demand platform to grow virus

A critical capability gap for rapidly addressing an emerging pathogen is growing sufficient quantities of that virus for initial testing. Specifically, a certain amount of virus is required (1) as 'bait' to pull antibody from complex mixture (serum) for discovery; (2) for testing the utility of gene-encoded antibodies in animal models; and (3) for running efficacy tests on selected antibodies. The effort to grow meaningful quantities of virus for testing is further complicated for previously unknown emerging viral threats; therefore, the growth platform developed in TA1 must be immediately adaptable, including for unidentified agents. Performers may opt to pursue various approaches including, but not limited to:

- Innovative ways to express virus from cell platforms through high-yield transfection strategies of rapidly generated recombinant genetic systems
- Engineering currently available cell types to grow virus
- Automating and optimizing the process of identifying a viral culture system, which is currently a manual and laborious process

Table 1 outlines metrics for this technical area.

Proposers must demonstrate the ability to achieve the goals and metrics defined in Table 1 (TA1) with five viruses. **The viruses must include at least one RNA virus, one DNA virus, and represent both (+)-sense and (-)-sense single stranded viral genomes.**

Proposers must provide the following in the proposal:

- The choice of study pathogens, demonstrating relevance to National Security and DoD force health protection
- The proposed methods for validation and completion for TA1, describing an adaptable platform able to grow a variety of different pathogens including unknown isolates
- The proposed transition plan describing the product/data pipeline from initial isolate input to final product deliverable to TA2/3 innovation teams

- The ability of the TA1 innovation, as part of the end-to-end platform, to address unknown pathogens

TA2: System to evolve antibodies

Current response capabilities are limited by their inability to rapidly identify highly potent and protective antibodies. Existing strategies have relied on obtaining antibodies from recently recovered patients. However, the body utilizes a broad polyclonal response targeting a number of distinct epitopes, sometimes of moderate potency, to eliminate the pathogen within days to weeks of initial infection. Conversely, antibody therapies are typically comprised of either a monoclonal or a minimal polyclonal mixture, thereby necessitating a stringent requirement for high potency. Recent experiences with Ebola have shown that months to years of antibody maturation inside the human body may be required to develop high-potency antibodies that are viable targets for therapy development. Innovation in TA2 is required to develop a platform technology for the rapid development of highly potent antibodies. It is anticipated that an *in vitro* antibody maturation platform may be required to achieve this goal; however, any approach meeting the time and quality metrics in Table 1 are encouraged. *In vitro* systems have the advantage of not being limited by the natural 'brakes' in the human body which slow the maturation process in order to prevent formation of autoimmunity or prolonged immune mediated pathology. The antibody evolution strategies should couple seamlessly with antibody discovery platforms using new technology or currently existing discovery platforms.

Potential technical innovations in the development of an *in vitro* antibody maturation platform include, but are not limited to:

- *In vitro* artificial immune system
- *In vitro* affinity maturation coupled to discovery technologies
- Computational approaches assisting in affinity maturation

Performers must demonstrate the ability to achieve the goals and metrics defined in TA2 (Table 1) by identifying and evolving highly potent antibodies against five viruses, representing at least one RNA virus, one DNA virus, and represent both (+)-sense and (-)-sense single stranded viral genomes. The antibody candidates generated can target some or all of the viruses selected in TA1.

Note: While the BAA focuses on antibody technology for TA2, proposals incorporating the use of other approaches will be considered as long as the program metrics defined in Table 1 are met.

Proposers must provide the following in the proposal:

- The choice of study pathogens, demonstrating relevance to National Security and DoD force health protection
- The proposed methods for validation and completion of TA2, demonstrating the ability to increase antibody/therapy potency greater than 100-fold during ex vivo maturation

- The detailed proposed method to define antibody/therapy potency and quantitative metrics of how potency will be measured
- The ability of the TA2 innovation, as part of the end-to-end platform, to address unknown pathogens
- The ability to integrate established technologies with key innovation areas (*e.g.*, antibody discovery)
- The proposed transition plan describing the product/data pipeline from initial antibody input to final product deliverable to TA3 innovation team

TA3: Deliver medical countermeasure(s)

Consistent countermeasure delivery has been a critical technical challenge for current gene-encoded antibody delivery platforms. The key bottleneck is delivering DNA or RNA such that it crosses the cell membrane for translation into a functional protein. The required metrics, defined in Table 1, must be achieved with a minimum of **five different genetic constructs**. Assessing differences in genetic backbone are appropriate, but they cannot be applied towards the five different genetic constructs metric (*e.g.*, different plasmids encoding the same antibody will be considered one genetic construct). Proposers must demonstrate the platform utility of their genetic constructs, with the ultimate goal of a genetic expression construct (backbone) that results in highly efficient expression in large animals or humans, regardless of the inserted gene of interest and without risking genome integration. Delivery of each of the five constructs must result in serum expression of at least 10 µg/mL in large animals. Proposers are also expected to articulate how their choice of constructs are expected to translate into a safe and effective response in humans as one of these constructs will be utilized in human clinical trials as part of the capability demonstrations. Additionally, protection must be demonstrated in animals (large or small models) for three different pathogens. While survival following lethal challenge is an ideal measure of protection from acute disease, oftentimes, lethal challenge models are not available. Performer teams must define quantitative metrics to determine protection in each animal model proposed. The constructs for expression in large animals and the protection models may be the same constructs. To ensure that the platform will be useful to **prevent or halt** the spread of an infectious outbreak at population scale, the performers must demonstrate the ability to achieve protection within 3 days of administration. Frequent administration of a medical countermeasure during an outbreak presents distribution and response complications; therefore, the number of doses in the final solution must be minimal, ideally a single administration. Finally, additional consideration will be given to proposals that address the use of antibody cocktails to prevent viral escape from a single monoclonal antibody and/or to provide cross-reactive protection against multiple strains/pathogens.

TA3 will encourage novel delivery approaches, including delivery mechanisms involving local electrical techniques (electroporation) or chemical approaches (liposomal or polymeric packaging of nucleic acid). Approaches that carry risk for genomic integration of the gene of interest and/or vector will not be considered; non-permanent, transient expression technologies are encouraged. Proposals are encouraged to utilize delivery methods that could

be most useful during an outbreak, even in austere settings. Although proposals utilizing intravenous administration are not excluded from consideration, proposers should be mindful of the practicality of field deployment of their solutions.

Performers must demonstrate the ability to achieve the goals and metrics defined in TA3 (Table 1) by attaining the specified serum concentration, protection from lethal challenge, and reproducibility in animal models for ultimate use in humans. Therefore, it is required that once the delivery technology is tested and validated in animal models, it will be tested in humans as part of phase I clinical studies.

Note: While the BAA focuses on gene-encoded antibody technology for TA3, proposals incorporating the use of other approaches will be considered as long as the appropriate program metrics defined in Table 1 are met.

Proposers must provide the following in the proposal:

- The proposed methods for validation and completion for TA3, describing an adaptable platform able to support the delivery of a variety of antibody genetic constructs seamlessly
- The detailed proposed method to define antibody/therapy potency and quantitative metrics of how will potency will be measured
- The detailed proposed study design of animal challenge model(s)
- The detailed proposed method to define protection, including quantitative metrics used to determine protection in each animal model proposed

Platform Integration and Testing

To ensure end-to-end functional platform integration, the performer will be required to transition the technological advances from the three TAs into capability demonstrations. A central component of this program will be the demonstration of an end-to-end platform functionality. The first capability demonstration will occur in month 12 of the program. Performer groups will be allotted 90 days to:

- Grow 10^{13} viral pathogen of their choice
- Discover pathogen specific antibodies
- Successfully transition both antibody/therapy and virus to the antibody evolution group
- Evolve antibody/therapy to 100-fold increased potency
- Deliver genetic sequence of highly potent antibody/therapy to the manufacturing group (data)
- Manufacture research grade material for animal studies
- Demonstrate both product delivery and protection from viral challenge in an animal model

At month 24, performers will conduct a capability demonstration with a pathogen of performer choice. At 36 months, performers will conduct a third 60-day capability demonstration with a DARPA selected pathogen. It is required that during one of the capability demonstrations, GMP manufacturing of clinical grade material will be completed for use in a human clinical trial. The proposal must include a discussion of the number of doses to be manufactured during the demonstration, and the time for manufacturing of these doses must fall within the 60-day timeline.

Using the lessons learned in the previous capability demonstrations, performers will conduct two final capability demonstrations with pathogens that are unidentified to the groups to simulate a "real–world" response to an unknown pathogen. The final deliverable will be the ability to produce at a minimum 1000 doses. The platform should be capable to scale-up manufacturing to 20,000 doses of gene-encoded antibody product for Phase I/II human clinical trials in <60 days, with a delivery platform able to achieve protective levels inside the body.

Finally, the proposal must include a discussion of where the capability platform will be located. It is anticipated that the platform will either be centrally located in one facility run by one of the performer teams, or that aspects of the platform will be physically located at different facilities which are integrated through a system of agreements allowing for the facile transfer of material between locations. In the latter case, the proposal must discuss the coordination and management of the decentralized platform.

Proposers must provide the following in the proposal:

- The proposed manufacturing plan, demonstrating the ability to GMP manufacture products in addition to being able to scale-up manufacturing and distribute 20,000 doses
- The proposed transition plan describing the product/data pipeline from receipt of pathogen and antibody input to final product
- The regulatory plan for phase I clinical safety trials

Table 1. Program metrics and milestones

Technical Metrics
TA 1: On-demand platform to grow virus 1. Amplify pathogen to a minimum 10^{13} infectious units (PFU or TCID50) within 72 hours post-inoculation 2. Pathogen genetic drift less than 0.1% after 5 successive culturing (growth) iterations (mutations per base pair per culturing cycle) 3. Progeny virus bio-identical to original isolate in lipid, protein, and carbohydrate content 4. Apply platform to five viruses representing at least one RNA virus, one DNA virus, and both (+)-sense and (-)-sense single stranded viral genomes

Technical Metrics
TA 2: System to evolve antibodies 1. Ready for use in less than 72 hours 2. Demonstrate the ability to couple antibody discovery capability with the antibody evolution capability either through new discovery technologies or physical coupling with existing technologies 3. Support rapid potency maturation improving antibody/therapy greater than 100-fold in under 8 days 4. Apply technology to mature five different antibodies/therapies targeting at a minimum three (3) different pathogens representing at least one RNA virus, one DNA virus, and both (+)-sense and (-)-sense single stranded viral genomes *While it is assumed that the development of antibody therapies may be necessary to achieve a protective countermeasure in <60 days, all innovative approaches that meet the time and quality metrics stated above will be considered.
TA 3: Deliver medical countermeasure(s) 1. Achieve greater than 10 µg/mL serum concentration of target medical countermeasure with peak production in <3 days post-injection in large animals 2. Minimally or low-invasive simple delivery method given at 1 delivery site 3. Demonstrate reproducible production (less than 10% variance in serum concentration) in large animals 4. Demonstrate 100% protection against performer defined pathogen challenge in animal models by day 3 post countermeasure administration 5. Demonstrate longevity of protection lasts for greater than 30 days *While it is assumed that the development of antibody therapies may be necessary to achieve a protective countermeasure in <60 days, all innovative approaches that meet the time and quality metrics stated above will be considered.
Platform Integration: All of the following metrics must be achieved within 60 days and must include full integration with the metrics for TA1-3: 1. Demonstrate the ability to identify a pathogen specific antibody/therapy for rapid affinity maturation in TA2 within 14 days 2. Demonstrate the ability to manufacture research grade genetic constructs for use in animal studies 3. Demonstrate the ability to manufacture clinical grade GMP material for human safety trials – this metric must be met for only one of the phase II demonstrations. The ability to scale-up manufacturing and distribution to 20,000 doses must also be

Technical Metrics
addressed as part of this metric 4. Demonstrate safety and efficacy in animal models and in human clinical trials

[1]Letter Report on Action Needed to Protect against Biological Attack; President's Council of Advisors on Science and Technology – Executive Office of the President. November 2016; https://www.whitehouse.gov/sites/default/files/microsites/ostp/PCAST/pcast_biodefense_letter_report_final.pdf

1.4. PROGRAM METRICS

In order for the Government to evaluate the effectiveness of a proposed solution in achieving the stated program objectives, proposers should note that the Government hereby promulgates the following program metrics that may serve as the basis for determining whether satisfactory progress is being made to warrant continued funding of the program. Although the program metrics are specified (Table 1, Section 1.3), proposers should note that the Government has identified these goals with the intention of bounding the scope of effort, while affording the maximum flexibility, creativity, and innovation in proposing solutions to the stated problem.

2. Award Information

2.1. GENERAL AWARD INFORMATION

Multiple awards are possible. The amount of resources made available under this BAA will depend on the quality of the proposals received and the availability of funds.

The Government reserves the right to select for negotiation all, some, one, or none of the proposals received in response to this solicitation and to make awards without discussions with proposers. The Government also reserves the right to conduct discussions if it is later determined to be necessary. If warranted, portions of resulting awards may be segregated into pre-priced options. Additionally, DARPA reserves the right to accept proposals in their entirety or to select only portions of proposals for award. In the event that DARPA desires to award only portions of a proposal, negotiations may be opened with that proposer. The Government reserves the right to fund proposals in phases with options for continued work, as applicable. The Government reserves the right to request any additional, necessary documentation once it makes the award instrument determination. Such additional information may include but is not limited to Representations and Certifications (see Section 6.B.2.3, "Representations and Certifications"). The Government reserves the right to remove proposers from award consideration should the parties fail to reach agreement on award terms, conditions, and/or cost/price within a reasonable time, and the proposer fails to timely provide requested additional information. Proposals identified for negotiation may result in a procurement contract, cooperative agreement, or other transaction, depending upon the nature of the work proposed, the required degree of interaction between parties, whether or not the research is classified as Fundamental Research, and other factors.

Proposers looking for innovative, commercial-like contractual arrangements are encouraged to consider requesting Other Transactions. To understand the flexibility and options associated with Other Transactions, consult www.darpa.mil/work-with-us/contract-management#OtherTransactions.

In all cases, the Government contracting officer shall have sole discretion to select award instrument type, regardless of instrument type proposed, and to negotiate all instrument terms and conditions with selectees. DARPA will apply publication or other restrictions, as necessary, if it determines that the research resulting from the proposed effort will present a high likelihood of disclosing performance characteristics of military systems or manufacturing technologies that are unique and critical to defense. Any award resulting from such a determination will include a requirement for DARPA permission before publishing any information or results on the program. For more information on publication restrictions, see the section below on Fundamental Research.

2.2. FUNDAMENTAL RESEARCH

It is DoD policy that the publication of products of fundamental research will remain unrestricted to the maximum extent possible. National Security Decision Directive (NSDD) 189 defines fundamental research as follows:

> 'Fundamental research' means basic and applied research in science and engineering, the results of which ordinarily are published and shared broadly within the scientific community, as distinguished from proprietary research and from industrial development, design, production, and product utilization, the results of which ordinarily are restricted for proprietary or national security reasons.

As of the date of publication of this BAA, the Government expects that program goals as described herein either cannot be met by proposers intending to perform fundamental research or the proposed research is anticipated to present a high likelihood of disclosing performance characteristics of military systems or manufacturing technologies that are unique and critical to defense. Therefore, the Government anticipates restrictions on the resultant research that will require the awardee[3] to seek DARPA permission before publishing any information or results relative to the program.

Proposers should indicate in their proposal whether they believe the scope of the research included in their proposal is fundamental or not. While proposers should clearly explain the intended results of their research, the Government shall have sole discretion to select award instrument type and to negotiate all instrument terms and conditions with selectees. Appropriate clauses will be included in resultant awards for non-fundamental research to prescribe publication requirements and other restrictions, as appropriate. This clause can be found at www.darpa.mil/work-with-us/additional-baa.

For certain research projects, it may be possible that although the research being performed by the awardee is restricted research, a subawardee may be conducting fundamental research. In those cases, it is the awardee's responsibility to explain in their proposal why its subawardee's effort is fundamental research

3. Eligibility Information

3.1. ELIGIBLE APPLICANTS

All responsible sources capable of satisfying the Government's needs may submit a proposal that shall be considered by DARPA.

---

[3] As used throughout this BAA, awardee refers to anyone who might receive a prime award from the Government, including recipients of procurement contracts, grants, cooperative agreements, Other Transactions, or any type of award. Subawardee refers to anyone who might receive a subaward from a prime awardee.

3.1.1. Federally Funded Research and Development Centers (FFRDCs) and Government Entities

FFRDCs

FFRDCs are subject to applicable direct competition limitations and cannot propose to this BAA in any capacity unless they meet the following conditions: (1) FFRDCs must clearly demonstrate that the proposed work is not otherwise available from the private sector. (2) FFRDCs must provide a letter on official letterhead from their sponsoring organization citing the specific authority establishing their eligibility to propose to Government solicitations and compete with industry, and their compliance with the associated FFRDC sponsor agreement's terms and conditions. This information is required for FFRDCs proposing to be awardees or subawardees.

Government Entities

Government Entities (e.g., Government/National laboratories, military educational institutions, etc.) are subject to applicable direct competition limitations. Government entities must clearly demonstrate that the work is not otherwise available from the private sector and provide written documentation citing the specific statutory authority and contractual authority, if relevant, establishing their ability to propose to Government solicitations.

Authority and Eligibility

At the present time, DARPA does not consider 15 U.S.C. § 3710a to be sufficient legal authority to show eligibility. While 10 U.S.C.§ 2539b may be the appropriate statutory starting point for some entities, specific supporting regulatory guidance, together with evidence of agency approval, will still be required to fully establish eligibility. DARPA will consider FFRDC and Government entity eligibility submissions on a case-by-case basis; however, the burden to prove eligibility for all team members rests solely with the proposer.

3.1.2. Non-U.S. Organizations

Non-U.S. organizations and/or individuals may participate to the extent that such participants comply with any necessary nondisclosure agreements, security regulations, export control laws, and other governing statutes applicable under the circumstances.

3.2. ORGANIZATIONAL CONFLICTS OF INTEREST

FAR 9.5 Requirements

In accordance with FAR 9.5, proposers are required to identify and disclose all facts relevant to potential OCIs involving the proposer's organization and *any* proposed team member (subawardee, consultant). Under this Section, the proposer is responsible for providing this disclosure with each proposal submitted to the BAA. The disclosure must include the proposer's, and as applicable, proposed team member's OCI mitigation plan. The OCI mitigation plan must include a description of the actions the proposer has taken, or intends to take, to prevent the existence of conflicting roles that might bias the proposer's judgment and to prevent the proposer from having unfair competitive advantage. The OCI mitigation plan will

specifically discuss the disclosed OCI in the context of each of the OCI limitations outlined in FAR 9.505-1 through FAR 9.505-4.

Agency Supplemental OCI Policy

In addition, DARPA has a supplemental OCI policy that prohibits contractors/performers from concurrently providing Scientific Engineering Technical Assistance (SETA), Advisory and Assistance Services (A&AS) or similar support services and being a technical performer. Therefore, as part of the FAR 9.5 disclosure requirement above, a proposer must affirm whether the proposer or *any* proposed team member (subawardee, consultant) is providing SETA, A&AS, or similar support to any DARPA office(s) under: (a) a current award or subaward; or (b) a past award or subaward that ended within one calendar year prior to the proposal's submission date.

If SETA, A&AS, or similar support is being or was provided to any DARPA office(s), the proposal must include:

- The name of the DARPA office receiving the support;
- The prime contract number;
- Identification of proposed team member (subawardee, consultant) providing the support; and
- An OCI mitigation plan in accordance with FAR 9.5.

Government Procedures

In accordance with FAR 9.503, 9.504 and 9.506, the Government will evaluate OCI mitigation plans to avoid, neutralize or mitigate potential OCI issues before award and to determine whether it is in the Government's interest to grant a waiver. The Government will only evaluate OCI mitigation plans for proposals that are determined selectable under the BAA evaluation criteria and funding availability.

The Government may require proposers to provide additional information to assist the Government in evaluating the proposer's OCI mitigation plan.

If the Government determines that a proposer failed to fully disclose an OCI; or failed to provide the affirmation of DARPA support as described above; or failed to reasonably provide additional information requested by the Government to assist in evaluating the proposer's OCI mitigation plan, the Government may reject the proposal and withdraw it from consideration for award.

3.3. COST SHARING/MATCHING

Cost sharing is not required; however, it will be carefully considered where there is an applicable statutory condition relating to the selected funding instrument. Cost sharing is encouraged where there is a reasonable probability of a potential commercial application related to the proposed research and development effort.

For more information on potential cost sharing requirements for Other Transactions for Prototype, see http://www.darpa.mil/work-with-us/contract-management#OtherTransactions

3.4. COLLABORATIVE EFFORTS

Proposer's must have expertise in integration, microbiology, immunology, regulatory, medical product development and the GMP manufacturing of medical countermeasures for use in human subjects.

4. Application and Submission Information

4.1. ADDRESS TO REQUEST APPLICATION PACKAGE

This announcement, any attachments, and any references to external websites herein constitute the total solicitation. If proposers cannot access the referenced material posted in the announcement found at http://www.darpa.mil, contact the administrative contact listed herein.

4.2. CONTENT AND FORM OF APPLICATION SUBMISSION

All submissions, including abstracts and proposals must be written in English with type not smaller than 12-point font. Smaller font may be used for figures, tables, and charts. Copies of all documents submitted must be clearly labeled with the DARPA BAA number, proposer organization, and proposal title/proposal short title.

4.2.1. Proposal Abstract Format

Proposers are strongly encouraged to submit an abstract in advance of a proposal to minimize effort and reduce the potential expense of preparing an out of scope proposal. The abstract is a concise version of the proposal comprising a maximum of **8** pages including all figures, tables, and charts. The (optional) submission letter is not included in the page count. All pages shall be formatted for printing on 8-1/2 by 11-inch paper with font size not smaller than 12-point and standard 1-inch margins. Smaller font sizes may be used for figures, tables, and charts.

Submissions must be written in English.

The page limit does not include:

1. Official submission letter (optional)
2. Cover Sheet
3. Resumes
4. Bibliography (optional)

Abstracts must include the following components:

A. Cover Sheet (does not count towards page limit): Include the administrative and technical points of contact (name, address, phone, fax, email, lead organization). Also

include the BAA number, title of the proposed project, primary subcontractors, estimated cost, duration of the project, and the label "ABSTRACT."

B. Goals and Impact: Clearly describe what is being proposed and what difference it will make (qualitatively and quantitatively), including brief answers to the following questions:

1. What is the proposed work attempting to accomplish or do?
2. How is it done today? And what are the limitations?
3. What is innovative in your approach and how does it compare to SOA?
4. What are the key technical challenges in your approach and how do you plan to overcome these?
5. Who will care and what will the impact be if you are successful?
6. How much will it cost and how long will it take?

C. Technical Plan: All three technical areas (TA's) much be addressed in the proposal. Summarize your plan for accomplishing the program goals for all three of the technical areas. Outline and address all technical challenges inherent in the approach and possible solutions for overcoming potential problems. This section should provide appropriate specific milestones (quantitative, if possible) at intermediate stages of the project to demonstrate progress, and a brief plan for accomplishment of the milestones.

D. Management and Capabilities: It is expected that proposals will involve multidisciplinary teams covering all of the technical areas. These teams must be fully functionally integrated. Provide a brief summary of expertise of the team, including subcontractors and key personnel and how each member will be integrated into a fully functional team.

A principal investigator for the project must be identified, and a description of the team's organization. Teams are encouraged to identify a program manager to serve as a primary point of contact to communicate with the DARPA program manager and Contracting Officer Representative, coordinate effort across performer teams, organize regular performer meetings or discussions, and ensure timely completion of milestones and deliverables.

Include a description of the team's organization including roles and responsibilities. Describe the organizational experience in this area, existing intellectual property required to complete the project, and any specialized facilities to be used as part of the project. List Government-furnished materials or data assumed to be available. If desired, include a brief bibliography with links to relevant papers, reports, or resumes of key performers.

E. Cost and Schedule: Provide a cost estimate for resources over the proposed timeline of the project, broken down by major cost items (e.g. labor, materials, equipment). Include cost estimates for each potential subcontractor (may be a rough or of magnitude).

G. CVs/Resumes: Include resumes of key team members (resumes do not count toward page limit).

H. Bibliography: If desired include a brief bibliography not to exceed two pages.

4.2.2. Proposal Format

All full proposals must be in the format given below. Proposals shall consist of two volumes: 1) **Volume I, Technical and Management Proposal**, and 2) **Volume II, Cost Proposal.** All pages shall be printed on 8-1/2 by 11-inch paper with type not smaller than 12-point and standard 1-inch margins. Smaller font may be used for figures, tables and charts. The page limitation for full proposals includes all figures, tables, and charts. Volume I, Technical and Management Proposal, may include an attached bibliography of relevant technical papers or research notes (published and unpublished) which document the technical ideas and approach upon which the proposal is based. Copies of not more than three (3) relevant papers may be included with the submission. The bibliography and attached papers are not included in the page counts given below. The submission of other supporting materials along with the proposals is strongly discouraged and will not be considered for review. **The maximum page count for Volume 1 is 40 pages**. A submission letter is optional and is not included in the page count. Volume I should include the following components:

NOTE: Non-conforming submissions that do not follow the instructions herein may be rejected without further review.

a. Volume I, Technical and Management Proposal

Section I. Administrative

A. Cover Sheet (LABELED "PROPOSAL: VOLUME I"):

1. BAA number (HR001117S0019);
2. Technical area;
3. Lead organization submitting proposal (prime contractor);
4. Type of organization, selected from among the following categories: "LARGE BUSINESS," "SMALL DISADVANTAGED BUSINESS," "OTHER SMALL BUSINESS," "HBCU," "MI," "OTHER EDUCATIONAL," OR "OTHER NONPROFIT";
5. Proposer's reference number (if any);
6. Other team members (if applicable) and type of business for each;
7. Proposal title;

8. Technical point of contact (Program Manager or Principle Investigator) to include: salutation, last name, first name, street address, city, state, zip code, telephone, fax, e-mail;
9. Administrative point of contact (Contracting Officer) to include: salutation, last name, first name, street address, city, state, zip code, telephone, fax, e-mail;
10. Award instrument requested: cost-plus-fixed-free (CPFF), cost-contract-no fee, firm-fixed-price (FFP), other type of procurement contract (specify), cooperative agreement, or other transaction;
11. Place(s) and period(s) of performance;
12. Proposal validity period;
13. Total funds requested from DARPA, and the amount of cost share (if any); AND
14. Date proposal was submitted.

Information on award instruments is available at http://www.darpa.mil/work-with-us/contract-management.

B. Official Transmittal Letter.

Section II. Detailed Proposal Information

A. Executive Summary: Provide a synopsis of the proposed project, including answers to the following questions:

- What is the proposed work attempting to accomplish or do?
- How is it done today, and what are the limitations?
- What is innovative in your approach?
- What are the key technical challenges in your approach and how do you plan to overcome these?
- Who or what will be affected and what will be the impact if the work is successful?
- How much will it cost, and how long will it take?

B. Goals and Impact: Clearly describe what the team is trying to achieve and the difference it will make (qualitatively and quantitatively) if successful. Describe the innovative aspects of the project in the context of existing capabilities and approaches, clearly delineating the uniqueness and benefits of this project in the context of the state of the art, alternative approaches, and other projects from the past and present. Describe how the proposed project is revolutionary and how it significantly rises above the current state of the art. Describe the deliverables associated with the proposed project and any plans to commercialize the technology, transition it to a customer, or further the work.

C. Technical Plan: Outline and address technical challenges inherent in the approach and possible solutions for overcoming potential problems. This section should provide appropriate measurable milestones (quantitative if possible) at intermediate stages of the program to demonstrate progress, and a plan for achieving the milestones. The technical plan should demonstrate a deep understanding of the technical challenges and present a credible (even if risky) plan to achieve the program goal. Discuss mitigation of technical risk.

Proposals should also address the following specific criteria:

1. The choice of study pathogens, demonstrating relevance to National Security and DoD force health protection.
2. The proposed methods for validation and completion for TA1, describing an adaptable platform able to grow a variety of different pathogens including unknown isolates.
3. The proposed methods for validation and completion of TA2, demonstrating the ability to increase antibody potency greater than 100-fold during *ex vivo* maturation.
4. The detailed proposed method to define antibody/therapy potency and quantitative metrics of how will potency will be measured.
5. The detailed proposed study design of animal challenge model(s)
6. The detailed proposed method to define protection, including quantitative metrics used to determine protection in each animal model proposed.
7. The proposed manufacturing plan, demonstrating the ability to GMP manufacture products in addition to being able to scale-up manufacturing and distribute 20,000 doses.
8. The ability of the end-to-end platform to address unknown pathogens.
9. The ability to integrate established technologies with key innovation areas.
10. The proposed transition plan describing the product/data pipeline from initial input to final product.

D. Management Plan: Provide a summary of expertise of the team, including any subcontractors, and key personnel who will be doing the work. Resumes count against the proposal page count. Identify a principal investigator for the project. Provide a clear description of the team's organization including an organization chart that includes, as applicable: the programmatic relationship of team members; the unique capabilities of team members; the task responsibilities of team members, the teaming strategy among the team members; and key personnel with the amount of effort to be expended by each person during each year. Provide a detailed plan for coordination including explicit guidelines for interaction among

collaborators/subcontractors of the proposed effort. Include risk management approaches. Describe any formal teaming agreements that are required to execute this program.

E. Capabilities: Describe organizational experience in relevant subject area(s), existing intellectual property, specialized facilities, and any Government-furnished materials or information. Discuss any work in closely related research areas and previous accomplishments.

F. Statement of Work (SOW): The SOW should provide a detailed task breakdown, citing specific tasks and their connection to the interim milestones and program metrics. Each phase of the program should be separately defined. The SOW must not include proprietary information.

For each task/subtask, provide:

- A detailed description of the approach to be taken to accomplish each defined task/subtask.
- Identification of the primary organization responsible for task execution (prime contractor, subcontractor(s), consultant(s), by name).
- A measurable milestone, i.e., a deliverable, demonstration, or other event/activity that marks task completion. Include quantitative metrics.
- A definition of all deliverables (e.g., data, reports, software) to be provided to the Government in support of the proposed tasks/subtasks.

G. Schedule and Milestones: Provide a detailed schedule showing tasks (task name, duration, work breakdown structure element as applicable, performing organization), milestones, and the interrelationships among tasks. The task structure must be consistent with that in the SOW. Measurable milestones should be clearly articulated and defined in time relative to the start of the project.

<u>Section III. Additional Information</u> (Note: Does not count towards page limit)

A brief bibliography of relevant technical papers and research notes (published and unpublished) which document the technical ideas upon which the proposal is based. Copies of not more than three (3) relevant papers can be included in the submission.

a. Volume II, Cost Management Proposal

Cover Sheet (LABELED “PROPOSAL: VOLUME II”):

1. BAA number;
2. Technical area;

3. Lead Organization Submitting proposal;
4. Type of organization, selected among the following categories: "LARGE BUSINESS", "SMALL DISADVANTAGED BUSINESS", "OTHER SMALL BUSINESS", "HBCU", "MI", "OTHER EDUCATIONAL", OR "OTHER NONPROFIT";
5. Proposer's reference number (if any);
6. Other team members (if applicable) and type of business for each;
7. Proposal title;
8. Technical point of contact (Program Manager or Principal Investigator) to include: salutation, last name, first name, street address, city, state, zip code, telephone, fax (if available), electronic mail (if available);
9. Administrative point of contact (Contracting Officer) to include: salutation, last name, first name, street address, city, state, zip code, telephone, fax (if available), and electronic mail (if available);
10. Award instrument requested: cost-plus-fixed-free (CPFF), cost-contract-no fee, firm-fixed-price (FFP), other type of procurement contract (*specify*), cooperative agreement, or other transaction;
11. Place(s) and period(s) of performance;
12. Total proposed cost separated by basic award and option(s) (if any);
13. Name, address, and telephone number of the proposer's cognizant Defense Contract Management Agency (DCMA) administration office (*if known)*;
14. Name, address, and telephone number of the proposer's cognizant Defense Contract Audit Agency (DCAA) audit office (*if known*);
15. Date proposal was prepared;
16. DUNS number (http://www.dnb.com/get-a-duns-number.html);
17. Taxpayer ID number (https://www.irs.gov/Individuals/International-Taxpayers/Taxpayer-Identification-Numbers-TIN);
18. CAGE code (https://www.dlis.dla.mil/bincs/FAQ.aspx);
19. Proposal validity period

Note that non-conforming proposals may be rejected without review.

Proposers that do not have a Cost Accounting Standards (CAS) complaint accounting system considered adequate for determining accurate costs that are negotiating a cost- type procurement contract must complete an SF 1408. For more information on CAS compliance, see http://www.dcaa.mil/cas.html. To facilitate this process, proposers should complete the SF 1408 found at http://www.gsa.gov/portal/forms/download/115778 and submit the completed form with the proposal. To complete the form, check the boxes on the second page, then provide a narrative explanation of your accounting system to supplement the checklist on page one. For more information, see (http://www.dcaa.mil/preaward_accounting_system_adequacy_checklist.html).

The Government strongly encourages that the proposer provide a detailed cost breakdown to include:

(1) Total program cost broken down by major cost items to include:
   i. Direct Labor – Including individual labor categories with associated labor hours and direct labor rates. If selected for award, be prepared to submit supporting documentation to justify labor rates. (i.e., screenshots of HR databases, comparison to NIH or other web-based salary database);
   ii. Consultants – If consultants are to be used, proposer must provide a copy of the consultant's proposed SOW as well as a signed consultant agreement or other document which verifies the proposed loaded daily / hourly rate, hours and any other proposed consultant costs (e.g., travel);
   iii. Indirect Costs – Including Fringe Benefits, Overhead, General and Administrative Expense, Cost of Money, Fee, etc. (must show base amount and rate), if available, provide current Forward Pricing Rate Agreement or Forward Pricing Rate Proposal. If not available, provide 2 years historical data to include pool and expense costs used to generate the rates. For academia, provide DHHS or ONR negotiated rate package or, if calculated by other than a rate, provide University documentation identifying G&A and fringe costs by position;
   iv. Travel – Provide the purpose of the trip, number of trips, number of days per trip, departure and arrival destinations, number of people, estimated rental car and airfare costs, and prevailing per diem rates as determined by gsa.gov, etc.; Quotes must be supported by screenshots from travel websites;
   v. Other Direct Costs – Itemized with costs including tuition remission, animal per diem rates, health insurance/fee; back-up documentation is to be submitted to support proposed costs;
   vi. Equipment Purchases – Itemization with individual and total costs, including quantities, unit prices, proposed vendors (if known), and the basis of estimate (e.g., quotes, prior purchases, catalog price lists, etc.); any item that exceeds $5,000 must be supported with back-up documentation such as a copy of catalog price lists or quotes prior to purchase (NOTE: For equipment purchases, include a letter stating why the proposer cannot provide the requested resources from its own funding), and;
   vii. Materials – Itemization with costs, including quantities, unit prices, proposed vendors (if known), and the basis of estimate (e.g., quotes, prior purchases, catalog price lists, etc.); any item that exceeds $5,000 must be supported with back-up documentation such as a copy of catalog price lists or quotes prior to purchase.
(2) A summary of major program tasks by Government Fiscal Year (GFY = Oct 1 – Sep 30)
(3) A summary of total program costs by phase and task;
(4) A summary of projected funding requirements by month;
(5) An itemization of any information technology (IT) purchase (including a letter stating why the proposer cannot provide the requested resources from its own funding), as defined in FAR Part 2.101;

(6) An itemization of Subcontracts. **All subcontractor cost proposal documentation must be prepared at the same level of detail as that required of the prime.** Subcontractor proposals should include Interdivisional Work Transfer Agreements (IWTA) or evidence of similar arrangements (an IWTA is an agreement between multiple divisions of the same organization);
(7) The source, nature, and amount of any industry cost-sharing. Where the effort consists of multiple portions which could reasonably be partitioned for purposes of funding, these should be identified as options with separate cost estimates for each;
(8) Identification of pricing assumptions of which may require incorporation into the resulting award instrument (e.g., use of Government Furnished Property/Facilities/Information, access to Government Subject Matter Expert/s, etc.);
(9) Any Forward Pricing Rate Agreement, DHHS rate agreement, other such approved rate information, or such documentation that may assist in expediting negotiations (if available); and
(10) Proposers with a Government acceptable accounting system who are proposing a cost-type contract, must submit the DCAA document approving the cost accounting system.

4.2.3. Additional Proposal Information

Proprietary Markings

Proposers are responsible for clearly identifying proprietary information. Submissions containing proprietary information must have the cover page and each page containing such information clearly marked with a label such as "Proprietary" or "Company Proprietary." NOTE: "Confidential" is a classification marking used to control the dissemination of U.S. Government National Security Information as dictated in Executive Order 13526 and should not be used to identify proprietary business information.

Unclassified Submissions

DARPA anticipates that submissions received under this BAA will be unclassified. However, should a proposer wish to submit classified information, an *unclassified* email must be sent to the BAA mailbox requesting submission instructions from the Technical Office PSO. If a determination is made that the award instrument may result in access to classified information, a SCG and/or DD Form 254 will be issued by DARPA and attached as part of the award.

Human Research Subjects/Animal Use

Proposers that anticipate involving Human Research Subjects or Animal Use must comply with the approval procedures detailed at www.darpa.mil/work-with-us/additional-baa.

Human Subjects Research

All research selected for funding involving human subjects, to include use of human biological specimens and human data, must comply with the federal regulations for human subjects

protection. Further, research involving human subjects that is conducted or supported by the DoD must comply with 32 CFR 219, *Protection of Human Subjects* (and DoD Instruction 3216.02, *Protection of Human Subjects and Adherence to Ethical Standards in DoD-Supported Research* (http://www.dtic.mil/whs/directives/corres/pdf/321602p.pdf).

Institutions awarded funding for research involving human subjects must provide documentation of a current Assurance of Compliance with federal regulations for human subjects protection, such as a Department of Health and Human Services, Office of Human Research Protection Federal Wide Assurance (http://www.hhs.gov/ohrp). All institutions engaged in human subjects research, to include subcontractors, must also hold a valid assurance. In addition, all personnel involved in human subjects research must provide documentation of completion of human subjects research training.

For all proposed research that will involve human subjects in the first year or phase of the project, the institution must provide evidence of or a plan for review by an Institutional Review Board (IRB) upon final proposal submission to DARPA as part of their proposal, prior to being selected for funding. The IRB conducting the review must be the IRB identified on the institution's Assurance of Compliance with human subjects protection regulations. The protocol, separate from the proposal, must include a detailed description of the research plan, study population, risks and benefits of study participation, recruitment and consent process, data collection, and data analysis. It is recommended that you consult the designated IRB for guidance on writing the protocol. The informed consent document must comply with federal regulations (32 CFR 219.116). A valid Assurance of Compliance with human subjects protection regulations along with evidence of completion of appropriate human subjects research training by all investigators and personnel involved with human subjects research should accompany the protocol for review by the IRB.

In addition to a local IRB approval, a headquarters-level human subjects administrative review and approval is required for all research conducted or supported by the DoD. The Army, Navy, or Air Force office responsible for managing the award can provide guidance and information about their component's headquarters-level review process. Note that confirmation of a current Assurance of Compliance with human subjects protection regulations and appropriate human subjects research training is required before headquarters-level approval can be issued.

The time required to complete the IRB review/approval process varies depending on the complexity of the research and the level of risk involved with the study. The IRB approval process can last between one and three months, followed by a DoD review that could last between three and six months. Ample time should be allotted to complete the approval process. DoD/DARPA funding cannot be used toward human subjects research until ALL approvals are granted.

Animal Use

Award recipients performing research, experimentation, or testing involving the use of animals shall comply with the rules on animal acquisition, transport, care, handling, and use as outlined in: (i) 9 CFR parts 1-4, Department of Agriculture rules that implement the Animal Welfare Act of 1966, as amended, (7 U.S.C. § 2131-2159); (ii) National Institutes of Health Publication No. 86-23, "Guide for the Care and Use of Laboratory Animals" (8th Edition); and (iii) DoD Instruction 3216.01, “Use of Animals in DoD Programs.”

For projects anticipating animal use, proposals should briefly describe plans for Institutional Animal Care and Use Committee (IACUC) review and approval. Animal studies in the program will be expected to comply with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, available at http://grants.nih.gov/grants/olaw/olaw.htm.

All award recipients must receive approval by a DoD-certified veterinarian, in addition to an IACUC approval. No animal studies may be conducted using DoD/DARPA funding until the United States Army Medical Research and Materiel Command (USAMRMC) Animal Care and Use Review Office (ACURO) or other appropriate DoD veterinary office(s) grant approval. As a part of this secondary review process, the award recipient will be required to complete and submit an ACURO Animal Use Appendix, which may be found at http://mrmc.amedd.army.mil/index.cfm?pageid=research_protections.acuro_animalappendix.

Small Business Subcontracting Plan

Pursuant to Section 8(d) of the Small Business Act (15 U.S.C. § 637(d)) and FAR 19.702(a)(1), each proposer who submits a contract proposal and includes subcontractors might be required to submit a subcontracting plan with their proposal. The plan format is outlined in FAR 19.704.

Section 508 of the Rehabilitation Act (29 U.S.C. § 749d)/FAR 39.2

All electronic and information technology acquired or created through this BAA must satisfy the accessibility requirements of Section 508 of the Rehabilitation Act (29 U.S.C. § 749d)/FAR 39.2.

Intellectual Property

All proposers must provide a good faith representation that the proposer either owns or possesses the appropriate licensing rights to all intellectual property that will be utilized under the proposed effort.

For Procurement Contracts

Proposers responding to this BAA requesting procurement contracts will need to complete the certifications at DFARS 252.227-7017. See http://www.darpa.mil/work-with-us/additional-baa for further information. If no restrictions are intended, the proposer should state “none.” The table below captures the requested information:

Technical Data Computer Software To be Furnished With Restrictions	Summary of Intended Use in the Conduct of the Research	Basis for Assertion	Asserted Rights Category	Name of Person Asserting Restrictions
(LIST)	(NARRATIVE)	(LIST)	(LIST)	(LIST)

For All Non-Procurement Contracts

Proposers responding to this BAA requesting a Cooperative Agreement, Technology Investment Agreement, or Other Transaction for Prototypes shall follow the applicable rules and regulations governing these various award instruments, but, in all cases, should appropriately identify any potential restrictions on the Government's use of any Intellectual Property contemplated under the award instrument in question. This includes both Noncommercial Items and Commercial Items. Proposers are encouraged to use a format similar to that described in the section above. If no restrictions are intended, then the proposer should state "NONE."

System for Award Management (SAM) and Universal Identifier Requirements

All proposers must be registered in SAM unless exempt per FAR 4.1102. FAR 52.204-7, "System for Award Management" and FAR 52.204-13, "System for Award Management Maintenance" are incorporated into this BAA. See www.darpa.mil/work-with-us/additional-baa for further information.

4.2.4. Submission Information

DARPA will acknowledge receipt of all submissions and assign an identifying control number that should be used in all further correspondence regarding the submission. DARPA intends to use electronic mail correspondence regarding HR001117S0019. Submissions may not be submitted by fax or e-mail; any so sent will be disregarded.

Submissions will not be returned. An electronic copy of each submission received will be retained at DARPA and all other non-required copies destroyed. A certification of destruction may be requested, provided the formal request is received by DARPA within 5 days after notification that a proposal was not selected.

For (abstract and) proposal submission dates, see Part I., Overview Information. Submissions received after these dates and times may not be reviewed.

For Proposers Submitting Proposal Abstracts or Full Proposals Requesting Procurement Contracts or OTs through DARPA's BAA Submission Portal:

Abstracts and Full Proposals sent in response to HR001117S0019 may be submitted via DARPA's BAA Website (https://baa.darpa.mil). Visit the website to complete the two-step registration process. Submitters will need to register for an Extranet account (via the form at the URL listed above) and wait for two separate e-mails containing a username and temporary password. After accessing the Extranet, submitters may then create an account for the DARPA BAA website (via the "Register your Organization" link along the left side of the homepage), view submission instructions, and upload/finalize the abstract. Proposers using the DARPA BAA Website may encounter heavy traffic on the submission deadline date; it is highly advised that submission process be started as early as possible.

All unclassified concepts submitted electronically through DARPA's BAA Website must be uploaded as zip files (.zip or .zipx extension). The final zip file should be no greater than 50 MB in size. Only one zip file will be accepted per submission. Classified submissions and proposals requesting assistance instruments (cooperative agreements) should NOT be submitted through DARPA's BAA Website (https://baa.darpa.mil), though proposers will likely still need to visit https://baa.darpa.mil to register their organization (or verify an existing registration) to ensure the BAA office can verify and finalize their submission.

Technical support for BAA Website may be reached at BAAT_Support@darpa.mil, and is typically available during regular business hours, (9:00 AM- 5:00 PM EST Monday – Friday).

Proposers using the DARPA BAA Website may encounter heavy traffic on the submission deadline date; it is highly advised that submission process be started as early as possible.

For Full Proposals Requesting Cooperative Agreements:

Proposers requesting cooperative agreements may submit proposals through one of the following methods: (1) hard copy mailed directly to DARPA; or (2) electronic upload per the instructions at http://www.grants.gov/applicants/apply-for-grants.html. Cooperative agreement proposals may not be submitted through any other means. If proposers intend to use Grants.gov as their means of submission, then they must submit their entire proposal through Grants.gov; applications cannot be submitted in part to Grants.gov and in part as a hard-copy. Proposers using the Grants.gov do not submit paper proposals in addition to the Grants.gov electronic submission.

Grants.gov Submissions: Grants.gov requires proposers to complete a one-time registration process before a proposal can be electronically submitted. First time registration can take between three business days and four weeks. For more information about registering for Grants.gov, see http://www.darpa.mil/work-with-us/additional-baa.

Hard-copy Submissions: Proposers electing to submit cooperative agreement proposals as hard copies must complete the SF 424 R&R form (Application for Federal Assistance,) available on the Grants.gov website http://apply07.grants.gov/apply/forms/sample/RR_SF424_2_0-V2.0.pdf

Failure to comply with the submission procedures may result in the submission not being evaluated. DARPA will acknowledge receipt of complete submissions via email and assign control numbers that should be used in all further correspondence regarding proposals.

4.3. FUNDING RESTRICTIONS

Not Applicable

4.4. OTHER SUBMISSION REQUIREMENTS

Not Applicable.

5. Application Review Information

5.1. EVALUATION CRITERIA

Proposals will be evaluated using the following criteria, listed in descending order of importance:
5.1.1 Overall Scientific and Technical Merit; 5.1.2 Potential Contribution and Relevance to the DARPA Mission; and 5.1.3 Cost Realism.

5.1.1. Overall Scientific and Technical Merit

The proposed technical approach is innovative, feasible, achievable, and complete.

The proposed technical team has the expertise and experience to accomplish the proposed tasks. Task descriptions and associated technical elements provided are complete and in a logical sequence with all proposed deliverables clearly defined such that a final outcome that achieves the goal can be expected as a result of award. The proposal identifies major technical risks and planned mitigation efforts are clearly defined and feasible.

5.1.2. Potential Contribution and Relevance to the DARPA Mission

The potential contributions of the proposed effort are relevant to the national technology base. Specifically, DARPA's mission is to make pivotal early technology investments that create or prevent strategic surprise for U.S. National Security.

5.1.3. Cost Realism

The proposed costs are realistic for the technical and management approach and accurately reflect the technical goals and objectives of the solicitation. The proposed costs are consistent with the proposer's Statement of Work and reflect a sufficient understanding of the costs and level of effort needed to successfully accomplish the proposed technical approach. The costs for the prime proposer and proposed subawardees are substantiated by the details provided in the proposal (e.g., the type and number of labor hours proposed per task, the types and

quantities of materials, equipment and fabrication costs, travel and any other applicable costs and the basis for the estimates).

It is expected that the effort will leverage all available relevant prior research in order to obtain the maximum benefit from the available funding. For efforts with a likelihood of commercial application, appropriate direct cost sharing may be a positive factor in the evaluation. DARPA recognizes that undue emphasis on cost may motivate proposers to offer low-risk ideas with minimum uncertainty and to staff the effort with junior personnel in order to be in a more competitive posture. DARPA discourages such cost strategies.

5.2. REVIEW OF PROPOSALS

Review Process

It is the policy of DARPA to ensure impartial, equitable, comprehensive proposal evaluations based on the evaluation criteria listed in Section V.A. and to select the source (or sources) whose offer meets the Government's technical, policy, and programmatic goals.

DARPA will conduct a scientific/technical review of each conforming proposal. Conforming proposals comply with all requirements detailed in this BAA; proposals that fail to do so may be deemed non-conforming and may be removed from consideration. Proposals will not be evaluated against each other since they are not submitted in accordance with a common work statement. DARPA's intent is to review proposals as soon as possible after they arrive; however, proposals may be reviewed periodically for administrative reasons

Award(s) will be made to proposers whose proposals are determined to be the most advantageous to the Government, consistent with instructions and evaluation criteria specified in the BAA herein, and availability of funding.

Handling of Source Selection Information

DARPA policy is to treat all submissions as source selection information (see FAR 2.101 and 3.104), and to disclose their contents only for the purpose of evaluation. Restrictive notices notwithstanding, during the evaluation process, submissions may be handled by support contractors for administrative purposes and/or to assist with technical evaluation. All DARPA support contractors performing this role are expressly prohibited from performing DARPA-sponsored technical research and are bound by appropriate nondisclosure agreements. Subject to the restrictions set forth in FAR 37.203(d), input on technical aspects of the proposals may be solicited by DARPA from non-Government consultants/experts who are strictly bound by the appropriate non-disclosure requirements.

Federal Awardee Performance and Integrity Information (FAPIIS)

Per 41 U.S.C. § 2313, as implemented by FAR 9.103 and 2 CFR § 200.205, prior to making an award above the simplified acquisition threshold, DARPA is required to review and consider any information available through the designated integrity and performance system (currently

FAPIIS). Awardees have the opportunity to comment on any information about themselves entered in the database, and DARPA will consider any comments, along with other information in FAPIIS or other systems prior to making an award.

6. Award Administration Information

6.1. SELECTION NOTICES

As soon as the evaluation of a proposal is complete, the proposers will be notified that (1) the proposal has been selected for funding pending contract negotiations, or (2) the proposal has not been selected. These official notifications will be sent via email to the Technical POC identified on the proposal coversheet.

6.1.1. Proposal Abstracts

DARPA will respond to abstracts with a statement as to whether DARPA is interested in the idea. If DARPA does not recommend the proposer submit a full proposal, DARPA will provide feedback to the proposer regarding the rationale for this decision. Regardless of DARPA's response to an abstract, proposers may submit a full proposal. DARPA will review all full proposals submitted using the published evaluation criteria and without regard to any comments resulting from the review of an abstract.

6.1.2. Full Proposals

As soon as the evaluation of a proposal is complete, the proposer will be notified that (1) the proposal has been selected for funding pending award negotiations, in whole or in part, or (2) the proposal has not been selected. These official notifications will be sent via e-mail to the Technical POC and/or Administrative POC identified on the proposal coversheet.

6.2. ADMINISTRATIVE AND NATIONAL POLICY REQUIREMENTS

6.2.1. Meeting and Travel Requirements

There will be a program kickoff meeting in the Arlington, VA vicinity and all key participants are required to attend. Performers should also anticipate regular program-wide PI meetings and periodic site visits at the Program Manager's discretion to the Arlington, VA vicinity. Proposers shall include within the content of their proposal details and costs of any travel or meetings they deem to be necessary throughout the course of the effort, to include periodic status reviews by the government.

6.2.1. FAR and DFARS Clauses

Solicitation clauses in the FAR and DFARS relevant to procurement contracts and FAR and DFARS clauses that may be included in any resultant procurement contracts are incorporated herein and can be found at http://www.darpa.mil/work-with-us/additional-baa.

6.2.2. Controlled Unclassified Information (CUI) on Non-DoD Information Systems

Further information on Controlled Unclassified Information on Non-DoD Information Systems is incorporated herein can be found at http://www.darpa.mil/work-with-us/additional-baa.

6.2.3. Representations and Certifications

If a procurement contract is contemplated, prospective awardees will need to be registered in the SAM database prior to award and complete electronic annual representations and certifications consistent with FAR guidance at 4.1102 and 4.1201; the representations and certifications can be found at www.sam.gov. Supplementary representations and certifications can be found at www.darpa.mil/work-with-us/additional-baa.

.

6.2.4. Terms and Conditions

A link to the DoD General Research Terms and Conditions for Grants and Cooperative Agreements and supplemental agency terms and conditions can be found at www.darpa.mil/work-with-us/contract-management#GrantsCooperativeAgreements..

6.3. REPORTING

The number and types of reports will be specified in the award document, but will include as a minimum monthly financial status reports and monthly technical status reports. The reports shall be prepared and submitted in accordance with the procedures contained in the award document and mutually agreed on before award. Reports and briefing material will also be required as appropriate to document progress in accomplishing program metrics. A Final Report that summarizes the project and tasks will be required at the conclusion of the performance period for the award, notwithstanding the fact that the research may be continued under a follow-on vehicle.

6.4. ELECTRONIC SYSTEMS

6.4.1. Wide Area Work Flow (WAWF)

Performers will be required to submit invoices for payment directly to https://wawf.eb.mil, unless an exception applies. Performers must register in WAWF prior to any award under this BAA.

6.4.2. i-EDISON

The award document for each proposal selected for funding will contain a mandatory requirement for patent reports and notifications to be submitted electronically through i-Edison (http://public.era.nih.gov/iedison).

7. Agency Contacts

Administrative, technical or contractual questions should be sent via e-mail to P3@darpa.mil.

Points of Contact
The BAA Coordinator for this effort may be reached at:
P3@darpa.mil
DARPA/BTO
ATTN: HR001117S0019
675 North Randolph Street
Arlington, VA 22203-2114

8. Other Information

The Biological Technologies Office (BTO) of the Defense Advanced Research Projects Agency (DARPA) is sponsoring two Proposers Days for the planned proposer community in support of the Pandemic Prevention Platform (P3) program. A Proposers Day will be held on February 22, 2017 at the Crown Plaza Tysons Corner McLean Hotel in McLean, VA and another on March 2, 2017 at the Doubletree by Hilton Hotel San Diego Downtown in San Diego, CA. Advanced registration is required.

The Proposers Day objectives are:

- To introduce the science and technology community (industry, academia, and government) to the P3 program vision and goals;
- To facilitate interaction between investigators that may have capabilities to develop elements of interest and relevance to P3 goals; and
- To encourage and promote teaming arrangements among organizations that have the relevant expertise, research facilities and capabilities for executing research and development responsive to the P3 program goals.

The Proposers Day will include brief overview presentations by government personnel, an information session to respond to questions from participants, "lightning" talks (five minutes, one PowerPoint slide) and posters for potential proposers to highlight technical capability or interest to promote teaming, and private sidebar meetings with the DARPA government team and potential proposer teams that can be scheduled beforehand on the registration website.

Attendance at this event is not a requirement for submission of a proposal or selection for funding. Information relayed during the Proposers Day will be made available on the BTO section of the DARPA Opportunities page: http://www.darpa.mil/work-with-us/opportunities

Participants are required to register no later than **February 16, 2017 at 12:00 PM ET** for the Washington D.C. proposers day and no later than **February 24, 2017 at 12:00 PM ET** for the San Diego proposers day.

There will not be a registration fee for this workshop. Registration is limited by the venue capacity, and early registration is strongly recommended. **Teams are limited to two attendees**.

The registration cutoff date is **February 16, 2017** (Washington D.C.) or **February 24, 2017** (San Diego) or once attendance capacity is met, whichever comes first. **There will be no on-site registration.** Because it is expected that a diverse technical team will be essential to a successful program, it is strongly suggested that teams able to attend both workshops should do so to increase opportunities to network with other interested groups.

An online registration form, foreign national visit request form, and various other meeting details can be found at the registration website, http://www.cvent.com/d/9vqy52. All attendees are required to present government-issued photo identification upon entry to the event. Non-U.S. citizens are required to submit a DARPA Form 60 "Foreign National Visit Request," which is available on the registration website. The completed DARPA Form 60 must be uploaded to the registration website by **February 16, 2017** (McLean, VA) or **February 24, 2017** (San Diego, CA) at 12:00 PM ET.

9. APPENDIX 1 – Volume II checklist

Volume II, Cost Proposal
Checklist and Sample Templates

The following checklist and sample templates are provided to assist the proposer in developing a complete and responsive cost volume. Full instructions appear in Section 4.2.2 beginning on Page 24 of HR001117S0019. This worksheet must be included with the coversheet of the Cost Proposal.

1. Are all items from the Volume II, Cost Proposal cover sheet format section of **HR001117S0019** included on your Cost Proposal cover sheet?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]
   If reply is "No", please explain:

2. Does your Cost Proposal include (1) a summary cost buildup by Phase, (2) a summary cost buildup by Year, and (3) a detailed cost buildup of for each Phase that breaks out each task and shows the cost per month?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

3. Does your cost proposal (detailed cost buildup #3 above in item 2) show a breakdown of the major cost items listed below:
   Direct Labor (Labor Categories, Hours, Rates)
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Indirect Costs/Rates (i.e., overhead charges, fringe benefits, G&A)
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Materials and/or Equipment
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Subcontracts/Consultants
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Other Direct Costs
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Travel
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

4. Have you provided documentation for proposed costs related to travel, to include purpose of trips, departure and arrival destinations and sample airfare?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

5. Does your cost proposal include a complete itemized list of <u>all</u> material and equipment items to be purchased (a priced bill-of-materials (BOM))?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

6. Does your cost proposal include vendor quotes or written engineering estimates (basis of estimate) for <u>all</u> material and equipment with a unit price exceeding $5000?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

7. Does your cost proposal include a clear justification for the cost of labor (written labor basis-of-estimate (BOE)) providing rationale for the labor categories and hours proposed for each task?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

8. Do you have subcontractors/consultants? If YES, continue to question 9. If NO, skip to question 13.
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

9. Does your cost proposal include copies of all subcontractor/consultant technical (to include Statement of Work) and cost proposals?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

10. Do all subcontract proposals include the required summary buildup, detailed cost buildup, and supporting documentation (SOW, Bill-of-Materials, Basis-of-Estimate, Vendor Quotes, etc.)?
    ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

    If reply is "No", please explain:

11. Does your cost proposal include copies of consultant agreements, if available?
○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

If reply is "No", please explain:

12. If requesting a FAR-based contract, does your cost proposal include a tech/cost analysis for all proposed subcontractors?
○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

If reply is "No", please explain:

13. Have all team members (prime and subcontractors) who are considered a Federally Funded Research & Development Center (FFRDC), included documentation that clearly demonstrates work is not otherwise available from the private sector AND provided a letter on letterhead from the sponsoring organization citing the specific authority establishing their eligibility to propose to government solicitations and compete with industry, and compliance with the associated FFRDC sponsor agreement and terms and conditions.
○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

If reply is "No", please explain:

14. Does your proposal include a response regarding Organizational Conflicts of Interest?
○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

If reply is "No", please explain:

15. Does your proposal include a completed Data Rights Assertions table/certification?
○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

If reply is "No", please explain:

Pandemic Prevention Platform (P3)

COL Matthew Hepburn, MD
Program Manager, Biological Technologies Office (BTO)

Briefing for:

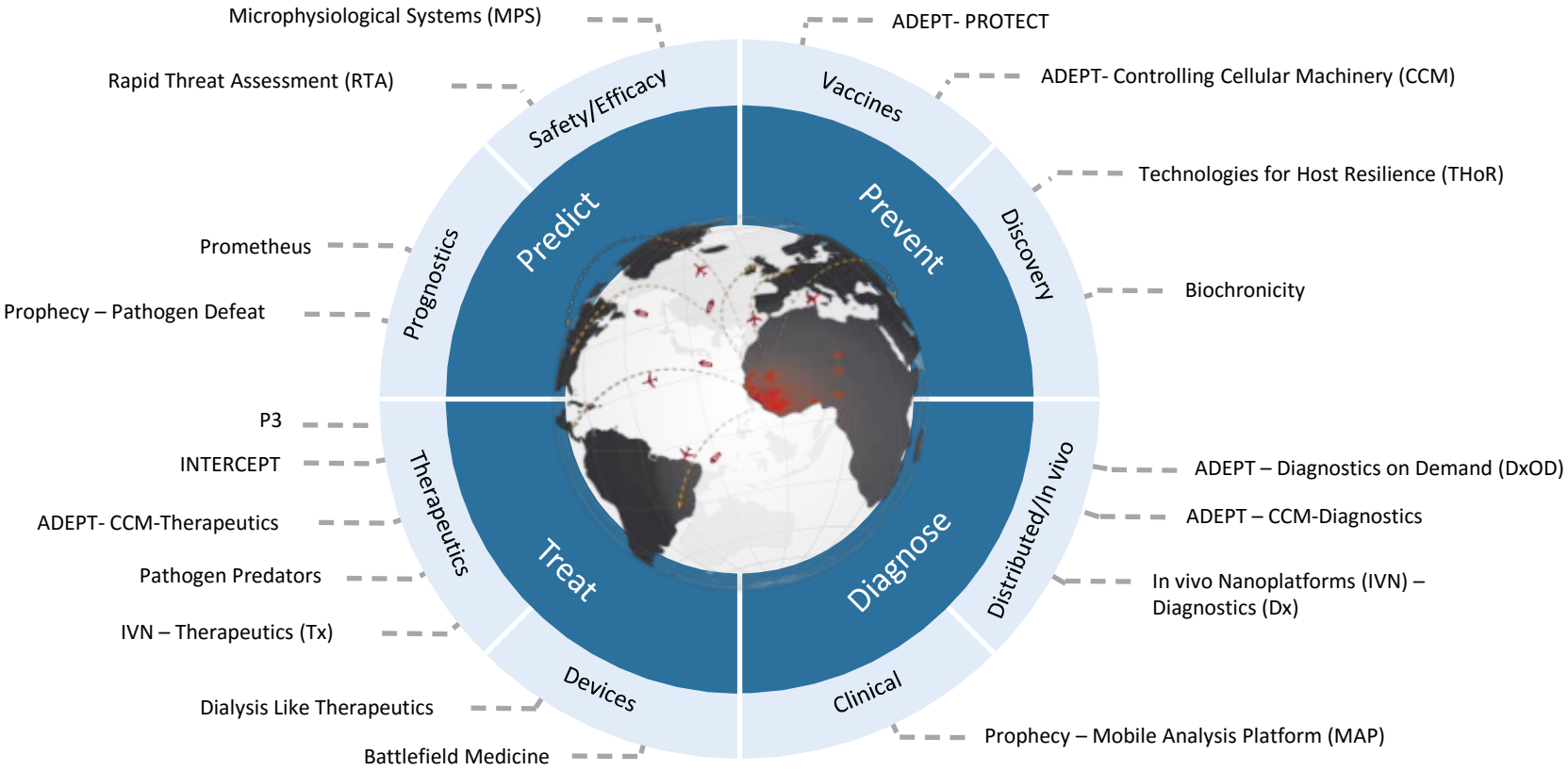
P3 Proposers Day

February 22, 2017

Approved for public release; distribution is unlimited.

BTO Vision for Pandemic Preparedness

Develop scalable operational technologies that have the capability to predict, prevent, diagnose, and treat pandemic infectious disease outbreaks



Approved for public release; distribution is unlimited.

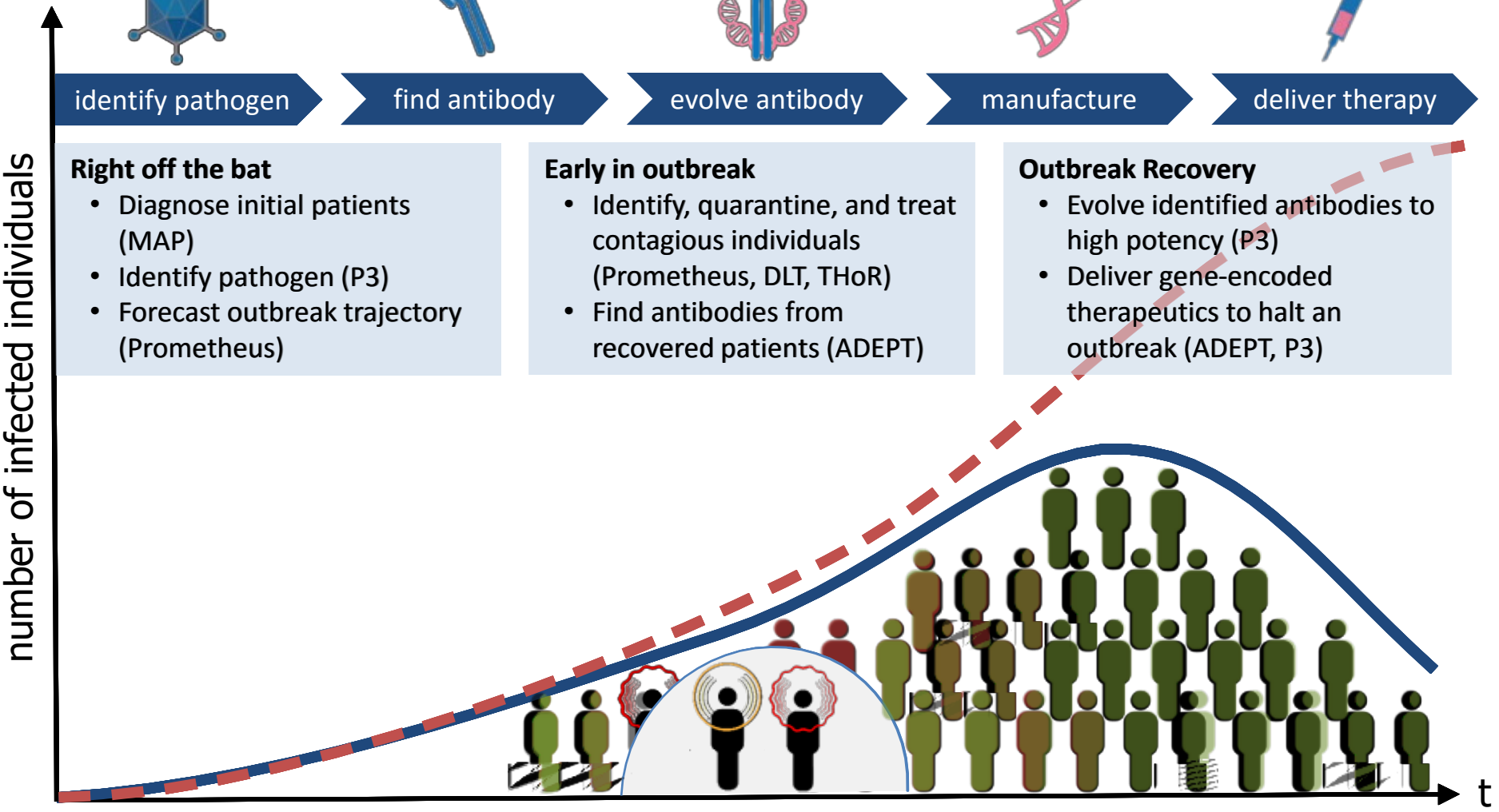
Pandemic Response Capabilities

identify pathogen → find antibody → evolve antibody → manufacture → deliver therapy

Right off the bat
- Diagnose initial patients (MAP)
- Identify pathogen (P3)
- Forecast outbreak trajectory (Prometheus)

Early in outbreak
- Identify, quarantine, and treat contagious individuals (Prometheus, DLT, THoR)
- Find antibodies from recovered patients (ADEPT)

Outbreak Recovery
- Evolve identified antibodies to high potency (P3)
- Deliver gene-encoded therapeutics to halt an outbreak (ADEPT, P3)



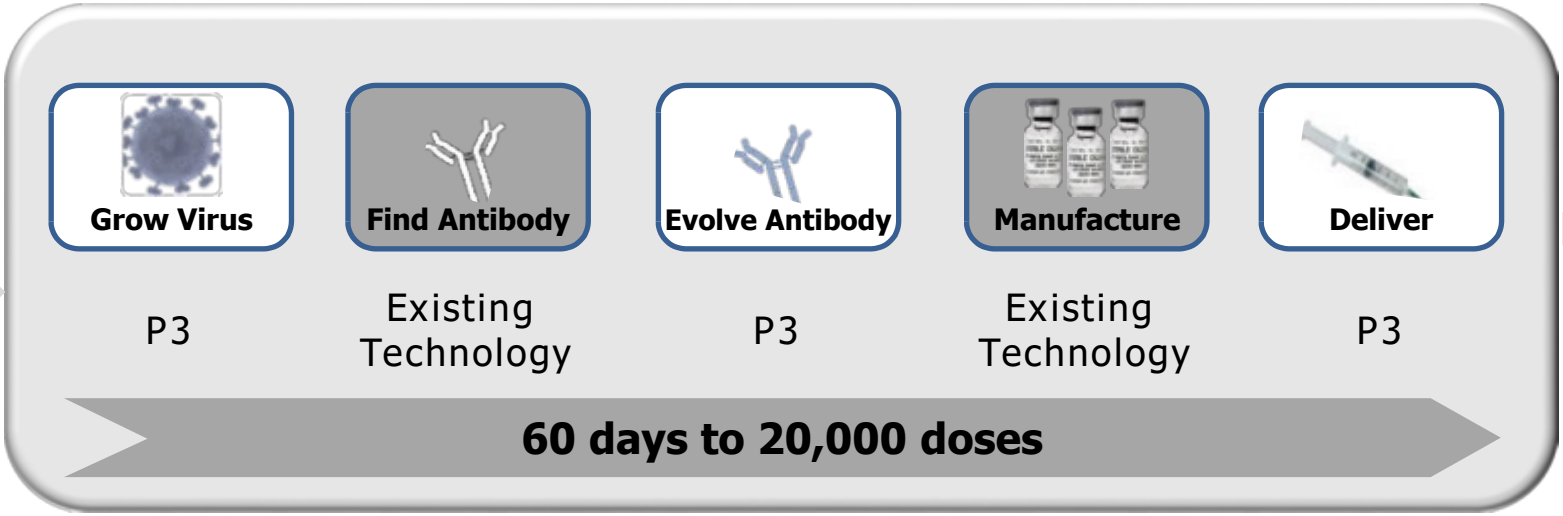
Manufacture and deliver gene-encoded antibody products for prophylaxis among at-risk populations

Approved for public release; distribution is unlimited.

Pandemic Prevention Platform (P3) Vision

Develop a functionally integrated platform to deliver pandemic prevention treatments in <60 days

Pandemic Outbreak
Any Virus



Treatment to Prevent Pandemic

Approved for public release; distribution is unlimited.

Capability Demonstrations

Grow Virus

Find Antibody

Evolve Antibody

Manufacture

Deliver

Grow enough virus for pre-clinical evaluation:

- Bait
- In vitro Testing and Evolution
- In vivo Testing and Evaluation

Identify an Antibody (or Countermeasure)

- Naïve or convalescent sample
- "Other" technology will be considered

Evolve Antibody (or Countermeasure)

- Increased potency (100-fold)
- Will be dependent on proposed pathogen

Research Grade Manufacturing

- Downstream in vivo testing

Clinical Grade Manufacturing

- 1000 doses for phase I clinical study – during **1** demo

Testing and Evaluation

- Test production and efficacy in small and large animals
- **1X** - Phase I safety study –

*<60 days does not apply to the Phase I study

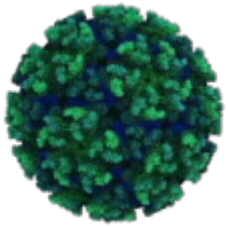
Complete the entire process, end-to-end, in <60 days

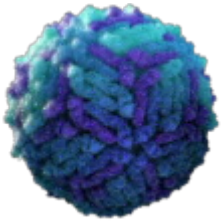
Approved for public release; distribution is unlimited.

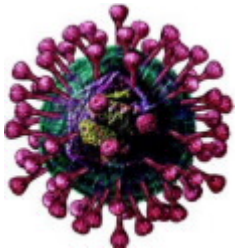
Program Considerations

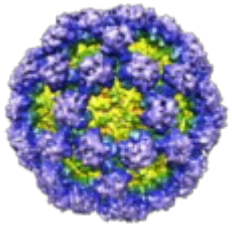
- **Pathogen Selection**

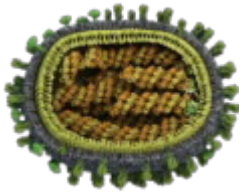
Example Pathogens:

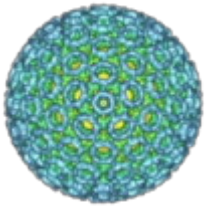

Chikungunya virus


Dengue virus


Coronavirus (MERS/SARS)


Noroviruses

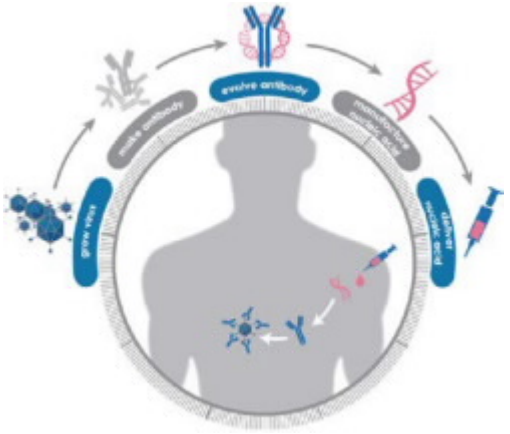

Influenza virus


Rift Valley Fever virus

- **Milestones and Metrics**

Time

Quality



Process Logistics

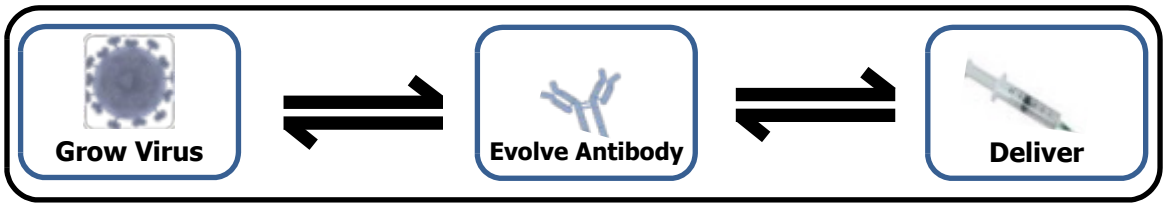
- **Approach and Teaming**

Approved for public release; distribution is unlimited.

Program Structure & Government Partners

Program Month:

0	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48



Platform Development (30 months)

Capability Demo #1

Capability Demo #2

Capability Demo #3

End-to-End Platform Demo/Testing

Fully Integrated Capability Demonstrations #4 and #5 (2x 60 day simulations)

Blinded pathogens

Approved for public release; distribution is unlimited.

The purpose of Modification 2 to HR001117S0019, the Pandemic Prevention Platform (P3) Broad Agency Announcement, is to change the requirement for the quantity of virus that must be grown as part of the effort. Specifically, the requirement to grow 10^{13} infectious viral units is no longer applicable. The new requirement states that performers must demonstrate the ability to grow pathogen quantities that are sufficient to complete medical countermeasure development, including "bait" for antibody or countermeasure discovery as well as *in vitro* and *in vivo* assays to determine countermeasure efficacy.

The corresponding changes can be found on pages 12 and 13 of the BAA and are bolded below:

Platform Integration and Testing

To ensure end-to-end functional platform integration, the performer will be required to transition the technological advances from the three TAs into capability demonstrations. A central component of this program will be the demonstration of an end-to-end platform functionality. The first capability demonstration will occur in month 12 of the program. Performer groups will be allotted 90 days to:

- **Grow pathogen quantities sufficient to complete medical countermeasure discovery, testing, and evaluation**
- Discover pathogen-specific antibodies
- Successfully transition both antibody/therapy and virus to the antibody evolution group
- Evolve antibody/therapy to 100-fold increased potency
- Deliver genetic sequence of highly potent antibody/therapy to the manufacturing group (data)
- Manufacture research grade material for animal studies
- Demonstrate both product delivery and protection from viral challenge in an animal model

At month 24, performers will conduct a capability demonstration with a pathogen of performer choice. At 36 months, performers will conduct a third 60-day capability demonstration with a DARPA selected pathogen. It is required that during one of the capability demonstrations, GMP manufacturing of clinical grade material will be completed for use in a human clinical trial. The proposal must include a discussion of the number of doses to be manufactured during the demonstration, and the time for manufacturing of these doses must fall within the 60-day timeline.

Using the lessons learned in the previous capability demonstrations, performers will conducted two final capability demonstrations with pathogens that are unidentified to the groups to simulate a "real–world" response to an unknown pathogen. The final deliverable will be the ability to produce at a minimum 1000 doses. The platform should be capable to scale-up manufacturing to 20,000 doses of gene-encoded antibody product for Phase I/II human clinical trials in <60 days, with a delivery platform able to achieve protective levels inside the body.

Finally, the proposal must include a discussion of where the capability platform will be located. It is anticipated that the platform will either be centrally located in one facility run by one of the performer teams, or that aspects of the platform will be physically located at different facilities which are integrated through a system of agreements allowing for the facile transfer of material between locations. In the latter case, the proposal must discuss the coordination and management of the decentralized platform.

Proposers must provide the following in the proposal:

- The proposed manufacturing plan, demonstrating the ability to GMP manufacture products in addition to being able to scale-up manufacturing and distribute 20,000 doses
- The proposed transition plan describing the product/data pipeline from receipt of pathogen and antibody input to final product
- The regulatory plan for phase I clinical safety trials

Table 1. Program metrics and milestones

Technical Metrics
TA 1: On-demand platform to grow virus **1. Grow pathogen quantities sufficient to complete medical countermeasure discovery, testing, and evaluation** 2. Pathogen genetic drift less than 0.1% after 5 successive culturing (growth) iterations (mutations per base pair per culturing cycle) 3. Progeny virus bio-identical to original isolate in lipid, protein, and carbohydrate content 4. Apply platform to five viruses representing at least one RNA virus, one DNA virus, and both (+)-sense and (-)-sense single stranded viral genomes
TA 2: System to evolve antibodies. 1. Ready for use in less than 72 hours 2. Demonstrate the ability to couple antibody discovery capability with the antibody evolution capability either through new discovery technologies or physical coupling with existing technologies 3. Support rapid potency maturation improving antibody/therapy greater than 100-fold in under 8 days 4. Apply technology to mature five different antibodies/therapies targeting at a minimum three (3) different pathogens representing at least one RNA virus, one DNA virus, and both (+)-sense and (-)-sense single stranded viral genomes *While it is assumed that the development of antibody therapies may be necessary to achieve a protective countermeasure in <60 days, all innovative approaches that meet the time and quality metrics stated above will be considered.

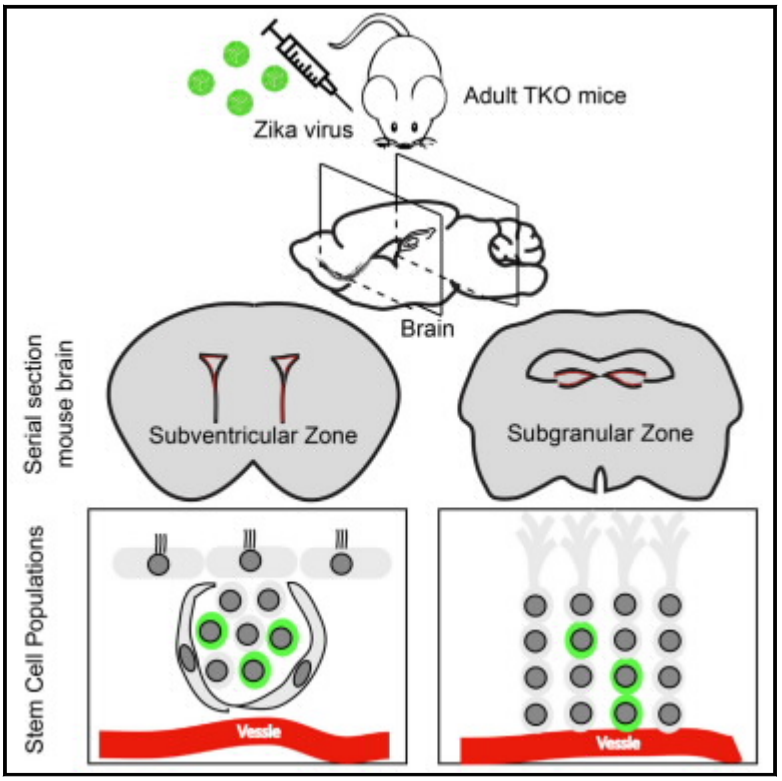
Technical Metrics
TA 3: Deliver medical countermeasure(s) 1. Achieve greater than 10 μg/mL serum concentration of target medical countermeasure with peak production in <3 days post-injection in large animals 2. Minimally or low-invasive simple delivery method given at 1 delivery site 3. Demonstrate reproducible production (less than 10% variance in serum concentration) in large animals 4. Demonstrate 100% protection against performer defined pathogen challenge in animal models by day 3 post countermeasure administration 5. Demonstrate longevity of protection lasts for greater than 30 days *While it is assumed that the development of antibody therapies may be necessary to achieve a protective countermeasure in <60 days, all innovative approaches that meet the time and quality metrics stated above will be considered.
Platform Integration: All of the following metrics must be achieved within 60 days and must include full integration with the metrics for TA1-3: 1. Demonstrate the ability to identify a pathogen specific antibody/therapy for rapid affinity maturation in TA2 within 14 days 2. Demonstrate the ability to manufacture research grade genetic constructs for use in animal studies 3. Demonstrate the ability to manufacture clinical grade GMP material for human safety trials – this metric must be met for only one of the phase II demonstrations. The ability to scale-up manufacturing and distribution to 20,000 doses must also be addressed as part of this metric 4. Demonstrate safety and efficacy in animal models and in human clinical trials

Brief Report

Cell Stem Cell

Zika Virus Infects Neural Progenitors in the Adult Mouse Brain and Alters Proliferation

Graphical Abstract



Highlights

- Zika virus (ZIKV) can infect neural progenitors in the adult mouse brain
- ZIKV-infected adult NPCs show evidence of cell death
- Cell proliferation is also impacted in ZIKV-infected adult NPC populations

Authors

Hongda Li, Laura Saucedo-Cuevas, Jose A. Regla-Nava, ..., Alexey V. Terskikh, Sujan Shresta, Joseph G. Gleeson

Correspondence

sujan@lji.org (S.S.), jogleeson@rockefeller.edu (J.G.G.)

In Brief

Li et al. show that peripheral ZIKV exposure in a mouse model can infect adult neural stem cells in the brain, leading to cell death and reduced proliferation. Thus, in addition to impacting fetal development, ZIKV infection may also have negative effects on the adult brain.

Li et al., 2016, Cell Stem Cell 19, 1–6
November 3, 2016 a 2016 Elsevier Inc.
http://dx.doi.org/10.1016/j.stem.2016.08.005

CellPress

Pleasecite this article in press as: Li et al., Zika Virus Infects Neural Progenitors in the Adult Mouse Brain and Alters Proliferation, Cell Stem Cell (2016), http://dx.doi.org/10.1016/j.stem.2016.08.005

Cell Stem Cell
Brief Report

CellPress

Zika Virus Infects Neural Progenitors in the Adult Mouse Brain and Alters Proliferation

Hongda Li,[1,2,5] Laura Saucedo-Cuevas,[1,2,5] Jose A. Regla-Nava,[3] Guoliang Chai,[1,2] Nicholas Sheets,[3] William Tang,[3] Alexey V. Terskikh,[4] Sujan Shresta,[3,*] and Joseph G. Gleeson[1,2,6,*]
[1]Laboratory for Pediatric Brain Diseases, Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10065, USA
[2]Department of Neurosciences, Rady Children's Institute for Genomic Medicine, University of California, San Diego, La Jolla, CA 92093, USA
[3]Division of Inflammation Biology, La Jolla Institute for Allergy & Immunology, La Jolla, CA 92037, USA
[4]Del. E. Webb Center for Neuroscience, Aging and Stem Cell Regeneration, Sanford Burnham Prebys Discovery Institute, La Jolla, CA 92037, USA
[5]Co-first author
[6]Lead Contact
*Correspondence: sujan@lji.org (S.S.), jogleeson@rockefeller.edu (J.G.G.)
http://dx.doi.org/10.1016/j.stem.2016.08.005

SUMMARY

Zika virus (ZIKV)-related neuropathology is an important global health concern. Several studies have shown that ZIKV can infect neural stem cells in the developing brain, but infection in the adult brain has not been examined. Two areas in the adult mouse brain contain neural stem cells: the subventricular zone of the anterior forebrain and the subgranular zone of the hippocampus. Here, using 6-week-old mice triply deficient in interferon regulatory factor (IRF) as a model, we show that blood-borne ZIKV administration can lead to pronounced evidence of ZIKV infection in these adult neural stem cells, leading to cell death and reduced proliferation. Our data therefore suggest that adult as well as fetal neural stem cells are vulnerable to ZIKV neuropathology. Thus, although ZIKV is considered a transient infection in adult humans without marked long-term effects, there may in fact be consequences of exposure in the adult brain.

Recent world attention has been drawn to a global Zika virus (ZIKV) outbreak and its link with devastating cases of microcephaly and Guillain-Barre syndrome. ZIKV infection is spreading rapidly within the Americas after originating from an outbreak in Brazil (Campos et al., 2015). Mounting evidence suggests that ZIKV infection in pregnant women can cause congenital abnormalities as well as fetal demise (Calvet et al., 2016; Cugola et al., 2016; Miner et al., 2016; Wu et al., 2016). Initial case descriptions of microcephaly and spontaneous abortions have been supported by evidence of viral RNA and antigen in the brains of congenitally infected fetuses and newborns (Martines et al., 2016; Mlakar et al., 2016).

The radial-glial-derived cortical neural stem cells (NSCs) in the fetal brain appear to be especially impacted by ZIKV infection, either through greater susceptibility to the viral infection or virus-induced cytotoxicity. This same population is affected by inherited forms of microcephaly, suggesting that loss of these cells is responsible for the microcephaly after ZIKV infection. Indeed recent work demonstrated that ZIKV can infect human cortical NSCs and attenuate their growth and survival, when applied directly to either monolayer culture (Tang et al., 2016) or cerebral organoids or neurospheres (Dang et al., 2016; Garcez et al., 2016; Nowakowski et al., 2016; Qian et al., 2016). Vertical transmission from ZIKV-infected murine dams to fetuses yielded virus in brain and histopathological evidence of cytotoxicity, supporting direct infection of NSCs. Effects of ZIKV on the placenta and secondary effects on brain may have also contributed (Miner et al., 2016).

Many vector-borne flaviviruses have to overcome host type I IFN responses to replicate and cause disease in vertebrates. Wild-type mice are resistant to parenteral infection with dengue virus (DENV), and unlike in human cells where the virus is able to block type I and type II IFN receptor signaling, murine cells do not show the same block (Aguirre et al., 2012; Ashour et al., 2010; Yu et al., 2012). Therefore, similar to DENV mouse models, current ZIKV models utilize mice lacking one or more components of the IFN signaling. These models include the use of the IFN regulatory factor (IRF) transcription factors IRF-3, -5, and -7 triple knockout strain (aka IRF-TKO) (Zellweger and Shresta, 2014), which lacks type I IFN production. These mouse models appear to reproduce key features of human ZIKV infection, including viremia and neuronal tissue tropism, and are proving to be valuable for answering fundamental questions about ZIKV pathogenesis.

In the adult brain, neurogenesis contracts after birth to just the anterior subventricular zone (SVZ) of the forebrain and the subgranular zone (SGZ) of the hippocampal dentate gyrus. These restricted niches contain progenitor cells that divide to produce neurons or glia, depending upon intrinsic and environmental cues. Neurogenic niches are characterized by a comparatively high vascular density and proximity to cerebrospinal fluid (CSF) (Stolp and Molnár, 2015), allowing not just communication through signaling molecules but also proximity to circulating viruses.

To identify direct target cells of ZIKV in the adult CNS, we infected 5- to 6-week-old *Irf3*$^{ff/ff}$ *Irf5*$^{ff/ff}$ *Irf7*$^{ff/ff}$ TKO mice with an Asian lineage ZIKV strain (FSS13025, 2010 Cambodian isolate) via retro-orbital injection (see Supplemental Experimental Procedures for section of strain rationale). Retro-orbital

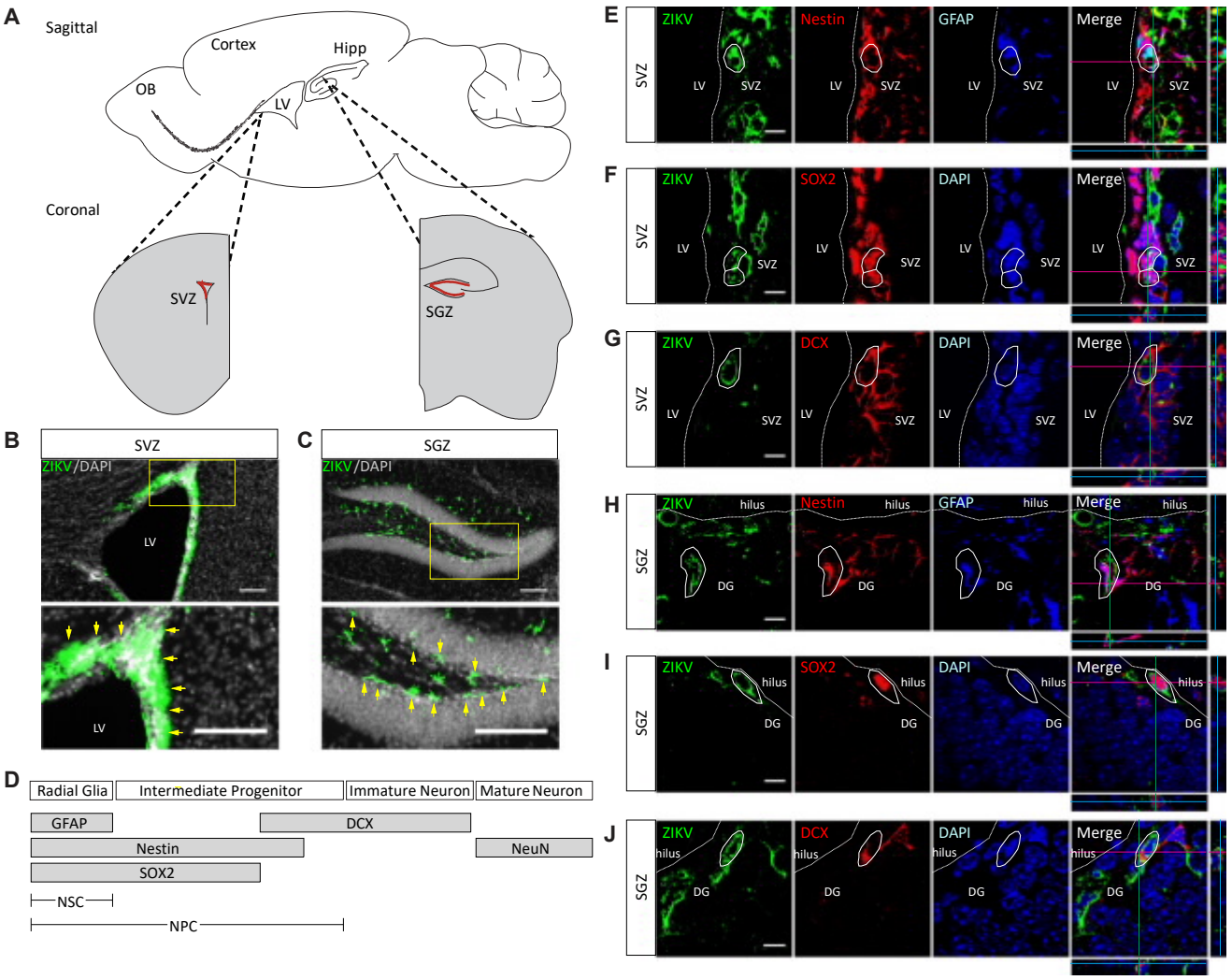


Figure 1. Systemic ZIKV Can Infect Neural Progenitor Cells in the Adult Brain

(A) Schematic of stem cell niches in adult mouse brain. Neural progenitor cells (NPCs) located in the subventricular zone (SVZ) in the anterior forebrain (red) adjacent to the cerebral ventricles contribute new neurons to the olfactory bulb. NPCs in the subgranular zone (SGZ) in the dorsal forebrain (red) contribute new neurons to the hippocampal dentate gyrus. LV, lateral ventricle; OB, olfactory bulb; Hipp, hippocampus.

(B and C) Adult TKO mice were infected with ZIKV and then sacrificed, their brains were serially sectioned, and they were immunostained for ZIKV envelope protein (green). Evidence of ZIKV was found in the SVZ and SGZ, with less expression elsewhere in adult brain. High-power inset below with arrows (yellow) highlights immunoreactive cells. LV, lateral ventricle. Scale bar, 100 mm.

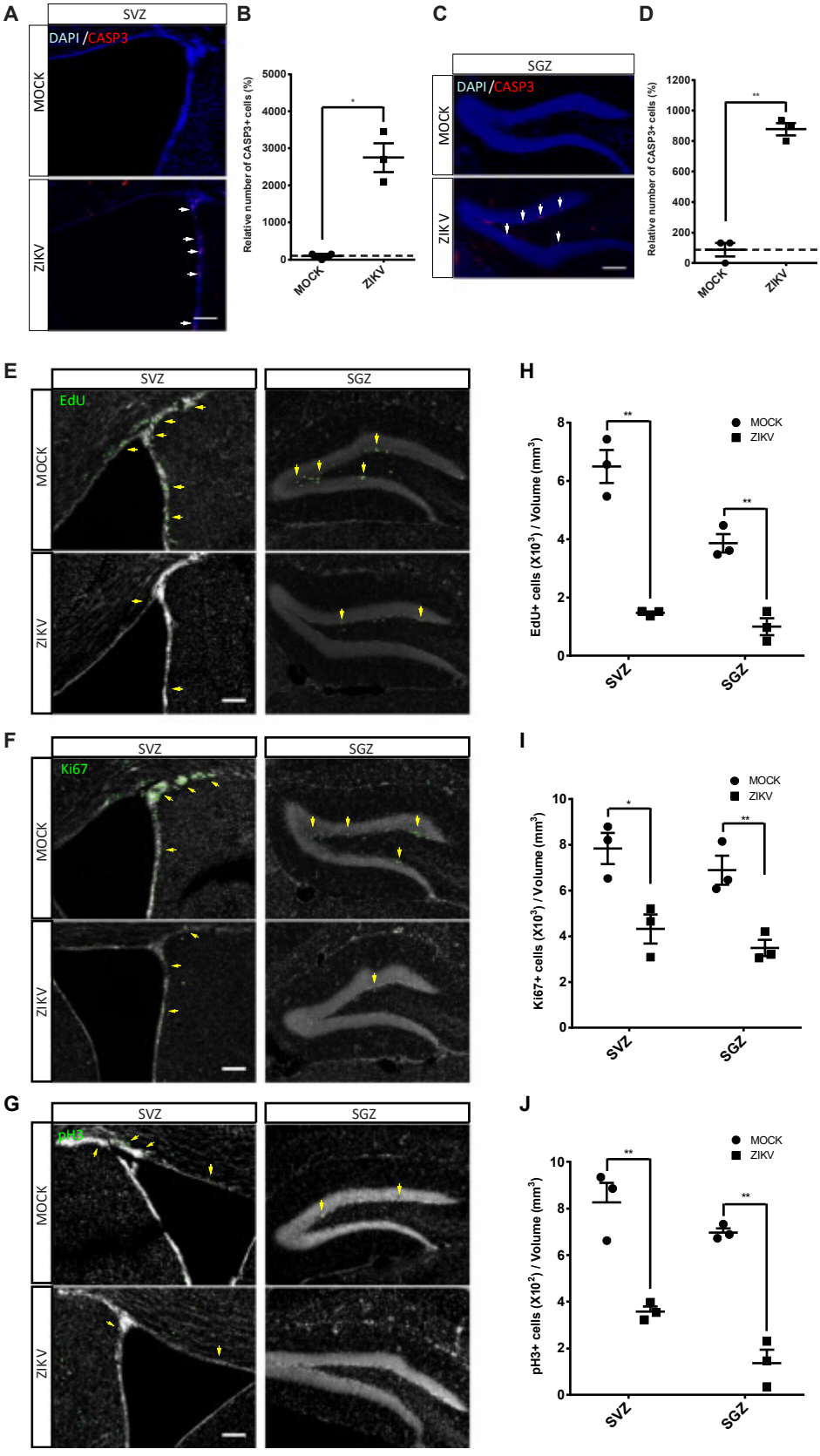
(D) Marker expression during neural stem cell (NSC) differentiation from radial glia to mature neuron.

(E–J) Confocal images and orthogonal projection of SVZ and SGZ regions co-stained for GFAP, Nestin, SOX2, and DCX with ZIKV envelop protein, evidencing ZIKV in NPCs and immature neuron populations. White circle outlines: infected cells. LV, lateral ventricle; SVZ, subventricular zone; DG, dentate gyrus. Scale bar, 10 mm.

injection was selected as a method to introduce virus into the peripheral circulation, rather than direct introduction into the brain, to model the blood-borne route of arbovirus transmission. In results similar to those of a previous report, TKO mice were vulnerable to ZIKV infection (Lazear et al., 2016) and began to exhibit ruffled fur and lethargy as evidence for viral illness by 3–4 days post-infection (DPI) and developed evidence of hindlimb weakness by 6 DPI.

To examine the potential for virus infection in the brain, we screened serial coronal sections of whole brain from infected and mock-treated mice with the monoclonal 4G2 antibody that reacts with the flavivirus-specific family envelope protein. We observed dramatic immunoreactivity in proximity to the SVZ of the anterior forebrain, as well as the SGZ of the hippocampus (Figures 1A–1C), the two regions in mouse that maintain stem cell populations throughout adulthood, in infected (but not mock-infected) mice. In contrast there was less immunoreactivity in other regions of the brain under these conditions (Figures S1A–S1C), suggesting a particular tropism of the virus for proliferative regions of the brain. Quantification across major brain regions showed statistically significant selective vulnerability to these proliferative zones (Figure S1D).

Pleasecite this article in press as: Li et al., Zika Virus Infects Neural Progenitors in the Adult Mouse Brain and Alters Proliferation, Cell Stem Cell (2016), http://dx.doi.org/10.1016/j.stem.2016.08.005



(legend on next page)

In adult SVZ and SGZ, radial-glia-like NSCs give rise to intermediate progenitor cells (IPCs), which then migrate to final destinations, where they express developmental-dependent markers and integrate into neuronal circuitry (Figure 1D, see Supplemental Information). For the remainder of this paper, neural progenitor cell (NPC) is used to refer to both NSCs and IPCs. To identify which cells were positive for 4G2, we co-stained them with different cell type markers. We detected the presence of ZIKV in GFAP- and Nestin-expressing NSCs, SOX2-expressing IPCs, and DCX-expressing immature neurons (Figures 1E–1J). 4G2 reactivity in sagittal sections confirmed ZIKV-infected DCX+ cells along the rostral migratory stream (Figure S1E). Consistent with previous reports where ZIKV was introduced directly into newborn and juvenile brain (Bell et al., 1971), we detected 4G2 reactivity in NeuN-expressing neurons and S100b-expressing astrocytes (Figures S2A–S2D), but much less than that in SOX2+ or DCX+ cells (Figures S2F and S2G). We rarely observed 4G2 reactivity in NG2-expressing oligodendrocytes (Figures S2E). We conclude that ZIKV has tropism for proliferative NPCs and immature neurons over terminal-differentiated cell population in the adult brain.

ZIKV infection can lead to caspase-3 (CASP3) activation in both NPCs differentiated from human ESCs/IPSCs and embryonic mouse brain (Cugola et al., 2016; Miner et al., 2016). In order to determine whether systemic ZIKV infection can induce cell death in adult NPC populations, we stained for cleaved (i.e., activated) CASP3 in NPC niches. Mock-infected TKO mice showed scant evidence of CASP3+ cells. In contrast abundant CASP3+ cells were detected in ZIKV-infected brains within these neurogenic niches (Figures 2A–2D), and were also usually positive for Nestin. Similarly, the ZIKV staining colocalized with CASP3 in NPCs in the SVZ and SGZ (Figures S2I and S2J), suggesting that ZIKV infection can induce apoptotic cell death in adult NPCs in these regions.

We assessed the impact of systemic ZIKV infection on cell proliferation in niches in adult brain using the thymidine analog EdU and a series of cell-cycle markers. We performed EdU pulse-labeling in TKO mice 6 days after ZIKV or mock infection. Quantitative analysis at 2 hr after EdU injection showed that, in both neurogenic regions, although the brain size and volume of the SGZ and SVZ was not notably different (Figure S2H), ZIKV-infected mice had approximately a 4- to 5-fold reduction in the number of EdU+ cells per section, compared with mock (Figures 2E and 2H).

Consistent with the EdU-labeling results, there were many fewer cells positive for the proliferation marker Ki67 in the SVZ and SGZ, which was reduced by approximately 2- to 3-fold (Figures 2F and 2I). Similarly, there were many fewer cells positive for the mitotic marker phospho-Histone H3 in the SVZ and SGZ, reduced by approximately 2- to 4-fold (Figures 2G and 2J). Results were statistically significant and consistent across the three infected and three mock-infected animals. These results indicate that ZIKV infection leads to decreased NPC proliferation in the adult SGZ and SVZ.

Here we demonstrate that ZIKV exposure in adult mice shows infection of brain with a predilection for neurogenic niches and is associated with cellular apoptosis and reduction of cellular proliferation. Based upon the presence of the ZIKV antigen following exposure, we conclude that the virus was able to infect SVZ and SGZ niche cells to a much greater degree than non-neurogenic regions. This infection correlated with evidence of apoptosis and reduced numbers of cells evidencing DNA synthesis or proliferation. However, the relative contribution of these features, as well as the long-term effects, on the NPC niches remains unknown. Our results suggest that ZIKV infection can enter the adult brain and lead to neuropathology in mammals.

The degree to which IFN-deficient mice model the extent and severity of flavivirus infection in humans is unknown. We recognize that healthy humans may be able to mount an effective antiviral response and prevent entry into the CNS, but it remains a possibility that some immunocompromised humans and even some apparently healthy humans may be susceptible in ways modeled by the TKO mice. It will be important to determine the extent of involvement of stem cell niches with less immunocompromised strains of mice. Brain inflammation in general, including IFN-a induction, can lead to reduction in adult neurogenesis (Kaneko et al., 2006; Kohman and Rhodes, 2013), and therefore the interaction between ZIKV infection and IFN signaling pathway and its impact on adult neurogenesis merit further investigation.

There are several caveats to our study, representing a single viral strain, single mouse strain, and single time point analysis. First, ZIKV infection can downregulate expression of neural progenitor markers like Nestin and SOX2 (Tang et al., 2016) and thus our analysis may underestimate the proportion of infected cells co-expressing these markers. Second, infected NPCs could have divided or differentiated between infection and harvest, and therefore future time course studies could be important. Third, we noticed that some ZIKV-infected cells in non-neurogenic regions of the brain, such as the hilus of the hippocampus (Figure S1B2), were not positive for NPC markers. Further examination will help identify whether these cells represent microglia or infiltrating neutrophils (Aliota et al., 2016) or some other cell type.

CNS involvement associated with other flavivirus infections in adults is receiving increasing attention. Although DENV infection

Figure 2. ZIKV-Infected Adult NPCs Undergo Cell Death and Show Reduced Proliferation

(A and C) Representative images of SVZ (A) or SGZ (C) region from ZIKV-infected brains showing more CASP3+ cells (arrows) compared to regions in mock-infected brains. Scale bar, 100 mm.

(B and D) Quantification of the number of CASP3+ cells relative to mock-infected brains. All data represent means ± SEM, n = 3 animals for each group. Student's t test, *p < 0.05, **p < 0.01.

(E) Adult TKO mice were infected with ZIKV or mock, injected with EdU after 6 days, then sacrificed after 2 hr. ZIKV-infected animals show reduced incorporation of EdU in both SVZ and SGZ, indicating reduced entry into S-phase. Scale bar, 100 mm.

(F) Reduced Ki67 staining in ZIKV-infected mice in both SVZ and SGZ, indicating reduced cell proliferation. Scale bar, 100 mm.

(G) Reduced phospho-histone H3 (pH3) staining in ZIKV-infected mice in both SVZ and SGZ, indicating reduced mitotic cells. Scale bar, 100 mm.

(H–J) Stereological quantification of the number of EdU+, Ki67+, and pH3+ cells in each mouse was performed in every sixth section of the entire brains from three pairs of ZIKV and mock-infected animals. All data represent means ± SEM, n = 3 animals for each group. Student's t test, *p < 0.05, **p < 0.01.

in humans usually produces a self-limited illness, CNS involvement is now considered criteria for severe dengue in the World Health Organization (WHO) classification (http://www.who.int/tdr/publications/documents/dengue-diagnosis.pdf), other criteria for which include but are not limited to cerebrospinal fluid viremia and encephalitis or meningoencephalitis, and was detected in nearly half of fatal dengue cases (Araújo et al., 2012). Approximately 1:150 cases of West Nile infection show neuroinvasive disease or encephalitis, with potential manifestations of flaccid paralysis, extrapyramidal findings, coarse tremor, myoclonus, and substantial cognitive difficulties. ZIKV can produce neurological findings to include demyelinating polyneuropathy and brachial plexopathy and recently acute meningoencephalitis (Carteaux et al., 2016). It will be important to evaluate the areas damaged following ZIKV CNS infection in humans, especially in neurogenic regions, and consider potential consequences on neurocognition.

Neurotropic viruses can gain entry into the CNS and cause disease through different means (Luethy et al., 2016), but how ZIKV gains entry into the brain remains unknown. Current models include entry of the virus directly across the blood-brain barrier (BBB), across synapses from peripheral nerves, or through entry of infected microglia. Once across the BBB, the means of entry into NPCs may be through specific transmembrane receptors, such as the candidate AXL receptor (Miner and Diamond, 2016; Nowakowski et al., 2016). The means by which infection leads to NPC death is also under active study. We hypothesize that the particular cell death in NPCs may be p53 mediated, in keeping with current models of human microcephaly (Pilaz et al., 2016; Tang et al., 2016).

Infection of NPCs in stem cell niches may relate to the emergent cases of ZIKA-linked GBS, an acute post-infectious immune-mediated polyradiculoneuropathy. The dramatically increased GBS incidence in ZIKV endemic regions (Watrin et al., 2016) and evidence of acute ZIKV infection in GBS patients from these regions (Beckham et al., 2016) suggests a causal relationship. A broad range of antibodies directed to neuronal glycolipids, possibly emerging from cross-reaction to viral proteins or release of neural antigens from damaged cells, are observed in some GBS cases. Although identified in many non-ZIKV-related GBS cases, less than 50% of sera at admission from ZIKV-related GBS showed significant auto-antibodies to glycolipids, and moreover complementary analysis did not show any competition between the glycolipid GA1 and ZIKV proteins, suggesting absence of antigenic mimicry (Beckham et al., 2016). Our data suggest that ZIKV-infected NPCs could release neural antigens as a possible source of auto-antibodies, although we cannot rule out that other neural cells are the target relevant to GBS.

A recent study using radioactive carbon dating reported a striking annual turnover rate of 1.75% in the human dentate gyrus, where approximately 700 new neurons are added every day from the SGZ to the pool of excitatory granule neuron population to support plasticity (Spalding et al., 2013). Furthermore, hippocampal neurogenesis deficits have been linked to cognitive deficits characteristic of depression (Patrício et al., 2013) and Alzheimer's disease (Demars et al., 2010; Rodríguez et al., 2011). SGZ neurogenesis is critical in rodents for hippocampal-dependent contextual conditioning learning (Saxe et al., 2006; Winocur et al., 2006), longer-term spatial memory (Deng et al., 2009), and pattern separation (Clelland et al., 2009; Sahay et al., 2011). Whether there are long-term effects of ZIKV on adult neurogenesis or cognition in rodents or humans will be an important question for the future.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes two figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2016.08.005 .

AUTHOR CONTRIBUTIONS

H.L. and L.S.-C. performed histological assessment and statistics; J.A.R.-N., N.S., and W.T. performed mouse breeding and infections; and G.C. and W.T. performed animal surgery and perfusions. H.L., L.S.-C., S.S., and J.G.G. designed the experiments and wrote the paper. A.V.T assisted with experimental design and edited the manuscript. S.S. and J.G.G supervised the project.

ACKNOWLEDGMENTS

This work was supported by the NIH (R01NS041537, R01NS048453, R01NS052455, P01HD070494, and P30NS047101), the Simons Foundation Autism Research Initiative (SFARI), the Howard Hughes Medical Institute, and California Institute of Regenerative Medicine (J.G.G.); NIH R01 AI116813 (S.S.); and a Druckenmiller Fellowship from New York Stem Cell Foundation (H.L.). We thank Alysson Muotri and Charles Rice for discussions.

Received: June 15, 2016
Revised: July 18, 2016
Accepted: August 2, 2016
Published: August 18, 2016

REFERENCES

Aguirre, S., Maestre, A.M., Pagni, S., Patel, J.R., Savage, T., Gutman, D., Maringer, K., Bernal-Rubio, D., Shabman, R.S., Simon, V., et al. (2012). DENV inhibits type I IFN production in infected cells by cleaving human STING. PLoS Pathog. *8*, e1002934.

Aliota, M.T., Caine, E.A., Walker, E.C., Larkin, K.E., Camacho, E., and Osorio, J.E. (2016). Characterization of Lethal Zika Virus Infection in AG129 Mice. PLoS Negl. Trop. Dis. *10*, e0004682.

Araújo, F.M., Araújo, M.S., Nogueira, R.M., Brilhante, R.S., Oliveira, D.N., Rocha, M.F., Cordeiro, R.A., Araújo, R.M., and Sidrim, J.J. (2012). Central nervous system involvement in dengue: a study in fatal cases from a dengue endemic area. Neurology *78*, 736–742.

Ashour, J., Morrison, J., Laurent-Rolle, M., Belicha-Villanueva, A., Plumlee, C.R., Bernal-Rubio, D., Williams, K.L., Harris, E., Fernandez-Sesma, A., Schindler, C., and García-Sastre, A. (2010). Mouse STAT2 restricts early dengue virus replication. Cell Host Microbe *8*, 410–421.

Beckham, J.D., Pastula, D.M., Massey, A., and Tyler, K.L. (2016). Zika Virus as an Emerging Global Pathogen: Neurological Complications of Zika Virus. J. Am. Med. Assoc. Neurol. *73*, 875–879.

Bell, T.M., Field, E.J., and Narang, H.K. (1971). Zika virus infection of the central nervous system of mice. Arch. Gesamte Virusforsch. *35*, 183–193.

Calvet, G., Aguiar, R.S., Melo, A.S., Sampaio, S.A., de Filippis, I., Fabri, A., Araujo, E.S., de Sequeira, P.C., de Mendonça, M.C., de Oliveira, L., et al. (2016). Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study. Lancet Infect. Dis. *16*, 653–660.

Campos, G.S., Bandeira, A.C., and Sardi, S.I. (2015). Zika Virus Outbreak, Bahia, Brazil. Emerg. Infect. Dis. *21*, 1885–1886.

Carteaux, G., Maquart, M., Bedet, A., Contou, D., Brugières, P., Fourati, S., Cleret de Langavant, L., de Broucker, T., Brun-Buisson, C., Leparc-Goffart,

I., and Mekontso Dessap, A. (2016). Zika Virus Associated with Meningoencephalitis. N. Engl. J. Med. *374*, 1595–1596.

Clelland, C.D., Choi, M., Romberg, C., Clemenson, G.D., Jr., Fragniere, A., Tyers, P., Jessberger, S., Saksida, L.M., Barker, R.A., Gage, F.H., and Bussey, T.J. (2009). A functional role for adult hippocampal neurogenesis in spatial pattern separation. Science *325*, 210–213.

Cugola, F.R., Fernandes, I.R., Russo, F.B., Freitas, B.C., Dias, J.L., Guimarães, K.P., Benazzato, C., Almeida, N., Pignatari, G.C., Romero, S., et al. (2016). The Brazilian Zika virus strain causes birth defects in experimental models. Nature *534*, 267–271.

Dang, J., Tiwari, S.K., Lichinchi, G., Qin, Y., Patil, V.S., Eroshkin, A.M., and Rana, T.M. (2016). Zika Virus Depletes Neural Progenitors in Human Cerebral Organoids through Activation of the Innate Immune Receptor TLR3. Cell Stem Cell *19*, 258–265.

Demars, M., Hu, Y.S., Gadadhar, A., and Lazarov, O. (2010). Impaired neurogenesis is an early event in the etiology of familial Alzheimer's disease in transgenic mice. J. Neurosci. Res. *88*, 2103–2117.

Deng, W., Saxe, M.D., Gallina, I.S., and Gage, F.H. (2009). Adult-born hippocampal dentate granule cells undergoing maturation modulate learning and memory in the brain. J. Neurosci. *29*, 13532–13542.

Garcez, P.P., Loiola, E.C., Madeiro da Costa, R., Higa, L.M., Trindade, P., Delvecchio, R., Nascimento, J.M., Brindeiro, R., Tanuri, A., and Rehen, S.K. (2016). Zika virus impairs growth in human neurospheres and brain organoids. Science *352*, 816–818.

Kaneko, N., Kudo, K., Mabuchi, T., Takemoto, K., Fujimaki, K., Wati, H., Iguchi, H., Tezuka, H., and Kanba, S. (2006). Suppression of cell proliferation by interferon-alpha through interleukin-1 production in adult rat dentate gyrus. Neuropsychopharmacology *31*, 2619–2626.

Kohman, R.A., and Rhodes, J.S. (2013). Neurogenesis, inflammation and behavior. Brain Behav. Immun. *27*, 22–32.

Lazear, H.M., Govero, J., Smith, A.M., Platt, D.J., Fernandez, E., Miner, J.J., and Diamond, M.S. (2016). A Mouse Model of Zika Virus Pathogenesis. Cell Host Microbe *19*, 720–730.

Luethy, L.N., Erickson, A.K., Jesudhasan, P.R., Ikizler, M., Dermody, T.S., and Pfeiffer, J.K. (2016). Comparison of three neurotropic viruses reveals differences in viral dissemination to the central nervous system. Virology *487*, 1–10.

Martines, R.B., Bhatnagar, J., Keating, M.K., Silva-Flannery, L., Muehlenbachs, A., Gary, J., Goldsmith, C., Hale, G., Ritter, J., Rollin, D., et al. (2016). Notes from the Field: Evidence of Zika Virus Infection in Brain and Placental Tissues from Two Congenitally Infected Newborns and Two Fetal Losses–Brazil, 2015. MMWR Morb. Mortal. Wkly. Rep. *65*, 159–160.

Miner, J.J., and Diamond, M.S. (2016). Understanding How Zika Virus Enters and Infects Neural Target Cells. Cell Stem Cell *18*, 559–560.

Miner, J.J., Cao, B., Govero, J., Smith, A.M., Fernandez, E., Cabrera, O.H., Garber, C., Noll, M., Klein, R.S., Noguchi, K.K., et al. (2016). Zika Virus Infection during Pregnancy in Mice Causes Placental Damage and Fetal Demise. Cell *165*, 1081–1091.

Mlakar, J., Korva, M., Tul, N., Popović, M., Poljšak-Prijatelj, M., Mraz, J., Kolenc, M., Resman Rus, K., Vesnaver Vipotnik, T., Fabjan Vodušek, V., et al. (2016). Zika Virus Associated with Microcephaly. N. Engl. J. Med. *374*, 951–958.

Nowakowski, T.J., Pollen, A.A., Di Lullo, E., Sandoval-Espinosa, C., Bershteyn, M., and Kriegstein, A.R. (2016). Expression Analysis Highlights AXL as a Candidate Zika Virus Entry Receptor in Neural Stem Cells. Cell Stem Cell *18*, 591–596.

Patrício, P., Mateus-Pinheiro, A., Sousa, N., and Pinto, L. (2013). Re-cycling paradigms: cell cycle regulation in adult hippocampal neurogenesis and implications for depression. Mol. Neurobiol. *48*, 84–96.

Pilaz, L.J., McMahon, J.J., Miller, E.E., Lennox, A.L., Suzuki, A., Salmon, E., and Silver, D.L. (2016). Prolonged Mitosis of Neural Progenitors Alters Cell Fate in the Developing Brain. Neuron *89*, 83–99.

Qian, X., Nguyen, H.N., Song, M.M., Hadiono, C., Ogden, S.C., Hammack, C., Yao, B., Hamersky, G.R., Jacob, F., Zhong, C., et al. (2016). Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure. Cell *165*, 1238–1254.

Rodríguez, J.J., Noristani, H.N., Olabarria, M., Fletcher, J., Somerville, T.D., Yeh, C.Y., and Verkhratsky, A. (2011). Voluntary running and environmental enrichment restores impaired hippocampal neurogenesis in a triple transgenic mouse model of Alzheimer's disease. Curr. Alzheimer Res. *8*, 707–717.

Sahay, A., Scobie, K.N., Hill, A.S., O'Carroll, C.M., Kheirbek, M.A., Burghardt, N.S., Fenton, A.A., Dranovsky, A., and Hen, R. (2011). Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature *472*, 466–470.

Saxe, M.D., Battaglia, F., Wang, J.W., Malleret, G., David, D.J., Monckton, J.E., Garcia, A.D., Sofroniew, M.V., Kandel, E.R., Santarelli, L., et al. (2006). Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. Proc. Natl. Acad. Sci. USA *103*, 17501–17506.

Spalding, K.L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H.B., Boström, E., Westerlund, I., Vial, C., Buchholz, B.A., et al. (2013). Dynamics of hippocampal neurogenesis in adult humans. Cell *153*, 1219–1227.

Stolp, H.B., and Molnár, Z. (2015). Neurogenic niches in the brain: help and hindrance of the barrier systems. Front. Neurosci. *9*, 20.

Tang, H., Hammack, C., Ogden, S.C., Wen, Z., Qian, X., Li, Y., Yao, B., Shin, J., Zhang, F., Lee, E.M., et al. (2016). Zika Virus Infects Human Cortical Neural Progenitors and Attenuates Their Growth. Cell Stem Cell *18*, 587–590.

Watrin, L., Ghawché, F., Larre, P., Neau, J.P., Mathis, S., and Fournier, E. (2016). Guillain-Barré Syndrome (42 Cases) Occurring During a Zika Virus Outbreak in French Polynesia. Medicine (Baltimore) *95*, e3257.

Winocur, G., Wojtowicz, J.M., Sekeres, M., Snyder, J.S., and Wang, S. (2006). Inhibition of neurogenesis interferes with hippocampus-dependent memory function. Hippocampus *16*, 296–304.

Wu, K.Y., Zuo, G.L., Li, X.F., Ye, Q., Deng, Y.Q., Huang, X.Y., Cao, W.C., Qin, C.F., and Luo, Z.G. (2016). Vertical transmission of Zika virus targeting the radial glial cells affects cortex development of offspring mice. Cell Res. *26*, 645–654.

Yu, C.Y., Chang, T.H., Liang, J.J., Chiang, R.L., Lee, Y.L., Liao, C.L., and Lin, Y.L. (2012). Dengue virus targets the adaptor protein MITA to subvert host innate immunity. PLoS Pathog. *8*, e1002780.

Zellweger, R.M., and Shresta, S. (2014). Mouse models to study dengue virus immunology and pathogenesis. Front. Immunol. *5*, 151.

REQUEST FOR QUOTATION/PROPOSAL (RFQ/P)

GENERAL PROVISIONS

We would like to take this opportunity to remind suppliers of our commitment to conduct business with uncompromising integrity. This commitment is clearly established in Leidos' Code of Conduct. Leidos expects suppliers to conduct themselves in a manner consistent with the principles of our code of conduct. In addition, we strongly encourage our suppliers to have proactive and meaningful ethics programs established within their organizations. We want our suppliers to understand, foster, and mirror the ethical conduct we expect from our employees in all business transactions. If you believe that Leidos or any of its employees or agents has acted improperly or unethically, please report such behavior to the Leidos ethics HOTLINE (855) 7LEIDOS.

1. **LATE OFFERS.** Formal offers, amendments, or requests for withdrawal of offers received after the date specified for submittal may not be considered.

2. **ALTERNATE PROPOSALS.** In addition to the offer solicited herein, the offeror is invited to submit an alternate proposal, which may be advantageous to Leidos ("Leidos" or "Buyer").

3. **DISCLOSURE OF PARTICIPANTS.** All lower-tier subcontractors must be disclosed with the proposal.

4. **COMPLETENESS.** All information required by RFQ/P must be supplied to constitute a responsive bid. Non-responsive offers may not be considered.

5. **BRAND NAMES.** Brand names and part numbers, when used, are for reference to indicate the performance or quality desired. Equal items will be considered provided that the offeror describes the article. Offers for equal items shall state the brand name and part number, or level of quality. The determination of the Buyer as to what items are equal shall be final and conclusive. When brand name, part number, or level of quality is not stated by the offeror, it is understood the offer is exactly as specified.

6. **COUNTERFEIT PRODUCTS.** For purposes of this clause, Goods are any tangible items, including without limitation the lowest level of separately identifiable items, such as parts, articles, components, and assemblies. "Counterfeit Goods" are Goods that are or contain items misrepresented as having been designed, produced, and/or sold by an authorized manufacturer and seller, including without limitation unauthorized copies, replicas, or substitutes. The term also includes authorized Goods that have reached a design life limit or have been damaged beyond possible repair, but are altered and misrepresented as acceptable.

   Offeror agrees and shall ensure that Counterfeit Goods are not delivered to Leidos. Goods delivered to Leidos or incorporated into other Goods and delivered to Leidos shall be new and shall be procured directly from the Original Component Manufacturer (OCM)/Original Equipment Manufacturer (OEM), or through an OCM/OEM authorized distributor chain. If requested by Leidos, bidder shall provide OCM/OEM documentation that authenticates products. Offeror shall report suspected or confirmed counterfeit items into the Government-Industry Data Exchange Program (GIDEP).

7. **NET PRICES.** Offered prices, unless otherwise specified, must be net, including transportation and handling charges, which shall be set forth as a separate line item on quotation/proposal. Transportation charges must be fully prepaid by offeror to destination, and subject only to cash discount for prompt payment of invoices.
   a. Prices should be quoted as "Unit" prices; do not quote "Lot" prices.
   b. Provide pricing schedule based on specified price breaks, if any.
   c. If applicable, furnish published price list with offer.

8. **EVALUATION.** Buyer reserves the right: (1) to award on the basis of individual items, or groups of items, or on the entire list of items; (2) to reject any or all offers, or any part thereof; (3) to waive any informality in the offers; and (4) to accept the offer that is in the best interest of Leidos. The Buyer's decision shall be final.

9. **NO BID.** In the event an offer cannot be submitted for the specified requirements as set forth in the RFQ/P, please provide an explanation as to why you are unable to bid on these requirements.

10. **TAXES.** Buyer may be exempt from the payment of any federal excise or any state sales tax. The price offered must be net, exclusive of taxes. However, when under established trade practice, any federal excise tax is included in the list price, offeror may quote the list price and shall show separately the amount of federal tax, either as a flat sum or as a percentage of the list price, which shall be deducted by Buyer.

11. **AWARD.** The order will be awarded to the lowest responsible and responsive offeror complying with all the provisions of the RFQ/P, provided the offered price is reasonable and is in the best interest of Buyer. The Buyer reserves the right to reject the offer of an offeror who has previously failed to perform properly or complete on time, contacts of similar nature, or the offer of an offeror who, under investigation shows is not in position to perform the order.

12. **ACCEPTANCE.** A written award mailed (or otherwise furnished) to the successful offeror shall be deemed to result in a binding contract without further action by either party.

13. **SERVICE LIFE.** When applicable, all offerors will state their company policy regarding the return of defective and unserviceable items or products as well as the minimum service life of the offered item or product.

14. **DELIVERY.** When applicable, all shipments are to be made F.O.B. to a specific destination as specified, or prepaid. Collect shipments can be accepted only with proper advance notification, and only with specific approval from the Buyer.

15. **PACKING SLIPS OR DELIVERY TICKETS.** When applicable, all shipments or deliveries shall be accompanied by Packing Slips or Delivery Tickets in duplicate and shall contain the following information for each item delivered:
    a. Purchase Order Number
    b. Item Number
    c. Description or Part Number
    d. Quantity Ordered
    e. Quantity Shipped
    f. Name of the Supplier
    g. The above requirement is extremely important when accepting shipments. Offerors are cautioned that failure to comply with these conditions shall be considered sufficient reason for refusal to accept the goods.

16. **LIABILITY.** Leidos is not obligated to pay for any expenses incurred as a result of your efforts to respond to this solicitation. Moreover, Leidos will not, by virtue of this solicitation, become obligated to award a purchase order. Leidos reserves the right to subsequently award the effort described herein to a respondent determined to be in compliance with the guidelines and in the overall best interest of Leidos and its customer.

    Leidos will not accept confidential or proprietary information without a signed Non-Disclosure Agreement. All information provided by you in response to this solicitation will remain the property of the Leidos.

    The Offeror shall hold Buyer, its officers, agents, servants, and employees, harmless from liability of any nature or kind because of use of any copyrighted, or uncopyrighted compositions; secret process, patented or unpatented invention; articles or appliances furnished or used under this bid, and agrees to defend, at Offeror's expense, any and all actions brought against Buyer, or itself because of the unauthorized use of such articles.

17. **GENERAL.** Any Purchase Order or Subcontract issued as a result of this Request for Quote/Proposal is subject to Buyer's Terms and Conditions regardless of offeror's terms and conditions submitted with the proposal. Specific exceptions are to be noted as part of offeror's proposal and if incorporated into Buyer's Purchase Order or Subcontract will become binding on the parties.

18. **TERMS and CONDITIONS.** This prospective order is subject to all attachments in this solicitation to include, but not limited to: Statement of Work (when applicable); Leidos' standard terms and conditions; Federal terms and Conditions, agency clauses, and/or any specific Customer clauses included; Supplier Representations and Certifications.

19. GRATUITIES. By acknowledgment of response to this RFQ/P, the offeror hereby certifies that no gratuities were offered by the offeror or solicited by any Leidos employee either directly or indirectly. Any situation where a gratuity is solicited should be reported immediately to the Leidos SVP, Contracts, Procurement and Pricing at 571-526-7051.

SUBCONTRACT AGREEMENT
FIRM FIXED PRICE (Federal)

SELLER:	**SUBCONTRACT NO:**
ADDRESS:	**DEFENSE PRIORITY ALLOCATION SYSTEM (DPAS) RATING:**
	FIRM FIXED PRICE:

INTRODUCTION

This Subcontract, effective **[Insert Date],** is made between Leidos, Inc. (hereinafter known as "Leidos" or "Buyer"), and **[insert Seller's name]** (hereinafter known as "Seller"). The work to be performed by Seller under this Subcontract will support Leidos' work under Prime Contract No. **[insert Prime Contract No.];** Task/Delivery Order No. **[insert Task Order No. if applicable]** that has been issued by **[insert agency's name]**. The work defined in Attachment I (Statement of Work and Schedule) shall be performed on a Firm Fixed Price basis in accordance with this Schedule A (Specific Terms and Conditions), and any document referenced herein.

SCHEDULE A – SPECIFIC TERMS AND CONDITIONS

1.0 PERIOD OF PERFORMANCE

The term of this Subcontract shall commence upon the effective date above and shall terminate on **[Insert Date].**

2.0 PRICE

The total, firm fixed price for the work to be performed under this Subcontract is **[Insert $ Amount]**. Unless the price of this Subcontract is amended in writing by mutual agreement of the parties, Leidos is not obligated to compensate Seller beyond the total price defined herein. The price set forth herein includes all applicable Federal, State and local taxes and duties including, but not limited to, sales, use and value-added taxes, arising out of or relating to Seller's delivery of goods or services to Leidos, or Seller's performance of this Subcontract.

3.0 INVOICES

Invoices shall be submitted to Leidos in accordance with Article 4.0 and shall contain the following information: LEIDOS as the billed-to name and address (11955 Freedom Drive, Reston, VA 20190), Seller's name and remit-to address, subcontract number, labor categories, project labor categories (as applicable), hourly rates, labor hours, and extended dollar totals by category. Material and other direct costs detail shall be separated from labor costs. Invoices will be delivered electronically to [Insert Buyer/SCA Name] at [Insert Buyer/SCA Email Address]. Invoices shall be submitted individually per email and the email's subject must be in the following format: Subcontract #, Invoice #, Invoice Amount.

Invoices shall clearly reference a unique invoice number on each invoice and the date of the invoice. Invoices shall include, as applicable, the "Amount Previously Billed" and the "Amount of this Invoice."

3.1 MINIMUM LABOR CATEGORY QUALIFICATION REQUIREMENTS [USE THIS ARTICLE IF APPLICABLE]

The following statement shall be included in all invoices that include costs for labor categories that are subject to minimum labor category qualification requirements for education, employment, licensing and/or professional certification for subcontractor personnel:

> "Seller has reviewed the qualifications of the individuals whose labor costs are being invoiced hereunder and hereby certify that all individuals meet the minimum labor category qualification requirements for education, employment, licensing and/ or professional certification for the specific labor categories for which his or her work is being billed."

Seller agrees that if this subcontract includes minimum labor category qualification requirements, Seller shall not invoice Leidos for any labor until subcontractor first verifies that each individual proposed to work satisfies the minimum labor category qualification requirements for each labor category. In instances where an individual does not meet all the minimum labor category qualification requirements, but Seller believes the individual is qualified to perform the work, Seller must request and receive a written approval from Buyer waiving some or all of the minimum labor category qualification requirements prior to permitting the individual to start work.

Seller agrees to promptly notify Leidos if it discovers, subsequent to assigning an individual to perform on a subcontract with minimum labor category qualification requirements, that a subcontractor employee does not meet one or more of the applicable minimum labor category qualification requirements.

Seller agrees to refund to Leidos all monies paid for any individual performing on a subcontract with minimum labor category qualification requirements where it is determined that the individual does not meet one or more of the applicable minimum labor category qualification requirements.

4.0 PAYMENT

Payment shall be made in accordance with the milestone schedule provided herein, Payment terms are Net 45 Days after acceptance of the delivered items or services and receipt of a proper invoice, unless otherwise specified in this Subcontract. Leidos may make any adjustments in Seller's invoices due to shortages, late delivery, rejections, or other failure to comply with the requirements of this Subcontract before payment. Cash Discounts will be taken from date of acceptance of delivered items, or date of a proper invoice, whichever is later. Progress, interim, or milestone payments shall not constitute final acceptance. Leidos may offset against any payment hereunder any amount owed to Leidos by Seller.

Milestone No.	Milestone Description	Due/Delivery Date	Payment Amount

Seller may select Automated Clearing House Credits ("ACH funds transfer"), as the means of settlement. With regard to such ACH funds transfer, a payment from Leidos to Seller shall be considered timely with respect to any payment due date contained herein if the ACH funds transfer is completed no later than four (4) business days after such payment due date. Leidos shall not be in breach of these terms and conditions, or suffer any loss of discount or other penalty, with respect to an ACH funds transfer that was initiated properly and timely by Leidos to the extent its completion is delayed because of failure or delay by the ACH funds transfer system, the operation of an ACH funds transfer system rule which could not be anticipated by Leidos, or rejection by the Seller's bank.

4.1 DELIVERY

Goods and services shall be delivered in accordance with Attachment I – Statement of Work and the Milestone Schedule provided in Section 4.0. Time is of the essence. All goods furnished under this Subcontract shall be delivered FOB Destination. Delivery shall not be deemed complete until all goods have been received and accepted by Leidos, notwithstanding delivery to any carrier. Services shall be deemed delivered after they have been performed, received, and accepted by Leidos.

5.0 AUDIT

At any time before final payment under this Subcontract, Leidos may request audit of the invoices or vouchers and supporting documentation. Seller shall accommodate any such Leidos request, but may request that Leidos and Seller execute a non-disclosure agreement to provide for the confidentiality of the information that is exchanged. Each payment previously made shall be subject to reduction to the extent of amounts, on preceding vouchers, that are found by Leidos not to have been properly payable and shall also be subject to reduction for overpayments or to increase for underpayments. Upon receipt and approval of the voucher designated by Seller as the "completion voucher" and supporting documentation, and upon compliance by Seller with all terms of this Subcontract, Leidos shall pay any balance due Seller.

5.1 REPRESENTATIONS AND CERTIFICATIONS

Seller attests to the accuracy of the Representations and Certifications incorporated into this order as attachment IV. The Seller shall provide immediate written notice to Leidos if, at any time prior to subcontract closeout, the Seller learns that its (i) certifications were erroneous; (ii) has become erroneous by reason of changed circumstances; or (iii) has been notified of any system deficiencies and/or related internal control structure or business system(s) that would affect its ability to report hours delivered accurately and completely, and bill costs according to FAR Part 31, Contract Cost Principles and Procedures. Failure to provide the required notice shall be a material breach of this Agreement and shall be grounds for immediate termination thereof by Leidos, in addition to any other remedies that may be available at law or in equity. Seller shall be responsible for submitting all periodic certifications to the cognizant Leidos Contractual POC when required by the referenced FAR regulation or when required by the terms of this agreement.

6.0 TECHNICAL AND CONTRACTUAL REPRESENTATIVES

The following authorized representatives are hereby designated for this Subcontract:

SELLER:		**LEIDOS:**	
TECHNICAL:	_____	TECHNICAL:	_____
PHONE:	_____	PHONE	_____
EMAIL:	_____	EMAIL:	_____

CONTRACTUAL:		CONTRACTUAL:	
PHONE:		PHONE:	
EMAIL:		EMAIL:	

All notices or other written communication required or permitted to be given under any provision of this Subcontract shall be in writing and shall be deemed to have been given by the notifying party if delivered by hand, facsimile (with confirmed receipt), electronic media (with confirmed receipt) or mailed by an overnight delivery service, to the receiving party's above-identified contractual representative.

7.0 PERSONNEL

(a) If applicable, personnel performing under this Subcontract shall meet or exceed the minimum qualification and experience requirements specified in Attachment II, Labor Categories/Qualifications.

(b) Seller shall designate "Key Personnel" who are essential to the successful completion and execution of this Subcontract. Key Personnel shall perform all work necessary for the timely and quality completion of the task to which they are assigned. Seller may not substitute or replace a Key Personnel without Leidos' prior written approval. Seller's Key Personnel are: **[INSERT NAMES HERE].**

(c) Leidos reserves the right to direct the removal of any individual assigned to this Subcontract.

8.0 WARRANTY

In addition to any other warranties specified herein or provided by the manufacturer, Seller warrants that; 1) the services provided under this Subcontract shall be performed with that degree of skill and judgment normally exercised by recognized professional firms performing services of the same or substantially similar nature; and 2) that any goods delivered under this Subcontract will be new, unless otherwise specified, and **[FOR A PERIOD OF X YEARS]** following acceptance be free from defects in design, material and workmanship. All goods and services will conform to applicable specifications, drawings, and standards of quality and performance. In the event of any breach of the foregoing warranties, Seller shall, at its own expense, at Leidos' election either: (1) re-perform the non-conforming services and/or correct the non-conforming goods to conform to this standard; or (2) refund to Leidos that portion of the amounts received by Seller attributable to the non-conforming services and/or goods. All warranties of Seller shall inure to the benefit of both Leidos and Leidos' customers. The foregoing warranties shall survive any delivery, inspection, acceptance or payment by Leidos.

9.0 INDEMNIFICATION

(a) Seller shall indemnify, defend and hold Leidos and Leidos' customers harmless from and against any and all damages, losses, liabilities and expenses (including reasonable attorneys' fees) arising out of or relating to any claims, causes of action, lawsuits or other proceedings, regardless of legal theory, that result, in whole or in part, from Seller's (or any of Seller's subcontractors, suppliers, employees, agents or representatives): (i) intentional misconduct, negligence, or fraud, (ii) breach of any representation, warranty or covenant made herein,; (iii) breach of the confidentiality or disclosure provisions herein; or (v) violation of any law or regulation. Notwithstanding the foregoing, Seller's obligations under this Article shall not apply to the extent that a claim is finally determined by a court of competent jurisdiction to be caused by the negligence or willful misconduct of Leidos.

(b) Leidos shall promptly notify Seller of any claim that is covered by this indemnification provision and shall authorize representatives of Seller to settle or defend any such claim or suit and to take charge of any litigation in connection therewith.

9.1 INFRINGEMENT INDEMNITY

Seller shall indemnify, defend and hold Leidos and Leidos' customers, as well as their respective officers, directors, employees and agents (collectively, the "Indemnified Parties"), harmless from and against any claim, suit or proceeding ("Claim") asserting that the goods or services, or any part thereof, furnished under this Subcontract, or the use (including resale) thereof, constitutes an infringement of any patent, trademark, trade secret, copyright or other intellectual property right, and Seller shall pay all damages and costs awarded against and reasonable expenses incurred by the Indemnified Parties in connection with such claim, including reasonable attorneys' fees. In the event such goods or services or use thereof are enjoined in whole or in part, Seller shall at its expense and Leidos' or its customer's option undertake one of the following: (i) obtain for Leidos and its customer the right to continue the use of such goods or services; (ii) in a manner acceptable to Leidos and its customer, substitute equivalent goods or services or make modifications thereto so as to avoid such infringement and extend this indemnity thereto; or (iii) refund to Leidos an amount equal to the purchase price for such goods or services plus any excess costs or expenses incurred in obtaining substitute goods or services from another source.

Notwithstanding the foregoing paragraph, when this Subcontract is performed under the Authorization and Consent of the U.S. Government to infringe U.S. patents, Seller's liability for infringement of such patents in such performance shall be limited to the extent of the obligation of Leidos to indemnify the U.S. Government.

10.0 INSURANCE

Without prejudice to Seller's liability to indemnify Leidos as stated in any Indemnification provision contained in this Subcontract, Seller shall procure at its expense and maintain for the duration of this Subcontract, and ensure that any of its subcontractors used in connection with this Subcontract procure and maintain, the insurance policies required below.

(a) **Workers' Compensation:** Coverage for statutory obligations imposed by laws of any State in which the work is to be performed. Where applicable, Seller shall provide evidence of coverage for the United States Longshore & Harborworkers' Act (USL&H) coverage for employees engaged in work on or near navigable waters of the United States, and Defense Base Act (DBA) including all employees working on U.S. Government contracts outside the United States. Such policy(ies) shall be endorsed to provide a waiver of subrogation in favor of Leidos, its directors, officers and employees, and Leidos' customer where required by Leidos' Prime Contract with its customer. Employer's Liability coverage of $1 million each accident shall also be maintained.

(b) **Commercial General Liability:** Coverage for third party bodily injury and property damage, including products and completed operations, contractual liability, and independent contractors' liability with limits not less than $1,000,000 per occurrence and $2,000,000 in the aggregate. Such policy(ies) shall be endorsed to name Leidos, its directors, officers and employees, and Leidos' customer where required by Leidos' Prime Contract with its customer, as Additional Insureds.

(c) **Business Automobile Liability**: Coverage for use of all owned, non-owned, and hired vehicles with limits of not less than $1,000,000 per accident combined single limit for bodily injury and property damage liability. Such policy(ies) shall be endorsed to name Leidos, its directors, officers and employees, and Leidos' customer where required by Leidos' Prime Contract with its customer, as Additional Insureds.

(d) **Professional Liability / Errors and Omissions:** If seller is performing any professional services, coverage for damages (including financial loss) caused by any acts, errors and omissions arising out of Seller's performance or failure to perform professional services with limits of not less than $1,000,000 per claim.

The Additional Insured coverages above shall be primary and non-contributing with respect to any other insurance that may be maintained by Leidos and notwithstanding any provision contained herein, the Seller, and its employees, agents, representatives, consultants, subcontractors and suppliers, are not insured by Leidos, and are not covered under any policy of insurance that Leidos has obtained or has in place.

Any self-insured retentions, deductibles and exclusions in coverage in the policies required under this Article shall be assumed by, for the account of, and at the sole risk of Seller. In no event shall the liability of Seller or any subcontractors be limited to the extent of any of insurance or the minimum limits required herein.

Prior to commencement of any work, and within 15 days of any policy renewal that occurs while any work is on-going under this Subcontract, Seller shall provide Leidos evidence of the insurance coverage required above, including evidence of additional insured status and waivers of subrogation where required. Failure of Buyer to demand such evidence or to identify any deficiency in the insurance provided shall not be construed as or deemed to be a waiver of Seller's, or its subcontractors', obligations to maintain the above insurance coverages.

11.0 LEIDOS FURNISHED ITEMS AND INTELLECTUAL PROPERTY

(a) All items furnished, loaned or bailed by Leidos to Seller hereunder, or purchased, or otherwise acquired by Seller for the performance of and specifically charged to Leidos under this Subcontract (collectively, the "Items"), are the property of Leidos (or, as directed by Leidos pursuant to the terms of its prime contract, the U.S. Government). Upon completion, expiration or termination of this Subcontract, Seller shall return all Items in good condition (reasonable wear only accepted) together with all spoiled and surplus Items to Leidos. In lieu of the return of Items to Leidos, Seller shall make such other disposition of all Items as directed in writing by Leidos. Seller agrees to replace, at its expense, all such Items not returned in accordance with this Section or returned in other than good condition. Seller shall not charge Leidos for any storage, maintenance or return of any Items. Except as provided for in any flow down clauses, Seller shall bear all risk of loss for all Items in Seller's possession or for which Seller is responsible. Seller also agrees to use any designs, data or other things contained or embodied in Items provided to or utilized by Seller under this Subcontract in accordance with any restrictive legends placed on such Items by Leidos or any third party. If Leidos furnishes any material (including but not limited to any computer software or other data) for fabrication pursuant to this Subcontract, Seller agrees: (i) not to substitute any other material for such fabrication without Leidos' prior written consent and (ii) that title to such material shall not be affected by incorporation in or attachment to any other property.

(b) Seller understands and agrees that each of the Intellectual Property-related clauses specified in Schedule B, which may include (but are not limited to) FAR 52.227-1, 52.227-11, 52.227-14, 52.227-15 and 52.227-16 (and/or DFARS 252.227-7013, 252.227-7014 and 252.227-7015 if Leidos' prime contract is with the DOD), are incorporated herein as though fully set forth and shall take precedence over any other terms in this Subcontract. For avoidance of doubt, Seller hereby grants to Leidos such intellectual property rights as Leidos needs in order to perform its obligations to Leidos' U.S. Government customers. Seller shall not assert any

intellectual property right provided to the U.S. Government in a manner inconsistent with Leidos' contract obligations to Leidos' U.S. Government customers.

(c) To the extent that Seller provides any commercial computer software under this Agreement, the Parties agree that any normal commercial terms governing such commercial items shall govern use of such commercial items, except to the extent that such normal commercial terms shall conflict or be inconsistent with applicable federal law or regulation. In the case of any conflict or inconsistency, the applicable federal law or regulation shall take precedence over any conflicting or inconsistent commercial term. The Parties further agree that the use of any commercial terms shall be contingent upon the acceptance of any commercial computer software by the U.S. Government. In addition, the Parties agree that to the extent that the U.S. Government is the end user of any commercial computer software provided by Seller, Leidos shall have the right to perform Leidos' contract obligations to its U.S. Government Customers using that commercial computer software.

11.1 GOVERNMENT PROPERTY REQUIREMENTS (AS APPLICABLE)

When Government Property is applicable to this subcontract, unless otherwise specified, any property acquired under this subcontract shall become Government property. Seller shall i) certify to the completion of a physical inventory (all government equipment and materials) and the results thereof NLT October 10, annually or as otherwise required by the agency applicable property clause incorporated in the Schedule B Part II; ii) submit disposition requests when Government property is no longer required on the subcontract; iii) report any loss, damage, destruction or theft of Government property immediately after discovery; and iv) comply with close out of all Government property requirements to include, but not limited to submission of a final Government property report, final inventory report, and certification of the inventory condition code. All notices and required written communication shall be sent to subkproperty@leidos.com.

If Seller does not have a Government approved property system, Seller shall also comply with the following: i) Document receipt of Government property accountable to this subcontract (A copy of the signed packing slip or other documentation received with the property, including issue signature, must be forwarded within 30 days of receipt, report any item fabricated for use on the subcontract which is to be retained by the Seller, and annotate the receipt documentation with any Government property ID numbers with which the property is tagged; ii) Submit an annual (year ending June 30) physical inventory of Government equipment and material by July 31 that includes the Control number, Noun description, Model number (if applicable), Part number (if applicable), Serial number, unit price, date of last use for taggable property and inventories for material items should also include the quantity of line items and a total price; iii) Immediately report changes to the information provided to Leidos upon receipt of Government property; iv) Store and maintain property to protect it against deteriorating agents, adverse environments, damage and theft; v) Allow site access upon request to facilitate property surveillance activities required by Leidos' prime contract; vi) Obtain advanced approval from Leidos for the acquisition of any non-deliverable, which would not be consumable or expendable property and movement of Government property from authorized plants.

Unless otherwise authorized in writing, Government property is to be utilized only on the subcontract for which it is acquired or furnished. Property which is no longer being utilized should be reported when it becomes excess to the Seller's requirements.

12.0 DISCLOSURE

During the term of this Subcontract and for a period of three (3) years thereafter, Seller shall not disclose information concerning work under this Subcontract to any third party, unless such disclosure is required by law or necessary for the performance of this Subcontract. No news releases, public announcement, denial or confirmation of any part of the subject matter of this Subcontract or any phase of any program hereunder shall be made without prior written consent of Leidos which shall not be unreasonably withheld.

13.0 COMPLIANCE WITH LAW

Seller agrees to comply with the applicable provisions of any federal, state or local law or ordinance and all orders, rules and regulations issued thereunder. To the extent that performance of this Agreement requires that Seller's employees be deployed outside the United States ("Deployed Personnel"), Seller shall at all times be responsible for its Deployed Personnel's compliance with applicable local, state, and host nation laws, and Seller shall bear all risks, costs, damages or liabilities that may result from any non-compliance. Should Seller wish that its Deployed Personnel be authorized to carry a weapon, Seller: (1) shall forward a request to the Contracting Officer (or other authorized Government official) for approval; (2) shall comply with the requirements of all Government instructions, regulations, directives, orders, and policies pertaining to the possession and use of weapons by contractor personnel; (3) shall bear all risks, costs, damages or liabilities that may result from the possession or use of weapons by its Deployed Personnel, and (3) shall indemnify Leidos for any and all claims or liabilities that may arising from or relating to such weapons use or possession. Seller acknowledges and agrees that Leidos neither authorizes or requires Seller's Deployed Personnel to possess weapons.

14.0 COUNTERFEIT PRODUCTS

For purposes of this clause, Goods are any tangible items delivered under this Agreement, including without limitation the lowest level of separately identifiable items, such as parts, articles, components, and assemblies. "Counterfeit Goods" are Goods that are or contain items misrepresented as having been designed, produced, and/or sold by an authorized manufacturer and seller, including without limitation unauthorized copies, replicas, or substitutes. The term also includes authorized Goods that have reached a design life limit or have been damaged beyond possible repair, but are altered and misrepresented as acceptable.

Seller agrees and shall ensure that Counterfeit Goods are not delivered to Buyer. Goods delivered to Buyer or incorporated into other Goods and delivered to Buyer shall be new and shall be procured directly from the Original Component Manufacturer (OCM)/Original Equipment Manufacturer (OEM), or through an OCM/OEM authorized distributor chain. Work shall not be acquired from independent distributors or brokers unless approved in advance in writing by Buyer. When requested by Buyer, Seller shall provide OCM/OEM documentation that authenticates traceability of the affected items to the applicable OCM/OEM.

In the event that Work delivered under this Agreement constitutes or includes Counterfeit Goods, Seller shall, at its expense, promptly replace such Counterfeit Goods with authentic Goods conforming to the requirements of this Agreement. Notwithstanding any other provision in this Agreement, Seller shall be liable for all costs relating to the removal and replacement of Counterfeit Goods, including without limitation Buyer's costs of removing Counterfeit Goods, of reinserting replacement Goods, and of any testing necessitated by the reinstallation of Goods after Counterfeit Goods have been exchanged. Seller shall include equivalent provisions in lower tier subcontracts for the delivery of items that will be included in or furnished as Goods to Buyer.

14.1 CONFLICT MINERALS

Leidos fully supports the goals and objectives of Section 1502 of the Dodd-Frank Wall Street Reform and Consumer Protection Act (the "Act") which aims to prevent the use of certain "Conflict Minerals" that directly or indirectly finance or benefit armed groups in the Democratic Republic of the Congo (DRC) or its adjoining countries (as defined in the Act). It is the expectation of Leidos that all our suppliers have a policy to reasonably assure that the tantalum, tin, tungsten and gold ("3TG") in the products they manufacture are conflict free. In connection with that policy, Leidos expects our suppliers to undertake appropriate due diligence measures to determine if the 3TG materials in their products are being sourced from certified conflict-free smelters validated as compliant with the Conflict-Free Smelter Program (CFS) protocol and found on the CFS Compliant Smelter List. By accepting this order, Seller agrees to participate in future due diligence surveys from Leidos. Leidos routinely evaluates our relationship with our suppliers and will consider the extent to which a supplier has failed to comply with these responsibilities under the Act. If it is determined that a supplier's efforts are deficient, we may take appropriate action, including terminating our relationship with the supplier.

15.0 EXPORT CONTROL COMPLIANCE

Seller shall comply with all applicable U.S. export laws and regulations, including International Traffic in Arms Regulations ("ITAR") and the Export Administration Regulations ("EAR"). The subject technology of this Subcontract (including data, services, software and hardware provided hereunder, defined as "Controlled Technology") may be controlled under these laws and regulations and may not be exported or re-exported without prior authorization in accordance with ITAR and EAR. Access to Controlled Technology by Foreign Persons as defined by 22CFR120.16 may require an export authorization. Seller shall have full responsibility for obtaining any export licenses or authorization required to fulfill its obligations under this Subcontract.

15.1 ENDING TRAFFICKING IN PERSONS

As required by FAR 22.1701, FAR 22.1702, FAR 22.1703, and FAR 22.1704, and, where applicable, Defense Federal Acquisition Regulation Supplement (DFARS), employees, consultants, subcontractors, vendors, and agents performing any work under this Subcontract shall not: (i) engage in or support trafficking in persons; (ii) procure commercial sex acts; (iii) use forced labor in the performance of the contract; (iv) destroy, conceal, confiscate, or otherwise deny access by an employee to the employee's identity documents, such as passports or driver's licenses; (v) use misleading or fraudulent recruitment practices during the recruitment of employees, or fail to disclose information about the key terms and conditions of employment, including wages and fringe benefits, the location of work, living conditions and housing (if housing is provided or arranged), any significant costs to be charged to the employee, and, if applicable, the hazardous nature of the work; (vi) use recruiters that do not comply with local labor laws; (vii) charge recruitment fees to employees; (vii) fail to provide or reimburse return transportation costs upon the end of employment for non-national employees brought into a country for the purpose of working on a U.S. government contract or subcontract; (viii) provide or arrange housing that fails to meet the host country housing and safety standards; and (ix) fail to provide a written agreement if required by law or contract, in the employee's native language at least five days before the employee parts from his or her country of origin.

Seller shall immediately report suspected violations of these requirements to the Leidos Hotline at 855-753-4367. Incidents of suspected human trafficking activity may also be made to the Global Human Trafficking Hotline at 1-844-888-FREE and help@befree.org.

Should Seller violate any of these requirements set forth in FAR Subpart 22.17 during the course of performance under this Subcontract, Buyer may terminate performance of work under this Subcontract in whole or in part. Upon request, Seller shall provide information to Buyer demonstrating its compliance with these requirements, including, where applicable, a compliance plan and any corrective actions implemented as a result of any violations of these requirements.

16.0 ORGANIZATIONAL CONFLICT OF INTEREST

Seller represents and warrants that its performance of this Subcontract does not constitute and will not create an organizational conflict of interest (OCI) as defined in FAR Part 9.5 or under any other applicable OCI clause or regulation. If during the course of performance, Seller becomes aware of any actual or potential organizational conflict of interest caused by its performance of this Subcontract, Seller shall promptly notify Leidos in writing of the nature of such actual or potential organizational conflict of interest.

17.0 CHANGES

Seller shall assert its right to an adjustment under the applicable Changes clause within 20 days from the date of receipt of the written order. Failure to agree to any adjustment will be a dispute under the Disputes clause of this Subcontract, provided, however, that nothing in this clause excuses the Seller from proceeding with the work as changed without interruption and without awaiting settlement of any such dispute.

18.0 RESERVED

19.0 GOVERNING LAW

This Subcontract shall be governed by and construed in accordance with the laws of the Commonwealth of Virginia.

20.0 DISPUTES

(a) Leidos and Seller agree to enter into negotiations to resolve any dispute arising out of or relating to this Subcontract. Both parties agree to negotiate in good faith to attempt to reach a mutually agreeable settlement within a reasonable amount of time.

(b) Subject to the disputes provision set forth in Schedule B, if negotiations are unsuccessful, the parties agree that the sole and exclusive venue for any legal proceeding arising out of or relating to this Subcontract shall be in the state or federal courts of the Commonwealth of Virginia. The parties expressly submit to the jurisdiction of the state and federal courts of the Commonwealth of Virginia and agree that any party that unsuccessfully challenges the enforceability of this forum selection clause shall reimburse the other party all reasonable attorneys' fees and costs incurred in defending such unsuccessful challenge.

21.0 SUBCONTRACT CLOSEOUT

If Seller has applied indirect rates to any material, travel and/or other direct cost, Seller shall submit the Attachment V, Closeout Package that can be downloaded from www.leidos.com/suppliers, with a completion invoice bearing the statement "COMPLETION INVOICE" within 90 days of completion of the work. Seller shall also submit its Final invoice within 60 days of subcontract end, or 60 after receipt of final indirect rates when applicable. The Final invoice shall reflect any final audited indirect rate adjustments for the period(s) of performance bearing the statement, "This FINAL INVOICE was prepared using final audited rates as applicable to material, travel and/or other direct costs."

If indirect rates do not apply, Seller shall submit the Attachment V, Closeout Package with a FINAL invoice bearing the statement "FINAL INVOICE" within 60 days of completion of the work.

Leidos may unilaterally close-out this Subcontract

22.0 ASSIGNMENTS AND SUBCONTRACTS

This Agreement may not be assigned, novated or otherwise transferred by operation of law or otherwise by Seller without prior written consent from Leidos, which consent shall not be unreasonably withheld; provided, however, that Leidos may deny consent where it is in Leidos' best interest to do so. Any change of control of Seller shall be deemed an assignment of this Agreement that requires the prior written consent of Leidos. For purposes of this Agreement, "change of control" means any merger, consolidation, sale of all or substantially all of Seller's assets or sale of a substantial block of Seller's stock. Any such assignment, novation or transfer not in accordance with this provision shall be a material breach of this Agreement and shall be grounds for immediate termination thereof by Leidos, in addition to any other remedies that may be available at law or in equity.

Seller shall obtain prior written consent before subcontracting any portion of the work covered in this agreement that was not disclosed at the time of Seller's proposal. Further, Seller shall notify the Buyer's Contractual POC in writing if the Seller changes the amount of any lower-tier subcontract effort such that, the sum of lower-tier subcontractor(s) performance exceeds 70 percent of the total cost of work to be performed by Seller under the Agreement. The notification shall identify the revised percentage of

Seller's effort and shall include verification that the Seller will provide added value as related to the work to be performed by the lower-tier subcontractor(s).

23.0 GENERAL RELATIONSHIP

Leidos shall be solely responsible for all liaison and coordination with Leidos' customer as it affects the applicable prime contract and this Subcontract. Seller's communications with Leidos' customer shall be limited to those necessary for the Seller's performance under this Subcontract. Any other communications between Seller and Leidos' customer requires the prior written approval of Leidos.

Seller is an independent contractor in all respects with regard to this Subcontract. Nothing contained in this Subcontract shall be deemed or construed to create a partnership, joint venture, agency, or other relationship other than that of contractor and customer.

24.0 NON-WAIVER OF RIGHTS

The failure of either party to insist upon strict performance of any of the terms and conditions in the Subcontract, or to exercise any rights or remedies, shall not be construed as a waiver of its rights to assert any of the same or to rely on any such terms or conditions at any time thereafter. The invalidity in whole or in part of any term or condition of this subcontract shall not affect the validity of other parts hereof.

25.0 STANDARDS OF BUSINESS ETHICS & CONDUCT

Leidos is committed to conducting its business fairly, impartially and in an ethical and proper manner. These characteristics make it imperative that Leidos employees adhere to a particularly high ethical standard in accordance with Leidos' Code of Conduct, which may be viewed at www.leidos.com. Leidos' expectation is that Seller also will conduct its business fairly, impartially and in an ethical and proper manner, consistent with the principles of the Leidos Code of Conduct. In addition, Leidos strongly encourages that Seller have proactive and meaningful ethics and compliance programs established within your organization. As evidence of our commitment, should Seller wish to review Leidos' ethics training for your organization, request a copy through Buyer's contractual point of contact. Leidos expects the Seller to understand, foster, and mirror the ethical conduct expected from our employees in all business transactions. If Seller has cause to believe that Leidos or any employee or agent of Leidos has acted improperly or unethically under this agreement/order, Seller shall report such behavior to the Leidos Ethics Hotline (855) 7LEIDOS. Leidos provides its Code of Conduct for informational purposes only, and makes no representations as to its appropriateness for use outside of Leidos.

26.0 DPAS PRIORITY RATING

If so identified, this Subcontract is a "rated order" certified for national defense use, and Seller's signature constitutes acceptance of requirements under the Defense Priorities and Allocation System Regulation (15 C.F.R. Part 700).

27.0 ORDER OF PRECEDENCE

The documents listed below are hereby incorporated by reference. In the event of an inconsistency or conflict between or among the provisions of this Subcontract, the inconsistency shall be resolved by giving precedence in the following order:

1. Schedule A: Specific Terms and Conditions (Rev. **[Insert Date]**)
2. Schedule B: U.S. Government Terms and Conditions, Part I – FAR Clauses (Rev. **[Insert Date]**)
3. Schedule B: Contract Clauses, Part II Clauses - **[Agency]**, (Rev. **[Insert Date]**)
4. Attachment I: Statement of Work (Rev. **[Insert Date]**)
5. Attachment II: Labor Categories/Qualification. **[Reserve if N/A]**
6. Attachment III: Small Business Subcontracting Plan **[Reserve if N/A]**.
7. Attachment IV: Seller's Representations and Certifications
8. Attachment V: Closeout Package (provided for at www.leidos.com/suppliers)

28.0 SURVIVAL

If this Subcontract expires, is completed, or is terminated, Seller shall not be relieved of those obligations contained in the following articles: 1.0, 2.0, 4.0, 4.1, 5.0, 5.1, 6.0, 8.0, 9.0, 9.1, 10.0, 11.0, 12.0, 13.0, 17.0, 19.0, 20.0, 23.0, 24.0, 26.0, 27.0, and 28.0.

29.0 ENTIRE AGREEMENT

The parties hereby agree that this Subcontract shall constitute the entire agreement and understanding between the parties hereto and shall supersede and replace any and all prior or contemporaneous representations, agreements or understandings of any kind, whether written or oral, relating to the subject matter hereof. In witness whereof, the duly authorized representatives of Leidos and the Seller have executed this Subcontract on the dates shown below.

By Seller's signature of this Subcontract, Seller certifies to the best of its knowledge and belief that no Federal appropriated funds have been paid or will be paid to any person for influencing or attempting to influence an officer or employee of any agency, a Member of Congress, an officer or employee of Congress, or an employee of a Member of Congress on its behalf in connection with the awarding of this subcontract.

Seller further certifies the organization or any of its Principals are not presently debarred, suspended, proposed for debarment or declared ineligible for the award of contracts by any Federal agency. In the event the Seller is proposed or debarred by any Federal agency during the performance term of this subcontract, Seller shall notify the Leidos contractual representative immediately in writing.

SELLER:	**LEIDOS, INC.**
(Company Name)	
x (Signature)	x (Signature)
NAME: (Type or Print)	**NAME:** (Type or Print)
TITLE:	**TITLE:**
DATE:	**DATE:**

SCHEDULE B – PART I – FAR CLAUSES

U.S. GOVERNMENT TERMS and CONDITIONS (FEDERAL – NON-COMMERCIAL ITEMS)

1. SCHEDULE B DEFINITIONS

Schedule B Definitions shall apply to all Schedule B terms incorporated into this subcontract (e.g., Part I, Part II). In the U.S. Government clauses contained in the Schedule B document(s), unless the context of the clause requires otherwise, the term "Contractor" shall mean Seller, the term "Contract" shall mean the Subcontract or Purchase Order, and the terms "Government," "Contracting Officer" and equivalent phrases shall mean Leidos and Leidos' Contractual Representative, respectively. It is intended that these clauses shall apply to Seller in such manner as is necessary to reflect the position of Seller as a subcontractor to Leidos, to insure Seller's obligations to Leidos and to the United States Government, and to enable Leidos to meet its obligations under its Prime Contract and the Subcontract. All references to Subcontract shall include purchase orders and unless otherwise stated, all definitions defined by FAR 2.101 shall be applied to the terms referenced herein.

2. DISPUTES

Notwithstanding any provisions herein to the contrary:

(a) If a decision relating to the Prime Contract is made by the Contracting Officer and such decision is also related to this Subcontract, said decision, if binding upon Leidos under the Prime Contract shall in turn be binding upon Leidos and Seller with respect to such matter; provided, however, that if Seller disagrees with any such decision made by the Contracting Officer and Leidos elects not to appeal such decision, Seller shall have the right reserved to Leidos under the Prime Contract with the Government to prosecute a timely appeal in the name of Leidos, as permitted by the Prime Contract or by law, Seller to bear its own legal and other costs. If Leidos elects not to appeal any such decision, Leidos agrees to notify Seller in a timely fashion after receipt of such decision and to assist Seller in its prosecution of any such appeal in every reasonable manner. If Leidos elects to appeal any such decision of the Contracting Officer, Leidos agrees to furnish Seller promptly with a copy of such appeal. Any decision upon appeal, if binding upon Leidos, shall in turn be binding upon Seller. Pending the making of any decision, either by the Contracting Officer or on appeal, Seller shall proceed diligently with performance of this Subcontract.

(b) If, as a result of any decision or judgment which is binding upon Seller and Leidos, as provided above, Leidos is unable to obtain payment or reimbursement from the Government under the Prime Contract for, or is required to refund or credit to the Government, any amount with respect to any item or matter for which Leidos has reimbursed or paid Seller, Seller shall, on demand, promptly repay such amount to Leidos. Additionally, pending the final conclusion of any appeal hereunder, Seller shall, on demand, promptly repay any such amount to Leidos. Leidos' maximum liability for any matter connected with or related to this Subcontract which was properly the subject of a claim against the Government under the Prime Contract shall not exceed the amount of Leidos' recovery from the Government.

(c) If this Subcontract is issued by Leidos under a Government subcontract rather than a Prime Contract, and if Leidos has the right under such Government subcontract to appeal a decision made by the Contracting Officer under the Prime Contract in the name of the Prime Contractor (or if Leidos is subject to any arbitrator's decision under the terms of its subcontract), and said decision is also related to this Subcontract, this Disputes Clause shall also apply to Seller in a manner consistent with its intent and similar to its application had this Subcontract been issued by Leidos under a Prime Contract with the Government.

(d) Seller agrees to provide certification that data supporting any claim made by Seller hereunder is made in good faith and that the supporting data is accurate and complete to the best of Seller's knowledge or belief, all in accordance with the requirements of the Contract Disputes Act of 1978 (41 USC 601-613) and implementing regulations. If any claim of Seller is determined to be based upon fraud or misrepresentation, Seller agrees to defend, indemnify and hold Leidos harmless for any and all liability, loss, cost or expense resulting therefrom.

(e) Any dispute not addressed in paragraph (a) above, will be subject to the disputes clause of Schedule A of the Subcontract or PO terms and conditions.

3. INDEMNIFICATION - COST OR PRICING DATA - COST ACCOUNTING STANDARDS – OTHER

Seller agrees to indemnify and hold Leidos harmless to the full extent of any cost or price reduction, plus interest, effected by Leidos' customer, which may result from (i) certified cost or pricing data submitted by Seller or its lower-tier subcontractors which is not accurate, current or complete as certified by Seller; (ii) the failure by Seller or its lower-tier subcontractors to disclose and consistently follow applicable cost accounting practices and standards or otherwise comply with pertinent parts of the FAR, applicable agency supplements thereto, and standards and regulations promulgated by the Cost Accounting Standards Board. Seller further agrees to indemnify, defend, and hold Buyer harmless from and against any losses, liabilities, offsets and expenses (including reasonable attorney's fees) arising out of or relating to Seller's failure to comply with (i) the provisions of the Anti-Kickback Act or FAR 52.203-7; or (ii) 52.203-13, Contractor Code of Business Ethics and Conduct, if applicable to this Subcontract.

4. CONTRACT COST PRINCIPLES AND PROCEDURES

Seller agrees that to the extent applicable, costs allocated to this Subcontract shall be in full compliance with Subpart 31.2 of FAR (Subpart 31.3 for Educational Institutions) and the applicable agency supplements thereto, if any, set forth in Part II hereof. In the event such compliance is not maintained, Seller agrees to compensate Leidos to the full extent of any prices or costs, including any penalties or interest that are determined by Leidos' customer to be unallowable or unreasonable or not allocable, under Leidos' contract with its customer.

5. FAR CLAUSES APPLICABLE TO THIS SUBCONTRACT

The U.S. Government terms or clauses in FAR Subpart 52.2 referenced herein are incorporated and made a part of this Subcontract, unless:

(a) The description is preceded by a selection box (☐), the FAR clause (and its alternate) shall only apply when selected;

(b) Unless otherwise specified for performance applicability; or

(c) A value (e.g., >$150k) is identified. Clauses are applicable when the subcontract ceiling value (to include all options) exceeds the specified values at the time of award or after modification unless otherwise specified. Note: References to "SAT" refers to the "Simplified Acquisition Threshold" as defined by FAR 2.101 and will be the threshold that is current on the date of the Subcontract award.

To the extent that the Prime Contract or higher-tier subcontract under which this Subcontract is issued, is modified (to include a newer version of any such clause), the date of the modified version of the clause as it appears in such Prime Contract or higher-tier subcontract shall be controlling and said version shall be incorporated herein.

SECTION I: APPLICABLE TO ALL - FEDERAL STANDARD & FEDERAL COMMERCIAL ITEMS	
52.203-6	Restrictions on Subcontractor Sales to the Government (>**SAT**) (**Alternate I** applies to Commercial Items)
52.203-7	Anti-Kickback Procedures (except paragraph (c) (1)) - (>$150k)
52.203-12	Limitation on Payments to Influence Certain Federal Transactions (>$150k)
52.203-13	Contractor Code of Business Ethics and Conduct (Applies when POP >120 days; and >**$5.5M**)
☐ 52.203-15	Whistleblower Protections Under the American Recovery and Reinvestment Act of 2009
52.203-16	Preventing Personal Conflicts of Interest (>$150k)
52.203-17	Contractor Employee Whistleblower Rights and Requirements to Inform Employees or Whistle Blower Rights (>**SAT**)
52.203-19	Prohibition on Requiring Certain Internal Confidentiality Agreements or Statements
52.204-9	Personal Identity Verification of Contractor Personnel (Applies when support requires routine access to Federally-controlled facilities or Federally-controlled information systems)
52.204-21	Basic Safeguarding of Covered Contractor Information Systems (Applies when the subcontractor may have Federal contract information residing in or transiting through its information system)
52.209-6	Protecting the Governments Interest When Subcontracting with Contractors Debarred, Suspended, or Proposed for Debarment (>$35k)
☐ 52.217-6	Option for Increased Quantity (Add " " for, "the period of time in which the Contracting Officer has to exercise the option)
☐ 52.217-7	Option for Increased Quantity—Separately Priced Line Item (Add " " for, "the period of time in which the Contracting Officer has to exercise the option)
☐ 52.217-8	Option to Extend Services (Add " " for, "the period of time within which the Contracting Officer may exercise the option.")
☐ 52.217-9	Option to Extend the Term of the Contract (Subparagraph (a) Add " " for, "the period of time within which the Contracting Officer may exercise the option," and, add " " for the number of days for written notice. Subparagraph (c) Add " " for the duration (specified in months or years) for total duration of this contract.)
52.219-8	Utilization of Small Business Concerns
52.219-9	Small Business Subcontracting Plan (>$700k; n/a to small business concern)
52.222-3	Convict Labor
52.222-4	Contract Work Hours and Safety Standards —Overtime Compensation (Applies to subcontracts that may require or involve the employment of laborers and mechanics)
52.222-17	Non-displacement of Qualified Workers (>**SAT**)
52.222-20	Walsh-Healey Public Contracts Act (Applies when manufacturing or furnishing materials, supplies, articles, or equipment and performed within the United States, Puerto Rico or the U.S. Virgin Islands; and >$15k)

52.222-21	Prohibition of Segregated Facilities
52.222-26	Equal Opportunity
52.222-35	Equal Opportunity for Veterans (>$150k).
52.222-36	Affirmative Action for Workers with Disabilities (>$15k)
52.222-37	Employment Reports on Special Disabled Veterans, Veterans of the Vietnam Era and Other Eligible Veterans (>$150k)
52.222-40	Notification of Employee Rights Under the National Labor Relation Acts (>$10,000 and subcontract will be performed wholly or partially in the United States)
52.222-41	Service Contract Labor Standards
52.222-42	Statement of Equivalent Rates for Federal Hires
52.222-43	Fair Labor Standards Act and Service Contract Act – Price Adjustment (Applies to Multiple Year and Option Contracts)
52.222-44	Fair Labor Standards Act and Service Contract Act – Price Adjustment
52.222-50	Combating Trafficking in Persons
FAR 52.222-41 through -44 are inapplicable if either FAR 52.222-51 or -53 is selected.	
☐ 52.222-51	Exemption from the Application of the Service Contract Act of Contracts for Maintenance, Calibration or Repair of Certain Equipment – Requirements
☐ 52.222-53	Exemption from the Application of the Service Contract Act to Contracts for Certain Services – Requirements
52.222-54	Employment Eligibility Verification (>$3,500, n/a if performance is wholly OCONUS)
52.222-55	Minimum Wages Under Executive Order 13658 (Applies to orders subject to the Service Contract Labor Standards statute or the Wage Rate Requirements (Construction) statute, and are to be performed in whole or in part in the United States)
52.222-56	Certification Regarding Trafficking in Persons Compliance Plan (Provision applies to non-COTS >$500K)
52.223-9	Estimate of Percentage of Recovered Material Content for EPA Designated Items (Applies to Awards that involve the use of EPA –designated items)
52.223-15	Energy Efficiency in Energy – Consuming Products
52.223-16	IEEE 1680 Standard for the Environmental Assessment of Personal Computer Products ☐ **Alternate I**
52.223-18	Encouraging Contractor Policies to Ban Text While Driving (>$3,500)
52.225-1	Buy American Act – Supplies (Applies unless 52.225-3 (with its alternates when applicable) or 52.222-5 is selected)
☐ 52.225-3	Buy American Act – Free Trade Agreements – Israeli Trade Act ☐ **Alternate I** ☐ **Alternate II** ☐ **Alternate III**
☐ 52.225-5	Trade Agreements
52.225-13	Restrictions on Certain Foreign Purchases
52.225-26	Contractors Performing Private Security Functions Outside the United States (Applies to support of combat operations or other significant military operations)
52.226-6	Promoting Excess Food Donation to on Profit Organizations (Applies when manufacturing or furnishing materials, supplies, articles, or equipment and performed within the United States, Puerto Rico or the U.S. Virgin Islands; and >$25k)
52.239-1	Privacy or Security Safeguards
52.244-6	Award of Commercial Items
52.245-1	Government Property (Applies when Government Property is applicable to the subcontract) ☐ **Alternate I** – Responsibility and Liability for Government Property (n/a to cost-reimbursement type Awards)
52.245-2	Government Property Installation Operation Services (Applies for fixed-price services)
52.247-64	Preference for Privately Owned U.S. Flag Commercial Vessels (Applies to ocean transfer of supplies) ☐ **Alternate I**
	Service Contract Reporting Requirements: First-tier subcontracts for services (including construction) valued at or above the thresholds set forth in 4.1703(a)(2) shall annually by October 15th provide a report to the Leidos Contractual POC that includes subcontract number, subcontractor name and DUNS number and the number of direct-labor hours expended on the services performed during the previous Government fiscal year (October 1-September 30). The subcontractor shall be advised that this information will be made available to the public as required by section 743 of Division C of the Consolidated Appropriations Act, 2010.

SECTION II: APPLICABLE TO FEDERAL ORDERS FOR NON-COMMERCIAL ITEMS

52.203-3	Gratuities
52.203-5	Covenant Against Contingent Fees
52.203-8	Cancellation, Rescission, and Recovery of Funds for Illegal or Improper Activity
52.203-10	Price or Fee Adjustment for Illegal or Improper Activity
52.203-14	Display of Hotline Poster(s) (>**$5.5M**)
52.204-2	Security Requirements (Applies to services where access to classified information is required) **Alternate I** (Applies to R&D or Educational Institutions) **Alternate II** (Applies to Architect Engineering Awards)
52.204-4	Printed or Copied Double-Sided on Recycled Paper
52.208-8	Required Sources for Helium and Helium Usage Data (Applies when support involves a major helium requirement)
52.211-5	Material Requirements
Negotiated Awards where the ceiling value exceeds the threshold in FAR 15.403-4(a)(1):	
52.214-26	Audit and Records-Sealed Bidding
52.214-28	Subcontractor Cost or Pricing Data - Modifications - Sealed Bidding
52.215-11	Price Reduction for Defective Cost or Pricing Data -Modifications
52.215-13	Subcontractor Cost or Pricing Data – Modifications
52.215-15	Pension Adjustments and Asset Reversions
52.215-21	Requirements for Cost or Pricing Data or Information Other Than Cost or Pricing Data – Modifications
Negotiated Awards where the ceiling value exceeds the threshold in FAR 15.403-4(a)(1) and when Buyer's submission of Certified Cost and Pricing Data was required.	
52.214-27	Price Reduction for Defective Cost or Pricing Data Modifications – Sealed Bidding
52.215-10	Price Reduction for Defective Cost or Pricing Data
52.215-12	Subcontractor Cost or Pricing Data
52.215-18	Reversion or Adjustment of Plans for Post-Retirement Benefits (PRB) Other Than Pensions
52.215-19	Notification of Ownership Changes
52.215-20	Requirements for Cost or Pricing Data or Information Other Than Cost or Pricing Data
52.215-2	Audit and Records-Negotiation (>SAT current at the time of award)
52.215-14	Integrity of Unit Prices (except paragraph (b)) (>SAT current at the time of award; except architect engineer services, utility services, services where supplies are not required, and petroleum products)
52.215-23	Limitations on Pass-Through Charges (Applicable to CR awards for non-DOD; and >SAT current at the time of award. If DoD, applies to all order types when >$750k)
52.217-8	Option to Extend Services
52.217-9	Option to Extend the Term of the Contract
52.219-16	Liquidated Damages – Subcontracting Plan (>$700k and when 52.219-9 is applicable)
52.222-1	Notice to the Government of Labor Disputes
52.222-38	Compliance with Veteran's Employment Reporting Requirements
52.223-3	Hazardous Material Identification and Material Safety Data (Applies to support of DOD programs involving Hazardous Material) **Alternate I** (Applies to support of Federal Civilian programs involving Hazardous Material)
52.223-5	Pollution Prevention and Right-to-Know Information (including **Alternate I and II**) - (Applies when performance is on a Federal facility)
52.223-6	Drug-Free Workplace
52.223-7	Notice of Radioactive Materials (Applies to subcontract performance containing use of radioactive materials)
52.223-11	Ozone – Depleting Substances (Applies to work containing ozone depleting substances)
52.224-1	Privacy Act Notification (Applies to awards for redesign, development, or operation of records on individuals that is subject to Privacy Act of 1974)
52.224-2	Privacy Act (Applies to awards for redesign, development, or operation of records on individuals that is subject to Privacy Act of 1974)
☐ 52.225-3	Buy American Act – Free Trade Agreements – Israeli Trade Act ☐ **Alternate I** ☐ **Alternate II** ☐ **Alternate III**
52.225-8	Duty-Free Entry

52.225-19	Contractor Personnel in a Designated Operational Area or Supporting a Diplomatic or Consular Mission Outside the United States (Applies to OCONUS performance)
52.227-1	Authorization and Consent **Alternate I** (Applies R&D awards) **Alternate II** (Applies when support involves communication services with a common carrier and the services are unregulated and not priced by a tariff schedule set by a regulatory body)
52.227-2	Notice and Assistance Regarding Patent and Copyright Infringement (>SAT current at the time of award)
52.227-3	Patent Indemnity
52.227-9	Refund of Royalties (Applies when royalties reported during negotiation of the subcontract >$250)
52.227-10	Filing of Patent Applications - Classified Subject Matter
52.227-11	Patent Rights - Ownership by the Contractor (Applies when support is experimental, developmental, or research performed by a small business or nonprofit organization)
52.227-13	Patent Rights – Ownership by the Government (Applies to experimental, developmental, or research work)
52.227-14	Rights in Data – General ☐ **Alternate I** ☐ **Alternate II** ☐ **Alternate III** ☐ **Alternate IV** ☐ **Alternate V**
☐ 52.227-17	Rights in Data - Special Works
☐ 52.227-18	Rights in Data - Existing Work
52.227-19	Commercial Computer Software License (Applies to any Computer Commercial Software delivered by Seller)
52.227-21	Technical Data Declaration, Revision & Withholding of Payment Major Systems (Applies when supporting the acquisition of a major system)
52.227-22	Major System - Minimum Rights (Applies when supporting the acquisition of Major Systems)
52.227-23	Rights to Proposal Data (Technical)
52.228-3	Worker's Compensation Insurance - DBA (Inapplicable when 52.228-4 is selected and OCONUS performance)
☐ 52.228-4	Worker's Compensation and War-Hazard Insurance Overseas (Applies to OCONUS performance)
52.228-5	Insurance - Work on a Government Installation (Applies when subcontract performance is on a Government installation)
52.229-3	Federal, State, and Local Taxes
52.229-6	Taxes - Foreign Fixed-Price Contracts (Applies to fixed-price OCONUS performance)
52.229-8	Taxes - Foreign Cost-Reimbursement Contracts (Applies to cost-reimbursement OCONUS performances)
52.230-X clauses apply (when selected) to negotiated awards >$750k at the time of award only.	
☒ 52.230-2	Cost Accounting Standards (except paragraph (b))
☐ 52.230-3	Disclosure and Consistency of Cost Accounting Practices (except p. (b))
☐ 52.230-4	Disclosure and Consistency of Cost Accounting Practices—Foreign Concerns
☐ 52.230-5	Cost Accounting Standards-Educational Institution (except para (b))
☐ 52.230-6	Administration of Cost Accounting Standards
52.232-16	Progress Payments (Notwithstanding paragraph 1. above, in paragraph 52.232-16(d),"Government" means the "U.S. Government" except in subdivision (d)(2)(iv).
52.232-17	Interest
52.232-40	Providing Accelerated Payments to Small Business Subcontractors (Applies to small businesses)
52.233-3	Protest After Award
52.234-1	Industrial Resources Developed Under Defense Production Act Title III (Applies to major systems and supply contracts)
52.234-4	Earned Value Management System (Applies when use of an Earned Value Management System (EVMS) is required by the subcontract)
52.237-2	Protection of Government Buildings, Equipment and Vegetation (Applies when services are not performed on Seller site)
52.237-7	Indemnification and Medical Liability Insurance (Applies to health care services)
52.242-13	Bankruptcy
52.242-15	Stop-Work Order
52.244-5	Competition in Subcontracting
52.246-16	Responsibility for Supplies (Applies to supply Awards)

52.247-3	Capability to Perform a Contract for the Relocation of a Federal Office (Applies when performance involves moving a Federal office)
52.247-63	Preference for U.S. Flag Air Carriers (Applies for the use of International Air Transportation)
52.248-1	Value Engineering ☐ **Alternate II** (Applies if VECP requirement is required by the SOW)

Clauses below are applicable at the specified subcontract type only. [1]T&M/LH = T&M subcontracts where indirect rates are not applied to materials [2]CR = Cost reimbursable subcontracts (e.g., CPFF) including any T&M subcontracts where indirect rates are applied to the materials portion		**T&M[1]/LH**	**CR[2]**	**FFP**
52.215-15	Pension Adjustments and Asset Reversions	X	X	
52.215-17	Waiver of Facilities Capital Cost of Money (Applies if FCCM was not proposed by the Subcontractor nor included in the subsequent subcontract award)		X	
52.216-7	Allowable Cost and Payment **Alternate II** (Applies to Educational Institutions) **Alternate IV** (Applies to non-profit organizations other than Educational Institutions)	X	X	
52.222-2	Payment for Overtime Premiums -Subparagraph (a) Add "0"	X	X	
52.229-10	State of New Mexico Gross Receipts and Compensating Tax (Applies to the acquisition of tangible personal property and title to such property passes directly to and vests in the United States upon delivery)	X	X	
52.232-20	Limitation of Cost (Applies when subcontract is fully-funded)		X	
52.232-22	Limitation of Funds (Applies when subcontract is incrementally funded)		X	
52.232-32	Performance-Based Payments (Applies when negotiated payment amounts are performance-based)			X
52.242-1	Notice of Intent to Disallow Costs	X	X	
52.243-1	Changes - Fixed Price - **Alternate II** **Alternate III** (Applies to Architect-Engineer Services) **Alternate IV** (Applies to Transportation Services)			X
52.243-2	Changes - Cost-Reimbursement - **Alternate II**		X	
52.243-3	Changes – Time and Material – Labor Hour	X		
52.246-2	Inspection of Supplies - Fixed Price (Applies to the purchase of supplies)			X
52.246-3	Inspection of Supplies – Cost Reimbursement		X	
52.246-4	Inspection of Services - Fixed-Price (Applies to services)			X
52.246-5	Inspection of Services - Cost-Reimbursement		X	
52.246-6	Inspection - Time-and- Material and Labor-Hour	X		
52.246-7	Inspection of Research and Development – Fixed-Price			X
52.246-8	Inspection of Research and Development - Cost-Reimbursement (Applies to R&D with fee) **Alternate I** (Applies to R&D awards without fee)		X	
Termination clauses 52.249-2 through -6 are modified to require termination proposals to be submitted within 30 days (in lieu of 1 year)				
52.249-2	Termination for Convenience (Fixed-Price)			X
52.249-3	Termination for Convenience (Applies to dismantling, demolition, or removal of improvements Awards)			X
52.249-5	Termination for Convenience (Applies to Educational and Other Nonprofit Institutions)			X
52.249-6	Termination (Cost-Reimbursement)		X	
52.249-6	Termination (Cost-Reimbursement) – Alternate IV	X		
52.249-7	Termination (Applies to Fixed-Price Architect-Engineer)			X
52.249-8	Default (Fixed-Price) **Alternate I** – (Applies to Transportation Services)			X

SCHEDULE B – PART II – DOD FAR SUPPLEMENTS
APPLICABLE TO ALL DEPARTMENT OF DEFENSE (DOD) ORDERS – NON COMMERCIAL ITEMS

1. DFAR SUPPLEMENT CLAUSES APPLICABLE TO THIS SUBCONTRACT

All references herein to "DFAR Supplement" or "DFAR SUPP" shall mean the Department of Defense Supplement to the Federal Acquisition Regulation. The clauses in DFAR Supplement Subpart 252.2 referenced herein are incorporated and made a part of this Subcontract, unless the Description is preceded by a selection box (☐), the FAR clause (and its alternate) shall only apply when selected. To the extent that the Prime Contract or higher-tier subcontract under which this Subcontract is issued, is modified (to include a newer version of any such clause), the date of the modified version of the clause as it appears in such Prime Contract or higher-tier subcontract shall be controlling and said version shall be incorporated herein.

2. CONTRACTOR MANPOWER REPORTING

☒ If selected, Seller shall report ALL subcontractor labor hours required for performance of services provided under this subcontract via the Government's secure data collection site. Seller is required to completely fill in all required data fields using the following web address: http://www.ecmra.mil/. Reporting inputs will be for the subcontract labor executed during the period of performance of this subcontract, during each Government fiscal year (FY), which runs October 1 through September 30. While inputs may be reported any time during the FY, all data shall be reported no later than October 31 of each calendar year. Subcontractors may direct questions to the help desk at help desk at: http://www.ecmra.mil.

SECTION I: APPLICABLE TO ALL – DOD STANDARD & COMMERCIAL ITEMS	
252.203-7000	Requirements Relating to Compensation of Former DOD Officials
252.204-7012	Safeguarding Covered Defense Information and Cyber Incident Reporting (Applies when seller is providing operationally critical support, or for which performance will involve a covered contractor information system) Seller shall provide the cognizant Leidos contractual representative, notification of any incident report number, that has been submitted (for any tier) in accordance with 252.204-7012.
☐ 252.204-7014	Limitations on the Use or Disclosure of Information by Litigation Support Contractors
252.204-7015	Notice of Authorized Disclosure of Information for Litigation Support
252.211-7003	Item Unique Identification and Valuation *(when Government Property applies)*
252.211-7007	Reporting of Government-Furnished Property, subsection (d). (When Government Property applies)
252.222-7007	Representation Regarding Combating Trafficking in Persons
252.225-7001	Buy American Act and Balance of Payments Program (Applies unless 252.225-7021, 252.225-7036 or 252.225-7045 is selected) **Alternate I** (Applies to support of operations in Afghanistan)
252.225-7008	Restriction on Acquisition of Specialty Metals
252.225-7009	Restriction on Acquisition of Certain Articles Containing Specialty Metals
252.225-7012	Preference for Certain Domestic Commodities
252.225-7015	Restriction on Acquisition of Hand or Measuring Tools (Applies to supply orders for delivery of measuring and hand tools)
252.225-7017	Photovoltaic Devices (Applies if order results in DoD ownership of photovoltaic devices)
☐ 252.225-7021	Trade Agreements **Alternate II** (Applies to support of operations in Afghanistan)
252.225-7028	Exclusionary Policies and Practices of Foreign Governments (for purchase of supplies and services for international military education training and FMS)
☐ 252.225-7036	Buy American Act – Free Trade Agreement-Balance of Payments Program ☐ Alternate I ☐ Alternate II ☐ Alternate III ☐ Alternate IV ☐ Alternate V
252.225-7038	Restriction on Acquisition of Air Circuit Breakers (Applies for orders or subcontract performance that require air circuit breakers for naval vessels)
225.225-7039	Defense Contractors Performing Private Security Functions Outside the United States (Services only when performance is OCONUS)

252.225-7040	Contractor Personnel Authorized to Accompany U.S. Armed Forces Deployed Outside the United States (Services only. Applies when performance is OCONUS and personnel are authorized to accompany US Armed Forces)
☐ 252.225-7045	Balance of Payments Program – Construction Material Under Trade Agreements ☐ Alternate I ☐ Alternate II ☐ Alternate III
252.225-7048	Export Controlled Items
252-226-7001	Utilization of Indian Organizations, Indian-Owned Economic Enterprises, and Native Hawaiian Small Business Concerns **(>$500K)**
252.227-7013	Rights in Technical Data - Noncommercial Items **Alternate II** (Applies when subcontractor support is for the development or delivery of a vessel design or any useful article embodying a vessel design)
252.227-7015	Technical Data – Commercial Items (Applies to the delivery of technical data pertaining to commercial items, components or processes) **Alternate I** (Applies to the development or delivery of a vessel design or any useful article embodying a vessel design)
252.227-7019	Validation of Asserted Restrictions -Computer Software (Applies to the delivery of computer software)
252.227-7037	Validation of Restrictive Markings on Technical Data (Applies for the delivery of technical data)
252.236-7013	Requirement for Competition Opportunity for American Steel Producers, Fabricators, and Manufacturers (Applies to subcontractor support that includes the acquisition of steel as a construction material)
252.237-7010	Prohibition on Interrogation of Detainees by Contractor Personnel (Services only, when performance includes interaction with detainees)
252.237-7019	Training for Contractor Personnel Interacting with Detainees ((Services only, when performance includes interaction with detainees)
252.239-7018	Supply Chain Risk
252.244-7000	Subcontracts for Commercial Items
252.246-7003	Notification of Potential Safety Issues
252.246-7004	Safety of Facilities, Infrastructure, and Equipment for Military Operations (Applies when subcontractor support is for the construction, installation, repair, maintenance, or operation of facilities, infrastructure, or for equipment configured for occupancy, planned for use by DoD military or civilian personnel during military operations)
252.246-7007	Contractor Counterfeit Electronic Part Detection and Avoidance System (applicable to the acquisition of electronic parts or assemblies containing electronic parts)
252.247-7003	Pass-Through of Motor Carrier Fuel Surcharge Adjustment to the Cost Bearer **(**Applies to subcontracts that include the use of motor carriers, brokers, or freight forwarders)
252.247-7023	Transportation of Supplies by Sea
252.247-7024	Notice of Transportation of Supplies by Sea

SECTION II: APPLICABLE TO DOD ORDERS FOR NON-COMMERCIAL ITEMS	
252.203-7001	Prohibition on Persons Convicted of Fraud or Other Defense-Contract Related Felonies - Commercial items exempt (**>$150k**)
252.203-7002	Requirement to Inform Employees of Whistleblower Rights
252.203-7004	Display of Online Posters - Commercial items exempt **(>$5.5M)**
252.204-7000	Disclosure of Information
252.204-7009	Limitations on the Use or Disclosure of Third-Party Contractor Reported Cyber Incident Information (Applies when performance requires safeguarding covered defense information and cyber incident reporting)
252.209-7004	Subcontracting with Firms that are Owned or Controlled by the Government of a Terrorist Country
☐ 252.211-7000	Acquisition Streamlining **(>$1.5M)**
252.215-7000	Pricing Adjustments (Applies when 52.215-11, 52.215-12, or 52.215-13 are applicable)
252.219-7003	Small Business Subcontracting Plan - Small businesses exempt **(>$700K)**
252.219-7004	Small Business Subcontracting Plan (Test Program) - Small businesses exempt **(>$700K)**
☐ 252.222-7000	Restrictions on Employment of Personnel (services only)
252.222-7006	Restrictions On The Use Of Mandatory Arbitration Agreements
252.223-7001	Hazard Warning Labels (Applies to support involving hazardous material)

252.223-7002	Safety Precautions for Ammunition and Explosives (Applies to support involving ammunition or explosives)
252.223-7003	Change in Place of Performance *(Applies to support involving* Ammunition or Explosives)
252.225-7004	Report of Intended Performance Outside the United States and Canada – OCONUS performance only **(>$700K)**
252.225-7007	Prohibition on Acquisition of United States Munitions List Items from Communist Chinese Military Companies (Applies for items covered by the US Munitions List)
252.225-7013	Duty-Free Entry
252.225-7016	Restriction on Acquisition of Ball and Roller Bearings (Commercial items exempt)
252.225-7043	Antiterrorism/Force Protection Policy for Defense Contractors Outside the United States (Applies to OCONUS performance)
252.227-7014	Rights in Noncommercial Computer Software and Noncommercial Computer Software Documentation (Applies to the delivery of computer software or computer software documentation)
252.227-7016	Rights in Bid or Proposal Information
☐ 252.227-7020	Rights in Special Works
☐ 252.227-7021	Rights in Data - Existing Works
☐ 252.227-7022	Government Rights (Unlimited)
☐ 252.227-7023	Drawings and Other Data to Become Property of Government
252.227-7025	Limitations on the Use or Disclosure of Government-Furnished Information Marked w/ Restrictive Legends (Applies if subcontractor is provided with technical data marked with another parties' restrictive legends)
252.227-7030	Technical Data - Withholding of Payment
252.227-7033	Rights in Shop Drawings (Applies to construction orders)
252.228-7005	Accident Reporting & Investigation Involving Aircraft, Missiles, and Space Launch Vehicles
252.231-7000	Supplemental Cost Principles
252.235-7002	Animal Welfare (Services only. Applies to research of live vertebrate animals)
252.235-7003	Frequency Authorization (Services only, for the development, production, construction, testing, or operation of a device for which a radio frequency authorization is required)
252.237-7023	Continuation of Essential Contractor Services (If Subcontract provides "Essential contractor service")
252.239-7000	Protection Against Compromising Emanations (Applies when information technology requires protection against compromising emanations.
252.239-7010	Cloud Computing Services (Applies when order involves cloud services)
252.239-7016	Telecommunications Security Equipment, Devices, Techniques and Services (Applies to orders securing telecommunications)
252.245-7001	Reports of Government Property (when government property applies)
252.246-7001	Warranty of Data **Alternate II** (Applies to Fixed-Price subcontracts)
252.249-7002	Notification of Anticipated Contract Termination or Reduction - Services only (**>$150k**)

Schedule C – HR001117S0019 DARPA Additional BAA Requirements/Clauses

Proprietary Markings

Proposers are responsible for clearly identifying proprietary information. Submissions containing proprietary information must have the cover page and each page containing such information clearly marked with a label such as "Proprietary" or "Company Proprietary." NOTE: "Confidential" is a classification marking used to control the dissemination of U.S. Government National Security Information as dictated in Executive Order 13526 and should not be used to identify proprietary business information.

Unclassified Submissions

DARPA anticipates that submissions received under this BAA will be unclassified. However, should a proposer wish to submit classified information, an *unclassified* email must be sent to the BAA mailbox requesting submission instructions from the Technical Office PSO. If a determination is made that the award instrument may result in access to classified information, a SCG and/or DD Form 254 will be issued by DARPA and attached as part of the award.

Human Research Subjects/Animal Use

Proposers that anticipate involving Human Research Subjects or Animal Use must comply with the approval procedures detailed at www.darpa.mil/work-with-us/additional-baa. protection. Further, research involving human subjects that is conducted or supported by the DoD must comply with 32 CFR 219, *Protection of Human Subjects* (and DoD Instruction 3216.02, *Protection of Human Subjects and Adherence to Ethical Standards in DoD-Supported Research* (http://www.dtic.mil/whs/directives/corres/pdf/321602p.pdf). Institutions awarded funding for research involving human subjects must provide documentation of a current Assurance of Compliance with federal regulations for human subjects protection, such as a Department of Health and Human Services, Office of Human Research Protection Federal Wide Assurance (http://www.hhs.gov/ohrp). All institutions engaged in human subjects research, to include subcontractors, must also hold a valid assurance. In addition, all personnel involved in human subjects research must provide documentation of completion of human subjects research training. For all proposed research that will involve human subjects in the first year or phase of the project, the institution must provide evidence of or a plan for review by an Institutional Review Board (IRB) upon final proposal submission to DARPA as part of their proposal, prior to being selected for funding. The IRB conducting the review must be the IRB identified on the institution's Assurance of Compliance with human subjects protection regulations. The protocol, separate from the proposal, must include a detailed description of the research plan, study population, risks and benefits of study participation, recruitment and consent process, data collection, and data analysis. It is recommended that you consult the designated IRB for guidance on writing the protocol. The informed consent document must comply with federal regulations (32 CFR 219.116). A valid Assurance of Compliance with human subjects protection regulations along with evidence of completion of appropriate human subjects research training by all investigators and personnel involved with human subjects research should accompany the protocol for review by the IRB.

In addition to a local IRB approval, a headquarters-level human subjects administrative review and approval is required for all research conducted or supported by the DoD. The Army, Navy, or Air Force office responsible for managing the award can provide guidance and information about their component's headquarters-level review process. Note that confirmation of a current Assurance of Compliance with human subjects protection regulations and appropriate human subjects research training is required before headquarters-level approval can be issued. The time required to complete the IRB review/approval process varies depending on the complexity of the research and the level of risk involved with the study. The IRB approval process can last between one and three months, followed by a DoD review that could last between three and six months. Ample time should be allotted to complete the approval process. DoD/DARPA funding cannot be used toward human subjects research until ALL approvals are granted.

Human Subjects Research and Animal Use

Human Subjects Research

All research selected for funding involving human subjects, to include use of human biological specimens and human data, must comply with the federal regulations for human subjects protection. Further, research involving human subjects that is conducted or supported by the DoD must comply with 32 CFR 219, Protection of Human Subjects (and DoD Instruction 3216.02, Protection of Human Subjects and Adherence to Ethical Standards in DoD-Supported Research (http://www.dtic.mil/whs/directives/corres/pdf/321602p.pdf).

Institutions awarded funding for research involving human subjects must provide documentation of a current Assurance of Compliance with federal regulations for human subjects protection, such as a Department of Health and Human Services, Office of Human Research Protection Federal Wide Assurance (http://www.hhs.gov/ohrp). All institutions engaged in human subjects research, to include subcontractors, must also hold a valid assurance. In addition, all personnel involved in human subjects research must provide documentation of completion of human subjects research training.

For all proposed research that will involve human subjects in the first year or phase of the project, the institution must provide evidence of or a plan for review by an Institutional Review Board (IRB) upon final proposal submission to DARPA as part of their proposal, prior to being selected for funding. The IRB conducting the review must be the IRB identified on the institution's Assurance of Compliance with human subjects protection regulations. The protocol, separate from the proposal, must include a detailed description of the research plan, study population, risks and benefits of study participation, recruitment and consent process, data collection, and data analysis. It is recommended that you consult the designated IRB for guidance on writing the protocol. The informed consent document must comply with federal regulations (32 CFR 219.116). A valid Assurance of Compliance with human subjects protection regulations along with evidence of completion of appropriate human subjects research training by all investigators and personnel involved with human subjects research should accompany the protocol for review by the IRB.

In addition to a local IRB approval, a headquarters-level human subjects administrative review and approval is required for all research conducted or supported by the DoD. The Army, Navy, or Air Force office responsible for managing the award can provide guidance and information about their component's headquarters-level review process. Note that confirmation of a current Assurance of Compliance with human subjects protection regulations and appropriate human subjects research training is required before headquarters-level approval can be issued.
The time required to complete the IRB review/approval process varies depending on the complexity of the research and the level of risk involved with the study. The IRB approval process can last between one and three months, followed by a DoD review that could last between three and six months. Ample time should be allotted to complete the approval process. DoD/DARPA funding cannot be used toward human subjects research until ALL approvals are granted.

Animal Use

Award recipients performing research, experimentation, or testing involving the use of animals shall comply with the rules on animal acquisition, transport, care, handling, and use as outlined in: (i) 9 CFR parts 1-4, Department of Agriculture rules that implement the Animal Welfare Act of 1966, as amended, (7 U.S.C. § 2131-2159); (ii) National Institutes of Health Publication No. 86-23, "Guide for the Care and Use of Laboratory Animals" (8th Edition); and (iii) DoD Instruction 3216.01, "Use of Animals in DoD Programs."

For projects anticipating animal use, proposals should briefly describe plans for Institutional Animal Care and Use Committee (IACUC) review and approval. Animal studies in the program will be expected to comply with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, available at http://grants.nih.gov/grants/olaw/olaw.htm.

All award recipients must receive approval by a DoD-certified veterinarian, in addition to an IACUC approval. No animal studies may be conducted using DoD/DARPA funding until the United States Army Medical Research and Materiel Command (USAMRMC) Animal Care and Use Review Office (ACURO) or other appropriate DoD veterinary office(s) grant approval. As a part of this secondary review process, the award recipient will be required to complete and submit an ACURO Animal Use Appendix, which may be found at http://mrmc.amedd.army.mil/index.cfm?pageid=research_protections.acuro_animalappendix.

Intellectual Property

All proposers must provide a good faith representation that the proposer either owns or possesses the appropriate licensing rights to all intellectual property that will be utilized under the proposed effort.

For Procurement Contracts

Proposers responding to this BAA requesting procurement contracts will need to complete the certifications at DFARS 252.227-7017. See http://www.darpa.mil/work-with-us/additional-baa for further information. If no restrictions are intended, the proposer should state "none." The table below captures the requested information:

Technical Data Computer Software To be Furnished With Restrictions	Summary of Intended Use in the Conduct of the Research	Basis for Assertion	Asserted Rights Category	Name of Person Asserting Restrictions
(LIST)	(NARRATIVE)	(LIST)	(LIST)	(LIST)

For All Non-Procurement Contracts

Proposers responding to this BAA requesting a Cooperative Agreement, Technology Investment Agreement, or Other Transaction for Prototypes shall follow the applicable rules and regulations governing these various award instruments, but, in all cases, should appropriately identify any potential restrictions on the Government's use of any Intellectual Property contemplated under the award instrument in question. This includes both Noncommercial Items and Commercial Items. Proposers are encouraged to use a format similar to that described in the section above. If no restrictions are intended, then the proposer should state "NONE."

Controlled Unclassified Information (CUI) on Non-DoD Information Systems

In addition to classified information, certain types of unclassified information also require application of access and distribution controls and protective measures for a variety of reasons. This information is referred to collectively as Controlled Unclassified Information (CUI). DoD CUI includes, but is not limited to: For Official Use Only (FOUO), Law Enforcement Sensitive (LES), DoD Unclassified Controlled Nuclear Information (DoD UCNI), and Limited Distribution, as well as some categories developed by other executive branch agencies. For further information, consult DoDM 5200.01, Volume 4, "DoD Information Security Program: Controlled Unclassified Information (CUI)."

All non-DoD entities doing business with DARPA are expected to adhere to the following procedural safeguards, in addition to any other relevant federal or DoD specific procedures, for submission of any proposals to DARPA and any potential business with DARPA:

- Do not process DARPA CUI on publicly available computers or post DARPA CUI to publicly available webpages or websites that have access limited only by domain or Internet protocol restriction.
- Ensure that all DARPA CUI is protected by a physical or electronic barrier when not under direct individual control of an authorized user and limit the transfer or DARPA CUI to subcontractors or teaming partners with a need to know and commitment to this level of protection.
- Ensure that DARPA CUI on mobile computing devices is identified and encrypted and all communications on mobile devices or through wireless connections are protected and encrypted.
- Overwrite media that has been used to process DARPA CUI before external release or disposal. Proposers with questions regarding CUI should contact the DARPA Security and Intelligence Directorate at (703) 526-1581.

Publication Restrictions on Non-Fundamental Research

National Security Decision Directive (NSDD) 189 defines fundamental research as follows:

"Fundamental research" means basic and applied research in science and engineering, the results of which ordinarily are published and shared broadly within the scientific community, as distinguished from proprietary research and from industrial development, design, production, and product utilization, the results of which ordinarily are restricted for proprietary or national security reasons.

The following clause or similar language will be included in all awards where the resultant research will not be considered fundamental research and there will be restrictions on publication:

"There shall be no dissemination or publication, except within and between the contractor and any subcontractors, of information developed under this contract or contained in the reports to be furnished pursuant to this contract without prior written approval of DARPA's Public Release Center (DARPA/PRC). All technical reports will be given proper review by appropriate authority to determine which distribution statement is to be applied prior to the initial distribution of these reports by the contractor. With regard to subcontractor proposals for fundamental research, papers resulting from unclassified fundamental research are exempt from prepublication controls and this review requirement, pursuant to DoD Instruction 5230.27 dated October 6, 1987.

When submitting material for written approval for open publication, the contractor/awardee must submit a request for public release to the DARPA/PRC and include the following information: (1) Document Information: document title, document author, short plain-language description of technology discussed in the material (approx. 30 words), number of pages (or minutes of video), and document type (e.g., briefing, report, abstract, article, or paper); (2)

Event Information: event type (conference, principal investigator meeting, article, or paper), event date, desired date for DARPA's approval; (3) DARPA Sponsor: DARPA program manager, DARPA office, and contract number; and (4) Contractor/Awardee's Information: POC name, e-mail, and phone. Allow three weeks for processing. Due dates under three weeks require a justification. Unusual electronic file formats may require additional processing time. Requests may be sent either via email to public_release_center@darpa.mil or by mail to 675 North Randolph Street, Arlington VA 22203-2114, telephone (571) 218-4235. Refer to the following for link for information about DARPA's public release process: http://www.darpa.mil/work-with-us/contract-management/public-release.

SUMMARY OF PROPOSED COSTS

LABOR	**BASE PERIOD**
Period Dates (estimate)	Estimated 4 Year Period
Direct Labor	
Overhead Cost	
G&A Cost	
Subtotal	
Fixed Fee Percentage	
Fixed Fee Cost	
Total (Labor Cost Plus Fixed Fee)	
OTHER DIRECT COSTS	
FFP Subcontractor Costs	
Estimated Travel	
Materials	
Equipment	
Total Other Direct Costs	
Total Proposed Cost Plus Fixed Fee	

DARPA-P3

Offeror:
Date:

INSTRUCTIONS: Fill in additional information for Direct Labor Costs in Columns E, F and G. Add columns to Include escalation if necessary. If subcontractor agreements are Firm Fixed Price they can be listed as ODCs. If subs are direct labor, include costs here. The subtotal column is intended to provide the rate without fee included so it can be identified as a separate amounnt in the award. The rates in the total column will be used to compare the proposed rates to the base contract maximum rates, which include fee. The Offeror is responsible for ensuring mathematical calculations are correct.

SUMMARY OF DIRECT LABOR COSTS (INCLUDING BUILDUP)

BASE PERIOD

	Labor Category	Base Contract Attch 7 Position #	Prime/Subcontractor	Base/Direct Labor Rate	Fringe Percentage Enter the % for each category in this column, not the dollar value.	Overhead Percentage Enter the % for each category in this column, not the dollar value.	G&A Percentage Enter the % for each category in this column, not the dollar value.	Loaded Rate Without Fee	Hours	Subtotal		Fee Enter the fee percentage in the yellow block. Calculate the dollar value by summing at the bottom of this column.	Fully Loaded Rate	Total
												%		
1				$0.00	%	%	%	$0.00	0.00	$0.00		$0.00	$0.00	$0.00
2				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
3				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
4				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
5				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
6				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
7				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
8				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
9				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
10				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
11				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
12				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
13				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
14				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
15				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
16				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
17				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
18				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
19				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
20				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
21				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
22				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
23				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
24				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
25				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
				$0.00				$0.00	0.00	$0.00		$0.00	$0.00	**$0.00**

SUMMARY OF MATERIALS AND SUPPLIES

FFP Subcontractor Costs

Subcontractor	Task	Price	Basis for Fair and Reasonable Determination (see FAR 15.404)

Estimated Travel

Add additional trips if necessary. If no travel is proposed, delete this information and state 'no travel is proposed.'

	Trip 1	Trip 2	Trip 3
City of Trip Origin			
City of Trip Destination			
Justification			
Traveler (prime or sub)			
Airfare Class			
Airline			
Airfare Cost	$0.00	$0.00	$0.00
Lodging Cost	0.00	0.00	0.00
Meal Cost	0.00	0.00	0.00
Incidentals	0.00	0.00	0.00
No. of days			
Total Tip Cost	0.00	0.00	0.00
No. Trips per Period			
No. of Persons			
Total Trip Cost	0.00	0.00	0.00

Total Travel Cost	$0.00

Government Furnished Contractor Acquired Property

Add and delete rows as necesasry. If none is proposed, delete this information and state 'none is proposed.'

Item	Item Description (including part numbers)	Need Date	Quantity	Cost (rent-free basis)

SUMMARY OF MATERIALS AND SUPPLIES

Materials

Add or delete rows below as necessary. If no materiels are proposed, delete this information and state 'no materials are proposed.'

Item	Item Description	Quantity	Cost per Unit	No. of Units
			$0.00	
			0.00	
			0.00	
			0.00	
			0.00	
Total Material Cost				0.00

DARPA-P3

Offeror:
Date:

INSTRUCTIONS: Propose any costs for Contractor Manpower Reporting here. If you are proposing as 'Not Separate

CONTRACTOR MANPOWER REPORTING

		10/31/2017	10/31/2018	10/31/2019	10/31/2020	10/31/2021
1	Contractor Manpower Reporting	$0.00	$0.00	$0.00	$0.00	$0.00

This requirement is due No Later Than October 31 of each year.

ly Priced,' enter $0.00 as the cost.

Title of Contract Effort	
Name of Organization providing Reference	
Name of POC Providing Reference **Telephone Number** **Email Address**	**Name:** **Telephone:** **Email:**
Contract/Delivery Order Number	
Contract Type	
Contract Value	Total contract value
Final Delivery Schedule (PoP)	Entire contract PoP
SCOPE OF WORK	
Description of Contract Work ***Rationale for Comparability to the Proposed Effort***	

April 7, 2017

Subject: Formal Request for Proposal (RFP)
In response refer to RFP No. LEIDOS-DARPA-P3 _____(Your Company Name)

References: DARPA-HR001117S0019

Team Member:

You are requested to submit a **Firm Fixed Price** proposal in response to this Request for Proposal (RFP). The Statement of Work (SOW) and Specifications will be provided shortly in an amendment to this RFP. Your proposal must be in accordance with the terms, conditions, and special provisions included herein with this RFP. You must complete and return the Suppliers Reps and Certs (Attachment G) and the pricing template (Attachment H). On the last page of this letter is a Proposal Check List for you to use to ensure that all required documentation has been included in your proposal.

Your response is due by 2:00 PM (EST) on Wednesday, April 19, 2017.

PROPOSAL INSTRUCTIONS AND INFORMATION

Leidos is not obligated to pay for any expenses incurred as a result of your efforts to respond to this solicitation. Moreover, Leidos will not, by virtue of this RFP, become obligated to award a subcontract. Leidos reserves the right to subsequently award the effort described herein to one or more respondents determined to be in the overall best interest of Leidos.

All confidential or proprietary information shall be provided in accordance with the Non-Disclosure Agreement (NDA). All information provided by you in response to this solicitation will remain the property of **Leidos unless agreed to otherwise in an NDA.**

You are cautioned to strictly adhere to the enclosed proposal instructions, with particular attention to the form, format, and content of your proposal. Failure to do so may result in our decision to eliminate your proposal from further consideration. However, alternative proposals, which contain products or services which meet the functional requirements will be considered as separate proposals.

I. PROPOSAL COVER LETTER

Leidos requires a Proposal Cover Letter. Your cover letter must contain all of the following:

- ✓ Dated on or before Wednesday, April 19, 2017;
- ✓ Signed by an official within your organization authorized to legally bind your Company;
- ✓ State any exceptions taken to the terms and conditions of the SOW or "Draft" Subcontract Agreement (Schedule A, Schedule B Part I and Schedule B Part II) or any of its attachments;
- ✓ State a Proposal Validity Period of one hundred and eighty (180) days.

II. STATEMENT OF WORK

The Statement of Work (SOW) will be provided later.

III. Draft Subcontract Agreement and Required Clauses

It is anticipated that the effort set forth in this RFP will be performed on a Firm Fixed (FFP) basis. Any resultant award will include all clauses required by Leidos' Prime Contract, all clauses required by law on the date of execution of the Subcontract, and any other mutually agreeable clauses, provisions and terms and conditions, including:

- "Draft" Firm Fixed (FFP) Subcontract: Schedule A (Attachment C)
- Subcontract: Schedule B Part I – FAR Flow-down clauses (Attachment D)
- Subcontract: Schedule B Part II – DFAR Flow-down clauses (Attachment E)
- Subcontract: Schedule C – DARPA Unique Prime Contract Flowdown and Specific Requirements (Attachment F)

IV. Period of Performance

The P3 program will be approximately a four-year (4) effort from date of award.

V. CERTIFICATE OF INSURANCE

The insurance requirements set forth in Leidos' Schedule A may exceed your company's current insurance levels. Please cross-reference your company's current insurance policy with the Leidos requirements. Please provide a copy of your company's Certificate of Insurance. Upon award of a subcontract by Leidos, it is required that Leidos be named as additionally insured with your insurance provider. (No Waivers will be granted).

VI. Cost/Price Proposal

Per Section II of the DARPA-P3 BAA – Detailed Proposal Information the following cost/pricing detailed breakdown is required:

The cost/price proposal **MUST** be submitted in sufficient detail and must provide such supporting documentation as appropriate and necessary to permit proper evaluation and analysis.

1. Labor Rates

SAMPLE:

Labor Categories	Total Labor Hours	Labor Rate	Fringe Rate %	Overhead Rate %	G&A Rate %	Fee %	Fully Burdened Labor Rate	Total Price per Labor Category
Administrative Assistant	345							
Clinical Nurse Specialist	657							
Technical Writer I	565							
Total	**1468**							

2. **Indirect Costs** – Including Fringe Benefits, Overhead, General and Administrative Expense, Cost of Money, Fee, etc. (must show base amount and rate), if available, provide current Forward Pricing Rate Agreement or Forward Pricing Rate Proposal. If not available, provide 2 years historical data to include pool and expense costs used to generate the rates. For academia, provide DHHS or ONR negotiated rate package or, if calculated by other than a rate, provide University documentation identifying G&A and fringe costs by position;

3. **Travel:**

All proposed travel must be in accordance with:

GSA website: (CONUS): http://www.gsa.gov/portal/content/101518

Defense Travel Management website: (OCONUS):
http://www.defensetravel.dod.mil/site/perdiemCalc.cfm

All other cost (i.e. airfare and rental car) must have supporting documentation.

Trips must be identified by:

a. Departure and Arrival Destinations
b. Number employees per trip
c. Number of Trips per Period
d. Number of Days/Nights per trip
e. Estimated car rental costs
f. Estimated airfare costs

All Travel costs must be supported with screenshots from the travel websites.

4. **Other Direct Costs (ODC's)**

Subcontractor must provide a list of ODC's that will be procured for this requirement and pricing documentation.

Other Direct Costs – Itemized with costs including tuition remission, animal per diem rates, health insurance/fee; back-up documentation is to be submitted to support proposed costs;

Equipment Purchases – Itemization with individual and total costs, including quantities, unit prices, proposed vendors (if known), and the basis of estimate (e.g., quotes, prior purchases, catalog price lists, etc.); any item that exceeds $5,000 must be supported with back-up documentation such as a copy of catalog price lists or quotes prior to purchase (NOTE: For equipment purchases, include a letter stating why the proposer cannot provide the requested resources from its own funding), and;

Materials – Itemization with costs, including quantities, unit prices, proposed vendors (if known), and the basis of estimate (e.g., quotes, prior purchases, catalog price lists, etc.); any item that exceeds $5,000 must be supported with back-up documentation such as a copy of catalog price lists or quotes prior to purchase.

All Proposed ODC's must be supported with screenshots from websites or other documentation.

This level of detail must be provided for FFP subcontracts.

VII. Cost Realism

DARPA intends to perform a Cost Realism analysis on this proposal.

Cost Realism Definition:
The proposed costs are realistic for the technical and management approach and accurately reflect the technical goals and objectives of the solicitation. The proposed costs are consistent with the proposer's Statement of Work and reflect a sufficient understanding of the costs and level of effort needed to successfully accomplish the proposed technical approach. The costs for the prime proposer and proposed subawardees are substantiated by the details provided in the proposal (e.g., the type and number of labor hours proposed per task, the types and quantities of materials, equipment and fabrication costs, travel and any other applicable costs and the basis for the estimates).

It is expected that the effort will leverage all available relevant prior research in order to obtain the maximum benefit from the available funding. For efforts with a likelihood of commercial application, appropriate direct cost sharing may be a positive factor in the evaluation. DARPA recognizes that undue emphasis on cost may motivate proposers to offer low-risk ideas with minimum uncertainty and to staff the effort with junior personnel in order to be in a more competitive posture. DARPA discourages such cost strategies.

VIII. Past Performance:

Subcontractor should provide __2___ past performance references that closely match the RFP requirement. Template is attached.

IX. REPRESENTATIONS AND CERTIFICATIONS & VENDOR MASTER TEMPLATE

Please complete the Supplier Representations and Certifications (Attachment G).

X. PROPOSAL VALIDITY PERIOD

Your proposal shall be valid for a period of one hundred and twenty (180) days.

XI. Proposal Submittal Requirements

1. Due Date: On or before 2:00 p.m. (EST) on Wednesday, April 19, 2017.

2. Method of Submission: One (1) electronic copy to: kristen.k.trump@leidos.com and Michael.a.younkins@leidos.com.

XII. ORGANIZATIONAL CONFLICTS OF INTEREST

FAR 9.5 Requirements - https://www.acquisition.gov/far/html/Subpart%209_5.html

In accordance with FAR 9.5, proposers are required to identify and disclose all facts relevant to potential OCIs involving the proposer's organization and any proposed team member. Under this Section, the proposer is responsible for providing this disclosure with each proposal submitted to the BAA. The

disclosure must include the proposer's, and as applicable, proposed team member's OCI mitigation plan. The OCI mitigation plan must include a description of the actions the proposer has taken, or intends to take, to prevent the existence of conflicting roles that might bias the proposer's judgment and to prevent the proposer from having unfair competitive advantage. The OCI mitigation plan will 18 HR001117S0019, Pandemic Prevention Platform (P3) specifically discuss the disclosed OCI in the context of each of the OCI limitations outlined in FAR 9.505-1 through FAR 9.505-4.

DARPA Agency Supplemental OCI Policy

In addition, DARPA has a supplemental OCI policy that prohibits contractors/performers from concurrently providing Scientific Engineering Technical Assistance (SETA), Advisory and Assistance Services (A&AS) or similar support services and being a technical performer. Therefore, as part of the FAR 9.5 disclosure requirement above, a proposer must affirm whether the proposer or any proposed team member is providing SETA, A&AS, or similar support to any DARPA office(s) under: (a) a current award or subaward; or (b) a past award or subaward that ended within one calendar year prior to the proposal's submission date.

If SETA, A&AS, or similar support is being or was provided to any DARPA office(s), the proposal must include:

- The name of the DARPA office receiving the support;
- The prime contract number;
- Identification of proposed team member (subawardee, consultant) providing the support; and
- An OCI mitigation plan in accordance with FAR 9.5.

Additional Requirements:

a. Identification of technical data previously delivered to the Government IAW DFARS 252.227-7028.
b. Provide permission for Leidos to use your LOGO
c. If you are a Large Business – you must provide a Small Business Subcontracting Plan with your proposal
d. All Subcontractors who are considered a Federally Funded Research & Development Center (FFRDC), must include documentation that clearly demonstrates work is not otherwise available from the private sector AND provided a letter on letterhead from the sponsoring organization citing the specific authority establishing their eligibility to propose to government solicitations and compete with industry, and compliance with the associated FFRDC sponsor agreement and terms and conditions.

Your proposal must be received by Leidos by Wednesday, April 19, 2017 and include a validity period of **180 days** from the due date. Please direct any questions, in writing, regarding this RFP to the undersigned.

Proposals will be evaluated against the following evaluation criteria: Award shall be made to the best value proposal considering price, schedule, technically acceptable proposal and appropriate facilities.

RESPONDING TO THIS REQUEST FOR PROPOSAL CERTIFIES THAT NO GRATUITIES WERE OFFERED BY THE SUPPLIER OR SOLICITED BY ANY LEIDOS EMPLOYEE. IF A GRATUITY WAS SOLICITED, IT SHOULD BE REPORTED IMMEDIATELY TO MR. KIM DENVER, SR. VICE PRESIDENT, AT (571) 526-7051. PLEASE REVIEW LEIDOS' CODE OF ETHICS AT https://www.leidos.com/sites/default/files/CodeOfConduct.pdf.

Kristen Trump
Subcontracts Administrator
240-529-0419

Attachment 1: RFP/RFQ Enclosures

ATTACHMENTS - RFP/RFQ ENCLOSURES

A.	Statement/Scope of Work and Schedule (TBD)
B.	RFQP General Provisions
C.	Schedule A – Federal - FFP
D.	Schedule B Part I – Federal Non-Commercial Items
E.	Schedule B Part II – DOD Non-Commercial Items
F.	Schedule C – DARPA Unique Prime Contract Flowdown Clauses and Requirements
G.	Supplier Reps and Certs
H.	Pricing Template (Sample)
I.	Past Performance Template

SUBMIT WITH YOUR PROPOSAL

1. Certificate of Insurance in accordance with Section 10.0 of the Subcontract Agreement – Schedule A, Federal, FFP IDIQ (Rev 2015-08) or Evidence of Coverage
2. Letter of Commitment on company letterhead
3. One electronic copy of the technical proposal
4. One electronic copy of the price proposal

Specific Terms and Conditions and Contract Clauses

It should be noted that any purchase order/subcontract resulting from this proposal shall include specific terms and conditions and contract clauses, which are listed by FAR reference, and which are identified herein as Attachment C, D, E, F respectively. All clauses required by the Prime Contract, all clauses required by law on the date of execution of the subcontract, and any mutually agreeable clauses, terms and conditions will be negotiated at time of award.

PROPOSAL CHECK LIST

1. Pricing Detail
    a. Forward Pricing Rate Agreement or Forward Pricing Rate Proposal
    b. If “a” above is not available – 2 Years of Historical Data to Include Pool and Expense Costs Used to Generate the Rates
    c. If Academia – Provide DHHS or ONR Negotiated Rates
    d. Salary Breakdown with all Indirect Rates
    e. Travel Breakdown and Documentation
    f. ODC Breakdown (Materials and Supplies)and Documentation (Description, Make, Model, quantity and Price per unit)
    g. Equipment Breakdown and Documentation (Description, Make, Model, quantity and price per unit)
    h. Suppliers Reps and Certs – Completed and Signed

2. OCI Certification

3. Past Performance

4. FFRDC Documentation

5. LOGO (High resolution jpeg) and permission for Leidos to use for proposal

6. Certificate of Insurance – will be required at time of award

7. Review Attachment C – Schedule A – Federal – FFP document, Schedule B Part I and II. These documents do not have to be returned by April 19, 2017 – however you do need to submit it within 15 days after the requested proposal date with any redlines.

SUMMARY OF PROPOSED COSTS

LABOR	**BASE PERIOD**
Period Dates (estimate)	Estimated 4 Year Period
Direct Labor	
Overhead Cost	
G&A Cost	
Subtotal	
Fixed Fee Percentage	
Fixed Fee Cost	
Total (Labor Cost Plus Fixed Fee)	
OTHER DIRECT COSTS	
FFP Subcontractor Costs	
Estimated Travel	
Materials	
Equipment	
Total Other Direct Costs	
Total Proposed Cost Plus Fixed Fee	

DARPA-P3

Offeror:
Date:

INSTRUCTIONS: Fill in additional information for Direct Labor Costs in Columns E, F and G. Add columns to Include escalation if necessary. If subcontractor agreements are Firm Fixed Price they can be listed as ODCs. If subs are direct labor, include costs here. The subtotal column is intended to provide the rate without fee included so it can be identified as a separate amounnt in the award. The rates in the total column will be used to compare the proposed rates to the base contract maximum rates, which include fee. The Offeror is responsible for ensuring mathematical calculations are correct.

SUMMARY OF DIRECT LABOR COSTS (INCLUDING BUILDUP)

BASE PERIOD

	Labor Category	Base Contract Attch 7 Position #	Prime/Subcontractor	Base/Direct Labor Rate	Fringe Percentage Enter the % for each category in this column, not the dollar value.	Overhead Percentage Enter the % for each category in this column, not the dollar value.	G&A Percentage Enter the % for each category in this column, not the dollar value.	Loaded Rate Without Fee	Hours	Subtotal		Fee Enter the fee percentage in the yellow block. Calculate the dollar value by summing at the bottom of this column.	Fully Loaded Rate	Total
												%		
1				$0.00	%	%	%	$0.00	0.00	$0.00		$0.00	$0.00	$0.00
2				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
3				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
4				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
5				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
6				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
7				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
8				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
9				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
10				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
11				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
12				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
13				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
14				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
15				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
16				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
17				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
18				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
19				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
20				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
21				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
22				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
23				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
24				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
25				0.00	%	%	%	0.00	0.00	0.00		0.00	0.00	0.00
				$0.00				$0.00	0.00	$0.00		$0.00	$0.00	**$0.00**

SUMMARY OF MATERIALS AND SUPPLIES

FFP Subcontractor Costs

Subcontractor	Task	Price	Basis for Fair and Reasonable Determination (see FAR 15.404)

Estimated Travel

Add additional trips if necessary. If no travel is proposed, delete this information and state 'no travel is proposed.'

	Trip 1	Trip 2	Trip 3
City of Trip Origin			
City of Trip Destination			
Justification			
Traveler (prime or sub)			
Airfare Class			
Airline			
Airfare Cost	$0.00	$0.00	$0.00
Lodging Cost	0.00	0.00	0.00
Meal Cost	0.00	0.00	0.00
Incidentals	0.00	0.00	0.00
No. of days			
Total Tip Cost	0.00	0.00	0.00
No. Trips per Period			
No. of Persons			
Total Trip Cost	0.00	0.00	0.00

Total Travel Cost	$0.00

Government Furnished Contractor Acquired Property

Add and delete rows as necesasry. If none is proposed, delete this information and state 'none is proposed.'

Item	Item Description (including part numbers)	Need Date	Quantity	Cost (rent-free basis)

SUMMARY OF MATERIALS AND SUPPLIES

Materials

Add or delete rows below as necessary. If no materiels are proposed, delete this information and state 'no materials are proposed.'

Item	Item Description	Quantity	Cost per Unit	No. of Units
			$0.00	
			0.00	
			0.00	
			0.00	
			0.00	
Total Material Cost				0.00

DARPA-P3

Offeror:
Date:

INSTRUCTIONS: Propose any costs for Contractor Manpower Reporting here. If you are proposing as 'Not Separate

CONTRACTOR MANPOWER REPORTING

		10/31/2017	10/31/2018	10/31/2019	10/31/2020	10/31/2021
1	Contractor Manpower Reporting	$0.00	$0.00	$0.00	$0.00	$0.00

This requirement is due No Later Than October 31 of each year.

ly Priced,' enter $0.00 as the cost.

leidos

Identification and Assertion of Restrictions on the Government's Use, Release, or Disclosure of Technical Data or Computer Software.

The Subcontractor asserts for itself, or the persons identified below, that the Government's rights to use, release, or disclose the following technical data or computer software should be restricted:

Technical Data or Computer Software to be Furnished With Restrictions*	Basis for Assertion**	Asserted Rights Category***	Name of Person Asserting Restrictions****
(LIST)*****	(LIST)	(LIST)	(LIST)

*For technical data (other than computer software documentation) pertaining to items, components, or processes developed at private expense, identify both the deliverable technical data and each such item, component, or process. For computer software or computer software documentation identify the software or documentation.

**Generally, development at private expense, either exclusively or partially, is the only basis for asserting restrictions. For technical data, other than computer software documentation, development refers to development of the item, component, or process to which the data pertain. The Government's rights in computer software documentation generally may not be restricted. For computer software, development refers to the software. Indicate whether development was accomplished exclusively or partially at private expense. If development was not accomplished at private expense, or for computer software documentation, enter the specific basis for asserting restrictions.

***Enter asserted rights category (e.g., government purpose license rights from a prior contract, rights in SBIR data generated under another contract, limited, restricted, or government purpose rights under this or a prior contract, or specially negotiated licenses).

****Corporation, individual, or other person, as appropriate.

*****Enter “none” when all data or software will be submitted without restrictions.

Date _____

Printed Name and Title _____

_____

Signature _____

(End of identification and assertion)

leidos

[redacted]

April 18, 2017

Re: Organizational Conflicts of Interest Certification (RFP No. LEIDOS-DARPA-P3)

Dear Ms. Trump:

We have reviewed the FAR 9.5 requirements. This is to affirm that [redacted] has no potential organizational conflicts of interest involving [redacted] and any proposed team member.

If you have any questions, please contact [redacted] or [redacted]. Thank you for your consideration of this proposal.

Sincerely,

[redacted]

[redacted]

SCHEDULE B – PART I – FAR CLAUSES

U.S. GOVERNMENT TERMS and CONDITIONS (FEDERAL – NON-COMMERCIAL ITEMS)

1. SCHEDULE B DEFINITIONS

Schedule B Definitions shall apply to all Schedule B terms incorporated into this subcontract (e.g., Part I, Part II). In the U.S. Government clauses contained in the Schedule B document(s), unless the context of the clause requires otherwise, the term "Contractor" shall mean Seller, the term "Contract" shall mean the Subcontract or Purchase Order, and the terms "Government," "Contracting Officer" and equivalent phrases shall mean Leidos and Leidos' Contractual Representative, respectively. It is intended that these clauses shall apply to Seller in such manner as is necessary to reflect the position of Seller as a subcontractor to Leidos, to insure Seller's obligations to Leidos and to the United States Government, and to enable Leidos to meet its obligations under its Prime Contract and the Subcontract. All references to Subcontract shall include purchase orders and unless otherwise stated, all definitions defined by FAR 2.101 shall be applied to the terms referenced herein.

2. DISPUTES

Notwithstanding any provisions herein to the contrary:

(a) If a decision relating to the Prime Contract is made by the Contracting Officer and such decision is also related to this Subcontract, said decision, if binding upon Leidos under the Prime Contract shall in turn be binding upon Leidos and Seller with respect to such matter; provided, however, that if Seller disagrees with any such decision made by the Contracting Officer and Leidos elects not to appeal such decision, Seller shall have the right reserved to Leidos under the Prime Contract with the Government to prosecute a timely appeal in the name of Leidos, as permitted by the Prime Contract or by law, Seller to bear its own legal and other costs. If Leidos elects not to appeal any such decision, Leidos agrees to notify Seller in a timely fashion after receipt of such decision and to assist Seller in its prosecution of any such appeal in every reasonable manner. If Leidos elects to appeal any such decision of the Contracting Officer, Leidos agrees to furnish Seller promptly with a copy of such appeal. Any decision upon appeal, if binding upon Leidos, shall in turn be binding upon Seller. Pending the making of any decision, either by the Contracting Officer or on appeal, Seller shall proceed diligently with performance of this Subcontract.

(b) If, as a result of any decision or judgment which is binding upon Seller and Leidos, as provided above, Leidos is unable to obtain payment or reimbursement from the Government under the Prime Contract for, or is required to refund or credit to the Government, any amount with respect to any item or matter for which Leidos has reimbursed or paid Seller, Seller shall, on demand, promptly repay such amount to Leidos. Additionally, pending the final conclusion of any appeal hereunder, Seller shall, on demand, promptly repay any such amount to Leidos. Leidos' maximum liability for any matter connected with or related to this Subcontract which was properly the subject of a claim against the Government under the Prime Contract shall not exceed the amount of Leidos' recovery from the Government.

(c) If this Subcontract is issued by Leidos under a Government subcontract rather than a Prime Contract, and if Leidos has the right under such Government subcontract to appeal a decision made by the Contracting Officer under the Prime Contract in the name of the Prime Contractor (or if Leidos is subject to any arbitrator's decision under the terms of its subcontract), and said decision is also related to this Subcontract, this Disputes Clause shall also apply to Seller in a manner consistent with its intent and similar to its application had this Subcontract been issued by Leidos under a Prime Contract with the Government.

(d) Seller agrees to provide certification that data supporting any claim made by Seller hereunder is made in good faith and that the supporting data is accurate and complete to the best of Seller's knowledge or belief, all in accordance with the requirements of the Contract Disputes Act of 1978 (41 USC 601-613) and implementing regulations. If any claim of Seller is determined to be based upon fraud or misrepresentation, Seller agrees to defend, indemnify and hold Leidos harmless for any and all liability, loss, cost or expense resulting therefrom.

(e) Any dispute not addressed in paragraph (a) above, will be subject to the disputes clause of Schedule A of the Subcontract or PO terms and conditions.

3. INDEMNIFICATION - COST OR PRICING DATA - COST ACCOUNTING STANDARDS – OTHER

Seller agrees to indemnify and hold Leidos harmless to the full extent of any cost or price reduction, plus interest, effected by Leidos' customer, which may result from (i) certified cost or pricing data submitted by Seller or its lower-tier subcontractors which is not accurate, current or complete as certified by Seller; (ii) the failure by Seller or its lower-tier subcontractors to disclose and consistently follow applicable cost accounting practices and standards or otherwise comply with pertinent parts of the FAR, applicable agency supplements thereto, and standards and regulations promulgated by the Cost Accounting Standards Board. Seller further agrees to indemnify, defend, and hold Buyer harmless from and against any losses, liabilities, offsets and expenses (including reasonable attorney's fees) arising out of or relating to Seller's failure to comply with (i) the provisions of the Anti-Kickback Act or FAR 52.203-7; or (ii) 52.203-13, Contractor Code of Business Ethics and Conduct, if applicable to this Subcontract.

4. CONTRACT COST PRINCIPLES AND PROCEDURES

Seller agrees that to the extent applicable, costs allocated to this Subcontract shall be in full compliance with Subpart 31.2 of FAR (Subpart 31.3 for Educational Institutions) and the applicable agency supplements thereto, if any, set forth in Part II hereof. In the event such compliance is not maintained, Seller agrees to compensate Leidos to the full extent of any prices or costs, including any penalties or interest that are determined by Leidos' customer to be unallowable or unreasonable or not allocable, under Leidos' contract with its customer.

5. FAR CLAUSES APPLICABLE TO THIS SUBCONTRACT

The U.S. Government terms or clauses in FAR Subpart 52.2 referenced herein are incorporated and made a part of this Subcontract, unless:

(a) The description is preceded by a selection box (☐), the FAR clause (and its alternate) shall only apply when selected;

(b) Unless otherwise specified for performance applicability; or

(c) A value (e.g., >$150k) is identified. Clauses are applicable when the subcontract ceiling value (to include all options) exceeds the specified values at the time of award or after modification unless otherwise specified. Note: References to "SAT" refers to the "Simplified Acquisition Threshold" as defined by FAR 2.101 and will be the threshold that is current on the date of the Subcontract award.

To the extent that the Prime Contract or higher-tier subcontract under which this Subcontract is issued, is modified (to include a newer version of any such clause), the date of the modified version of the clause as it appears in such Prime Contract or higher-tier subcontract shall be controlling and said version shall be incorporated herein.

SECTION I: APPLICABLE TO ALL - FEDERAL STANDARD & FEDERAL COMMERCIAL ITEMS	
52.203-6	Restrictions on Subcontractor Sales to the Government (>**SAT**) (**Alternate I** applies to Commercial Items)
52.203-7	Anti-Kickback Procedures (except paragraph (c) (1)) - (>$150k)
52.203-12	Limitation on Payments to Influence Certain Federal Transactions (>$150k)
52.203-13	Contractor Code of Business Ethics and Conduct (Applies when POP >120 days; and >**$5.5M**)
☐ 52.203-15	Whistleblower Protections Under the American Recovery and Reinvestment Act of 2009
52.203-16	Preventing Personal Conflicts of Interest (>$150k)
52.203-17	Contractor Employee Whistleblower Rights and Requirements to Inform Employees or Whistle Blower Rights (>**SAT**)
52.203-19	Prohibition on Requiring Certain Internal Confidentiality Agreements or Statements
52.204-9	Personal Identity Verification of Contractor Personnel (Applies when support requires routine access to Federally-controlled facilities or Federally-controlled information systems)
52.204-21	Basic Safeguarding of Covered Contractor Information Systems (Applies when the subcontractor may have Federal contract information residing in or transiting through its information system)
52.209-6	Protecting the Governments Interest When Subcontracting with Contractors Debarred, Suspended, or Proposed for Debarment (>$35k)
☐ 52.217-6	Option for Increased Quantity (Add " " for, "the period of time in which the Contracting Officer has to exercise the option)
☐ 52.217-7	Option for Increased Quantity—Separately Priced Line Item (Add " " for, "the period of time in which the Contracting Officer has to exercise the option)
☐ 52.217-8	Option to Extend Services (Add " " for, "the period of time within which the Contracting Officer may exercise the option.")
☐ 52.217-9	Option to Extend the Term of the Contract (Subparagraph (a) Add " " for, "the period of time within which the Contracting Officer may exercise the option," and, add " " for the number of days for written notice. Subparagraph (c) Add " " for the duration (specified in months or years) for total duration of this contract.)
52.219-8	Utilization of Small Business Concerns
52.219-9	Small Business Subcontracting Plan (>$700k; n/a to small business concern)
52.222-3	Convict Labor
52.222-4	Contract Work Hours and Safety Standards —Overtime Compensation (Applies to subcontracts that may require or involve the employment of laborers and mechanics)
52.222-17	Non-displacement of Qualified Workers (>**SAT**)
52.222-20	Walsh-Healey Public Contracts Act (Applies when manufacturing or furnishing materials, supplies, articles, or equipment and performed within the United States, Puerto Rico or the U.S. Virgin Islands; and >$15k)

52.222-21	Prohibition of Segregated Facilities
52.222-26	Equal Opportunity
52.222-35	Equal Opportunity for Veterans (>$150k).
52.222-36	Affirmative Action for Workers with Disabilities (>$15k)
52.222-37	Employment Reports on Special Disabled Veterans, Veterans of the Vietnam Era and Other Eligible Veterans (>$150k)
52.222-40	Notification of Employee Rights Under the National Labor Relation Acts (>$10,000 and subcontract will be performed wholly or partially in the United States)
52.222-41	Service Contract Labor Standards
52.222-42	Statement of Equivalent Rates for Federal Hires
52.222-43	Fair Labor Standards Act and Service Contract Act – Price Adjustment (Applies to Multiple Year and Option Contracts)
52.222-44	Fair Labor Standards Act and Service Contract Act – Price Adjustment
52.222-50	Combating Trafficking in Persons
FAR 52.222-41 through -44 are inapplicable if either FAR 52.222-51 or -53 is selected.	
☐ 52.222-51	Exemption from the Application of the Service Contract Act of Contracts for Maintenance, Calibration or Repair of Certain Equipment – Requirements
☐ 52.222-53	Exemption from the Application of the Service Contract Act to Contracts for Certain Services – Requirements
52.222-54	Employment Eligibility Verification (>$3,500, n/a if performance is wholly OCONUS)
52.222-55	Minimum Wages Under Executive Order 13658 (Applies to orders subject to the Service Contract Labor Standards statute or the Wage Rate Requirements (Construction) statute, and are to be performed in whole or in part in the United States)
52.222-56	Certification Regarding Trafficking in Persons Compliance Plan (Provision applies to non-COTS >$500K)
52.223-9	Estimate of Percentage of Recovered Material Content for EPA Designated Items (Applies to Awards that involve the use of EPA –designated items)
52.223-15	Energy Efficiency in Energy – Consuming Products
52.223-16	IEEE 1680 Standard for the Environmental Assessment of Personal Computer Products ☐ **Alternate I**
52.223-18	Encouraging Contractor Policies to Ban Text While Driving (>$3,500)
52.225-1	Buy American Act – Supplies (Applies unless 52.225-3 (with its alternates when applicable) or 52.222-5 is selected)
☐ 52.225-3	Buy American Act – Free Trade Agreements – Israeli Trade Act ☐ **Alternate I** ☐ **Alternate II** ☐ **Alternate III**
☐ 52.225-5	Trade Agreements
52.225-13	Restrictions on Certain Foreign Purchases
52.225-26	Contractors Performing Private Security Functions Outside the United States (Applies to support of combat operations or other significant military operations)
52.226-6	Promoting Excess Food Donation to on Profit Organizations (Applies when manufacturing or furnishing materials, supplies, articles, or equipment and performed within the United States, Puerto Rico or the U.S. Virgin Islands; and >$25k)
52.239-1	Privacy or Security Safeguards
52.244-6	Award of Commercial Items
52.245-1	Government Property (Applies when Government Property is applicable to the subcontract) ☐ **Alternate I** – Responsibility and Liability for Government Property (n/a to cost-reimbursement type Awards)
52.245-2	Government Property Installation Operation Services (Applies for fixed-price services)
52.247-64	Preference for Privately Owned U.S. Flag Commercial Vessels (Applies to ocean transfer of supplies) ☐ **Alternate I**
	Service Contract Reporting Requirements: First-tier subcontracts for services (including construction) valued at or above the thresholds set forth in 4.1703(a)(2) shall annually by October 15th provide a report to the Leidos Contractual POC that includes subcontract number, subcontractor name and DUNS number and the number of direct-labor hours expended on the services performed during the previous Government fiscal year (October 1-September 30). The subcontractor shall be advised that this information will be made available to the public as required by section 743 of Division C of the Consolidated Appropriations Act, 2010.

SECTION II: APPLICABLE TO FEDERAL ORDERS FOR NON-COMMERCIAL ITEMS

52.203-3	Gratuities
52.203-5	Covenant Against Contingent Fees
52.203-8	Cancellation, Rescission, and Recovery of Funds for Illegal or Improper Activity
52.203-10	Price or Fee Adjustment for Illegal or Improper Activity
52.203-14	Display of Hotline Poster(s) (>**$5.5M**)
52.204-2	Security Requirements (Applies to services where access to classified information is required) **Alternate I** (Applies to R&D or Educational Institutions) **Alternate II** (Applies to Architect Engineering Awards)
52.204-4	Printed or Copied Double-Sided on Recycled Paper
52.208-8	Required Sources for Helium and Helium Usage Data (Applies when support involves a major helium requirement)
52.211-5	Material Requirements
Negotiated Awards where the ceiling value exceeds the threshold in FAR 15.403-4(a)(1):	
52.214-26	Audit and Records-Sealed Bidding
52.214-28	Subcontractor Cost or Pricing Data - Modifications - Sealed Bidding
52.215-11	Price Reduction for Defective Cost or Pricing Data -Modifications
52.215-13	Subcontractor Cost or Pricing Data – Modifications
52.215-15	Pension Adjustments and Asset Reversions
52.215-21	Requirements for Cost or Pricing Data or Information Other Than Cost or Pricing Data – Modifications
Negotiated Awards where the ceiling value exceeds the threshold in FAR 15.403-4(a)(1) and when Buyer's submission of Certified Cost and Pricing Data was required.	
52.214-27	Price Reduction for Defective Cost or Pricing Data Modifications – Sealed Bidding
52.215-10	Price Reduction for Defective Cost or Pricing Data
52.215-12	Subcontractor Cost or Pricing Data
52.215-18	Reversion or Adjustment of Plans for Post-Retirement Benefits (PRB) Other Than Pensions
52.215-19	Notification of Ownership Changes
52.215-20	Requirements for Cost or Pricing Data or Information Other Than Cost or Pricing Data
52.215-2	Audit and Records-Negotiation (>SAT current at the time of award)
52.215-14	Integrity of Unit Prices (except paragraph (b)) (>SAT current at the time of award; except architect engineer services, utility services, services where supplies are not required, and petroleum products)
52.215-23	Limitations on Pass-Through Charges (Applicable to CR awards for non-DOD; and >SAT current at the time of award. If DoD, applies to all order types when >$750k)
52.217-8	Option to Extend Services
52.217-9	Option to Extend the Term of the Contract
52.219-16	Liquidated Damages – Subcontracting Plan (>$700k and when 52.219-9 is applicable)
52.222-1	Notice to the Government of Labor Disputes
52.222-38	Compliance with Veteran's Employment Reporting Requirements
52.223-3	Hazardous Material Identification and Material Safety Data (Applies to support of DOD programs involving Hazardous Material) **Alternate I** (Applies to support of Federal Civilian programs involving Hazardous Material)
52.223-5	Pollution Prevention and Right-to-Know Information (including **Alternate I and II**) - (Applies when performance is on a Federal facility)
52.223-6	Drug-Free Workplace
52.223-7	Notice of Radioactive Materials (Applies to subcontract performance containing use of radioactive materials)
52.223-11	Ozone – Depleting Substances (Applies to work containing ozone depleting substances)
52.224-1	Privacy Act Notification (Applies to awards for redesign, development, or operation of records on individuals that is subject to Privacy Act of 1974)
52.224-2	Privacy Act (Applies to awards for redesign, development, or operation of records on individuals that is subject to Privacy Act of 1974)
☐ 52.225-3	Buy American Act – Free Trade Agreements – Israeli Trade Act ☐ **Alternate I** ☐ **Alternate II** ☐ **Alternate III**
52.225-8	Duty-Free Entry

52.225-19	Contractor Personnel in a Designated Operational Area or Supporting a Diplomatic or Consular Mission Outside the United States (Applies to OCONUS performance)
52.227-1	Authorization and Consent **Alternate I** (Applies R&D awards) **Alternate II** (Applies when support involves communication services with a common carrier and the services are unregulated and not priced by a tariff schedule set by a regulatory body)
52.227-2	Notice and Assistance Regarding Patent and Copyright Infringement (>SAT current at the time of award)
52.227-3	Patent Indemnity
52.227-9	Refund of Royalties (Applies when royalties reported during negotiation of the subcontract >$250)
52.227-10	Filing of Patent Applications - Classified Subject Matter
52.227-11	Patent Rights - Ownership by the Contractor (Applies when support is experimental, developmental, or research performed by a small business or nonprofit organization)
52.227-13	Patent Rights – Ownership by the Government (Applies to experimental, developmental, or research work)
52.227-14	Rights in Data – General ☐ **Alternate I** ☐ **Alternate II** ☐ **Alternate III** ☐ **Alternate IV** ☐ **Alternate V**
☐ 52.227-17	Rights in Data - Special Works
☐ 52.227-18	Rights in Data - Existing Work
52.227-19	Commercial Computer Software License (Applies to any Computer Commercial Software delivered by Seller)
52.227-21	Technical Data Declaration, Revision & Withholding of Payment Major Systems (Applies when supporting the acquisition of a major system)
52.227-22	Major System - Minimum Rights (Applies when supporting the acquisition of Major Systems)
52.227-23	Rights to Proposal Data (Technical)
52.228-3	Worker's Compensation Insurance - DBA (Inapplicable when 52.228-4 is selected and OCONUS performance)
☐ 52.228-4	Worker's Compensation and War-Hazard Insurance Overseas (Applies to OCONUS performance)
52.228-5	Insurance - Work on a Government Installation (Applies when subcontract performance is on a Government installation)
52.229-3	Federal, State, and Local Taxes
52.229-6	Taxes - Foreign Fixed-Price Contracts (Applies to fixed-price OCONUS performance)
52.229-8	Taxes - Foreign Cost-Reimbursement Contracts (Applies to cost-reimbursement OCONUS performances)
52.230-X clauses apply (when selected) to negotiated awards >$750k at the time of award only.	
☒ 52.230-2	Cost Accounting Standards (except paragraph (b))
☐ 52.230-3	Disclosure and Consistency of Cost Accounting Practices (except p. (b))
☐ 52.230-4	Disclosure and Consistency of Cost Accounting Practices—Foreign Concerns
☐ 52.230-5	Cost Accounting Standards-Educational Institution (except para (b))
☐ 52.230-6	Administration of Cost Accounting Standards
52.232-16	Progress Payments (Notwithstanding paragraph 1. above, in paragraph 52.232-16(d),"Government" means the "U.S. Government" except in subdivision (d)(2)(iv).
52.232-17	Interest
52.232-40	Providing Accelerated Payments to Small Business Subcontractors (Applies to small businesses)
52.233-3	Protest After Award
52.234-1	Industrial Resources Developed Under Defense Production Act Title III (Applies to major systems and supply contracts)
52.234-4	Earned Value Management System (Applies when use of an Earned Value Management System (EVMS) is required by the subcontract)
52.237-2	Protection of Government Buildings, Equipment and Vegetation (Applies when services are not performed on Seller site)
52.237-7	Indemnification and Medical Liability Insurance (Applies to health care services)
52.242-13	Bankruptcy
52.242-15	Stop-Work Order
52.244-5	Competition in Subcontracting
52.246-16	Responsibility for Supplies (Applies to supply Awards)

52.247-3	Capability to Perform a Contract for the Relocation of a Federal Office (Applies when performance involves moving a Federal office)
52.247-63	Preference for U.S. Flag Air Carriers (Applies for the use of International Air Transportation)
52.248-1	Value Engineering ☐ **Alternate II** (Applies if VECP requirement is required by the SOW)

Clauses below are applicable at the specified subcontract type only. [1]T&M/LH = T&M subcontracts where indirect rates are not applied to materials [2]CR = Cost reimbursable subcontracts (e.g., CPFF) including any T&M subcontracts where indirect rates are applied to the materials portion		**T&M[1]/LH**	**CR[2]**	**FFP**
52.215-15	Pension Adjustments and Asset Reversions	X	X	
52.215-17	Waiver of Facilities Capital Cost of Money (Applies if FCCM was not proposed by the Subcontractor nor included in the subsequent subcontract award)		X	
52.216-7	Allowable Cost and Payment **Alternate II** (Applies to Educational Institutions) **Alternate IV** (Applies to non-profit organizations other than Educational Institutions)	X	X	
52.222-2	Payment for Overtime Premiums -Subparagraph (a) Add "0"	X	X	
52.229-10	State of New Mexico Gross Receipts and Compensating Tax (Applies to the acquisition of tangible personal property and title to such property passes directly to and vests in the United States upon delivery)	X	X	
52.232-20	Limitation of Cost (Applies when subcontract is fully-funded)		X	
52.232-22	Limitation of Funds (Applies when subcontract is incrementally funded)		X	
52.232-32	Performance-Based Payments (Applies when negotiated payment amounts are performance-based)			X
52.242-1	Notice of Intent to Disallow Costs	X	X	
52.243-1	Changes - Fixed Price - **Alternate II** **Alternate III** (Applies to Architect-Engineer Services) **Alternate IV** (Applies to Transportation Services)			X
52.243-2	Changes - Cost-Reimbursement - **Alternate II**		X	
52.243-3	Changes – Time and Material – Labor Hour	X		
52.246-2	Inspection of Supplies - Fixed Price (Applies to the purchase of supplies)			X
52.246-3	Inspection of Supplies – Cost Reimbursement		X	
52.246-4	Inspection of Services - Fixed-Price (Applies to services)			X
52.246-5	Inspection of Services - Cost-Reimbursement		X	
52.246-6	Inspection - Time-and- Material and Labor-Hour	X		
52.246-7	Inspection of Research and Development – Fixed-Price			X
52.246-8	Inspection of Research and Development - Cost-Reimbursement (Applies to R&D with fee) **Alternate I** (Applies to R&D awards without fee)		X	
Termination clauses 52.249-2 through -6 are modified to require termination proposals to be submitted within 30 days (in lieu of 1 year)				
52.249-2	Termination for Convenience (Fixed-Price)			X
52.249-3	Termination for Convenience (Applies to dismantling, demolition, or removal of improvements Awards)			X
52.249-5	Termination for Convenience (Applies to Educational and Other Nonprofit Institutions)			X
52.249-6	Termination (Cost-Reimbursement)		X	
52.249-6	Termination (Cost-Reimbursement) – Alternate IV	X		
52.249-7	Termination (Applies to Fixed-Price Architect-Engineer)			X
52.249-8	Default (Fixed-Price) **Alternate I** – (Applies to Transportation Services)			X

SCHEDULE B – PART II – DOD FAR SUPPLEMENTS
APPLICABLE TO ALL DEPARTMENT OF DEFENSE (DOD) ORDERS – NON COMMERCIAL ITEMS

1. DFAR SUPPLEMENT CLAUSES APPLICABLE TO THIS SUBCONTRACT

All references herein to "DFAR Supplement" or "DFAR SUPP" shall mean the Department of Defense Supplement to the Federal Acquisition Regulation. The clauses in DFAR Supplement Subpart 252.2 referenced herein are incorporated and made a part of this Subcontract, unless the Description is preceded by a selection box (☐), the FAR clause (and its alternate) shall only apply when selected. To the extent that the Prime Contract or higher-tier subcontract under which this Subcontract is issued, is modified (to include a newer version of any such clause), the date of the modified version of the clause as it appears in such Prime Contract or higher-tier subcontract shall be controlling and said version shall be incorporated herein.

2. CONTRACTOR MANPOWER REPORTING

☒ If selected, Seller shall report ALL subcontractor labor hours required for performance of services provided under this subcontract via the Government's secure data collection site. Seller is required to completely fill in all required data fields using the following web address: http://www.ecmra.mil/. Reporting inputs will be for the subcontract labor executed during the period of performance of this subcontract, during each Government fiscal year (FY), which runs October 1 through September 30. While inputs may be reported any time during the FY, all data shall be reported no later than October 31 of each calendar year. Subcontractors may direct questions to the help desk at help desk at: http://www.ecmra.mil.

SECTION I: APPLICABLE TO ALL – DOD STANDARD & COMMERCIAL ITEMS	
252.203-7000	Requirements Relating to Compensation of Former DOD Officials
252.204-7012	Safeguarding Covered Defense Information and Cyber Incident Reporting (Applies when seller is providing operationally critical support, or for which performance will involve a covered contractor information system) Seller shall provide the cognizant Leidos contractual representative, notification of any incident report number, that has been submitted (for any tier) in accordance with 252.204-7012.
☐ 252.204-7014	Limitations on the Use or Disclosure of Information by Litigation Support Contractors
252.204-7015	Notice of Authorized Disclosure of Information for Litigation Support
252.211-7003	Item Unique Identification and Valuation *(when Government Property applies)*
252.211-7007	Reporting of Government-Furnished Property, subsection (d). (When Government Property applies)
252.222-7007	Representation Regarding Combating Trafficking in Persons
252.225-7001	Buy American Act and Balance of Payments Program (Applies unless 252.225-7021, 252.225-7036 or 252.225-7045 is selected) **Alternate I** (Applies to support of operations in Afghanistan)
252.225-7008	Restriction on Acquisition of Specialty Metals
252.225-7009	Restriction on Acquisition of Certain Articles Containing Specialty Metals
252.225-7012	Preference for Certain Domestic Commodities
252.225-7015	Restriction on Acquisition of Hand or Measuring Tools (Applies to supply orders for delivery of measuring and hand tools)
252.225-7017	Photovoltaic Devices (Applies if order results in DoD ownership of photovoltaic devices)
☐ 252.225-7021	Trade Agreements **Alternate II** (Applies to support of operations in Afghanistan)
252.225-7028	Exclusionary Policies and Practices of Foreign Governments (for purchase of supplies and services for international military education training and FMS)
☐ 252.225-7036	Buy American Act – Free Trade Agreement-Balance of Payments Program ☐ Alternate I ☐ Alternate II ☐ Alternate III ☐ Alternate IV ☐ Alternate V
252.225-7038	Restriction on Acquisition of Air Circuit Breakers (Applies for orders or subcontract performance that require air circuit breakers for naval vessels)
225.225-7039	Defense Contractors Performing Private Security Functions Outside the United States (Services only when performance is OCONUS)

252.225-7040	Contractor Personnel Authorized to Accompany U.S. Armed Forces Deployed Outside the United States (Services only. Applies when performance is OCONUS and personnel are authorized to accompany US Armed Forces)
☐ 252.225-7045	Balance of Payments Program – Construction Material Under Trade Agreements ☐ Alternate I ☐ Alternate II ☐ Alternate III
252.225-7048	Export Controlled Items
252-226-7001	Utilization of Indian Organizations, Indian-Owned Economic Enterprises, and Native Hawaiian Small Business Concerns **(>$500K)**
252.227-7013	Rights in Technical Data - Noncommercial Items **Alternate II** (Applies when subcontractor support is for the development or delivery of a vessel design or any useful article embodying a vessel design)
252.227-7015	Technical Data – Commercial Items (Applies to the delivery of technical data pertaining to commercial items, components or processes) **Alternate I** (Applies to the development or delivery of a vessel design or any useful article embodying a vessel design)
252.227-7019	Validation of Asserted Restrictions -Computer Software (Applies to the delivery of computer software)
252.227-7037	Validation of Restrictive Markings on Technical Data (Applies for the delivery of technical data)
252.236-7013	Requirement for Competition Opportunity for American Steel Producers, Fabricators, and Manufacturers (Applies to subcontractor support that includes the acquisition of steel as a construction material)
252.237-7010	Prohibition on Interrogation of Detainees by Contractor Personnel (Services only, when performance includes interaction with detainees)
252.237-7019	Training for Contractor Personnel Interacting with Detainees ((Services only, when performance includes interaction with detainees)
252.239-7018	Supply Chain Risk
252.244-7000	Subcontracts for Commercial Items
252.246-7003	Notification of Potential Safety Issues
252.246-7004	Safety of Facilities, Infrastructure, and Equipment for Military Operations (Applies when subcontractor support is for the construction, installation, repair, maintenance, or operation of facilities, infrastructure, or for equipment configured for occupancy, planned for use by DoD military or civilian personnel during military operations)
252.246-7007	Contractor Counterfeit Electronic Part Detection and Avoidance System (applicable to the acquisition of electronic parts or assemblies containing electronic parts)
252.247-7003	Pass-Through of Motor Carrier Fuel Surcharge Adjustment to the Cost Bearer **(**Applies to subcontracts that include the use of motor carriers, brokers, or freight forwarders)
252.247-7023	Transportation of Supplies by Sea
252.247-7024	Notice of Transportation of Supplies by Sea

SECTION II: APPLICABLE TO DOD ORDERS FOR NON-COMMERCIAL ITEMS	
252.203-7001	Prohibition on Persons Convicted of Fraud or Other Defense-Contract Related Felonies - Commercial items exempt (**>$150k**)
252.203-7002	Requirement to Inform Employees of Whistleblower Rights
252.203-7004	Display of Online Posters - Commercial items exempt **(>$5.5M)**
252.204-7000	Disclosure of Information
252.204-7009	Limitations on the Use or Disclosure of Third-Party Contractor Reported Cyber Incident Information (Applies when performance requires safeguarding covered defense information and cyber incident reporting)
252.209-7004	Subcontracting with Firms that are Owned or Controlled by the Government of a Terrorist Country
☐ 252.211-7000	Acquisition Streamlining **(>$1.5M)**
252.215-7000	Pricing Adjustments (Applies when 52.215-11, 52.215-12, or 52.215-13 are applicable)
252.219-7003	Small Business Subcontracting Plan - Small businesses exempt **(>$700K)**
252.219-7004	Small Business Subcontracting Plan (Test Program) - Small businesses exempt **(>$700K)**
☐ 252.222-7000	Restrictions on Employment of Personnel (services only)
252.222-7006	Restrictions On The Use Of Mandatory Arbitration Agreements
252.223-7001	Hazard Warning Labels (Applies to support involving hazardous material)

252.223-7002	Safety Precautions for Ammunition and Explosives (Applies to support involving ammunition or explosives)
252.223-7003	Change in Place of Performance *(Applies to support involving* Ammunition or Explosives)
252.225-7004	Report of Intended Performance Outside the United States and Canada – OCONUS performance only **(>$700K)**
252.225-7007	Prohibition on Acquisition of United States Munitions List Items from Communist Chinese Military Companies (Applies for items covered by the US Munitions List)
252.225-7013	Duty-Free Entry
252.225-7016	Restriction on Acquisition of Ball and Roller Bearings (Commercial items exempt)
252.225-7043	Antiterrorism/Force Protection Policy for Defense Contractors Outside the United States (Applies to OCONUS performance)
252.227-7014	Rights in Noncommercial Computer Software and Noncommercial Computer Software Documentation (Applies to the delivery of computer software or computer software documentation)
252.227-7016	Rights in Bid or Proposal Information
☐ 252.227-7020	Rights in Special Works
☐ 252.227-7021	Rights in Data - Existing Works
☐ 252.227-7022	Government Rights (Unlimited)
☐ 252.227-7023	Drawings and Other Data to Become Property of Government
252.227-7025	Limitations on the Use or Disclosure of Government-Furnished Information Marked w/ Restrictive Legends (Applies if subcontractor is provided with technical data marked with another parties' restrictive legends)
252.227-7030	Technical Data - Withholding of Payment
252.227-7033	Rights in Shop Drawings (Applies to construction orders)
252.228-7005	Accident Reporting & Investigation Involving Aircraft, Missiles, and Space Launch Vehicles
252.231-7000	Supplemental Cost Principles
252.235-7002	Animal Welfare (Services only. Applies to research of live vertebrate animals)
252.235-7003	Frequency Authorization (Services only, for the development, production, construction, testing, or operation of a device for which a radio frequency authorization is required)
252.237-7023	Continuation of Essential Contractor Services (If Subcontract provides "Essential contractor service")
252.239-7000	Protection Against Compromising Emanations (Applies when information technology requires protection against compromising emanations.
252.239-7010	Cloud Computing Services (Applies when order involves cloud services)
252.239-7016	Telecommunications Security Equipment, Devices, Techniques and Services (Applies to orders securing telecommunications)
252.245-7001	Reports of Government Property (when government property applies)
252.246-7001	Warranty of Data **Alternate II** (Applies to Fixed-Price subcontracts)
252.249-7002	Notification of Anticipated Contract Termination or Reduction - Services only (**>$150k**)

Schedule C – HR001117S0019 DARPA Additional BAA Requirements/Clauses

Proprietary Markings

Proposers are responsible for clearly identifying proprietary information. Submissions containing proprietary information must have the cover page and each page containing such information clearly marked with a label such as "Proprietary" or "Company Proprietary." NOTE: "Confidential" is a classification marking used to control the dissemination of U.S. Government National Security Information as dictated in Executive Order 13526 and should not be used to identify proprietary business information.

Unclassified Submissions

DARPA anticipates that submissions received under this BAA will be unclassified. However, should a proposer wish to submit classified information, an *unclassified* email must be sent to the BAA mailbox requesting submission instructions from the Technical Office PSO. If a determination is made that the award instrument may result in access to classified information, a SCG and/or DD Form 254 will be issued by DARPA and attached as part of the award.

Human Research Subjects/Animal Use

Proposers that anticipate involving Human Research Subjects or Animal Use must comply with the approval procedures detailed at www.darpa.mil/work-with-us/additional-baa. protection. Further, research involving human subjects that is conducted or supported by the DoD must comply with 32 CFR 219, *Protection of Human Subjects* (and DoD Instruction 3216.02, *Protection of Human Subjects and Adherence to Ethical Standards in DoD-Supported Research* (http://www.dtic.mil/whs/directives/corres/pdf/321602p.pdf). Institutions awarded funding for research involving human subjects must provide documentation of a current Assurance of Compliance with federal regulations for human subjects protection, such as a Department of Health and Human Services, Office of Human Research Protection Federal Wide Assurance (http://www.hhs.gov/ohrp). All institutions engaged in human subjects research, to include subcontractors, must also hold a valid assurance. In addition, all personnel involved in human subjects research must provide documentation of completion of human subjects research training. For all proposed research that will involve human subjects in the first year or phase of the project, the institution must provide evidence of or a plan for review by an Institutional Review Board (IRB) upon final proposal submission to DARPA as part of their proposal, prior to being selected for funding. The IRB conducting the review must be the IRB identified on the institution's Assurance of Compliance with human subjects protection regulations. The protocol, separate from the proposal, must include a detailed description of the research plan, study population, risks and benefits of study participation, recruitment and consent process, data collection, and data analysis. It is recommended that you consult the designated IRB for guidance on writing the protocol. The informed consent document must comply with federal regulations (32 CFR 219.116). A valid Assurance of Compliance with human subjects protection regulations along with evidence of completion of appropriate human subjects research training by all investigators and personnel involved with human subjects research should accompany the protocol for review by the IRB.

In addition to a local IRB approval, a headquarters-level human subjects administrative review and approval is required for all research conducted or supported by the DoD. The Army, Navy, or Air Force office responsible for managing the award can provide guidance and information about their component's headquarters-level review process. Note that confirmation of a current Assurance of Compliance with human subjects protection regulations and appropriate human subjects research training is required before headquarters-level approval can be issued. The time required to complete the IRB review/approval process varies depending on the complexity of the research and the level of risk involved with the study. The IRB approval process can last between one and three months, followed by a DoD review that could last between three and six months. Ample time should be allotted to complete the approval process. DoD/DARPA funding cannot be used toward human subjects research until ALL approvals are granted.

Human Subjects Research and Animal Use

Human Subjects Research

All research selected for funding involving human subjects, to include use of human biological specimens and human data, must comply with the federal regulations for human subjects protection. Further, research involving human subjects that is conducted or supported by the DoD must comply with 32 CFR 219, Protection of Human Subjects (and DoD Instruction 3216.02, Protection of Human Subjects and Adherence to Ethical Standards in DoD-Supported Research (http://www.dtic.mil/whs/directives/corres/pdf/321602p.pdf).

Institutions awarded funding for research involving human subjects must provide documentation of a current Assurance of Compliance with federal regulations for human subjects protection, such as a Department of Health and Human Services, Office of Human Research Protection Federal Wide Assurance (http://www.hhs.gov/ohrp). All institutions engaged in human subjects research, to include subcontractors, must also hold a valid assurance. In addition, all personnel involved in human subjects research must provide documentation of completion of human subjects research training.

For all proposed research that will involve human subjects in the first year or phase of the project, the institution must provide evidence of or a plan for review by an Institutional Review Board (IRB) upon final proposal submission to DARPA as part of their proposal, prior to being selected for funding. The IRB conducting the review must be the IRB identified on the institution's Assurance of Compliance with human subjects protection regulations. The protocol, separate from the proposal, must include a detailed description of the research plan, study population, risks and benefits of study participation, recruitment and consent process, data collection, and data analysis. It is recommended that you consult the designated IRB for guidance on writing the protocol. The informed consent document must comply with federal regulations (32 CFR 219.116). A valid Assurance of Compliance with human subjects protection regulations along with evidence of completion of appropriate human subjects research training by all investigators and personnel involved with human subjects research should accompany the protocol for review by the IRB.

In addition to a local IRB approval, a headquarters-level human subjects administrative review and approval is required for all research conducted or supported by the DoD. The Army, Navy, or Air Force office responsible for managing the award can provide guidance and information about their component's headquarters-level review process. Note that confirmation of a current Assurance of Compliance with human subjects protection regulations and appropriate human subjects research training is required before headquarters-level approval can be issued.
The time required to complete the IRB review/approval process varies depending on the complexity of the research and the level of risk involved with the study. The IRB approval process can last between one and three months, followed by a DoD review that could last between three and six months. Ample time should be allotted to complete the approval process. DoD/DARPA funding cannot be used toward human subjects research until ALL approvals are granted.

Animal Use

Award recipients performing research, experimentation, or testing involving the use of animals shall comply with the rules on animal acquisition, transport, care, handling, and use as outlined in: (i) 9 CFR parts 1-4, Department of Agriculture rules that implement the Animal Welfare Act of 1966, as amended, (7 U.S.C. § 2131-2159); (ii) National Institutes of Health Publication No. 86-23, "Guide for the Care and Use of Laboratory Animals" (8th Edition); and (iii) DoD Instruction 3216.01, "Use of Animals in DoD Programs."

For projects anticipating animal use, proposals should briefly describe plans for Institutional Animal Care and Use Committee (IACUC) review and approval. Animal studies in the program will be expected to comply with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, available at http://grants.nih.gov/grants/olaw/olaw.htm.

All award recipients must receive approval by a DoD-certified veterinarian, in addition to an IACUC approval. No animal studies may be conducted using DoD/DARPA funding until the United States Army Medical Research and Materiel Command (USAMRMC) Animal Care and Use Review Office (ACURO) or other appropriate DoD veterinary office(s) grant approval. As a part of this secondary review process, the award recipient will be required to complete and submit an ACURO Animal Use Appendix, which may be found at http://mrmc.amedd.army.mil/index.cfm?pageid=research_protections.acuro_animalappendix.

Intellectual Property

All proposers must provide a good faith representation that the proposer either owns or possesses the appropriate licensing rights to all intellectual property that will be utilized under the proposed effort.

For Procurement Contracts

Proposers responding to this BAA requesting procurement contracts will need to complete the certifications at DFARS 252.227-7017. See http://www.darpa.mil/work-with-us/additional-baa for further information. If no restrictions are intended, the proposer should state "none." The table below captures the requested information:

Technical Data Computer Software To be Furnished With Restrictions	Summary of Intended Use in the Conduct of the Research	Basis for Assertion	Asserted Rights Category	Name of Person Asserting Restrictions
(LIST)	(NARRATIVE)	(LIST)	(LIST)	(LIST)

For All Non-Procurement Contracts

Proposers responding to this BAA requesting a Cooperative Agreement, Technology Investment Agreement, or Other Transaction for Prototypes shall follow the applicable rules and regulations governing these various award instruments, but, in all cases, should appropriately identify any potential restrictions on the Government's use of any Intellectual Property contemplated under the award instrument in question. This includes both Noncommercial Items and Commercial Items. Proposers are encouraged to use a format similar to that described in the section above. If no restrictions are intended, then the proposer should state "NONE."

Controlled Unclassified Information (CUI) on Non-DoD Information Systems

In addition to classified information, certain types of unclassified information also require application of access and distribution controls and protective measures for a variety of reasons. This information is referred to collectively as Controlled Unclassified Information (CUI). DoD CUI includes, but is not limited to: For Official Use Only (FOUO), Law Enforcement Sensitive (LES), DoD Unclassified Controlled Nuclear Information (DoD UCNI), and Limited Distribution, as well as some categories developed by other executive branch agencies. For further information, consult DoDM 5200.01, Volume 4, "DoD Information Security Program: Controlled Unclassified Information (CUI)."

All non-DoD entities doing business with DARPA are expected to adhere to the following procedural safeguards, in addition to any other relevant federal or DoD specific procedures, for submission of any proposals to DARPA and any potential business with DARPA:

- Do not process DARPA CUI on publicly available computers or post DARPA CUI to publicly available webpages or websites that have access limited only by domain or Internet protocol restriction.
- Ensure that all DARPA CUI is protected by a physical or electronic barrier when not under direct individual control of an authorized user and limit the transfer or DARPA CUI to subcontractors or teaming partners with a need to know and commitment to this level of protection.
- Ensure that DARPA CUI on mobile computing devices is identified and encrypted and all communications on mobile devices or through wireless connections are protected and encrypted.
- Overwrite media that has been used to process DARPA CUI before external release or disposal. Proposers with questions regarding CUI should contact the DARPA Security and Intelligence Directorate at (703) 526-1581.

Publication Restrictions on Non-Fundamental Research

National Security Decision Directive (NSDD) 189 defines fundamental research as follows:

"Fundamental research" means basic and applied research in science and engineering, the results of which ordinarily are published and shared broadly within the scientific community, as distinguished from proprietary research and from industrial development, design, production, and product utilization, the results of which ordinarily are restricted for proprietary or national security reasons.

The following clause or similar language will be included in all awards where the resultant research will not be considered fundamental research and there will be restrictions on publication:

"There shall be no dissemination or publication, except within and between the contractor and any subcontractors, of information developed under this contract or contained in the reports to be furnished pursuant to this contract without prior written approval of DARPA's Public Release Center (DARPA/PRC). All technical reports will be given proper review by appropriate authority to determine which distribution statement is to be applied prior to the initial distribution of these reports by the contractor. With regard to subcontractor proposals for fundamental research, papers resulting from unclassified fundamental research are exempt from prepublication controls and this review requirement, pursuant to DoD Instruction 5230.27 dated October 6, 1987.

When submitting material for written approval for open publication, the contractor/awardee must submit a request for public release to the DARPA/PRC and include the following information: (1) Document Information: document title, document author, short plain-language description of technology discussed in the material (approx. 30 words), number of pages (or minutes of video), and document type (e.g., briefing, report, abstract, article, or paper); (2)

Event Information: event type (conference, principal investigator meeting, article, or paper), event date, desired date for DARPA's approval; (3) DARPA Sponsor: DARPA program manager, DARPA office, and contract number; and (4) Contractor/Awardee's Information: POC name, e-mail, and phone. Allow three weeks for processing. Due dates under three weeks require a justification. Unusual electronic file formats may require additional processing time. Requests may be sent either via email to public_release_center@darpa.mil or by mail to 675 North Randolph Street, Arlington VA 22203-2114, telephone (571) 218-4235. Refer to the following for link for information about DARPA's public release process: http://www.darpa.mil/work-with-us/contract-management/public-release.

Title of Contract Effort	
Name of Organization providing Reference	
Name of POC Providing Reference **Telephone Number** **Email Address**	**Name:** **Telephone:** **Email:**
Contract/Delivery Order Number	
Contract Type	
Contract Value	Total contract value
Final Delivery Schedule (PoP)	Entire contract PoP
SCOPE OF WORK	
Description of Contract Work ***Rationale for Comparability to the Proposed Effort***	

April 7, 2017

Subject: Formal Request for Proposal (RFP)
In response refer to RFP No. LEIDOS-DARPA-P3 _____(Your Company Name)

References: DARPA-HR001117S0019

Team Member:

You are requested to submit a **Firm Fixed Price** proposal in response to this Request for Proposal (RFP). The Statement of Work (SOW) and Specifications will be provided shortly in an amendment to this RFP. Your proposal must be in accordance with the terms, conditions, and special provisions included herein with this RFP. You must complete and return the Suppliers Reps and Certs (Attachment G) and the pricing template (Attachment H). On the last page of this letter is a Proposal Check List for you to use to ensure that all required documentation has been included in your proposal.

Your response is due by 2:00 PM (EST) on Wednesday, April 19, 2017.

PROPOSAL INSTRUCTIONS AND INFORMATION

Leidos is not obligated to pay for any expenses incurred as a result of your efforts to respond to this solicitation. Moreover, Leidos will not, by virtue of this RFP, become obligated to award a subcontract. Leidos reserves the right to subsequently award the effort described herein to one or more respondents determined to be in the overall best interest of Leidos.

All confidential or proprietary information shall be provided in accordance with the Non-Disclosure Agreement (NDA). All information provided by you in response to this solicitation will remain the property of **Leidos unless agreed to otherwise in an NDA.**

You are cautioned to strictly adhere to the enclosed proposal instructions, with particular attention to the form, format, and content of your proposal. Failure to do so may result in our decision to eliminate your proposal from further consideration. However, alternative proposals, which contain products or services which meet the functional requirements will be considered as separate proposals.

I. PROPOSAL COVER LETTER

Leidos requires a Proposal Cover Letter. Your cover letter must contain all of the following:

- ✓ Dated on or before Wednesday, April 19, 2017;
- ✓ Signed by an official within your organization authorized to legally bind your Company;
- ✓ State any exceptions taken to the terms and conditions of the SOW or "Draft" Subcontract Agreement (Schedule A, Schedule B Part I and Schedule B Part II) or any of its attachments;
- ✓ State a Proposal Validity Period of one hundred and eighty (180) days.

II. STATEMENT OF WORK

The Statement of Work (SOW) will be provided later.

III. Draft Subcontract Agreement and Required Clauses

It is anticipated that the effort set forth in this RFP will be performed on a Firm Fixed (FFP) basis. Any resultant award will include all clauses required by Leidos' Prime Contract, all clauses required by law on the date of execution of the Subcontract, and any other mutually agreeable clauses, provisions and terms and conditions, including:

- "Draft" Firm Fixed (FFP) Subcontract: Schedule A (Attachment C)
- Subcontract: Schedule B Part I – FAR Flow-down clauses (Attachment D)
- Subcontract: Schedule B Part II – DFAR Flow-down clauses (Attachment E)
- Subcontract: Schedule C – DARPA Unique Prime Contract Flowdown and Specific Requirements (Attachment F)

IV. Period of Performance

The P3 program will be approximately a four-year (4) effort from date of award.

V. CERTIFICATE OF INSURANCE

The insurance requirements set forth in Leidos' Schedule A may exceed your company's current insurance levels. Please cross-reference your company's current insurance policy with the Leidos requirements. Please provide a copy of your company's Certificate of Insurance. Upon award of a subcontract by Leidos, it is required that Leidos be named as additionally insured with your insurance provider. (No Waivers will be granted).

VI. Cost/Price Proposal

Per Section II of the DARPA-P3 BAA – Detailed Proposal Information the following cost/pricing detailed breakdown is required:

The cost/price proposal **MUST** be submitted in sufficient detail and must provide such supporting documentation as appropriate and necessary to permit proper evaluation and analysis.

1. Labor Rates

SAMPLE:

Labor Categories	Total Labor Hours	Labor Rate	Fringe Rate %	Overhead Rate %	G&A Rate %	Fee %	Fully Burdened Labor Rate	Total Price per Labor Category
Administrative Assistant	345							
Clinical Nurse Specialist	657							
Technical Writer I	565							
Total	**1468**							

2. **Indirect Costs** – Including Fringe Benefits, Overhead, General and Administrative Expense, Cost of Money, Fee, etc. (must show base amount and rate), if available, provide current Forward Pricing Rate Agreement or Forward Pricing Rate Proposal. If not available, provide 2 years historical data to include pool and expense costs used to generate the rates. For academia, provide DHHS or ONR negotiated rate package or, if calculated by other than a rate, provide University documentation identifying G&A and fringe costs by position;

3. **Travel:**

All proposed travel must be in accordance with:

GSA website: (CONUS): http://www.gsa.gov/portal/content/101518

Defense Travel Management website: (OCONUS):
http://www.defensetravel.dod.mil/site/perdiemCalc.cfm

All other cost (i.e. airfare and rental car) must have supporting documentation.

Trips must be identified by:

a. Departure and Arrival Destinations
b. Number employees per trip
c. Number of Trips per Period
d. Number of Days/Nights per trip
e. Estimated car rental costs
f. Estimated airfare costs

All Travel costs must be supported with screenshots from the travel websites.

4. **Other Direct Costs (ODC's)**

Subcontractor must provide a list of ODC's that will be procured for this requirement and pricing documentation.

Other Direct Costs – Itemized with costs including tuition remission, animal per diem rates, health insurance/fee; back-up documentation is to be submitted to support proposed costs;

Equipment Purchases – Itemization with individual and total costs, including quantities, unit prices, proposed vendors (if known), and the basis of estimate (e.g., quotes, prior purchases, catalog price lists, etc.); any item that exceeds $5,000 must be supported with back-up documentation such as a copy of catalog price lists or quotes prior to purchase (NOTE: For equipment purchases, include a letter stating why the proposer cannot provide the requested resources from its own funding), and;

Materials – Itemization with costs, including quantities, unit prices, proposed vendors (if known), and the basis of estimate (e.g., quotes, prior purchases, catalog price lists, etc.); any item that exceeds $5,000 must be supported with back-up documentation such as a copy of catalog price lists or quotes prior to purchase.

All Proposed ODC's must be supported with screenshots from websites or other documentation.

This level of detail must be provided for FFP subcontracts.

VII. Cost Realism

DARPA intends to perform a Cost Realism analysis on this proposal.

Cost Realism Definition:
The proposed costs are realistic for the technical and management approach and accurately reflect the technical goals and objectives of the solicitation. The proposed costs are consistent with the proposer's Statement of Work and reflect a sufficient understanding of the costs and level of effort needed to successfully accomplish the proposed technical approach. The costs for the prime proposer and proposed subawardees are substantiated by the details provided in the proposal (e.g., the type and number of labor hours proposed per task, the types and quantities of materials, equipment and fabrication costs, travel and any other applicable costs and the basis for the estimates).

It is expected that the effort will leverage all available relevant prior research in order to obtain the maximum benefit from the available funding. For efforts with a likelihood of commercial application, appropriate direct cost sharing may be a positive factor in the evaluation. DARPA recognizes that undue emphasis on cost may motivate proposers to offer low-risk ideas with minimum uncertainty and to staff the effort with junior personnel in order to be in a more competitive posture. DARPA discourages such cost strategies.

VIII. Past Performance:

Subcontractor should provide __2___ past performance references that closely match the RFP requirement. Template is attached.

IX. REPRESENTATIONS AND CERTIFICATIONS & VENDOR MASTER TEMPLATE

Please complete the Supplier Representations and Certifications (Attachment G).

X. PROPOSAL VALIDITY PERIOD

Your proposal shall be valid for a period of one hundred and twenty (180) days.

XI. Proposal Submittal Requirements

1. Due Date: On or before 2:00 p.m. (EST) on Wednesday, April 19, 2017.

2. Method of Submission: One (1) electronic copy to: kristen.k.trump@leidos.com and Michael.a.younkins@leidos.com.

XII. ORGANIZATIONAL CONFLICTS OF INTEREST

FAR 9.5 Requirements - https://www.acquisition.gov/far/html/Subpart%209_5.html

In accordance with FAR 9.5, proposers are required to identify and disclose all facts relevant to potential OCIs involving the proposer's organization and any proposed team member. Under this Section, the proposer is responsible for providing this disclosure with each proposal submitted to the BAA. The

disclosure must include the proposer's, and as applicable, proposed team member's OCI mitigation plan. The OCI mitigation plan must include a description of the actions the proposer has taken, or intends to take, to prevent the existence of conflicting roles that might bias the proposer's judgment and to prevent the proposer from having unfair competitive advantage. The OCI mitigation plan will 18 HR001117S0019, Pandemic Prevention Platform (P3) specifically discuss the disclosed OCI in the context of each of the OCI limitations outlined in FAR 9.505-1 through FAR 9.505-4.

DARPA Agency Supplemental OCI Policy

In addition, DARPA has a supplemental OCI policy that prohibits contractors/performers from concurrently providing Scientific Engineering Technical Assistance (SETA), Advisory and Assistance Services (A&AS) or similar support services and being a technical performer. Therefore, as part of the FAR 9.5 disclosure requirement above, a proposer must affirm whether the proposer or any proposed team member is providing SETA, A&AS, or similar support to any DARPA office(s) under: (a) a current award or subaward; or (b) a past award or subaward that ended within one calendar year prior to the proposal's submission date.

If SETA, A&AS, or similar support is being or was provided to any DARPA office(s), the proposal must include:

- The name of the DARPA office receiving the support;
- The prime contract number;
- Identification of proposed team member (subawardee, consultant) providing the support; and
- An OCI mitigation plan in accordance with FAR 9.5.

Additional Requirements:

a. Identification of technical data previously delivered to the Government IAW DFARS 252.227-7028.
b. Provide permission for Leidos to use your LOGO
c. If you are a Large Business – you must provide a Small Business Subcontracting Plan with your proposal
d. All Subcontractors who are considered a Federally Funded Research & Development Center (FFRDC), must include documentation that clearly demonstrates work is not otherwise available from the private sector AND provided a letter on letterhead from the sponsoring organization citing the specific authority establishing their eligibility to propose to government solicitations and compete with industry, and compliance with the associated FFRDC sponsor agreement and terms and conditions.

Your proposal must be received by Leidos by Wednesday, April 19, 2017 and include a validity period of **180 days** from the due date. Please direct any questions, in writing, regarding this RFP to the undersigned.

Proposals will be evaluated against the following evaluation criteria: Award shall be made to the best value proposal considering price, schedule, technically acceptable proposal and appropriate facilities.

RESPONDING TO THIS REQUEST FOR PROPOSAL CERTIFIES THAT NO GRATUITIES WERE OFFERED BY THE SUPPLIER OR SOLICITED BY ANY LEIDOS EMPLOYEE. IF A GRATUITY WAS SOLICITED, IT SHOULD BE REPORTED IMMEDIATELY TO MR. KIM DENVER, SR. VICE PRESIDENT, AT (571) 526-7051. PLEASE REVIEW LEIDOS' CODE OF ETHICS AT https://www.leidos.com/sites/default/files/CodeOfConduct.pdf.

Kristen Trump
Subcontracts Administrator
240-529-0419

Attachment 1: RFP/RFQ Enclosures

ATTACHMENTS - RFP/RFQ ENCLOSURES

A.	Statement/Scope of Work and Schedule (TBD)
B.	RFQP General Provisions
C.	Schedule A – Federal - FFP
D.	Schedule B Part I – Federal Non-Commercial Items
E.	Schedule B Part II – DOD Non-Commercial Items
F.	Schedule C – DARPA Unique Prime Contract Flowdown Clauses and Requirements
G.	Supplier Reps and Certs
H.	Pricing Template (Sample)
I.	Past Performance Template

SUBMIT WITH YOUR PROPOSAL

1. Certificate of Insurance in accordance with Section 10.0 of the Subcontract Agreement – Schedule A, Federal, FFP IDIQ (Rev 2015-08) or Evidence of Coverage
2. Letter of Commitment on company letterhead
3. One electronic copy of the technical proposal
4. One electronic copy of the price proposal

Specific Terms and Conditions and Contract Clauses

It should be noted that any purchase order/subcontract resulting from this proposal shall include specific terms and conditions and contract clauses, which are listed by FAR reference, and which are identified herein as Attachment C, D, E, F respectively. All clauses required by the Prime Contract, all clauses required by law on the date of execution of the subcontract, and any mutually agreeable clauses, terms and conditions will be negotiated at time of award.

PROPOSAL CHECK LIST

1. Pricing Detail
    a. Forward Pricing Rate Agreement or Forward Pricing Rate Proposal
    b. If “a” above is not available – 2 Years of Historical Data to Include Pool and Expense Costs Used to Generate the Rates
    c. If Academia – Provide DHHS or ONR Negotiated Rates
    d. Salary Breakdown with all Indirect Rates
    e. Travel Breakdown and Documentation
    f. ODC Breakdown (Materials and Supplies)and Documentation (Description, Make, Model, quantity and Price per unit)
    g. Equipment Breakdown and Documentation (Description, Make, Model, quantity and price per unit)
    h. Suppliers Reps and Certs – Completed and Signed

2. OCI Certification

3. Past Performance

4. FFRDC Documentation

5. LOGO (High resolution jpeg) and permission for Leidos to use for proposal

6. Certificate of Insurance – will be required at time of award

7. Review Attachment C – Schedule A – Federal – FFP document, Schedule B Part I and II. These documents do not have to be returned by April 19, 2017 – however you do need to submit it within 15 days after the requested proposal date with any redlines.

REQUEST FOR QUOTATION/PROPOSAL (RFQ/P)

GENERAL PROVISIONS

We would like to take this opportunity to remind suppliers of our commitment to conduct business with uncompromising integrity. This commitment is clearly established in Leidos' Code of Conduct. Leidos expects suppliers to conduct themselves in a manner consistent with the principles of our code of conduct. In addition, we strongly encourage our suppliers to have proactive and meaningful ethics programs established within their organizations. We want our suppliers to understand, foster, and mirror the ethical conduct we expect from our employees in all business transactions. If you believe that Leidos or any of its employees or agents has acted improperly or unethically, please report such behavior to the Leidos ethics HOTLINE (855) 7LEIDOS.

1. **LATE OFFERS.** Formal offers, amendments, or requests for withdrawal of offers received after the date specified for submittal may not be considered.

2. **ALTERNATE PROPOSALS.** In addition to the offer solicited herein, the offeror is invited to submit an alternate proposal, which may be advantageous to Leidos ("Leidos" or "Buyer").

3. **DISCLOSURE OF PARTICIPANTS.** All lower-tier subcontractors must be disclosed with the proposal.

4. **COMPLETENESS.** All information required by RFQ/P must be supplied to constitute a responsive bid. Non-responsive offers may not be considered.

5. **BRAND NAMES.** Brand names and part numbers, when used, are for reference to indicate the performance or quality desired. Equal items will be considered provided that the offeror describes the article. Offers for equal items shall state the brand name and part number, or level of quality. The determination of the Buyer as to what items are equal shall be final and conclusive. When brand name, part number, or level of quality is not stated by the offeror, it is understood the offer is exactly as specified.

6. **COUNTERFEIT PRODUCTS.** For purposes of this clause, Goods are any tangible items, including without limitation the lowest level of separately identifiable items, such as parts, articles, components, and assemblies. "Counterfeit Goods" are Goods that are or contain items misrepresented as having been designed, produced, and/or sold by an authorized manufacturer and seller, including without limitation unauthorized copies, replicas, or substitutes. The term also includes authorized Goods that have reached a design life limit or have been damaged beyond possible repair, but are altered and misrepresented as acceptable.

   Offeror agrees and shall ensure that Counterfeit Goods are not delivered to Leidos. Goods delivered to Leidos or incorporated into other Goods and delivered to Leidos shall be new and shall be procured directly from the Original Component Manufacturer (OCM)/Original Equipment Manufacturer (OEM), or through an OCM/OEM authorized distributor chain. If requested by Leidos, bidder shall provide OCM/OEM documentation that authenticates products. Offeror shall report suspected or confirmed counterfeit items into the Government-Industry Data Exchange Program (GIDEP).

7. **NET PRICES.** Offered prices, unless otherwise specified, must be net, including transportation and handling charges, which shall be set forth as a separate line item on quotation/proposal. Transportation charges must be fully prepaid by offeror to destination, and subject only to cash discount for prompt payment of invoices.
   a. Prices should be quoted as "Unit" prices; do not quote "Lot" prices.
   b. Provide pricing schedule based on specified price breaks, if any.
   c. If applicable, furnish published price list with offer.

8. **EVALUATION.** Buyer reserves the right: (1) to award on the basis of individual items, or groups of items, or on the entire list of items; (2) to reject any or all offers, or any part thereof; (3) to waive any informality in the offers; and (4) to accept the offer that is in the best interest of Leidos. The Buyer's decision shall be final.

9. **NO BID.** In the event an offer cannot be submitted for the specified requirements as set forth in the RFQ/P, please provide an explanation as to why you are unable to bid on these requirements.

10. **TAXES.** Buyer may be exempt from the payment of any federal excise or any state sales tax. The price offered must be net, exclusive of taxes. However, when under established trade practice, any federal excise tax is included in the list price, offeror may quote the list price and shall show separately the amount of federal tax, either as a flat sum or as a percentage of the list price, which shall be deducted by Buyer.

11. **AWARD.** The order will be awarded to the lowest responsible and responsive offeror complying with all the provisions of the RFQ/P, provided the offered price is reasonable and is in the best interest of Buyer. The Buyer reserves the right to reject the offer of an offeror who has previously failed to perform properly or complete on time, contacts of similar nature, or the offer of an offeror who, under investigation shows is not in position to perform the order.

12. **ACCEPTANCE.** A written award mailed (or otherwise furnished) to the successful offeror shall be deemed to result in a binding contract without further action by either party.

13. **SERVICE LIFE.** When applicable, all offerors will state their company policy regarding the return of defective and unserviceable items or products as well as the minimum service life of the offered item or product.

14. **DELIVERY.** When applicable, all shipments are to be made F.O.B. to a specific destination as specified, or prepaid. Collect shipments can be accepted only with proper advance notification, and only with specific approval from the Buyer.

15. **PACKING SLIPS OR DELIVERY TICKETS.** When applicable, all shipments or deliveries shall be accompanied by Packing Slips or Delivery Tickets in duplicate and shall contain the following information for each item delivered:
    a. Purchase Order Number
    b. Item Number
    c. Description or Part Number
    d. Quantity Ordered
    e. Quantity Shipped
    f. Name of the Supplier
    g. The above requirement is extremely important when accepting shipments. Offerors are cautioned that failure to comply with these conditions shall be considered sufficient reason for refusal to accept the goods.

16. **LIABILITY.** Leidos is not obligated to pay for any expenses incurred as a result of your efforts to respond to this solicitation. Moreover, Leidos will not, by virtue of this solicitation, become obligated to award a purchase order. Leidos reserves the right to subsequently award the effort described herein to a respondent determined to be in compliance with the guidelines and in the overall best interest of Leidos and its customer.

    Leidos will not accept confidential or proprietary information without a signed Non-Disclosure Agreement. All information provided by you in response to this solicitation will remain the property of the Leidos.

    The Offeror shall hold Buyer, its officers, agents, servants, and employees, harmless from liability of any nature or kind because of use of any copyrighted, or uncopyrighted compositions; secret process, patented or unpatented invention; articles or appliances furnished or used under this bid, and agrees to defend, at Offeror's expense, any and all actions brought against Buyer, or itself because of the unauthorized use of such articles.

17. **GENERAL.** Any Purchase Order or Subcontract issued as a result of this Request for Quote/Proposal is subject to Buyer's Terms and Conditions regardless of offeror's terms and conditions submitted with the proposal. Specific exceptions are to be noted as part of offeror's proposal and if incorporated into Buyer's Purchase Order or Subcontract will become binding on the parties.

18. **TERMS and CONDITIONS.** This prospective order is subject to all attachments in this solicitation to include, but not limited to: Statement of Work (when applicable); Leidos' standard terms and conditions; Federal terms and Conditions, agency clauses, and/or any specific Customer clauses included; Supplier Representations and Certifications.

19. GRATUITIES. By acknowledgment of response to this RFQ/P, the offeror hereby certifies that no gratuities were offered by the offeror or solicited by any Leidos employee either directly or indirectly. Any situation where a gratuity is solicited should be reported immediately to the Leidos SVP, Contracts, Procurement and Pricing at 571-526-7051.

I. OVERVIEW OF THE FUNDING OPPORTUNITY

Program Announcement for the Department of Defense

Defense Health Program

Congressionally Directed Medical Research Programs

Peer Reviewed Medical Research Program

Focused Program Award

Announcement Type: Initial

Funding Opportunity Number: W81XWH-17-PRMRP-FPA

Catalog of Federal Domestic Assistance Number: 12.420 Military Medical Research and Development

SUBMISSION AND REVIEW DATES AND TIMES

- **Pre-Application Submission Deadline:** 5:00 p.m. Eastern time (ET), July 20, 2017
- **Invitation to Submit an Application:** August 2017
- **Application Submission Deadline:** 11:59 p.m. ET, October 26, 2017
- **End of Application Verification Period:** 5:00 p.m. ET, October 31, 2017
- **Peer Review:** December 2017
- **Programmatic Review:** February 2018

This Program Announcement must be read in conjunction with the General Application Instructions, version 20170516. The General Applications Instructions document is available for downloading from the Grants.gov funding opportunity announcement by selecting the "Package" tab, clicking "Preview," and then selecting "Download Instructions."

TABLE OF CONTENTS

I. OVERVIEW OF THE FUNDING OPPORTUNITY 1

II. DETAILED INFORMATION ABOUT THE FUNDING OPPORTUNITY 4

II.A. Program Description 4

II.A.1. FY17 PRMRP Topic Areas 4

II.B. Award Information 6

II.C. Eligibility Information 9

II.C.1. Eligible Applicants 9

II.C.2. Cost Sharing 10

II.C.3. Other 10

II.D. Application and Submission Information 10

II.D.1. Address to Request Application Package 11

II.D.2. Content and Form of the Application Submission 11

II.D.3. Dun and Bradstreet Universal Numbering System (DUNS) Number and System for Award Management (SAM) 29

II.D.4. Submission Dates and Times 29

II.D.5. Funding Restrictions 30

II.D.6. Other Submission Requirements 31

II.E. Application Review Information 31

II.E.1. Criteria 31

II.E.2. Application Review and Selection Process 36

II.E.3. Integrity and Performance Information 37

II.E.4. Anticipated Announcement and Federal Award Dates 37

II.F. Federal Award Administration Information 37

II.F.1. Federal Award Notices 37

II.F.2. Administrative and National Policy Requirements 39

II.F.3. Reporting 39

II.G. Federal Awarding Agency Contacts 40

II.G.1. CDMRP Help Desk 40

II.G.2. Grants.gov Contact Center 40

II.H. Other Information 40

II.H.1. Program Announcement and General Application Instructions Versions 40

II.H.2. Administrative Actions 40

II.H.3. Application Submission Checklist 43

APPENDIX 1 ACRONYM LIST 45
APPENDIX 2 AREAS OF ENCOURAGEMENT 46
APPENDIX 3 DOD AND VA WEBSITES 65

II. DETAILED INFORMATION ABOUT THE FUNDING OPPORTUNITY

II.A. Program Description

Applications to the Fiscal Year 2017 (FY17) Peer Reviewed Medical Research Program (PRMRP) are being solicited for the Defense Health Agency (DHA) J9, Research and Development Directorate, by the U.S. Army Medical Research Acquisition Activity (USAMRAA) using delegated authority provided by United States Code, Title 10, Section 2358 (10 USC 2358). As directed by the Office of the Assistant Secretary of Defense for Health Affairs (OASD[HA]), the DHA manages the Defense Health Program (DHP) Research, Development, Test, and Evaluation (RDT&E) appropriation. The execution management agent for this Program Announcement is the Congressionally Directed Medical Research Programs (CDMRP). The PRMRP was initiated in 1999 to provide support for military health-related research of exceptional scientific merit. Appropriations for the PRMRP from FY99 through FY16 totaled $1.371 billion. The FY17 appropriation is $300 million (M).

The vision of the FY17 PRMRP is to improve the health and well-being of all military Service members, Veterans, and beneficiaries. The PRMRP challenges the scientific and clinical communities to address at least one of the FY17 Topic Areas with original ideas that foster new directions along the entire spectrum of research and clinical care. The program seeks applications in laboratory, clinical, behavioral, epidemiologic, and other areas of research to advance knowledge in disease etiology, improve prevention, detection, diagnosis, treatment, and quality of life for those affected by a relevant disease or condition, and to develop and validate clinical care or public health guidelines.

II.A.1. FY17 PRMRP Topic Areas

All applications for PRMRP funding must specifically address at least one of the Topic Areas as directed by Congress and must be directly relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries. If the proposed research does not specifically address at least one of the FY17 PRMRP Topic Areas, the Government will administratively withdraw the application. The Government reserves the right to reassign the application's Topic Area if submitted under an inappropriate Topic Area. The FY17 PRMRP Topic Areas are listed below.

- Acute Lung Injury
- Antimicrobial Resistance
- Arthritis
- Burn Pit Exposure
- Chronic Migraine and Post-Traumatic Headache
- Congenital Heart Disease
- Constrictive Bronchiolitis
- Diabetes
- Diarrheal Diseases
- Dystonia
- Early Trauma Thermal Regulation
- Eating Disorders

- Emerging Infectious Diseases
- Epidermolysis Bullosa
- Focal Segmental Glomerulosclerosis
- Fragile X
- Guillain-Barré Syndrome
- Hepatitis B and C
- Hereditary Angioedema
- Hydrocephalus
- Immunomonitoring of Intestinal Transplants
- Inflammatory Bowel Diseases
- Influenza
- Integrative Medicine
- Interstitial Cystitis
- Malaria
- Metals Toxicology
- Mitochondrial Disease
- Musculoskeletal Disorders
- Nanomaterials for Bone Regeneration
- Non-Opioid Pain Management
- Pancreatitis
- Pathogen-Inactivated Dried Cryoprecipitate
- Polycystic Kidney Disease
- Post-Traumatic Osteoarthritis
- Pulmonary Fibrosis
- Respiratory Health
- Rett Syndrome
- Rheumatoid Arthritis
- Scleroderma
- Sleep Disorders
- Spinal Muscular Atrophy
- Sustained-Release Drug Delivery
- Tinnitus
- Tuberculosis
- Vaccine Development for Infectious Disease
- Vascular Malformations
- Women's Heart Disease

Research relevant to one or more FY17 PRMRP Topic Areas may be considered for funding. ***Applicants should select the FY17 PRMRP Program Announcement most appropriate to the stage of the proposed research.*** Areas of Encouragement related to the FY17 PRMRP Topic Areas have been identified by the Department of Defense (DoD), the Department of Veterans Affairs (VA), and other relevant stakeholders (Appendix 2). Applicants are urged to read and consider these Areas of Encouragement before preparing their applications. ***The information provided is not exhaustive, and applicants are not restricted to submitting applications that address an Area of Encouragement on this list.***

II.B. Award Information

The PRMRP Focused Program Award mechanism is intended to optimize research and accelerate the solution for a critical question related to at least one of the Congressionally directed FY17 PRMRP Topic Areas through a synergistic, multidisciplinary research program. The anticipated total costs budgeted for the entire period of performance for an FY17 PRMRP Focused Program Award will not exceed **$10M**. Refer to Section II.D.5, Funding Restrictions, for detailed funding information.

Key aspects of this award include:

Overarching Challenge: Focused Program Award applications must describe a unifying, overarching challenge that will be addressed by a set of research projects. The overarching challenge must be relevant to a critical problem or question in the field of research and/or patient care in at least one of the FY17 PRMRP Topic Areas.

Research Projects: Applications shall include multiple, distinct research projects led by individual project leaders that address complementary aspects of the overarching challenge. Applicants are strongly encouraged to submit a minimum of four research projects; additional studies are allowed. While individual projects must be capable of standing on their own high scientific merits, they must also be interrelated and synergistic with the other proposed projects and advance a solution beyond what would be possible through individual efforts. The exploration of multiple hypotheses or viewpoints of the same line of questioning is encouraged. This award mechanism is not intended to support a series of research projects that are dependent on the success of any other project. Each project should propose a unique approach to addressing the overarching challenge and be capable of producing research findings with potential to impact the field and/or patient care. Individual research projects may range from exploratory, hypothesis-developing studies through small-scale clinical trials (i.e., up to and including Phase II or equivalent). There should be a clear intent to progress toward translational/ clinical work over the course of the effort.

Implementation: The research strategy to address the overarching challenge must be supported by a detailed plan that identifies critical milestones; outlines the knowledge, resources, and technical innovations that will be utilized to achieve the milestones; and explains how the outcomes will be translated to patients. A robust statistical plan and statistical expertise should be included where applicable. A plan for assessing individual project performance and progress toward addressing the overarching challenge must be included in the implementation plan. Plans to include an External Advisory Board are encouraged; however, applicants must be careful to avoid potential conflicts of interest (COIs) during review of the application by ensuring no contact with, recruiting of, or naming of specific members in the application. For multi-institutional collaborations, plans for communication and data transfer among the collaborating institutions, as well as how data, specimens, and/or imaging products obtained during the study will be handled, must be included. An intellectual and material property plan agreed to by participating organizations is required in the application's supporting documentation.

Research Team: The overall effort will be led by a Principal Investigator (PI) with demonstrated success in leading large, focused projects. The PI is required to devote a minimum

of 20% effort to this award. The PI should create an environment that fosters and supports collaboration and innovation in a way that engages all members of the team in all aspects of the research plan. The research team assembled by the PI should be highly qualified and multidisciplinary, with identified project leaders for each of the complementary and synergistic research projects. The resources and expertise brought to the team by each project leader should combine to create a robust, synergistic collaboration. The PRMRP Science Officer assigned to a resulting award must be invited to participate in periodic research team meetings. The plan for such meetings should be noted in the application.

Milestone Meeting: The PI will be required to present an update on progress toward accomplishing the goals of the award at a Milestone Meeting to be held in the National Capital Region after the conclusion of Year 2 of the period of performance. The PI may bring up to three additional members of the research team to the meeting. The Milestone Meeting will be attended by members of the PRMRP Programmatic Panel, CDMRP staff, and the USAMRAA Grants Officer.

Military Relevance: Relevance to the healthcare needs of military Service members, Veterans, and beneficiaries is a key feature of this award. Investigators are encouraged to consider the following characteristics as examples of how a project may demonstrate military relevance:

- Explanation of how the project addresses an aspect of the target disease/condition that has direct relevance to military Service members, Veterans, or other military health system beneficiaries

- Description of how the knowledge, information, products, or technologies gained from the proposed research could be implemented in a dual-use capacity to benefit the civilian population and also address a military need

- Use of military or Veteran populations or datasets in the proposed research, if appropriate to the proposed research project

- Involvement of military consultants (Army, Air Force) or specialty leaders (Navy, Marine Corps) to the Surgeons General in a relevant specialty area

PIs are strongly encouraged to integrate and/or align their research projects with DoD and/or VA research laboratories and programs. Collaboration with the DoD or VA is also encouraged. A list of websites that may be useful in identifying additional information about ongoing DoD and VA areas of research interest or potential opportunities for collaboration within the FY17 PRMRP Topic Areas can be found in Appendix 3.

Use of Active Duty Military and VA Populations: If the proposed research plan involves access to active duty military and/or VA patient populations or resources, the PI is responsible for establishing such access. If possible, access to target active duty military and/or VA patient populations/resources should be confirmed at the time of application submission by inclusion of a letter of support, signed by the lowest ranking person with approval authority, for studies involving active duty military Service members, Veterans, military- and/or VA-controlled study materials, and military and/or VA databases. If access cannot be confirmed at the time of application submission, the Government reserves the right to withhold or revoke funding until

the PI has demonstrated support for and access to the relevant population(s) and/or resources. Note that access to a Veteran population for clinical studies may only be obtained by either collaboration with a VA investigator where the VA investigator has a substantial role in the research or by advertising to the general public.

Research Involving Human Anatomical Substances, Human Subjects, or Human Cadavers: All DoD-funded research involving new and ongoing research with human anatomical substances, human subjects, or human cadavers must be reviewed and approved by the U.S. Army Medical Research and Materiel Command (USAMRMC) Office of Research Protections (ORP), Human Research Protection Office (HRPO) prior to research implementation. This administrative review requirement is in addition to the local Institutional Review Board (IRB) or Ethics Committee (EC) review. Local IRB/EC approval at the time of submission is ***not*** required. The HRPO is mandated to comply with specific laws and requirements governing all research involving human anatomical substances, human subjects, or human cadavers that is supported by the DoD. These laws and requirements will necessitate information in addition to that supplied to the IRB/EC. ***Allow a minimum of 2 to 3 months for HRPO regulatory review and approval processes***. When possible, protocols should be written for research with human subjects and/or human anatomical substances that are specific to the DoD-supported effort outlined in the submitted application. Submission to HRPO of protocols covering more than the scope of work in the DoD-funded award will require HRPO review of the entire protocol as DoD-supported research and may include extensive modifications to meet DoD human subjects protection requirements. Refer to the General Application Instructions, Appendix 1, and the Human Subject Resource Document available on the electronic Biomedical Research Application Portal (eBRAP) "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) for additional information.

A clinical trial is defined as a prospective accrual of patients (human subjects) in whom an intervention (e.g., device, drug, biologic, surgical procedure, rehabilitative modality, behavioral intervention, or other) is tested for a measurable outcome with respect to safety, effectiveness, and/or efficacy. This outcome represents a direct effect on the subject of that intervention or interaction.

Research Involving Animals: All projects should adhere to a core set of standards for rigorous study design and reporting to maximize the reproducibility and translational potential of preclinical research. The standards are described in Landis, S.C., et al. A call for transparent reporting to optimize the predictive value of preclinical research. *Nature* 2012, 490:187-191 (www.nature.com/nature/journal/v490/n7419/full/nature11556.html). While these standards are written for preclinical studies, the basic principles of randomization, blinding, sample-size estimation, and data handling derive from well-established best practices in clinical studies. Applicants should consult the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines to ensure relevant aspects of rigorous animal research are adequately planned for and, ultimately, reported. The ARRIVE guidelines can be found at http://www.elsevier.com/__data/promis_misc/622936arrive_guidelines.pdf

All DoD-funded research involving new and ongoing research with animals must be reviewed and approved by the USAMRMC ORP Animal Care and Use Review Office (ACURO), in addition to the local Institutional Animal Care and Use Committee (IACUC) of record. IACUC

approval at the time of submission is ***not*** required. Specific documents relating to the use of animals in the proposed research will be requested **if the application is selected for funding**. The ACURO must review and approve all animal use prior to the start of working with animals, including amendments to ongoing projects. PIs must submit the institutional animal use protocol, IACUC approval of that protocol, and a version of the animal use appendix titled "Research Involving Animals." ***Allow at least 2 to 3 months for ACURO regulatory review and approval processes for animal studies.*** Refer to the General Application Instructions, Appendix 1, for additional information.

The CDMRP intends that information, data, and research resources generated under awards funded by this Program Announcement be made available to the research community (which includes both scientific and consumer advocacy communities) and to the public at large. For additional guidance, refer to the General Application Instructions, Appendix 2, Section K.

Awards will be made no later than September 30, 2018. For additional information refer to Section II.F.1, Federal Award Notices.

II.C. Eligibility Information

II.C.1. Eligible Applicants

II.C.1.a. Organization: All organizations, including international organizations, are eligible to apply.

Government Agencies within the United States: Local, state, and Federal Government agencies are eligible to the extent that applications do not overlap with their fully funded internal programs. Such agencies are required to explain how their applications do not overlap with their internal programs.

As applications for this Program Announcement may be submitted by extramural and intramural organizations, these terms are defined below.

Extramural Organization: An eligible non-DoD organization. Examples of extramural organizations include academia, biotechnology companies, foundations, Government, and research institutes. *Extramural Submission: Application submitted by a non-DoD organization to Grants.gov.*

Intramural DoD Organization: A DoD laboratory, DoD military treatment facility, and/or DoD activity embedded within a civilian medical center. *Intramural Submission: Application submitted by a DoD organization for an intramural investigator who is a DoD military or civilian employee working within a DoD laboratory or military treatment facility or in a DoD activity embedded within a civilian medical center.*

Note: Applications from an intramural organization or from an extramural non-DoD Federal organization may be submitted through a research foundation.

The USAMRAA makes awards to eligible organizations, not to individuals.

II.C.1.b. Principal Investigator:

- The PI must be an independent investigator at or above the level of Full Professor (or equivalent).
    - Project leaders for each of the complementary and synergistic research projects must be at or above the level of Assistant Professor (or equivalent).

An eligible PI, regardless of ethnicity, nationality, or citizenship status, must be employed by, or affiliated with, an eligible organization.

The CDMRP encourages all PIs to participate in a digital identifier initiative through Open Researcher and Contributor ID, Inc. (ORCID). Registration for a unique ORCID identifier can be done online at http://orcid.org/.

II.C.2. Cost Sharing

Cost sharing/matching is not an eligibility requirement.

II.C.3. Other

Extramural organizations must be able to access **.gov** and **.mil** websites in order to fulfill the financial and technical deliverable requirements of the award and submit invoices for payment.

There are no limitations on the number of applications for which an investigator may be named as a PI.

For general information on required qualifications for award recipients, refer to the General Application Instructions, Appendix 3.

Refer to Section II.H.2, Administrative Actions, for a list of administrative actions that may be taken if a pre-application or application does not meet the administrative, eligibility, or ethical requirements defined in this Program Announcement.

II.D. Application and Submission Information

Submission of applications that are essentially identical or propose essentially the same research project to different funding opportunities within the same program and fiscal year is prohibited and will result in administrative withdrawal of the duplicative application(s). ***As an exception, applicants may submit a research project described in their FY17 PRMRP Focused Program Award to the Discovery Award (Funding Opportunity Number: W81XWH-17-PRMRP-DA), Investigator-Initiated Research Award (Funding Opportunity Number: W81XWH-17-PRMRP-IIRA), and/or Clinical Trial Award (Funding Opportunity Number: W81XWH-17-PRMRP-CTA); however, accepting multiple awards to support the same project will not be allowed.***

Extramural Submission is defined as an application submitted by a non-DoD organization to Grants.gov.

Intramural Submission is defined as an application submission by a DoD organization for an intramural investigator, who is a DoD military or civilian employee working within a DoD laboratory or military treatment facility, or working in a DoD activity embedded within a civilian medical center.

II.D.1. Address to Request Application Package

Submitting Extramural and Intramural Organizations: Pre-application content and forms can be accessed at eBRAP (https://eBRAP.org).

Submitting Extramural Organizations: Full application packages can be accessed at Grants.gov.

Submitting Intramural DoD Organizations: Full application packages can be accessed at eBRAP.org.

Contact information for the CDMRP Help Desk and the Grants.gov Contact Center can be found in Section II.G, Federal Awarding Agency Contacts.

II.D.2. Content and Form of the Application Submission

Submission is a two-step process requiring both ***pre-application*** and ***full application*** as indicated below. The submission process should be started early to avoid missing deadlines. There are no grace periods.

Pre-application Submission: All pre-applications for both extramural and intramural organizations must be submitted through eBRAP (https://eBRAP.org/).

eBRAP is a multifunctional web-based system that allows PIs to submit their pre-applications electronically through a secure connection, to view and edit the content of their pre-applications and full applications, to receive communications from the CDMRP, and to submit documentation during award negotiations and period of performance.

Full Application Submission: Full applications must be submitted through the online portals as described below.

***Submitting Extramural Organizations*:** Full applications from extramural organizations must be submitted through Grants.gov. Applications submitted by extramural organizations (e.g., research foundations) on behalf of intramural DoD or other Federal organizations or investigators will be considered extramural submissions.

Submitting Intramural DoD Organizations: Intramural DoD organizations may submit full applications to either eBRAP or Grants.gov. Intramural DoD organizations that are unable to submit to Grants.gov should submit through eBRAP. Intramural DoD organizations with the capability to submit through Grants.gov may submit following the instructions for extramural submissions through Grants.Gov or may submit to eBRAP. Applications from extramural organizations, including non-DoD Federal organizations, received through eBRAP will be withdrawn. See definitions in Section II.C.1, Eligible Applicants.

For both Extramural and Intramural applicants: A key feature of eBRAP is the ability of an organization's representatives and PIs to view and modify the full application submissions associated with them. eBRAP will validate full application files against the specific Program Announcement requirements and discrepancies will be noted in an email to the PI and in the Full Application Files tab in eBRAP. It is the applicant's responsibility to review all application components for accuracy as well as ensure proper ordering as specified in this Program Announcement.

The application title, eBRAP log number, and all information for the PI, Business Official(s), performing organization, and contracting organization must be consistent throughout the entire pre-application and application submission process. Inconsistencies may delay application processing and limit or negate the ability to view, modify, and verify the application in eBRAP. If any changes need to be made, the applicant should contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507 prior to the application deadline.

II.D.2.a. Step 1: Pre-Application Submission Content

During the pre-application process, each submission is assigned a unique log number by eBRAP. This unique eBRAP log number will be needed during the full application submission process.

To begin the pre-application process, first select whether the submitting organization is extramural or intramural, then confirm your selection or cancel. **Incorrect selection of extramural or intramural submission type may result in delays in processing.**

If an error has been made in the selection of extramural versus intramural and the pre-application submission deadline has passed, the PI or Business Official must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

All pre-application components must be submitted by the PI through eBRAP (https://eBRAP.org/). Because the invitation to submit an application is based on the contents of the pre-application, investigators should not change the title or research objectives after the pre-application is submitted.

PIs and organizations identified in the pre-application should be the same as those intended for the subsequent application submission. If any changes are necessary after submission of the pre-application, the PI must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

The pre-application consists of the following components, which are organized in eBRAP by separate tabs (refer to the General Application Instructions, Section II.B, for additional information on pre-application submission):

- **Tab 1 – Application Information**

  Select the FY17 PRMRP Topic Area addressed by the proposed research. If the proposed research project is aligned with more than one FY17 PRMRP Topic Area, select the Topic Area of highest relevance as the required first choice.

- **Tab 2 – Application Contacts**

  Enter contact information for the PI. Enter the organization's Business Official responsible for sponsored program administration (the "person to be contacted on matters involving this application" in Block 5 of the Grants.gov SF424 (R&R) Form for extramural submissions). The Business Official must be either selected from the eBRAP list or invited in order for the pre-application to be submitted.

  Select the performing organization (site at which the PI will perform the proposed work) and the contracting organization (organization submitting on behalf of the PI, which corresponds to Block 5 on the Grants.gov SF424 (R&R) Form for extramural submissions), and click on "*Add Organizations to this Pre-application.*" The organizations must be either selected from the eBRAP drop-down list or invited in order for the pre-application to be submitted.

  It is recommended that PIs identify an Alternate Submitter in the event that assistance with pre-application submission is needed.

- **Tab 3 – Collaborators and Key Personnel**

  Enter the name, organization, and role of all collaborators and key personnel associated with the application.

  FY17 PRMRP Programmatic Panel members should not be involved in any pre-application or application. For questions related to Panel members and pre-applications or applications, refer to Section II.H.2.c, Withdrawal, or contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

  To preserve the integrity of its peer and programmatic review processes, the CDMRP discourages inclusion of any employee of its review contractors having any role in pre-application or application preparation, research, or other duties for submitted pre-applications or applications. For FY17, the identities of the peer review contractor and the programmatic review contractor may be found at the CDMRP website (http://cdmrp.army.mil/about/2tierRevProcess). Pre-applications or applications that include names of personnel from either of these companies will be administratively withdrawn unless plans to manage COIs are provided and deemed appropriate by the Grants Officer. Refer to the General Application Instructions, Appendix 3, for detailed information.

- **Tab 4 – Conflicts of Interest**

  List all individuals other than collaborators and key personnel who may have a COI in the review of the application (including those with whom the PI has a personal or professional relationship). Refer to the General Application Instructions, Appendix 3, Section C, for further information regarding COIs.

- **Tab 5 – Pre-Application Files**

  Note: Upload documents as individual PDF files unless otherwise noted. eBRAP will not allow a file to be uploaded if the number of pages exceeds the limit specified below.

- **Preproposal Narrative (five-page limit):** The Preproposal Narrative page limit applies to text and non-text elements (e.g., figures, tables, graphs, photographs, diagrams, chemical structures, drawings) used to describe the project. Inclusion of URLs that provide additional information to expand the Preproposal Narrative and could confer an unfair competitive advantage is prohibited and may result in administrative withdrawal of the pre-application.

  The Preproposal Narrative should include the following:

  - **Topic Area:** Describe how the proposed program relates to at least one of the FY17 PRMRP Topic Areas. If applicable, describe how the proposed research program addresses an FY17 PRMRP Area of Encouragement (Appendix 2).

  - **Overarching Challenge:** Describe the unifying challenge or question to be addressed and how it is relevant to a critical problem or question in the field of research and/or patient care in at least one of the FY17 PRMRP Topic Area(s). Clearly articulate the rationale for the overarching challenge; include relevant preliminary data and literature citations.

  - **Research Strategy:** The FY17 PRMRP Focused Program Award strongly encourages a minimum of four individual but complementary research projects addressing the overarching challenge. For each proposed project, state the hypothesis to be tested, the specific aims, and the objectives to be reached. Briefly describe the experimental approach. Describe how the projects are interrelated to and synergistic with each other and align with the overarching challenge.

  - **Impact:** Describe the potential short-term and long-term impact of the proposed research on at least one of the FY17 PRMRP Topic Areas and its related research field(s), and patient population(s). Explain how the effort is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

  - **Research Team:** Briefly describe the composition, expertise, and organization of the research team. Identify the project leaders and describe each team member's role in the projects, with additional emphasis on the leadership role of the PI. Briefly describe how these features will facilitate the success of the key aspects of the projects.

  - **Clinical Trial (if applicable):** If the proposed research includes a clinical trial(s), briefly state the clinical intervention(s), subject population(s), and the type and phase of the clinical trial(s). Describe the objectives of the clinical trial(s), how it addresses the overarching challenge, and how it complements the other proposed projects. ***Only small-scale (i.e., up to and including Phase II or equivalent) clinical trials are allowed.***

- **Pre-Application Supporting Documentation:** The items to be included as supporting documentation for the pre-application ***must be uploaded as individual files*** and are limited to the following:

- References Cited (one-page limit): List the references cited (including URLs if available) in the Preproposal Narrative using a standard reference format that includes the full citation (i.e., author[s], year published, reference title, and reference source, including volume, chapter, page numbers, and publisher, as appropriate).

- List of Abbreviations, Acronyms, and Symbols: Provide a list of abbreviations, acronyms, and symbols used in the Preproposal Narrative.

- Key Personnel Biographical Sketches (five-page limit per individual). ***All biographical sketches should be uploaded as a single combined file.*** Biographical sketches should be used to demonstrate background and expertise through education, positions, publications, and previous work accomplished.

- **Tab 6 – Submit Pre-Application**

  This tab must be completed for the pre-application to be accepted and processed.

Pre-Application Screening

Pre-Application Screening Criteria

To determine the technical merits of the pre-application and the relevance to the mission of the DHP and the PRMRP, pre-applications will be screened based on the following criteria:

- **Overarching Challenge:** How well the unifying challenge or question addresses a critical problem or question in the field of research and/or patient care of one or more FY17 PRMRP Topic Area(s). How well the rationale supports the overarching challenge.

- **Research Strategy:** How well a hypothesis and specific aims are defined for each proposed project and to what extent each project's approach will address them. How well the proposed projects complement each other and synergistically address the overarching challenge to advance a solution beyond what would be possible through individual efforts.

- **Impact:** Whether the potential immediate and long-range outcome(s)/product(s) (intellectual and/or material) of the proposed research, if successful, will impact a central critical problem or question in the field of research and/or patient care in the FY17 PRMRP Topic Area(s) addressed. To what degree the project is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

- **Research Team:** To what degree the background and expertise of the PI, project leaders, and key personnel are appropriate with respect to their abilities to successfully complete the projects and the extent to which the PI is well prepared and committed to lead the research team and proposed projects.

Notification of Pre-Application Screening Results

Following the pre-application screening, PIs will be notified as to whether or not they are invited to submit applications; however, they will not receive feedback (e.g., a critique of strengths and

weaknesses) on their pre-application. The estimated time frame for notification of invitation to submit an application is indicated in Section I, Overview of the Funding Opportunity. Invitations to submit a full application are based on the Pre-Application Screening Criteria listed above.

II.D.2.b. Step 2: Full Application Submission Content

Applications will not be accepted unless the PI has received notification of invitation.

All contributors and administrators to the application must use matching compatible versions of Adobe software when editing and preparing application components. The use of different software versions will result in corruption of the submitted file. Refer to the General Application Instructions, Section III, for details on compatible Adobe software.

The CDMRP cannot make allowances/exceptions to its policies for submission problems encountered by the applicant organization using system-to-system interfaces with Grants.gov.

Each application submission must include the completed full application package for this Program Announcement. The full application package is submitted by the Authorized Organizational Representative through Grants.gov (http://www.grants.gov/) for extramural organizations or through eBRAP (https://ebrap.org/) for intramural organizations. See Table 1 below for more specific guidelines.

II.D.2.b.i. Full Application Guidelines

Extramural organizations, including non-DoD Federal agencies, must submit full applications through Grants.gov. Submissions of extramural applications through eBRAP may be withdrawn.

Table 1. Full Application Submission Guidelines

Extramural Submissions	Intramural DoD Submissions
Application Package Location	
Download application package components for W81XWH-17-PRMRP-FPA from Grants.gov (http://www.grants.gov).	Download application package components for W81XWH-17-PRMRP-FPA from eBRAP (https://ebrap.org).
Full Application Package Components	
SF424 (R&R) Application for Federal Assistance Form: Refer to the General Application Instructions, Section III.A.1, for detailed information.	**Tab 1 – Summary:** Provide a summary of the application information. **Tab 2 – Application Contacts:** This tab will be pre-populated by eBRAP; add Authorized Organizational Representative.

Extramural Submissions	Intramural DoD Submissions
Descriptions of each required file can be found under Full Application Submission Components: • Attachments • Research & Related Senior/Key Person Profile (Expanded) • Research & Related Budget • Project/Performance Site Location(s) Form • R&R Subaward Budget Attachment(s) Form (if applicable)	**Tab 3 – Full Application Files:** Upload files under each Application Component in eBRAP. Descriptions of each required file can be found under Full Application Submission Components: • Attachments • Key Personnel • Budget • Performance Sites **Tab 4 – Application and Budget Data:** Review and edit proposed project start date, proposed end date, and budget data pre-populated from the Budget Form.
Application Package Submission	
Submit package components to Grants.gov (https://www.grants.gov/). If either the Project Narrative or the budget fails eBRAP validation or if the Project Narrative or the budget need to be modified, an updated Grants.gov application package must be submitted via Grants.gov as a "Changed/Corrected Application" with the previous Grants.gov Tracking ID prior to the application submission deadline.	**Submit package components to eBRAP (https://ebrap.org).** **Tab 5 – Submit/Request Approval Full Application:** After all components are uploaded and prior to the full application submission deadline, enter your password in the space provided "Enter Your Password Here" and press the "Submit Full Application" button. eBRAP will notify your Resource Manager/Comptroller or equivalent Business Official by email to log into eBRAP to review and to approve prior to the application submission deadline.
Application Verification Period	
The full application package submitted to Grants.gov may be viewed and modified in eBRAP until the end of the application verification period. During the application verification period, the full application package, ***with the exception of the Project Narrative and Budget Form,*** may be modified.	After eBRAP has processed the full application, the organizational Resource Manager/ Comptroller or equivalent Business Official and PI(s) will receive an email notification of this status and will be able to view and modify application components in eBRAP. During the application verification period, the full application package, ***with the exception of the Project Narrative and Budget Form,*** may be modified.
Further Information	
Refer to the General Application Instructions, Section III, for further information regarding Grants.gov requirements.	Refer to the General Application Instructions, Section IV, for further information regarding eBRAP requirements.

The organization's Business Official or Authorized Organization Representative (or Resource Manager/Comptroller) should approve/verify the full application submission prior to the application verification deadline.

Application viewing, modification, and verification in eBRAP are strongly recommended, but not required. ***The Project Narrative and Budget cannot be changed after the application submission deadline.*** Prior to the full application deadline, a corrected or modified full application package may be submitted. Other application components may be changed until the end of the application verification period. After the end of the application verification period, the full application cannot be modified.

Material submitted after the end of the application verification period, unless specifically requested by the Government, will not be forwarded for processing.

The full application package must be submitted using the unique eBRAP log number to avoid delays in application processing.

II.D.2.b.ii. Full Application Submission Components:

- **Extramural Applications Only –**

  SF424 (R&R) Application for Federal Assistance Form: Refer to the General Application Instructions, Section III.A.1, for detailed information.

- **Extramural and Intramural Applications –**

  Attachments:

  Each attachment to the full application components must be uploaded as an individual file in the format specified and in accordance with the formatting guidelines listed in the General Application Instructions, Appendix 4.

  For all attachments, ensure that the file names are consistent with the guidance. Attachments will be rejected if the file names are longer than 50 characters or incorrect file names that contain characters other than the following: A-Z, a-z, 0-9, underscore, hyphen, space, and period. In addition, there are file size limits that may apply in some circumstances. Individual attachments may not exceed 20 MB and the file size for the entire full application package may not exceed 200 MB.

  - **Attachment 1: Project Narrative (40-page limit):** Upload as "ProjectNarrative.pdf." The page limit of the Project Narrative applies to text and non-text elements (e.g., figures, tables, graphs, photographs, diagrams, chemical structures, drawings) used to describe the project. Inclusion of URLs that provide additional information to expand the Project Narrative and could confer an unfair competitive advantage is prohibited and may result in administrative withdrawal of the application.

    Describe the proposed project in detail using the outline below.

Overall Program: Provide a description of the comprehensive effort using the following outline. Applicants are strongly encouraged to submit a minimum of four research projects; additional studies are allowed. Emphasize areas of synergy throughout the narrative.

- **Overarching Challenge:** Describe the unifying, overarching challenge or question to be addressed and how it is relevant to a critical problem or question in the field of research and/or patient care in one or more FY17 PRMRP Topic Areas. Clearly articulate the rationale for the overarching challenge; include relevant literature citations. Clearly describe how the proposed research projects are interrelated and synergistic and will advance toward a solution through a multidisciplinary research program. Describe how each project will address the overarching challenge in a unique but complementary way and how the combined efforts of the projects will address the overarching challenge more effectively than if the projects were conducted independently.

- **Leadership:** Describe how the PI's research experience, leadership skills, and commitment to making an impact in his/her field of research and/or patient care demonstrate substantial qualifications to coordinate this collaborative effort. Describe the PI's demonstrated success in leading large, focused projects and outline the PI's responsibilities during the conduct of the award. Discuss the qualifications of the research team being brought together by the PI and how the assembled expertise will create a robust, synergistic collaboration necessary to address the overarching challenge and enable the success of the proposed research.

- **Implementation Plan and Environment:** Provide an overall strategic plan for completing the proposed projects that identifies critical milestones. Outline the knowledge, expertise, and technical innovations that the investigative team will utilize to make decisions, allocate resources, and accomplish the milestones. Describe and/or provide evidence that the research can be initiated without delay once the award is made. Present an overall management plan to facilitate consistent and intensive interactions by all team members, including aspects such as adherence to regulatory requirements, administrative support, and oversight to accelerate translation of the projects' outcomes to patients and/or for clinical use. Describe the research environment(s) and how the facilities and resources will support the research requirements and the collaboration. Outline shared resources and/or cores that will be created and/or leveraged through the award. Describe plans for communication, data transfer among the collaborating institutions, and how data, specimens, and/or imaging products obtained during the study will be handled. If applicable, describe how Standard Operating Procedures will be created, reviewed, implemented, and modified during the course of the award. Describe how individual project performance will be assessed during the course of the award, including progression toward defined milestones, realization of study objectives, and addressing the overarching challenge. If an External Advisory Board (EAB) is to be utilized, describe the role of the board and the expertise to be sought in its members. To avoid potential COIs in the review of the application, potential candidates for an EAB should not be contacted, recruited, or named during the application process.

Research Plan: Provide the following details for each proposed research project, organizing each project clearly and separately. Start each project on a separate page:

- **Title:** Provide a title for each project.

- **Project Leader:** Identify the project leader and any key personnel, as appropriate, describing each person's qualifications and specific contributions to the project.

- **Background:** Briefly describe the ideas and reasoning on which the proposed work is based. Provide sufficient preliminary data to support the feasibility of work proposed. If the project is exploratory/hypothesis-developing, preliminary data are not required. For each project, the leader must demonstrate logical reasoning and provide a sound scientific rationale for the proposed project as established through a critical review and analysis of published literature. If proposing translational or clinical research, it is important to describe the project showing proof of concept and, if applicable, efficacy in an in-vivo system(s) to support the translational feasibility and promise of the approach.

- **Hypothesis/Objective:** State the hypothesis to be tested and/or the objective(s) to be reached.

- **Specific Aims:** Concisely explain each project's specific aims. The specific aims should align with the overall goal of the program and associated tasks described in the Statement of Work.

- **Research Strategy:** Describe the experimental design, methods, and analyses, in sufficient detail for analysis. Provide a description of how the study will be controlled and how the study variables will be measured. If the project is a clinical trial, define the primary and secondary endpoints and how they will be assessed. Explain how the research strategy will address the overarching challenge and meet appropriate milestones. Address potential problem areas and present alternative methods and approaches. Clearly describe the statistical plan and the rationale for the statistical methodology as well as an appropriate power analysis, if applicable. If animal studies are proposed, describe how they will be conducted in accordance with the ARRIVE guidelines (http://www.elsevier.com/__data/promis_misc/622936arrive_guidelines.pdf). Justify how the model system or human subjects/samples are appropriate to the proposed research project. If human subjects or human biological samples will be used, describe the study population and include a detailed plan for the recruitment of human subjects or the acquisition of samples. Describe the availability of the proposed study population and past successes in recruiting similar populations. If active duty military, military families, and/or Veteran population(s) or datasets will be used in the proposed research project, describe how access to the population(s)/dataset(s) will be obtained. If applicable, describe how data will be reported and how it will be assured that the documentation will support a regulatory filing with the U.S. Food and Drug Administration (FDA).

- **Statistical Plan:** Clearly describe a statistical plan appropriate to the type of study; provide the rationale for the statistical methodology. Define the number of samples and/or subjects (animal and/or human) to be used, and include a power analysis to demonstrate that the sample size is appropriate to meet the objectives of the study.

- **Impact:** Describe the anticipated outcome(s)/product(s) (intellectual and/or material) that will be directly attributed to the results of the proposed research. Explain the anticipated long-term gains from this research. Compare to the information known/products currently available, if applicable.

- **Clinical Trial (if applicable):** ***Only small-scale (e.g., up to and including Phase II or equivalent) clinical trials are allowed.*** Provide detailed plans for initiating and conducting the clinical trial during the course of this award. As appropriate, outline a plan for applying for and obtaining Investigational New Drug/Investigational Device Exemption (IND/IDE) status (or other FDA approvals). Describe the type of clinical trial to be performed (e.g., prospective, randomized, controlled) and indicate the phase of trial and/or class of device, as appropriate. Outline the regulatory strategy. Provide preclinical and/or clinical evidence to support the safety of the intervention.

    - Identify the intervention to be tested and describe the projected outcomes. Describe how the proposed intervention compares with currently available interventions and/or standards of care. Include a discussion of any current clinical use of the intervention under investigation, and/or details of its study in clinical trials for other indications (as appropriate).

    - Describe the methods that will be used to recruit a sample of human subjects from the accessible population (e.g., convenience, simple random, stratified random). Provide information on and justification for the inclusion and exclusion criteria.

    - Describe the process for obtaining informed consent and any screening procedures required to determine eligibility for study participation.

    - Describe the human subject-to-group assignment process (e.g., randomization, block randomization, stratified randomization, age-matched controls, alternating group, or other procedures), if applicable. Explain the specific actions to accomplish the group assignment (e.g., computer assignment, use of table of random numbers). If multiple site studies are involved, state the approximate number of subjects to be enrolled at each site.

    - Provide evidence to document the availability of and access to all critical reagents, including the intervention itself, if applicable, for the duration of the proposed trial.

    - Describe how quality control will be addressed. Describe how compliance with Good Laboratory Practices (GLP), Good Manufacturing Practices (GMP), and Good Clinical Practices (GCP) guidelines will be established, monitored, and maintained, as applicable.

- Describe the composition of the clinical trial team. Provide details on how the team (including investigator(s), study coordinator, and statistician) possesses the appropriate expertise in conducting clinical trials.

- **Attachment 2: Supporting Documentation:** Combine and upload as a single file named "Support.pdf." Start each document on a new page. If documents are scanned to PDF, the lowest resolution (100 to 150 dpi) should be used. The Supporting Documentation attachment should not include additional information such as figures, tables, graphs, photographs, diagrams, chemical structures, or drawings. These items should be included in the Project Narrative. Any additional material viewed as an extension of the Project Narrative will be removed or may result in administrative withdrawal of the application.

  There are no page limits for any of these components unless otherwise noted. Include only those components described below; inclusion of items not requested will result in the removal of those items or may result in administrative withdrawal of the application.

  - References Cited: List the references cited (including URLs, if available) in the Project Narrative using a standard reference format that includes the full citation (i.e., author[s], year published, title of reference, source of reference, volume, chapter, page numbers, and publisher, as appropriate).

  - List of Abbreviations, Acronyms, and Symbols: Provide a list of abbreviations, acronyms, and symbols.

  - Facilities, Existing Equipment, and Other Resources: Describe the facilities and equipment available for performance of the proposed project and any additional facilities or equipment proposed for acquisition at no cost to the award. Indicate whether or not Government-furnished facilities or equipment are proposed for use. If so, reference should be made to the original or present Government award under which the facilities or equipment items are now accountable. There is no form for this information.

  - Publications and/or Patents: Include a list of relevant publication URLs and/or patent abstracts. If publications are not publicly available, then copies of up to eight published manuscripts may be included in Attachment 2. Extra items will not be reviewed.

  - Letters of Organizational Support: Provide a letter (or letters, if applicable), signed by the Department Chair or appropriate organization official, confirming the laboratory space, equipment, and other resources available for the project. Letters of support not requested in the Program Announcement, such as those from members of Congress, do not impact application review or funding decisions.

  - Letters of Collaboration: Provide a signed letter from each collaborating individual or organization that will demonstrate that the PI has the support or resources necessary for the proposed work. If an investigator at an intramural organization is

named as a collaborator on an application submitted through an extramural organization, the application must include a letter from the collaborator's Commander or Commanding Officer at the intramural organization that authorizes the collaborator's involvement.

- Letters of Commitment (if applicable): If the proposed study involves use of a commercially produced investigational drug, device, or biologic, provide a letter of commitment from the commercial entity indicating availability of the product for the duration of the study, support for the proposed phase of research, and support for the indication to be tested.

- Letters Confirming Access to Military or VA Patient Populations or Resources (if applicable): If the proposed research plan involves access to active duty military and/or VA patient populations or resources, include a letter of support, signed by the lowest ranking person with approval authority, confirming such access. If access cannot be confirmed at the time of application submission, the Government reserves the right to withhold or revoke funding until the PI has demonstrated support for and access to the relevant population(s) and/or resources.

- Intellectual Property: Information can be found in Code of Federal Regulations, Title 2, Part 200.315 (2 CFR 200.315), "Intangible Property."

  - Intellectual and Material Property Plan (if applicable): Provide a plan for resolving intellectual and material property issues among participating organizations.

- Data and Research Resources Sharing Plan: Describe how data and resources generated during the performance of the project will be shared with the research community. Refer to the General Application Instructions, Appendix 2, Section K, for more information about the CDMRP expectations for making data and research resources publicly available.

- **Attachment 3: Technical Abstract (one-page limit):** Upload as "TechAbs.pdf." The technical abstract is used by all reviewers. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information.*** Use only characters available on a standard QWERTY keyboard. Spell out all Greek letters, other non-English letters, and symbols. Graphics are not allowed.

  Clarity and completeness within the space limits of the technical abstract are highly important. Describe the proposed research effort including the following elements:

  - **Overarching Challenge:** Identify the unifying, overarching challenge or question that will be addressed by the research plan and describe how it relates to a critical problem or question in one or more of the FY17 PRMRP Topic Areas.

  - **Background:** Briefly articulate the rationale for the overarching challenge and the proposed research.

- **Research Plan:** Provide a brief description of the studies proposed, including hypotheses, objectives, and scientific approach.

- **Impact:** Briefly describe the potential short-term and long-term impact of the results of the proposed research on at least one of the FY17 PRMRP Topic Areas and its related research field(s) and patient population(s).

- **Military Relevance:** Explain how the effort is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

- **Attachment 4: Lay Abstract (one-page limit):** Upload as "LayAbs.pdf." The lay abstract is used by all reviewers. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information***. Use only characters available on a standard QWERTY keyboard. Spell out all Greek letters, other non-English letters, and symbols. Graphics are not allowed.

  State the FY17 PRMRP Topic Area(s) addressed by the proposed research program. Include a comprehensive overview of the effort that can be ***readily understood by readers without a background in science or medicine.*** Clearly describe the central critical problem or question to be addressed and the ultimate applicability and impact of the research. Do not duplicate the technical abstract.

- **Attachment 5: Statement of Work (SOW) (eight-page limit):** Upload as "SOW.pdf." The suggested SOW format and examples specific to different types of research projects are available on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm). For the Focused Program Award mechanism, use the SOW format example titled "SOW for Collaborative Projects ." The SOW must be in PDF format prior to attaching.

  The SOW should include a list of major tasks that support the proposed specific aims, followed by a series of subtasks outlined related to the major tasks and milestones within the period of performance. The SOW should describe only the work for which funding is being requested by this application and, as applicable, should also:

  Include the name(s) of the key personnel and contact information for each study site/ subaward site.

  Indicate the number (and type, if applicable) of research subjects (animal or human) and/or human anatomical samples projected or required for each task and at each site. Refer to the General Application Instructions, Appendix 1, for additional information regarding regulatory requirements.

  Briefly state the methods to be used.

  For studies with prospective accrual of human subjects, indicate quarterly enrollment targets.

Identify cell line(s) and commercial or organizational source(s) to be used. If human anatomical substances (including cell lines) will be used, specify whether or not identifiable information is accessible to the research team by any means.

If applicable, indicate timelines required for regulatory approvals relevant to human subjects research (e.g., IND/IDE applications) by the FDA or other Government agency.

- **Attachment 6: Impact Statement (two-page limit): Upload as "Impact.pdf."**

  - Explain why the proposed program is important and relevant to understanding the cause or progression of the disease or condition, and/or to developing improvements in prevention, detection, diagnosis, treatment, or quality of life in the FY17 PRMRP Topic Area(s) addressed. Describe how the overarching challenge addresses a central critical problem or question in the relevant Topic Area(s). If applicable, describe how the project addresses an FY17 PRMRP Area of Encouragement (Appendix 2). In addition to articulating the potential impact of the overall program, address the potential impact of each individual project. Explain how the various projects' outcomes will ultimately be translated to patients.

  - ***Describe the short-term impact:*** Detail the anticipated outcome(s)/product(s) (intellectual and/or material) that will be directly attributed to the results of the proposed research.

  - ***Describe the long-term impact:*** Explain the anticipated long-term gains from this research. Compare to the information known/products currently available, if applicable. Explain the long-range vision for how the research will impact the field of study and/or patient care.

- **Attachment 7: Military Relevance Statement (one-page limit): Upload as "MilRel.pdf."**

  - Describe how the proposed effort is responsive to the healthcare needs of military Service members, Veterans, and/or beneficiaries. Provide information about the incidence and/or prevalence of the disease or condition to be studied in the general population as well as in military Service members, Veterans, and/or beneficiaries.

  - If active duty military, military families, and/or Veteran population(s) or dataset(s) will be used in the proposed research project, describe the population(s)/dataset(s) and the appropriateness of the population(s)/dataset(s) for the proposed study. If a non-military population will be used for the proposed research project, explain how the population simulates the targeted population (i.e., military Service members, Veterans, and/or beneficiaries).

  - If applicable, show how the proposed research project aligns with DoD and/or VA areas of research interest and/or patient care. Provide a description of how the knowledge, information, products, or technologies gained from the research could be implemented in a dual-use capacity to benefit the civilian population and address a military need, as appropriate.

- **Attachment 8: Transition Plan and Regulatory Strategy (three-page limit): Upload as "Transition.pdf."**

  - Provide information on the methods and strategies proposed to move the product or knowledge outcomes of the program to the next phases of development and to eventual clinical use. Articulate this information for the overall effort as well as the individual projects. Applicants are encouraged to work with their organization's Technology Transfer Office (or equivalent) to develop the transition plan. The transition plan should include the components listed below, as appropriate:

  - A description of the outcomes expected upon completion of the proposed research efforts. Outcomes should be specific, measurable, and should include the intended end user.

  - Details of the funding strategy that will be used to bring the outcomes to the next phase of development and/or delivery to market or incorporation into patient care (e.g., specific potential industry partners, specific funding opportunities to be applied for).

  - Details of the development plan and FDA regulatory strategy, to include considerations for compliance with GMP, GLP, and GCP guidelines (if appropriate).

  - For knowledge outcomes, a description of how the knowledge will be further developed, disseminated, and incorporated into clinical/patient care.

  - A description of collaborations and other resources that will be used to provide continuity of development.

  - A brief schedule and milestones for bringing the outcomes to the next phase of development (e.g., further research, clinical trials, transition to industry, delivery to the market, incorporation into clinical practice, approval by the FDA).

  - If applicable, ownership rights and/or access to the intellectual property necessary for the development and/or commercialization of products or technologies supported with this award and the Government's ability to access such products or technologies in the future.

- **Attachment 9: Data and Research Resource Sharing Plan (one-page limit): Upload as "Sharing.pdf."**

  - Describe how data and resources generated during the performance of the proposed research projects will be shared with the research community. This includes cases where pre-existing data or research resources will be utilized and/or modified during the course of the proposed projects. Specifically describe a plan to make animal models, tissue samples, and other resources developed as part of the proposed research projects available to the scientific community. If there are limitations associated with a pre-existing agreement for the original data or research resources that preclude subsequent sharing, the applicant should explain this in the data- and/or

research resource-sharing plan. Refer to the General Application Instructions, Appendix 2, Section K, for more information about the CDMRP expectations for making data and research resources publicly available.

- In preparing requested budgets, applicants may include anticipated costs associated with data- and research resource-sharing (i.e., making a large dataset available to the public or developing an important resource for the scientific community).

- **Attachment 10: IND/IDE Documentation:** ***Only applicable for applications that include a clinical trial(s).*** If submitting multiple documents, start each document on a new page. **Combine and upload as a single file named "IND-IDE.pdf."** The IND/IDE Documentation Form located on the eBRAP website may ***not*** be used in place of this information:

  - State whether the trial(s) requires regulation by the FDA. If FDA regulation is required, describe the planned indication for the proposed product and whether an IND/IDE is necessary.

  - If an IND or IDE application is required, indicate when it was submitted to the FDA and provide an explanation of the status of the IND or IDE application (e.g., past the critical 30-day period, pending response to questions raised by the Agency, on clinical hold). If the IND or IDE application has not been submitted to the FDA yet, provide a detailed timeline with appropriate milestones for application preparation and submission. Identify any consultants or experts who will assist in the regulatory application, if applicable, and include a copy of any curricula vitae or biographical sketches in the Key Personnel Biographical Sketches section of the application. Provide a summary of previous meetings with the FDA on development of this product, if appropriate. A copy of the Agency meeting minutes should be included if available. Provide copies of communications from the FDA relevant to the most recent status of the IND or IDE application.

  - If an IND or IDE is not required for the proposed trial(s), or if it qualifies for an abbreviated IDE, provide evidence in the form of communication from the FDA or the IRB of record to that effect. Devices qualifying for an abbreviated IDE must comply with the abbreviated IDE requirements but do not require the submission of an IDE application to the FDA.

- **Attachment 11: DoD Military Budget Form(s), if applicable: Upload as "MFBudget.pdf."** If a military facility (military health system facility, research laboratory, treatment facility, dental treatment facility, or a DoD activity embedded with a civilian medical center) will be a collaborator in performance of the project, complete the DoD Military Budget Form, available for download on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm), including a budget justification, for each military facility as instructed. The costs per year should be included on the Grants.gov Research and Related Budget form under subaward costs. Refer to the General Application Instructions, Section III.A.7, for detailed information.

- **Extramural and Intramural Applications –**

  Research & Related Senior/Key Person Profile (Expanded): For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.3, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.2, for detailed information.

  - PI Biographical Sketch (five-page limit): Upload as "Biosketch_LastName.pdf." The suggested biographical sketch format is available on the "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) in eBRAP. The National Institutes of Health Biographical Sketch may also be used. All biographical sketches should be submitted in the portable document format (PDF) that is not editable.

  - PI Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."

  - Key Personnel Biographical Sketches (five-page limit each): Upload as "Biosketch_LastName.pdf."

  - Key Personnel Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."

  Research & Related Budget: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.4, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.3, for detailed information.

  Budget Justification (no page limit): Upload as "BudgetJustification.pdf." The budget justification for the entire period of performance must be uploaded to the Research & Related Budget after completion of the budget for Period 1.

  Project/Performance Site Location(s) Form: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.5, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.4, for detailed information.

- **Extramural Applications Only –**

  R&R Subaward Budget Attachment(s) Form (if applicable): Refer to the General Application Instructions, Section III.A.6, for detailed information.

  - **Extramural Subaward:** Complete the Research & Related Subaward Budget Form through Grants.gov. (Refer to the General Application Instructions, Section III.A.6, for detailed information.)

  - **Intramural DoD Collaborator(s):** Complete the DoD Military Budget Form and upload to Grants.gov as Attachment 11. (Refer to the General Application Instructions, Section III.A.7, for detailed information.) Intramural DoD Collaborator(s) costs per year

should be included on the Grants.gov Research and Related Budget form under subaward costs. **DoD Military Budget Form:** A military facility collaborating in the performance of the project should be treated as a subaward for budget purposes. However, do not complete the Grants.Gov R&R Subaward Budget Attachment Form; instead, complete the DoD Military Budget Form (Attachment 11) to show all direct and indirect costs. The costs per year should be included on the Grants.gov Research & Related Budget Form under subaward costs. Refer to the General Application Instructions, Section III.A.7, for detailed information.

II.D.3. Dun and Bradstreet Universal Numbering System (DUNS) Number and System for Award Management (SAM)

Applicant organizations and all subrecipient organizations must have a DUNS number to submit applications to Grants.gov. The applicant organization must also be registered in the Entity Management functional area of the SAM with an "Active" status to submit applications through the Grants.gov portal. Verify the status of the applicant's organization's Entity registration in SAM well in advance of the application submission deadline. Allow 3 to 4 weeks to complete the entire SAM registration process. If an applicant has not fully complied with the requirements by the time the Federal awarding agency is ready to make a Federal award, the Federal awarding agency may determine that the applicant is not qualified to receive a Federal award and use that determination as a basis for making a Federal award to another applicant. Refer to the General Application Instructions, Section III, for further information regarding Grants.gov requirements.

II.D.4. Submission Dates and Times

All submission dates and times are indicated in Section I, Overview of the Funding Opportunity. Pre-application and application submissions are required. The pre-application and application submission process should be started early to avoid missing deadlines. There are no grace periods. Failure to meet either of these deadlines will result in submission rejection.

Applicant Verification of Full Application Submission in eBRAP

Prior to the end of the application verification period, PIs and organizational representatives can review and modify in eBRAP certain components of a submitted application. Following retrieval and processing of the full application, eBRAP will notify the organizational representatives and PI by email to log into eBRAP to review, modify, and verify the full application submission. eBRAP will validate retrieved files against the specific Program Announcement requirements and discrepancies will be noted in both the email and in the Full Application Files tab in eBRAP. eBRAP does not confirm the accuracy of file content. It is the applicant's responsibility to review all application components and ensure proper ordering as specified in the Program Announcement. ***If either the Project Narrative or the budget fails eBRAP validation or needs to be modified, an updated full application package must be submitted prior to the application submission deadline.*** The Project Narrative and Budget Form cannot be changed after the application submission deadline.

II.D.5. Funding Restrictions

The maximum period of performance is **4** years.

The anticipated total costs budgeted for the entire period of performance will not exceed **$10M**. If indirect cost rates have been negotiated, indirect costs are to be budgeted in accordance with the organization's negotiated rate. No budget will be approved by the Government exceeding **$10M** total costs or using an indirect cost rate exceeding the organization's negotiated rate.

All direct and indirect costs of any subaward or contract must be included in the total direct costs of the primary award.

The applicant may request the entire maximum funding amount for a project that may have a period of performance less than the maximum **4** years.

For this award mechanism, direct costs must be requested for:

Travel costs for the PI and up to three additional members of the research team to attend a 1-day Milestone Meeting to be held in the National Capital Area during the award period of performance. This meeting will be held to provide a presentation on progress. Costs associated with travel to this meeting should be included in Year 3 of the budget. These travel costs are in addition to those allowed for annual scientific/technical meetings.

May be requested for (not all-inclusive):

- Salary
- Research supplies
- Equipment
- Research-related subject costs
- Clinical research costs
- Support for multidisciplinary collaborations, including travel
- Travel costs for up to four investigators to travel to one scientific/technical meeting per year in addition to the required meeting described above

Extramural (non-Federal) awards will consist solely of assistance agreements (Cooperative Agreements and Grants). For extramural awards with an intragovernmental component, direct transfer of funds from an extramural award recipient to a DoD or other Federal agency is not allowed except under very limited circumstances. Funding to intramural DoD and other Federal agencies will be managed through a direct fund transfer. Intragovernmental only funding to intramural DoD and other Federal agencies will be managed through a direct fund transfer. Intramural applicants are responsible for coordinating through their agency's procedures the use

of contractual or assistance funding awards or other appropriate agreements to support extramural collaborators.

Refer to the General Application Instructions, Section III.A.4, for budget regulations and instructions for the Research & Related Budget. ***For Federal agencies or organizations collaborating with Federal agencies, budget restrictions apply as are noted in the General Application Instructions, Section III.A.4***.

The CDMRP expects to allot approximately $60M of the $300M FY17 appropriation to fund approximately six Focused Program Award applications, depending on the quality and number of applications received. Funding of applications received in response to this Program Announcement is contingent upon the availability of Federal funds for this program.

II.D.6. Other Submission Requirements

Refer to the General Application Instructions, Appendix 4, for detailed formatting guidelines.

II.E. Application Review Information

II.E.1. Criteria

II.E.1.a. Peer Review

To determine technical merit, all applications will be evaluated according to the following scored criteria, which are of equal importance:

Scored Review Criteria for the Overall Program:

- **Overall Impact**
    - To what extent the overarching challenge impacts a critical problem or question in the designated FY17 PRMRP Topic Area(s).
    - To what degree the proposed program could, if successful, make a significant impact on the lives of relevant patient populations in the short term or long term.
    - How well the research projects are integrated, complement each other, and provide a synergistic, multidisciplinary approach to solving a critical problem.
    - How well the research program will, if successful:
        - Make important scientific advances in the relevant field of research
        - Promote greater understanding of the causes and progression of the relevant disease(s)/condition(s), or
        - Promote the development of improvements in prevention, detection, diagnosis, treatment, or quality of life

- **Implementation Plan**
    - How the proposed projects are supported by a detailed plan that identifies critical milestones and explains how these milestones will be achieved.
    - How well resources and/or cores that will be created or leveraged will be utilized and shared.
    - To what extent the plans to assess individual project performance during the course of the award are appropriate.
    - How well the overall management plan will facilitate consistent and intensive interactions by all team members in the projects.
    - How the proposed plans for communication, data and specimen collection, data transfer, and periodic meetings are appropriate and robust.
    - To what extent the plans for creating, reviewing, implementing, and modifying Standard Operating Procedures are appropriate, if applicable.
- **Leadership and Environment**
    - To what degree the PI is experienced in successfully leading large, focused projects and is therefore well-positioned to lead the research team in achieving the overarching goal of the proposed effort.
    - How well the PI demonstrates experience, leadership skills, and commitment to making an impact in the relevant field of research and/or patient care.
    - Whether the PI will devote a minimum of 20% effort to this award.
    - To what degree the scientific environment(s) is appropriate for the proposed research.
    - How well the research requirements are supported by the availability of and accessibility to facilities and resources (including patient populations, samples, and collaborative arrangements).
    - To what degree the quality and extent of organizational support are appropriate for the proposed research.
- **Transition Plan and Regulatory Strategy**
    - The degree to which the strategy proposed to bring the outcomes to the next level of development, including funding, milestones, and schedule, is realistic.
    - Whether appropriate collaborations and other resources for providing continuity of development are established and/or well described.
    - How the regulatory strategy and development plan are appropriate and well described.

- How well the application identifies intellectual property ownership, and whether there is sufficient evidence of a plan to resolve intellectual and material property issues, if applicable.

- Whether the applicant has demonstrated that they have access to all intellectual property rights necessary for development and commercialization and evidence that the Government has the ability to access such products or technologies, if applicable.

- If applicable, whether data will be appropriately reported and documented to support a regulatory filing with the FDA.

Scored Review Criteria for Individual Research Projects <u>without</u> a clinical trial:

- **Impact**

  - To what extent the individual project impacts the overarching challenge.

  - To what degree the individual project could, if successful, make a significant impact on the lives of relevant patient populations in the short term or long term.

  - How well the individual project will, if successful, make important scientific advances in the relevant field of research.

- **Research Strategy and Feasibility**

  - How well the scientific rationale supports the research and its feasibility, as demonstrated by a critical review and analysis of the literature, the presentation of preliminary data (where applicable), and logical reasoning.

  - How well the hypothesis, objectives, and aims are developed.

  - To what degree the experimental approach, methods, endpoints, and analyses support completion of the aims and are designed to achieve rigorous and reproducible results.

  - How well the choice of model (animal, cell line, or other) is justified and whether it is appropriate.

  - If applicable, to what degree the statistical plan and power analysis, including sample size projections, are appropriate for the proposed project.

  - Whether there is sufficient evidence to support availability and accessibility of the populations, samples, or other resources required for the study, if applicable.

  - How well potential problems are acknowledged and alternative approaches are addressed.

- **Personnel**

  - To what degree the project team's background and expertise are appropriate with respect to its ability to perform the proposed work, including whether there is evidence of

sufficient expertise for all aspects of the work and whether there is evidence of strong commitment to the projects.

- To what degree the levels of effort are appropriate for successful conduct of the proposed work.

Scored Review Criteria for Individual Research Projects <u>with</u> clinical trials:

- **Impact**
  - To what extent the individual project impacts the overarching challenge.
  - How well the individual project will, if successful, make important scientific advances in the relevant field of research.
  - How well the sample population represents the targeted patient population that might benefit from the proposed intervention.
  - How the potential outcomes of the proposed clinical trial will provide/improve short-term benefits for individuals.
  - How significantly the long-term benefits for implementation of the intervention may impact patient care and/or quality of life.
- **Research Strategy and Feasibility**
  - How well the scientific rationale supports the research and its feasibility, as demonstrated by a critical review and analysis of the literature, the presentation of preliminary data, and logical reasoning.
  - How well the hypothesis and/or objectives and specific aims are developed.
  - To what degree the experimental approach, methods, endpoints, and analyses support completion of the aims and are designed to achieve rigorous and reproducible results.
  - To what degree the statistical plan and power analysis, including sample size projections, are appropriate for the proposed trial and any proposed correlative studies.
  - How well the application demonstrates the availability of and access to the appropriate patient population(s), as well as the ability to accrue a sufficient number of subjects.
  - How well potential problems and delays (e.g., slow accrual, attrition) are acknowledged and alternative approaches are addressed.
- **Clinical Strategy**
  - How the intervention compares with currently available interventions and/or standards of care.

- Whether there is evidence of support, indicating availability of the intervention from its source, for the duration of the proposed clinical trial (if applicable).
- To what degree the PI has provided preclinical and/or clinical evidence to support the safety of the intervention.
- How well the inclusion and exclusion criteria meet the needs of the proposed clinical trial.
- The degree to which the informed consent, screening, and retention processes for human subjects will meet the needs of the proposed clinical trial.
- Whether a member of the study team holds the IND/IDE for the indication proposed or whether the timeline proposed for obtaining the IND/IDE is appropriate (if applicable).
- For investigator-sponsored INDs, whether there is evidence of appropriate institutional support, including capabilities to ensure monitoring as required by the FDA.
- Whether plans to comply with GMP, GLP, and GCP guidelines are appropriate.
- Whether measures are described to ensure the consistency of dosing of active ingredients for nutritional supplements (if applicable).

- **Personnel**
  - To what degree the project team's background and expertise are appropriate with respect to its ability to perform the proposed work, including whether there is evidence of sufficient expertise for all aspects of the work and whether there is evidence of strong commitment to the projects.
  - How well the project leader has assembled an appropriate and robust clinical team with the combined backgrounds and expertise needed to enable successful conduct of the clinical trial.
  - To what degree the levels of effort are appropriate for successful conduct of the proposed work.

In addition, the following unscored criteria will also contribute to the overall evaluation of the application:

- **Data and Resource Sharing**
  - To what degree the plan for sharing of project data and research resources is appropriate and reasonable to facilitate use by the wider research community.
- **Budget**
  - Whether the budget is appropriate for the proposed research and within the limitations of this Program Announcement.

- **Environment**
    - If applicable, to what degree the intellectual and material property plan is appropriate.
- **Application Presentation**
    - To what extent the writing, clarity, and presentation of the application components influence the review.

II.E.1.b. Programmatic Review

To make funding recommendations and select the application(s) that, individually or collectively, will best achieve the program objectives, the following criteria are used by programmatic reviewers:

- Ratings and evaluations of the peer reviewers
- Relevance to the mission of the DHP and FY17 PRMRP, as evidenced by the following:
    - Adherence to the intent of the award mechanism
    - Military relevance
    - Program portfolio composition
    - Relative impact

II.E.2. Application Review and Selection Process

All applications are evaluated by scientists, clinicians, and consumers in a two-tier review process. The first tier is peer review of applications against established criteria for determining technical merit. Each application is evaluated for its own merit, independent of other applications. The second tier is a programmatic review that makes recommendations for funding to the Commanding General, USAMRMC, on behalf of the DHA and the OASD(HA), based on technical merit, the relevance to the mission of the DHP and PRMRP, the specific intent of the award mechanism, and to other specified evaluation criteria in the Program Announcement. Programmatic review is a comparison-based process in which applications with scientific and technical merit compete in a common pool. ***The highest-scoring applications from the first tier of review are not automatically recommended for funding. Funding recommendations depend on various factors as described in Section II.E.1.b, Programmatic Review.*** Additional information about the two-tier process used by the CDMRP can be found at http://cdmrp.army.mil/about/fundingprocess.

All CDMRP review processes are conducted confidentially to maintain the integrity of the merit-based selection process. Panel members sign a statement that application and evaluation information will not be disclosed outside the panel. Violations of confidentiality can result in the dissolving of a panel(s) and other corrective actions. In addition, personnel at the applicant or collaborating organizations are prohibited from contacting persons involved in the review

process to gain protected evaluation information or to influence the evaluation process. Violations of these prohibitions will result in the administrative withdrawal of the organization's application. Violations by panel members or applicants that compromise the confidentiality of the review process may also result in suspension or debarment from Federal awards. Furthermore, the unauthorized disclosure of confidential information of one party to another third party is a crime in accordance with 18 USC 1905.

II.E.3. Integrity and Performance Information

Prior to making an assistance agreement award where the Federal share is expected to exceed the simplified acquisition threshold (currently $150,000) over the period of performance, the Federal awarding agency is required to review and consider any information about the applicant that is available in the Federal Awardee Performance and Integrity Information System (FAPIIS).

An applicant, at its option, may review FAPIIS, accessible through SAM, and submit comments to FAPIIS on any information about itself that a Federal awarding agency previously entered and is currently available in FAPIIS.

The Federal awarding agency will consider any comments by the applicant, in addition to other information in the designated integrity and performance system, in making a judgment about the applicant's integrity, business ethics and record of performance under Federal awards when determining a recipient's qualification prior to award, according to the qualification standards of the Department of Defense Grant and Agreement Regulations (DoDGAR), Section 22.415.

II.E.4. Anticipated Announcement and Federal Award Dates

All application review dates and times are indicated in Section I, Overview of the Funding Opportunity.

Each PI and organization will receive email notification of posting of the funding recommendation in eBRAP. Each PI will receive a peer review summary statement on the strengths and weaknesses of the application.

II.F. Federal Award Administration Information

II.F.1. Federal Award Notices

Awards will be made no later than September 30, 2018. Refer to the General Application Instructions, Appendix 2, for additional award administration information.

Awards are made to organizations, not to individual PIs. The types of awards made under the Program Announcement will be assistance agreements (grants or cooperative agreements). The level of involvement on the part of DoD during project performance is the key factor in determining whether to award a grant or cooperative agreement.

Extramural Organizations: An assistance agreement (grant or cooperative agreement) is appropriate when the Federal Government transfers a "thing of value," to a "state, local

government," or "other recipient," to carry out a public purpose of support or stimulation authorized by a law of the United States, instead of acquiring property or service for the direct benefit and use of the U.S. Government. An assistance agreement can take the form of a grant or cooperative agreement. If "no substantial involvement" on the part of the funding agency is anticipated, a grant award will be made (31 USC 6304). Conversely, if substantial involvement on the part of the funding agency is anticipated, a cooperative agreement will be made (31 USC 6305). Substantial involvement may include collaboration, participation, or intervention in the research to be performed under the award. The award type, along with the start date, will be determined during the negotiation process.

After email notification of application review results through the eBRAP, and if selected for funding, a representative from the USAMRAA will contact the business official authorized to negotiate on behalf of the PI's organization.

Only an appointed USAMRAA Grants Officer may obligate the Government to the expenditure of funds. No commitment on the part of the Government should be inferred from discussions with any other individual. The award document signed by the Grants Officer is the official authorizing documents.

Intramural Organizations: Awards to Federal Government organizations (to include intramural DoD organizations) will be executed through the Military Interdepartmental Purchase Request (MIPR) or Funding Authorization Document (FAD) process. Transfer of funds is contingent upon appropriate safety and administrative approvals. Intramural applicants and collaborators are reminded to coordinate receipt and commitment of funds through their respective resource managers (RM).

After email notification of application review results through the eBRAP, and if selected for funding, a representative from the CDMRP will contact the business official authorized to negotiate on behalf of the PI's organization.

II.F.1.a. Award Transfers

Changes in PI are not allowed, except under extenuating circumstances that will be evaluated on a case-by-case basis and at the discretion of the Grants Officer. An organizational transfer of an award will not be allowed in the last year of the (original) period of performance or any extension thereof.

The organizational transfer of an award supporting a clinical trial is strongly discouraged and in most cases will not be allowed. Approval of a transfer request will be on a case-by-case basis at the discretion of the Grants Officer.

Refer to the General Application Instructions, Appendix 2, Section B, for general information on organization or PI changes.

II.F.2. Administrative and National Policy Requirements

Applicable requirements in the DoDGAR found in 32 CFR, Chapter 1, Subchapter C, and 2 CFR, Chapter XI, apply to grants and cooperative agreements resulting from this Program Announcement.

Refer to the General Application Instructions, Appendix 2, for general information regarding administrative requirements.

Refer to the General Application Instructions, Appendix 5, for general information regarding national policy requirements.

Refer to full text of the USAMRAA General Research Terms and Conditions for Institutions of Higher Education, Hospitals, and Non-Profit Organizations and the USAMRAA General Research Terms and Conditions with For-Profit Organizations for further information.

II.F.3. Reporting

Refer to the General Application Instructions, Appendix 2, Section A, for general information on reporting requirements. Annual progress reports as well as a final progress report will be required.

Quarterly technical progress reports will be required. In addition to written progress reports, in-person presentations may be requested.

Award Chart: Complete the Award Chart template, a one-page PowerPoint file that must be downloaded from the CDMRP eBRAP System at https://ebrap.org/eBRAP/public/Program.htm, and submit to eBRAP at the time of award.

Award Expiration Transition Plan: Complete the Transition Plan template, a one-page Word document that must be submitted with the Final Progress Report. The Transition Plan must outline if and how the research supported by this award will progress, and include source(s) of funding, either known or pending.

Awards resulting from this Program Announcement will incorporate additional reporting requirements related to recipient integrity and performance matters. Recipient organizations that have Federal contract, grant, and cooperative agreement awards with a cumulative total value greater than $10,000,000 are required to provide information to FAPIIS about certain civil, criminal, and administrative proceedings that reached final disposition within the most recent 5-year period and that were connected with performance of a Federal award. Recipients are required to disclose semiannually information about criminal, civil, and administrative proceedings as specified in the applicable Terms and Conditions. The applicable Terms and Conditions for institutions of higher education, hospitals, and nonprofit organizations are available in OAR Article I, Section B, in July 2016 R&D General Terms and Conditions. The applicable Terms and Conditions for for-profit organizations are available in Section 34 of the February 2017 USAMRAA General Research Terms and Conditions with For-Profit Organizations.

II.G. Federal Awarding Agency Contacts

II.G.1. CDMRP Help Desk

Questions related to Program Announcement content or submission requirements as well as questions related to the pre-application or intramural application submission through eBRAP should be directed to the CDMRP Help Desk, which is available Monday through Friday from 8:00 a.m. to 5:00 p.m. ET. Response times may vary depending upon the volume of inquiries.

Phone: 301-682-5507

Email: help@eBRAP.org

II.G.2. Grants.gov Contact Center

Questions related to extramural application submission through Grants.gov portal should be directed to the Grants.gov Contact Center, which is available 24 hours a day, 7 days a week (closed on U.S. Federal holidays). Note that the CDMRP Help Desk is unable to provide technical assistance with Grants.gov submission.

Phone: 800-518-4726; International 1-606-545-5035

Email: support@grants.gov

Sign up on Grants.gov for "send me change notification emails" by following the link on the Synopsis page for the Program Announcement or by responding to the prompt provided by Grants.gov when first downloading the Grants.gov application package. If the Grants.gov application package is updated or changed, the original version of the application package may not be accepted by Grants.gov.

II.H. Other Information

II.H.1. Program Announcement and General Application Instructions Versions

Questions related to this Program Announcement should refer to the Program name, the Program Announcement name, and the Program Announcement version code 20170516b. The Program Announcement numeric version code will match the General Applications Instructions version code 20170516.

II.H.2. Administrative Actions

After receipt of pre-applications or applications, the following administrative actions may occur:

II.H.2.a. Rejection

The following will result in administrative rejection of the pre-application:

- Preproposal Narrative is missing.

The following will result in administrative rejection of the application:

- Submission of an application for which a letter of invitation was not received.
- Project Narrative exceeds page limit.
- Project Narrative is missing.
- Budget is missing.
- Submission of the same research project to different Funding Opportunities within the same program and fiscal year. Refer to Section II.D, Application and Submission Information, for exceptions.

II.H.2.b. Modification

- Pages exceeding the specific limits will be removed prior to review for all documents other than the Project Narrative.
- Documents not requested will be removed.

II.H.2.c. Withdrawal

The following may result in administrative withdrawal of the pre-application or application:

- An FY17 PRMRP Programmatic Panel member is named as being involved in the research proposed or is found to have assisted in the pre-application or application processes including, but not limited to, concept design, application development, budget preparation, and the development of any supporting documentation. ***A list of the FY17 PRMRP Programmatic Panel members can be found at http://cdmrp.army.mil/prmrp/panels/panels17.shtml***.
- The application fails to conform to this Program Announcement description to the extent that appropriate review cannot be conducted.
- Inclusion of URLs, with the exception of links in References Cited and Publication and/or Patent Abstract sections.
- Page size is larger than 8.5 inches x 11.0 inches (approximately 21.59 cm x 27.94 cm).
- To preserve the integrity of its peer and programmatic review processes, the CDMRP discourages inclusion of any employee of its review contractors having any role in the preparation, research or other duties for submitted applications. For FY17, the identities of the peer review contractor and the programmatic review contractor may be found at the

CDMRP website (http://cdmrp.army.mil/about/2tierRevProcess). Applications that include names of personnel from either of these companies will be administratively withdrawn unless plans to manage COIs are provided and deemed appropriate by the Grants Officer. Refer to the General Application Instructions, Appendix 3, for detailed information.

- Personnel from applicant or collaborating organizations are found to have contacted persons involved in the review process to gain protected evaluation information or to influence the evaluation process.
- Applications from extramural organizations, including non-DoD Federal agencies, received through eBRAP may be withdrawn.
- Applications submitted by an intramural DoD organization may be withdrawn if the intramural organization cannot coordinate the use of contractual, assistance, or other appropriate agreements to provide funds to extramural collaborators.
- The proposed research project does not address at least one of the Congressionally directed FY17 PRMRP Topic Areas.
- An application for which the PI does not meet the eligibility criteria will be withdrawn.

II.H.2.d. Withhold

Applications that appear to involve research misconduct will be administratively withheld from further consideration pending organizational investigation. The organization will be required to provide the findings of the investigation to the USAMRAA Grants Officer for a determination of the final disposition of the application.

II.H.3. Application Submission Checklist

Application Components	Action	Completed
SF424 (R&R) Application for Federal Assistance **(Extramural submissions only)**	Complete form as instructed.	
Summary (Tab 1) and Application Contacts (Tab 2) **(Intramural submissions only)**	Complete these tabs as instructed.	
Attachments	Project Narrative: Upload as Attachment 1 with file name "ProjectNarrative.pdf."	
	Supporting Documentation: Upload as Attachment 2 with file name "Support.pdf."	
	Technical Abstract: Upload as Attachment 3 with file name "TechAbs.pdf."	
	Lay Abstract: Upload as Attachment 4 with file name "LayAbs.pdf."	
	Statement of Work: Upload as Attachment 5 with file name "SOW.pdf."	
	Impact Statement: Upload as Attachment 6 with file name "Impact.pdf."	
	Military Relevance: Upload as Attachment 7 with file name "MilRel.pdf."	
	Transition Plan and Regulatory Strategy: Upload as Attachment 8 with file name "Transition.pdf."	
	Data and Research Resources Sharing Plan: Upload as Attachment 9 with file name "Sharing.pdf."	
	IND/IDE Documentation: Upload as Attachment 10 with file name "IND-IDE.pdf," if applicable.	
	DoD Military Budget Form(s): Upload as Attachment 11 with file name "MFBudget.pdf," if applicable.	
Research & Related Senior/Key Person Profile (Expanded)	Attach PI Biographical Sketch (Biosketch_LastName.pdf) to the appropriate field.	
	Attach PI Previous/Current/Pending Support (Support_LastName.pdf) to the appropriate field.	
	Attach Biographical Sketch (Biosketch_LastName.pdf) for each senior/key person to the appropriate field.	
	Attach Previous/Current/Pending (Support_LastName.pdf) for each senior/key person to the appropriate field.	

Application Components	Action	Completed
Research & Related Budget **(Extramural submissions only)**	Complete as instructed. Attach Budget Justification (BudgetJustification.pdf) to the appropriate field.	
Budget **(Intramural submissions only)**	Complete the DoD Military Budget Form and justification.	
Project/Performance Site Location(s) Form	Complete form as instructed.	
R&R Subaward Budget Attachment(s) Form, if applicable	Complete form as instructed.	
Additional Application Components	**Action**	**Completed**
Confidential Letters of Recommendation	Confirm upload to eBRAP.	

APPENDIX 1
ACRONYM LIST

ACURO	Animal Care and Use Review Office
CDMRP	Congressionally Directed Medical Research Programs
CFR	Code of Federal Regulations
DHA	Defense Health Agency
DHP	Defense Health Program
DoD	Department of Defense
DoDGAR	Department of Defense Grant and Agreement Regulations
DUNS	Data Universal Numbering System
eBRAP	Electronic Biomedical Research Application Portal
EC	Ethics Committee
ET	Eastern Time
FAPIIS	Federal Awardee Performance and Integrity Information System
FY	Fiscal Year
GCP	Good Clinical Practices
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
HRPO	Human Research Protection Office
IACUC	Institutional Animal Care and Use Committee
IRB	Institutional Review Board
M	Million
OASD(HA)	Office of the Assistant Secretary of Defense for Health Affairs
OMB	Office of Management and Budget
ORP	Office of Research Protections
PI	Principal Investigator
PRMRP	Peer Reviewed Medical Research Program
RDT&E	Research, Development, Test, and Evaluation
RM	Resource Manager
SAM	System for Award Management
SOW	Statement of Work
USAMRAA	U.S. Army Medical Research Acquisition Activity
USAMRMC	U.S. Army Medical Research and Materiel Command
USC	United States Code
VA	Department of Veterans Affairs

APPENDIX 2
AREAS OF ENCOURAGEMENT

Applications addressing any of the FY17 PRMRP Topic Areas are of interest to the program. ***Any aspect of research relevant to one or more FY17 PRMRP Topic Areas may be considered for funding.*** Areas of encouragement related to the FY17 PRMRP Topic Areas have been identified by the Department of Defense, Department of Veterans Affairs, and other relevant stakeholders. Applicants are urged to read and consider these areas of encouragement before preparing their applications. ***The information provided is not exhaustive, and applicants are not restricted to submitting applications that address an area of encouragement in this list.***

Acute Lung Injury

- Research on the etiology and prevention of acute respiratory distress syndrome caused by the host's or immune system's responses to infectious disease or septic shock.
- Preventive techniques, novel and/or innovative detection technologies, and therapeutics to reduce the incidence and/or severity of acute respiratory distress syndrome and/or other lung injury secondary to trauma, transfusion, burns, hemorrhagic shock, inhalation, and/or oxygen exposure.
- Development of a clinical laboratory-based device for manufacturing/amplification of stem cells to treat acute lung injury/acute respiratory distress syndrome due to inhalation injury, trauma, or other causes.
- Clinical studies to test cellular therapies in the treatment of acute lung injury/acute respiratory distress syndrome due to inhalation injury, trauma, or other causes.
- Metrics to associate the health outcomes of acute lung injury with physiological and physical performance, as well as the psychological health of military Service members.
- Strategies to stabilize and support the safe transport of patients with lung injury in order to optimize therapeutic interventions.
- Studies to identify the prevalence and associated morbidity and mortality of blast overpressure lung injury in forward operating environments.

Antimicrobial Resistance

- Identification and evaluation of novel and/or innovative antifungals against resistant fungal infections, particularly for use as topical therapies for wounds and for surgical and post-surgical therapies.
- Identification of novel and/or innovative targets for multi-drug resistant pathogens.
- Development of novel and/or innovative interventions, diagnostics, and treatment for multi-drug-resistant pathogens.
- Development of novel and/or innovative therapies that interrupt biofilm production in wound infections and infected hardware models.
- Development and testing of treatment protocols and/or diagnostic tests to limit prescribing antibiotics for conditions that are commonly viral in nature or conditions that would resolve themselves without antibiotic treatment (e.g., sinusitis, bronchitis, viral upper respiratory infections, and acute gastroenteritis).

- Development of rapid, rugged, sensitive, and specific diagnostics for antimicrobial resistance in remote environments.
- Research to improve understanding of the impact of travelers' diarrhea, as well as antibiotic treatment for the colonization of multi-drug-resistant organisms.

Arthritis

- Studies that define the risk of contracting septic arthritis; reactive arthritis; arthritis caused by a gastrointestinal, genitourinary, or viral infection (e.g., Lyme disease, Campylobacter, chikungunya); and other arthritis types in active duty military personnel.
- Research toward the development of clinical practice guidelines to prevent, identify, and treat arthritis, particularly that resulting from traumatic injury, surgery, and/or infectious diseases.
- Research to establish activity recommendations for maximal joint life following joint repair, particularly in young patient populations.
- Strategies for repairing focal cartilage defects using cell-based therapies.
- Research quantifying the impacts of obesity, weight loss, physical fitness (all components—cardiovascular, strength, flexibility, balance), and dietary factors on the development of or prevention/risk reduction of arthritis.
- Identification and/or validation of biomarkers for early psoriatic arthritis.
- Basic and translational research to identify treatments to mitigate and/or reverse osteoarthritis, particularly in the knee, hip, ankle, and shoulder.
- Studies to examine using existing regenerative medicine techniques and therapies to treat osteoarthritis, including dose response information and the frequency and timing of application.
- Development of novel and/or innovative treatments or combination therapies for psoriatic arthritis.

Burn Pit Exposure

- Research on the etiology and treatment of adverse health events related to military deployment to Iraq and Afghanistan that are associated with exposure to airborne hazards and open pit burning of solid waste and other materials.
- Toxicological studies to ascertain the toxicity of natural dust, burn pit combustion products and interactions between pollutants, as well as their mechanisms of action.
- Characterization of emissions from open air burns, burn boxes, and incinerators.
- Development and validation of instruments for assessing levels of exposure to airborne hazards for use in research and for occupational and environmental exposure monitoring.

Chronic Migraine and Post-Traumatic Headache

- Research to investigate, develop, and validate biomarkers that are useful in diagnosing and monitoring traumatic brain injury patients with chronic migraine or post-traumatic headache.
- Epidemiological/natural history studies to characterize specific types of post-traumatic headache, the pathobiology of these headaches (such as the role of cortical spreading

depression acutely after injury as a risk factor for chronic headaches of a migrainous type), and the risk factors that might predispose people to certain types of post-traumatic headache.

- Double-blind, placebo-controlled trials in the post-traumatic headache population in order to determine whether similar phenotypes in primary headache disorders and post-traumatic headache will respond similarly to treatment.
- Research on the optimal approaches to effective management of co-occurring psychological health disorders and acute and chronic pain management for chronic migraine and post-traumatic headache, with a focus on assessing and eliminating adverse outcomes and decreasing polypharmacy.
- Research on the utility of the Patient-Centered Medical Home model of care for patients with chronic migraine or post-traumatic headache.
- Research on the treatment and prevention of acute flares of chronic migraine and post-traumatic headaches with an emphasis on non-opioid medications.
- Evaluation of the use of mechanical stimulation and/or other non-pharmaceutical treatments to reduce acute and chronic migraines and headaches.
- Precision medicine research to investigate, develop, and validate biomarkers that are not only useful in diagnosing and monitoring traumatic brain injury patients with chronic migraine or post-traumatic headache, but can also identify individual responses to treatment.
- Evaluation of the differences in etiology, diagnosis, treatment plan, and prevention of migraine headaches between men and women.

Congenital Heart Disease

- Population-based and outcomes-based research projects to assess the health outcomes of individuals with congenital heart disease across their life spans.
- Research on tissue engineering approaches to patches, grafts, and transplantation that provide structural support, restore native activity, allow for tissue growth, and prevent the need for reoperation.
- Research to improve understanding of the causes of congenital heart defects, including genomic, proteomic, and metabolomic profiling.
- Research on transition of the care of congenital heart disease patients from pediatric to adult providers.
- Research both on the risk of neurologic injury and on enhanced neuroprotection before, during, and after surgery for congenital heart disease.
- Research to design and implement disease-in-a-dish and/or microfluidic models with an established phenotype to increase the efficacy of finding novel and/or innovative drug targets, screen existing drugs, perform cardiotoxicity testing, and/or uncover pathogenesis.

Constrictive Bronchiolitis

- Clinical assessments to determine the prevalence and severity of constrictive bronchiolitis and related respiratory diseases in previously deployed military Service members and/or Veterans.

- Development and testing of less invasive and non-invasive approaches for diagnosing constrictive bronchiolitis.
- Research to develop novel and/or innovative therapeutics to slow or reverse the progression of constrictive bronchiolitis.
- Development and/or validation of animal models for constrictive bronchiolitis.
- Research to understand the role of environmental exposures, including mineralized dust and other particulates, in the etiology of constrictive bronchiolitis.

Diabetes

- Development of biomarkers to identify and monitor at-risk individuals and assess treatment response.
- Research on interventions to prevent diabetes complications, including diabetic retinopathy and diabetic neuropathy.
- Research to better understand the heterogeneity of diabetes.
- Research on the transplantation of allogenic or autologous pancreatic islet cells for long-term natural insulin production, including current good manufacturing practices for cell line development.
- Identification and/or evaluation of interventions to reduce the development of diabetes among individuals meeting the clinical criteria for pre-diabetes.
- Development and evaluation of an inpatient glycemic management program with strategies to improve in-hospital dysglycemia, improve short-term and long-term morbidity/mortality, reduce the cost of health care, and promote a seamless transition of care between inpatient and outpatient arenas.
- Research to design and implement disease-in-a-dish and/or microfluidic models to model pancreatic islets to uncover pathogenesis and improve the efficiency of drug discovery.

Diarrheal Diseases

- Development and testing of vaccines, including multi-pathogen combination approaches, against the most common causes of bacterial and viral travelers' diarrhea, including *Campylobacter jejuni, Shigella,* and Enterotoxigenic *E. coli*.
- Development and evaluation of antibiotic and non-antibiotic therapies to treat all-cause travelers' diarrhea to improve the time to clinical cure, minimize resistance acquisition, and mitigate the chronic health consequences of these infections.
- Research to improve understanding of host responses and develop therapies to treat acute bacterial and viral infections that are associated with travel and may lead to chronic functional bowel and rheumatologic disorders.
- Development of evidence necessary to demonstrate the need, impact, and potential market value of a therapeutic for post-infectious functional bowel disorders to include preventive, treatment, or vaccine technology strategies.
- Development and evaluation of point-of-care diagnostics for acute diarrheal illness that distinguishes between bacterial, viral, protozoal, and toxin-mediated etiologies and leads to actionable public health or individual management interventions.

- Investigations into the relationship between existing gastrointestinal flora (microbiome) and the susceptibility, duration, and severity of diarrheal disease, as well as the impacts of dietary modification and/or probiotic use.
- Research to identify, reduce, or eliminate sources of infection causing diarrheal diseases.

Dystonia

- Research on the risk, incidence, and etiology of generalized dystonia, focal dystonia, multifocal dystonia, segmental dystonia, and/or hemidystonia.
- Research on interventions to prevent, slow the progression of, or treat dystonia.
- Research to improve identification of delayed onset dystonia following traumatic brain injury.

Early Trauma Thermal Regulation

- Research into regional cooling for different environments, including prolonged care, as a therapeutic for secondary ischemic injury (e.g., head with traumatic brain injury, limb with tourniquet).
- Development of a tunable support mechanism for thermal homeostasis for use in prolonged field care, extended evacuation, or unmanned evacuation.
- Development and evaluation of a novel and/or innovative technology and techniques to reduce the impact of hypothermia.
- Research to determine the benefits of invasive warming/cooling during patient movement.
- Research to validate the attenuating effects of (early) external head cooling on concussive brain injuries.
- Research into therapeutic cooling or targeted temperature management as a neuroprotective treatment for traumatic brain injury.

Eating Disorders

- Investigations into the prevalence, diagnosis, and treatment patterns of eating disorders in Service members and their families, including potential relationships with military-unique behaviors or conditions.
- Assessment of patterns of comorbidity between eating disorders and other mental health conditions, including an examination of whether eating disorders are more likely to precede or follow the development of other mental health conditions.
- Studies to identify the most effective treatments (and order of treatment) for patients with an eating disorder and a comorbid disorder.
- Research to advance the understanding of the biological, genetic, and environmental factors that influence eating disorders.
- Studies to elucidate risk factors for the onset or recurrence of eating disorders.
- Research to develop screening tools and valid biomarkers that accurately identify individuals with binge eating disorder versus healthy controls in order to monitor at-risk populations and tailor treatments for disordered eating.

- Research to better understand subclinical disordered eating and other specified feeding and eating disorders and the potential for these to develop into full-blown eating disorders.

Emerging Infectious Diseases

- Methods to minimize risk from arboviruses among military Service members and their families deployed to/living in high-risk areas, including personal protective equipment and environmental controls.
- Investigation of revaccination strategies and identification of correlates of protection for currently available vaccines (especially mumps).
- Targeted vector management of *Aedes aegypti, Anopheline sp.* and related mosquito species, including focus areas on novel and/or innovative adult surveillance devices, pathogen identification, spatial repellents, and barrier treatments.
- Research toward understanding the potential influence of dietary intake as a potential repellent to mosquito vectors and vector behavior.
- Evaluation of non-vaccine prophylactics or therapeutics to prevent/treat dengue or Zika virus disease.
- Characterization of health emergency relevant infectious disease threats in West Africa.
- Development of biomarker-guided clinical management strategies for severe infectious disease in the austere African setting.
- Validation of airborne and droplet precaution recommendations in the medical transport environment.
- Development of new testing methods for emerging pathogens and pathogen reduction technologies for blood-borne pathogens, including Zika, malaria, dengue, and others, in blood products.
- Modeling of epidemics and development of strategies to counter risks in emerging infectious diseases.
- Rapid, rugged, sensitive, and specific screening and diagnostics, including biomarkers for infectious threats such as Zika and other clinically relevant bacterial, viral, or protozoal pathogens in clinical samples or blood products that can be transmitted sexually or by blood transmission.
- Development of broad-acting detection systems to identify emerging members of a known viral family (for example, a broad Coronavirus panel that can detect Middle Eastern Respiratory Syndrome).
- Determination of the human health effects, host preference, vector competence, and co-infection status of the dengue, chikungunya, Zika, and Mayaro viruses in *Aedes aegypti* and related mosquito species.

Epidermolysis Bullosa

- Research to provide further insight into those cellular pathways that might be corrected or bypassed that otherwise promote the development of squamous cell carcinomas in recessive dystrophic and junctional epidermolysis bullosa.

- Research, including randomized controlled clinical trials, focused on systemic drugs that prevent, delay the onset, or modify the aggressiveness of squamous cell carcinoma in patients with recessive and junctional epidermolysis bullosa.
- Research, including clinical trials, focused on medications (topical or systemic) or dressings that might enhance wound healing in inherited epidermolysis bullosa.

Focal Segmental Glomerulosclerosis

- Development of a curative therapy or treatments to delay or halt the progression of focal segmental glomerulosclerosis and/or prevent post-transplantation recurrence.
- Research to determine the efficacy of medications used off-label (outside the FDA-approved indication) to treat focal segmental glomerulosclerosis.
- Research to improve understanding of the causes of primary and secondary focal segmental glomerulosclerosis, especially genetic mutations.
- Development of non-invasive methods to diagnose focal segmental glomerulosclerosis and its variants.
- Research on the impact of dietary modifications on the progression of renal disease.

Fragile X

- Research to advance the understanding of the pathophysiology of fragile X syndrome.
- Identification and validation of functional measures of the manifestations of fragile X syndrome across the life span.
- Identification and testing of novel and/or innovative targets for fragile X syndrome therapeutics.
- Development and evaluation of novel, innovative, or existing therapeutics for the treatment of fragile X syndrome.

Guillain-Barré Syndrome

- Research to elucidate the characteristics of various exposures (e.g., viruses, bacteria, vaccinations, surgery) associated with Guillain-Barré syndrome and their effects on the immune system.
- Research on the immune system cell types and molecular mechanisms responsible for the disease pathology of Guillain-Barré syndrome.
- Research to prevent or reduce the effects of residual weakness, relapse of muscle weakness, and other neurological symptoms of Guillain-Barré syndrome to improve patients' quality of life and increase their independence.
- Development of new treatments and refinement of existing treatments for Guillain-Barré syndrome.

Hepatitis B and C

- Research on vaccination options for those persons unable to mount immunity to the hepatitis B virus.

- Development of strategies for reliable, non-invasive, early detection of hepatitis-related liver disease and hepatocellular carcinoma.
- Research on strategies to promote reversal of liver fibrosis and/or assess the associated clinical and pathological outcomes.
- Natural history or other studies to establish risk indicators of progression from asymptomatic or inactive chronic hepatitis infection to symptomatic disease and parameters for the initiation and duration of disease treatment.
- Clinical studies to evaluate combination or curative therapies for treatment of hepatitis infection.
- Identification and reduction of hepatitis in blood products for transfusion.
- Research on strategies to reduce vertical (mother-to-child) transmission of hepatitis B virus and hepatitis C virus.
- Development of a vaccine against hepatitis C.
- Development of treatment protocols for patients with hepatitis B and C co-infections.

Hereditary Angioedema

- Research to improve early diagnosis of hereditary angioedema.
- Evaluation of existing, innovative, or novel therapeutics in pediatric hereditary angioedema patients.
- Research toward development of a cure for hereditary angioedema.
- Development and/or validation of novel and/or innovative therapeutic strategies for the treatment and/or prevention of hereditary angioedema attacks.

Hydrocephalus

- Research on the etiology, prevention, diagnosis, and treatment of post-traumatic hydrocephalus.
- Discovery or validation of novel and/or innovative therapies and therapeutic targets for the treatment of hydrocephalus and its sequelae, including therapies directed at myelin regeneration and repair.
- Research on approaches to lessening the impact of brain damage caused by hydrocephalus.
- Development or validation of biomarkers and imaging techniques, particularly multimodal approaches, to aid in diagnosis, prognosis, and monitoring of therapeutic efficacy.
- Research on the prevention of shunt failure.
- Studies to better understand the causes and/or pathogenesis of hydrocephalus.
- Development or validation of improved hydrocephalus model systems.

Immunomonitoring of Intestinal Transplants

- Studies to elucidate the role of the mucosal immune system, other host-derived factors, or gut microbiota-derived factors in maintaining intestinal transplant viability.

- Development and evaluation of intestinal implant strategies that circumvent the induction of immunity against the transplant through the use of autologous stem cells or organoids derived from intestinal stem cells.
- Development and evaluation of evidence-based approaches that focus on dampening the regional immune response against intestinal transplants without inducing global immune suppression.
- Development and evaluation of implant-associated materials (e.g., scaffolds) with anti-inflammatory properties that protect the intestinal transplant from immune attack.
- Development and evaluation of strategies for inducing and maintaining populations of anti-inflammatory regulatory immune cell populations at the transplant site.
- Studies to improve immunomonitoring of recipient immune responses after intestinal transplantation, with a focus on prospective leukocyte profiling, to aid in diagnosis and treatment of immunological and immunosuppression-related complications.
- Development and/or validation of precise, real-time implanted monitoring devices to improve individualized patient outcomes after intestinal transplantation.

Inflammatory Bowel Diseases

- Clinical studies in human subjects directed toward understanding how acute enteric infections may trigger chronic bowel diseases with acute and sub-acute inflammatory bowel disease, including systems biology approaches.
- Mechanistic studies in animal models designed to understand how enteric infection may trigger inflammatory bowel disease, including genomic, microbiomic, and immune mechanisms.
- Epidemiological studies of post-infectious acute and sub-acute inflammatory bowel diseases that define risk and provide estimates of illness-associated disability, health care costs, and symptom duration from a military health system and societal perspective.
- Studies to understand the interaction between acute/chronic stress and infection and the development of inflammatory bowel disease.
- Research to explore whether the travel patterns of active duty personnel increase the risk of developing inflammatory bowel disease by means of exposure to changes in intestinal microflora.
- Research to better characterize the association between the use of drugs, such as isotretinoin and long-term doxycycline, and the development of inflammatory bowel disease.
- Research on the role of diet in the development and progression of inflammatory bowel disease.
- Research on the influence of the microbiome on inflammatory bowel disease.
- Research on hyperbaric oxygen treatment of inflammatory bowel disease.
- Research on treatment strategies for patients whose inflammatory bowel disease is refractory to standard care medications or who positively respond to standard care medications, but become nonresponsive over time.

Influenza

- Development of a diagnostic test for influenza drug resistance.
- Development and testing of a universal influenza vaccine.
- Development and evaluation of novel, innovative, and/or combination influenza therapies.
- Research to improve the understanding of host responses to influenza infection and the mechanisms of drug resistance.
- Research on the factors that contribute to and/or reduce the risk of the emergence of zoonotic and pandemic influenza viruses.

Integrative Medicine

- Research on the use of integrative medicine strategies, such as meditation, tai chi, acupuncture, and/or herbal medicine/nutritional supplementation, to improve the psychological health and quality of life in cancer patients and survivors.
- Research on the use of integrative medicine in treatment and management of chronic pain disorders, including comparative efficacy studies relative to standard of care.
- Rigorous longitudinal studies of integrative medicine approaches for enhancing resilience and treating psychological health issues and co-occurring disorders.
- Precision medicine research to investigate, develop, and validate biomarkers that can help determine an individual's response to integrative medicine treatments for pain.
- Development of outcomes tools and measures to evaluate the effectiveness of integrative medicine pain management approaches.
- Definitive studies to determine the effectiveness of self-care, meditation-based treatments, courses, and training programs in improving resiliency to stress in a military environment; in austere operational platforms such as submarines and small-deck ships; or in combat-fatigued military health care providers.

Interstitial Cystitis

- Studies that define the risk, prevalence, and operational impact of interstitial cystitis among active duty personnel.
- Identification of biological markers for making a definitive diagnosis of interstitial cystitis.
- Evaluation and assessment of novel and/or innovative treatment options for interstitial cystitis patients, including intravesical therapy.
- Research on the etiology of interstitial cystitis to inform targeted therapy development.

Malaria

- Investigation of mechanisms of drug resistance in malaria, to include host and pathogen, against drugs used for treatment and prophylaxis.
- Development of a vaccine that induces high levels of long-lived, broadly protective immunity against *Plasmodium falciparum* and/or *P. vivax*.
- Operational research to optimize application of existing malaria drugs to reduce impact of artemisinin resistant *P. falciparum* in military forces.

- Operational research to optimize deployment of pharmacovigilance systems to ensure capture of adverse events related to malaria chemoprophylaxis and treatment.
- Operational research to assess efficacy and spread of artemisinin combination therapies across the Greater Mekong Subregion's military forces.
- Development of malaria prophylactic regimens that encourage higher compliance and methods to monitor compliance in deployed Service members.
- Identification of novel and/or innovative malaria drug targets for blood and liver stage malaria parasites.
- Development of new, or improvement of existing, strategies for culturing relapsing malaria parasites, with emphasis on *Plasmodium vivax, P. ovale,* and *P. cynomolgi* in vitro.
- Development, testing, and evaluation of spatial repellents to provide protection against Anopheline vectors.
- Identification of a correlate of immunity against malaria and development of a surrogate test of protection.
- Structurally optimize validated malaria antigens by engineering protective epitopes to elicit specific and enhanced immune responses.

Metals Toxicology

- Identification and validation of biomarkers to evaluate military Service members' acute exposure to toxic metals in an operational environment and predict potential consequent health risks and associated health outcomes.
- Retrospective studies to evaluate risk and exposure among workers at industrial facilities.
- Research on the toxicity of metal combinations and the interaction between different metal components.
- Research on the additive effects of multiple exposures to metal(s).
- Research on underlying diet quality and micronutrient sufficiency and the risk for metals toxicity.
- Research on the toxicity of metal-based engineered nanomaterials.

Mitochondrial Disease

- Research on the basic biology and physiology of mitochondria to better understand the pathology of primary mitochondrial diseases.
- Development of improved tools and animal models to study primary mitochondrial disease and evaluate therapeutics.
- Identification and testing of non-invasive techniques and biomarkers to monitor mitochondrial function, aid in diagnosis, and/or evaluate therapeutic efficacy.
- Research on novel and/or innovative treatments to alleviate symptoms or slow down the progression of mitochondrial diseases.

Musculoskeletal Disorders

- Research to increase understanding, diagnosis, prevention, and/or treatments of chronic overuse musculoskeletal disorders.
- Research on measures (e.g., clinical biomarkers, novel/innovative interventions, therapeutics) to diagnose, predict, reduce the incidence of, or optimize health outcomes in military training- and Service-related musculoskeletal disorders.
- Research on the validation and/or optimization of rehabilitation strategies for Service-related musculoskeletal disorders.
- Research to prevent, control, and/or optimize musculoskeletal health outcomes for work-related musculoskeletal disorders.
- Research on lower back pain strategies to prevent surgery and recurrence of symptoms, identify factors that predict optimal treatment response for different patients, and encourage self-management as a treatment.
- Research to determine whether tailored psychosocial education interventions are effective in improving psychological resilience to musculoskeletal pain.
- Research on standardized approaches for early identification and prevention of chronic musculoskeletal pain.

Nanomaterials for Bone Regeneration

- Research on nanomaterials-based methods to facilitate recruitment of endogenous cell populations for enhanced bone regeneration and osseointegration.
- Technologies addressing segmental/large bone defects in craniomaxillofacial and/or load-bearing regions.
- Development of controlled release/extended release of growth factors for bone regeneration.
- Development of technologies that repair the soft tissue envelope to enhance bone regeneration.

Non-Opioid Pain Management

- Research on pain management strategies for patients with limited access to skilled providers and resources, including battlefield, prolonged field, transport, and other resource-limited environments.
- Development of population-based outcomes, tools, and measures to evaluate the effectiveness of pain management approaches across time and environments.
- Research on treatments for chronic pain management, particularly in complex patients (i.e., chronic, high-utilization, polypharmacy patients).
- Research to increase understanding of and preventative treatments for conversion of acute to chronic pain.
- Research that provides evidence of which pain management strategies work for whom and under what conditions.
- Research to identify and address biopsychosocial aspects of pain to reduce or eliminate the use of opioid pain medication(s).

- Comparative studies evaluating the efficacy of different pain management strategies, including complementary and alternative medicine approaches.
- Identification and development of non-opioid pain management techniques that promote positive psychological health-related outcomes.
- Development of non-opioid pain medicine that can be given via inhalation or intramuscularly, submucosally, or intravenously on the battlefield to provide adequate relief of pain without affecting the cardiorespiratory systems.
- Research on case management strategies designed to optimize the effectiveness of pain clinics, support use of non-opioid pain therapies, and reduce dependence on opioid therapies.
- Research that will directly obtain an FDA analgesic indication for oral transmucosal or intramuscular ketamine.

Pancreatitis

- Retrospective studies to determine the risk and incidence of pancreatitis among former and current active duty personnel.
- Development and testing of novel and/or innovative therapeutics for acute and/or chronic pancreatitis.
- Research on the basic biology and physiology of the pancreas to better understand the etiology and pathology of pancreatitis.

Pathogen-Inactivated Dried Cryoprecipitate

- Development or validation of technology to produce pathogen-reduced dried cryoprecipitate in military or civilian donor centers that is acceptable for FDA licensure in support of combat or contingency operations.

Polycystic Kidney Disease

- Development of improved treatment strategies for polycystic kidney disease, including approaches to identify and monitor patients at higher risk for progressing to end-stage renal disease.
- Research on the underlying pathobiology and molecular mechanisms of polycystic kidney disease, including studies of genetic factors, cyst formation and growth, the role of cilia, and factors that modify disease progression and/or severity.
- Research on the lifestyle factors that may modify the progression of polycystic kidney disease.

Post-Traumatic Osteoarthritis

- Research to establish activity recommendations for maximal joint life following joint repair, particularly in young patient populations.
- Research into cell-based approaches for treatment or prevention of post-traumatic osteoarthritis.
- Studies to evaluate and develop best practices for multidisciplinary team approaches and treatment algorithms for post-traumatic osteoarthritis.

- Development or validation of novel and/or innovative approaches to restoring joint stability after injury.
- Sustained release, intra-articular injectable steroidal, non-steroidal, or disease-modifying therapies that offer two or more months of symptomatic relief of pain and/or inflammation in a single injection.
- Research on therapies that target multiple phases of the cellular response pathways that are implicated in the development of post-traumatic osteoarthritis, including cell death, inflammation, matrix changes, and changes in catabolic and anabolic responses.
- Development and/or validation of predictive models for early onset post-traumatic osteoarthritis in extremity-injured patients for the purpose of tailoring rehabilitation programs toward prevention, delay, or mitigation of onset.

Pulmonary Fibrosis

- Retrospective studies to determine the risk and incidence of pulmonary fibrosis among military Service members, including Veterans.
- Identification of biomarkers of pulmonary injury or early predictors of interstitial lung disease.
- Research into the pathobiology and molecular mechanisms underlying the development of pulmonary fibrosis.
- Development and/or testing of novel and/or innovative treatments to delay or modify the progression of pulmonary fibrosis.
- Development and/or validation of improved tools and animal models to study pulmonary fibrosis and evaluate therapeutics.

Respiratory Health (excludes lung cancer and mesothelioma)

- Research on the causes, treatment, and prevention of chronic obstructive pulmonary disease (COPD), including identification and validation of biomarkers and disease phenotypes, as well as employing personalized medicine in clinical research and disease management.
- Research investigating the treatment of patients with chronic mild hypoxia.
- Research on the cause, treatment, and prevention of respiratory symptoms and ailments possibly associated with deployed and redeployed military personnel, including acute eosinophilic pneumonia, asthma, allergies, and other chronic lung diseases and breathing problems.
- Research to evaluate the impact of deployment on the prevalence and severity of respiratory disease in military Service members and Veterans.
- Development and/or validation of methods to detect volatile organic hydrocarbons from the breath of individuals exposed to toxic chemicals or other agents that can cause lung injury, which can be utilized for diagnosis and prognosis of lung injury or disease.
- Identification of biochemical, physiological, or combined biomarkers for evaluating risk or extent of injury from either acute or long-term toxic occupational or environmental exposures.

- Research investigating exposure rates, detection, and treatment of diseases related to inhalation of mold and fungi, such as coccidioidomycosis.

Rett Syndrome

- Identification and/or validation of novel and/or innovative biological targets for the treatment of Rett syndrome.
- Development and testing of interventions to improve the neurological symptoms of Rett syndrome.
- Research to understand the relationship between genetic mutations, physical traits, and symptoms in individuals with Rett syndrome.
- Research on the pathobiology of the MeCP2 gene and protein.
- Research to understand Rett syndrome's commonalities with and differences from classic autism and regressive autism.

Rheumatoid Arthritis

- Research to better understand the relationship between genetic risk and environmental triggers, such as infection or smoking, in developing rheumatoid arthritis.
- Studies that identify or validate biomarkers or personalized medicine strategies that allow for individualized medication choice based on the patient's underlying biology or disease state.
- Research on the long-term use of immunosuppressants in patients with rheumatoid arthritis and the likelihood of developing infections.
- Research to establish activity recommendations following joint replacement for maximal joint life.

Scleroderma

- Research on the molecular mechanisms and pathogenesis of scleroderma.
- Development and/or testing of novel and/or innovative therapies and identification of novel and/or innoavtive therapeutic targets in scleroderma.
- Research on biomarkers and other approaches to diagnose scleroderma, monitor disease progression, and/or assess response to treatment.
- Epidemiologic studies investigating the impact of localized scleroderma (morphea) on duty performance, use of personal protective equipment, and deployability.
- Research on the efficacy of newer immunosuppressive agents, including biologics and small molecule inhibitors, in the treatment of morphea and scleroderma (systemic sclerosis).
- Research on the efficacy of UVA1 for topical treatment in localized scleroderma and systemic sclerosis.

Sleep Disorders

- Research on how the disruption of normal sleep and circadian biological rhythms adversely affects the physical and psychological health, safety, performance, and productivity of military and civilian populations.
- Studies to assess sleep disturbance and clinical sleep disorders in female military personnel.

- Research on the association between sleep disorders and post-traumatic stress disorder, traumatic brain injury, depression, and/or anxiety and suicidal behaviors.
- Research to assess clinical algorithms that improve adherence to continuous positive airway pressure (CPAP).
- Investigations into non-CPAP-based treatment regimens that enhance readiness and deployability in active duty military personnel.
- Research focused on investigating how psychiatric disorders (post-traumatic stress disorder in particular), combined with the disruption/degradation of sleep quality, impact long-term physical health through changes in hormonal physiology.
- Development and/or testing of non-pharmacological treatments for sleep disorders.
- Research on the impact of sleep deprivation on the efficacy of psychological treatments for post-traumatic stress disorder, depression, and other mood disorders.
- Research on the prevention and/or mitigation of sleep disorders that are associated with long aeromedical evacuation flights for both clinical team members and patients.
- Development of tools and technologies that allow rapid and objective measurement of Service member performance in the face of fragmented sleep and/or sleep maintenance difficulty.
- Research examining the broader effects of Warfighters' sleep disorders on the sleep, behavioral health, and productivity of their family members, as well as the readiness of their military unit.

Spinal Muscular Atrophy

- Research into phenotyping the spinal muscular atrophy disease state.
- Research into which molecular deficiency imposed by a deficiency of SMN, a motor neuron protein, causes motor neuron deficits.
- Exploration of the form and function of SMN-depleted neuromuscular junctions at ultrastructural (e.g., dysregulation of endocytosis), transcriptomic, and proteomic levels, particularly the mildest SMN reduction that leads to consistent quantifiable motor neuron loss.
- Research to find non-SMN-altering spinal muscular atrophy modifying genes in cellular, small animal, and human models that may lead to identification of novel and/or innovative therapeutic targets.
- Research to further understand SMN gene regulation and post-transcriptional mechanisms leading to synergistic SMN-repleting approaches, as well as to determine whether boosting SMN induction maximizes efficacy.
- Research to determine whether SMN can be repleted to physiologic levels in all cells in the spinal muscular atrophy model immediately post-natally and whether there is a limit to the therapeutic response.
- Clinical research to determine what clinical findings persist even with treatment; whether these findings are related to drug timing, dosage, biodistribution, or a combination of factors; and whether these factors are modifiable.

- Research involving dietary/nutritional studies in spinal muscular atrophy to identify the optimal anabolic diet.
- Research to determine mitochondrial involvement and astrocytic and other non-neuronal contributions to motor neuron vulnerability.

Sustained-Release Drug Delivery

- Development of technology that can provide a sustained-release delivery of drugs for a minimum of 1 week or more. Potential applications of this technology could include long-acting therapies for post-traumatic stress, opiate dependence, low-dose pain control, allergies, attention deficit/hyperactivity disorder, chemoprophylaxis, and other conditions.
- Research into the use of printable, sustained-release scaffolds for predictable delivery of prescription and non-prescription medications.
- Research into novel and/or innovative approaches to developing bioavailable and sustained-release oral formulations of intravenous broad-spectrum fungicidal and anti-microbial medications.

Tinnitus

- Research to understand the mechanisms of tinnitus, its relationship to noise-induced hearing loss, and progression to chronic tinnitus, with the focus on developing interventions for tinnitus.
- Research to increase knowledge of the prevalence, incidence, natural history, and occupational and sex-related differences of tinnitus and its possible relation to individual blast/noise exposures.
- Research to understand the operational readiness impacts of tinnitus on the military.
- Development and validation of objective tools/methods to diagnose and characterize tinnitus (e.g., imaging techniques to identify functional and structural changes in the brain, biomarkers of resiliency, and susceptibility to tinnitus).
- Identification of novel and/or innovative therapies for interventions to prevent, manage, and treat tinnitus, including behavioral approaches, new uses for existing drugs, nutritional and pharmaceutical strategies, and acoustic, electrical, and other stimulation technologies.

Tuberculosis

- Development of a diagnostic assay that can be used at the point of care to rapidly and accurately diagnose tuberculosis.
- Development of novel and/or innovative tuberculosis vaccines or optimization of current tuberculosis vaccines.
- Identification and/or validation of biomarkers that can be used to assess vaccine efficacy and protection against tuberculosis disease.
- Research to understand, diagnose, or treat multi-drug-resistant tuberculosis or extensively drug-resistant tuberculosis.
- Research to determine the appropriate precautions to use for transport of active tuberculosis patients.

- A sustained release formulation of anti-tuberculosis drugs that would facilitate long-term treatment and reduce the emergence of resistance due to poor compliance.

Vaccine Development for Infectious Disease

- Development of a therapeutic vaccine and/or other strategies to achieve HIV remission.
- Evaluation of passive immunization strategies to use in conjunction with dengue and Zika vaccinations.
- Research leading to a better understanding of the immune mechanisms involved in clearance of the dengue and Zika viruses and the mechanisms of immune enhancement that lead to more severe clinical disease.
- Development of flexible vaccine technologies that can be used to rapidly respond to emerging and re-emerging infectious diseases threats.
- Evaluation of humoral and cellular immune responses after vaccination compared to natural infection.
- Development of a safe, effective dengue human challenge model for clinical trials.
- Development of a non-human primate model to study Zika virus-induced microcephaly/fetal neuropathology as a tool to measure the safety and efficacy of vaccines and antivirals.
- Development and fielding of vaccines to prevent U.S. Service members from becoming ill from endemic disease exposure during operational deployments. This includes, but is not limited to, arthropod-borne diseases (Zika virus, dengue, chikungunya, hantavirus hemorrhagic fever with renal/pulmonary syndrome, rickettsioses, and trypanosomiasis), leptospirosis, HIV, norovirus, Middle East Respiratory Syndrome coronavirus, schistosomiasis, leishmaniasis, Nipah virus, Lassa fever, and West Nile fever.

Vascular Malformations

- Studies into the natural history, genetics, and pathogenesis of vascular malformations.
- Research to discover or develop novel and/or innovative therapeutic targets and treatments to regress or prevent vascular malformations.
- Research to improve methods to diagnose and manage vascular malformations.
- Development of non-invasive or minimally invasive technologies or approaches for the control of internal bleeding associated with vascular malformations.
- Studies to identify risk and/or establish standard practices for the treatment of hemorrhage from brain arteriovenous malformations.
- Research on the prevention or treatment of complications associated with vascular malformations such as pain, infection, seizures, tissue breakdown, tissue overgrowth, or airway obstruction.
- Studies to elucidate the progression of vascular malformations in disease-in-a-dish/microfluidic models for the purpose of identifying novel and/or innovative drug targets, screening existing drugs, and/or elucidating the pathogenesis of the disease.

Women's Heart Disease

- Retrospective studies to determine the risk and incidence of heart disease among former and current female active duty personnel, including Reservists and National Guard members.
- Research focused on elucidating the potential relationship between post-traumatic stress disorder and women's heart disease.
- Identification of sex-specific approaches to increase the effectiveness of diagnosis, treatment, and/or cardiac rehabilitation programs.
- Research on factors to predict and prevent the long-term impacts of gestational diabetes, gestational hypertension, and preeclampsia on the cardiovascular health of women.
- Research on trauma-induced cardiac arrest secondary to hemorrhage and polytrauma in the female population.

APPENDIX 3
DOD AND VA WEBSITES

PIs are encouraged to integrate and/or align their research projects with Department of Defense (DoD) and/or Department of Veterans Affairs (VA) research laboratories and programs. Collaboration with DoD or VA investigators is also encouraged. Below is a list of websites that may be useful in identifying additional information about DoD and VA areas of research interest, ongoing research or potential opportunities for collaboration within the FY17 PRMRP Topic Areas.

Air Force Office of Scientific Research
http://www.wpafb.af.mil/afrl/afosr/

Air Force Research Laboratory
http://www.wpafb.af.mil/afrl

Armed Forces Radiobiology Research Institute
http://www.usuhs.edu/afrri/

Clinical and Rehabilitative Medicine Research Program
https://crmrp.amedd.army.mil

Combat Casualty Care Research Program
https://ccc.amedd.army.mil

Congressionally Directed Medical Research Programs
http://cdmrp.army.mil

Defense Advanced Research Projects Agency
http://www.darpa.mil/

Defense Technical Information Center
http://www.dtic.mil

Defense Threat Reduction Agency
http://www.dtra.mil/

Military Health System Research Symposium
https://mhsrs.amedd.army.mil/SitePages/Home.aspx

Military Infectious Diseases Research Program
https://midrp.amedd.army.mil

Military Operational Medicine Research Program
https://momrp.amedd.army.mil

Naval Health Research Center
http://www.med.navy.mil/sites/nhrc

Navy and Marine Corps Public Health Center
http://www.nmcphc.med.navy.mil/

Office of Naval Research
http://www.med.navy.mil/

Office of the Under Secretary of Defense for Acquisition, Technology and Logistics
http://www.acq.osd.mil/

Telemedicine and Advanced Technology Research Center
http://www.tatrc.org/

Uniformed Services University of the Health Sciences
http://www.usuhs.edu/research

U.S. Army Institute of Surgical Research
http://www.usaisr.amedd.army.mil/

U.S. Army Research Institute of Environmental Medicine
http://www.usariem.army.mil/

U.S. Army Medical Research Institute of Infectious Diseases
http://www.usamriid.army.mil/

U.S. Army Medical Research and Materiel Command
http://mrmc.amedd.army.mil

U.S. Army Research Laboratory
http://www.arl.army.mil

U.S. Department of Defense Blast Injury Research Program
https://blastinjuryresearch.amedd.army.mil/

U.S. Department of Veterans Affairs, Office of Research and Development
http://www.research.va.gov

U.S. Naval Research Laboratory
http://www.nrl.navy.mil

Walter Reed Army Institute of Research
http://www.wrair.army.mil/

Biographical Sketch

Provide the following information for each individual included in the Research & Related Senior/Key Person Profile (Expanded) Form.			
NAME	POSITION TITLE		
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training).			
INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List in chronological order the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 5 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INDIVIDUAL.

RESEARCH AND PROFESSIONAL EXPERIENCE (CONTINUED). PAGE LIMITATIONS APPLY. DO NOT EXCEED 5 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INDIVIDUAL.

RESEARCH AND PROFESSIONAL EXPERIENCE (CONTINUED). PAGE LIMITATIONS APPLY. DO NOT EXCEED 5 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INDIVIDUAL.

RESEARCH AND PROFESSIONAL EXPERIENCE (CONTINUED). PAGE LIMITATIONS APPLY. DO NOT EXCEED 5 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INDIVIDUAL.

RESEARCH AND PROFESSIONAL EXPERIENCE (CONTINUED). PAGE LIMITATIONS APPLY. DO NOT EXCEED 5 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INDIVIDUAL.

RENAISSANCE®
CHICAGO O'HARE SUITES HOTEL

8500 West Bryn Mawr Ave
Chicago, IL 60631
(773) 380-9600

HOW TO GET TO THE CHICAGO RENAISSANCE O'HARE SUITES HOTEL:

BUS/SHUTTLE FROM O'HARE INT'L AIRPORT: From your gate go to Baggage Claim. Follow the signs labeled "Bus/Shuttle Center" to the nearest underground pedestrian walkway. In the elevator press the Bus/Shuttle Center button. Once in the center, wait by Door 2. The vans arrive every 30 minutes between 6:00am-11:00pm. Beyond then, to request pickup, please call the hotel at ***773/380-9600***. ******Please note when guest arrive in the International Terminals and need shuttle transportation they must call for pick-up the shuttle bus does not run in that area, guests may utilize the courtesy phone at 5E and a shuttle will be dispatched******

DRIVING FROM O'HARE INT'L AIRPORT: Take I-90 East, exit at Cumberland Ave South. Turn right at West Bryn Mawr Ave. The hotel is a ½ mile down on your right midway down the block.

DRIVING FROM MIDWAY AIRPORT: Go North on Cicero Avenue to I-55 South to I-294 North. Remain on I-294 North to I-90 Ease (toward Chicago not O'Hare). Exit at Cumberland Ave South. Turn right at West Bryn Mawr Ave. The hotel is a ½ mile down on your right midway down the block.

DRIVING FROM DOWNTOWN CHICAGO (EAST): Take the Kennedy Expressway (I-90 West) towards O'Hare. Exit at Cumberland Ave South. Merge over to the far right lane. The next light is Bryn Mawr. Turn right at West Bryn Mawr Ave. The hotel is a ½ mile down on your right midway down the block.

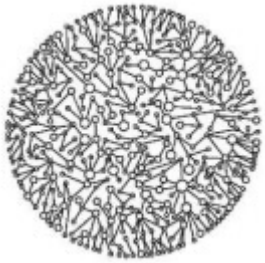
DRIVING FROM THE WEST: Take the Northwest Tollway (I-90 East) past O'Hare International Airport. Exit at Cumberland Ave South. Turn right at West Bryn Mawr Ave. The hotel is a ½ mile down on your right midway down the block.

DRIVING FROM THE NORTH: Take the Tri-State (I-294 South) to I-90 East (toward Chicago: not O'Hare). Exit at Cumberland Ave South. Turn right at West Bryn Mawr Ave. The hotel is a ½ mile down on your right midway down the block.

DRIVING FROM THE SOUTH: Take the Tri-State (I-294 North) to I-90 East (toward Chicago: not O'Hare). Exit at Cumberland South. Turn right at West Bryn Mawr Ave. The hotel is a ½ mile down on your right midway down the block.

Annual Programmatic Meeting

National Institute of Allergy and Infectious Diseases (NIAID)
Systems Biology for Infectious Diseases (SBID) Research
May 7th – 10th, 2017
Renaissance Chicago O'Hare Suites



INSTRUCTIONS FOR SPEAKERS

The organizers sincerely appreciate your participation in this meeting. Due to the high level of interest in this meeting and the sizeable number of speakers on the agenda, it is very important that you plan your presentation (including transition to and from the lectern and time for questions) to fit within your allotted time. Session chairs have been asked to keep the presentations on schedule and may curtail your presentation if it exceeds the allotted time.

LOGISTICS

Prior to Your Talk

1. Slides must be saved in either **PowerPoint** or **PDF** format.
2. **All presentations must be saved on a memory stick and loaded onto the presentation laptop before 8:30 am CDT on the morning of the presentation.** The presentation laptop will be available in the Renaissance Ballroom during the Welcome Reception on Sunday evening (6:00 pm – 7:30 pm CDT, May 7th); from 7:30 am – 8:15 am CDT each day of the meeting (May 8th – May 10th); or during any lunch, dinner or refreshment break.

Day of Your Talk

3. Please be in **Renaissance Ballroom** at least 5 minutes before the start of your session.
4. At the beginning of your session, the session Chair(s) will stand at the lectern, introduce herself/himself, and announce the title of the session.
5. PLENARY SESSION SPEAKERS: Session Chair(s) will introduce you and your presentation title before the start of your talk.
6. FLASH PRESENTATION SPEAKERS: Given the very short length of Flash Presentation talks, speakers should plan to introduce yourself and your presentation title.

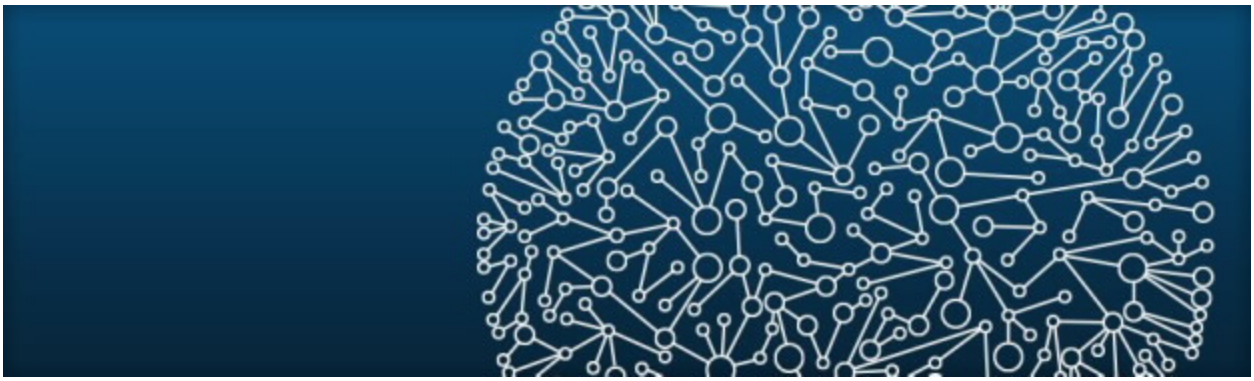
During Your Talk

7. If you experience problems accessing your slides, the moderator and/or a technical support staff member will be available to assist you.
8. **We ask that you please adhere strictly to the time allotted for your presentation.** Chair(s) will time each presentation and will notify you when 5 minutes are remaining (Plenary Session speakers only).
9. Your presentation will be projected (by a laptop connected to a projector) onto a single screen adjacent to the lectern. You will operate the laptop and advance the slides.
10. A laser pointer will be provided to help you emphasize key points.
11. If time remains at the conclusion of your talk, the Chair will open the floor for questions and/or discussion.

After Your Talk

12. Return to your seat and be ready to return to the stage at the end of the session.
13. At the end of the session, return to the stage for a panel discussion and Q&A.

If you have any questions, please do not hesitate to contact
Amie Eisfeld (amie.eisfeld@wisc.edu) or Amy Kuehn (aeschuster@wisc.edu).



Annual Programmatic Meeting

National Institute of Allergy and Infectious Diseases (NIAID)
Systems Biology for Infectious Diseases (SBID) Research

May 7-10, 2017
Chicago, IL

Meeting Information and Abstract Book

General Information

Venue
Renaissance Chicago O'Hare Suites
8500 West Bryn Mawr Avenue
Chicago, Illinois 60631 USA
Phone: +1-773-380-9600
http://www.marriott.com/hotels/travel/chibr-renaissance-chicago-ohare-suites-hotel/

Airport Shuttle
From your gate, go to Baggage Claim. Follow the signs labeled "Bus/Shuttle Center" to the nearest underground pedestrian walkway. In the elevator press the Bus/Shuttle Center button. Once in the center, wait by Door 2. The vans arrive every 30 minutes between 6:00 am and 11:00 pm. Beyond then, to request pickup, please call the hotel at 773-380-9600.

Meeting Location
Renaissance Ballroom

Registration
Please stop at the Registration Table in front of the Renaissance Ballroom on Sunday Evening during the Welcome Reception (6:00 pm – 7:30 pm) or any other time during the meeting.

Internet Access
Wi-Fi login credentials will be made available to all meeting participants for use in the Renaissance Ballroom.

Presenters
Presentations should be saved on a memory stick and loaded onto the presentation laptop by 8:30 am on the morning of the presentation.

Posters
The Poster Session will be held in the Renaissance Ballroom on Tuesday afternoon. Participants are encouraged to hang posters upon arrival on Sunday or Monday. Poster numbers are indicated in the table on page 74.

Happy Hour
The hotel hosts a happy hour on Monday through Thursday evenings (5:30 pm – 7:30 pm) in the hotel lobby. Complimentary cocktails are available.

Agenda

Sunday, May 7, 2017	
Time	**Agenda Item**
01:00 pm - 06:00 pm	Arrival and Check-In
06:00 pm - 07:30 pm	**Welcome Reception**

Monday, May 8, 2017	
Time	**Agenda Item**
07:30 am - 08:30 am	**Breakfast**

WORKSHOP - Modeling and Data Dissemination Working Groups

08:30 am - 09:25 am	Modeling Case Study 1 Christian Forst (FluDyNeMo) and Richard Scheurmann (ViPR/IRD)
09:25 am - 10:20 am	Modeling Case Study 2 Katrina Waters (OMICS-LHV) and Chris Plaisier (OMICS4TB)
10:20 am - 10:30 am	**Refreshment Break**
10:30 am - 10:45 am	Data Dissemination Updates: PATRIC + OMICS4TB
10:45 am - 11:15 am	Data Dissemination Updates: ViPR/IRD + FluDyNeMo, FluOMICS, OMICS-LHV
11:15 am - 11:30 am	Data Dissemination Updates: EuPath + MaHPIC
11:30 am - 12:00 pm	Discussion of the potential for joint publication(s) on data and model sharing by the SysBio Centers and BRCs
12:00 pm - 01:00 pm	**Lunch**

OPENING REMARKS

01:00 pm - 01:05 pm	Welcome and Meeting Overview Yoshi Kawaoka (OMICS-LHV)
01:05 pm - 01:30 pm	NIAID Overview: Accomplishments and Future Directions of the Systems Biology Program Marciela DeGrace (NIAID)

Monday, May 8, 2017 (cont.)	
Time	**Agenda Item**
PLENARY SESSION 1 - Progress Overviews - Virology Centers	
01:30 pm - 02:00 pm	OMICS-LHV Yoshi Kawaoka
02:00 pm - 02:30 pm	Fluomics Adolfo Garcia-Sastre
02:30 pm - 03:00 pm	FluDyNeMo Elodie Ghedin
03:00 pm - 03:30 pm	**Refreshment Break**
PLENARY SESSION 2 - Progress Overviews - Malaria and TB Centers	
03:30 pm - 04:00 pm	MaHPIC Mary Galinski and Jessie Kissinger
04:00 pm - 04:30 pm	OMICS4TB David Sherman
04:30 pm - 04:45 pm	**Panel-Led Discussion for All Centers**
PLENARY SESSION 3 - Research Highlights: Extended Talks - Part 1 **Moderators: Andrew Patterson and Brian Brunk**	
04:45 pm - 05:05 pm	Trim14 Mediates the Innate Immune Response to SARS-CoV Infection Lisa Gralinski (OMICS-LHV)
05:05 pm - 05:25 pm	Systems-based analysis of RIG-I-dependent signaling identifies KHSRP as an inhibitor of RIG-I receptor activation Sumit Chanda (FluOMICS)
05:25 pm - 05:45 pm	A blood-based transcriptional signature in a novel murine tuberculosis model predicts risk of human tuberculosis progression Kevin Urdhal (OMICS4TB)
05:45 pm - 06:00 pm	**Panel-Led Discussion**
06:00 pm - 08:00 pm	**Dinner**

Tuesday, May 9, 2017	
Time	**Agenda Item**
07:30 am - 08:30 am	**Breakfast**

PLENARY SESSION 4 - Research Highlights: Extended Talks - Part 2
Moderators: Raul Andino and Michelle Schwalbe

08:30 am - 08:50 am	Glycomic Analysis of Host-Response Reveals New Potential Mechanisms Governing Severity Lara Mahal (FluDyNeMo)
08:50 am - 09:10 am	A TRIP into the Wilds of TB Pathogenesis Tige Rustad (OMICS4TB)
09:10 am - 09:30 am	The nuclear RNA exosome is co-opted to enhance host:viral RNA hybrids that propel influenza virus ribogenesis and infectivity Ivan Marazzi (FluOMICS)
09:30 am - 09:50 am	Global Mapping of Molecular Networks Revealed by Integrative Omics Analysis Highlights Potential Mechanisms in a Non-Human Primate Model of Malaria Mark Styczynski (MaHPIC)
09:50 am - 10:15 am	**Panel-Led Discussion**
10:15 am - 10:40 am	**Refreshment Break**

PLENARY SESSION 5 - Pilot, Supplement and Collaborative Project Highlights - Part 1
Moderators: Andy Pekosz and Michael Zhang

10:40 am - 10:55 am	Human Ebola Virus Disease Pathogenesis Revealed by Multi-Platform 'Omics Analysis Amie Eisfeld (OMICS-LHV)
10:55 am - 11:10 am	A Comparative Study of Molecular Responses to Influenza Infection in Mice, Ferrets and Humans Christian Forst (FluDyNeMo)
11:10 am - 11:25 am	A Novel Method for tracking Host Transcriptome Stability in the Context of an Active Viral Infection Kris White (FluOMICS)
11:25 am - 11:40 am	Multi-omics signatures of disease progression and protective immunity in RhCMV/MTB-vaccinated macaques after MTB challenge Daniel Zak (OMICS4TB)
11:40 am - 12:00 pm	**Panel-Led Discussion**
12:00 pm - 01:00 pm	**Lunch**

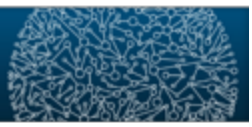
Tuesday, May 9, 2017 (cont.)	
Time	**Agenda Item**
PLENARY SESSION 6 - Research Highlights: Short Talks (selected from poster abstracts) **Moderators: Michael Gribskov and Shannon McWeeney**	
01:00 pm - 01:15 pm	Modeling host-parasite blood-stage interactions in malaria-infected rhesus macaques Luis Fonseca (MaHPIC)
01:15 pm - 01:30 pm	Lipid Signatures of Survival in Humans Infected with Ebola Virus during the Sierra Leone Outbreak Jennifer Kyle (OMICS-LHV)
01:30 pm - 01:45 pm	Microbial community composition and functions in the nasopharynx of patients infected with influenza viruses Lingdi Zhang (FluDyNeMo)
01:45 pm - 02:00 pm	Systematic Assessment of Drug Susceptibility in *Mycobacterium tuberculosis* Shuyi Ma (OMICS4TB)
02:00 pm - 02:15 pm	High-resolution metabolomics of plasma from human malaria cases reveals differences based on stage and species of the malaria parasite Regina Joice Cordy (MaHPIC)
FLASH PRESENTATIONS FROM POSTERS	
02:15 pm - 02:17 pm	Transcription factor induction in *Mycobacterium tuberculosis* identifies infection stress phenotypes Marshall J Davis (OMICS4TB)
02:17 pm - 02:19 pm	Single-cell analyses reveal the effects of cell cycle on influenza virus replication Chang Wang (FluDyNeMo)
02:19 pm - 02:21 pm	Identification and Analysis of Ebola Virus and Host Protein Interactions Danielle Westhoff-Smith (OMICS-LHV)
02:21 pm - 02:23 pm	A Multi-Omic Data Analysis Platform for Mining & Visualization of Host-Viral Response Lindsey Anderson (OMICS-LHV)
02:23 pm - 02:25 pm	Destroying the “Non-self”: Defining Influenza A Viral Endonuclease Mechanisms for Host Shutoff Kelsey Haas (FluOMICS)
02:25 pm - 02:27 pm	Analysis of Multi-omics Datasets Using Human Genome-Scale Metabolic Network Mingyuan Tian (OMICS-LHV)
02:27 pm - 02:29 pm	Effects of Influenza Infection on the Respiratory Mycobiome in Ferrets Yixuan Ma (FluDyNeMo)
02:29 pm - 02:31 pm	Modeling the Interplay between Viral Replication, Immune Response and Pathogenic Outcomes in the Mouse Lung Paritosh Pande (OMICS-LHV)

Tuesday, May 9, 2017 (cont.)	
Time	**Agenda Item**
FLASH PRESENTATIONS FROM POSTERS	
02:31 pm - 02:33 pm	RNAi Screen to Identify Ebola-Host PPI Partners Modulating Host Cell Responses Makoto Kuroda (OMICS-LHV)
02:33 pm - 02:35 pm	Universal full-length genomic amplification of Zika virus facilitates sequencing and reverse genetics Tsui-wen Chou (FluDyNeMo)
02:35 pm - 02:37 pm	A system-level transcriptional regulatory model reveals regulation underlying intracellular growth of *Mycobacterium tuberculosis* Eliza Peterson (OMICS4TB)
02:37 pm - 02:39 pm	Compounds Identified by a System Biology Approach Regulate the Pathogenicity of Influenza A Virus Shufang Fan (OMICS-LHV)
02:39 pm - 02:41 pm	Comprehensive Pan-Virus, Pan-OMICS Data Analysis for Discovery of Common and Unique Host Responses Hugh Mitchell (OMICS-LHV)
02:45 pm - 03:15 pm	**Panel-Led Discussion**
03:15 pm - 05:00 pm	**POSTER SESSION** **Refreshment Break**
PLENARY SESSION 7 - Modeling and Data Working Groups: Summary of Activities	
05:00 pm - 05:20 pm	Modeling Summary
05:20 pm - 05:40 pm	Data Dissemination Summary
05:40 pm - 06:00 pm	**Panel-Led Discussion**
06:00 pm - 08:00 pm	**Dinner**

Wednesday, May 10, 2017	
Time	**Agenda Item**
07:30 am - 08:30 am	**Breakfast**
PLENARY SESSION 8 - Pilot, Supplement and Collaborative Project Highlights - Part 2 **Moderators: Stephen Smale and Michael Gillette**	
08:30 am - 08:45 am	Host transcription and genome 3D structure perturbation by influenza A NS1 protein Sven Heinz (FluOMICS)
08:45 am - 09:00 am	Discovery of network motifs mediating the transition to dormancy in *Mycobacterium tuberculosis* Eliza Peterson (OMICS4TB)
09:00 am - 09:15 am	The Epigenetic Landscape during Coronavirus Infection Alexandra Schafer (OMICS-LHV)
09:15 am - 09:30 am	Deciphering influenza virus-host interactions using single-cell RNA sequencing Bin Zhou (FluDyNeMo)
09:30 am - 09:45 am	*Plasmodium knowlesi* – Macaque Infection Models and Systems Biology Mary Galinski (MaHPIC)
09:45 am - 10:00 am	**Panel-Led Discussion**
10:00 am - 10:20 am	**Refreshment Break**
PLENARY SESSION 9 - Research Highlights: Extended Talks - Part 3 **Moderators: Ishwar Chandramouliswaran and Richard Scheuermann**	
10:20 am - 10:40 am	Influenza virus networks of pathogenesis inferred from the integration of multiple large-scale datasets David Jimenez-Morales (FluOMICS)
10:40 am - 11:00 am	Multi-omics modeling of different clinical manifestation of *Plasmodium vivax* infection Shuzhao Li (MaHPIC)
11:00 am - 11:20 pm	Integration of Transcriptomic, Epigenetic and Glycomic Data in Ferret Models of Influenza Infection Reveals Coordinated Molecular Responses Elodie Ghedin (FluDyNeMo)
11:20 am - 11:35 am	**Panel-Led Discussion**
11:35 am - 12:00 pm	Final Remarks
12:00 pm - 1:00 pm	**Lunch**

Wednesday, May 10, 2017 (cont.)	
Time	**Agenda Item**
EXECUTIVE SESSION	
01:00 pm - 2:30 pm	NIAID personnel, Steering Committee members, and SB Center PIs and PMs
CLOSED SESSION	
02:30 pm - 4:00 pm	NIAID personnel and Steering Committee members

Plenary Session Abstracts



Note: Abstracts for each Plenary Session are listed in the order they appear in the agenda.

PLENARY SESSION 3
Presentation Type: Research Highlight Talk
Center: OmicsLHV

Trim14 Mediates the Innate Immune Response to SARS-CoV Infection

Lisa E. Gralinski[1], Vineet D. Menachery[1], Jacob Kocher[1], Michael S. Diamond[2], Yoshihiro Kawaoka[3] and Ralph S. Baric[1]

[1]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; [2]Washington University School of Medicine, St. Louis, MO 63110, USA; [3]Department of Pathobiological Sciences, University of Wisconsin-Madison, Madison, WI, 53711, USA

Identifying host factors important for viral pathogenesis may reveal new targets for antiviral treatment. Members of the tripartite motif (TRIM) family of proteins play important roles in innate immunity and antiviral defenses to a broad range of viruses. We identified TRIM14 as an interferon stimulated gene based in IFN-beta treatment of Calu3 airway epithelial cells. TRIM14 expression increased over time in Calu3 cells infected with Severe Acute Respiratory Syndrome (SARS) Coronavirus but decreased in cells infected with Middle East Respiratory Syndrome (MERS) Coronavirus. Expression is also upregulated in the lungs of SARS-CoV infected mice in a dose-dependent manner. We next examined the potential antiviral role of TRIM14 by examining its effect on SARS-CoV and MERS-CoV virus replication. siRNA knockdown of TRIM14 resulted in a modest decrease in SARS-CoV titer at 24 hours post infection but no change in MERS-CoV titers. To examine the role of this gene in and *in vivo* infection, CRISPR/Cas9-mediated TRIM14 knockout mice were generated on a C57BL/6J background and infected with SARS-CoV. Knockout mice were more susceptible to SARS-CoV infection as measured by increased weight loss and higher titers in the lung at days two and four post infection. Importantly, TRIM14 knockout mice had lower levels of MCP-1/CCL2, G-CSF and RANTES/CCL5 proteins in their lungs than C57BL/6J control mice at two days post infection. Together these data indicate that the host anti-viral response to SARS-CoV infection is impaired in the absence of TRIM14, possibly due to impaired NF-kB signaling.

PLENARY SESSION 3
Presentation Type: Research Highlight Talk
Center: FluOmics

Systems -based analysis of RIG -I-dependent signaling identifies KHSRP as an inhibitor of RIG -I receptor activation

Stephen Soonthornvacharin [1,2,3], Ariel Rodriguez -Frandsen1 [,2], Yingyao Zhou [4], Felipe Galvez [1,2], Nicholas J. Huffmast er[1,2], Shashank Tripathi [5,6], Vinod R. M. T. Balasubramaniam [5,6], Atsushi Inoue [1], Elisa de Castro [5,6], Hong Moulton [7], David A. Stein [7], María Teresa Sánchez -Aparicio [5,6], Paul D. De Jesus [1,2], Quy Nguyen [1,2], Renate König [1,8], Nevan J. Krogan [9], Adolfo García -Sastr e[5,6,10,] Sunnie M. Yoh [1,2]* and Sumit K. Chanda [1,2]*

[1]Immunity and Pathogenesis Program, Infectious and Inflammatory Disease Center, Sanford Burnham Prebys Medical Discovery Institute, 10901 North Torrey Pines Road, La Jolla, California 92037, USA; [2]The San Diego Center for Systems Biology (SDCSB), La Jolla, California 92093, USA; [3]Graduate School of Biomedical Sciences, Sanford Burnham Prebys Medical Discovery Institute, 10901 North Torrey Pines Road, La Jolla, California 92037, USA; [4]Genomics Institute of the Novartis Research Foundation, 10675 John Jay Hopkins Drive, S an Diego, California 92121, USA; [5]Department of Microbiology, Icahn School of Medicine at Mount Sinai, 1468 Madison Avenue , New York, New York 10029, USA; [6]Global Health and Emerging Pathogen Institute, Icahn School of Medicine at Mount Sinai, 1468 Madison Avenue , New York, New York 10029, USA; [7]Department of Biomedical Sciences, College of Veterinary Medicine,Oregon State University Corvallis, 450 SW 30th Street, Oregon 97331, USA; [8]Host -Path ogen - Interactions, Paul -Ehrlich -Institute, German Center for Infection Research (DZIF), Paul - Ehrlich -Stra ße 51 –59, 63225 Langen, Germany; [9]Department of Cellular and Molecular Pharmacology, University of California San Francisco, 1700 4th Street, Byers Hal l 308D, Box 2530, San Francisco, California 94158, U SA; [10]Department of Medicine, Division of Infectious Diseases, Icahn School of Medicine at Mount Sinai, 1468 Madison Avenue, New York, New York 10029, USA

Retinoic acid -inducible gene I (RIG -I) receptor recognizes 5′ -triphosphorylated RNA and triggers a signaling cascade that results in the induction of type -I interferon (IFN) - dependent responses. Its precise regulation represents a pivotal balance between antiviral defenses and autoimmunity. To elucidate the cellular cofactors that regulate RIG -I signaling, we performed two global RNA interference analyses to identify both positive and negative regulatory nodes operating on the signaling pathway during virus infection. These factors were integrated with e xperimentally and computationally derived interactome data to build a RIG -I protein interaction network. Our analysis revealed diverse cellular processes, including the unfolded protein response, Wnt signaling and RNA metabolism, as critical cellular compo nents governing innate responses to non -self RNA species. Importantly, we identified K -Homology Splicing Regulatory Protein (KHSRP) as a negative regulator of this pathway. We find that KHSRP associates with the regulatory domain of RIG -I to maintain the r eceptor in an inactive state and attenuate its sensing of viral RNA (vRNA). Consistent with increased RIG -I antiviral signaling in the absence of KHSRP, viral replication is reduced when KHSRP expression is knocked down both in vitro and in vivo. Taken tog ether, these data indicate that KHSRP functions as a checkpoint regulator of the innate immune response to pathogen challenge. The authors acknowledge support from the National Institutes of Health (U19 AI106754 and P50 GM085764).

PLENARY SESSION 3
Presentation Type: Research Highlight Talk
Center: Omics4TB

A blood-based transcriptional signature in a novel murine tuberculosis model predicts risk of human tuberculosis progression

Courtney Plumlee, Jared Delahaye, Sara Cohen, Benjamin Gern, Jackie Braun, Jessica Farrow-Johnson, Tige Rustad, David Sherman, Daniel Zak, and Kevin Urdahl

Center for Infectious Disease Research (formerly Seattle Biomedical Research Institute), Seattle, Washington

Tuberculosis (TB) is a highly heterogeneous human disease that develops in some, but not all, individuals who inhale aerosolized water droplets containing small numbers of *Mycobacterium tuberculosis* (Mtb). Using advanced machine-learning algorithms, we discovered a blood transcriptional signature that identifies Mtb-exposed individuals in the process of progressing to active TB up to 18 months before they exhibit clinical symptoms. This signature may help prevent TB disease by identifying high risk individuals who could benefit from early intervention, but is limited because it fails to identify ~30% of TB progressors and does not reveal insights into the diverse outcome-governing pathways within the Mtb-infected lung that underlie TB progression. To develop a tractable system that would enable blood-based signatures to be linked to mechanistic pathways occurring within the lung, we pioneered a novel murine TB model that recapitulates key aspects of human Mtb infection. We find that mice infected with a physiologic, ultra-low dose (ULD) (i.e., 1-3 CFUs) of aerosolized Mtb exhibit a broad range of outcomes, with bacterial burdens ranging from less than 10 to ~10^6 CFUs within individual lungs. Mice that contain Mtb exhibit Mtb well-circumscribed granulomatous structures that share many features with human Mtb granulomas. Furthermore, we have identified two distinct early blood transcriptional signatures that distinguish “controller” and “progressor” mice. Remarkably, these mouse-derived signatures predict TB risk in human populations as well as our previously identified human-derived signature, confirming the model’s relevance to human disease. This finding brings the unmatched tools and tractability of the mouse system to bear on questions central to TB pathogenesis in a clinically relevant model. This project was funded in part with funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, grant U19AI106761.

PLENARY SESSION 4
Presentation Type: Research Highlight Talk
Center: FluDyNeMo

Glycomic Analysis of Host-Response Reveals New Potential Mechanisms Governing Severity

Lauren Zhang[1], Brian Kasper[1], Sujeethraj Koppolu[1], Lawrence Meche[1], Christopher Vaiana[1], Daniel Heindel[1], Clayton Wiley[2], Stephanie Bissel[2], Chalise Bloom[3], Ted Ross[3], Bin Zhang[4], Elodie Ghedin[5] and Lara K. Mahal[1]

[1]Biomedical Research Institute, Department of Chemistry, New York University, NY; [2]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA; [3]Center for Vaccines and Immunology, University of Georgia, GA; [4]Department of Genetics and Genomic Sciences, Icahn Institute of Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, NY; [5]Center for Genomics & Systems Biology, Department of Biology, and College of Global Public Health, New York University, NY

Influenza is a major public health concern, causing an annual death rate of about 7.5% in the United States together with pneumonia (www.cdc.gov/flu/weekly/nchs.htm). Clinical outcomes of influenza vary significantly ranging from mild infections treatable at home to severe infections leading to hospitalization and death. The major molecular drivers of specific influenza outcomes are not known. Influenza infection is initiated in the upper airway through adhesion of viral hemagglutinin (HA) to specific glycan receptors found on the surface of the upper respiratory tract (α2,6-linked sialic acids in humans and ferrets). Propagation of the virus requires cleavage from sialic acid containing receptors through expression of a sialidase (neuraminidase), the current target of anti-viral therapies. Glycosylation patterns are not static, although there is limited data on how glycosylation changes post-infection. Using a ferret model, we identified specific glycan changes that map onto the pathology of influenza with our lectin microarray analysis. We discovered that high mannose levels vary significantly among uninfected, moderately, and severely infected ferret samples. This motif represents a new infection associated molecular pattern on host cells, which may guide immune responses via the lectin-mediated complement system (MBL2), leading to the immune storm associated with severity.

PLENARY SESSION 4
Presentation Type: Research Highlight Talk
Center: Omics4TB

A TRIP into the Wilds of TB Pathogenesis

Tige Rustad[1], Jessica Farrow-Johnson[1], Jenny Lohmiller[1], David Sherman[1,2]

[1]Center for Infectious Disease Research, Seattle, WA; [2]Department of Pathobiology, University of Washington, Seattle, Washington

M. tuberculosis adapts to the shifting micro-environmental stresses encountered during infection by triggering nuanced bacterial responses that are primarily mediated through a complex network of transcriptional changes. To design more effective TB therapies, we must understand the systems-scale consequences of these stress response networks under different conditions. One way to probe the function and control of these adaptations is by perturbing the transcription factors (TFs) that translate environmental signals into transcriptional responses that have evolve to generate adaptive phenotypes. To do this we have developed a novel **T**F- **r**egulon **i**nduction **p**henotypic (TRIP) screen to identify MTB regulons that influence clinically relevant bacterial phenotypes. In combination with a previously generated transcriptional regulatory network, TRIP screens have identified bacterial regulons critical for growth and survival in the mouse lung. To confirm that these phenotypes are specific to adaptation to the host we assessed the phenotype of these TFs in log phase in vitro culture and found twelve TFs with a log or greater larger defect in the mouse. We expect that integrating stress response phenotypes with TFs and their gene networks at a genome-scale will advance efforts to understand TB pathogenesis and develop faster acting interventions.

PLENARY SESSION 4
Presentation Type: Research Highlight Talk
Center: FluOmics

The nuclear RNA exosome is co-opted to enhance host:viral RNA hybrids that propel influenza virus ribogenesis and infectivity

Rialdi Alexander,[1] Hultquist Judd,[2] Jimenez Morales David,[2] Peralta Zuleyma,[3] Campisi Laura,[1] Fenouil Romain,[3] Moshkina Natasha,[1] Wang Zhen Zhen,[1] Laffleur Brice,[4] Michael Robyn,[2] Haas Kelsey,[2] Pefanis Evangelos,[5] Albrecht A. Randy,[1] Pache Lars,[6] Chanda Sumit,[6] Jen Joanna,[7], Ochando Jordi,[8] Byun Minji,[8] Basu Uttiya,[4] Garcia-Sastre Adolfo,[9] Krogan Nevan, van Bakel Harm, Marazzi Ivan[10]

[1]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, USA; [2]Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA 94158-2140, USA; [3]Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY 10029-6574, USA; [4]Department of Microbiology and Immunology, College of Physicians and Surgeons, Columbia University, New York, New York 10032, USA; [5]Regeneron Pharmaceuticals and Regeneron Genetics Center, Tarrytown, NY 10591, USA; [6]Burnham Institute for Medical Research, La Jolla, California, 92037; [7]Department of Neurology, University of California, Los Angeles, California, USA; [8]Department of Immunology, Icahn School of Medicine at Mount Sinai, New York, NY 10029; [9]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY 10029-6574, USA; Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029-6574, USA; Division of Infectious Diseases, Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, NY 10029-6574, USA; [10]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY 10029-6574, USA; Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029-6574, USA

The nuclear RNA exosome is an essential multi-subunit complex that controls RNA homeostasis. Congenital mutations in exosome genes are associated with neurodegenerative diseases. Here, we show that transient depletion of nuclear RNA exosome subunits in epithelial cells inhibits influenza virus replication. Similarly, viral biogenesis was suppressed in cells derived from mice with conditional ablation of the RNA exosome subunit Exosc3. Furthermore, patient-derived cells with a congenital EXOSC3 mutation were less susceptible to influenza virus infection. Using proteomics and next generation sequencing during infection, we show that the viral polymerase complex (PA, PB2, PB1) co-opts the nuclear RNA exosome complex and cellular RNAs en route to 3' end degradation. Mechanistically, the nuclear RNA exosome coordinates the initial steps of viral transcription with RNAPII at host promoters. Exosome deficiency uncouples chromatin targeting of the viral polymerase complex and the formation of cellular:viral RNA hybrids, which are essential RNA intermediates that license transcription of antisense genomic viral RNAs.

Overall, we discovered a critical nexus between an essential component of the influenza virus (polymerase) and an essential component of the cell (exosome), alteration of which leads to breakage of host-pathogen symmetry and a lose-lose scenario (viral impairment and neurodegeneration).

PLENARY SESSION 4
Presentation Type: Research Highlight Talk
Center: MaHPIC

Global Mapping of Molecular Networks Revealed by Integrative Omics Analysis Highlights Potential Mechanisms in a Non-Human Primate Model of Malaria

Mark Styczynksi

Malaria is a complex disease affecting multiple organs, and the systemic host response to the disease is not well-understood on a molecular level; hence, unveiling mechanisms across molecular layers is crucial for systematically characterizing pathology in affected organs. Here, we report an integrative analysis of transcriptomics, metabolomics, and lipidomics in peripheral blood (PB) and in bone marrow (BM) using a Non-Human Primate (NHP) animal model infected with *Plasmodium cynomolgi* (a model for *Plasmodium vivax* infection in humans) over 100 days. We developed a framework to integrate multiple longitudinal "omics" data types that accounts for potential nonlinear relationships among the heterogeneous data types using a mutual information-based machine learning approach. An atlas of tissue-specific (PB and BM) network modules of malaria was generated. These modules implicated the mTOR pathway and PD1 as being involved in the host response; correlation of these modules with immunological profiles and clinical traits identified associations to a distinct subset of immune cells. These results were corroborated by the analysis of transcriptional and metabolic data from an infection of the same host organism by a different malaria parasite, as well by the analysis of existing transcriptional data for human malarial infections. Importantly, some of the findings identified by this integrative approach were too weak to detect with analyses of the individual omics datasets, highlighting the utility of our integrative approach. To our knowledge, this is the first report of large-scale, longitudinal multi-omics experimental datasets generated using a NHP malaria model. This global multi-omics map of malaria pathology provides a unique resource for malaria study, and illustrates the power of integrative multi-omics analysis to characterize key processes in malaria.

PLENARY SESSION 5
Presentation Type: Pilot, Supplement or Collaborative Talk
Center: OmicsLHV

Human Ebola Virus Disease Pathogenesis Revealed by Multi-Platform 'Omics Analysis

Amie J. Eisfeld[1,‡], Peter J. Halfmann[1,‡], Jason P. Wendler[2,‡], Jennifer E. Kyle[2,‡], Kristin E. Burnum-Johnson[2,‡], Zuleyma Peralta[3,‡], Tadashi Maemura[4], Kevin B. Walters[1,†], Tokiko Watanabe[4], Satoshi Fukuyama[4], Makoto Yamashita[4], Jon M. Jacobs[2], Young-Mo Kim[2], Cameron P. Casey[2], Kelly G. Stratton[5], Bobbie-Jo Webb-Robertson[5], Marina A. Gritsenko[2], Matthew E. Monroe[2], Karl K. Weitz[2], Anil K. Shukla[2], Mingyuan Tian[6], Gabriele Neumann[1], Jennifer L. Reed[6], Harm van Bakel[3,7], Thomas O. Metz[2], Richard D. Smith[2], Katrina M. Waters[8], Alhaji N'jai[1,9], Foday Sahr[10], and Yoshihiro Kawaoka[1,4,11]

[1]Department of Pathobiological Sciences, University of Wisconsin-Madison, Madison, WI, 53711, USA; [2]Biological Sciences Division, Earth and Biological Sciences Directorate, Pacific Northwest National Laboratory, Richland, WA, 99352, USA; [3]Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York City, NY, 10029, USA; [4]Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo, 108-8639, Japan; [5]Applied Statistics & Computational Modeling, Pacific Northwest National Laboratory, Richland, WA 99352, USA; [6]Department of Chemical and Biological Engineering, University of Wisconsin-Madison, Madison, WI, 53706, USA; [7]Icahn Institute for Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, New York City, NY, 10029, USA; [8]Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA, 99352, USA; [9]Department of Biological Sciences, Fourah Bay College, College of Medicine & Allied Health Sciences, University of Sierra Leone, Freetown, Sierra Leone; [10]34th Regimental Military Hospital at Wilberforce, Freetown, Sierra Leone; [11]International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan; [‡]These authors contributed equally to the work presented in this manuscript; [†]Current affiliation: Drug Development Division, Southern Research, Frederick, MD, 21701, USA

Ebola virus (EBOV) causes high mortality in humans, yet the mechanisms of EBOV disease (EVD) pathogenesis remain ill-defined. To clarify how host responses contribute to EVD, we performed multi-platform 'omics analysis of peripheral blood mononuclear cells and plasma from EVD patients. Our results indicate that EVD signatures overlap with those of sepsis, suggest that pancreatic enzymes contribute to tissue damage in fatal EVD, and show that EBOV infection induces aberrant neutrophils whose activity may explain hallmarks of fatal EVD. Moreover, integrated biomarker prediction identified putative biomarkers from different platforms that differentiated survivors and fatalities early after infection. This work reveals novel insights into EVD pathogenesis, suggests an effective approach for biomarker identification, and provides an important community resource for further analysis of human EVD severity. This project was funded in part by an administrative supplement to grant U19AI106772, provided by the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

PLENARY SESSION 5
Presentation Type: Pilot, Supplement or Collaborative Talk
Center: FluDyNeMo

A Comparative Study of Molecular Responses to Influenza infection in Mice, Ferrets and Humans

Christian V. Forst[1], Minghui Wang[1], Won-min Song[1], Xianxiao Zhou[1], Adam Geber[6], Chalise Bloom[2], Ted M Ross[2], Adolfo García-Sastre[3,4,5], Elodie Ghedin[6], and Bin Zhang[1]

[1]Department of Genetics and Genomic Sciences, Icahn Institute of Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, NY 10029*, USA; [2]Center for Vaccines and Immunology, University of Georgia, GA. [3]Department of Microbiology, Icahn School of Medicine at Mount Sinai, NY; [4]Department of Medicine, Division of Infectious Diseases, Icahn School of Medicine at Mount Sinai, NY; [5]Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, NY. [6]Center for Genomics & Systems Biology, Department of Biology, and College of Global Public Health, New York University, NY*

Influenza A viruses infect a number of mammalian hosts, but not all respond similarly to the infection. To better understand the core molecular responses and identify unique aspects in each species, we performed a comparative study of the transcriptomic data derived from human blood cells, human primary epithelial cells, ferret blood and lung tissues, and mouse lung tissues infected with influenza A virus. Differential expression analysis revealed interferon signaling as the most common molecular pathway activated across all 3 organisms and different tissues. Not surprisingly, *Rig-I (DDX5), IFITM3, MX1/MX2, OASL,* and *OAS3* were among the most differentially expressed genes (DEGs) in all three organisms. Surprisingly, the inter-organismal immune system level response was better conserved than intra-organism response (i.e., when comparing different tissues from the same species). For example, we observed better overlap of DEGs between almost any combination of human blood, ferret lung and blood, as well as mouse lung data without human cell data, than with human cell data included. Immune functions, such as cytokine/chemokine, IFN, and toll like receptor signaling dominate most of the significant intersections of the DEG signatures. The DEGs unique to mouse were enriched for the cytochrome P450 detoxification pathway genes. Similarly, the human host seemed to possess unique antigen processing and presentation functions not observed in mice or ferrets. Highly conserved co-expression network modules across all organisms included well-known functions, such as cell-cycle, interferon signaling, protein metabolism, cellular localization, and cell activation. Only a limited number of small modules were found to be unique to each species, such as a human specific SOX3/VIK-1(ZNF655) module or a T-cell relevant semaphorin module in ferrets. However, a more detailed network analysis based on comparative node degree analysis and module differential connectivity presented a slightly different picture. Some coexpressed gene modules with potentially similar functions, based on gene set enrichment analysis as well as correlations to infection related traits, showed different connectivity between different organisms. For example, a module with a reciprocal DNA recombination function that was relevant for antigen repertoire generation, seemed important in ferrets but differently connected in human cells. In summary, our comparative multiscale analysis revealed molecular processes common to the 3 organisms as well as specific to each during influenza infection. Our cross-organism comparative study provides insights into the adaptability of the virus to different organisms and to the spectrum of host-defense mechanisms.

PLENARY SESSION 5
Presentation Type: Pilot, Supplement or Collaborative Talk
Center: FluOmics

A Novel Method for tracking Host Transcriptome Stability in the Context of an Active Viral Infection

Kris White, Pablo Abreu, Benjamin tenOever and Adolfo Garcia-Sastre

Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA.

The stability of the host transcriptome during a viral infection has been the subject of limited studies to date. The main obstacle being the lack of tools to probe this essential host function during an active viral infection. Actinomycin D is commonly used to separate mRNA transcription from mRNA stability for study, but this drug also inhibits viral infection. Here we describe for the first time a method which can track host mRNA stability on a transcriptomics scale during influenza A virus infection. To do this we transfected human A549 cells with purified polyA+ mRNA from a separate species, in this case Gallus Gallus (DF1 cells). Do to the fact that Gallus Gallus mRNA is not transcribed within A549 cells, this allowed us to track the abundance of the transfected Gallus Gallus transcriptome as a function of mRNA stability. Following mRNA transfection, we infected the A549 cells with H5N1 (Halo) influenza virus and tracked mRNA stability compared to mock infected cells via illumine sequencing. Our data analysis technique was developed to ensure that the significant homology between the species did not impair the conclusions. Raw reads were first aligned to the human genome. Those reads which aligned were discarded and those which did not were used in de novo genome guided assembly of the Gallus Gallus transcriptome. In our analysis we found that influenza A infection has a global stabilizing effect on mRNA, with 76.66% of mapped transcripts showing increased stability over time in infected cells compared to mock. We have also identified pathways in which mRNA stability is specifically targeted by influenza A virus.

PLENARY SESSION 5
Presentation Type: Pilot, Supplement or Collaborative Talk
Center: Omics4TB

Multi-omics signatures of disease progression and protective immunity in RhCMV/MTB-vaccinated macaques after MTB challenge: Supplement to OTB U19 AI106761

Daniel Zak[1], Smitha Shankar[1], Jackie Braun[1], Joe Valvo[1], Lynn Amon[1], Scott Hansen[2], Alan Aderem[1], Louis Picker[2]

[1]Center for Infectious Disease Research, Seattle, WA 98109; [2]Oregon Health and Science University, Portland, OR 97239

The immune responses that a vaccine must induce to protect against tuberculosis (TB) disease are presently unknown. Rhesus cytomegalovirus (RhCMV) has emerged as a novel vaccine vector capable of inducing long-lived mucosal effector memory T cell responses and has demonstrated protective efficacy against SIV and MTB in rigorous non-human primate (NHP) challenge models. In two supplements to the Omics for TB (OTB) program (U19 AI106761), we have undertaken multi-omics (transcriptomics, metabolomics, and proteomics) analysis of peripheral blood samples from two independent MTB challenge trials evaluating RhCMV vectors expressing MTB antigens (RhCMV/MTB). In both trials, RhCMV/MTB-vaccinated NHPs exhibited significantly reduced TB disease compared to controls. Multi-omics analysis of post-vaccination/pre-challenge samples from these trials has potential to reveal correlates of protective immunity against MTB and analysis of post-challenge samples can reveal correlates of TB disease progression and refinement of multi-omics signatures of risk identified from analysis of human cohorts. Currently, we have completed whole blood transcriptomics (RNA-Seq) analysis of the first study and plasma metabolomics (Metabolon DiscoveryHD4) and plasma proteomics (Somalogic SOMAscan) analyses of the 2nd trial. Transcriptomics of the 2nd trial and metabolomics of the 1st trial are underway (supported by independent programs). RNA-Seq of the 1st trial revealed a signature present on the day of MTB challenge that correlates with vaccine-induced protection against extrapulmonary TB, observed months later at necropsy. This signature involves genes in IL-1B, Oxidative stress, and Hypoxia pathways, with expression of PTX3, NALP3, IL-1B, NRF2, and HIF1A on the day of challenge being protective. Interferon response-associated genes, which we have shown are associated with risk for tuberculosis disease progression in humans, were absent from the protection signature. While we are currently blinded to the protection status of animals from the 2nd trial, analysis of proteomic and metabolomic responses of the unvaccinated NHPs to MTB challenge revealed responses consistent with signatures of TB disease risk that we identified in human cohorts. These include robust induction of circulating STAT1 and GAPDH proteins as well as cortisol and 3−ureidopropionate during TB progression. Ongoing analyses include cross-platform and cross-trial prediction and integration to identify robust signatures of protection and disease.

PLENARY SESSION 6
Presentation Type: Poster Talk
Center: MaHPIC

Modeling host-parasite blood-stage interactions in malaria-infected rhesus macaques

Luis L. Fonseca[1,2], Chester Joyner[1,3], The MaHPIC Consortium[1], Alberto Moreno[1,3], John W. Barnwell[1,4], Mary R. Galinski[1,3] and Eberhard O. Voit[1,2]

[1]Malaria Host–Pathogen Interaction Center, Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, Atlanta 30322,USA; [2]The Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta 30332, USA; [3]Division of Infectious Diseases, Department of Medicine, Emory University, Atlanta 30322,USA; [4]Malaria Branch, Division of Parasitic Diseases and Malaria, Centers for Disease Control and Prevention, Atlanta 30329, USA.

Malaria is globally the most deadly parasitic disease in humans, and the long-time coexistence with malaria has left indelible marks in the human genome that are the causes of a variety of genetic disorders. Although anemia is a common clinical complication of malaria, the root causes and mechanisms involved in the pathogenesis of malarial anemia are unclear and difficult to study in humans. Non-human primate (NHP) model systems enable the mechanistic study and quantification of underlying causative factors of malarial anemia, and particularly the onset of severe anemia.

Data obtained in the course of *Plasmodium coatneyi* and *P. cynomolgi* infections in malaria-naïve rhesus macaques (*Macaca mulatta*), were used to parameterize a discrete recursive model of the host-parasite blood-stage interactions. The model accounts for reticulocytes, red blood cells (RBCs), and infected RBCs and, using a mechanistic approach, allows predictions regarding the effects of varying levels of erythropoietic output, reticulocyte lifespan, RBC removal, and the immune response against the parasite.

Through optimization of the model parameters against the readouts of each individual macaque, we were able to infer the macaque's temporal response against the infection. Analysis of these results showed that: removal of RBCs occurs primarily by bystander effect and not by parasite invasion; in *P. cynomolgi*, reticulocytes were prematurely released before infection patency, which may be correlated with this parasite's preference for reticulocytes; and, under healthy conditions, rhesus reticulocytes have a lifespan in circulation of 24h, similar to humans.
We will show how the model has evolved and where it will go in the future.

This project has been funded in whole or in part with Federal funds from the National Institute of Allergy and Infectious Diseases; National Institutes of Health, Department of Health and Human Services [Contract No. HHSN272201200031C] and the National Center for Research Resources [ORIP/OD P51OD011132].

PLENARY SESSION 6
Presentation Type: Poster Talk
Center: OmicsLHV

Lipid Signatures of Survival in Humans Infected with Ebola Virus during the Sierra Leone Outbreak

Jennifer E. Kyle[1], Jason P. Wendler[1], Kristin E. Burnum-Johnson[1], Amie. J. Eisfeld[2], Pete J. Halfmann[2], Kelly G. Stratton[3], Cameron P. Casey[1], Karl K. Weitz[1], Richard D. Smith[1], Yoshihiro Kawaoka[2,4,5], Katrina M.Waters[1], Thomas O. Metz[1]

[1]Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA, 99352, USA; [2]Department of Pathobiological Sciences, University of Wisconsin-Madison, Madison, WI, 53711, USA; [3] Applied Statistics & Computational Modeling, Pacific Northwest National Laboratory, Richland, WA 99352, USA; [4]Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo, 108-8639, Japan; [5]International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

Much of what is known about EBOV pathogenicity has been elucidated from *in vitro* and *in vivo* models. This includes the role of lipids. Here we show plasma lipidomics of human EBOV disease (EVD) reveals striking trends in patients that survived compared with fatalities. Samples were obtained from 11 EVD survivors, 9 EVD fatalities, and 7 healthy Sierra Leonean volunteers. Plasma lipids were extracted and subjected to liquid chromatography-tandem mass spectrometry. A total of 423 lipids were identified and quantified across 4 lipid categories and 19 subclasses. Subclass trends include decreased diacylglycerophophocholine (PC), lyso-PC (LPC) and diacylglycerophophoinositol (PI) lipids among fatalities compared to survivors, whereas diacylglycerophophoethanolamine (PE), diacylglycerophophoserine (PS), and diacylglycerophophoglycerol (PG) were increased. Of the lipids that underwent the greatest change (> 5 log2 fold change (FC)), ceramides (CER) that contained 16:0 or 18:0 fatty acids increased in those that succumbed over EVD progression, whereas PIs containing 18:3 or 20:3 fatty acids increased in those that survived. Of the 41 lipids above this 5-FC cutoff, 50% were PC and LPCs, 9.5% CER, 9.5% PI, and 7% PS, with a p-value of at least 10E-4. Certain PS lipids (e.g., PS(18:0_18:1)) also revealed a trend for survivors vs fatalities.

Multi-organ dysfunction with EVD is well-known. Here we show the probable impact of liver dysfunction on the lipidome, along with other signatures of EVD pathogenesis. The decrease in PC and LPC suggests the liver is not actively secreting VLDL. The increase in CER containing 16:0 and 18:0 fatty acids over time indicates apoptosis is more prevalent in those that succumbed. Taken together these lipid signatures are also reminiscent of choline deficiency (resulting from nutrient deprivation); however, the apoptosis signature along with the increase in PSs and PEs may also result from virus pathogenesis. Lastly, PIs containing 18:3 or 20:3 fatty acids were noted to increase in survivors. 18:3 (n-6) is a precursor to 20:3 (n-6), which has anti-inflammatory and anti-proliferative roles. This study was funded by an administrative supplement to grant U19AI106772, provided by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (USA).

PLENARY SESSION 6
Presentation Type: Poster Talk
Center: FluDyNeMo

Microbial community composition and functions in the nasopharynx of patients infected with influenza viruses

Lingdi Zhang[1], Gabrielle M. Gussin[1], Adam Geber[1], Alan Twaddle[1], Lauren Lashua[1], Aubree Gordon[2], Richard Bonneau[1], Elodie Ghedin[1]

[1]Center for Genomics and Systems Biology, New York University, NY; [2]Department of Epidemiology, School of Public Health, University of Michigan, MI

The human upper respiratory tract is a reservoir of diverse microbial communities that include bacteria that can become pathogenic. Influenza virus infection can alter the respiratory tract microbiota and increase susceptibility to the colonization from these pathogenic bacteria. The emergence and spread of antibiotic resistance strains of respiratory bacteria is a significant global burden and contributes to mortality associated with influenza virus and bacteria co-infection. To determine how the microbiome of the upper respiratory tract is affected by influenza virus infections (nvH1N1, H3N2, or influenza B virus) and antibiotic treatment, we analyzed nasopharyngeal samples from index and contact cases part of an influenza household transmission study in Nicaragua. We used metatranscriptome analysis and 16S rRNA target gene sequencing to characterize the microbial community structure and gene expression profile. From the metatranscriptome data, we found that half of our subjects (15/35) carried *Streptococcus*, and that all samples had significant expression of different classes of antibiotic resistance genes. By unsupervised clustering of 16S rRNA data, samples partitioned into 3 community types and we identified several genes as differentially expressed between the 3 partitions. These included cardiolipin synthase, which is associated with daptomycin resistance, and phenicol resistance genes. We integrated host transcriptome and metatranscriptome data to understand the interactions between the host, the virus and the microbiome, as well as the role antibiotic resistance genes play in the interactions.

PLENARY SESSION 6
Presentation Type: Poster Talk and Poster
Center: Omics4TB

Systematic Assessment of Drug Susceptibility in *Mycobacterium tuberculosis*

Shuyi Ma[1,2], Tige R. Rustad[1,2], and David R. Sherman[1,2]

[1]Center for Infectious Disease Research, formerly Seattle Biomedical Research Institute, Seattle WA, USA; [2]Pathobiology Program, Department of Global Health, University of Washington, Seattle WA, USA

Designing optimal combination TB therapies requires a systems-level understanding of *M. tuberculosis* (MTB) response to drugs under different conditions. We probed the effects of perturbing of genetic context on MTB drug response by harnessing a genome-scale experimental screening strategy. We exposed a comprehensive pooled library of transcription factor (TF) overexpression mutant strains to 8 clinically relevant anti-TB agents and identified drug-specific sets of regulators that influence MTB susceptibility. In the case of isoniazid (INH), we identified susceptibility-altering TFs, which included a regulator previously known to confer decreased susceptibility to INH (Rv1909c, *furA*). We successfully validated another TF (Rv1963c, *mce3R*), which increased susceptibility to INH when overexpressed. To further probe mechanisms underlying *mce3R* hypersusceptibility, we combined known mce3R regulon information with MTB drug response transcriptomes and identified putative effector genes. We selected one of its repressed target genes, Rv1469, as potentially contributing to mce3R's increased INH susceptibility. Functional testing with the Rv1469 transposon knockout strain confirmed our hypothesis. Integrating drug response phenotypes with TFs and their gene networks at a genome-scale advances efforts to design TB therapies by enabling systematic interrogation of new mechanisms and informing rational design of complementary drug combinations.

PLENARY SESSION 6
Presentation Type: Poster Talk
Center: MaHPIC

High-resolution metabolomics of plasma from human malaria cases reveals differences based on stage and species of the malaria parasite

Cordy RJ[1], Banton S[2], Uppal K[2], Luvira V[3,4], Tran V[2], MaHPIC Consortium[1], Prachumsri JS[3,4], Patrapuvich R[3,4], Li S[2], Jones DP[2], Galinski MR[1,5]

[1]International Center for Malaria Research, Education and Development, Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, Atlanta, GA, USA; [2]Division of Pulmonary, Allergy and Critical Care Medicine, School of Medicine, Emory University, Atlanta, GA, USA; [3]Malaria Vivax Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [4]Department of Clinical of Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [5]Division of Infectious Diseases, Department of Medicine, Emory University, Atlanta, GA, USA; WUSTL

Malaria-causing parasites *Plasmodium falciparum* and *Plasmodium vivax* cause the highest morbidity and mortality of the *Plasmodium* species that infect humans. High-resolution metabolomics was performed on plasma samples from adults with *P. vivax*, *P. falciparum*, and those co-infected with the two species, alongside healthy and febrile non-malarial cases from the Kanchanaburi and Tak provinces of Thailand. Comparative analyses revealed specific metabolites and metabolic pathways that differentiated the groups. Global metabolic differences were observed in the plasma metabolome of malaria-infected individuals compared to non-malaria-infected individuals. These changes included increased levels of multiple classes of lipids in the plasma of individuals with malaria. Within the malaria cases, differences were also observed in the plasma of individuals harboring different developmental stages of the parasite (e.g. asexual and sexual stages). These findings suggest that specific processes involved in parasite development and in clinical disease progression are associated with global shifts in the host metabolic profile during malaria infection.

PLENARY SESSION 8
Presentation Type: Pilot, Supplement or Collaborative Talk
Center: FluOmics

Host transcription and genome 3D structure perturbation b y influenza A NS1 protein

Sven Heinz [1], Mathew Urbanowski [2], Max Chang [1], Michael Hayes [1], Lorane Texari [1], Alexander Rialdi [2], Kris White [2], Ivan Marazzi [2,3], Randolf Albrecht [2], Megan Shaw [2], Adolfo Garcia -Sastre [2,3,4], Christopher Benner [1]

[1]Department of Medicine, University of California, San Diego, La Jolla, CA 92093, USA; [2]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, New York, USA; [3]Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, New York, USA; [4]Department of Medicine, Division of Infectious Diseases, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

The spatial organization of the genome within the nucleus plays a crucial role in transcription and epigenetic reg ulation by organizing sets of regulatory elements into domains and contributing to cellular identity. Influenza A (IA) infection induces dramatic changes in genome 3D organization in the nuclei of host cells. Using Hi -C, a sequencing -based method that capt ures chromosome conformation, we found early -onset loss of genome structure following IA infection in both primary human tracheobronchial epithelial cells (HTBE) and monocyte -derived macrophages (MDM). In many cases CTCF -anchored loops were lost, resulting in dissolution of topologically associated domains (TADs). Transcriptome analysis by RNA -seq indicated that IA -induced loss of genome 3D interactions occurs where defects in transcription termination at 3' UTRs of highly transcribed genes leads to RNA pol ymerase II (Pol II) elongating hundreds of kilobases past the transcription termination sites observed in uninfected cells. Genome -wide RNA polymerase II location analysis by ChIP -Seq revealed striking dysregulation of all aspects of transcription, from in itiation to elongation to transcription termination, as early as 6 h post -infection.

The IA non -structural protein 1 (NS1) is known to inhibit transcript cleavage and polyadenylation by interacting with and inactivating the host factor CPSF30, the 30 kDa subunit of cleavage and polyadenylation specificity factor of the host cell. Infection of MDM with NS1 -deficient IA and transient short -term overexpression of NS1 protein in THP -1 cells established that NS1 is necessary and sufficient for the striking glob al effects of IA infection on transcription and host 3D genome structure. Transfection with NS1 variants bearing mutations that abolish its interaction with CPSF30 demonstrated that these properties of NS1 are mediated by its interactions with CPSF30. Our data indicate that NS1 compromises all stages of gene transcription of a large number of host genes, and that transcription through structural domain boundaries severely impacts host genome 3D structure and function. These results reinforce the potential o f targeting the NS1 -CPSF30 interaction as a promising strategy for pharmaceutical intervention during IA infection.

PLENARY SESSION 8
Presentation Type: Pilot, Supplement or Collaborative Talk
Center: Omics4TB

Discovery of network motifs mediating the transition to dormancy in *Mycobacterium tuberculosis*

Abrar Abidi[1], Eliza Peterson[1], Christopher Plaisier[1], Nitin Baliga[1]

[1]*Institute for Systems Biology, Seattle, WA*

Transcription factors (TFs) modulate the expression of their downstream target genes through the simple functions of activation or repression. Over time, evolution has joined these simple functions into new emergent behaviors by linking together TFs into sub-circuits known as network motifs. Presumably, these TF-TF interactions have evolved because they are capable of providing behaviors that are advantageous to the organism: increase (or decrease) of response times, filter noise, add robustness, or act as a switch between different cell states. The transition between cell states is critically important to *Mycobacterium tuberculosis* (MTB) during the transition into a dormant phenotype. This lead us to hypothesize that network motifs control this transition into dormancy through regulation of a temporal genetic program. Using the genome-wide ChIP-seq dataset that was generated by the Omics4TB group, we have discovered a certain class of network motif, known as the feedforward loop (FFL), that involves many genes which are known to be differentially expressed during hypoxia or aeration. Focusing on hypoxia-induced dormancy, we have identified a circuit model that controls this crucial cell state transition. We ran extensive permutation tests to confirm the significance of the network motifs and used our Environment and Gene Regulatory Influence Network (EGRIN) model to confirm conditional associations. Further validation of our circuit model requires high-resolution expression measurements during MTB's transition into and out of hypoxia. However, the prevailing experimental strategy for this transition has limited user control which restricts the resolution of the time-course data. To address this problem, we have designed a far more sophisticated and precise experimental setup that allows us to control the oxygen content and continuously monitor dissolved oxygen levels. This approach will allow us to collect the appropriate expression measurements that will then be compared to the theoretical model that is described by our network motif structure. Future studies will also involve the perturbation of critical nodes and observation of changes in cell states (expression profiles and dormancy phenotype) as a result of the re-wired regulatory circuit.

PLENARY SESSION 8
Presentation Type: Pilot, Supplement or Collaborative Talk
Center: OmicsLHV

The Epigenetic Landscape during Coronavirus Infection

Alexandra Schäfer[1], Vineet D. Menachery[1], Katrina M. Waters[2], Amy C. Sims[1], Timothy P. Sheahan[1], and Ralph S. Baric [1]

[1]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC; [2]Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA

Coronaviruses comprise a large group of emerging human and animal pathogens, including the highly pathogenic SARS-CoV and MERS-CoV strains. The molecular mechanisms regulating emerging coronavirus pathogenesis are complex and include virus-host interactions associated with entry, replication, egress and innate immune control. The current study was designed to identify host epigenetic processes that play a crucial role in controlling transcriptional regulatory networks that antagonize or promote SARS-CoV and MERS-CoV infection and pathogenesis.
More recently, a growing body of evidence supports the hypothesis that viruses, even lytic RNA viruses that replicate in the cytoplasm, have developed intricate, highly evolved, and well-coordinated processes that are designed to regulate the host epigenome, and control host innate immune antiviral defense processes, thereby promoting robust virus replication and pathogenesis. To test this hypothesis, we used ChIP-Seq to study the histone modification status of immune response genes during the early course of SARS-CoV and MERS-CoV infection. In addition to ChIP-Seq, we used MeDIP-Seq to evaluate the role of MHC expression after MERS-CoV infection, defining alternative yet complementary approaches for eliciting global gene expression control. We identified significant epigenetic signature differences regarding histone modification and STAT1-binding patterns that correlate with expression outcomes genome-wide. The study revealed that infection with SARS-CoV results in enrichment of H3K4me3 incorporation and STAT1 binding of ISG promoters. In contrast, MERS-CoV infection alters the histone modification patterns of ISG towards the repressive H3K27me3 and inhibits binding of STAT1, causing a repressed state of transcription and thereby inhibiting the expression of those genes early during infection. MeDIP-Seq analysis suggested that DNA methylation rather than histone modification plays a crucial role in MERS-CoV mediated antagonism of antigen presentation genes. By combining measures of epigenome reorganization with transcriptomic and proteomic datasets, we articulate a spatial-temporal data integration approach to identify regulatory genomic clusters and regions that play a crucial role in the host's innate immune response, thereby defining a new viral antagonism mechanism following emerging coronavirus infection.

This project is funded in whole with funds from the NIAID, NIH, grant U19AI106772.

PLENARY SESSION 8
Presentation Type: Pilot, Supplement or Collaborative Talk
Center: FluDyNeMo

Deciphering influenza virus -host interactions using single -cell RNA sequencing

Bin Zhou [1], Chang Wang [1], Erming Wang [2], Minghui Wang [2], Wissam Hanou [2], Tsui-wen Chou [1], Melissa Smith [2], Robert Sebra [2], Bin Zhang [2], and Elodie Ghedin [1,3]

[1]Center for Genomics and Systems Biology, Department of Biology, New York University; [2]Department of Genetics and Genomic Sciences, Icahn Institute of Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai; [3]College of Global Public Health, New York University, New York, New York

The interaction between the virus and host factors ultimately determines the outcome of an infection and the fate of the disease. At the single -cell level, whether an infection is productive or abortive depends both on the composition of the viral particle and the state of the cell it infects. While the genetic heterogeneity of the influenza virus population ("quasi-species") has been studied in the context of viral replication efficiency, the heterogeneity of the infected cells has rarely been analyzed. We infected human A549 cells and canine MDCK cells with multiple influenza A strains and compared virus yields at the single -cell level. The results showed that even within a relatively homogeneous cell line (compared to primary cultures), the viral yields are highly variable from cell to cell. To determine the underlying mechanisms for this variability, we used single -cell RNA sequencing to simultaneously quantify viral replication and profile the cellular transcriptome in both cell lines. Hundreds to thousands of differentially expressed genes were identified under various experimental conditions, some of which were undiscoverable by conventional population -level bulk RNA sequencing. We also performed network analyses, including weighted gene co-expression network analysis (WGCNA), to identify key drivers involved in influenza virus replication. By establishing a correlation between viral replication and the host transcriptome at a single -cell resolution, we were able to identify a) factors that support high vs. low virus replication in a single cell infection; b) shared and unique host factors related to the different replication competence of various viral strains; c) host responses that lead to the different capacity of each cell line (MDCK vs. A549) to support viral replication; and d) effects of infected cells on the neighboring uninfected cells. This study paves the way and assures a bright future of applying the single -cell sequencing strategy to animal models and primary human tissues to better understand disease progress and severity.

PLENARY SESSION 8
Presentation Type: Pilot, Supplement or Collaborative Talk
Center: MaHPIC

Plasmodium knowlesi – Macaque Infection Models and Systems Biology

Mary R. Galinski [1], Juan B. Gutierrez [2], Rabindra Tirouvanziam [1], Regina Joice Cordy [1], Ebru Karpuzoglu Belgin [1], Jeremy D. DeBarry [2], Mustafa Nural [2], Shuzhao Li [1], Mark Styczynski [3], Luis L. Fonseca [3], Eberhard O. Voit [3], Alberto Moreno [1], Jessica C. Kissinger [2] and the MaHPIC Consortium [1,2,3]

[1]Emory University, Atlanta, GA, USA; [2]University of Georgia, Athens, GA, USA; [3]Georgia Institute of Technology, Atlanta, GA, USA

The MaHPIC has developed collaborative systems biology experiments with the Defense Advanced Research Project Agency (DARPA) to study *Plasmodium knowlesi* infections in two species of macaques. *Plasmodium knowlesi* infection of *Macaca mulatta* (rhesus macaques) is highly virulent and typically deadly. In contrast, *P. knowlesi* infection in *Macaca fascicularis* (long-tailed macaques) is mild and typically self-controlled. In our recent infection studies, we have observed mild disease in *M. fascicularis*, with reduced hemoglobin levels and altered blood chemistry, followed by recovery without antimalarial treatment. Compared to rhesus macaques infected with *P. knowlesi*, these animals developed comparatively low parasitemias that plateaued and then resolved into a chronic state. The MaHPIC has planned to investigate the basic differences in the pathogenesis and immune responses in these hosts through the analysis and integration of multi-omic datasets generated over the course of longitudinal infections. The DARPA collaboration has enabled the addition of telemetry for real-time continuous physiological assessments of temperature, activity levels, heart rate and blood pressure, targeted proteomics and metabolomics, and pathology analysis of tissues upon necropsy. The DARPA goals are complementary and additive, being focused on identifying the underlying mechanisms of host resilience shown by the *M. fascicularis*, and aiming to develop host-directed therapies that can induce this resilience in individuals that would otherwise become severely ill. This project has been funded in whole or in part with Federal funds from the National Institute of Allergy and Infectious Diseases; National Institutes of Health, Department of Health and Human Services [Contract No. HHSN272201200031C], DARPA [Contract No. W911NF-16-C-0008], and the National Center for Research Resources [ORIP/OD P51OD011132].

PLENARY SESSION 9
Presentation Type: Research Highlight Talk
Center: FluOmics

Influenza virus networks of pathogenesis inferred from the integration of multiple large-scale datasets

David Jimenez-Morales[1,2], Judd Hultquist[2], John Von Dollen[1,2], Jeff Johnson[1,2], Hannes Braberg[1], and Nevan J Krogan[1,2]

[1]Department of Cellular and Molecular Pharmacology, University of California at San Francisco, San Francisco, California. [2]Gladstone Institute of Virology & Immunology, The J. David Gladstone Institutes, San Francisco, California

Influenza A virus (IAV) has a large social and economical impact worldwide. The lack of effective treatments demands an ambitious strategy to facilitate therapeutic intervention. With the goal of identifying and validating groups of genes involved in early IAV infection and pathogenesis, the FluOMICs consortium is employing a systems biology approach to compare three clinically relevant influenza virus strains that differ in virulence (H1N1, H3N2, H5N1), in both mice and humans. To enable the comparison and identify events that explain the diverse outcomes between IAV strains, we have generated a wide variety of large-scale -OMICS datasets, including transcriptomics, proteomics (protein abundance, phosphorylation, ubiquitination), and metabolomics (lipids and polar metabolites). Altogether, we quantified the functional activity of more than 6,000 genes/proteins at multiple time points during the course of infection, providing an unprecedented, detailed view of the infection landscape. For example, the phosphoproteome allowed us to infer a map of kinase activity unveiling key differences between strains. We found that JAK2, an important mediator for signaling events in both innate and adaptive immunity, increases activity during the course of H1N1 infection, while remaining invariant during H5N1 infection. In contrast, the RAF family of kinases involved in the regulation of cell growth and differentiation, increased their activity specifically during H5N1 infection. Moreover, functional analysis of the ubiquitome suggests more prominent ubiquitination-mediated degradation of immune signaling proteins during H5N1 infection. Finally, following a network module identification approach, we have developed an integrative analysis for the detection of active subnetworks that predict pathogenicity. This approach is based on the identification within the network of correlation patterns, densely connected regions, and clusters with significant changes in expression levels. As a result, the collection of predicted pathogenic modules represents an integrated -OMICS signature of pathogenicity. Ongoing perturbation experiments will refine the predicted signatures of pathogenesis.

This work is supported by the National Institutes of Health National Institute of Allergy and Infectious Diseases under grant U19 AI106754 (Flu-OMICs). The Modeling Core is directed by Nevan Krogan.

PLENARY SESSION 9
Presentation Type: Research Highlight Talk
Center: MaHPIC

Multi-omics modeling of different clinical manifestation of *Plasmodium vivax* infection

Luiz Gustavo Gardinassi[1,2], Regina Joice Cordy[2,3], Myriam Arévalo-Herrera[4,5], Sócrates Herrera[4,6], ViLinh Tran[1,2], Ken Liu[1], Dean P. Jones[1,2], Mary R. Galinski[2,3,7], Shuzhao Li[1,2]

1-Division of Pulmonary, Allergy and Critical Care Medicine, School of Medicine, Emory University, Atlanta, GA, USA; 2-Malaria Host–Pathogen Interaction Center, Atlanta, GA, USA; 3-International Center for Malaria Research, Education and Development, Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, Atlanta, GA, USA; 4-Malaria Vaccine and Drug Development Center (MVDC), Cali, Colombia; 5-Faculty of Health, Universidad del Valle, Cali, Colombia; 6-Caucaseco Scientific Research Center, Cali, Colombia; 7-Division of Infectious Diseases, Department of Medicine, Emory University, Atlanta, GA, USA

Peripheral blood is the most common access to understand the blood chemistry and immune response during an infection. Recent advancement in flow cytometry, transcriptomics and metabolomics brings rich information from the analysis of peripheral blood samples. Our group has developed computational tools in this area, including blood transcription modules for transcriptomics analysis and mummichog for metabolomics analysis. We combined these novel tools with a controlled human malaria infection trial, which consisted of patients that were naïve or semi-immune to *Plasmodium vivax* infection. The blood transcription modules revealed that the two groups of patients had different expression of interferon related genes, neutrophil and NK signatures at baseline; they went on with distinct kinetic profiles after infection. The metabolomics data also indicated different responses in amino acid pathways and lipid metabolism between the two groups. We have recently developed a new framework of “multiscale, multifactorial response network” (MMRN) to integrate different modalities in immune response. Current efforts are under way to integrate multi-omics data in malaria infection using this MMRN approach.

This project has been funded in whole or in part with Federal funds from the National Institute of Allergy and Infectious Diseases; National Institutes of Health, Department of Health and Human Services [Contract No. HHSN272201200031C] and the National Center for Research Resources [ORIP/OD P51OD011132].

PLENARY SESSION 9
Presentation Type: Research Highlight Talk
Center: FluDyNeMo

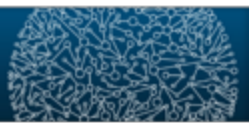
Integration of Transcriptomic, Epigenetic and Glycomic Data in Ferret Models of Influenza Infection Reveals Coordinated Molecular Responses

Christian V. Forst[1], Won-min Song[1], Minghui Wang[1], Bin Zhou[2], Adam Geber[2], Lauren Lashua[2], Xianxiao Zhou[1], Chalise Bloom[3], Tsui-Wen Chou[2], Guy Mason[2], Christopher Vaiana[4], Lawrence Meche[4], Brian Kasper[4], Clayton Wiley[5], Stephanie Bissel[5], Eric Schadt[1], Alyson Kelvin[6], Lara Mahal[4], Ted Ross[3], Bin Zhang[1$] and Elodie Ghedin[2]

[1]Department of Genetics and Genomic Sciences, Icahn Institute of Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, NY; [2]Center for Genomics & Systems Biology, Department of Biology, and College of Global Public Health, New York University, NY; [3]Center for Vaccines and Immunology, University of Georgia, GA; [4]Department of Chemistry, New York University, NY; [5]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA; [6]Immune Diagnostics and Research, and University Health Network, Toronto, ON

To build predictive models of influenza severity, we developed a large cohort of multi-Omics and physiological data from 48 ferrets in two distinct sampling studies of 24 ferrets each, including a sequential study following individual ferrets from pre-infection day until day 8, and a serial/longitudinal study, where different animals were infected and samples collected at different time points. For the serial study of both lower and upper lungs, ANOVA time-series analysis identified 8,366 mRNAs, 38 long non-coding RNAs (lncRNAs), 99 miRNAs, 6 glycans and 3 cytokines with significant responses across the time course. Most prominent molecular targets include well known host-defense genes such as *IFIT3*, *IFIT1*, *OAS1*, *Rig-I* (*DDX58*), *IFI44* and *MX1*/*MX2*, miRNA *mir*-1271 and lncRNA ENSMPUT00000020969 as well as snRNA *RN7SK*. Chemokines CXCL10, CCL2 and lectin Calsepa also showed differential expression. The Jonckheere trend analysis revealed that *APLNR*, *WNT5B*, *WIF1* and *PVRIG* were among the most significantly upregulated targets along the time-course while *PARP14*, *GCA*, *SAMD9L* and *TRIM5* were down-regulated. We further employed Multiscale Embedded Gene Co-expression Network Analysis (MEGENA) to identify highly coexpressed modules in the upper and lower lung locations. We rank ordered the modules in each location based on their response to the infections, i.e., the enrichment for the differentially expressed genes, microRNAs and lncRNAs. The top ranked modules in both locations were associated with the interferon signaling, cell-cycle and ribosome pathways. However, the lower lung was more responsive as it had far more network modules significantly enriched by the differentially expressed molecules (37.9%) than the upper lung (11.4%), indicating a higher infection rate in the lower lung. A similar big difference between upper and lower lung was observed based on the module-trait correlations, i.e., more modules in the lower lung were significantly correlated with traits than those in the upper lung. This study further identified well-known as well as promising novel key-drivers and pathways for further investigation in the fight against influenza infections.

Poster Abstracts



Notes

Poster abstracts have been organized into the following sections:

- Models and Biosignatures of Infectious Disease
- Single Cell Analyses
- Perturbation and Validation Studies
- New Tools
- Collaborations

Poster numbers for the Tuesday Poster Session are provided at the beginning of each poster abstract section, and all poster numbers are listed together on page 74.

Poster Abstracts

Models and Biosignatures of Infectious Disease

Poster	Presenter	Title
1	Tian	Analysis of Multi-omics Datasets Using Human Genome-Scale Metabolic Network
2	Cahalane	Metabolic Profiling of Influenza Infection
3	Pande	Modeling the Interplay between Viral Replication, Immune Response and Pathogenic Outcomes in the Mouse Lung
4	Mitchell	Comprehensive Pan-Virus, Pan-OMICS Data Analysis for Discovery of Common and Unique Host Responses
5	Peterson	A system-level transcriptional regulatory model reveals regulation underlying intracellular growth of *Mycobacterium tuberculosis*
6	Forst	Integrative Network Analysis Reveals Novel Sex Specific Key Processes and Drivers in Response to Influenza Infection and Vaccination
7	Zhang	Multiscale Systems Approaches Identify Key Signatures, Intrinsic Networks and Causal Regulators for Influenza Infection and Vaccination
8	Wang	Age- Dependent Molecular Responses to Influenza Vaccination
9	Yan	Multi-omic analysis of severity of infection in Macaca mulatta infected with *Plasmodium cynomolgi*. A multi-omic approach powered by the SKED framework.
10	Hultquist	Proteomic Profiling of Influenza A Virus Infection Reveals Strain-specific Rewiring of Host Cell Networks
11	Haas	Destroying the “Non-self”: Defining Influenza A Viral Endonuclease Mechanisms for Host Shutoff
12	Ziehr	Polysome Sequencing During Influenza Infection
13	Ma	Effects of Influenza Infection on the Respiratory Mycobiome in Ferrets
14	Ding	Role of the respiratory microbiome in influenza disease severity
15	Kelvin	Influenza strain specificity and microbiome function influences mortality in infants

Presentation Type: Flash Talk and Poster
Center: OmicsLHV
Section: Models and Biosignatures of Infectious Disease
Poster 1

Analysis of Multi -omics D atasets Using Human Genome -Scale Metabolic Network

Mingyuan Tian [1], Jennifer Reed [1]

[1]*Chemical and Biological Engineering, University of Wisconsin -Madison , US*

The reconstructed human genome -scale metabolic network is a powerful tool to analyze multi -omics dataset, including transcriptomic, proteomic, metabolomic and lipidomic data. The most recent human metabolic reconstruction, *iHsa* contains 2315 genes, 8263 reactions, and 5620 metabolites. We performed flux coupling a nalysis and metabolite coupling analysis on *iHsa* to identify metabolic modules containing reactions (and their associated genes) and/or metabolites . These modules contain metabolites and reactions where reaction flux or metabolite production changes in one reaction or metabolite imply similar flux or production changes in all other members of the module . Reactions in the module were then converted to genes via gene -protein -reaction associations in the reconstructed network.

We identified 330 gene modules, which account for 1050 genes . Each gene module includes genes whose associated reaction fluxes are coupled due to the topology of human genome -scale metabolic network . Functional enrichment analysis was then performed using these gene modules. Enrichment analysis for transcriptomic data in peripheral blood mononuclear cell s from Ebola patients found that pathways involved with heparan sulfate proteoglycan, a glycosaminoglycan utilized by filo viruses to attach to host cells, were significantly differentiall y expressed at the early stage of infection between survivors and non -survivors.

This project was funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, grant U19AI106772 .

Presentation Type: Poster
Center: FluOmics
Section: Models and Biosignatures of Infectious Disease
Poster 2

Metabolic Profiling of Influenza Infection

Celina Cahalane[1], David Jimenez-Morales[2], John VonDollen[2], Hannah Baumann[1], Matthew Urbanowksi[3], Megan Shaw[3], Giorgi Metreveli[3,4], Nevan Krogan[2], Adolfo Garcia-Sastre[3,4], Oswald Quehenberger[5], Edward Dennis[5], and Leah Shriver[1]

[1]Departments of Chemistry and Biology, University of Akron, Akron, Ohio; [2]Department of Cellular and Molecular Phamacology, University of California, San Francisco, San Francisco, California; [3]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, New York [4] Global Health & Emerging Pathogens Institute, Mount Sinai School of Medicine, New York, New York; [5] Department of Chemistry and Biochemistry, University of California, San Diego, San Diego, California

Influenza virus is a negative-strand RNA virus that infects cells of the respiratory tract and leads to significant morbidity and mortality. In order to identify more effective antiviral targets, a multi-laboratory OMICs collaboration (FluOMICs) was formed in order to determine virus-dependent host pathways that could be targeted to prevent influenza infection or replication. Metabolomics is an emerging systems biology approach that provides a global view of changes in endogenous small molecules including amino acids, nucleic acids, and glycolytic intermediates involved in cellular function and signaling. We analyzed changes in cellular metabolism associated with influenza infection with a global profiling platform that uses hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS). In addition, we have developed an in-house bioinformatics platform, Metabolomics Identification through Orthologous Datasets (MIDTOD), to increase the throughput of our platform and facilitate the integration of metabolomics with proteomics and genomics. This software processes metabolomic data using MSstats and the output, including metabolite identifications, are automatically linked to alterations in the genome and proteome. Using this approach, we have analyzed human cells and mice infected with the H1N1, H5N1, and H3N2 strains of influenza virus. We find both time-dependent and strain-dependent changes in glycerophospholipid metabolism, one carbon metabolism associated with methylation, and amino acid metabolism. Our data suggest that dynamic changes in host metabolism occur after infection and provide new targets for therapeutic intervention.

Presentation Type: Flash Talk and Poster
Center: OmicsLHV
Section: Models and Biosignatures of Infectious Disease
Poster 3

Modeling the Interplay between Viral Replication, Immune Response and Pathogenic Outcomes in the Mouse Lung

Paritosh Pande[1], Dennis Thomas[1], Hugh Mitchell[1], Jason Wendler[1], Jeremy Teuton[1], Ben Ziehr[2], Shiho Chiba[2], Amy Sims[3], Amie Eisfeld[2], and Katrina Waters[1]

[1]Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA, USA; [2]Influenza Research Institute, Department of Pathobiological Sciences, University of Wisconsin-Madison, Madison, WI, 53711, USA; [3]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Mathematical models describing the kinetics of viral infections are useful in furthering our understanding of the dynamics of viral infections by providing quantitative insights into the mechanisms of the infection and the interactions between the infection and the host immune response. The knowledge gleaned from such models can not only provide a quantitative understanding of the underlying biological phenomena but could also aid in predicting the differences in host-response to different strains, and to generate and test hypotheses about antiviral mechanisms. We are currently developing mechanistic mathematical models to study the dynamics of Influenza A virus (IAV) and Severe Acute Respiratory Syndrome virus (SARS-CoV) infection in an *in vivo* mouse model. We first compare the dynamics of IAV and SARS-CoV based on the biologically relevant parameters of a target cell-limited model of viral kinetics proposed by Baccam *et al*. To further investigate the specific role of various components of the immune system, and to elucidate the differences in the host-response to low pathogenic and high pathogenic strains of IAV, we report the development of a more detailed and complex computational model, which integrates inflammatory, humoral, and cellular response of the immune system. Additionally, we also propose to use machine learning approaches to identify the critical host factors associated with phenotype, leading towards the development of parsimonious models to better understand the individual sub-processes of the overall complex model. We believe that our modeling approach will not only provide quantitative insights into the known mechanisms of immune response to viral infections, but will also enable generation of novel hypotheses pertaining to the role of various key elements, such as, host genetic factors, immunosenescence, and coinfection in viral infection kinetics, which would in turn be used for designing further validation experiments. This study was funded by grant U19AI106772, provided by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (USA).

Presentation Type: Flash Talk and Poster
Center: OmicsLHV
Section: Models and Biosignatures of Infectious Disease
Poster 4

Comprehensive Pan -Virus, Pan -OMICS Data Analysis for Discovery of Common and Unique Host Responses

Hugh Mitchell [1], Jason Wendler [1], Amie Eisfeld [2], Amy Sims [3], Larissa Thackray [4], Michael Diamond [4], Ralph Baric [3], Yoshihiro Kawaoka [2,6], and Katrina Waters [1]

[1]Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA, USA; [2]Influenza Research Institute, Department of Pathobiological Sciences, University of Wisconsin-Madison School of Veterinary Medicine, Madison, Wisconsin, USA; [3]Departments of Epidemiology and Microbiology & Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA; [4]Departments of Medicine, Molecular Microbiology, Pathology & Immunology, Washington University School of Medicine, St. Louis, MO, USA; [6]Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

Highly pathogenic viruses are a serious public health threat due to their propensity for genetic variability and evolutionary drive to manipulate host pathways to their advantage. In order to determine the most effective intervention strategies, the host-response system must be thoroughly characterized and molecular networks used to identify targets which interrupt the pathogenesis program Comparing the host response of several lethal viruses in a side-by-side manner demonstrates the value of this strategy as the unique and common cellular programs required for virus replication and pathogenesis are revealed. We have collected data from Influenza virus, West Nile Virus, MERS coronavirus, SARS coronavirus and Ebola virus, from in vivo, ex vivo and in vitro biological systems, with transcriptomics, proteomics, metabolomics and lipidomics measurements performed in parallel. To identify important patterns in these large datasets, we employed a functional enrichment approach that ranks features by significance from differential expression statistics, rather than using an arbitrary significance cutoff, thereby increasing consistency in our analysis. By representing the results of this approach visually, we can identify critical host factors common to multiple viruses, which helps in identifying potential pan-virus therapy approaches. Additionally, the comparison allows the identification of unique responses to individual viruses whose importance is not evident from studying a single virus in isolation, thus providing insight into virus-specific host interactions. We also compared results from multiple OMICs data types in the same experimental condition, which allows for a more complete picture of the host response than is available from a single data type (e.g. transcriptomics) because multiple lines of evidence can be combined to increase confidence in our findings. Here we identify metabolic pathway perturbations that span multiple conditions and virus types and that are supported by multiple OMICs platforms. We also highlight responses unique to individual viruses that provides candidate critical host factors on a virus-specific level. This study was funded by grant U19AI106772, provided by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (USA).

Presentation Type: Flash Talk and Poster
Center: Omics4TB
Section: Models and Biosignatures of Infectious Disease
Poster 5

A system-level transcriptional regulatory model reveals regulation underlying intracellular growth of *Mycobacterium tuberculosis*

Eliza JR Peterson[1], Aaron Brooks[1], David J. Reiss[1], Wei-Ju Wu[1], Abrar Abidi[1], Amardeep Kaur[1], Nitin S. Baliga[1]

[1]*Institute for Systems Biology, Seattle, WA*

We present the most detailed transcriptional regulatory network model of the bacterium, *Mycobacterium tuberculosis* (MTB), responsible for today's deadliest infectious disease. MTB is an extraordinarily successful pathogen, infecting one-third of the world's population, and the success of MTB is largely due to adaptations that allow for persistence within the host. The regulatory mechanisms underlying these adaptations in MTB (and most microbes) are poorly understood. To address this problem, we have developed a generalized framework for inferring mechanistically detailed, and predictive Environment and Gene Regulatory Influence Network (**EGRIN**) models. In addition, for MTB we have developed an **EGRIN 2.0** model that is a statistical ensemble of multiple context-specific EGRIN models. The ensemble of EGRIN models was mined for co-regulated modules or 'corems' – recurring associations among genes, gene regulatory elements (GREs), transcription factors (TFs), environmental factors, and the environmental conditions affiliated with those associations. Importantly, the MTB EGRIN 2.0 model extends well beyond the standard static regulatory network model to predict dynamic, context-dependent regulation. Exploring the EGRIN 2.0 model, we identified corems with predicted regulation by MtrA (Rv3246c). MtrA is the response regulator of the essential two-component signal transduction system, MtrAB, which has not been extensively investigated. Model predictions of target genes and environmental conditions has led to further experiments (transcriptomics, ChIP-seq, MtrA perturbation strains, lipid analyses) that suggest MtrAB functions during intracellular growth, by regulating modifications of cell wall structure. Moreover, it is likely the phosphorylation status of MtrA is dependent on environmental conditions and is constitutively active under low pH. Future studies could identify small molecules that inhibit MtrA phosphorylation and thus inhibit intracellular growth of MTB.

Presentation Type: Poster
Center: FluDyNeMo
Section: Models and Biosignatures of Infectious Disease
Poster 6

Integrative Network Analysis Reveals Novel Sex Specific Key Processes and Drivers in Response to Influenza Infection and Vaccination

Christian V. Forst[1], Minghui Wang[1], Won-min Song[1], Yongzhong Zhao[1], Xianxiao Zhou[1], Andrew McKenzie[1], Igor Katsyv[1], Michael Schotsaert[2], Chi-Jene Chen[2], Rong Hai[2], Chalise E. Bloom[3], Lauren Lashua[4], Adam Geber[4], Bin Zhou[4], John Tsang[5,6], Andrew Kasarskis[1], Adolfo García-Sastre[2,7,8], Ted M Ross[3], Elodie Ghedin[4], Eric Schadt[1] and Bin Zhang[1]

[1]Department of Genetics and Genomic Sciences, Icahn Institute of Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, NY*; [2]Department of Microbiology, Icahn School of Medicine at Mount Sinai,* NY*; [3]Center for Vaccines and Immunology, University of Georgia, GA. [4]Center for Genomics & Systems Biology, Department of Biology, and College of Global Public Health, New York University, NY,; [5]Systems Genomics and Bioinformatics Unit, Laboratory of Systems Biology, National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD; [6]Trans-NIH Center for Human Immunology, Autoimmunity, and Inflammation (CHI), National Institutes of Health, Bethesda, MD; [7]Department of Medicine, Division of Infectious Diseases, Icahn School of Medicine at Mount Sinai,* NY*; [8]Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai,* NY

The immune response in women is generally more robust than in men. Consequently, women are often less compromised by infections and more responsive to vaccination. The exact mechanisms underlying women's privileged immune response are not well understood. In this study, we developed a multi-scale network analysis framework to decipher the difference in influenza infection and vaccination response between females and males. We integrated data from multiple experimental readouts, including transcription and immunological responses. We found vast sex differences at all levels, from physiological traits to gene expression and to functional gene networks. We revealed stronger and more specific responses to influenza infection and vaccination in females compared to males. For example, more than twice as many genes are significantly responding across the time-series in females compared to males. The unique molecular response in females involves specific adaptive immune system functions, including early B/T-cell activation that strongly associates with immune cell response and virus neutralization titers. In contrast, males show a more moderate innate immune response enriched for generic cellular processes due to later up-regulated immunoglobulins. We further identified sex-specific gene coexpression and regulatory networks and their key regulators as novel predictive biomarkers and putative targets for augmenting vaccine responses and/or for therapeutic intervention. Most top ranked gene co-expression modules in the female networks are related to the immune response while there are very few immune related modules in the male networks that are correlated to phenotypic traits. Key causal regulators of the sex-specific modules provide additional insight into the mechanisms underlying sex differences in the response to infection and vaccination. Therefore, this study depicts a global landscape of gender differences at the level of gene expression and gene regulation in response to influenza infection and vaccination.

Presentation Type: Poster
Center: FluDyNeMo
Section: Models and Biosignatures of Infectious Disease
Poster 7

Multiscale Systems Approaches Identify Key Signatures, Intrinsic Networks and Causal Regulators for Influenza Infection and Vaccination

Christian V. Forst[1], Won-min Song[1], Minghui Wang[1], Bin Zhou[2], Adam Geber[2], Lauren Lashua[2], Chalise Bloom[3], Tsui-Wen Chou[2], Guy Mason[2], Clayton Wiley[4], Stephanie Bissel[4], Alyson Kelvin[5], Ted Ross[3], Elodie Ghedin[2] and Bin Zhang[1]

[1]Department of Genetics and Genomic Sciences, Icahn Institute of Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, NY; [2]Center for Genomics & Systems Biology, Department of Biology, and College of Global Public Health, New York University, NY; [3]Center for Vaccines and Immunology, University of Georgia, GA; [4]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA; [5]Immune Diagnostics and Research, and University Health Network, Toronto, ON

Influenza infection activates a number of host defense pathways, including the innate and adaptive immune responses, and the induction of cytokines and activation of apoptosis. To decode the complexity of the molecular responses to influenza infection and vaccination, we developed multiscale systems biology approaches to model and validate high order molecular interactions and the signaling of host responses. We first developed a spectrum of distinct differential analysis techniques to identify molecular signatures. These include i) significant response analysis (SRA) and significant Jonckheere trend analysis (JTA) for single time-series datasets; and ii) differential response analysis (DRA) for 2 time series datasets under 2 conditions, such as infections with different viruses, during the temporal development of transcriptional or protein expression in response to infection. To further identify higher-order molecular interactions, we developed novel multiscale coexpression network analysis approaches including Multiscale Embedded Gene coExpression Network Analysis (MEGENA) and multiScale Weighted Interaction Network Analysis (SWINA) by integrating multi-resolution clustering, planar filtering and weighted coexpression techniques. To quantify the changes in molecular interactions between 2 scenarios, we developed differential correlation and network analyses. These novel systems biology approaches were applied to multiple influenza infection and vaccination cohorts to systematically identify key signatures, intrinsic networks and causal regulators of host responses to virus in multiple model systems. We identified the dedicator of cytokinesis 5 (*DOCK5*) as a novel host factor involved in viral replication. CRISPR/Cas9 knockout of *DOCK5* significantly reduced influenza virus replication, indicating that *DOCK5* is required in the viral life cycle. We also revealed fundamental gene network structures and key regulators in influenza vaccination, as well as age-dependent molecular responses to influenza vaccination. Such multiscale systems biology approaches are completely data-driven and present global and unbiased maps of regulatory relationships involving hundreds of thousands of interactions, with significantly improved power to uncover novel host-virus pathways and driver genes.

Presentation Type: Poster
Center: FluDyNeMo
Section: Models and Biosignatures of Infectious Disease
Poster 8

Age- Dependent Molecular Responses to Influenza Vaccination

Christian V. Forst[1*], Minghui Wang[1*], Won-min Song[1], Elodie Ghedin[2] and Bin Zhang[1]

[1]Department of Genetics and Genomic Sciences, Icahn Institute of Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, NY; [2]Center for Genomics & Systems Biology, Department of Biology, and College of Global Public Health, New York University, NY

The elderly are more prone to infectious diseases and experience greater disease severity. Vaccine response is also poor, providing suboptimal protection in an older population. To characterize the age-related molecular responses to vaccination, we employed our multiscale network analysis approach to several transcriptomic datasets for multi-year studies of influenza vaccine response. We separated participants into 2 groups including a group with subjects 40 years old or younger, and a group with participants of 60 years old or older. A number of differentially expressed genes (DEGs) between the “young” and “elderly” groups were typical immune response genes such as *STAT1, GBP1/2/4*, chemokine *CXCL10*, immunglobulins (*IGHA1, IGHM, IGL,* and *IGLL1*), complement regulatory factor *SERPING1*, FC fragment of IgG receptor 1b (*FCGR1B*), and a TNF receptor *TNFRSF17*. However, the two groups have a wide difference in the number of DEGs between the pre-vaccination time point and any other post-vaccination time point. The “young” group had 30 times more DEGs than the ”elderly” group. The most prominent DEGs specific to the young group included IFN responsive *PARP9*, *IFIT2/3*, ER-stress responding *FBXO6*, vesicle transporting *VAMP5*, and proteasomal genes (*PSME1*, *PSMB8/9*). We further performed gene coexpression network analysis to identify high-order modular structures of gene-gene interactions in each group and then rank ordered each set of modules based on their enrichment for the DEG signatures. The top ranked modules were conserved between the two groups and they were associated with inferferon signaling, cell-cycle, and mRNA processing, indicating that co-regulation is better conserved between the age groups. However, a number of functional modules were specific to each group. For example, modules associated with *ATP6V* mediated vacuolar trafficking, the spliceosome, and the *GAB1* signalosome, were exclusive to the young group. In summary, this study systematically identified age-dependent gene signatures, networks and key regulators in response to influenza vaccination and thus paves the way for developing high-performance predictive models of influenza vaccine response.

Presentation Type: Poster
Center: MaHPIC
Section: Models and Biosignatures of Infectious Disease
Poster 9

Multi-omic analysis of severity of infection in *Macaca mulatta* infected with *Plasmodium cynomolgi*. A multi-omic approach powered by the SKED framework.

Yi H. Yan [1], Eliza beth D. Trippe [1], Diego M. Moncada [2], Mary R. Galinski [3], Alberto Moreno [3], Jessica C. Kissinger [1], Rabindra Tirouvanziam [3], Juan B. Gutierrez [1], and the MaHPIC Consortium

[1]*University of Georgia,* [2]*Universidad del Quindio (Colombia),* [3]*Emory University*

Severe anemia accounts for a large portion of the complications associated with malaria. This phenomenon is multifactorial, and the mechanisms that cause it remain elusive. A multi-omic approach comprised of immunology, transcriptomics, metabolomics, lipidomics, and proteomics was used to study five *M. mulatta* infected with *P. cynomolgi*. This experiment resulted in one death, two severe infections, and 2 mild infections. The constraints of small cohorts, noisy and incomplete data, different sampling rates between platforms, and technical variability pose tremendous challenges to the integration of these data sets. Our group designed the SKED framework (Scientific Knowledge Extracted from Data) to simplify data harmonization, reproducibility of results, and reusability of analytic tools. Using data primitives (a small set of minimalistic data structures to which all data sets can be reduced), we were able to: (i) generate novel, reusable, and reproducible methods that aid in the detection of subtle signals usually ignored by traditional bioinformatics methods, and (ii) identify novel innate immunity mechanisms by which a malaria infection results in severe anemia. Particularly, we discovered that TLR3 and RIG-I pathways in macrophages and dendritic cells, conventionally linked to host antiviral responses, are upregulated in severe malaria. These pathways cross-talk, and their interaction had not been clearly implicated before in severe malaria. Associated with the activation of these pathways are changes in the protein effector proteins IL1, EP1, MX1, MX2, OAS1-3, and PML. In conclusion, the SKED framework allowed us to simplify the process of multi-omic data analysis, and fine-tune analytical methods to discern subtle signals that had been previously ignored. The biological knowledge obtained with these methods will advance knowledge both in the world of malaria, and in the field of quantitative biology. This project has been funded in whole or in part with Federal funds from the National Institute of Allergy and Infectious Diseases; National Institutes of Health, Department of Health and Human Services [Contract No. HHSN272201200031C] and the National Center for Research Resources [ORIP/OD P51OD011132].

Presentation Type: Poster
Center: FluOmics
Section: Models and Biosignatures of Infectious Disease
Poster 10

Proteomic Profiling of Influenza A Virus Infection Reveals Strain-specific Rewiring of Host Cell Networks

Judd F. Hultquist[1,2], David Jimenez-Morales[1,2], Michael M. McGregor[1,2], Kelsey Haas[1,2], Jeffrey Johnson[1,2], William Newton[1,2], Giorgi Metreveli[3,4], Matthew Urbanowski[3,4], Megan Shaw[3,4], Ivan Marazzi[3,4], Randy A. Albrecht[3,4], Adolfo Garcia-Sastre[3,4], and Nevan J. Krogan[1,2]

[1]Quantitative Biosciences Institute, Department of Cellular & Molecular Pharmacology, University of California – San Francisco, San Francisco, CA 94158, USA; [2]J. David Gladstone Institutes, San Francisco, CA 94158, USA; [3]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA; [4]Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

Despite their shared ancestry, different strains of influenza A virus (IAV) display extraordinary divergence in both their replicative potential and pathogenic outcome. We hypothesize that this diversity and the propensity to manifest as severe disease is linked directly to differential engagement of the host response during early infection. To test this hypothesis, we employed a multi-OMICs approach to map changes to the host cell transcriptome, proteome, and metabolome during the course of infection with three different IAV strains both *in vivo* and *ex vivo*. Critically, we found that while changes to host gene expression were highly correlated between strains during early infection, changes in post-translational modifications were highly strain specific. H5N1 IAV, for example, resulted in the specific dephosphorylation of several small GTPase signaling modules. Predictive modeling of phosphorylated peptide motifs mapped these differences to a few specific kinases, whose activity changes in a strain specific manner during the course of infection. While changes to the host ubiquitome are similarly as strain-specific, these changes cannot yet be mapped back to the activity of specific E3 ligases. However, concurrent measurement of mRNA and protein abundance has allowed for the identification of key cellular targets marked for degradation. Furthermore, protein interaction mapping across the proteome of these three viruses suggests critical host-pathogen interfaces that may initiate these downstream processes and ultimately dictate both infection and pathogenesis. As proteomic capabilities continue to evolve, we believe that these approaches will be critical for understanding the molecular mechanisms of disease, identifying actionable biomarkers, and developing the next generation of small molecule therapeutics.

This work is supported by the National Institutes of Health National Institute of Allergy and Infectious Diseases under grant U19 AI106754 (Flu-OMICs) to A.G.; the Proteomics Core is directed by N.K.

Presentation Type: Flash Talk and Poster
Center: FluOmics
Section: Models and Biosignatures of Infectious Disease
Poster 11

Destroying the "Non-self": Defining Influenza A Viral Endonuclease Mechanisms for Host Shutoff

Kelsey Haas, Michael McGregor, David Jimenez-Morales, Judd Hultquist and Nevan Krogan

Department of Cellular & Molecular Pharmacology, University of California – San Francisco; Quantitative Biosciences Institute, University of California – San Francisco; J. David Gladstone Institutes, San Francisco, California

Viruses cannot replicate outside of a host cell and are efficient at infecting and converting cells into virus-producing factories. Influenza A Virus (IAV), a commonly encountered respiratory virus, shuts down host gene transcription and translation to prioritize viral gene translation. The resulting accumulation of viral proteins and global reduction of host proteins is termed host shutoff. One IAV protein that mediates host shutoff is viral endonuclease, PA-X. In infected cells, PA-X selectively reduces host mRNA levels while viral mRNA levels remain high. It is unclear how PA-X discriminates between host and viral mRNAs to target host mRNA for degradation. We hypothesize that PA-X selectively targets host mRNA by interacting with host transcription machinery and mRNA processing factors. To determine the host factors that interact with PA-X, we adopted a two-pronged systems biology approach relying on (1) quantitative functional genomics to identify functional host interactors and (2) proteomics to identify physical interactors of PA-X. We found a subset of transcription, mRNA processing and mRNA decay proteins that are functional and physical interactors of PA-X. We are validating the host factors' role in PA-X mediated host shutoff using a high-throughput mRNA degradation assay in IAV-infected cells. Understanding how PA-X discerns host and viral mRNA can provide deeper insight into IAV pathogenicity, facilitate development of novel antiviral therapeutics, and highlight key themes for recognizing "self" and "non-self" molecules. This work is supported by the National Science Foundation under grant 1650113 to K.H. and the National Institutes of Health National Institute of Allergy and Infectious Diseases under grant U19 AI106754 to N.K.

Presentation Type: Poster
Center: OmicsLHV
Section: Models and Biosignatures of Infectious Disease
Poster 12

Polysome Sequencing During Influenza Infection

Benjamin Ziehr[1], Amie J. Eisfeld[1], Yoshihiro Kawaoka[1,2,3,4]

[1]*Influenza Research Institute, Department of Pathobiological Sciences, University of Wisconsin-Madison School of Veterinary Medicine, Madison, Wisconsin;* [2]*Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan;* [3]*Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan;* [4]*International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan.*

During viral infections mRNA translation is key in determining whether viral replication or the innate immune response will be successful. Current omics technologies interrogate either end of mRNA translation, through microarrays or proteomics. However, relying on mRNA and protein abundance misses a critical level of control, mRNA translation, as mRNA abundance doesn't always correlate with protein abundance. To define the complement of mRNAs undergoing translation during influenza infection we performed polysome fractionation followed by deep sequencing, polysome fractionation splits mRNAs based on their level of translation. The extent of translation for an mRNA is dependent on the number of ribosomes bound to the mRNA. mRNAs bound by few ribosomes are considered to be in monosomes, while mRNAs bound by many ribosomes and translated the most are associated with polysomes. To determine the efficiency of mRNA translation for each mRNA observed by deep sequencing we measured the distribution of that mRNA between monosomes and polysomes relative to its cytoplasmic abundance. The resulting ratio represents the level of translation for each mRNA or its Translation Efficiency (TE). This approach yields not only sequencing data on mRNA splicing, transcription start site usage and total abundance, but also how that data correlates to the efficiency of mRNA translation for each mRNA. Comparing the TE of mRNAs from mock and influenza infected cells provides a method to investigate the effect of influenza infection on the level of translation for every cellular mRNA. With this data we hope to answer questions related to the extent of host shut off during influenza infection and which if any host mRNAs are selected for increased mRNA translation during infection. Interestingly, we also observed significant changes in which mRNA isoforms are expressed during infection, specifically for mRNAs related to the immune response. Leveraging mRNA sequencing technology to interrogate the efficiency of mRNA translation provides a unique vantage point to observe how viral infection alters global mRNA translation.

This project is funded in whole with funds from NIAID, NIH, grant U19AI106772.

Presentation Type: Flash Talk and Poster
Center: FluDyNeMo
Section: Models and Biosignatures of Infectious Disease
Poster 13

Effects of Influenza Infection on the Respiratory Mycobiome in Ferrets

Yixuan Ma[1], Tao Ding[1], Adam Geber[1], Lauren Lashua[1], Alan Twaddle[1], Chalise E. Bloom[2], Stephanie Bissel[3], Clayton Wiley[3], Ted Ross[2], Minghui Wang[4], Bin Zhang[4], Elodie Ghedin[1]

[1]Center for Genomics and Systems Biology, New York University, NY; [2]Center for Vaccines and Immunology, University of Georgia, GA; [3]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA; [4]Department of Genetics and Genomic Sciences, Icahn Institute of Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, NY

The fungal composition—also called the mycobiome—in the respiratory tract has been associated with health and disease due to its interactions with the bacterial population and the host. We used a ferret model of influenza infection to survey the changes that occur in the influenza-associated respiratory mycobiome over the course of the infection. Nasal washes and bronchoalveolar lavages (BAL) were collected prior to and throughout infection. Target gene sequencing of the internal transcribed spacer (ITS) region was used to profile the mycobiome composition. Our analysis shows that the respiratory mycobiota changed significantly over the course of the infection with fungal features associated with differences pre-infection and post-infection. We also observed differences in the composition between the BAL and nasal washes, reflective of upper respiratory and lower respiratory tract variations. We evaluated the association between the mycobiome and disease severity based on physiological and pathological scores. This research contributes to a broader knowledge of the respiratory microbial environment in the context of a virus infection.

Presentation Type: Poster
Center: FluDyNeMo
Section: Models and Biosignatures of Infectious Disease
Poster 14

Role of the respiratory microbiome in influenza disease severity

Tao Ding[1], Yixuan Ma[1], Adam Geber[1], Lauren Lashua[1], Alan Twaddle[1], Chalise E. Bloom[2], Stephanie Bissel[3], Clayton Wiley[3], Ted Ross[2], Minghui Wang[4], Bin Zhang[4], Elodie Ghedin[1]

[1]Center for Genomics and Systems Biology, New York University, NY; [2]Center for Vaccines and Immunology, University of Georgia, GA; [3]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA; [4]Department of Genetics and Genomic Sciences, Icahn Institute of Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, NY

Severe cases of influenza infection are often associated with secondary bacterial co-infections with opportunistic pathogens, such as *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes*. To understand the role of the respiratory microbiome in determining disease severity in influenza, we characterized the microbiota in respiratory samples from ferrets infected with nvH1N1. We collected samples pre-infection and at multiple time points post-infection for two sets of experiments. In one experiment nasal washes were collected longitudinally for the same ferrets (N=33); in the second design animals were sacrificed at each time point (N=48) so that bronchoalveolar lavages (BAL), lung tissue, as well as nasal washes were collected. Target gene sequencing of the 16S rRNA V4 hypervariable region and of the internal transcribed spacer 1 (ITS1) was performed to profile the bacterial and fungal compositions, respectively. Total RNA extracted from lung tissue was also used for metatranscriptome sequencing to profile microbial gene expression. Our analysis shows that there was, as expected, drastic modulation of the respiratory microbiota over the course of the infection, with a decrease in diversity. More importantly, we observed a significant difference in community structure before infection between ferrets who developed severe disease and those with mild disease, indicating that the microbiota can potentially be predictive of disease severity. We identified specific bacteria and fungi that were characteristic of these differences and were key species in cross-kingdom microbial interaction networks. Using the metatranscriptome data we identified co-expression modules that consisted of host and microbial genes, providing further evidence of the association between microbial functions and the host immune response to the viral infection. This study integrates different microbiome data layers and provides new insight into the modeling of disease severity in influenza infection.

Presentation Type: Poster
Center: FluDyNeMo
Section: Models and Biosignatures of Infectious Disease
Poster 15

Influenza strain specificity and microbiome function influences mortality in infants

Alyson A. Kelvin[1], Adnan Khan[1], David Kelvin[1], Luoling Xu[1], Amber Farooqui[1]

[1]Division of Experimental Therapeutics, Toronto General Hospital Research Institute, University Health Network, Toronto, Ontario, Canada; Institute of Medical Science, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada

Infants are highly susceptible to severe respiratory disease following influenza virus infection but the mechanisms of disease severity and the role of the microbiome are poorly understood. Previously, we developed a mother-infant dyad model using ferrets to explore the host-pathogen dynamics of influenza infection in infants and their mothers and to understand influenza pathogenesis in the infant age group. We found that infection with pandemic 2009 H1N1 virus in infant ferrets led to severe disease and mortality as well as viral transmission to the mother causing infection in her respiratory tract and her mammary glands. Here we investigate the interplay of the microbiome and the influenza virus with the aim of uncovering mechanisms leading to mortality and severe disease in the infant age group. Metagenomic analysis showed differential changes in microbial species in infant ferret lungs compared to those in adults during 2009 H1N1 infection. Infants had higher frequencies of staphylococcus species whereas adults had increased streptococcus species after infection suggesting a role for the host microbiome in influenza severity. We went on to further investigate the role of the microbiome during influenza infection in infants by treating infant ferrets with the antibiotic clavamox (amoxicillin derivative) to manipulate the resident microbial species. We then infected clavamox treated infant ferrets with an influenza B virus, B/Brisbane/60/2008 (B/Bris), at (10^6 TCID50). We have previously shown that B/Bris causes a mild disease in ferrets compared to pandemic 2009 H1N1 strains. During B/Bris infection untreated infants had minimal weight loss and small temperature increases. Moreover, untreated infants regained a typical growth curve within days of inoculation and had 100% survival at the end of the study (day 14 post-infection). Infants treated with clavamox and infected with the same inoculum of B/Bris developed severe disease and 60% of treated-infected infants a reached weight cut-off or succumbed to disease. We are currently analyzing the microbial changes in concert with host transcriptome analysis of immune responses to dissect the role of microbial species in influenza pathogenesis in infants. These results suggest a role of the microbiome in influenza disease severity and mortality in infants.

Poster Abstracts

Single Cell Analyses

Poster	Presenter	Title
16	Wang	Single-cell analyses reveal the effects of cell cycle on influenza virus replication
17	Sherman	Development of Parallel Microscopic Determination of Fitness; a time-lapse microscopy platform for Mycobacterium tuberculosis
18	Bissel	In situ localization and distribution of differentially expressed innate immune genes during influenza infection in ferret lungs
19	Wang	Analysis of in vivo dynamics of H5N1 influenza virus infection in mice using fluorescent reporter viruses

Presentation Type: Flash Talk and Poster
Center: FluDyNeMo
Section: Single Cell Analyses
Poster 16

Single-cell analyses reveal the effects of cell cycle on influenza virus replication

Chang Wang[1], Bin Zhou[1] and Elodie Ghedin[1,2]

[1]Center for Genomics and Systems Biology, Department of Biology, New York University, [2]College of Global Public Health, New York University, New York, New York 10003, USA

Replication of the influenza virus relies on numerous cellular functions that are related to the host cell cycle. Systematic yeast two-hybrid arrays, RNA interference screenings, and biochemical assays have suggested a link between influenza virus replication and host cell cycle regulation. Although population heterogeneity, including cell-to-cell variation due to different phases of the cell cycle, may have confounded the detection and interpretation of the observation, results from these population-level analyses warranted an in-depth investigation of cellular mechanisms associated with cell cycle control upon viral infection. Fast-evolving single-cell technologies have enabled the assessment of gene expression with respect to different cell states. A recent single-cell viral replication study suggested independence between the host cell cycle and virus replication due to the absence of a correlation between cell size and virus titers. However, without gene expression profiling and identification of cell states, the relationship between virus replication and host cell cycle at the single-cell level remains elusive. Here we performed single-cell transcriptome profiling of individual cells to elucidate the disruption of the host cell cycle in response to viral exposure in the early infection stage. We quantified virus replication and host response in parallel by using A549 cells infected with two influenza A virus strains (PR8 and H1N1pdm09). Differential expression analyses and co-expression network reconstruction identified several cell-cycle related genes that control phase transitions in the host cell and are involved in the cellular response to infection. The regulated expression of some of these genes with respect to virus replication are likely to be mediated by other genes that are involved in homeostatic and metabolic regulation, and represent an indirect regulatory link to the cell cycle. Results from this study allow a better understanding of the host-virus interaction, and the casual relationship between virus replication and cell cycles.

Presentation Type: Poster
Center: Omics4TB
Section: Single Cell Analyses
Poster 17

Development of Parallel Microscopic Determination of Fitness; a time-lapse microscopy platform for *Mycobacterium tuberculosis*

Magdalena Donczew, Kyle Minch, Bailey Marshall, and David R. Sherman

Center for Infectious Disease Research, Seattle, WA, 98109

With 10.4 million new cases of active disease annually and 30% of the world latently infected, tuberculosis (TB) disease is a major public health crisis and, despite decades of research, treatment options remain suboptimal. To augment current laboratory approaches to TB research, we developed a new time-lapse microscopy platform called Parallel Microscopic Determination of Fitness (PMDF). PMDF allows us to assay hundreds to thousands of individual MTB microcolonies per experiment by tracking growth of individual cells. In each experiment we are able to measure median growth rate, death rate and the extent of heterogeneity of response across the population. To better understand MTB responses to drug treatments we treated cells with a range of concentrations of anti-tubercular agents. Using this approach, we have identified drug-and strain-specific bacterial behaviors that are providing insight into important MTB physiological responses.

Presentation Type: Poster
Center: FluDyNeMo
Section: Single Cell Analyses
Poster 18

In situ localization and distribution of differentially expressed innate immune genes during influenza infection in ferret lungs

Stephanie J Bissel[1,3], Guoji Wang[1], Chalise E Bloom[2], Ted M Ross[2,3], Clayton A Wiley[1,3]

[1] Department of Pathology, University of Pittsburgh, Pittsburgh, PA; [2] Center for Vaccines and Immunology and Department of Infectious Diseases, University of Georgia, Athens, GA; [3]FluDyNeMo Center

Influenza infection triggers an innate immune response in ferret lung tissue. To probe abundance, cell type and regional distribution of innate immune gene expression during infection, lungs from newly weaned and young adult ferrets infected with pandemic H1N1 2009 influenza A virus were probed to visualize RNA *in situ* at a single cell level. This approach provides morphological context of dynamic gene expression changes occurring over the course of influenza infection (0, 1, 3, 5, 8 and 14 days post-infection) in the context of age and severity of disease. Differentially expressed innate immune genes were chosen based on published gene expression profiles. Gene expression will be correlated with distribution of virus. Differential clinical outcome to influenza infection between young adult and newly weaned ferrets can be related to differential local innate immune response. This project was funded in part with funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, grant U01AI111598.

Presentation Type: Poster
Center: FluOmics
Section: Single Cell Analyses
Poster 19

Analysis of in vivo dynamics of H5N1 influenza virus infection in mice using fluorescent reporter viruses

Guojun Wang [1], Ying Chen [1,4], Michael Schotsaert [1], Adolfo Garcia-Sastre [1,2,3]

[1]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA; [2]Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA; [3]Department of Medicine, Division of Infectious Diseases, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA; [4]College of Animal Science and Technology, Guangxi University, No.100 Daxue Road, Nanning 530004, China

Influenza A virus is a major pathogen responsible for human and animal health. Highly pathogenic avian influenza viruses (HPAIVs), like H5N1, continue to evolve in nature and repeatedly infect humans, threatening public health. However, cells types infected in vivo by H5N1 are poorly understood. The ability to visualize influenza viruses offer powerful tool to study influenza virus replication, tropism and pathogenesis. Here, we infected mice with H5N1GFP virus and tracked the dynamics of H5N1 virus infection progression in mice by flow cytometry. In myeloid panel, we found high level of GFP-postive cells, such as epithelial cells, alveolar macrophages, DCs, eosinophils, and neutrophils, and reached the peak at 3 dpi. In addition, a significant proportion of NK cells, T cells and B cells were GFP postive, suggesting highly infection of these cells. Our results demonstrate high levels of immune cells harboring H5N1 influenza virus antigen during viral infection, which furthers our knowledge of infection of H5N1 influenza virus in mammals.

Poster Abstracts

Perturbation and Validation Studies

Poster	Presenter	Title
20	Davis	Transcription factor induction in Mycobacterium tuberculosis identifies infection stress phenotypes
21	Westhoff Smith	Identification and Characterization of Ebola Virus and Host Protein Interactions
22	Kuroda	RNAi Screen to Identify Ebola-Host PPI Partners Modulating Host Cell Responses
23	Fan	Compounds Identified by a System Biology Approach Regulate the Pathogenicity of Influenza A Virus
24	Ma	Systematic Assessment of Drug Susceptibility in Mycobacterium tuberculosis
25	VanBlargen	Investigation of West Nile virus replication and pathogenesis by in vivo transcriptomics
26	Martin-Sancho	Comparative siRNA screen identifies conserved and strain-specific host factors for Influenza A virus replication
27	Rodriguez-Frandsen	Identification of new host restriction factors affecting influenza A virus replication in primary human respiratory epithelial cells
28	Stein	PPMO-knockdown of cellular genes inhibits influenza virus infections

Presentation Type: Flash Talk and Poster
Center: Omics4TB
Section: Perturbation and Validation Studies
Poster 20

Transcription factor induction in *Mycobacterium tuberculosis* identifies infection stress phenotypes

Marshall J. Davis[1,2], Jessica Farrow-Johnson[1], Jessica Winkler[1], Kyle J. Minch[1], Tige R. Rustad[1], David R. Sherman[1,2*]

[1]Center for Infectious Disease Research, Seattle, WA, USA, 98109; [2]Pathobiology Program, University of Washington, Department of Global Health, Seattle, WA, USA 98195

We have developed a systematic method to evaluate the regulatory role of each transcription factor (TF) in Mycobacterium tuberculosis during infection. We created a TF-induction (TFI) library by cloning 207 of the estimated 214 MTB TFs into an anhydrotetracycline-inducible expression vector and transforming these into wildtype H37Rv to create a panel of merodiploid strains. The resultant mutants conditionally perturb effector gene expression in coordinated groups defined by direct binding of the TF in promoter regions. In comparison to gene knockout systems, this method also preserves complex downstream interactions over an extended period between the regulatory network and effector gene outputs driven by the initial binding of the induced regulator. To identify novel roles for TF-effector gene interactions in response to infection we combined all TFI mutants into a pooled culture and subjected the system to various stress conditions including infection of THP-1 cells, C57BL/6 mice, and a hypoxia/reaeration time course. The relative growth defects promoted by TF-induction were quantified by PCR amplification and high-throughput sequencing of each mutant's unique genetic tag. Eight induction mutants displayed growth defects in macrophages greater than when these same regulators were induced in vitro, and five TFs possess a defect specific to their adaptation to hypoxia and reaeration. These phenotypic data complement and inform our ongoing gene expression, TF-binding site, and growth defect studies, contributing to a functional genetic regulatory network of the MTB adaptation to infection. Regulators possessing defects across multiple screened infectious contexts are of particular interest moving forward. The causal interactions driving specific phenotypes will be pursued based on established function and potentially novel interactions indicated by our published gene expression and TF-binding site data.

Presentation Type: Flash Talk and Poster
Center: OmicsLHV
Section: Perturbation and Validation Studies
Poster 21

Identification and Characterization of Ebola Virus and Host Protein Interactions

Danielle Westhoff Smith[1], Amie J. Eisfeld[1], Kohei Oishi[2], Seiya Yamayoshi[2], Kei Takahashi[2], Hiroko Kozuka-Hata[3], Masaaki Oyama[3], Gabriele Neumann[1], Peter Halfmann[1] and Yoshihiro Kawaoka[1,2,4]

[1]Influenza Research Institute, Department of Pathobiological Sciences, University of Wisconsin-Madison School of Veterinary Medicine, Madison, Wisconsin, USA; [2]Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan; [3]Medical Proteomics Laboratory, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan, [4]Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

Despite encoding only seven known structural proteins, Ebola virus causes severe infections resulting in mortality rates as high as 90% depending on the species of Ebola virus. Due to its limited number of encoded proteins, Ebola virus must associate with cellular proteins to efficiently replicate and cause disease. To gain a better understanding of the functional interactions between host and Ebola virus proteins, we performed immunoprecipitation using tagged viral proteins followed by mass spectrometry to identify host-virus protein interactions. We identified 550 cellular proteins that interact with at least one tagged Ebola virus protein. 198 cellular proteins were eliminated from further analysis based on non-specific interactions and known toxicity due to knock-down by RNA inference. The remaining 350 cellular proteins were evaluated for their role in Ebola virus replication in human liver cells (Huh 7.0 cells) by use of a set of four small interfering RNAs (siRNAs) against each cellular target. We identified 53 host proteins that are essential for virus replication (i.e those whose knockdown decrease virus titers relative to control siRNA treatment) and 43 host proteins with anti-viral functions (i.e those whose knockdown increased titers relative to control siRNA treatment). Among those factors necessary for virus replication are microtubule components that interact with Ebola virus nucleoprotein as well as components of the spliceosome that interact with the RNA-dependent RNA polymerase. Ongoing experiments seek to further define the role and function of the host factors in the Ebola virus life cycle as well as identify novel Ebola anti-viral compounds.

This project is funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, grant U19AI106772.

Presentation Type: Flash Talk and Poster
Center: OmicsLHV
Section: Perturbation and Validation Studies
Poster 22

RNAi Screen to Identify Ebola-Host PPI Partners Modulating Host Cell Responses

Makoto Kuroda[1], Seiya Yamayoshi[2], Kohei Oishi[2], Kei Takahashi[2], Takashi Ishii[2], Hiroko Kozuka-Hata[3], Masaki Oyama[3], Danielle Westhoff Smith[1], Amie J Eisfeld[1], Gabriele Neumann[1], Peter Halfmann[1] and Yoshihiro Kawaoka[1,2,4,5]

[1]Influenza Research Institute, Department of Pathobiological Sciences, University of Wisconsin-Madison School of Veterinary Medicine, Madison, Wisconsin; [2]Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan; [3]Medical Proteomics Laboratory, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan; [4]Department of Special Pathogens, International Research Center for Infectious Disease, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan; [5]International Research Center for Infectious Disease, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

Viruses exploit multiple host cellular machinery for their efficient replication though a protein-protein interaction (PPI) with host proteins. Ebola virus encodes at least seven structural proteins, some of which have been reported to suppress antiviral responses, causing severe infections. To better understand viral and cellular determinants that could affect cellular events governing replication and pathogenicity of Ebola virus, we previously identified 351 host proteins exhibiting PPI with Ebola protein(s) by performing immunoprecipitation using tagged viral proteins and mass spectrometry. Here, we performed two RNAi screens using a set of 4 siRNAs targeting the 351 Ebola-host PPI partners to determine whether these target proteins can modulate cellular signaling (i.e., inflammatory and apoptosis signaling pathways). To evaluate knockdown effects on inflammatory signaling mediated by NF-□B, we assessed TNF-induced NF-□B activation by using a hepatocyte cell line (IHH) stably expressing an NF-□B-dependent luciferase reporter gene. We found 20 genes whose knockdown induced more than 5-fold change in NF-□B activation. Knockdown effects on apoptosis signaling were assessed by measuring staurosporine-induced caspase3/7 activation in HeLa S3 cells. We found 22 genes whose knockdown caused more than 2-fold change in caspase3/7 activation after staurosporine treatment. Further studies of these target proteins will provide a new insight into the role and function of the PPI partners and provide a potential cellular target for antiviral compounds.

Presentation Type: Flash Talk and Poster
Center: OmicsLHV
Section: Perturbation and Validation Studies
Poster 23

Compounds Identified by a System Biology Approach Regulate the Pathogenicity of Influenza A Virus

Shufang Fan[1], Amie J. Eisfeld[1], Takeo Gorai[1], Tiago Lopes[1,2,3], Gabriele Neumann[1] and Yoshihiro Kawaoka[1,2,3]

[1]Influenza Research Institute, Department of Pathobiological Sciences, University of Wisconsin-Madison School of Veterinary Medicine, Madison, Wisconsin, USA; [2]Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan; [3]International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

Influenza A viruses (IAVs) cause seasonal epidemics with significant global morbidity and mortality and substantial economic burdens, and sporadic pandemics in which these effects are typically more severe. Two classes of antiviral drugs are available for treatment of human IAV infections: adamantans, which impair viral M2 ion channel activity, and neuraminidase inhibitors, such as oseltamavir and zanamivir. However, the emergence of IAV mutants that are resistant to the antiviral activity of one or both drug classes is a significant problem. Previously, using a systems biology approach, we identified host factors whose knockdown dramatically affected influenza virus replication, suggesting that drugs that block the expression of these factors may interfere with influenza virus disease outcome. Here, we used an in-house computational drug discovery pipeline to identify more than three hundred drugs that have putative roles on regulating the activity or the expression of these host factors, screened these drugs for effects on single-cycle and multiple-cycle IAV replication, and calculated the median inhibitory concentration (IC_{50}) and the median cytotoxic concentration (CC_{50}) *in vitro*. Finally, we focused on fourteen high priority compounds with strong antiviral function and limited cytotoxicity *in vitro*, for which detailed information is available on pharmacological usages in humans and mice, and we assessed the effects of these drugs on pathogenicity of a 2009 pandemic H1N1 (pH1N1) virus in mice. Three compounds protected mice against pH1N1 pathogenicity, while 4 compounds increased pH1N1 pathogenicity. Current work is focused on determining how these drugs affect host response networks and identifying the mechanisms through which they affect pH1N1 pathogenicity. *This project is funded in whole with funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, grant U19AI106772.*

Presentation Type: Poster Talk and Poster
Center: Omics4TB
Section: Perturbation and Validation Studies
Poster 24

Systematic Assessment of Drug Susceptibility in *Mycobacterium tuberculosis*

Shuyi Ma[1,2], Tige R. Rustad[1,2], and David R. Sherman[1,2]

[1]Center for Infectious Disease Research, formerly Seattle Biomedical Research Institute, Seattle WA, USA; [2]Pathobiology Program, Department of Global Health, University of Washington, Seattle WA, USA

Designing optimal combination TB therapies requires a systems-level understanding of *M. tuberculosis* (MTB) response to drugs under different conditions. We probed the effects of perturbing of genetic context on MTB drug response by harnessing a genome-scale experimental screening strategy. We exposed a comprehensive pooled library of transcription factor (TF) overexpression mutant strains to 8 clinically relevant anti-TB agents and identified drug-specific sets of regulators that influence MTB susceptibility. In the case of isoniazid (INH), we identified susceptibility-altering TFs, which included a regulator previously known to confer decreased susceptibility to INH (Rv1909c, *furA*). We successfully validated another TF (Rv1963c, *mce3R*), which increased susceptibility to INH when overexpressed. To further probe mechanisms underlying *mce3R* hypersusceptibility, we combined known mce3R regulon information with MTB drug response transcriptomes and identified putative effector genes. We selected one of its repressed target genes, Rv1469, as potentially contributing to mce3R's increased INH susceptibility. Functional testing with the Rv1469 transposon knockout strain confirmed our hypothesis. Integrating drug response phenotypes with TFs and their gene networks at a genome-scale advances efforts to design TB therapies by enabling systematic interrogation of new mechanisms and informing rational design of complementary drug combinations.

Presentation Type: Poster
Center: OmicsLHV
Section: Perturbation and Validation Studies
Poster 25

Investigation of West Nile virus replication and pathogenesis by *in vivo* transcriptomics

Laura A. VanBlargan[1], Hugh D. Mitchell[2], Qing Tan[1], Allison M. Thompson[2], Amie J. Eisfeld[3], Katrina M. Waters[2], Larissa B. Thackray[1], and Michael S. Diamond[1]

[1]Washington University School of Medicine, St. Louis, MO 63110, USA; [2]Pacific Northwest National Laboratory, Richland, WA, 99352, USA; [3]University of Wisconsin-Madison, Madison, WI 53711, USA

The outcome of West Nile virus (WNV) infection in humans ranges from asymptomatic, to mild fever, to potentially fatal, neurological disease. Determinants of WNV disease outcome remain incompletely defined but include advanced age, immunocompromised status, and genetic polymorphisms in several innate immune molecules. To gain a better understanding of WNV pathogenesis *in vivo*, we generated transcriptome datasets from draining lymph nodes (after subcutaneous inoculation) and brain tissues (after intracranial inoculation) at 1, 2, 4, or 6 days post-infection from mice infected with a virulent WNV strain or attenuated mutant. In draining lymph nodes, the number of differentially expressed genes (DEGs)(>1.5 fold, $P<0.05$) relative to mock infection peaked at day 2 for both strains (>6,800 genes). In the cerebral cortex, the number of DEGs peaked at day 6 for the virulent strain (>14,700 genes), but at day 2 for the attenuated strain (>7,100 genes). DEGs were prioritized for further analyses with a network inference approach that employed five network metrics to rank potential regulatory genes based on their topological position in tissue-specific networks, followed by gene set enrichment analysis. Several genes prioritized by this approach have been implicated previously in the control of viral pathogenesis (including IFIT1, Rsad2, and Oas1a), but the majority of prioritized genes have not yet been characterized in the context of virus infections. This novel list of genes is being used to guide *in vitro* and *in vivo* investigations of WNV replication and pathogenesis, including studies employing ectopic expression or CRISPR/Cas9-mediated depletion of target genes. Initial CRISPR/Cas9 experiments have identified several potential pro-viral host genes that are currently undergoing further validation and characterization.

This project is funded in whole with funds from NIAID, NIH, grant U19AI106772.

Presentation Type: Poster
Center: FluOmics
Section: Perturbation and Validation Studies
Poster 26

Comparative siRNA screen identifies conserved and strain-specific host factors for Influenza A virus replication

Laura Martin-Sancho and Sumit K. Chanda

Infectious and Inflammatory Disease Center, Sanford Burnham Prebys Medical Discovery Institute, 10901 North Torrey Pines Road. La Jolla, California, USA

Influenza A virus (IAV) is a major public health burden, responsible for annual epidemics and sporadic pandemics that cause millions of deaths worldwide. The genome of IAV consists of eight single-stranded, negative-sense RNA segments, which encode up to 14 known viral proteins. To complete its infectious life cycle, IAV must not only exploit cellular factors for replication but also evade host immune responses. In recent years, large-scale loss-of-function screens have identified several host factors essential for IAV replication. However, most of these screens did not attempt to identify restriction factors or to investigate the presence of strain-specific factors. Here we carried out comparative genome-wide siRNA screens in THP-1 cells infected with the seasonal IAV strain H3N2, and the highly pathogenic H5N1. In addition to novel strain-specific restriction factors, we also identified factors that affected both strains, including IRF9, STAT2 or ADAP2. This comparative siRNA screen will provide new insights into the role of cellular restriction factors in viral adaptation, improving our knowledge of viral infection and will appoint novel target candidates for drug therapy.

Presentation Type: Poster
Center: FluOmics
Section: Perturbation and Validation Studies
Poster 27

Identification of new host restriction factors affecting influenza A virus replication in primary human respiratory epithelial cells

Ariel Rodriguez-Frandsen[1], Laura Martin-Sancho[1], Paul De Jesus[1], Stephen Soonthornvacharin[1], Numana Bhat[1], Randy Albrecht[2], Adolfo Garcia-Sastre[3], Sumit Chanda[1]

[1]Sanford-Burnham-Prebys Medical Discovery Institute, Infectious and Inflammatory Disease Center, 10901 North Torrey Pines Road, La Jolla, CA 92037; [2]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA; Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA; [3]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA; Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA; Department of Medicine, Division of Infectious Diseases, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

Influenza virus infections are a major cause of respiratory disease in humans. Influenza A viruses (IAV) are responsible for annual epidemics and occasional pandemics with potentially fatal outcome. The differences in the severity of the disease among individuals infected by IAV could be due to pre-existing health conditions, genetic factors, differences in the virulence of the circulating viruses or a combination of these elements. Thus, a comprehensive understanding of viral-host interactions, host innate immune responses and viral evasion strategies is pivotal to understand viral pathogenesis.

In recent years, genome-wide loss-of-function screens have identified several host factors required for IAV replication. However, these screens did not attempt to identify restriction factors or to investigate the presence of conserved and strain-specific host factors. In order to identify key early genes involved in the restriction of IAV infection, we have performed functional genomic cDNA overexpression as well as siRNA knockdown screens with a set of around 1000 interferon-stimulated genes (ISGs) in primary human respiratory epithelial HTBE cells. Furthermore, we hypothesize that strains of IAV with different levels of virulence interact with the host in a strain-specific fashion. Therefore, we infected the HTBE cells with two IAV strains of different pathogenicity (a seasonal H3N2 and the highly pathogenic H5N1) with the aim to identify cellular factors that influence viral replication and are common to or exclusive of these IAV strains. The screens were performed in the presence and absence of Type I IFN with the objective to pinpoint proteins involved in the IFN-mediated immune response or with restriction activity *per se*, respectively. We have identified several proteins that restrict H5N1 replication, whereas for the H3N2 virus we identified factors that restrict replication as well as proteins that increase its replication. We have selected some of these hits for further characterization and we are currently performing functional studies in order to identify the step in the viral life cycle affected as well as the mechanism of action for these IAV restriction factors.

Presentation Type: Poster
Center: FluOmics
Section: Perturbation and Validation Studies
Poster 28

PPMO -knockdown of cellular genes inhibits influenza virus infections

David S tein [1], Eva Böttcher -Friebertsh äuser [2], Shashank Tripathi [3], Hong Moulton [1], Adolfo García -Sastre [3]

[1]Oregon State University, Corvallis, OR ; [2]Philipps University, Marburg, Germany ; [3]Ichan School of Medicine at Mt. Sinai, New York, NY

Diminishing the expressi on of a specific host gene during viral infection can help elucidate its involvement in virus growth and pathogenesis. We used antisense peptide -conjugated morpholino oligomers (PPMO) to reduce the expression of the cellular genes TMPRSS2 and UBR4, then ev aluated the effect on influenza virus growth. A PPMO (T -ex5) designed to interfere with pre -mRNA splicing of TMPRSS2 was effective at inhibiting the HA cleavage of several influenza A viruses known to infect humans. Treatment of Calu -3 airway epithelial c ell line with T -ex5 markedly reduced the growth and spread of H1N1 and H3N2 viruses, at concentrations that were not cytotoxic to uninfected cells under similar conditions. In influenza -infected mice, PPMO -knockdown of UBR4 resulted in reduced virus growth in the lungs, while increasing overall survivorship. These studies further establish TMPRSS2 and UBR4 as important to viral replication and pathogenesis, and demonstrate the efficacy of PPMO as effective knockdown reagents for in vitro and in vivo studies .

Poster Abstracts

New Tools

Poster	Presenter	Title
29	Metz	Ion Mobility Separations: A Paradigm Shift in Mass-Spectrometry-based Omics Analyses
30	Anderson	A Multi-Omic Data Analysis Platform for Mining & Visualization of Host-Viral Response
31	DeBarry	Strategies for Supporting Systems Biology Data Management and Cyberinfrastructure
32	Chou	Universal full-length genomic amplification of Zika virus facilitates sequencing and reverse genetics
33	Chen	Novel reassortant swine influenza viruses with H1N1/2009 human pandemic and Eurasian Avian-origin genes in Guangxi, China

Presentation Type: Poster
Center: OmicsLHV
Section: New Tools
Poster 29

Ion Mobility Separations: A Paradigm Shift in Mass-Spectrometry-based Omics Analyses

Thomas O. Metz[1], Kristin E. Burnum-Johnson[1], Ryan S. Renslow[1], Jason P. Wendler[1], Amie J. Eisfeld[2], Yoshi Kawaoka[2,3,4], Erin S. Baker[1], Richard D. Smith[1]

[1]Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA, 99352, USA; [2]Department of Pathobiological Sciences, University of Wisconsin-Madison, Madison, WI, 53711, USA; [3]Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo, 108-8639, Japan; [4]International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

Ion mobility (IM) provides gas-phase separation of ionized molecules based on their size and shape. IM separations occur on a time scale of milliseconds, and can be easily nested between conventional liquid chromatography (LC) and mass spectrometry (MS), allowing for greater coverage of sample constituents for the same LC separation, or for decreasing the LC separation length (or time) needed while maintaining the same measurement coverage. Importantly, IM separations are much more repeatable than LC, facilitating reliable molecular feature alignment across multiple samples, and improving the ability to identify species.

An exciting aspect of IM-MS measurements is the ability to directly measure the chemistry and composition of molecules, based on their collision cross section (CCS) and accurate mass information, respectively. Through innovations in computational chemistry, we have overcome a significant, long standing obstacle in the field of metabolomics: the absence of methods for accurate and comprehensive identification of small molecules without relying on data from analysis of authentic chemical standards. A paradigm shift in metabolomics, we are developing reference libraries of predicted CCS to use in conjunction with very high accuracy mass information provided by MS for metabolites to enable 'standards-free' metabolomics. Additionally, we have developed a new approach that performs both discovery and targeted monitoring (DTM) in a single analysis using LC-IM-MS, providing an effective convergence of the attractive properties (i.e. sensitivity, confidence of identifications, and accuracy of quantification) of these powerful approaches. In DTM, heavy labeled target peptides are spiked into tryptic digests and both the labeled and unlabeled peptides are detected using LC-IM-MS. Compared with conventional LC-MS, DTM yields greater proteome coverage and detects lower abundance species, and also achieves detection limits similar to targeted selected reaction monitoring measurements. We initially applied LC-IM-MS in proteomics analyses of blood plasma from individuals with EBOV disease (EVD), including 11 EVD survivors, 9 EVD fatalities, and 7 healthy Sierra Leonean volunteers. LC-IM-MS provided 2-3-fold higher numbers of protein identifications compared to the conventional LC-MS.

This study was funded in part by grant U19AI106772, provided by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (USA).

Presentation Type: Flash Talk and Poster
Center: OmicsLHV
Section: New Tools
Poster 30

A Multi-Omic Data Analysis Platform for Mining & Visualization of Host-Viral Response

Lindsey Anderson[1], Hugh Mitchell[1], Kelly Stratton[2], Markus Kobold[2], Bobbie-Jo Webb-Robertson[2], and Katrina Waters[1]

[1]Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA, USA; [2]Computing & Analytics Division, Pacific Northwest National Laboratory, Richland, WA, USA

The advent of high-throughput OMICs technologies has made available large quantities of data, transforming molecular biology into a remarkably data-rich science. Researchers now find themselves having to deal with huge datasets and face challenges extracting and interpreting the wealth of information hidden in these data. As such, computational approaches for data mining, integration and interpretation are often the limiting factor in discovery-based science, and standardized approaches for data normalization and statistical processing are critical for cross-experiment and cross-system comparisons. In an effort to mediate these challenges, we are leveraging an interactive statistical web-based software platform, PMart (http://pmart.azurewebsites.net/pmart), which was developed to enable the analysis and exploration of publicly available cancer proteomics datasets for biomarker discovery. The PMart Virology tool uses pre-loaded OMICS-LHV data (transcriptomic, proteomic, metabolomic, and lipidomic data) in standardized format for subsequent integration, statistical and/or visual analytics. A user-friendly, web-based interface allows for expansive and customizable navigation of host-response data, unbiased to species, tissue type, viral strain, time point, and/or condition. The tool will incorporate workflows for functional enrichment analysis along with visualizations of the results, identifying pathways and processes that are perturbed across data types or across different viruses. The workflows will provide functional annotation clustering, to interrogate broad or narrow functional categories, as well as view the expression behavior of features associated with individual pathways. In addition, links will be included for exploring interactive metabolic pathway-specific maps to gain useful insights into the biochemical context of critical host factors. This software platform is designed to facilitate the re-analysis of multi-omics data for new hypothesis generation, thus providing significant benefit to those interested in mining a public repository without having to download raw data files and reprocess them. The data and enrichment pipelines used to demonstrate this web-based platform were funded by grant U19AI106772, provided by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (USA).

Presentation Type: Poster
Center: MaHPIC
Section: New Tools
Poster 31

Strategies for Supporting Systems Biology Data Management and Cyberinfrastructure

Jeremy D. DeBarry[1], Jay Humphrey[1], Nicolas Lackman[1], Mustafa Nural[1], Suman B. Pakala[1], Jessica C. Kissinger[123], Mary R. Galinski[4], The MaHPIC Consortium[4]

[1]Institute of Bioinformatics, [2]Center for Tropical and Emerging Global Diseases, [3]Department of Genetics University of Georgia, Athens, GA 30602; [4]Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, Atlanta, GA, USA

Because systems biology is, by nature, transdisciplinary, its success requires the effective integration of people and ideas as much as data and technologies. It follows that successful systems biology data management requires facilitating communication, education, and a host of other sociological factors, in addition to establishing and maintaining a system of hardware and software. This is challenging, because the same essential diversity that makes systems biology projects possible is also a potential hindrance, due to an inherent 'Tower of Babel' scenario where no two groups speak quite the same language, with a dangerous and contrasting array of mental imagery associated with technical words and phrases. This affects all aspects of communication, from the biological to the computational. Any group that agrees to serve as the computational and 'informatics' support team for a large transdisciplinary project should expect to be called upon to meet these challenges by producing a set of 'Rosetta Stones' to guide the effort and provide a unifying force to counteract the most dangerous illusion of team communication, namely, that it has taken place at all. We will discuss the strategies developed by the Malaria Host-Pathogen Interaction Center to identify and solve issues that run the gamut from the technical to the sociological, and from experimental design to public data deposition.

This project has been funded in whole or in part with Federal funds from the National Institute of Allergy and Infectious Diseases; National Institutes of Health, Department of Health and Human Services [Contract No. HHSN272201200031C] and the National Center for Research Resources [ORIP/OD P51OD011132].

Presentation Type: Flash Talk and Poster
Center: FluDyNeMo
Section: New Tools
Poster 32

Universal full-length genomic amplification of Zika virus facilitates sequencing and re verse genetics

Tsui-wen Chou[1], Boya Zhao[1], Chang Wang[1], Bin Zhou[1], and Elodie Ghedin[1,2]

[1]Center for Genomics and Systems Biology, Department of Biology, New York University; [2]College of Global Public Health, New York University, New York, New York

Zika virus contains a single-stranded positive-sense RNA genome of ~10,800 nucleotides long. It was first isolated in monkeys and mosquitos in the 1940's in Uganda. It was then confirmed in human infections in the 1950's and spread to equatorial Africa and to Asia in the following decades. The current widespread Zika outbreak in the Americas is caused by strains of the Asian lineage. Genome sequencing is crucial for monitoring the evolution and understanding the biology of the Zika virus. However, the 2 currently widely used genomic amplification methods have severe limitations. The first method uses a random priming strategy to amplify all RNA molecules in a sample, including both host and viral RNAs. It is not a scalable high-throughput method as the viral RNAs constitute only a small percentage of the total RNA, thus requiring a deep sequencing approach to capture a limited number of virus sequences. The second method uses a large number (34-36) of viral sequence-specific primers to amplify short overlapping regions of ~400 bp to cover the whole genome. It suffers from uneven coverage of different regions and is vulnerable to strain variations, inherent to a rapid spreading and evolving virus. We developed a full-length genomic amplification method using only two primers targeting the conserved sequences in the 5' and 3' terminal regions of the Zika genome making this approach universally applicable to all Zika strains of both African and Asian lineages. Multiple optimizations—from RNA isolation to reverse transcription to PCR—were performed to increase the sensitivity and robustness of the assay. The long amplicons were successfully sequenced on the Illumina platform for assembly of full-length genome sequences of the Zika virus. This full-length amplicon approach is particularly appropriate for single-molecule sequencing techniques, such as Oxford Nanopore and PacBio, and could be used for haplotyping mutations of individual viral genomes. In addition, we further developed this method to generate recombinant Zika viruses directly from PCR products, averting the many difficulties in the time-consuming process of cloning. For its usefulness in sequencing and reverse genetics, this universal genomic amplification method will accelerate the surveillance and research on Zika disease.

Presentation Type: Poster
Center: FluOmics
Section: New Tools
Poster 33

Novel reassortant swine influenza viruses with H1N1/2009 human pandemic and Eurasian Avian-origin genes in Guangxi, China

Ying Chen[1,4], Guojun Wang[4], Adolfo García-Sastre[2,3,4]

[1]College of Animal Science and Technology, Guangxi University, No.100 Daxue Road, Nanning 530004, China; [2] Department of Medicine, Division of infectious Disease, [3]and Global Health and Emerging Pathogens Institute, [4]Department of Medicine, Division Icahn School of Medicine at Mount Sinai, New York, New York,10029, USA

After the multiple introductions and reassortments of H1N1/2009 human pandemic viruses (H1N1/2009) into pig population, the genetic diversity of swine influenza viruses has increasing significantly in China. We report the isolation and genetic analysis of 11 novel reassortant H1N1 and H3N2 viruses, 2 whole Eurasian avian-like (EA-like) and 1 H1N1/2009-origin virus from pigs in Guangxi during 2013 to 2015. Phylogenetic analysis showed that these novel reassortant variants contained the surface genes (HA and NA gene) derived from EA-like H1N1 or seasonal human-like H3N2, H1N1/2009-origin internal genes (PB2, PB1, PA and NP) and classical swine-origin NS genes. Notably, the novel EA-like H1N1 viruses owned different M genes, either from H1N1/2009 or EA-like lineage, which were associated with the human infection, implying the potential zoonosis between swine to human. Besides, the persistence and prevalence of human-like H3N2 reassortant viruses with H1N1/2009-origin internal genes and classical swine-origin NS genes will increase the human adaptation with the potential pandemic. Further investigation will help to assess the potential risk of these novel reassortant viruses to humans.

Collaborations

Poster	Presenter	Title
34	Peterson	Collaborative research in Mycobacterium tuberculosis stemming from the SysBio of Infectious Disease Program

Presentation Type: Poster
Center: Omics4TB
Section: Collaborations
Poster 34

Eliza Peterson[1], Serdar Turkarslan[1], Nitin Baliga[1]

[1]*Institute for Systems Biology, Seattle, WA*

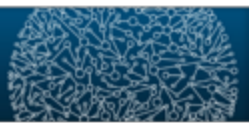
The large-scale data sets and predictive models generated by the Omics4TB group have created community-wide resources for studying TB. In addition to publications that facilitate exposure of these resources, the Omics4TB group has developed web portals for gathering Program resources and enabling access to the scientific community. The web portals have been viewed by more than 10,000 users in over 100 countries and many of these users have reached out to us seeking advice and/or collaboration. Among these users, we have pursued further research endeavors with four TB groups (in four countries). In collaboration with Tanya Parish (Infectious Disease Research Institute, Seattle, WA), we have generated promoter reporter strains of specific transcription factor (TF) target genes that are involved in tolerance to antitubercular treatment. Using these strains we are developing a screening assay to identify small-molecules that inhibit the tolerance TFs and synergize with antitubercular treatment. We are also working with Mario Alberto Flores-Valdez (State Center for Research and Assistance in Technology and Science, Jalisco, Mexico) to characterize transcriptional changes in an MTB mutant that has attenuated outcome during mouse infection. Del Besra (University of Birmingham, Birmingham, UK) also reached out to us to better understand the transcriptional network of PhoP, a response regulator found with mutations in an MTB strain that is resistant to a novel antibiotic. Finally, we are also working with Lindsay Eltis (University of Biritsh Columbia, Vancouver, Canada), who is primarily interested in MTB pathways responsible for the degradation of aromatic compounds and steroids, particularly cholesterol. MTB has the unique capability of utilizing host-derived cholesterol as both an energy source and as a precursor molecule for the biosynthesis of cell wall fatty acids. Collaborating with Prof. Eltis, we have used our regulatory network models to predict additional enzymes involved in cholesterol degradation, which his group is working to experimentally validate. These four research projects are just an example of the large and increasing impact the SysBio of Infectious Disease Program has had on the broader scientific community.

Poster Numbers

Poster	Presenter	Title
1	Tian	Analysis of Multi-omics Datasets Using Human Genome-Scale Metabolic Network
2	Cahalane	Metabolic Profiling of Influenza Infection
3	Pande	Modeling the Interplay between Viral Replication, Immune Response and Pathogenic Outcomes in the Mouse Lung
4	Mitchell	Comprehensive Pan-Virus, Pan-OMICS Data Analysis for Discovery of Common and Unique Host Responses
5	Peterson	A system-level transcriptional regulatory model reveals regulation underlying intracellular growth of *Mycobacterium tuberculosis*
6	Forst	Integrative Network Analysis Reveals Novel Sex Specific Key Processes and Drivers in Response to Influenza Infection and Vaccination
7	Zhang	Multiscale Systems Approaches Identify Key Signatures, Intrinsic Networks and Causal Regulators for Influenza Infection and Vaccination
8	Wang	Age- Dependent Molecular Responses to Influenza Vaccination
9	Gutierrez	Multi-omic analysis of severity of infection in Macaca mulatta infected with *Plasmodium cynomolgi*. A multi-omic approach powered by the SKED framework.
10	Hultquist	Proteomic Profiling of Influenza A Virus Infection Reveals Strain-specific Rewiring of Host Cell Networks
11	Haas	Destroying the “Non-self”: Defining Influenza A Viral Endonuclease Mechanisms for Host Shutoff
12	Ziehr	Polysome Sequencing During Influenza Infection
13	Ma	Effects of Influenza Infection on the Respiratory Mycobiome in Ferrets
14	Ding	Role of the respiratory microbiome in influenza disease severity
15	Kelvin	Influenza strain specificity and microbiome function influences mortality in infants
16	Wang	Single-cell analyses reveal the effects of cell cycle on influenza virus replication
17	Sherman	Development of Parallel Microscopic Determination of Fitness; a time-lapse microscopy platform for Mycobacterium tuberculosis
18	Bissel	In situ localization and distribution of differentially expressed innate immune genes during influenza infection in ferret lungs
19	Wang	Analysis of in vivo dynamics of H5N1 influenza virus infection in mice using fluorescent reporter viruses

Poster	Presenter	Title
20	Davis	Transcription factor induction in Mycobacterium tuberculosis identifies infection stress phenotypes
21	Westhoff Smith	Identification and Characterization of Ebola Virus and Host Protein Interactions
22	Kuroda	RNAi Screen to Identify Ebola-Host PPI Partners Modulating Host Cell Responses
23	Fan	Compounds Identified by a System Biology Approach Regulate the Pathogenicity of Influenza A Virus
24	Ma	Systematic Assessment of Drug Susceptibility in Mycobacterium tuberculosis
25	VanBlargen	Investigation of West Nile virus replication and pathogenesis by in vivo transcriptomics
26	Martin-Sancho	Comparative siRNA screen identifies conserved and strain-specific host factors for Influenza A virus replication
27	Rodriguez-Frandsen	Identification of new host restriction factors affecting influenza A virus replication in primary human respiratory epithelial cells
28	Stein	PPMO-knockdown of cellular genes inhibits influenza virus infections
29	Metz	Ion Mobility Separations: A Paradigm Shift in Mass-Spectrometry-based Omics Analyses
30	Anderson	A Multi-Omic Data Analysis Platform for Mining & Visualization of Host-Viral Response
31	DeBarry	Strategies for Supporting Systems Biology Data Management and Cyberinfrastructure
32	Chou	Universal full-length genomic amplification of Zika virus facilitates sequencing and reverse genetics
33	Chen	Novel reassortant swine influenza viruses with H1N1/2009 human pandemic and Eurasian Avian-origin genes in Guangxi, China
34	Peterson	Collaborative research in Mycobacterium tuberculosis stemming from the SysBio of Infectious Disease Program

Contact Information



First Name	Last Name	Email	Affiliation
Stephanie	Bissel	stephaniebissel@pitt.edu	FluDyNeMo
Tsui-Wen	Chou	twc298@nyu.edu	FluDyNeMo
Tao	Ding	tao.ding@nyu.edu	FluDyNeMo
Christian	Forst	christian.forst@mssm.edu	FluDyNeMo
Elodie	Ghedin	elodie.ghedin@nyu.edu	FluDyNeMo
Alyson	Kelvin	akelvin@jidc.org	FluDyNeMo
Lauren	Lashua	lauren.lashua@nyu.edu	FluDyNeMo
Yixuan	Ma	ym1165@nyu.edu	FluDyNeMo
Lara	Mahal	lkmahal@nyu.edu	FluDyNeMo
Ted	Ross	tedross@uga.edu	FluDyNeMo
Chang	Wang	cw2505@nyu.edu	FluDyNeMo
Minghui	Wang	minghui.wang@mssm.edu	FluDyNeMo
Bin	Zhang	bin.zhang@mssm.edu	FluDyNeMo
Lingdi	Zhang	lz967@nyu.edu	FluDyNeMo
Bin	Zhou	bin.zhou@nyu.edu	FluDyNeMo
Randy	Albrecht	randy.albrecht@mssm.edu	Fluomics
Christopher	Benner	cbenner@ucsd.edu	Fluomics
Ryan	Camping	ryan.camping@mssm.edu	Fluomics
Sumit	Chanda	alexakat@sbpdiscovery.org	Fluomics
Max	Chang	mchang@ucsd.edu	Fluomics
Ying	Chen	ying.chen@mssm.edu	Fluomics
Edward	Dennis	edennis@ucsd.edu	Fluomics
Adolfo	Garcia-Sastre	adolfo.garcia-sastre@mssm.edu	Fluomics
Kelsey	Haas	kelsey.haas@ucsf.edu	Fluomics
Sven	Heinz	sheinz@ucsd.edu	Fluomics
Judd	Hultquist	judd.hultquist@gladstone.ucsf.edu	Fluomics
David	Jimenez-Morales	biodavidjm@gmail.com	Fluomics
Eun-Yong	Kim	e-kim@northwestern.edu	Fluomics
Ivan	Marazzi	ivan.marazzi@mssm.edu	Fluomics
Laura	Martin-Sancho	llmartin@sbpdiscovery.org	Fluomics
Giorgi	Metreveli	giorgi.metreveli@mssm.edu	Fluomics
Hong	Moultron	hong.moulton@oregonstate.edu	Fluomics
Henrietta	Nymark-McMahon	henrietta@sbpdiscovery.org	Fluomics
Lars	Pache	lpache@sbpdiscovery.org	Fluomics
Oswald	Quehenberger	oquehenberger@ucsd.edu	Fluomics
Ariel	Rodriguez	arodriguez@sbpdiscovery.org	Fluomics
Megan	Shaw	megan.shaw@mssm.edu	Fluomics
Leah	Shriver	lshriver@uakron.edu	Fluomics

First Name	Last Name	Email	Affiliation
David	Stein	dave.stein@oregonstate.edu	Fluomics
Shashank	Tripathi	shashank.mssm@gmail.com	Fluomics
Guojun	Wang	guojun.wang@mssm.edu	Fluomics
Kris	White	kris.white@mssm.edu	Fluomics
Steven	Wolinsky	s-wolinsky@northwestern.edu	Fluomics
Jessica	Kissinger	jkissing@uga.edu	MaHPIC
Jeremy	DeBarry	jdebarry@uga.edu	MaHPIC
Luis	Fonseca	llfonseca@gatech.edu	MaHPIC
Mary	Galinski	Mary.Galinski@emory.edu	MaHPIC
Juan	Gutierrez	jgutierr@uga.edu	MaHPIC
Regina	Joice	rjoice@emory.edu	MaHPIC
Shuzhao	Li	sli49@emory.edu	MaHPIC
Mark	Styczynski	marksty@gatech.edu	MaHPIC
Eberhard	Voit	eberhard.voit@bme.gatech.edu	MaHPIC
Alan	Aderem	alan.aderem@cidresearch.org	Omics4TB
Nitin	Baliga	nitin.baliga@systemsbiology.org	Omics4TB
Drew	Dover	drew.dover@cidresearch.org	Omics4TB
Shuyi	Ma	shuyi.ma@cidresearch.org	Omics4TB
Eliza	Peterson	Eliza.Peterson@systemsbiology.org	Omics4TB
Chris	Plaisier	cplaisier@systemsbiology.org	Omics4TB
Courtney	Plumlee	courtney.plumlee@cidresearch.org	Omics4TB
Tige	Rustad	tige.rustad@cidresearch.org	Omics4TB
David	Sherman	david.sherman@cidresearch.org	Omics4TB
Serdar	Turkarslan	Serdar.Turkarslan@systemsbiology.org	Omics4TB
Kevin	Urdahl	kevin.urdahl@cidresearch.org	Omics4TB
Daniel	Zak	daniel.zak@cidresearch.org	Omics4TB
Lindsey	Anderson	Lindsey.Anderson@pnnl.gov	Omics-LHV
Ralph	Baric	rbaric@email.unc.edu	Omics-LHV
Kristin	Burnum-Johnson	kristin.burnum-johnson@pnnl.gov	Omics-LHV
Shiho	Chiba	shiho.chiba@wisc.edu	Omics-LHV
Michelle	Craft	mcraft@discovery.wisc.edu	Omics-LHV
Michael	Diamond	diamond@wusm.wustl.edu	Omics-LHV
Amie	Eisfeld	amie.eisfeld@wisc.edu	Omics-LHV
Shufang	Fan	shufang.fan@wisc.edu	Omics-LHV
Lisa	Gralinski	lgralins@email.unc.edu	Omics-LHV
Peter	Halfmann	peter.halfmann@wisc.edu	Omics-LHV
Yoshihiro	Kawaoka	yoshihiro.kawaoka@wisc.edu	Omics-LHV
Amy	Kuehn	amy.kuehn@wisc.edu	Omics-LHV
Makoto	Kuroda	makoto.kuroda@wisc.edu	Omics-LHV
Jennifer	Kyle	jennifer.kyle@pnnl.gov	Omics-LHV
Miron	Livny	miron@cs.wisc.edu	Omics-LHV
Tom	Metz	thomas.metz@pnnl.gov	Omics-LHV
Hugh	Mitchell	hugh.mitchell@pnnl.gov	Omics-LHV

First Name	Last Name	Email	Affiliation
Gabriele	Neumann	gabriele.neumann@wisc.edu	Omics-LHV
Paritosh	Pande	Paritosh.Pande@pnnl.gov	Omics-LHV
Alexandra	Schaefer	aschaefe@email.unc.edu	Omics-LHV
Amy	Sims	sims0018@ad.unc.edu	Omics-LHV
Larissa	Thackray	thackray@wustl.edu	Omics-LHV
Mingyuan	Tian	mtian29@wisc.edu	Omics-LHV
Laura	VanBlargan	lavanblargan@wustl.edu	Omics-LHV
Katrina	Waters	katrina.waters@pnnl.gov	Omics-LHV
Jason	Wendler	jason.wendler@pnnl.gov	Omics-LHV
Danielle	Westhoff Smith	danielle.westhoffsmith@wisc.edu	Omics-LHV
Ben	Ziehr	bziehr@wisc.edu	Omics-LHV
Raul	Andino	raul.andino@ucsf.edu	Steering Committee
Michael	Gillette	gillette@broadinstitute.org	Steering Committee
Michael	Gribskov	gribskov@purdue.edu	Steering Committee
Shannon	McWeeney	mcweeney@ohsu.edu	Steering Committee
Andrew	Patterson	adp117@psu.edu	Steering Committee
Andrew	Pekosz	apekosz@jhsph.edu	Steering Committee
Stephen	Smale	smale@mednet.ucla.edu	Steering Committee
Michael	Zhang	michael.zhang@utdallas.edu	Steering Committee
Brian	Brunk	brunkb@upenn.edu	EupathDB/FungiDB
Thomas	Brettin	brettin@anl.gov	PATRIC
Brian	Aevermann	baeverma@jcvi.org	ViPR/IRD
Richard	Scheuermann	rscheuermann@jcvi.org	ViPR/IRD
Michelle	Schwalbe	mschwalbe@nas.edu	NASEM
Ishwar	Chandramouliswaran	ishwarc@nih.gov	NIH/NIAID
Marciela	DeGrace	marciela.degrace@nih.gov	NIH/NIAID
Emily	Erbelding	emily.erbelding@nih.gov	NIH/NIAID
Maria	Giovanni	mgiovanni@niaid.nih.gov	NIH/NIAID
Megan	Hoffmann	megan.hoffmann@nih.gov	NIH/NIAID

Menu

Breakfast:

- Chilled orange, apple, cranberry and grapefruit juices
- Hand-cut seasonal fruit
- Assorted breads and coffee cakes
- Variety of organic yogurt and granola

Mid-Morning Breaks:

- Seasonal whole fruit
- Whole roasted almonds
- Assorted granola bars
- Watermelon-lime, strawberry-lemon, and ginger-mango shots

Mid-Afternoon Breaks:

- Fresh-baked brownies
- Hummus with toasted pita bread
- Seasonal berries
- Artisan cheese and crackers

Throughout the Day:

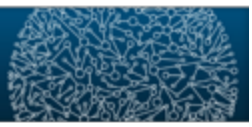
- Assorted soft drinks
- Iced teas
- Mineral water
- Coffee and tea

Lunches and Dinners:

Day	Meal	Type	Selections
Sunday	Dinner	Hor d'oeuvres	Fruit display[1]
Sunday	Dinner	Hor d'oeuvres	Garden fresh vegetable crudites[1]
Sunday	Dinner	Hor d'oeuvres	Warm spinach artichoke dip with toasted pita chips
Sunday	Dinner	Hor d'oeuvres	Vietnamese spring roll with fresh mint, soy and sweet chili sauce
Sunday	Dinner	Hor d'oeuvres	Pork pot stickers with soy chili sauce
Sunday	Dinner	Hor d'oeuvres	Peanut chicken satay
Monday	Lunch	Buffet	Garden salad with croutons and parmesan cheese
Monday	Lunch	Buffet	Potato salad
Monday	Lunch	Buffet	Soup du jour
Monday	Lunch	Buffet	Artisan sandwich rolls; cheddar, Swiss and provolone cheeses; condiments
Monday	Lunch	Buffet	Sandwich fillers: chicken salad (with cranberries and cashews), shaved roast beef with horseradish cream, grilled veggies, or roast turkey
Monday	Lunch	Buffet	Fresh-baked cookies and brownies
Monday	Dinner	Station	Salad Station[1]
Monday	Dinner	Station	Quesadilla Station[1]
Monday	Dinner	Station	Pasta Station[1]
Tuesday	Lunch	Buffet	Tossed green salad
Tuesday	Lunch	Buffet	Tomato and mozzarella salad
Tuesday	Lunch	Buffet	Herb-seared chicken with Cipolline onions and jus lie
Tuesday	Lunch	Buffet	Grilled flat iron steak, wild mushroom ragout
Tuesday	Lunch	Buffet	Roasted Yukon potatoes with fresh herbs
Tuesday	Lunch	Buffet	Green beans with toasted almond butter
Tuesday	Lunch	Buffet	Cheesecake: chocolate chip, raspberry and cappuccino
Tuesday	Dinner	Hor d'oeuvres	Garden fresh vegetable crudites[1]
Tuesday	Dinner	Hor d'oeuvres	International and domestic cheese[1]
Tuesday	Dinner	Hor d'oeuvres	Tomato and mozzarella bruschetta
Tuesday	Dinner	Hor d'oeuvres	Smoked salmon on toasted pita, dill cream, micro-celery greens
Tuesday	Dinner	Hor d'oeuvres	Mini Beef Wellington
Tuesday	Dinner	Hor d'oeuvres	Andouille sausage in puff pastry
Wednesday	Lunch	Buffet	Garden salad with croutons and parmesan cheese
Wednesday	Lunch	Buffet	Quinoa and roasted vegetable salad
Wednesday	Lunch	Buffet	Beluga lentil salad
Wednesday	Lunch	Buffet	Wraps (turkey club with bacon; roast beef with horseradish cream and marinated onions; or spring mix, grilled veggies, avocado and whole wheat tortilla)
Wednesday	Lunch	Buffet	Flash fried potato chips with Southport onion dip
Wednesday	Lunch	Buffet	Oatmeal raisin cookies
Wednesday	Lunch	Buffet	Lemon blueberry parfaits

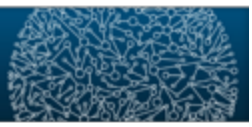
[1]Additional Details:
Hor d'oeuvres:
Garden fresh vegetable crudites Seasonal raw vegetables served with a variety of dipping sauces
Fruit display Sliced seasonal and tropical fruits with berries and dipping sauces
International and domestic cheese Camembert with Calvados (apple brandy), Caprino Cremoso with truffle, Blue Affine, and Pleasant Ridge Reserve, garnished with seasonal fruit and assorted crackers
Stations:
Salad Station Caesar salad (romaine lettuce, parmesan cheese, croutons and Caesar dressing) Mesclun green salad (assorted salad greens, shredded carrots, cucumber, cherry tomato and Balsamic dressing)
Quesadilla Station Made to order: soft tortillas and choice of fajita-marinated beef or chicken, farmer's cheese and cilantro Served with: salsa, sour cream, guacamole
Pasta Station Pastas: wild mushroom ravioli, cavatappi (helical tubes) or bowtie Sauces: roasted garlic cream, bolognese or pesto cream Served with: shaved asiago and parmesan cheeses and fresh-baked garlic breadsticks

Notes

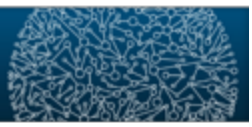


Notes

Notes



Notes



I. OVERVIEW OF THE FUNDING OPPORTUNITY

Program Announcement for the Department of Defense

Defense Health Program

Congressionally Directed Medical Research Programs

Peer Reviewed Medical Research Program

Focused Program Award

Announcement Type: Initial

Funding Opportunity Number: W81XWH-17-PRMRP-FPA

Catalog of Federal Domestic Assistance Number: 12.420 Military Medical Research and Development

SUBMISSION AND REVIEW DATES AND TIMES

- **Pre-Application Submission Deadline:** 5:00 p.m. Eastern time (ET), July 20, 2017
- **Invitation to Submit an Application:** August 2017
- **Application Submission Deadline:** 11:59 p.m. ET, October 26, 2017
- **End of Application Verification Period:** 5:00 p.m. ET, October 31, 2017
- **Peer Review:** December 2017
- **Programmatic Review:** February 2018

This Program Announcement must be read in conjunction with the General Application Instructions, version 20170516. The General Applications Instructions document is available for downloading from the Grants.gov funding opportunity announcement by selecting the "Package" tab, clicking "Preview," and then selecting "Download Instructions."

TABLE OF CONTENTS

I. OVERVIEW OF THE FUNDING OPPORTUNITY 1

II. DETAILED INFORMATION ABOUT THE FUNDING OPPORTUNITY 4

II.A. Program Description 4

II.A.1. FY17 PRMRP Topic Areas 4

II.B. Award Information 6

II.C. Eligibility Information 9

II.C.1. Eligible Applicants 9

II.C.2. Cost Sharing 10

II.C.3. Other 10

II.D. Application and Submission Information 10

II.D.1. Address to Request Application Package 11

II.D.2. Content and Form of the Application Submission 11

II.D.3. Dun and Bradstreet Universal Numbering System (DUNS) Number and System for Award Management (SAM) 29

II.D.4. Submission Dates and Times 29

II.D.5. Funding Restrictions 30

II.D.6. Other Submission Requirements 31

II.E. Application Review Information 31

II.E.1. Criteria 31

II.E.2. Application Review and Selection Process 36

II.E.3. Integrity and Performance Information 37

II.E.4. Anticipated Announcement and Federal Award Dates 37

II.F. Federal Award Administration Information 37

II.F.1. Federal Award Notices 37

II.F.2. Administrative and National Policy Requirements 39

II.F.3. Reporting 39

II.G. Federal Awarding Agency Contacts 40

II.G.1. CDMRP Help Desk 40

II.G.2. Grants.gov Contact Center 40

II.H. Other Information 40

II.H.1. Program Announcement and General Application Instructions Versions 40

II.H.2. Administrative Actions 40

II.H.3. Application Submission Checklist 43

APPENDIX 1 ACRONYM LIST 45
APPENDIX 2 AREAS OF ENCOURAGEMENT 46
APPENDIX 3 DOD AND VA WEBSITES 65

II. DETAILED INFORMATION ABOUT THE FUNDING OPPORTUNITY

II.A. Program Description

Applications to the Fiscal Year 2017 (FY17) Peer Reviewed Medical Research Program (PRMRP) are being solicited for the Defense Health Agency (DHA) J9, Research and Development Directorate, by the U.S. Army Medical Research Acquisition Activity (USAMRAA) using delegated authority provided by United States Code, Title 10, Section 2358 (10 USC 2358). As directed by the Office of the Assistant Secretary of Defense for Health Affairs (OASD[HA]), the DHA manages the Defense Health Program (DHP) Research, Development, Test, and Evaluation (RDT&E) appropriation. The execution management agent for this Program Announcement is the Congressionally Directed Medical Research Programs (CDMRP). The PRMRP was initiated in 1999 to provide support for military health-related research of exceptional scientific merit. Appropriations for the PRMRP from FY99 through FY16 totaled $1.371 billion. The FY17 appropriation is $300 million (M).

The vision of the FY17 PRMRP is to improve the health and well-being of all military Service members, Veterans, and beneficiaries. The PRMRP challenges the scientific and clinical communities to address at least one of the FY17 Topic Areas with original ideas that foster new directions along the entire spectrum of research and clinical care. The program seeks applications in laboratory, clinical, behavioral, epidemiologic, and other areas of research to advance knowledge in disease etiology, improve prevention, detection, diagnosis, treatment, and quality of life for those affected by a relevant disease or condition, and to develop and validate clinical care or public health guidelines.

II.A.1. FY17 PRMRP Topic Areas

All applications for PRMRP funding must specifically address at least one of the Topic Areas as directed by Congress and must be directly relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries. If the proposed research does not specifically address at least one of the FY17 PRMRP Topic Areas, the Government will administratively withdraw the application. The Government reserves the right to reassign the application's Topic Area if submitted under an inappropriate Topic Area. The FY17 PRMRP Topic Areas are listed below.

- Acute Lung Injury
- Antimicrobial Resistance
- Arthritis
- Burn Pit Exposure
- Chronic Migraine and Post-Traumatic Headache
- Congenital Heart Disease
- Constrictive Bronchiolitis
- Diabetes
- Diarrheal Diseases
- Dystonia
- Early Trauma Thermal Regulation
- Eating Disorders

- Emerging Infectious Diseases
- Epidermolysis Bullosa
- Focal Segmental Glomerulosclerosis
- Fragile X
- Guillain-Barré Syndrome
- Hepatitis B and C
- Hereditary Angioedema
- Hydrocephalus
- Immunomonitoring of Intestinal Transplants
- Inflammatory Bowel Diseases
- Influenza
- Integrative Medicine
- Interstitial Cystitis
- Malaria
- Metals Toxicology
- Mitochondrial Disease
- Musculoskeletal Disorders
- Nanomaterials for Bone Regeneration
- Non-Opioid Pain Management
- Pancreatitis
- Pathogen-Inactivated Dried Cryoprecipitate
- Polycystic Kidney Disease
- Post-Traumatic Osteoarthritis
- Pulmonary Fibrosis
- Respiratory Health
- Rett Syndrome
- Rheumatoid Arthritis
- Scleroderma
- Sleep Disorders
- Spinal Muscular Atrophy
- Sustained-Release Drug Delivery
- Tinnitus
- Tuberculosis
- Vaccine Development for Infectious Disease
- Vascular Malformations
- Women's Heart Disease

Research relevant to one or more FY17 PRMRP Topic Areas may be considered for funding. ***Applicants should select the FY17 PRMRP Program Announcement most appropriate to the stage of the proposed research.*** Areas of Encouragement related to the FY17 PRMRP Topic Areas have been identified by the Department of Defense (DoD), the Department of Veterans Affairs (VA), and other relevant stakeholders (Appendix 2). Applicants are urged to read and consider these Areas of Encouragement before preparing their applications. ***The information provided is not exhaustive, and applicants are not restricted to submitting applications that address an Area of Encouragement on this list.***

II.B. Award Information

The PRMRP Focused Program Award mechanism is intended to optimize research and accelerate the solution for a critical question related to at least one of the Congressionally directed FY17 PRMRP Topic Areas through a synergistic, multidisciplinary research program. The anticipated total costs budgeted for the entire period of performance for an FY17 PRMRP Focused Program Award will not exceed **$10M**. Refer to Section II.D.5, Funding Restrictions, for detailed funding information.

Key aspects of this award include:

Overarching Challenge: Focused Program Award applications must describe a unifying, overarching challenge that will be addressed by a set of research projects. The overarching challenge must be relevant to a critical problem or question in the field of research and/or patient care in at least one of the FY17 PRMRP Topic Areas.

Research Projects: Applications shall include multiple, distinct research projects led by individual project leaders that address complementary aspects of the overarching challenge. Applicants are strongly encouraged to submit a minimum of four research projects; additional studies are allowed. While individual projects must be capable of standing on their own high scientific merits, they must also be interrelated and synergistic with the other proposed projects and advance a solution beyond what would be possible through individual efforts. The exploration of multiple hypotheses or viewpoints of the same line of questioning is encouraged. This award mechanism is not intended to support a series of research projects that are dependent on the success of any other project. Each project should propose a unique approach to addressing the overarching challenge and be capable of producing research findings with potential to impact the field and/or patient care. Individual research projects may range from exploratory, hypothesis-developing studies through small-scale clinical trials (i.e., up to and including Phase II or equivalent). There should be a clear intent to progress toward translational/ clinical work over the course of the effort.

Implementation: The research strategy to address the overarching challenge must be supported by a detailed plan that identifies critical milestones; outlines the knowledge, resources, and technical innovations that will be utilized to achieve the milestones; and explains how the outcomes will be translated to patients. A robust statistical plan and statistical expertise should be included where applicable. A plan for assessing individual project performance and progress toward addressing the overarching challenge must be included in the implementation plan. Plans to include an External Advisory Board are encouraged; however, applicants must be careful to avoid potential conflicts of interest (COIs) during review of the application by ensuring no contact with, recruiting of, or naming of specific members in the application. For multi-institutional collaborations, plans for communication and data transfer among the collaborating institutions, as well as how data, specimens, and/or imaging products obtained during the study will be handled, must be included. An intellectual and material property plan agreed to by participating organizations is required in the application's supporting documentation.

Research Team: The overall effort will be led by a Principal Investigator (PI) with demonstrated success in leading large, focused projects. The PI is required to devote a minimum

of 20% effort to this award. The PI should create an environment that fosters and supports collaboration and innovation in a way that engages all members of the team in all aspects of the research plan. The research team assembled by the PI should be highly qualified and multidisciplinary, with identified project leaders for each of the complementary and synergistic research projects. The resources and expertise brought to the team by each project leader should combine to create a robust, synergistic collaboration. The PRMRP Science Officer assigned to a resulting award must be invited to participate in periodic research team meetings. The plan for such meetings should be noted in the application.

Milestone Meeting: The PI will be required to present an update on progress toward accomplishing the goals of the award at a Milestone Meeting to be held in the National Capital Region after the conclusion of Year 2 of the period of performance. The PI may bring up to three additional members of the research team to the meeting. The Milestone Meeting will be attended by members of the PRMRP Programmatic Panel, CDMRP staff, and the USAMRAA Grants Officer.

Military Relevance: Relevance to the healthcare needs of military Service members, Veterans, and beneficiaries is a key feature of this award. Investigators are encouraged to consider the following characteristics as examples of how a project may demonstrate military relevance:

- Explanation of how the project addresses an aspect of the target disease/condition that has direct relevance to military Service members, Veterans, or other military health system beneficiaries

- Description of how the knowledge, information, products, or technologies gained from the proposed research could be implemented in a dual-use capacity to benefit the civilian population and also address a military need

- Use of military or Veteran populations or datasets in the proposed research, if appropriate to the proposed research project

- Involvement of military consultants (Army, Air Force) or specialty leaders (Navy, Marine Corps) to the Surgeons General in a relevant specialty area

PIs are strongly encouraged to integrate and/or align their research projects with DoD and/or VA research laboratories and programs. Collaboration with the DoD or VA is also encouraged. A list of websites that may be useful in identifying additional information about ongoing DoD and VA areas of research interest or potential opportunities for collaboration within the FY17 PRMRP Topic Areas can be found in Appendix 3.

Use of Active Duty Military and VA Populations: If the proposed research plan involves access to active duty military and/or VA patient populations or resources, the PI is responsible for establishing such access. If possible, access to target active duty military and/or VA patient populations/resources should be confirmed at the time of application submission by inclusion of a letter of support, signed by the lowest ranking person with approval authority, for studies involving active duty military Service members, Veterans, military- and/or VA-controlled study materials, and military and/or VA databases. If access cannot be confirmed at the time of application submission, the Government reserves the right to withhold or revoke funding until

the PI has demonstrated support for and access to the relevant population(s) and/or resources. Note that access to a Veteran population for clinical studies may only be obtained by either collaboration with a VA investigator where the VA investigator has a substantial role in the research or by advertising to the general public.

Research Involving Human Anatomical Substances, Human Subjects, or Human Cadavers: All DoD-funded research involving new and ongoing research with human anatomical substances, human subjects, or human cadavers must be reviewed and approved by the U.S. Army Medical Research and Materiel Command (USAMRMC) Office of Research Protections (ORP), Human Research Protection Office (HRPO) prior to research implementation. This administrative review requirement is in addition to the local Institutional Review Board (IRB) or Ethics Committee (EC) review. Local IRB/EC approval at the time of submission is ***not*** required. The HRPO is mandated to comply with specific laws and requirements governing all research involving human anatomical substances, human subjects, or human cadavers that is supported by the DoD. These laws and requirements will necessitate information in addition to that supplied to the IRB/EC. ***Allow a minimum of 2 to 3 months for HRPO regulatory review and approval processes***. When possible, protocols should be written for research with human subjects and/or human anatomical substances that are specific to the DoD-supported effort outlined in the submitted application. Submission to HRPO of protocols covering more than the scope of work in the DoD-funded award will require HRPO review of the entire protocol as DoD-supported research and may include extensive modifications to meet DoD human subjects protection requirements. Refer to the General Application Instructions, Appendix 1, and the Human Subject Resource Document available on the electronic Biomedical Research Application Portal (eBRAP) "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) for additional information.

A clinical trial is defined as a prospective accrual of patients (human subjects) in whom an intervention (e.g., device, drug, biologic, surgical procedure, rehabilitative modality, behavioral intervention, or other) is tested for a measurable outcome with respect to safety, effectiveness, and/or efficacy. This outcome represents a direct effect on the subject of that intervention or interaction.

Research Involving Animals: All projects should adhere to a core set of standards for rigorous study design and reporting to maximize the reproducibility and translational potential of preclinical research. The standards are described in Landis, S.C., et al. A call for transparent reporting to optimize the predictive value of preclinical research. *Nature* 2012, 490:187-191 (www.nature.com/nature/journal/v490/n7419/full/nature11556.html). While these standards are written for preclinical studies, the basic principles of randomization, blinding, sample-size estimation, and data handling derive from well-established best practices in clinical studies. Applicants should consult the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines to ensure relevant aspects of rigorous animal research are adequately planned for and, ultimately, reported. The ARRIVE guidelines can be found at http://www.elsevier.com/__data/promis_misc/622936arrive_guidelines.pdf

All DoD-funded research involving new and ongoing research with animals must be reviewed and approved by the USAMRMC ORP Animal Care and Use Review Office (ACURO), in addition to the local Institutional Animal Care and Use Committee (IACUC) of record. IACUC

approval at the time of submission is ***not*** required. Specific documents relating to the use of animals in the proposed research will be requested **if the application is selected for funding**. The ACURO must review and approve all animal use prior to the start of working with animals, including amendments to ongoing projects. PIs must submit the institutional animal use protocol, IACUC approval of that protocol, and a version of the animal use appendix titled "Research Involving Animals." ***Allow at least 2 to 3 months for ACURO regulatory review and approval processes for animal studies.*** Refer to the General Application Instructions, Appendix 1, for additional information.

The CDMRP intends that information, data, and research resources generated under awards funded by this Program Announcement be made available to the research community (which includes both scientific and consumer advocacy communities) and to the public at large. For additional guidance, refer to the General Application Instructions, Appendix 2, Section K.

Awards will be made no later than September 30, 2018. For additional information refer to Section II.F.1, Federal Award Notices.

II.C. Eligibility Information

II.C.1. Eligible Applicants

II.C.1.a. Organization: All organizations, including international organizations, are eligible to apply.

Government Agencies within the United States: Local, state, and Federal Government agencies are eligible to the extent that applications do not overlap with their fully funded internal programs. Such agencies are required to explain how their applications do not overlap with their internal programs.

As applications for this Program Announcement may be submitted by extramural and intramural organizations, these terms are defined below.

Extramural Organization: An eligible non-DoD organization. Examples of extramural organizations include academia, biotechnology companies, foundations, Government, and research institutes. *Extramural Submission: Application submitted by a non-DoD organization to Grants.gov.*

Intramural DoD Organization: A DoD laboratory, DoD military treatment facility, and/or DoD activity embedded within a civilian medical center. *Intramural Submission: Application submitted by a DoD organization for an intramural investigator who is a DoD military or civilian employee working within a DoD laboratory or military treatment facility or in a DoD activity embedded within a civilian medical center.*

Note: Applications from an intramural organization or from an extramural non-DoD Federal organization may be submitted through a research foundation.

The USAMRAA makes awards to eligible organizations, not to individuals.

II.C.1.b. Principal Investigator:

- The PI must be an independent investigator at or above the level of Full Professor (or equivalent).
    - Project leaders for each of the complementary and synergistic research projects must be at or above the level of Assistant Professor (or equivalent).

An eligible PI, regardless of ethnicity, nationality, or citizenship status, must be employed by, or affiliated with, an eligible organization.

The CDMRP encourages all PIs to participate in a digital identifier initiative through Open Researcher and Contributor ID, Inc. (ORCID). Registration for a unique ORCID identifier can be done online at http://orcid.org/.

II.C.2. Cost Sharing

Cost sharing/matching is not an eligibility requirement.

II.C.3. Other

Extramural organizations must be able to access **.gov** and **.mil** websites in order to fulfill the financial and technical deliverable requirements of the award and submit invoices for payment.

There are no limitations on the number of applications for which an investigator may be named as a PI.

For general information on required qualifications for award recipients, refer to the General Application Instructions, Appendix 3.

Refer to Section II.H.2, Administrative Actions, for a list of administrative actions that may be taken if a pre-application or application does not meet the administrative, eligibility, or ethical requirements defined in this Program Announcement.

II.D. Application and Submission Information

Submission of applications that are essentially identical or propose essentially the same research project to different funding opportunities within the same program and fiscal year is prohibited and will result in administrative withdrawal of the duplicative application(s). ***As an exception, applicants may submit a research project described in their FY17 PRMRP Focused Program Award to the Discovery Award (Funding Opportunity Number: W81XWH-17-PRMRP-DA), Investigator-Initiated Research Award (Funding Opportunity Number: W81XWH-17-PRMRP-IIRA), and/or Clinical Trial Award (Funding Opportunity Number: W81XWH-17-PRMRP-CTA); however, accepting multiple awards to support the same project will not be allowed.***

Extramural Submission is defined as an application submitted by a non-DoD organization to Grants.gov.

Intramural Submission is defined as an application submission by a DoD organization for an intramural investigator, who is a DoD military or civilian employee working within a DoD laboratory or military treatment facility, or working in a DoD activity embedded within a civilian medical center.

II.D.1. Address to Request Application Package

Submitting Extramural and Intramural Organizations: Pre-application content and forms can be accessed at eBRAP (https://eBRAP.org).

Submitting Extramural Organizations: Full application packages can be accessed at Grants.gov.

Submitting Intramural DoD Organizations: Full application packages can be accessed at eBRAP.org.

Contact information for the CDMRP Help Desk and the Grants.gov Contact Center can be found in Section II.G, Federal Awarding Agency Contacts.

II.D.2. Content and Form of the Application Submission

Submission is a two-step process requiring both ***pre-application*** and ***full application*** as indicated below. The submission process should be started early to avoid missing deadlines. There are no grace periods.

Pre-application Submission: All pre-applications for both extramural and intramural organizations must be submitted through eBRAP (https://eBRAP.org/).

eBRAP is a multifunctional web-based system that allows PIs to submit their pre-applications electronically through a secure connection, to view and edit the content of their pre-applications and full applications, to receive communications from the CDMRP, and to submit documentation during award negotiations and period of performance.

Full Application Submission: Full applications must be submitted through the online portals as described below.

***Submitting Extramural Organizations*:** Full applications from extramural organizations must be submitted through Grants.gov. Applications submitted by extramural organizations (e.g., research foundations) on behalf of intramural DoD or other Federal organizations or investigators will be considered extramural submissions.

Submitting Intramural DoD Organizations: Intramural DoD organizations may submit full applications to either eBRAP or Grants.gov. Intramural DoD organizations that are unable to submit to Grants.gov should submit through eBRAP. Intramural DoD organizations with the capability to submit through Grants.gov may submit following the instructions for extramural submissions through Grants.Gov or may submit to eBRAP. Applications from extramural organizations, including non-DoD Federal organizations, received through eBRAP will be withdrawn. See definitions in Section II.C.1, Eligible Applicants.

For both Extramural and Intramural applicants: A key feature of eBRAP is the ability of an organization's representatives and PIs to view and modify the full application submissions associated with them. eBRAP will validate full application files against the specific Program Announcement requirements and discrepancies will be noted in an email to the PI and in the Full Application Files tab in eBRAP. It is the applicant's responsibility to review all application components for accuracy as well as ensure proper ordering as specified in this Program Announcement.

The application title, eBRAP log number, and all information for the PI, Business Official(s), performing organization, and contracting organization must be consistent throughout the entire pre-application and application submission process. Inconsistencies may delay application processing and limit or negate the ability to view, modify, and verify the application in eBRAP. If any changes need to be made, the applicant should contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507 prior to the application deadline.

II.D.2.a. Step 1: Pre-Application Submission Content

During the pre-application process, each submission is assigned a unique log number by eBRAP. This unique eBRAP log number will be needed during the full application submission process.

To begin the pre-application process, first select whether the submitting organization is extramural or intramural, then confirm your selection or cancel. **Incorrect selection of extramural or intramural submission type may result in delays in processing.**

If an error has been made in the selection of extramural versus intramural and the pre-application submission deadline has passed, the PI or Business Official must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

All pre-application components must be submitted by the PI through eBRAP (https://eBRAP.org/). Because the invitation to submit an application is based on the contents of the pre-application, investigators should not change the title or research objectives after the pre-application is submitted.

PIs and organizations identified in the pre-application should be the same as those intended for the subsequent application submission. If any changes are necessary after submission of the pre-application, the PI must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

The pre-application consists of the following components, which are organized in eBRAP by separate tabs (refer to the General Application Instructions, Section II.B, for additional information on pre-application submission):

- **Tab 1 – Application Information**

  Select the FY17 PRMRP Topic Area addressed by the proposed research. If the proposed research project is aligned with more than one FY17 PRMRP Topic Area, select the Topic Area of highest relevance as the required first choice.

- **Tab 2 – Application Contacts**

  Enter contact information for the PI. Enter the organization's Business Official responsible for sponsored program administration (the "person to be contacted on matters involving this application" in Block 5 of the Grants.gov SF424 (R&R) Form for extramural submissions). The Business Official must be either selected from the eBRAP list or invited in order for the pre-application to be submitted.

  Select the performing organization (site at which the PI will perform the proposed work) and the contracting organization (organization submitting on behalf of the PI, which corresponds to Block 5 on the Grants.gov SF424 (R&R) Form for extramural submissions), and click on "*Add Organizations to this Pre-application.*" The organizations must be either selected from the eBRAP drop-down list or invited in order for the pre-application to be submitted.

  It is recommended that PIs identify an Alternate Submitter in the event that assistance with pre-application submission is needed.

- **Tab 3 – Collaborators and Key Personnel**

  Enter the name, organization, and role of all collaborators and key personnel associated with the application.

  FY17 PRMRP Programmatic Panel members should not be involved in any pre-application or application. For questions related to Panel members and pre-applications or applications, refer to Section II.H.2.c, Withdrawal, or contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

  To preserve the integrity of its peer and programmatic review processes, the CDMRP discourages inclusion of any employee of its review contractors having any role in pre-application or application preparation, research, or other duties for submitted pre-applications or applications. For FY17, the identities of the peer review contractor and the programmatic review contractor may be found at the CDMRP website (http://cdmrp.army.mil/about/2tierRevProcess). Pre-applications or applications that include names of personnel from either of these companies will be administratively withdrawn unless plans to manage COIs are provided and deemed appropriate by the Grants Officer. Refer to the General Application Instructions, Appendix 3, for detailed information.

- **Tab 4 – Conflicts of Interest**

  List all individuals other than collaborators and key personnel who may have a COI in the review of the application (including those with whom the PI has a personal or professional relationship). Refer to the General Application Instructions, Appendix 3, Section C, for further information regarding COIs.

- **Tab 5 – Pre-Application Files**

  Note: Upload documents as individual PDF files unless otherwise noted. eBRAP will not allow a file to be uploaded if the number of pages exceeds the limit specified below.

- **Preproposal Narrative (five-page limit):** The Preproposal Narrative page limit applies to text and non-text elements (e.g., figures, tables, graphs, photographs, diagrams, chemical structures, drawings) used to describe the project. Inclusion of URLs that provide additional information to expand the Preproposal Narrative and could confer an unfair competitive advantage is prohibited and may result in administrative withdrawal of the pre-application.

  The Preproposal Narrative should include the following:

  - **Topic Area:** Describe how the proposed program relates to at least one of the FY17 PRMRP Topic Areas. If applicable, describe how the proposed research program addresses an FY17 PRMRP Area of Encouragement (Appendix 2).

  - **Overarching Challenge:** Describe the unifying challenge or question to be addressed and how it is relevant to a critical problem or question in the field of research and/or patient care in at least one of the FY17 PRMRP Topic Area(s). Clearly articulate the rationale for the overarching challenge; include relevant preliminary data and literature citations.

  - **Research Strategy:** The FY17 PRMRP Focused Program Award strongly encourages a minimum of four individual but complementary research projects addressing the overarching challenge. For each proposed project, state the hypothesis to be tested, the specific aims, and the objectives to be reached. Briefly describe the experimental approach. Describe how the projects are interrelated to and synergistic with each other and align with the overarching challenge.

  - **Impact:** Describe the potential short-term and long-term impact of the proposed research on at least one of the FY17 PRMRP Topic Areas and its related research field(s), and patient population(s). Explain how the effort is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

  - **Research Team:** Briefly describe the composition, expertise, and organization of the research team. Identify the project leaders and describe each team member's role in the projects, with additional emphasis on the leadership role of the PI. Briefly describe how these features will facilitate the success of the key aspects of the projects.

  - **Clinical Trial (if applicable):** If the proposed research includes a clinical trial(s), briefly state the clinical intervention(s), subject population(s), and the type and phase of the clinical trial(s). Describe the objectives of the clinical trial(s), how it addresses the overarching challenge, and how it complements the other proposed projects. ***Only small-scale (i.e., up to and including Phase II or equivalent) clinical trials are allowed.***

- **Pre-Application Supporting Documentation:** The items to be included as supporting documentation for the pre-application ***must be uploaded as individual files*** and are limited to the following:

- References Cited (one-page limit): List the references cited (including URLs if available) in the Preproposal Narrative using a standard reference format that includes the full citation (i.e., author[s], year published, reference title, and reference source, including volume, chapter, page numbers, and publisher, as appropriate).

- List of Abbreviations, Acronyms, and Symbols: Provide a list of abbreviations, acronyms, and symbols used in the Preproposal Narrative.

- Key Personnel Biographical Sketches (five-page limit per individual). ***All biographical sketches should be uploaded as a single combined file.*** Biographical sketches should be used to demonstrate background and expertise through education, positions, publications, and previous work accomplished.

- **Tab 6 – Submit Pre-Application**

  This tab must be completed for the pre-application to be accepted and processed.

Pre-Application Screening

Pre-Application Screening Criteria

To determine the technical merits of the pre-application and the relevance to the mission of the DHP and the PRMRP, pre-applications will be screened based on the following criteria:

- **Overarching Challenge:** How well the unifying challenge or question addresses a critical problem or question in the field of research and/or patient care of one or more FY17 PRMRP Topic Area(s). How well the rationale supports the overarching challenge.

- **Research Strategy:** How well a hypothesis and specific aims are defined for each proposed project and to what extent each project's approach will address them. How well the proposed projects complement each other and synergistically address the overarching challenge to advance a solution beyond what would be possible through individual efforts.

- **Impact:** Whether the potential immediate and long-range outcome(s)/product(s) (intellectual and/or material) of the proposed research, if successful, will impact a central critical problem or question in the field of research and/or patient care in the FY17 PRMRP Topic Area(s) addressed. To what degree the project is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

- **Research Team:** To what degree the background and expertise of the PI, project leaders, and key personnel are appropriate with respect to their abilities to successfully complete the projects and the extent to which the PI is well prepared and committed to lead the research team and proposed projects.

Notification of Pre-Application Screening Results

Following the pre-application screening, PIs will be notified as to whether or not they are invited to submit applications; however, they will not receive feedback (e.g., a critique of strengths and

weaknesses) on their pre-application. The estimated time frame for notification of invitation to submit an application is indicated in Section I, Overview of the Funding Opportunity. Invitations to submit a full application are based on the Pre-Application Screening Criteria listed above.

II.D.2.b. Step 2: Full Application Submission Content

Applications will not be accepted unless the PI has received notification of invitation.

All contributors and administrators to the application must use matching compatible versions of Adobe software when editing and preparing application components. The use of different software versions will result in corruption of the submitted file. Refer to the General Application Instructions, Section III, for details on compatible Adobe software.

The CDMRP cannot make allowances/exceptions to its policies for submission problems encountered by the applicant organization using system-to-system interfaces with Grants.gov.

Each application submission must include the completed full application package for this Program Announcement. The full application package is submitted by the Authorized Organizational Representative through Grants.gov (http://www.grants.gov/) for extramural organizations or through eBRAP (https://ebrap.org/) for intramural organizations. See Table 1 below for more specific guidelines.

II.D.2.b.i. Full Application Guidelines

Extramural organizations, including non-DoD Federal agencies, must submit full applications through Grants.gov. Submissions of extramural applications through eBRAP may be withdrawn.

Table 1. Full Application Submission Guidelines

Extramural Submissions	Intramural DoD Submissions
Application Package Location	
Download application package components for W81XWH-17-PRMRP-FPA from Grants.gov (http://www.grants.gov).	Download application package components for W81XWH-17-PRMRP-FPA from eBRAP (https://ebrap.org).
Full Application Package Components	
SF424 (R&R) Application for Federal Assistance Form: Refer to the General Application Instructions, Section III.A.1, for detailed information.	**Tab 1 – Summary:** Provide a summary of the application information. **Tab 2 – Application Contacts:** This tab will be pre-populated by eBRAP; add Authorized Organizational Representative.

Extramural Submissions	Intramural DoD Submissions
Descriptions of each required file can be found under Full Application Submission Components: • Attachments • Research & Related Senior/Key Person Profile (Expanded) • Research & Related Budget • Project/Performance Site Location(s) Form • R&R Subaward Budget Attachment(s) Form (if applicable)	**Tab 3 – Full Application Files:** Upload files under each Application Component in eBRAP. Descriptions of each required file can be found under Full Application Submission Components: • Attachments • Key Personnel • Budget • Performance Sites **Tab 4 – Application and Budget Data:** Review and edit proposed project start date, proposed end date, and budget data pre-populated from the Budget Form.
Application Package Submission	
Submit package components to Grants.gov (https://www.grants.gov/). If either the Project Narrative or the budget fails eBRAP validation or if the Project Narrative or the budget need to be modified, an updated Grants.gov application package must be submitted via Grants.gov as a "Changed/Corrected Application" with the previous Grants.gov Tracking ID prior to the application submission deadline.	**Submit package components to eBRAP (https://ebrap.org).** **Tab 5 – Submit/Request Approval Full Application:** After all components are uploaded and prior to the full application submission deadline, enter your password in the space provided "Enter Your Password Here" and press the "Submit Full Application" button. eBRAP will notify your Resource Manager/Comptroller or equivalent Business Official by email to log into eBRAP to review and to approve prior to the application submission deadline.
Application Verification Period	
The full application package submitted to Grants.gov may be viewed and modified in eBRAP until the end of the application verification period. During the application verification period, the full application package, ***with the exception of the Project Narrative and Budget Form,*** may be modified.	After eBRAP has processed the full application, the organizational Resource Manager/ Comptroller or equivalent Business Official and PI(s) will receive an email notification of this status and will be able to view and modify application components in eBRAP. During the application verification period, the full application package, ***with the exception of the Project Narrative and Budget Form,*** may be modified.
Further Information	
Refer to the General Application Instructions, Section III, for further information regarding Grants.gov requirements.	Refer to the General Application Instructions, Section IV, for further information regarding eBRAP requirements.

The organization's Business Official or Authorized Organization Representative (or Resource Manager/Comptroller) should approve/verify the full application submission prior to the application verification deadline.

Application viewing, modification, and verification in eBRAP are strongly recommended, but not required. ***The Project Narrative and Budget cannot be changed after the application submission deadline.*** Prior to the full application deadline, a corrected or modified full application package may be submitted. Other application components may be changed until the end of the application verification period. After the end of the application verification period, the full application cannot be modified.

Material submitted after the end of the application verification period, unless specifically requested by the Government, will not be forwarded for processing.

The full application package must be submitted using the unique eBRAP log number to avoid delays in application processing.

II.D.2.b.ii. Full Application Submission Components:

- **Extramural Applications Only –**

  SF424 (R&R) Application for Federal Assistance Form: Refer to the General Application Instructions, Section III.A.1, for detailed information.

- **Extramural and Intramural Applications –**

  Attachments:

  Each attachment to the full application components must be uploaded as an individual file in the format specified and in accordance with the formatting guidelines listed in the General Application Instructions, Appendix 4.

  For all attachments, ensure that the file names are consistent with the guidance. Attachments will be rejected if the file names are longer than 50 characters or incorrect file names that contain characters other than the following: A-Z, a-z, 0-9, underscore, hyphen, space, and period. In addition, there are file size limits that may apply in some circumstances. Individual attachments may not exceed 20 MB and the file size for the entire full application package may not exceed 200 MB.

  - **Attachment 1: Project Narrative (40-page limit):** Upload as "ProjectNarrative.pdf." The page limit of the Project Narrative applies to text and non-text elements (e.g., figures, tables, graphs, photographs, diagrams, chemical structures, drawings) used to describe the project. Inclusion of URLs that provide additional information to expand the Project Narrative and could confer an unfair competitive advantage is prohibited and may result in administrative withdrawal of the application.

    Describe the proposed project in detail using the outline below.

Overall Program: Provide a description of the comprehensive effort using the following outline. Applicants are strongly encouraged to submit a minimum of four research projects; additional studies are allowed. Emphasize areas of synergy throughout the narrative.

- **Overarching Challenge:** Describe the unifying, overarching challenge or question to be addressed and how it is relevant to a critical problem or question in the field of research and/or patient care in one or more FY17 PRMRP Topic Areas. Clearly articulate the rationale for the overarching challenge; include relevant literature citations. Clearly describe how the proposed research projects are interrelated and synergistic and will advance toward a solution through a multidisciplinary research program. Describe how each project will address the overarching challenge in a unique but complementary way and how the combined efforts of the projects will address the overarching challenge more effectively than if the projects were conducted independently.

- **Leadership:** Describe how the PI's research experience, leadership skills, and commitment to making an impact in his/her field of research and/or patient care demonstrate substantial qualifications to coordinate this collaborative effort. Describe the PI's demonstrated success in leading large, focused projects and outline the PI's responsibilities during the conduct of the award. Discuss the qualifications of the research team being brought together by the PI and how the assembled expertise will create a robust, synergistic collaboration necessary to address the overarching challenge and enable the success of the proposed research.

- **Implementation Plan and Environment:** Provide an overall strategic plan for completing the proposed projects that identifies critical milestones. Outline the knowledge, expertise, and technical innovations that the investigative team will utilize to make decisions, allocate resources, and accomplish the milestones. Describe and/or provide evidence that the research can be initiated without delay once the award is made. Present an overall management plan to facilitate consistent and intensive interactions by all team members, including aspects such as adherence to regulatory requirements, administrative support, and oversight to accelerate translation of the projects' outcomes to patients and/or for clinical use. Describe the research environment(s) and how the facilities and resources will support the research requirements and the collaboration. Outline shared resources and/or cores that will be created and/or leveraged through the award. Describe plans for communication, data transfer among the collaborating institutions, and how data, specimens, and/or imaging products obtained during the study will be handled. If applicable, describe how Standard Operating Procedures will be created, reviewed, implemented, and modified during the course of the award. Describe how individual project performance will be assessed during the course of the award, including progression toward defined milestones, realization of study objectives, and addressing the overarching challenge. If an External Advisory Board (EAB) is to be utilized, describe the role of the board and the expertise to be sought in its members. To avoid potential COIs in the review of the application, potential candidates for an EAB should not be contacted, recruited, or named during the application process.

Research Plan: Provide the following details for each proposed research project, organizing each project clearly and separately. Start each project on a separate page:

- **Title:** Provide a title for each project.

- **Project Leader:** Identify the project leader and any key personnel, as appropriate, describing each person's qualifications and specific contributions to the project.

- **Background:** Briefly describe the ideas and reasoning on which the proposed work is based. Provide sufficient preliminary data to support the feasibility of work proposed. If the project is exploratory/hypothesis-developing, preliminary data are not required. For each project, the leader must demonstrate logical reasoning and provide a sound scientific rationale for the proposed project as established through a critical review and analysis of published literature. If proposing translational or clinical research, it is important to describe the project showing proof of concept and, if applicable, efficacy in an in-vivo system(s) to support the translational feasibility and promise of the approach.

- **Hypothesis/Objective:** State the hypothesis to be tested and/or the objective(s) to be reached.

- **Specific Aims:** Concisely explain each project's specific aims. The specific aims should align with the overall goal of the program and associated tasks described in the Statement of Work.

- **Research Strategy:** Describe the experimental design, methods, and analyses, in sufficient detail for analysis. Provide a description of how the study will be controlled and how the study variables will be measured. If the project is a clinical trial, define the primary and secondary endpoints and how they will be assessed. Explain how the research strategy will address the overarching challenge and meet appropriate milestones. Address potential problem areas and present alternative methods and approaches. Clearly describe the statistical plan and the rationale for the statistical methodology as well as an appropriate power analysis, if applicable. If animal studies are proposed, describe how they will be conducted in accordance with the ARRIVE guidelines (http://www.elsevier.com/__data/promis_misc/622936arrive_guidelines.pdf). Justify how the model system or human subjects/samples are appropriate to the proposed research project. If human subjects or human biological samples will be used, describe the study population and include a detailed plan for the recruitment of human subjects or the acquisition of samples. Describe the availability of the proposed study population and past successes in recruiting similar populations. If active duty military, military families, and/or Veteran population(s) or datasets will be used in the proposed research project, describe how access to the population(s)/dataset(s) will be obtained. If applicable, describe how data will be reported and how it will be assured that the documentation will support a regulatory filing with the U.S. Food and Drug Administration (FDA).

- **Statistical Plan:** Clearly describe a statistical plan appropriate to the type of study; provide the rationale for the statistical methodology. Define the number of samples and/or subjects (animal and/or human) to be used, and include a power analysis to demonstrate that the sample size is appropriate to meet the objectives of the study.

- **Impact:** Describe the anticipated outcome(s)/product(s) (intellectual and/or material) that will be directly attributed to the results of the proposed research. Explain the anticipated long-term gains from this research. Compare to the information known/products currently available, if applicable.

- **Clinical Trial (if applicable):** ***Only small-scale (e.g., up to and including Phase II or equivalent) clinical trials are allowed.*** Provide detailed plans for initiating and conducting the clinical trial during the course of this award. As appropriate, outline a plan for applying for and obtaining Investigational New Drug/Investigational Device Exemption (IND/IDE) status (or other FDA approvals). Describe the type of clinical trial to be performed (e.g., prospective, randomized, controlled) and indicate the phase of trial and/or class of device, as appropriate. Outline the regulatory strategy. Provide preclinical and/or clinical evidence to support the safety of the intervention.

    - Identify the intervention to be tested and describe the projected outcomes. Describe how the proposed intervention compares with currently available interventions and/or standards of care. Include a discussion of any current clinical use of the intervention under investigation, and/or details of its study in clinical trials for other indications (as appropriate).

    - Describe the methods that will be used to recruit a sample of human subjects from the accessible population (e.g., convenience, simple random, stratified random). Provide information on and justification for the inclusion and exclusion criteria.

    - Describe the process for obtaining informed consent and any screening procedures required to determine eligibility for study participation.

    - Describe the human subject-to-group assignment process (e.g., randomization, block randomization, stratified randomization, age-matched controls, alternating group, or other procedures), if applicable. Explain the specific actions to accomplish the group assignment (e.g., computer assignment, use of table of random numbers). If multiple site studies are involved, state the approximate number of subjects to be enrolled at each site.

    - Provide evidence to document the availability of and access to all critical reagents, including the intervention itself, if applicable, for the duration of the proposed trial.

    - Describe how quality control will be addressed. Describe how compliance with Good Laboratory Practices (GLP), Good Manufacturing Practices (GMP), and Good Clinical Practices (GCP) guidelines will be established, monitored, and maintained, as applicable.

- Describe the composition of the clinical trial team. Provide details on how the team (including investigator(s), study coordinator, and statistician) possesses the appropriate expertise in conducting clinical trials.

- **Attachment 2: Supporting Documentation:** Combine and upload as a single file named "Support.pdf." Start each document on a new page. If documents are scanned to PDF, the lowest resolution (100 to 150 dpi) should be used. The Supporting Documentation attachment should not include additional information such as figures, tables, graphs, photographs, diagrams, chemical structures, or drawings. These items should be included in the Project Narrative. Any additional material viewed as an extension of the Project Narrative will be removed or may result in administrative withdrawal of the application.

  There are no page limits for any of these components unless otherwise noted. Include only those components described below; inclusion of items not requested will result in the removal of those items or may result in administrative withdrawal of the application.

  - References Cited: List the references cited (including URLs, if available) in the Project Narrative using a standard reference format that includes the full citation (i.e., author[s], year published, title of reference, source of reference, volume, chapter, page numbers, and publisher, as appropriate).

  - List of Abbreviations, Acronyms, and Symbols: Provide a list of abbreviations, acronyms, and symbols.

  - Facilities, Existing Equipment, and Other Resources: Describe the facilities and equipment available for performance of the proposed project and any additional facilities or equipment proposed for acquisition at no cost to the award. Indicate whether or not Government-furnished facilities or equipment are proposed for use. If so, reference should be made to the original or present Government award under which the facilities or equipment items are now accountable. There is no form for this information.

  - Publications and/or Patents: Include a list of relevant publication URLs and/or patent abstracts. If publications are not publicly available, then copies of up to eight published manuscripts may be included in Attachment 2. Extra items will not be reviewed.

  - Letters of Organizational Support: Provide a letter (or letters, if applicable), signed by the Department Chair or appropriate organization official, confirming the laboratory space, equipment, and other resources available for the project. Letters of support not requested in the Program Announcement, such as those from members of Congress, do not impact application review or funding decisions.

  - Letters of Collaboration: Provide a signed letter from each collaborating individual or organization that will demonstrate that the PI has the support or resources necessary for the proposed work. If an investigator at an intramural organization is

named as a collaborator on an application submitted through an extramural organization, the application must include a letter from the collaborator's Commander or Commanding Officer at the intramural organization that authorizes the collaborator's involvement.

- Letters of Commitment (if applicable): If the proposed study involves use of a commercially produced investigational drug, device, or biologic, provide a letter of commitment from the commercial entity indicating availability of the product for the duration of the study, support for the proposed phase of research, and support for the indication to be tested.

- Letters Confirming Access to Military or VA Patient Populations or Resources (if applicable): If the proposed research plan involves access to active duty military and/or VA patient populations or resources, include a letter of support, signed by the lowest ranking person with approval authority, confirming such access. If access cannot be confirmed at the time of application submission, the Government reserves the right to withhold or revoke funding until the PI has demonstrated support for and access to the relevant population(s) and/or resources.

- Intellectual Property: Information can be found in Code of Federal Regulations, Title 2, Part 200.315 (2 CFR 200.315), "Intangible Property."

  - Intellectual and Material Property Plan (if applicable): Provide a plan for resolving intellectual and material property issues among participating organizations.

- Data and Research Resources Sharing Plan: Describe how data and resources generated during the performance of the project will be shared with the research community. Refer to the General Application Instructions, Appendix 2, Section K, for more information about the CDMRP expectations for making data and research resources publicly available.

- **Attachment 3: Technical Abstract (one-page limit):** Upload as "TechAbs.pdf." The technical abstract is used by all reviewers. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information.*** Use only characters available on a standard QWERTY keyboard. Spell out all Greek letters, other non-English letters, and symbols. Graphics are not allowed.

  Clarity and completeness within the space limits of the technical abstract are highly important. Describe the proposed research effort including the following elements:

  - **Overarching Challenge:** Identify the unifying, overarching challenge or question that will be addressed by the research plan and describe how it relates to a critical problem or question in one or more of the FY17 PRMRP Topic Areas.

  - **Background:** Briefly articulate the rationale for the overarching challenge and the proposed research.

- **Research Plan:** Provide a brief description of the studies proposed, including hypotheses, objectives, and scientific approach.

- **Impact:** Briefly describe the potential short-term and long-term impact of the results of the proposed research on at least one of the FY17 PRMRP Topic Areas and its related research field(s) and patient population(s).

- **Military Relevance:** Explain how the effort is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

- **Attachment 4: Lay Abstract (one-page limit):** Upload as "LayAbs.pdf." The lay abstract is used by all reviewers. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information***. Use only characters available on a standard QWERTY keyboard. Spell out all Greek letters, other non-English letters, and symbols. Graphics are not allowed.

  State the FY17 PRMRP Topic Area(s) addressed by the proposed research program. Include a comprehensive overview of the effort that can be ***readily understood by readers without a background in science or medicine.*** Clearly describe the central critical problem or question to be addressed and the ultimate applicability and impact of the research. Do not duplicate the technical abstract.

- **Attachment 5: Statement of Work (SOW) (eight-page limit):** Upload as "SOW.pdf." The suggested SOW format and examples specific to different types of research projects are available on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm). For the Focused Program Award mechanism, use the SOW format example titled "SOW for Collaborative Projects ." The SOW must be in PDF format prior to attaching.

  The SOW should include a list of major tasks that support the proposed specific aims, followed by a series of subtasks outlined related to the major tasks and milestones within the period of performance. The SOW should describe only the work for which funding is being requested by this application and, as applicable, should also:

  Include the name(s) of the key personnel and contact information for each study site/ subaward site.

  Indicate the number (and type, if applicable) of research subjects (animal or human) and/or human anatomical samples projected or required for each task and at each site. Refer to the General Application Instructions, Appendix 1, for additional information regarding regulatory requirements.

  Briefly state the methods to be used.

  For studies with prospective accrual of human subjects, indicate quarterly enrollment targets.

Identify cell line(s) and commercial or organizational source(s) to be used. If human anatomical substances (including cell lines) will be used, specify whether or not identifiable information is accessible to the research team by any means.

If applicable, indicate timelines required for regulatory approvals relevant to human subjects research (e.g., IND/IDE applications) by the FDA or other Government agency.

- **Attachment 6: Impact Statement (two-page limit): Upload as "Impact.pdf."**

  - Explain why the proposed program is important and relevant to understanding the cause or progression of the disease or condition, and/or to developing improvements in prevention, detection, diagnosis, treatment, or quality of life in the FY17 PRMRP Topic Area(s) addressed. Describe how the overarching challenge addresses a central critical problem or question in the relevant Topic Area(s). If applicable, describe how the project addresses an FY17 PRMRP Area of Encouragement (Appendix 2). In addition to articulating the potential impact of the overall program, address the potential impact of each individual project. Explain how the various projects' outcomes will ultimately be translated to patients.

  - ***Describe the short-term impact:*** Detail the anticipated outcome(s)/product(s) (intellectual and/or material) that will be directly attributed to the results of the proposed research.

  - ***Describe the long-term impact:*** Explain the anticipated long-term gains from this research. Compare to the information known/products currently available, if applicable. Explain the long-range vision for how the research will impact the field of study and/or patient care.

- **Attachment 7: Military Relevance Statement (one-page limit): Upload as "MilRel.pdf."**

  - Describe how the proposed effort is responsive to the healthcare needs of military Service members, Veterans, and/or beneficiaries. Provide information about the incidence and/or prevalence of the disease or condition to be studied in the general population as well as in military Service members, Veterans, and/or beneficiaries.

  - If active duty military, military families, and/or Veteran population(s) or dataset(s) will be used in the proposed research project, describe the population(s)/dataset(s) and the appropriateness of the population(s)/dataset(s) for the proposed study. If a non-military population will be used for the proposed research project, explain how the population simulates the targeted population (i.e., military Service members, Veterans, and/or beneficiaries).

  - If applicable, show how the proposed research project aligns with DoD and/or VA areas of research interest and/or patient care. Provide a description of how the knowledge, information, products, or technologies gained from the research could be implemented in a dual-use capacity to benefit the civilian population and address a military need, as appropriate.

- **Attachment 8: Transition Plan and Regulatory Strategy (three-page limit): Upload as "Transition.pdf."**

  - Provide information on the methods and strategies proposed to move the product or knowledge outcomes of the program to the next phases of development and to eventual clinical use. Articulate this information for the overall effort as well as the individual projects. Applicants are encouraged to work with their organization's Technology Transfer Office (or equivalent) to develop the transition plan. The transition plan should include the components listed below, as appropriate:

  - A description of the outcomes expected upon completion of the proposed research efforts. Outcomes should be specific, measurable, and should include the intended end user.

  - Details of the funding strategy that will be used to bring the outcomes to the next phase of development and/or delivery to market or incorporation into patient care (e.g., specific potential industry partners, specific funding opportunities to be applied for).

  - Details of the development plan and FDA regulatory strategy, to include considerations for compliance with GMP, GLP, and GCP guidelines (if appropriate).

  - For knowledge outcomes, a description of how the knowledge will be further developed, disseminated, and incorporated into clinical/patient care.

  - A description of collaborations and other resources that will be used to provide continuity of development.

  - A brief schedule and milestones for bringing the outcomes to the next phase of development (e.g., further research, clinical trials, transition to industry, delivery to the market, incorporation into clinical practice, approval by the FDA).

  - If applicable, ownership rights and/or access to the intellectual property necessary for the development and/or commercialization of products or technologies supported with this award and the Government's ability to access such products or technologies in the future.

- **Attachment 9: Data and Research Resource Sharing Plan (one-page limit): Upload as "Sharing.pdf."**

  - Describe how data and resources generated during the performance of the proposed research projects will be shared with the research community. This includes cases where pre-existing data or research resources will be utilized and/or modified during the course of the proposed projects. Specifically describe a plan to make animal models, tissue samples, and other resources developed as part of the proposed research projects available to the scientific community. If there are limitations associated with a pre-existing agreement for the original data or research resources that preclude subsequent sharing, the applicant should explain this in the data- and/or

research resource-sharing plan. Refer to the General Application Instructions, Appendix 2, Section K, for more information about the CDMRP expectations for making data and research resources publicly available.

- In preparing requested budgets, applicants may include anticipated costs associated with data- and research resource-sharing (i.e., making a large dataset available to the public or developing an important resource for the scientific community).

- **Attachment 10: IND/IDE Documentation:** ***Only applicable for applications that include a clinical trial(s).*** If submitting multiple documents, start each document on a new page. **Combine and upload as a single file named "IND-IDE.pdf."** The IND/IDE Documentation Form located on the eBRAP website may ***not*** be used in place of this information:

  - State whether the trial(s) requires regulation by the FDA. If FDA regulation is required, describe the planned indication for the proposed product and whether an IND/IDE is necessary.

  - If an IND or IDE application is required, indicate when it was submitted to the FDA and provide an explanation of the status of the IND or IDE application (e.g., past the critical 30-day period, pending response to questions raised by the Agency, on clinical hold). If the IND or IDE application has not been submitted to the FDA yet, provide a detailed timeline with appropriate milestones for application preparation and submission. Identify any consultants or experts who will assist in the regulatory application, if applicable, and include a copy of any curricula vitae or biographical sketches in the Key Personnel Biographical Sketches section of the application. Provide a summary of previous meetings with the FDA on development of this product, if appropriate. A copy of the Agency meeting minutes should be included if available. Provide copies of communications from the FDA relevant to the most recent status of the IND or IDE application.

  - If an IND or IDE is not required for the proposed trial(s), or if it qualifies for an abbreviated IDE, provide evidence in the form of communication from the FDA or the IRB of record to that effect. Devices qualifying for an abbreviated IDE must comply with the abbreviated IDE requirements but do not require the submission of an IDE application to the FDA.

- **Attachment 11: DoD Military Budget Form(s), if applicable: Upload as "MFBudget.pdf."** If a military facility (military health system facility, research laboratory, treatment facility, dental treatment facility, or a DoD activity embedded with a civilian medical center) will be a collaborator in performance of the project, complete the DoD Military Budget Form, available for download on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm), including a budget justification, for each military facility as instructed. The costs per year should be included on the Grants.gov Research and Related Budget form under subaward costs. Refer to the General Application Instructions, Section III.A.7, for detailed information.

- **Extramural and Intramural Applications –**

  Research & Related Senior/Key Person Profile (Expanded): For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.3, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.2, for detailed information.

  - PI Biographical Sketch (five-page limit): Upload as "Biosketch_LastName.pdf." The suggested biographical sketch format is available on the "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) in eBRAP. The National Institutes of Health Biographical Sketch may also be used. All biographical sketches should be submitted in the portable document format (PDF) that is not editable.

  - PI Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."

  - Key Personnel Biographical Sketches (five-page limit each): Upload as "Biosketch_LastName.pdf."

  - Key Personnel Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."

  Research & Related Budget: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.4, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.3, for detailed information.

  Budget Justification (no page limit): Upload as "BudgetJustification.pdf." The budget justification for the entire period of performance must be uploaded to the Research & Related Budget after completion of the budget for Period 1.

  Project/Performance Site Location(s) Form: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.5, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.4, for detailed information.

- **Extramural Applications Only –**

  R&R Subaward Budget Attachment(s) Form (if applicable): Refer to the General Application Instructions, Section III.A.6, for detailed information.

  - **Extramural Subaward:** Complete the Research & Related Subaward Budget Form through Grants.gov. (Refer to the General Application Instructions, Section III.A.6, for detailed information.)

  - **Intramural DoD Collaborator(s):** Complete the DoD Military Budget Form and upload to Grants.gov as Attachment 11. (Refer to the General Application Instructions, Section III.A.7, for detailed information.) Intramural DoD Collaborator(s) costs per year

should be included on the Grants.gov Research and Related Budget form under subaward costs. **DoD Military Budget Form:** A military facility collaborating in the performance of the project should be treated as a subaward for budget purposes. However, do not complete the Grants.Gov R&R Subaward Budget Attachment Form; instead, complete the DoD Military Budget Form (Attachment 11) to show all direct and indirect costs. The costs per year should be included on the Grants.gov Research & Related Budget Form under subaward costs. Refer to the General Application Instructions, Section III.A.7, for detailed information.

II.D.3. Dun and Bradstreet Universal Numbering System (DUNS) Number and System for Award Management (SAM)

Applicant organizations and all subrecipient organizations must have a DUNS number to submit applications to Grants.gov. The applicant organization must also be registered in the Entity Management functional area of the SAM with an "Active" status to submit applications through the Grants.gov portal. Verify the status of the applicant's organization's Entity registration in SAM well in advance of the application submission deadline. Allow 3 to 4 weeks to complete the entire SAM registration process. If an applicant has not fully complied with the requirements by the time the Federal awarding agency is ready to make a Federal award, the Federal awarding agency may determine that the applicant is not qualified to receive a Federal award and use that determination as a basis for making a Federal award to another applicant. Refer to the General Application Instructions, Section III, for further information regarding Grants.gov requirements.

II.D.4. Submission Dates and Times

All submission dates and times are indicated in Section I, Overview of the Funding Opportunity. Pre-application and application submissions are required. The pre-application and application submission process should be started early to avoid missing deadlines. There are no grace periods. Failure to meet either of these deadlines will result in submission rejection.

Applicant Verification of Full Application Submission in eBRAP

Prior to the end of the application verification period, PIs and organizational representatives can review and modify in eBRAP certain components of a submitted application. Following retrieval and processing of the full application, eBRAP will notify the organizational representatives and PI by email to log into eBRAP to review, modify, and verify the full application submission. eBRAP will validate retrieved files against the specific Program Announcement requirements and discrepancies will be noted in both the email and in the Full Application Files tab in eBRAP. eBRAP does not confirm the accuracy of file content. It is the applicant's responsibility to review all application components and ensure proper ordering as specified in the Program Announcement. ***If either the Project Narrative or the budget fails eBRAP validation or needs to be modified, an updated full application package must be submitted prior to the application submission deadline.*** The Project Narrative and Budget Form cannot be changed after the application submission deadline.

II.D.5. Funding Restrictions

The maximum period of performance is **4** years.

The anticipated total costs budgeted for the entire period of performance will not exceed **$10M**. If indirect cost rates have been negotiated, indirect costs are to be budgeted in accordance with the organization's negotiated rate. No budget will be approved by the Government exceeding **$10M** total costs or using an indirect cost rate exceeding the organization's negotiated rate.

All direct and indirect costs of any subaward or contract must be included in the total direct costs of the primary award.

The applicant may request the entire maximum funding amount for a project that may have a period of performance less than the maximum **4** years.

For this award mechanism, direct costs must be requested for:

Travel costs for the PI and up to three additional members of the research team to attend a 1-day Milestone Meeting to be held in the National Capital Area during the award period of performance. This meeting will be held to provide a presentation on progress. Costs associated with travel to this meeting should be included in Year 3 of the budget. These travel costs are in addition to those allowed for annual scientific/technical meetings.

May be requested for (not all-inclusive):

- Salary
- Research supplies
- Equipment
- Research-related subject costs
- Clinical research costs
- Support for multidisciplinary collaborations, including travel
- Travel costs for up to four investigators to travel to one scientific/technical meeting per year in addition to the required meeting described above

Extramural (non-Federal) awards will consist solely of assistance agreements (Cooperative Agreements and Grants). For extramural awards with an intragovernmental component, direct transfer of funds from an extramural award recipient to a DoD or other Federal agency is not allowed except under very limited circumstances. Funding to intramural DoD and other Federal agencies will be managed through a direct fund transfer. Intragovernmental only funding to intramural DoD and other Federal agencies will be managed through a direct fund transfer. Intramural applicants are responsible for coordinating through their agency's procedures the use

of contractual or assistance funding awards or other appropriate agreements to support extramural collaborators.

Refer to the General Application Instructions, Section III.A.4, for budget regulations and instructions for the Research & Related Budget. ***For Federal agencies or organizations collaborating with Federal agencies, budget restrictions apply as are noted in the General Application Instructions, Section III.A.4***.

The CDMRP expects to allot approximately $60M of the $300M FY17 appropriation to fund approximately six Focused Program Award applications, depending on the quality and number of applications received. Funding of applications received in response to this Program Announcement is contingent upon the availability of Federal funds for this program.

II.D.6. Other Submission Requirements

Refer to the General Application Instructions, Appendix 4, for detailed formatting guidelines.

II.E. Application Review Information

II.E.1. Criteria

II.E.1.a. Peer Review

To determine technical merit, all applications will be evaluated according to the following scored criteria, which are of equal importance:

Scored Review Criteria for the Overall Program:

- **Overall Impact**
    - To what extent the overarching challenge impacts a critical problem or question in the designated FY17 PRMRP Topic Area(s).
    - To what degree the proposed program could, if successful, make a significant impact on the lives of relevant patient populations in the short term or long term.
    - How well the research projects are integrated, complement each other, and provide a synergistic, multidisciplinary approach to solving a critical problem.
    - How well the research program will, if successful:
        - Make important scientific advances in the relevant field of research
        - Promote greater understanding of the causes and progression of the relevant disease(s)/condition(s), or
        - Promote the development of improvements in prevention, detection, diagnosis, treatment, or quality of life

- **Implementation Plan**
    - How the proposed projects are supported by a detailed plan that identifies critical milestones and explains how these milestones will be achieved.
    - How well resources and/or cores that will be created or leveraged will be utilized and shared.
    - To what extent the plans to assess individual project performance during the course of the award are appropriate.
    - How well the overall management plan will facilitate consistent and intensive interactions by all team members in the projects.
    - How the proposed plans for communication, data and specimen collection, data transfer, and periodic meetings are appropriate and robust.
    - To what extent the plans for creating, reviewing, implementing, and modifying Standard Operating Procedures are appropriate, if applicable.
- **Leadership and Environment**
    - To what degree the PI is experienced in successfully leading large, focused projects and is therefore well-positioned to lead the research team in achieving the overarching goal of the proposed effort.
    - How well the PI demonstrates experience, leadership skills, and commitment to making an impact in the relevant field of research and/or patient care.
    - Whether the PI will devote a minimum of 20% effort to this award.
    - To what degree the scientific environment(s) is appropriate for the proposed research.
    - How well the research requirements are supported by the availability of and accessibility to facilities and resources (including patient populations, samples, and collaborative arrangements).
    - To what degree the quality and extent of organizational support are appropriate for the proposed research.
- **Transition Plan and Regulatory Strategy**
    - The degree to which the strategy proposed to bring the outcomes to the next level of development, including funding, milestones, and schedule, is realistic.
    - Whether appropriate collaborations and other resources for providing continuity of development are established and/or well described.
    - How the regulatory strategy and development plan are appropriate and well described.

- How well the application identifies intellectual property ownership, and whether there is sufficient evidence of a plan to resolve intellectual and material property issues, if applicable.

- Whether the applicant has demonstrated that they have access to all intellectual property rights necessary for development and commercialization and evidence that the Government has the ability to access such products or technologies, if applicable.

- If applicable, whether data will be appropriately reported and documented to support a regulatory filing with the FDA.

Scored Review Criteria for Individual Research Projects <u>without</u> a clinical trial:

- **Impact**

  - To what extent the individual project impacts the overarching challenge.

  - To what degree the individual project could, if successful, make a significant impact on the lives of relevant patient populations in the short term or long term.

  - How well the individual project will, if successful, make important scientific advances in the relevant field of research.

- **Research Strategy and Feasibility**

  - How well the scientific rationale supports the research and its feasibility, as demonstrated by a critical review and analysis of the literature, the presentation of preliminary data (where applicable), and logical reasoning.

  - How well the hypothesis, objectives, and aims are developed.

  - To what degree the experimental approach, methods, endpoints, and analyses support completion of the aims and are designed to achieve rigorous and reproducible results.

  - How well the choice of model (animal, cell line, or other) is justified and whether it is appropriate.

  - If applicable, to what degree the statistical plan and power analysis, including sample size projections, are appropriate for the proposed project.

  - Whether there is sufficient evidence to support availability and accessibility of the populations, samples, or other resources required for the study, if applicable.

  - How well potential problems are acknowledged and alternative approaches are addressed.

- **Personnel**

  - To what degree the project team's background and expertise are appropriate with respect to its ability to perform the proposed work, including whether there is evidence of

sufficient expertise for all aspects of the work and whether there is evidence of strong commitment to the projects.

- To what degree the levels of effort are appropriate for successful conduct of the proposed work.

Scored Review Criteria for Individual Research Projects <u>with</u> clinical trials:

- **Impact**
  - To what extent the individual project impacts the overarching challenge.
  - How well the individual project will, if successful, make important scientific advances in the relevant field of research.
  - How well the sample population represents the targeted patient population that might benefit from the proposed intervention.
  - How the potential outcomes of the proposed clinical trial will provide/improve short-term benefits for individuals.
  - How significantly the long-term benefits for implementation of the intervention may impact patient care and/or quality of life.
- **Research Strategy and Feasibility**
  - How well the scientific rationale supports the research and its feasibility, as demonstrated by a critical review and analysis of the literature, the presentation of preliminary data, and logical reasoning.
  - How well the hypothesis and/or objectives and specific aims are developed.
  - To what degree the experimental approach, methods, endpoints, and analyses support completion of the aims and are designed to achieve rigorous and reproducible results.
  - To what degree the statistical plan and power analysis, including sample size projections, are appropriate for the proposed trial and any proposed correlative studies.
  - How well the application demonstrates the availability of and access to the appropriate patient population(s), as well as the ability to accrue a sufficient number of subjects.
  - How well potential problems and delays (e.g., slow accrual, attrition) are acknowledged and alternative approaches are addressed.
- **Clinical Strategy**
  - How the intervention compares with currently available interventions and/or standards of care.

- Whether there is evidence of support, indicating availability of the intervention from its source, for the duration of the proposed clinical trial (if applicable).
- To what degree the PI has provided preclinical and/or clinical evidence to support the safety of the intervention.
- How well the inclusion and exclusion criteria meet the needs of the proposed clinical trial.
- The degree to which the informed consent, screening, and retention processes for human subjects will meet the needs of the proposed clinical trial.
- Whether a member of the study team holds the IND/IDE for the indication proposed or whether the timeline proposed for obtaining the IND/IDE is appropriate (if applicable).
- For investigator-sponsored INDs, whether there is evidence of appropriate institutional support, including capabilities to ensure monitoring as required by the FDA.
- Whether plans to comply with GMP, GLP, and GCP guidelines are appropriate.
- Whether measures are described to ensure the consistency of dosing of active ingredients for nutritional supplements (if applicable).

- **Personnel**
  - To what degree the project team's background and expertise are appropriate with respect to its ability to perform the proposed work, including whether there is evidence of sufficient expertise for all aspects of the work and whether there is evidence of strong commitment to the projects.
  - How well the project leader has assembled an appropriate and robust clinical team with the combined backgrounds and expertise needed to enable successful conduct of the clinical trial.
  - To what degree the levels of effort are appropriate for successful conduct of the proposed work.

In addition, the following unscored criteria will also contribute to the overall evaluation of the application:

- **Data and Resource Sharing**
  - To what degree the plan for sharing of project data and research resources is appropriate and reasonable to facilitate use by the wider research community.
- **Budget**
  - Whether the budget is appropriate for the proposed research and within the limitations of this Program Announcement.

- **Environment**
    - If applicable, to what degree the intellectual and material property plan is appropriate.
- **Application Presentation**
    - To what extent the writing, clarity, and presentation of the application components influence the review.

II.E.1.b. Programmatic Review

To make funding recommendations and select the application(s) that, individually or collectively, will best achieve the program objectives, the following criteria are used by programmatic reviewers:

- Ratings and evaluations of the peer reviewers
- Relevance to the mission of the DHP and FY17 PRMRP, as evidenced by the following:
    - Adherence to the intent of the award mechanism
    - Military relevance
    - Program portfolio composition
    - Relative impact

II.E.2. Application Review and Selection Process

All applications are evaluated by scientists, clinicians, and consumers in a two-tier review process. The first tier is peer review of applications against established criteria for determining technical merit. Each application is evaluated for its own merit, independent of other applications. The second tier is a programmatic review that makes recommendations for funding to the Commanding General, USAMRMC, on behalf of the DHA and the OASD(HA), based on technical merit, the relevance to the mission of the DHP and PRMRP, the specific intent of the award mechanism, and to other specified evaluation criteria in the Program Announcement. Programmatic review is a comparison-based process in which applications with scientific and technical merit compete in a common pool. ***The highest-scoring applications from the first tier of review are not automatically recommended for funding. Funding recommendations depend on various factors as described in Section II.E.1.b, Programmatic Review.*** Additional information about the two-tier process used by the CDMRP can be found at http://cdmrp.army.mil/about/fundingprocess.

All CDMRP review processes are conducted confidentially to maintain the integrity of the merit-based selection process. Panel members sign a statement that application and evaluation information will not be disclosed outside the panel. Violations of confidentiality can result in the dissolving of a panel(s) and other corrective actions. In addition, personnel at the applicant or collaborating organizations are prohibited from contacting persons involved in the review

process to gain protected evaluation information or to influence the evaluation process. Violations of these prohibitions will result in the administrative withdrawal of the organization's application. Violations by panel members or applicants that compromise the confidentiality of the review process may also result in suspension or debarment from Federal awards. Furthermore, the unauthorized disclosure of confidential information of one party to another third party is a crime in accordance with 18 USC 1905.

II.E.3. Integrity and Performance Information

Prior to making an assistance agreement award where the Federal share is expected to exceed the simplified acquisition threshold (currently $150,000) over the period of performance, the Federal awarding agency is required to review and consider any information about the applicant that is available in the Federal Awardee Performance and Integrity Information System (FAPIIS).

An applicant, at its option, may review FAPIIS, accessible through SAM, and submit comments to FAPIIS on any information about itself that a Federal awarding agency previously entered and is currently available in FAPIIS.

The Federal awarding agency will consider any comments by the applicant, in addition to other information in the designated integrity and performance system, in making a judgment about the applicant's integrity, business ethics and record of performance under Federal awards when determining a recipient's qualification prior to award, according to the qualification standards of the Department of Defense Grant and Agreement Regulations (DoDGAR), Section 22.415.

II.E.4. Anticipated Announcement and Federal Award Dates

All application review dates and times are indicated in Section I, Overview of the Funding Opportunity.

Each PI and organization will receive email notification of posting of the funding recommendation in eBRAP. Each PI will receive a peer review summary statement on the strengths and weaknesses of the application.

II.F. Federal Award Administration Information

II.F.1. Federal Award Notices

Awards will be made no later than September 30, 2018. Refer to the General Application Instructions, Appendix 2, for additional award administration information.

Awards are made to organizations, not to individual PIs. The types of awards made under the Program Announcement will be assistance agreements (grants or cooperative agreements). The level of involvement on the part of DoD during project performance is the key factor in determining whether to award a grant or cooperative agreement.

Extramural Organizations: An assistance agreement (grant or cooperative agreement) is appropriate when the Federal Government transfers a "thing of value," to a "state, local

government," or "other recipient," to carry out a public purpose of support or stimulation authorized by a law of the United States, instead of acquiring property or service for the direct benefit and use of the U.S. Government. An assistance agreement can take the form of a grant or cooperative agreement. If "no substantial involvement" on the part of the funding agency is anticipated, a grant award will be made (31 USC 6304). Conversely, if substantial involvement on the part of the funding agency is anticipated, a cooperative agreement will be made (31 USC 6305). Substantial involvement may include collaboration, participation, or intervention in the research to be performed under the award. The award type, along with the start date, will be determined during the negotiation process.

After email notification of application review results through the eBRAP, and if selected for funding, a representative from the USAMRAA will contact the business official authorized to negotiate on behalf of the PI's organization.

Only an appointed USAMRAA Grants Officer may obligate the Government to the expenditure of funds. No commitment on the part of the Government should be inferred from discussions with any other individual. The award document signed by the Grants Officer is the official authorizing documents.

Intramural Organizations: Awards to Federal Government organizations (to include intramural DoD organizations) will be executed through the Military Interdepartmental Purchase Request (MIPR) or Funding Authorization Document (FAD) process. Transfer of funds is contingent upon appropriate safety and administrative approvals. Intramural applicants and collaborators are reminded to coordinate receipt and commitment of funds through their respective resource managers (RM).

After email notification of application review results through the eBRAP, and if selected for funding, a representative from the CDMRP will contact the business official authorized to negotiate on behalf of the PI's organization.

II.F.1.a. Award Transfers

Changes in PI are not allowed, except under extenuating circumstances that will be evaluated on a case-by-case basis and at the discretion of the Grants Officer. An organizational transfer of an award will not be allowed in the last year of the (original) period of performance or any extension thereof.

The organizational transfer of an award supporting a clinical trial is strongly discouraged and in most cases will not be allowed. Approval of a transfer request will be on a case-by-case basis at the discretion of the Grants Officer.

Refer to the General Application Instructions, Appendix 2, Section B, for general information on organization or PI changes.

II.F.2. Administrative and National Policy Requirements

Applicable requirements in the DoDGAR found in 32 CFR, Chapter 1, Subchapter C, and 2 CFR, Chapter XI, apply to grants and cooperative agreements resulting from this Program Announcement.

Refer to the General Application Instructions, Appendix 2, for general information regarding administrative requirements.

Refer to the General Application Instructions, Appendix 5, for general information regarding national policy requirements.

Refer to full text of the USAMRAA General Research Terms and Conditions for Institutions of Higher Education, Hospitals, and Non-Profit Organizations and the USAMRAA General Research Terms and Conditions with For-Profit Organizations for further information.

II.F.3. Reporting

Refer to the General Application Instructions, Appendix 2, Section A, for general information on reporting requirements. Annual progress reports as well as a final progress report will be required.

Quarterly technical progress reports will be required. In addition to written progress reports, in-person presentations may be requested.

Award Chart: Complete the Award Chart template, a one-page PowerPoint file that must be downloaded from the CDMRP eBRAP System at https://ebrap.org/eBRAP/public/Program.htm, and submit to eBRAP at the time of award.

Award Expiration Transition Plan: Complete the Transition Plan template, a one-page Word document that must be submitted with the Final Progress Report. The Transition Plan must outline if and how the research supported by this award will progress, and include source(s) of funding, either known or pending.

Awards resulting from this Program Announcement will incorporate additional reporting requirements related to recipient integrity and performance matters. Recipient organizations that have Federal contract, grant, and cooperative agreement awards with a cumulative total value greater than $10,000,000 are required to provide information to FAPIIS about certain civil, criminal, and administrative proceedings that reached final disposition within the most recent 5-year period and that were connected with performance of a Federal award. Recipients are required to disclose semiannually information about criminal, civil, and administrative proceedings as specified in the applicable Terms and Conditions. The applicable Terms and Conditions for institutions of higher education, hospitals, and nonprofit organizations are available in OAR Article I, Section B, in July 2016 R&D General Terms and Conditions. The applicable Terms and Conditions for for-profit organizations are available in Section 34 of the February 2017 USAMRAA General Research Terms and Conditions with For-Profit Organizations.

II.G. Federal Awarding Agency Contacts

II.G.1. CDMRP Help Desk

Questions related to Program Announcement content or submission requirements as well as questions related to the pre-application or intramural application submission through eBRAP should be directed to the CDMRP Help Desk, which is available Monday through Friday from 8:00 a.m. to 5:00 p.m. ET. Response times may vary depending upon the volume of inquiries.

Phone: 301-682-5507

Email: help@eBRAP.org

II.G.2. Grants.gov Contact Center

Questions related to extramural application submission through Grants.gov portal should be directed to the Grants.gov Contact Center, which is available 24 hours a day, 7 days a week (closed on U.S. Federal holidays). Note that the CDMRP Help Desk is unable to provide technical assistance with Grants.gov submission.

Phone: 800-518-4726; International 1-606-545-5035

Email: support@grants.gov

Sign up on Grants.gov for "send me change notification emails" by following the link on the Synopsis page for the Program Announcement or by responding to the prompt provided by Grants.gov when first downloading the Grants.gov application package. If the Grants.gov application package is updated or changed, the original version of the application package may not be accepted by Grants.gov.

II.H. Other Information

II.H.1. Program Announcement and General Application Instructions Versions

Questions related to this Program Announcement should refer to the Program name, the Program Announcement name, and the Program Announcement version code 20170516b. The Program Announcement numeric version code will match the General Applications Instructions version code 20170516.

II.H.2. Administrative Actions

After receipt of pre-applications or applications, the following administrative actions may occur:

II.H.2.a. Rejection

The following will result in administrative rejection of the pre-application:

- Preproposal Narrative is missing.

The following will result in administrative rejection of the application:

- Submission of an application for which a letter of invitation was not received.
- Project Narrative exceeds page limit.
- Project Narrative is missing.
- Budget is missing.
- Submission of the same research project to different Funding Opportunities within the same program and fiscal year. Refer to Section II.D, Application and Submission Information, for exceptions.

II.H.2.b. Modification

- Pages exceeding the specific limits will be removed prior to review for all documents other than the Project Narrative.
- Documents not requested will be removed.

II.H.2.c. Withdrawal

The following may result in administrative withdrawal of the pre-application or application:

- An FY17 PRMRP Programmatic Panel member is named as being involved in the research proposed or is found to have assisted in the pre-application or application processes including, but not limited to, concept design, application development, budget preparation, and the development of any supporting documentation. ***A list of the FY17 PRMRP Programmatic Panel members can be found at http://cdmrp.army.mil/prmrp/panels/panels17.shtml***.
- The application fails to conform to this Program Announcement description to the extent that appropriate review cannot be conducted.
- Inclusion of URLs, with the exception of links in References Cited and Publication and/or Patent Abstract sections.
- Page size is larger than 8.5 inches x 11.0 inches (approximately 21.59 cm x 27.94 cm).
- To preserve the integrity of its peer and programmatic review processes, the CDMRP discourages inclusion of any employee of its review contractors having any role in the preparation, research or other duties for submitted applications. For FY17, the identities of the peer review contractor and the programmatic review contractor may be found at the

CDMRP website (http://cdmrp.army.mil/about/2tierRevProcess). Applications that include names of personnel from either of these companies will be administratively withdrawn unless plans to manage COIs are provided and deemed appropriate by the Grants Officer. Refer to the General Application Instructions, Appendix 3, for detailed information.

- Personnel from applicant or collaborating organizations are found to have contacted persons involved in the review process to gain protected evaluation information or to influence the evaluation process.
- Applications from extramural organizations, including non-DoD Federal agencies, received through eBRAP may be withdrawn.
- Applications submitted by an intramural DoD organization may be withdrawn if the intramural organization cannot coordinate the use of contractual, assistance, or other appropriate agreements to provide funds to extramural collaborators.
- The proposed research project does not address at least one of the Congressionally directed FY17 PRMRP Topic Areas.
- An application for which the PI does not meet the eligibility criteria will be withdrawn.

II.H.2.d. Withhold

Applications that appear to involve research misconduct will be administratively withheld from further consideration pending organizational investigation. The organization will be required to provide the findings of the investigation to the USAMRAA Grants Officer for a determination of the final disposition of the application.

II.H.3. Application Submission Checklist

Application Components	Action	Completed
SF424 (R&R) Application for Federal Assistance **(Extramural submissions only)**	Complete form as instructed.	
Summary (Tab 1) and Application Contacts (Tab 2) **(Intramural submissions only)**	Complete these tabs as instructed.	
Attachments	Project Narrative: Upload as Attachment 1 with file name "ProjectNarrative.pdf."	
	Supporting Documentation: Upload as Attachment 2 with file name "Support.pdf."	
	Technical Abstract: Upload as Attachment 3 with file name "TechAbs.pdf."	
	Lay Abstract: Upload as Attachment 4 with file name "LayAbs.pdf."	
	Statement of Work: Upload as Attachment 5 with file name "SOW.pdf."	
	Impact Statement: Upload as Attachment 6 with file name "Impact.pdf."	
	Military Relevance: Upload as Attachment 7 with file name "MilRel.pdf."	
	Transition Plan and Regulatory Strategy: Upload as Attachment 8 with file name "Transition.pdf."	
	Data and Research Resources Sharing Plan: Upload as Attachment 9 with file name "Sharing.pdf."	
	IND/IDE Documentation: Upload as Attachment 10 with file name "IND-IDE.pdf," if applicable.	
	DoD Military Budget Form(s): Upload as Attachment 11 with file name "MFBudget.pdf," if applicable.	
Research & Related Senior/Key Person Profile (Expanded)	Attach PI Biographical Sketch (Biosketch_LastName.pdf) to the appropriate field.	
	Attach PI Previous/Current/Pending Support (Support_LastName.pdf) to the appropriate field.	
	Attach Biographical Sketch (Biosketch_LastName.pdf) for each senior/key person to the appropriate field.	
	Attach Previous/Current/Pending (Support_LastName.pdf) for each senior/key person to the appropriate field.	

Application Components	Action	Completed
Research & Related Budget **(Extramural submissions only)**	Complete as instructed. Attach Budget Justification (BudgetJustification.pdf) to the appropriate field.	
Budget **(Intramural submissions only)**	Complete the DoD Military Budget Form and justification.	
Project/Performance Site Location(s) Form	Complete form as instructed.	
R&R Subaward Budget Attachment(s) Form, if applicable	Complete form as instructed.	
Additional Application Components	**Action**	**Completed**
Confidential Letters of Recommendation	Confirm upload to eBRAP.	

APPENDIX 1
ACRONYM LIST

ACURO	Animal Care and Use Review Office
CDMRP	Congressionally Directed Medical Research Programs
CFR	Code of Federal Regulations
DHA	Defense Health Agency
DHP	Defense Health Program
DoD	Department of Defense
DoDGAR	Department of Defense Grant and Agreement Regulations
DUNS	Data Universal Numbering System
eBRAP	Electronic Biomedical Research Application Portal
EC	Ethics Committee
ET	Eastern Time
FAPIIS	Federal Awardee Performance and Integrity Information System
FY	Fiscal Year
GCP	Good Clinical Practices
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
HRPO	Human Research Protection Office
IACUC	Institutional Animal Care and Use Committee
IRB	Institutional Review Board
M	Million
OASD(HA)	Office of the Assistant Secretary of Defense for Health Affairs
OMB	Office of Management and Budget
ORP	Office of Research Protections
PI	Principal Investigator
PRMRP	Peer Reviewed Medical Research Program
RDT&E	Research, Development, Test, and Evaluation
RM	Resource Manager
SAM	System for Award Management
SOW	Statement of Work
USAMRAA	U.S. Army Medical Research Acquisition Activity
USAMRMC	U.S. Army Medical Research and Materiel Command
USC	United States Code
VA	Department of Veterans Affairs

APPENDIX 2
AREAS OF ENCOURAGEMENT

Applications addressing any of the FY17 PRMRP Topic Areas are of interest to the program. ***Any aspect of research relevant to one or more FY17 PRMRP Topic Areas may be considered for funding.*** Areas of encouragement related to the FY17 PRMRP Topic Areas have been identified by the Department of Defense, Department of Veterans Affairs, and other relevant stakeholders. Applicants are urged to read and consider these areas of encouragement before preparing their applications. ***The information provided is not exhaustive, and applicants are not restricted to submitting applications that address an area of encouragement in this list.***

Acute Lung Injury

- Research on the etiology and prevention of acute respiratory distress syndrome caused by the host's or immune system's responses to infectious disease or septic shock.
- Preventive techniques, novel and/or innovative detection technologies, and therapeutics to reduce the incidence and/or severity of acute respiratory distress syndrome and/or other lung injury secondary to trauma, transfusion, burns, hemorrhagic shock, inhalation, and/or oxygen exposure.
- Development of a clinical laboratory-based device for manufacturing/amplification of stem cells to treat acute lung injury/acute respiratory distress syndrome due to inhalation injury, trauma, or other causes.
- Clinical studies to test cellular therapies in the treatment of acute lung injury/acute respiratory distress syndrome due to inhalation injury, trauma, or other causes.
- Metrics to associate the health outcomes of acute lung injury with physiological and physical performance, as well as the psychological health of military Service members.
- Strategies to stabilize and support the safe transport of patients with lung injury in order to optimize therapeutic interventions.
- Studies to identify the prevalence and associated morbidity and mortality of blast overpressure lung injury in forward operating environments.

Antimicrobial Resistance

- Identification and evaluation of novel and/or innovative antifungals against resistant fungal infections, particularly for use as topical therapies for wounds and for surgical and post-surgical therapies.
- Identification of novel and/or innovative targets for multi-drug resistant pathogens.
- Development of novel and/or innovative interventions, diagnostics, and treatment for multi-drug-resistant pathogens.
- Development of novel and/or innovative therapies that interrupt biofilm production in wound infections and infected hardware models.
- Development and testing of treatment protocols and/or diagnostic tests to limit prescribing antibiotics for conditions that are commonly viral in nature or conditions that would resolve themselves without antibiotic treatment (e.g., sinusitis, bronchitis, viral upper respiratory infections, and acute gastroenteritis).

- Development of rapid, rugged, sensitive, and specific diagnostics for antimicrobial resistance in remote environments.
- Research to improve understanding of the impact of travelers' diarrhea, as well as antibiotic treatment for the colonization of multi-drug-resistant organisms.

Arthritis

- Studies that define the risk of contracting septic arthritis; reactive arthritis; arthritis caused by a gastrointestinal, genitourinary, or viral infection (e.g., Lyme disease, Campylobacter, chikungunya); and other arthritis types in active duty military personnel.
- Research toward the development of clinical practice guidelines to prevent, identify, and treat arthritis, particularly that resulting from traumatic injury, surgery, and/or infectious diseases.
- Research to establish activity recommendations for maximal joint life following joint repair, particularly in young patient populations.
- Strategies for repairing focal cartilage defects using cell-based therapies.
- Research quantifying the impacts of obesity, weight loss, physical fitness (all components—cardiovascular, strength, flexibility, balance), and dietary factors on the development of or prevention/risk reduction of arthritis.
- Identification and/or validation of biomarkers for early psoriatic arthritis.
- Basic and translational research to identify treatments to mitigate and/or reverse osteoarthritis, particularly in the knee, hip, ankle, and shoulder.
- Studies to examine using existing regenerative medicine techniques and therapies to treat osteoarthritis, including dose response information and the frequency and timing of application.
- Development of novel and/or innovative treatments or combination therapies for psoriatic arthritis.

Burn Pit Exposure

- Research on the etiology and treatment of adverse health events related to military deployment to Iraq and Afghanistan that are associated with exposure to airborne hazards and open pit burning of solid waste and other materials.
- Toxicological studies to ascertain the toxicity of natural dust, burn pit combustion products and interactions between pollutants, as well as their mechanisms of action.
- Characterization of emissions from open air burns, burn boxes, and incinerators.
- Development and validation of instruments for assessing levels of exposure to airborne hazards for use in research and for occupational and environmental exposure monitoring.

Chronic Migraine and Post-Traumatic Headache

- Research to investigate, develop, and validate biomarkers that are useful in diagnosing and monitoring traumatic brain injury patients with chronic migraine or post-traumatic headache.
- Epidemiological/natural history studies to characterize specific types of post-traumatic headache, the pathobiology of these headaches (such as the role of cortical spreading

depression acutely after injury as a risk factor for chronic headaches of a migrainous type), and the risk factors that might predispose people to certain types of post-traumatic headache.

- Double-blind, placebo-controlled trials in the post-traumatic headache population in order to determine whether similar phenotypes in primary headache disorders and post-traumatic headache will respond similarly to treatment.
- Research on the optimal approaches to effective management of co-occurring psychological health disorders and acute and chronic pain management for chronic migraine and post-traumatic headache, with a focus on assessing and eliminating adverse outcomes and decreasing polypharmacy.
- Research on the utility of the Patient-Centered Medical Home model of care for patients with chronic migraine or post-traumatic headache.
- Research on the treatment and prevention of acute flares of chronic migraine and post-traumatic headaches with an emphasis on non-opioid medications.
- Evaluation of the use of mechanical stimulation and/or other non-pharmaceutical treatments to reduce acute and chronic migraines and headaches.
- Precision medicine research to investigate, develop, and validate biomarkers that are not only useful in diagnosing and monitoring traumatic brain injury patients with chronic migraine or post-traumatic headache, but can also identify individual responses to treatment.
- Evaluation of the differences in etiology, diagnosis, treatment plan, and prevention of migraine headaches between men and women.

Congenital Heart Disease

- Population-based and outcomes-based research projects to assess the health outcomes of individuals with congenital heart disease across their life spans.
- Research on tissue engineering approaches to patches, grafts, and transplantation that provide structural support, restore native activity, allow for tissue growth, and prevent the need for reoperation.
- Research to improve understanding of the causes of congenital heart defects, including genomic, proteomic, and metabolomic profiling.
- Research on transition of the care of congenital heart disease patients from pediatric to adult providers.
- Research both on the risk of neurologic injury and on enhanced neuroprotection before, during, and after surgery for congenital heart disease.
- Research to design and implement disease-in-a-dish and/or microfluidic models with an established phenotype to increase the efficacy of finding novel and/or innovative drug targets, screen existing drugs, perform cardiotoxicity testing, and/or uncover pathogenesis.

Constrictive Bronchiolitis

- Clinical assessments to determine the prevalence and severity of constrictive bronchiolitis and related respiratory diseases in previously deployed military Service members and/or Veterans.

- Development and testing of less invasive and non-invasive approaches for diagnosing constrictive bronchiolitis.
- Research to develop novel and/or innovative therapeutics to slow or reverse the progression of constrictive bronchiolitis.
- Development and/or validation of animal models for constrictive bronchiolitis.
- Research to understand the role of environmental exposures, including mineralized dust and other particulates, in the etiology of constrictive bronchiolitis.

Diabetes

- Development of biomarkers to identify and monitor at-risk individuals and assess treatment response.
- Research on interventions to prevent diabetes complications, including diabetic retinopathy and diabetic neuropathy.
- Research to better understand the heterogeneity of diabetes.
- Research on the transplantation of allogenic or autologous pancreatic islet cells for long-term natural insulin production, including current good manufacturing practices for cell line development.
- Identification and/or evaluation of interventions to reduce the development of diabetes among individuals meeting the clinical criteria for pre-diabetes.
- Development and evaluation of an inpatient glycemic management program with strategies to improve in-hospital dysglycemia, improve short-term and long-term morbidity/mortality, reduce the cost of health care, and promote a seamless transition of care between inpatient and outpatient arenas.
- Research to design and implement disease-in-a-dish and/or microfluidic models to model pancreatic islets to uncover pathogenesis and improve the efficiency of drug discovery.

Diarrheal Diseases

- Development and testing of vaccines, including multi-pathogen combination approaches, against the most common causes of bacterial and viral travelers' diarrhea, including *Campylobacter jejuni, Shigella,* and Enterotoxigenic *E. coli*.
- Development and evaluation of antibiotic and non-antibiotic therapies to treat all-cause travelers' diarrhea to improve the time to clinical cure, minimize resistance acquisition, and mitigate the chronic health consequences of these infections.
- Research to improve understanding of host responses and develop therapies to treat acute bacterial and viral infections that are associated with travel and may lead to chronic functional bowel and rheumatologic disorders.
- Development of evidence necessary to demonstrate the need, impact, and potential market value of a therapeutic for post-infectious functional bowel disorders to include preventive, treatment, or vaccine technology strategies.
- Development and evaluation of point-of-care diagnostics for acute diarrheal illness that distinguishes between bacterial, viral, protozoal, and toxin-mediated etiologies and leads to actionable public health or individual management interventions.

- Investigations into the relationship between existing gastrointestinal flora (microbiome) and the susceptibility, duration, and severity of diarrheal disease, as well as the impacts of dietary modification and/or probiotic use.
- Research to identify, reduce, or eliminate sources of infection causing diarrheal diseases.

Dystonia

- Research on the risk, incidence, and etiology of generalized dystonia, focal dystonia, multifocal dystonia, segmental dystonia, and/or hemidystonia.
- Research on interventions to prevent, slow the progression of, or treat dystonia.
- Research to improve identification of delayed onset dystonia following traumatic brain injury.

Early Trauma Thermal Regulation

- Research into regional cooling for different environments, including prolonged care, as a therapeutic for secondary ischemic injury (e.g., head with traumatic brain injury, limb with tourniquet).
- Development of a tunable support mechanism for thermal homeostasis for use in prolonged field care, extended evacuation, or unmanned evacuation.
- Development and evaluation of a novel and/or innovative technology and techniques to reduce the impact of hypothermia.
- Research to determine the benefits of invasive warming/cooling during patient movement.
- Research to validate the attenuating effects of (early) external head cooling on concussive brain injuries.
- Research into therapeutic cooling or targeted temperature management as a neuroprotective treatment for traumatic brain injury.

Eating Disorders

- Investigations into the prevalence, diagnosis, and treatment patterns of eating disorders in Service members and their families, including potential relationships with military-unique behaviors or conditions.
- Assessment of patterns of comorbidity between eating disorders and other mental health conditions, including an examination of whether eating disorders are more likely to precede or follow the development of other mental health conditions.
- Studies to identify the most effective treatments (and order of treatment) for patients with an eating disorder and a comorbid disorder.
- Research to advance the understanding of the biological, genetic, and environmental factors that influence eating disorders.
- Studies to elucidate risk factors for the onset or recurrence of eating disorders.
- Research to develop screening tools and valid biomarkers that accurately identify individuals with binge eating disorder versus healthy controls in order to monitor at-risk populations and tailor treatments for disordered eating.

- Research to better understand subclinical disordered eating and other specified feeding and eating disorders and the potential for these to develop into full-blown eating disorders.

Emerging Infectious Diseases

- Methods to minimize risk from arboviruses among military Service members and their families deployed to/living in high-risk areas, including personal protective equipment and environmental controls.
- Investigation of revaccination strategies and identification of correlates of protection for currently available vaccines (especially mumps).
- Targeted vector management of *Aedes aegypti, Anopheline sp.* and related mosquito species, including focus areas on novel and/or innovative adult surveillance devices, pathogen identification, spatial repellents, and barrier treatments.
- Research toward understanding the potential influence of dietary intake as a potential repellent to mosquito vectors and vector behavior.
- Evaluation of non-vaccine prophylactics or therapeutics to prevent/treat dengue or Zika virus disease.
- Characterization of health emergency relevant infectious disease threats in West Africa.
- Development of biomarker-guided clinical management strategies for severe infectious disease in the austere African setting.
- Validation of airborne and droplet precaution recommendations in the medical transport environment.
- Development of new testing methods for emerging pathogens and pathogen reduction technologies for blood-borne pathogens, including Zika, malaria, dengue, and others, in blood products.
- Modeling of epidemics and development of strategies to counter risks in emerging infectious diseases.
- Rapid, rugged, sensitive, and specific screening and diagnostics, including biomarkers for infectious threats such as Zika and other clinically relevant bacterial, viral, or protozoal pathogens in clinical samples or blood products that can be transmitted sexually or by blood transmission.
- Development of broad-acting detection systems to identify emerging members of a known viral family (for example, a broad Coronavirus panel that can detect Middle Eastern Respiratory Syndrome).
- Determination of the human health effects, host preference, vector competence, and co-infection status of the dengue, chikungunya, Zika, and Mayaro viruses in *Aedes aegypti* and related mosquito species.

Epidermolysis Bullosa

- Research to provide further insight into those cellular pathways that might be corrected or bypassed that otherwise promote the development of squamous cell carcinomas in recessive dystrophic and junctional epidermolysis bullosa.

- Research, including randomized controlled clinical trials, focused on systemic drugs that prevent, delay the onset, or modify the aggressiveness of squamous cell carcinoma in patients with recessive and junctional epidermolysis bullosa.
- Research, including clinical trials, focused on medications (topical or systemic) or dressings that might enhance wound healing in inherited epidermolysis bullosa.

Focal Segmental Glomerulosclerosis

- Development of a curative therapy or treatments to delay or halt the progression of focal segmental glomerulosclerosis and/or prevent post-transplantation recurrence.
- Research to determine the efficacy of medications used off-label (outside the FDA-approved indication) to treat focal segmental glomerulosclerosis.
- Research to improve understanding of the causes of primary and secondary focal segmental glomerulosclerosis, especially genetic mutations.
- Development of non-invasive methods to diagnose focal segmental glomerulosclerosis and its variants.
- Research on the impact of dietary modifications on the progression of renal disease.

Fragile X

- Research to advance the understanding of the pathophysiology of fragile X syndrome.
- Identification and validation of functional measures of the manifestations of fragile X syndrome across the life span.
- Identification and testing of novel and/or innovative targets for fragile X syndrome therapeutics.
- Development and evaluation of novel, innovative, or existing therapeutics for the treatment of fragile X syndrome.

Guillain-Barré Syndrome

- Research to elucidate the characteristics of various exposures (e.g., viruses, bacteria, vaccinations, surgery) associated with Guillain-Barré syndrome and their effects on the immune system.
- Research on the immune system cell types and molecular mechanisms responsible for the disease pathology of Guillain-Barré syndrome.
- Research to prevent or reduce the effects of residual weakness, relapse of muscle weakness, and other neurological symptoms of Guillain-Barré syndrome to improve patients' quality of life and increase their independence.
- Development of new treatments and refinement of existing treatments for Guillain-Barré syndrome.

Hepatitis B and C

- Research on vaccination options for those persons unable to mount immunity to the hepatitis B virus.

- Development of strategies for reliable, non-invasive, early detection of hepatitis-related liver disease and hepatocellular carcinoma.
- Research on strategies to promote reversal of liver fibrosis and/or assess the associated clinical and pathological outcomes.
- Natural history or other studies to establish risk indicators of progression from asymptomatic or inactive chronic hepatitis infection to symptomatic disease and parameters for the initiation and duration of disease treatment.
- Clinical studies to evaluate combination or curative therapies for treatment of hepatitis infection.
- Identification and reduction of hepatitis in blood products for transfusion.
- Research on strategies to reduce vertical (mother-to-child) transmission of hepatitis B virus and hepatitis C virus.
- Development of a vaccine against hepatitis C.
- Development of treatment protocols for patients with hepatitis B and C co-infections.

Hereditary Angioedema

- Research to improve early diagnosis of hereditary angioedema.
- Evaluation of existing, innovative, or novel therapeutics in pediatric hereditary angioedema patients.
- Research toward development of a cure for hereditary angioedema.
- Development and/or validation of novel and/or innovative therapeutic strategies for the treatment and/or prevention of hereditary angioedema attacks.

Hydrocephalus

- Research on the etiology, prevention, diagnosis, and treatment of post-traumatic hydrocephalus.
- Discovery or validation of novel and/or innovative therapies and therapeutic targets for the treatment of hydrocephalus and its sequelae, including therapies directed at myelin regeneration and repair.
- Research on approaches to lessening the impact of brain damage caused by hydrocephalus.
- Development or validation of biomarkers and imaging techniques, particularly multimodal approaches, to aid in diagnosis, prognosis, and monitoring of therapeutic efficacy.
- Research on the prevention of shunt failure.
- Studies to better understand the causes and/or pathogenesis of hydrocephalus.
- Development or validation of improved hydrocephalus model systems.

Immunomonitoring of Intestinal Transplants

- Studies to elucidate the role of the mucosal immune system, other host-derived factors, or gut microbiota-derived factors in maintaining intestinal transplant viability.

- Development and evaluation of intestinal implant strategies that circumvent the induction of immunity against the transplant through the use of autologous stem cells or organoids derived from intestinal stem cells.
- Development and evaluation of evidence-based approaches that focus on dampening the regional immune response against intestinal transplants without inducing global immune suppression.
- Development and evaluation of implant-associated materials (e.g., scaffolds) with anti-inflammatory properties that protect the intestinal transplant from immune attack.
- Development and evaluation of strategies for inducing and maintaining populations of anti-inflammatory regulatory immune cell populations at the transplant site.
- Studies to improve immunomonitoring of recipient immune responses after intestinal transplantation, with a focus on prospective leukocyte profiling, to aid in diagnosis and treatment of immunological and immunosuppression-related complications.
- Development and/or validation of precise, real-time implanted monitoring devices to improve individualized patient outcomes after intestinal transplantation.

Inflammatory Bowel Diseases

- Clinical studies in human subjects directed toward understanding how acute enteric infections may trigger chronic bowel diseases with acute and sub-acute inflammatory bowel disease, including systems biology approaches.
- Mechanistic studies in animal models designed to understand how enteric infection may trigger inflammatory bowel disease, including genomic, microbiomic, and immune mechanisms.
- Epidemiological studies of post-infectious acute and sub-acute inflammatory bowel diseases that define risk and provide estimates of illness-associated disability, health care costs, and symptom duration from a military health system and societal perspective.
- Studies to understand the interaction between acute/chronic stress and infection and the development of inflammatory bowel disease.
- Research to explore whether the travel patterns of active duty personnel increase the risk of developing inflammatory bowel disease by means of exposure to changes in intestinal microflora.
- Research to better characterize the association between the use of drugs, such as isotretinoin and long-term doxycycline, and the development of inflammatory bowel disease.
- Research on the role of diet in the development and progression of inflammatory bowel disease.
- Research on the influence of the microbiome on inflammatory bowel disease.
- Research on hyperbaric oxygen treatment of inflammatory bowel disease.
- Research on treatment strategies for patients whose inflammatory bowel disease is refractory to standard care medications or who positively respond to standard care medications, but become nonresponsive over time.

Influenza

- Development of a diagnostic test for influenza drug resistance.
- Development and testing of a universal influenza vaccine.
- Development and evaluation of novel, innovative, and/or combination influenza therapies.
- Research to improve the understanding of host responses to influenza infection and the mechanisms of drug resistance.
- Research on the factors that contribute to and/or reduce the risk of the emergence of zoonotic and pandemic influenza viruses.

Integrative Medicine

- Research on the use of integrative medicine strategies, such as meditation, tai chi, acupuncture, and/or herbal medicine/nutritional supplementation, to improve the psychological health and quality of life in cancer patients and survivors.
- Research on the use of integrative medicine in treatment and management of chronic pain disorders, including comparative efficacy studies relative to standard of care.
- Rigorous longitudinal studies of integrative medicine approaches for enhancing resilience and treating psychological health issues and co-occurring disorders.
- Precision medicine research to investigate, develop, and validate biomarkers that can help determine an individual's response to integrative medicine treatments for pain.
- Development of outcomes tools and measures to evaluate the effectiveness of integrative medicine pain management approaches.
- Definitive studies to determine the effectiveness of self-care, meditation-based treatments, courses, and training programs in improving resiliency to stress in a military environment; in austere operational platforms such as submarines and small-deck ships; or in combat-fatigued military health care providers.

Interstitial Cystitis

- Studies that define the risk, prevalence, and operational impact of interstitial cystitis among active duty personnel.
- Identification of biological markers for making a definitive diagnosis of interstitial cystitis.
- Evaluation and assessment of novel and/or innovative treatment options for interstitial cystitis patients, including intravesical therapy.
- Research on the etiology of interstitial cystitis to inform targeted therapy development.

Malaria

- Investigation of mechanisms of drug resistance in malaria, to include host and pathogen, against drugs used for treatment and prophylaxis.
- Development of a vaccine that induces high levels of long-lived, broadly protective immunity against *Plasmodium falciparum* and/or *P. vivax*.
- Operational research to optimize application of existing malaria drugs to reduce impact of artemisinin resistant *P. falciparum* in military forces.

- Operational research to optimize deployment of pharmacovigilance systems to ensure capture of adverse events related to malaria chemoprophylaxis and treatment.
- Operational research to assess efficacy and spread of artemisinin combination therapies across the Greater Mekong Subregion's military forces.
- Development of malaria prophylactic regimens that encourage higher compliance and methods to monitor compliance in deployed Service members.
- Identification of novel and/or innovative malaria drug targets for blood and liver stage malaria parasites.
- Development of new, or improvement of existing, strategies for culturing relapsing malaria parasites, with emphasis on *Plasmodium vivax, P. ovale,* and *P. cynomolgi* in vitro.
- Development, testing, and evaluation of spatial repellents to provide protection against Anopheline vectors.
- Identification of a correlate of immunity against malaria and development of a surrogate test of protection.
- Structurally optimize validated malaria antigens by engineering protective epitopes to elicit specific and enhanced immune responses.

Metals Toxicology

- Identification and validation of biomarkers to evaluate military Service members' acute exposure to toxic metals in an operational environment and predict potential consequent health risks and associated health outcomes.
- Retrospective studies to evaluate risk and exposure among workers at industrial facilities.
- Research on the toxicity of metal combinations and the interaction between different metal components.
- Research on the additive effects of multiple exposures to metal(s).
- Research on underlying diet quality and micronutrient sufficiency and the risk for metals toxicity.
- Research on the toxicity of metal-based engineered nanomaterials.

Mitochondrial Disease

- Research on the basic biology and physiology of mitochondria to better understand the pathology of primary mitochondrial diseases.
- Development of improved tools and animal models to study primary mitochondrial disease and evaluate therapeutics.
- Identification and testing of non-invasive techniques and biomarkers to monitor mitochondrial function, aid in diagnosis, and/or evaluate therapeutic efficacy.
- Research on novel and/or innovative treatments to alleviate symptoms or slow down the progression of mitochondrial diseases.

Musculoskeletal Disorders

- Research to increase understanding, diagnosis, prevention, and/or treatments of chronic overuse musculoskeletal disorders.
- Research on measures (e.g., clinical biomarkers, novel/innovative interventions, therapeutics) to diagnose, predict, reduce the incidence of, or optimize health outcomes in military training- and Service-related musculoskeletal disorders.
- Research on the validation and/or optimization of rehabilitation strategies for Service-related musculoskeletal disorders.
- Research to prevent, control, and/or optimize musculoskeletal health outcomes for work-related musculoskeletal disorders.
- Research on lower back pain strategies to prevent surgery and recurrence of symptoms, identify factors that predict optimal treatment response for different patients, and encourage self-management as a treatment.
- Research to determine whether tailored psychosocial education interventions are effective in improving psychological resilience to musculoskeletal pain.
- Research on standardized approaches for early identification and prevention of chronic musculoskeletal pain.

Nanomaterials for Bone Regeneration

- Research on nanomaterials-based methods to facilitate recruitment of endogenous cell populations for enhanced bone regeneration and osseointegration.
- Technologies addressing segmental/large bone defects in craniomaxillofacial and/or load-bearing regions.
- Development of controlled release/extended release of growth factors for bone regeneration.
- Development of technologies that repair the soft tissue envelope to enhance bone regeneration.

Non-Opioid Pain Management

- Research on pain management strategies for patients with limited access to skilled providers and resources, including battlefield, prolonged field, transport, and other resource-limited environments.
- Development of population-based outcomes, tools, and measures to evaluate the effectiveness of pain management approaches across time and environments.
- Research on treatments for chronic pain management, particularly in complex patients (i.e., chronic, high-utilization, polypharmacy patients).
- Research to increase understanding of and preventative treatments for conversion of acute to chronic pain.
- Research that provides evidence of which pain management strategies work for whom and under what conditions.
- Research to identify and address biopsychosocial aspects of pain to reduce or eliminate the use of opioid pain medication(s).

- Comparative studies evaluating the efficacy of different pain management strategies, including complementary and alternative medicine approaches.
- Identification and development of non-opioid pain management techniques that promote positive psychological health-related outcomes.
- Development of non-opioid pain medicine that can be given via inhalation or intramuscularly, submucosally, or intravenously on the battlefield to provide adequate relief of pain without affecting the cardiorespiratory systems.
- Research on case management strategies designed to optimize the effectiveness of pain clinics, support use of non-opioid pain therapies, and reduce dependence on opioid therapies.
- Research that will directly obtain an FDA analgesic indication for oral transmucosal or intramuscular ketamine.

Pancreatitis

- Retrospective studies to determine the risk and incidence of pancreatitis among former and current active duty personnel.
- Development and testing of novel and/or innovative therapeutics for acute and/or chronic pancreatitis.
- Research on the basic biology and physiology of the pancreas to better understand the etiology and pathology of pancreatitis.

Pathogen-Inactivated Dried Cryoprecipitate

- Development or validation of technology to produce pathogen-reduced dried cryoprecipitate in military or civilian donor centers that is acceptable for FDA licensure in support of combat or contingency operations.

Polycystic Kidney Disease

- Development of improved treatment strategies for polycystic kidney disease, including approaches to identify and monitor patients at higher risk for progressing to end-stage renal disease.
- Research on the underlying pathobiology and molecular mechanisms of polycystic kidney disease, including studies of genetic factors, cyst formation and growth, the role of cilia, and factors that modify disease progression and/or severity.
- Research on the lifestyle factors that may modify the progression of polycystic kidney disease.

Post-Traumatic Osteoarthritis

- Research to establish activity recommendations for maximal joint life following joint repair, particularly in young patient populations.
- Research into cell-based approaches for treatment or prevention of post-traumatic osteoarthritis.
- Studies to evaluate and develop best practices for multidisciplinary team approaches and treatment algorithms for post-traumatic osteoarthritis.

- Development or validation of novel and/or innovative approaches to restoring joint stability after injury.
- Sustained release, intra-articular injectable steroidal, non-steroidal, or disease-modifying therapies that offer two or more months of symptomatic relief of pain and/or inflammation in a single injection.
- Research on therapies that target multiple phases of the cellular response pathways that are implicated in the development of post-traumatic osteoarthritis, including cell death, inflammation, matrix changes, and changes in catabolic and anabolic responses.
- Development and/or validation of predictive models for early onset post-traumatic osteoarthritis in extremity-injured patients for the purpose of tailoring rehabilitation programs toward prevention, delay, or mitigation of onset.

Pulmonary Fibrosis

- Retrospective studies to determine the risk and incidence of pulmonary fibrosis among military Service members, including Veterans.
- Identification of biomarkers of pulmonary injury or early predictors of interstitial lung disease.
- Research into the pathobiology and molecular mechanisms underlying the development of pulmonary fibrosis.
- Development and/or testing of novel and/or innovative treatments to delay or modify the progression of pulmonary fibrosis.
- Development and/or validation of improved tools and animal models to study pulmonary fibrosis and evaluate therapeutics.

Respiratory Health (excludes lung cancer and mesothelioma)

- Research on the causes, treatment, and prevention of chronic obstructive pulmonary disease (COPD), including identification and validation of biomarkers and disease phenotypes, as well as employing personalized medicine in clinical research and disease management.
- Research investigating the treatment of patients with chronic mild hypoxia.
- Research on the cause, treatment, and prevention of respiratory symptoms and ailments possibly associated with deployed and redeployed military personnel, including acute eosinophilic pneumonia, asthma, allergies, and other chronic lung diseases and breathing problems.
- Research to evaluate the impact of deployment on the prevalence and severity of respiratory disease in military Service members and Veterans.
- Development and/or validation of methods to detect volatile organic hydrocarbons from the breath of individuals exposed to toxic chemicals or other agents that can cause lung injury, which can be utilized for diagnosis and prognosis of lung injury or disease.
- Identification of biochemical, physiological, or combined biomarkers for evaluating risk or extent of injury from either acute or long-term toxic occupational or environmental exposures.

- Research investigating exposure rates, detection, and treatment of diseases related to inhalation of mold and fungi, such as coccidioidomycosis.

Rett Syndrome

- Identification and/or validation of novel and/or innovative biological targets for the treatment of Rett syndrome.
- Development and testing of interventions to improve the neurological symptoms of Rett syndrome.
- Research to understand the relationship between genetic mutations, physical traits, and symptoms in individuals with Rett syndrome.
- Research on the pathobiology of the MeCP2 gene and protein.
- Research to understand Rett syndrome's commonalities with and differences from classic autism and regressive autism.

Rheumatoid Arthritis

- Research to better understand the relationship between genetic risk and environmental triggers, such as infection or smoking, in developing rheumatoid arthritis.
- Studies that identify or validate biomarkers or personalized medicine strategies that allow for individualized medication choice based on the patient's underlying biology or disease state.
- Research on the long-term use of immunosuppressants in patients with rheumatoid arthritis and the likelihood of developing infections.
- Research to establish activity recommendations following joint replacement for maximal joint life.

Scleroderma

- Research on the molecular mechanisms and pathogenesis of scleroderma.
- Development and/or testing of novel and/or innovative therapies and identification of novel and/or innoavtive therapeutic targets in scleroderma.
- Research on biomarkers and other approaches to diagnose scleroderma, monitor disease progression, and/or assess response to treatment.
- Epidemiologic studies investigating the impact of localized scleroderma (morphea) on duty performance, use of personal protective equipment, and deployability.
- Research on the efficacy of newer immunosuppressive agents, including biologics and small molecule inhibitors, in the treatment of morphea and scleroderma (systemic sclerosis).
- Research on the efficacy of UVA1 for topical treatment in localized scleroderma and systemic sclerosis.

Sleep Disorders

- Research on how the disruption of normal sleep and circadian biological rhythms adversely affects the physical and psychological health, safety, performance, and productivity of military and civilian populations.
- Studies to assess sleep disturbance and clinical sleep disorders in female military personnel.

- Research on the association between sleep disorders and post-traumatic stress disorder, traumatic brain injury, depression, and/or anxiety and suicidal behaviors.
- Research to assess clinical algorithms that improve adherence to continuous positive airway pressure (CPAP).
- Investigations into non-CPAP-based treatment regimens that enhance readiness and deployability in active duty military personnel.
- Research focused on investigating how psychiatric disorders (post-traumatic stress disorder in particular), combined with the disruption/degradation of sleep quality, impact long-term physical health through changes in hormonal physiology.
- Development and/or testing of non-pharmacological treatments for sleep disorders.
- Research on the impact of sleep deprivation on the efficacy of psychological treatments for post-traumatic stress disorder, depression, and other mood disorders.
- Research on the prevention and/or mitigation of sleep disorders that are associated with long aeromedical evacuation flights for both clinical team members and patients.
- Development of tools and technologies that allow rapid and objective measurement of Service member performance in the face of fragmented sleep and/or sleep maintenance difficulty.
- Research examining the broader effects of Warfighters' sleep disorders on the sleep, behavioral health, and productivity of their family members, as well as the readiness of their military unit.

Spinal Muscular Atrophy

- Research into phenotyping the spinal muscular atrophy disease state.
- Research into which molecular deficiency imposed by a deficiency of SMN, a motor neuron protein, causes motor neuron deficits.
- Exploration of the form and function of SMN-depleted neuromuscular junctions at ultrastructural (e.g., dysregulation of endocytosis), transcriptomic, and proteomic levels, particularly the mildest SMN reduction that leads to consistent quantifiable motor neuron loss.
- Research to find non-SMN-altering spinal muscular atrophy modifying genes in cellular, small animal, and human models that may lead to identification of novel and/or innovative therapeutic targets.
- Research to further understand SMN gene regulation and post-transcriptional mechanisms leading to synergistic SMN-repleting approaches, as well as to determine whether boosting SMN induction maximizes efficacy.
- Research to determine whether SMN can be repleted to physiologic levels in all cells in the spinal muscular atrophy model immediately post-natally and whether there is a limit to the therapeutic response.
- Clinical research to determine what clinical findings persist even with treatment; whether these findings are related to drug timing, dosage, biodistribution, or a combination of factors; and whether these factors are modifiable.

- Research involving dietary/nutritional studies in spinal muscular atrophy to identify the optimal anabolic diet.
- Research to determine mitochondrial involvement and astrocytic and other non-neuronal contributions to motor neuron vulnerability.

Sustained-Release Drug Delivery

- Development of technology that can provide a sustained-release delivery of drugs for a minimum of 1 week or more. Potential applications of this technology could include long-acting therapies for post-traumatic stress, opiate dependence, low-dose pain control, allergies, attention deficit/hyperactivity disorder, chemoprophylaxis, and other conditions.
- Research into the use of printable, sustained-release scaffolds for predictable delivery of prescription and non-prescription medications.
- Research into novel and/or innovative approaches to developing bioavailable and sustained-release oral formulations of intravenous broad-spectrum fungicidal and anti-microbial medications.

Tinnitus

- Research to understand the mechanisms of tinnitus, its relationship to noise-induced hearing loss, and progression to chronic tinnitus, with the focus on developing interventions for tinnitus.
- Research to increase knowledge of the prevalence, incidence, natural history, and occupational and sex-related differences of tinnitus and its possible relation to individual blast/noise exposures.
- Research to understand the operational readiness impacts of tinnitus on the military.
- Development and validation of objective tools/methods to diagnose and characterize tinnitus (e.g., imaging techniques to identify functional and structural changes in the brain, biomarkers of resiliency, and susceptibility to tinnitus).
- Identification of novel and/or innovative therapies for interventions to prevent, manage, and treat tinnitus, including behavioral approaches, new uses for existing drugs, nutritional and pharmaceutical strategies, and acoustic, electrical, and other stimulation technologies.

Tuberculosis

- Development of a diagnostic assay that can be used at the point of care to rapidly and accurately diagnose tuberculosis.
- Development of novel and/or innovative tuberculosis vaccines or optimization of current tuberculosis vaccines.
- Identification and/or validation of biomarkers that can be used to assess vaccine efficacy and protection against tuberculosis disease.
- Research to understand, diagnose, or treat multi-drug-resistant tuberculosis or extensively drug-resistant tuberculosis.
- Research to determine the appropriate precautions to use for transport of active tuberculosis patients.

- A sustained release formulation of anti-tuberculosis drugs that would facilitate long-term treatment and reduce the emergence of resistance due to poor compliance.

Vaccine Development for Infectious Disease

- Development of a therapeutic vaccine and/or other strategies to achieve HIV remission.
- Evaluation of passive immunization strategies to use in conjunction with dengue and Zika vaccinations.
- Research leading to a better understanding of the immune mechanisms involved in clearance of the dengue and Zika viruses and the mechanisms of immune enhancement that lead to more severe clinical disease.
- Development of flexible vaccine technologies that can be used to rapidly respond to emerging and re-emerging infectious diseases threats.
- Evaluation of humoral and cellular immune responses after vaccination compared to natural infection.
- Development of a safe, effective dengue human challenge model for clinical trials.
- Development of a non-human primate model to study Zika virus-induced microcephaly/fetal neuropathology as a tool to measure the safety and efficacy of vaccines and antivirals.
- Development and fielding of vaccines to prevent U.S. Service members from becoming ill from endemic disease exposure during operational deployments. This includes, but is not limited to, arthropod-borne diseases (Zika virus, dengue, chikungunya, hantavirus hemorrhagic fever with renal/pulmonary syndrome, rickettsioses, and trypanosomiasis), leptospirosis, HIV, norovirus, Middle East Respiratory Syndrome coronavirus, schistosomiasis, leishmaniasis, Nipah virus, Lassa fever, and West Nile fever.

Vascular Malformations

- Studies into the natural history, genetics, and pathogenesis of vascular malformations.
- Research to discover or develop novel and/or innovative therapeutic targets and treatments to regress or prevent vascular malformations.
- Research to improve methods to diagnose and manage vascular malformations.
- Development of non-invasive or minimally invasive technologies or approaches for the control of internal bleeding associated with vascular malformations.
- Studies to identify risk and/or establish standard practices for the treatment of hemorrhage from brain arteriovenous malformations.
- Research on the prevention or treatment of complications associated with vascular malformations such as pain, infection, seizures, tissue breakdown, tissue overgrowth, or airway obstruction.
- Studies to elucidate the progression of vascular malformations in disease-in-a-dish/microfluidic models for the purpose of identifying novel and/or innovative drug targets, screening existing drugs, and/or elucidating the pathogenesis of the disease.

Women's Heart Disease

- Retrospective studies to determine the risk and incidence of heart disease among former and current female active duty personnel, including Reservists and National Guard members.
- Research focused on elucidating the potential relationship between post-traumatic stress disorder and women's heart disease.
- Identification of sex-specific approaches to increase the effectiveness of diagnosis, treatment, and/or cardiac rehabilitation programs.
- Research on factors to predict and prevent the long-term impacts of gestational diabetes, gestational hypertension, and preeclampsia on the cardiovascular health of women.
- Research on trauma-induced cardiac arrest secondary to hemorrhage and polytrauma in the female population.

APPENDIX 3
DOD AND VA WEBSITES

PIs are encouraged to integrate and/or align their research projects with Department of Defense (DoD) and/or Department of Veterans Affairs (VA) research laboratories and programs. Collaboration with DoD or VA investigators is also encouraged. Below is a list of websites that may be useful in identifying additional information about DoD and VA areas of research interest, ongoing research or potential opportunities for collaboration within the FY17 PRMRP Topic Areas.

Air Force Office of Scientific Research
http://www.wpafb.af.mil/afrl/afosr/

Air Force Research Laboratory
http://www.wpafb.af.mil/afrl

Armed Forces Radiobiology Research Institute
http://www.usuhs.edu/afrri/

Clinical and Rehabilitative Medicine Research Program
https://crmrp.amedd.army.mil

Combat Casualty Care Research Program
https://ccc.amedd.army.mil

Congressionally Directed Medical Research Programs
http://cdmrp.army.mil

Defense Advanced Research Projects Agency
http://www.darpa.mil/

Defense Technical Information Center
http://www.dtic.mil

Defense Threat Reduction Agency
http://www.dtra.mil/

Military Health System Research Symposium
https://mhsrs.amedd.army.mil/SitePages/Home.aspx

Military Infectious Diseases Research Program
https://midrp.amedd.army.mil

Military Operational Medicine Research Program
https://momrp.amedd.army.mil

Naval Health Research Center
http://www.med.navy.mil/sites/nhrc

Navy and Marine Corps Public Health Center
http://www.nmcphc.med.navy.mil/

Office of Naval Research
http://www.med.navy.mil/

Office of the Under Secretary of Defense for Acquisition, Technology and Logistics
http://www.acq.osd.mil/

Telemedicine and Advanced Technology Research Center
http://www.tatrc.org/

Uniformed Services University of the Health Sciences
http://www.usuhs.edu/research

U.S. Army Institute of Surgical Research
http://www.usaisr.amedd.army.mil/

U.S. Army Research Institute of Environmental Medicine
http://www.usariem.army.mil/

U.S. Army Medical Research Institute of Infectious Diseases
http://www.usamriid.army.mil/

U.S. Army Medical Research and Materiel Command
http://mrmc.amedd.army.mil

U.S. Army Research Laboratory
http://www.arl.army.mil

U.S. Department of Defense Blast Injury Research Program
https://blastinjuryresearch.amedd.army.mil/

U.S. Department of Veterans Affairs, Office of Research and Development
http://www.research.va.gov

U.S. Naval Research Laboratory
http://www.nrl.navy.mil

Walter Reed Army Institute of Research
http://www.wrair.army.mil/

I. OVERVIEW OF THE FUNDING OPPORTUNITY

Program Announcement for the Department of Defense

Defense Health Program

Congressionally Directed Medical Research Programs

Peer Reviewed Medical Research Program

Investigator-Initiated Research Award

Announcement Type: Initial

Funding Opportunity Number: W81XWH-17-PRMRP-IIRA

Catalog of Federal Domestic Assistance Number: 12.420 Military Medical Research and Development

SUBMISSION AND REVIEW DATES AND TIMES

- **Pre-Application Submission Deadline:** 5:00 p.m. Eastern time (ET), July 13, 2017
- **Invitation to Submit an Application:** August 2017
- **Application Submission Deadline:** 11:59 p.m. ET, October 18, 2017
- **End of Application Verification Period:** 5:00 p.m. ET, October 23, 2017
- **Peer Review:** December 2017
- **Programmatic Review:** February 2018

This Program Announcement must be read in conjunction with the General Application Instructions, version 20170516. The General Applications Instructions document is available for downloading from the Grants.gov funding opportunity announcement by selecting the "Package" tab, clicking "Preview," and then selecting "Download Instructions."

TABLE OF CONTENTS

I. OVERVIEW OF THE FUNDING OPPORTUNITY 1

II. DETAILED INFORMATION ABOUT THE FUNDING OPPORTUNITY 4

II.A. Program Description 4

II.A.1. FY17 PRMRP Topic Areas 4

II.B. Award Information 6

II.C. Eligibility Information 9

II.C.1. Eligible Applicants 9

II.C.2. Cost Sharing 10

II.C.3. Other 10

II.D. Application and Submission Information 10

II.D.1. Address to Request Application Package 11

II.D.2. Content and Form of the Application Submission 11

II.D.3. Dun and Bradstreet Universal Numbering System (DUNS) Number and System for Award Management (SAM) 27

II.D.4. Submission Dates and Times 27

II.D.5. Funding Restrictions 28

II.D.6. Other Submission Requirements 29

II.E. Application Review Information 30

II.E.1. Criteria 30

II.E.2. Application Review and Selection Process 32

II.E.3. Integrity and Performance Information 33

II.E.4. Anticipated Announcement and Federal Award Dates 33

II.F. Federal Award Administration Information 33

II.F.1. Federal Award Notices 33

II.F.2. Administrative and National Policy Requirements 34

II.F.3. Reporting 35

II.G. Federal Awarding Agency Contacts 35

II.G.1. CDMRP Help Desk 35

II.G.2. Grants.gov Contact Center 36

II.H. Other Information 36

II.H.1. Program Announcement and General Application Instructions Versions 36

II.H.2. Administrative Actions 36

II.H.3. Application Submission Checklist 39

APPENDIX 1 ACRONYM LIST 41
APPENDIX 2 AREAS OF ENCOURAGEMENT..... 42
APPENDIX 3 DOD AND VA WEBSITES..... 61

II. DETAILED INFORMATION ABOUT THE FUNDING OPPORTUNITY

II.A. Program Description

Applications to the Fiscal Year 2017 (FY17) Peer Reviewed Medical Research Program (PRMRP) are being solicited for the Defense Health Agency (DHA) J9, Research and Development Directorate, by the U.S. Army Medical Research Acquisition Activity (USAMRAA) using delegated authority provided by United States Code, Title 10, Section 2358 (10 USC 2358). As directed by the Office of the Assistant Secretary of Defense for Health Affairs (OASD[HA]), the DHA manages the Defense Health Program (DHP) Research, Development, Test, and Evaluation (RDT&E) appropriation. The execution management agent for this Program Announcement is the Congressionally Directed Medical Research Programs (CDMRP). The PRMRP was initiated in 1999 to provide support for military health-related research of exceptional scientific merit. Appropriations for the PRMRP from FY99 through FY16 totaled $1.371 billion. The FY17 appropriation is $300 million (M).

The vision of the FY17 PRMRP is to improve the health and well-being of all military Service members, Veterans, and beneficiaries. The PRMRP challenges the scientific and clinical communities to address at least one of the FY17 Topic Areas with original ideas that foster new directions along the entire spectrum of research and clinical care. The program seeks applications in laboratory, clinical, behavioral, epidemiologic, and other areas of research to advance knowledge in disease etiology, improve prevention, detection, diagnosis, treatment, and quality of life for those affected by a relevant disease or condition, and to develop and validate clinical care or public health guidelines.

II.A.1. FY17 PRMRP Topic Areas

All applications for PRMRP funding must specifically address at least one of the Topic Areas as directed by Congress and must be directly relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries. If the proposed research does not specifically address at least one of the FY17 PRMRP Topic Areas, the Government will administratively withdraw the application. The Government reserves the right to reassign the application's Topic Area if submitted under an inappropriate Topic Area. The FY17 PRMRP Topic Areas are listed below.

- Acute Lung Injury
- Antimicrobial Resistance
- Arthritis
- Burn Pit Exposure
- Chronic Migraine and Post-Traumatic Headache
- Congenital Heart Disease
- Constrictive Bronchiolitis
- Diabetes
- Diarrheal Diseases
- Dystonia
- Early Trauma Thermal Regulation
- Eating Disorders

- Emerging Infectious Diseases
- Epidermolysis Bullosa
- Focal Segmental Glomerulosclerosis
- Fragile X
- Guillain-Barré Syndrome
- Hepatitis B and C
- Hereditary Angioedema
- Hydrocephalus
- Immunomonitoring of Intestinal Transplants
- Inflammatory Bowel Diseases
- Influenza
- Integrative Medicine
- Interstitial Cystitis
- Malaria
- Metals Toxicology
- Mitochondrial Disease
- Musculoskeletal Disorders
- Nanomaterials for Bone Regeneration
- Non-Opioid Pain Management
- Pancreatitis
- Pathogen-Inactivated Dried Cryoprecipitate
- Polycystic Kidney Disease
- Post-Traumatic Osteoarthritis
- Pulmonary Fibrosis
- Respiratory Health
- Rett Syndrome
- Rheumatoid Arthritis
- Scleroderma
- Sleep Disorders
- Spinal Muscular Atrophy
- Sustained-Release Drug Delivery
- Tinnitus
- Tuberculosis
- Vaccine Development for Infectious Disease
- Vascular Malformations
- Women's Heart Disease

Research relevant to one or more FY17 PRMRP Topic Areas may be considered for funding. ***Applicants should select the FY17 Program Announcement most appropriate to the stage of the proposed research.*** Areas of Encouragement related to the FY17 PRMRP Topic Areas have been identified by the Department of Defense (DoD), the Department of Veterans Affairs (VA), and other relevant stakeholders (Appendix 2). Applicants are urged to read and consider these Areas of Encouragement before preparing their applications. ***The information provided is not exhaustive, and applicants are not restricted to submitting applications that address an Area of Encouragement on this list.***

II.B. Award Information

The PRMRP Investigator-Initiated Research Award is intended to support studies that will make an important contribution toward research and/or patient care for a disease or condition related to at least one of the Congressionally directed FY17 PRMRP Topic Areas.

The rationale for a research idea may be derived from a laboratory discovery, population-based studies, a clinician's first-hand knowledge of patients, or anecdotal data. Applications must include relevant data that support the rationale for the proposed study. These data may be unpublished or from the published literature.

Impact: The Investigator-Initiated Research Award is designed to support research with the potential to yield highly impactful data that could lead to critical discoveries or major advancements. It is the responsibility of the Principal Investigator (PI) to clearly articulate the project's potential immediate and long-range outcome(s)/product(s) (knowledge and/or materiel) and how they will impact a central critical problem or question in the field of research and/or patient care in the FY17 PRMRP Topic Area(s) addressed.

Research projects may focus on any phase of research from basic laboratory research through translational research, including preclinical studies in animal models and human subjects, as well as correlative studies associated with an existing clinical trial. Research involving human subjects and human anatomical substances is permitted; ***however, this award may not be used to conduct clinical trials.*** A clinical trial is defined as a prospective accrual of patients (human subjects) in whom an intervention (e.g., device, drug, biologic, surgical procedure, rehabilitative modality, behavioral intervention, or other) is tested for a measurable outcome with respect to safety, effectiveness, and/or efficacy. This outcome represents a direct effect on the subject of that intervention or interaction. For more information on how to distinguish clinical research from clinical trials, see the Human Subject Resource Document at https://ebrap.org/eBRAP/public/Program.htm. PIs seeking funding for a clinical trial should apply to the FY17 PRMRP Clinical Trial Award mechanism (W81XWH-17-PRMRP-CTA).

The anticipated direct costs budgeted for the entire period of performance for a single PI FY17 PRMRP IIRA award will not exceed **$1.2M**. The anticipated direct costs budgeted for the entire period of performance for an FY17 PRMRP IIRA award with the Partnering PI Option will not exceed **$1.5M**. Refer to Section II.D.5, Funding Restrictions, for detailed funding information.

Military Relevance: Relevance to the healthcare needs of military Service members, Veterans, and beneficiaries is a key feature of this award. Investigators are encouraged to consider the following characteristics as examples of how a project may demonstrate military relevance:

- Explanation of how the project addresses an aspect of the target disease/condition that has direct relevance to military Service members, Veterans, or other military health system beneficiaries
- Description of how the knowledge, information, products, or technologies gained from the proposed research could be implemented in a dual-use capacity to benefit the civilian population and also address a military need

- Use of military or Veteran populations or datasets in the proposed research, if appropriate to the proposed research project

- Involvement of military consultants (Army, Air Force) or specialty leaders (Navy, Marine Corps) to the Surgeons General in a relevant specialty area

PIs are encouraged to integrate and/or align their research projects with DoD and/or VA research laboratories and programs. Collaboration with the DoD or VA is also encouraged. A list of websites that may be useful in identifying additional information about ongoing DoD and VA areas of research interest or potential opportunities for collaboration within the FY17 PRMRP Topic Areas can be found in Appendix 3.

Partnering PI Option: As a method to bring new perspectives in addressing the critical problem or question and facilitate progress in the field through collaborative efforts, the FY17 PRMRP is offering a Partnering PI Option for this award mechanism. The results of this partnering project should significantly advance the research beyond what would be possible through individual efforts. The Partnering PI Option is structured so that two investigators, each of whom will be designated as a PI, work synergistically on a single project. Each PI should bring complementary skills and perspectives to the research project. Developing the research plan should involve a reciprocal flow of ideas and information between the partners. The application should clearly demonstrate that both PIs have equal intellectual input into the des ign of the project and will devote equivalent levels of effort to the conduct of the project. Each PI must demonstrate that he/she possesses the research experience and resources to function as a PI and must also exhibit an appropriate level of authority and responsibility to direct the project supported by the awards. ***New and multi-institutional collaborative efforts are strongly encouraged.*** PIs should include plans for communication between investigators at different organizations, if applicable. Additionally, participating organizations must be willing to resolve potential intellectual and material property issues and to remove any barriers that might interfere with achieving high levels of cooperation to ensure successful completion of the proposed research project. As noted above, collaborations with DoD or VA researchers and clinicians are encouraged.

For the application process, one PI will be identified as the Initiating PI and will be responsible for the majority of the administrative tasks associated with application submission. The other PI will be identified as the Partnering PI. Initiating and Partnering PIs each have different submission requirements as described in Section II.D.2.b, Full Application Submission Content. If recommended for funding, each PI will receive his/her own award. ***A separate application submission is required for each partner, even if both PIs are at the same organization.*** Additional collaborators may be included in the application without being designated as PIs.

Applications submitted under the Partnering PI Option are allowed to request a higher direct cost budget than applications submitted with a single PI (see Section II.D.5, Funding Restrictions, for details). Costs should be divided as evenly as possible between both partners unless appropriately justified, but no more than 75% (or no less than 25%) of the proposed direct costs may be requested by a single partner. Submission of a Partnership Statement, Attachment 8, is required for applications submitted under the Partnering PI Option.

Use of Active Duty Military and VA Populations: If the proposed research plan involves access to active duty military and/or VA patient populations or resources, the PI is responsible for establishing such access. If possible, access to target active duty military and/or VA patient populations/resources should be confirmed at the time of application submission by inclusion of a letter of support, signed by the lowest ranking person with approval authority, for studies involving active duty military Service members, Veterans, military- and/or VA-controlled study materials, and military and/or VA databases. If access cannot be confirmed at the time of application submission, the Government reserves the right to withhold or revoke funding until the PI has demonstrated support for and access to the relevant population(s) and/or resources. Note that access to a Veteran population for clinical studies may only be obtained by either collaboration with a VA investigator where the VA investigator has a substantial role in the research or by advertising to the general public.

Research Involving Human Anatomical Substances, Human Subjects, or Human Cadavers: All DoD-funded research involving new and ongoing research with human anatomical substances, human subjects, or human cadavers must be reviewed and approved by the U.S. Army Medical Research and Materiel Command (USAMRMC) Office of Research Protections (ORP), Human Research Protection Office (HRPO) prior to research implementation. This administrative review requirement is in addition to the local Institutional Review Board (IRB) or Ethics Committee (EC) review. Local IRB/EC approval at the time of submission is ***not*** required. The HRPO is mandated to comply with specific laws and requirements governing all research involving human anatomical substances, human subjects, or human cadavers that is supported by the DoD. These laws and requirements will necessitate information in addition to that supplied to the IRB/EC. ***Allow a minimum of 2 to 3 months for HRPO regulatory review and approval processes***. When possible, protocols should be written for research with human subjects and/or human anatomical substances that are specific to the DoD-supported effort outlined in the submitted application. Submission to HRPO of protocols covering more than the scope of work in the DoD-funded award will require HRPO review of the entire protocol as DoD-supported research and may include extensive modifications to meet DoD human subjects protection requirements. Refer to the General Application Instructions, Appendix 1, and the Human Subject Resource Document available on the electronic Biomedical Research Application Portal (eBRAP) "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) for additional information.

Research Involving Animals: All projects should adhere to a core set of standards for rigorous study design and reporting to maximize the reproducibility and translational potential of preclinical research. The standards are described in Landis, S.C., et al. A call for transparent reporting to optimize the predictive value of preclinical research. *Nature* 2012, 490:187-191 (www.nature.com/nature/journal/v490/n7419/full/nature11556.html). While these standards are written for preclinical studies, the basic principles of randomization, blinding, sample-size estimation, and data handling derive from well-established best practices in clinical studies. Applicants should consult the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines to ensure relevant aspects of rigorous animal research are adequately planned for and, ultimately, reported. The ARRIVE guidelines can be found at http://www.elsevier.com/__data/promis_misc/622936arrive_guidelines.pdf.

All DoD-funded research involving new and ongoing research with animals must be reviewed and approved by the USAMRMC ORP Animal Care and Use Review Office (ACURO), in addition to the local Institutional Animal Care and Use Committee (IACUC) of record. IACUC approval at the time of submission is ***not*** required. Specific documents relating to the use of animals in the proposed research will be requested **if the application is selected for funding**. The ACURO must review and approve all animal use prior to the start of working with animals, including amendments to ongoing projects. PIs must submit the institutional animal use protocol, IACUC approval of that protocol, and a version of the animal use appendix titled "Research Involving Animals." ***Allow at least 2 to 3 months for ACURO regulatory review and approval processes for animal studies.*** Refer to the General Application Instructions, Appendix 1, for additional information.

The CDMRP intends that information, data, and research resources generated under awards funded by this Program Announcement be made available to the research community (which includes both scientific and consumer advocacy communities) and to the public at large. For additional guidance, refer to the General Application Instructions, Appendix 2, Section K.

Awards will be made no later than September 30, 2018. For additional information refer to Section II.F.1, Federal Award Notices.

II.C. Eligibility Information

II.C.1. Eligible Applicants

II.C.1.a. Organization: All organizations, including international organizations, are eligible to apply.

Government Agencies within the United States: Local, state, and Federal Government agencies are eligible to the extent that applications do not overlap with their fully funded internal programs. Such agencies are required to explain how their applications do not overlap with their internal programs.

As applications for this Program Announcement may be submitted by extramural and intramural organizations, these terms are defined below.

Extramural Organization: An eligible non-DoD organization. Examples of extramural organizations include academia, biotechnology companies, foundations, Government, and research institutes. *Extramural Submission: Application submitted by a non-DoD organization to Grants.gov.*

Intramural DoD Organization: A DoD laboratory, DoD military treatment facility, and/or DoD activity embedded within a civilian medical center. *Intramural Submission: Application submitted by a DoD organization for an intramural investigator who is a DoD military or civilian employee working within a DoD laboratory or military treatment facility or in a DoD activity embedded within a civilian medical center.*

Note: Applications from an intramural organization or from an extramural non-DoD Federal organization may be submitted through a research foundation.

The USAMRAA makes awards to eligible organizations, not to individuals.

II.C.1.b. Principal Investigator:

- Investigators at or above the level of Assistant Professor (or equivalent) are eligible to submit applications.
    - **Partnering PI Option:** Investigators at or above the level of Assistant Professor (or equivalent) that bring complementary skills and perspectives to the research project.

An eligible PI, regardless of ethnicity, nationality, or citizenship status, must be employed by, or affiliated with, an eligible organization.

The CDMRP encourages all PIs to participate in a digital identifier initiative through Open Researcher and Contributor ID, Inc. (ORCID). Registration for a unique ORCID identifier can be done online at http://orcid.org/.

II.C.2. Cost Sharing

Cost sharing/matching is not an eligibility requirement.

II.C.3. Other

Extramural organizations must be able to access **.gov** and **.mil** websites in order to fulfill the financial and technical deliverable requirements of the award and submit invoices for payment.

There are no limitations on the number of applications for which an investigator may be named as a PI or Partnering PI.

For general information on required qualifications for award recipients, refer to the General Application Instructions, Appendix 3.

Refer to Section II.H.2, Administrative Actions, for a list of administrative actions that may be taken if a pre-application or application does not meet the administrative, eligibility, or ethical requirements defined in this Program Announcement.

II.D. Application and Submission Information

Submission of applications that are essentially identical or propose essentially the same research project to different funding opportunities within the same program and fiscal year is prohibited and will result in administrative withdrawal of the duplicative application(s). As an exception, applicants may submit the research project described in their Investigator-Initiated Research Award application as part of an application to the FY17 PRMRP Focused Program Award (W81XWH-17-PRMRP-FPA); however, accepting multiple awards to support the same project will not be allowed.

Extramural Submission is defined as an application submitted by a non-DoD organization to Grants.gov.

Intramural Submission is defined as an application submission by a DoD organization for an intramural investigator, who is a DoD military or civilian employee working within a DoD laboratory or military treatment facility, or working in a DoD activity embedded within a civilian medical center.

II.D.1. Address to Request Application Package

Submitting Extramural and Intramural Organizations: Pre-application content and forms can be accessed at eBRAP (https://eBRAP.org).

Submitting Extramural Organizations: Full application packages can be accessed at Grants.gov.

Submitting Intramural DoD Organizations: Full application packages can be accessed at eBRAP.org.

Contact information for the CDMRP Help Desk and the Grants.gov Contact Center can be found in Section II.G, Federal Awarding Agency Contacts.

II.D.2. Content and Form of the Application Submission

Submission is a two-step process requiring both ***pre-application*** and ***full application*** as indicated below. The submission process should be started early to avoid missing deadlines. There are no grace periods.

Pre-application Submission: All pre-applications for both extramural and intramural organizations must be submitted through eBRAP (https://eBRAP.org/).

eBRAP is a multifunctional web-based system that allows PIs to submit their pre-applications electronically through a secure connection, to view and edit the content of their pre-applications and full applications, to receive communications from the CDMRP, and to submit documentation during award negotiations and period of performance.

Full Application Submission: Full applications must be submitted through the online portals as described below.

Submitting Extramural Organizations: Full applications from extramural organizations must be submitted through Grants.gov. Applications submitted by extramural organizations (e.g., research foundations) on behalf of intramural DoD or other Federal organizations or investigators will be considered extramural submissions.

Submitting Intramural DoD Organizations: Intramural DoD organizations may submit full applications to either eBRAP or Grants.gov. Intramural DoD organizations that are unable to submit to Grants.gov should submit through eBRAP. Intramural DoD organizations with the capability to submit through Grants.gov may submit following the instructions for extramural

submissions through Grants.Gov or may submit to eBRAP. Applications from extramural organizations, including non-DoD Federal organizations, received through eBRAP will be withdrawn. See definitions in Section II.C.1, Eligible Applicants.

eBRAP allows intramural organizations to submit full applications following pre-application submission.

For both Extramural and Intramural applicants: A key feature of eBRAP is the ability of an organization's representatives and PIs to view and modify the full application submissions associated with them. eBRAP will validate full application files against the specific Program Announcement requirements and discrepancies will be noted in an email to the PI and in the Full Application Files tab in eBRAP. It is the applicant's responsibility to review all application components for accuracy as well as ensure proper ordering as specified in this Program Announcement.

The application title, eBRAP log number, and all information for the PI, Business Official(s), performing organization, and contracting organization must be consistent throughout the entire pre-application and application submission process. Inconsistencies may delay application processing and limit or negate the ability to view, modify, and verify the application in eBRAP. If any changes need to be made, the applicant should contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507 prior to the application deadline.

The Investigator-Initiated Research Award mechanism is structured to accommodate two PIs. One PI will be identified as the Initiating PI and will be responsible for the majority of the administrative tasks associated with application submission. The other PI will be identified as the Partnering PI. Initiating and Partnering PIs each have different submission requirements; however, both PIs should contribute significantly to the development of the proposed research project including the Project Narrative, Statement of Work, and other required components.

The Initiating PI must complete the pre-application submission process and submit the contact information for the Partnering PI. The Partnering PI will then be notified of the pre-application submission separately by email. ***The Partnering PI must follow the link in this email in order to associate his/her full application package with that of the Initiating PI. After following the link, the Partnering PI must verify their contact information, organization, and designation as an extramural or intramural submission within eBRAP.*** If not previously registered, the Partnering PI must register in eBRAP. A new pre-application based on this research project should not be initiated by the Partnering PI. Do not delay completing these steps. If they are not completed, the Partnering PI will not be able to view and modify his/her application during the verification period in eBRAP.

II.D.2.a. Step 1: Pre-Application Submission Content

During the pre-application process, each submission is assigned a unique log number by eBRAP. This unique eBRAP log number will be needed during the full application submission process.

To begin the pre-application process, first select whether the submitting organization is extramural or intramural, then confirm your selection or cancel. **Incorrect selection of extramural or intramural submission type may result in delays in processing.**

If an error has been made in the selection of extramural versus intramural and the pre-application submission deadline has passed, the PI or Business Official must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

All pre-application components must be submitted by the PI (for single PI applicants) or Initiating PI (for applicants submitting under the Partnering PI Option) through eBRAP (https://eBRAP.org/). Because the invitation to submit an application is based on the contents of the pre-application, investigators should not change the title or research objectives after the pre-application is submitted.

PIs and organizations identified in the pre-application should be the same as those intended for the subsequent application submission. If any changes are necessary after submission of the pre-application, the PI must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

The pre-application consists of the following components, which are organized in eBRAP by separate tabs (refer to the General Application Instructions, Section II.B, for additional information on pre-application submission):

- **Tab 1 – Application Information**

  Select the FY17 PRMRP Topic Area addressed by the proposed research. If the proposed research project is aligned with more than one FY17 PRMRP Topic Area, select the Topic Area of highest relevance as the required first choice.

- **Tab 2 – Application Contacts**

  Enter contact information for the PI. Enter the organization's Business Official responsible for sponsored program administration (the "person to be contacted on matters involving this application" in Block 5 of the Grants.gov SF424 (R&R) Form of extramural submissions). The Business Official must be either selected from the eBRAP list or invited in order for the pre-application to be submitted.

  Select the performing organization (site at which the PI will perform the proposed work) and the contracting organization (organization submitting on behalf of the PI, which corresponds to Block 5 on the Grants.gov SF424 (R&R) Form of extramural submissions), and click on "*Add Organizations to this Pre-application.*" The organizations must be either selected from the eBRAP drop-down list or invited in order for the pre-application to be submitted.

It is recommended that PIs identify an Alternate Submitter in the event that assistance with pre-application submission is needed.

- **Tab 3 – Collaborators and Key Personnel**

  Enter the name, organization, and role of all collaborators and key personnel associated with the application.

  FY17 PRMRP Programmatic Panel members should not be involved in any pre-application or application. For questions related to Panel members and pre-applications or applications, refer to Section II.H.2.c, Withdrawal, or contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

  The Initiating PI must enter the contact information for the Partnering PI in the Partnering PI section.

  To preserve the integrity of its peer and programmatic review processes, the CDMRP discourages inclusion of any employee of its review contractors having any role in pre-application or application preparation, research, or other duties for submitted pre-applications or applications. For FY17, the identities of the peer review contractor and the programmatic review contractor may be found at the CDMRP website (http://cdmrp.army.mil/about/2tierRevProcess). Pre-applications or applications that include names of personnel from either of these companies will be administratively withdrawn unless plans to manage conflicts of interest (COIs) are provided and deemed appropriate by the Grants Officer. Refer to the General Application Instructions, Appendix 3, for detailed information.

- **Tab 4 – Conflicts of Interest (COIs).**

  List all individuals other than collaborators and key personnel who may have a COI in the review of the application (including those with whom the PI has a personal or professional relationship). Refer to the General Application Instructions, Appendix 3, Section C, for further information regarding COIs.

- **Tab 5 – Pre-Application Files.**

  Note: Upload documents as individual PDF files unless otherwise noted. eBRAP will not allow a file to be uploaded if the number of pages exceeds the limit specified below.

  - **Preproposal Narrative (four-page limit):** The Preproposal Narrative page limit applies to text and non-text elements (e.g., figures, tables, graphs, photographs, diagrams, chemical structures, drawings) used to describe the project. Inclusion of URLs that provide additional information to expand the Preproposal Narrative and could confer an unfair competitive advantage is prohibited and may result in administrative withdrawal of the pre-application.

The Preproposal Narrative should include the following:

- **Topic Area:** Describe how the proposed project relates to at least one of the FY17 PRMRP Topic Areas. If applicable, describe how the proposed research project addresses an FY17 PRMRP Area of Encouragement (Appendix 2).
- **Research Idea:** Clearly articulate the rationale for the project by presenting the ideas and reasoning behind the proposed research; include relevant literature citations.
- **Research Strategy:** State the hypothesis to be tested and the objective(s) to be reached. State the project's specific aims and briefly describe the experimental approach. If applying under the Partnering PI Option, describe how the collaborative effort will provide synergy and enhance the research.
- **Impact:** Describe the potential short-term and long-term impact of the results of the proposed study on at least one of the FY17 PRMRP Topic Areas, and its related research field(s) and/or patient population(s).
- **Military Relevance:** Explain how the project is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

- **Pre-Application Supporting Documentation:** The items to be included as supporting documentation for the pre-application ***must be uploaded as individual files*** and are limited to the following:
  - References Cited (one-page limit): List the references cited (including URLs if available) in the Preproposal Narrative using a standard reference format that includes the full citation (i.e., author[s], year published, reference title, and reference source, including volume, chapter, page numbers, and publisher, as appropriate).
  - List of Abbreviations, Acronyms, and Symbols: Provide a list of abbreviations, acronyms, and symbols used in the Preproposal Narrative.
  - Key Personnel Biographical Sketches (five-page limit per individual). ***All biographical sketches should be uploaded as a single combined file.*** Biographical sketches should be used to demonstrate background and expertise through education, positions, publications, and previous work accomplished.

- **Tab 6 – Submit Pre-Application.**

  This tab must be completed for the pre-application to be accepted and processed.

Pre-Application Screening

Pre-Application Screening Criteria

To determine the technical merits of the pre-application and the relevance to the mission of the DHP and the PRMRP, pre-applications will be screened based on the following criteria:

- **Research Idea:** How well the rationale for the research idea is supported by presented background information.

- **Research Strategy:** How well the specific aims and proposed methodology support the research idea and objectives. If applying under the Partnering PI Option, how the partnership will advance the research beyond what would be possible through individual efforts.

- **Impact:** Whether the potential immediate and long-range outcome(s)/product(s) (knowledge and/or materiel) of the proposed research, if successful, will impact a central critical problem or question in the field of research and/or patient care in the FY17 PRMRP Topic Area(s) addressed.

- **Programmatic Relevance:** Whether the proposed research idea supports the objectives of the PRMRP. To what degree the project is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

Notification of Pre-Application Screening Results

Following the pre-application screening, PIs (or Initiating PIs, if applying under the Partnering PI Option) will be notified as to whether or not they are invited to submit applications; however, they will not receive feedback (e.g., a critique of strengths and weaknesses) on their pre-application. The estimated time frame for notification of invitation to submit an application is indicated in Section I, Overview of the Funding Opportunity. Invitations to submit a full application are based on the Pre-Application Screening Criteria listed above.

II.D.2.b. Step 2: Full Application Submission Content

Applications will not be accepted unless the PI or Initiating PI has received notification of invitation.

All contributors and administrators to the application must use matching compatible versions of Adobe software when editing and preparing application components. The use of different software versions will result in corruption of the submitted file. Refer to the General Application Instructions, Section III, for details on compatible Adobe software.

The CDMRP cannot make allowances/exceptions to its policies for submission problems encountered by the applicant organization using system-to-system interfaces with Grants.gov.

Each application submission must include the completed full application package for this Program Announcement. The full application package is submitted by the Authorized Organizational Representative through Grants.gov (http://www.grants.gov/) for extramural

organizations or through eBRAP (https://ebrap.org/) for intramural organizations. See Table 1 below for more specific guidelines.

II.D.2.b.i. Full Application Guidelines

Extramural organizations, including non-DoD Federal agencies, must submit full applications through Grants.gov. Submissions of extramural applications through eBRAP may be withdrawn.

Table 1. Full Application Submission Guidelines

Extramural Submissions	Intramural DoD Submissions
Application Package Location	
Download application package components for W81XWH-17-PRMRP-IIRA from Grants.gov (https://www.grants.gov).	Download application package components for W81XWH-17-PRMRP-IIRA from eBRAP (https://ebrap.org).
Full Application Package Components	
SF424 (R&R) Application for Federal Assistance Form: Refer to the General Application Instructions, Section III.A.1, for detailed information.	**Tab 1 – Summary:** Provide a summary of the application information. **Tab 2 – Application Contacts:** This tab will be pre-populated by eBRAP; add Authorized Organizational Representative.
Descriptions of each required file can be found under Full Application Submission Components: • Attachments • Research & Related Senior/Key Person Profile (Expanded) • Research & Related Budget • Project/Performance Site Location(s) Form • R&R Subaward Budget Attachment(s) Form (if applicable)	**Tab 3 – Full Application Files:** Upload files under each Application Component in eBRAP. Descriptions of each required file can be found under Full Application Submission Components: • Attachments • Key Personnel • Budget • Performance Sites **Tab 4 – Application and Budget Data:** Review and edit proposed project start date, proposed end date, and budget data pre-populated from the Budget Form.
Application Package Submission	
Submit package components to Grants.gov (https://www.grants.gov). If either the Project Narrative or the budget fails eBRAP validation or if the Project Narrative or the budget need to be modified, an updated Grants.gov application package must be submitted via Grants.gov as a "Changed/Corrected Application" with the previous Grants.gov Tracking ID prior to the application submission deadline.	**Submit package components to eBRAP (https://ebrap.org).** **Tab 5 – Submit/Request Approval Full Application:** After all components are uploaded and prior to the full application submission deadline, enter your password in the space provided "Enter Your Password Here" and press the "Submit Full Application" button. eBRAP will notify your Resource Manager/Comptroller or

Extramural Submissions	Intramural DoD Submissions
	equivalent Business Official by email to log into eBRAP to review and to approve prior to the application submission deadline.
Application Verification Period	
The full application package submitted to Grants.gov may be viewed and modified in eBRAP until the end of the application verification period. During the application verification period, the full application package, ***with the exception of the Project Narrative and Budget Form,*** may be modified.	After eBRAP has processed the full application, the organizational Resource Manager/ Comptroller or equivalent Business Official and PI(s) will receive an email notification of this status and will be able to view and modify application components in eBRAP. During the application verification period, the full application package, ***with the exception of the Project Narrative and Budget Form,*** may be modified.
Further Information	
Refer to the General Application Instructions, Section III, for further information regarding Grants.gov requirements.	Refer to the General Application Instructions, Section IV, for further information regarding eBRAP requirements.

The organization's Business Official or Authorized Organization Representative (or Resource Manager/Comptroller) should approve/verify the full application submission prior to the application verification deadline.

Application viewing, modification, and verification in eBRAP are strongly recommended, but not required. ***The Project Narrative and Budget cannot be changed after the application submission deadline.*** Prior to the full application deadline, a corrected or modified full application package may be submitted. Other application components may be changed until the end of the application verification period. After the end of the application verifi cation period, the full application cannot be modified.

Material submitted after the end of the application verification period, unless specifically requested by the Government, will not be forwarded for processing.

Partnering PI Option: The CDMRP requires separate full application package submissions for the Initiating PI and Partnering PI, even if the PIs are located within the same organization. Initiating and Partnering PIs will each be assigned a unique eBRAP log number. Each full application package must be submitted using the unique eBRAP log number. ***Note: All associated applications (Initiating and Partnering PIs') must be submitted by the full application submission deadline.***

Application Components for the PI (for single PI applicants) or the Initiating PI (if applying under the Partnering PI Option)

The full application package must be submitted using the unique eBRAP log number to avoid delays in application processing.

II.D.2.b.ii. Full Application Submission Components:

- **Extramural Applications Only –**

  SF424 (R&R) Application for Federal Assistance Form: Refer to the General Application Instructions, Section III.A.1, for detailed information.

- **Extramural and Intramural Applications –**

  Attachments:

  Each attachment to the full application components must be uploaded as an individual file in the format specified and in accordance with the formatting guidelines listed in the General Application Instructions, Appendix 4.

  For all attachments, ensure that the file names are consistent with the guidance. Attachments will be rejected if the file names are longer than 50 characters or incorrect file names that contain characters other than the following: A-Z, a-z, 0-9, underscore, hyphen, space, and period. In addition, there are file size limits that may apply in some circumstances. Individual attachments may not exceed 20 MB and the file size for the entire full application package may not exceed 200 MB.

  - **Attachment 1: Project Narrative (15-page limit):** Upload as "ProjectNarrative.pdf." The page limit of the Project Narrative applies to text and non-text elements (e.g., figures, tables, graphs, photographs, diagrams, chemical structures, drawings) used to describe the project. Inclusion of URLs that provide additional information to expand the Project Narrative and could confer an unfair competitive advantage is prohibited and may result in administrative withdrawal of the application.

    Describe the proposed project in detail using the outline below.

    - **Background:** Describe how the proposed research project addresses one or more of the FY17 PRMRP Topic Areas. Present the ideas and reasoning behind the proposed work. Cite relevant literature. Describe previous experience most pertinent to the project. Include relevant preliminary data; these data may be unpublished or from the published literature.

    - **Hypothesis:** State the hypothesis to be tested.

    - **Specific Aims:** Concisely explain the project's specific aims and the objective(s) to be reached. These aims should agree with the primary aims and associated tasks

described in the Statement of Work. If the proposed work is part of a larger study, present only aims that this DoD award would fund.

- **Research Strategy:** Describe the experimental design, methods, and analyses, including appropriate controls, in sufficient detail for analysis. Address potential problem areas and present alternative methods and approaches. Clearly describe the statistical plan and the rationale for the statistical methodology as well as an appropriate power analysis, if applicable. If animal studies are proposed, describe how they will be conducted in accordance with the ARRIVE guidelines (http://www.elsevier.com/__data/promis_misc/622936arrive_guidelines.pdf).

- If human subjects or human biological samples will be used, describe the study population and include a detailed plan for the recruitment of human subjects or the acquisition of samples. Describe the availability of the proposed study population and past successes in recruiting similar populations. If active duty military, military families, and/or Veteran population(s) or datasets will be used in the proposed research project, describe the feasibility of accessing the population(s)/dataset(s). ***Clinical trials are not allowed under the Investigator-Initiated Research Award.***

- If applicable, describe how data will be reported and how it will be assured that the documentation will support a regulatory filing with the U.S. Food and Drug Administration (FDA).

- **Attachment 2: Supporting Documentation:** Combine and upload as a single file named "Support.pdf." Start each document on a new page. If documents are scanned to PDF, the lowest resolution (100 to 150 dpi) should be used. The Supporting Documentation attachment should not include additional information such as figures, tables, graphs, photographs, diagrams, chemical structures, or drawings. These items should be included in the Project Narrative. Any additional material viewed as an extension of the Project Narrative will be removed or may result in administrative withdrawal of the application.

  There are no page limits for any of these components unless otherwise noted. Include only those components described below; inclusion of items not requested will result in the removal of those items or may result in administrative withdrawal of the application.

  - References Cited: List the references cited (including URLs, if available) in the Project Narrative using a standard reference format that includes the full citation (i.e., author[s], year published, title of reference, source of reference, volume, chapter, page numbers, and publisher, as appropriate).

  - List of Abbreviations, Acronyms, and Symbols: Provide a list of abbreviations, acronyms, and symbols.

  - Facilities, Existing Equipment, and Other Resources: Describe the facilities and equipment available for performance of the proposed project and any additional facilities or equipment proposed for acquisition at no cost to the award. Indicate

whether or not Government-furnished facilities or equipment are proposed for use. If so, reference should be made to the original or present Government award under which the facilities or equipment items are now accountable. There is no form for this information.

- Publications and/or Patents: Include a list of relevant publication URLs and/or patents. If publications are not publicly available, then copies of up to five published manuscripts may be included in Attachment 2. Extra items will not be reviewed.

- Letters of Organizational Support: Provide a letter (or letters, if applicable), signed by the Department Chair or appropriate organization official, confirming the laboratory space, equipment, and other resources available for the project. Letters of support not requested in the Program Announcement, such as those from members of Congress, do not impact application review or funding decisions.

- Letters of Collaboration: Provide a signed letter from each collaborating individual or organization that will demonstrate that the PI has the support or resources necessary for the proposed work.

- Letters Confirming Access to Military or VA Patient Populations or Resources (if applicable): If the proposed research plan involves access to active duty military and/or VA patient populations or resources, include a letter of support, signed by the lowest ranking person with approval authority, confirming such access. If access cannot be confirmed at the time of application submission, the Government reserves the right to withhold or revoke funding until the PI has demonstrated support for and access to the relevant population(s) and/or resources.

- Intellectual Property: Information can be found in Code of Federal Regulations, Title 2, Part 200.315 (2 CFR 200.315), "Intangible Property."

  - Intellectual and Material Property Plan (if applicable): Provide a plan for resolving intellectual and material property issues among participating organizations.

  - Commercialization Strategy (if applicable): Describe the commercialization plan. The plan should include intellectual property, market size, financial analysis, strengths and weaknesses, barriers to the market, competitors, and management team. Discuss the significance of this development effort, when it can be anticipated, and the potential commercial use for the technology being developed.

- Data and Research Resources Sharing Plan: Describe how data and resources generated during the performance of the project will be shared with the research community. Refer to the General Application Instructions, Appendix 2, Section K, for more information about the CDMRP expectations for making data and research resources publicly available.

- **Attachment 3: Technical Abstract (one-page limit):** Upload as "TechAbs.pdf." The technical abstract is used by all reviewers. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information.*** Use only characters available on a standard QWERTY keyboard. Spell out all Greek letters, other non-English letters, and symbols. Graphics are not allowed.

  - The technical abstract is used by all reviewers. Of particular importance, programmatic reviewers typically do not have access to the full application and therefore rely on the technical abstract for appropriate description of the project's key aspects. Therefore, clarity and completeness within the space limits of the technical abstract are highly important.

  - Describe the proposed research project including the following elements: background, rationale, hypothesis, specific aims and objectives, study design, long-term and short-term impact to the relevant research field and patient population(s), and the relevance of the project to at least one FY17 PRMRP Topic Area.

- **Attachment 4: Lay Abstract (one-page limit):** Upload as "LayAbs.pdf." The lay abstract is used by all reviewers. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information***. Use only characters available on a standard QWERTY keyboard. Spell out all Greek letters, other non-English letters, and symbols. Graphics are not allowed.

  - Describe how the proposed research project addresses one or more of the FY17 PRMRP Topic Areas. Include a comprehensive overview of the proposed research project that will be ***readily understood by readers without a background in science or medicine.*** Clearly describe the central critical problem or question to be addressed and the ultimate applicability and impact of the research. ***Do not duplicate the technical abstract.***

- **Attachment 5: Statement of Work (SOW) (three-page limit):** Upload as "SOW.pdf." The suggested SOW format and examples specific to different types of research projects are available on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm). For the Investigator-Initiated Research Award mechanism, use the SOW format example titled "SOW (Statement of Work) Generic Format." The SOW must be in PDF format prior to attaching.

  The SOW should include a list of major tasks that support the proposed specific aims, followed by a series of subtasks outlined related to the major tasks and milestones within the period of performance. The SOW should describe only the work for which funding is being requested by this application and, as applicable, should also:

  Include the name(s) of the key personnel and contact information for each study site/ subaward site.

  Indicate the number (and type, if applicable) of research subjects (animal or human) and/or human anatomical samples projected or required for each task and at each site.

Refer to the General Application Instructions, Appendix 1, for additional information regarding regulatory requirements.

Briefly state the methods to be used.

For studies with prospective accrual of human subjects, indicate quarterly enrollment targets.

Identify cell line(s) and commercial or organizational source(s) to be used. If human anatomical substances (including cell lines) will be used, specify whether or not identifiable information is accessible to the research team by any means.

If applicable, indicate timelines required for regulatory approvals relevant to human subjects research (e.g., Investigational New Drug and Investigational Device Exemption applications) by the FDA or other Government agency.

Partnering PI Option: The Initiating and partnering PIs must submit an identical copy of a jointly created SOW. The contributions of the Initiating PI and Partnering PI should be noted for each task.

- **Attachment 6: Impact Statement (one-page limit): Upload as "Impact.pdf."**

  Explain why the proposed research project is important and relevant to understanding the cause or progression of the disease or condition, or to developing improvements in prevention, detection, diagnosis, treatment, patient care, or quality of life in the FY17 PRMRP Topic Area(s) addressed. Describe how the study will address a central critical problem or question in the relevant Topic Area(s). If applicable, describe how the project addresses an FY17 PRMRP Area of Encouragement (Appendix 2).

  - ***Describe the short-term impact:*** Detail the anticipated outcome(s)/product(s) (knowledge and/or material) that will be directly attributed to the results of the proposed research.

  - ***Describe the long-term impact:*** Explain the anticipated long-term gains from this research. Compare to the information known/products currently available, if applicable. Explain the long-range vision for how the research will impact the field of study and/or clinical care.

- **Attachment 7: Military Relevance Statement (one-page limit): Upload as "MilRel.pdf."**

  Describe how the proposed study is responsive to the healthcare needs of military Service members, Veterans, and/or beneficiaries. Provide information about the incidence and/or prevalence of the disease or condition in the general population as well as in military Service members, Veterans, and/or beneficiaries.

  If active duty military, military families, and/or Veteran population(s) or datasets will be used in the proposed research project, describe the population(s)/dataset(s) and the

appropriateness of the population(s)/dataset(s) for the proposed study. If a non-military population will be used for the proposed research project, explain how the population simulates the targeted population (i.e., military Service members, Veterans, and/or beneficiaries).

If applicable, show how the proposed research project aligns with DoD and/or VA areas of research interest. Provide a description of how the knowledge, information, products, or technologies gained from the research could be implemented in a dual-use capacity to benefit the civilian population and address a military need, as appropriate.

- **Attachment 8: Partnership Statement (one-page limit): Upload as "Partnership.pdf."** ***This attachment is only required for applications submitted under the Partnering PI Option.***

  Describe the expertise of the Initiating and Partnering PIs and how each will bring different strengths to the proposed project. Describe how the combined effort will be synergistic and produce an outcome greater than any that could be achieved by independent efforts. Outline the contribution and time commitment of each partner and how each will have equal intellectual input on the design, conduct, and analysis of the project. Describe how the PIs will manage the collaboration and workflow to optimize research efforts.

- **Attachment 9: DoD Military Budget Form(s), if applicable: Upload as "MFBudget.pdf."** If a military facility (military health system facility, research laboratory, treatment facility, dental treatment facility, or a DoD activity embedded with a civilian medical center) will be a collaborator in performance of the project, complete the DoD Military Budget Form, available for download on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm), including a budget justification, for each military facility as instructed. The costs per year should be included on the Grants.gov Research and Related Budget form under subaward costs. Refer to the General Application Instructions, Section III.A.7, for detailed information.

- **Extramural and Intramural Applications –**

  Research & Related Senior/Key Person Profile (Expanded): For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.3, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.2, for detailed information.

  - PI Biographical Sketch (five-page limit): Upload as "Biosketch_LastName.pdf." The suggested biographical sketch format is available on the "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) in eBRAP. The National Institutes of Health Biographical Sketch may also be used. All biographical sketches should be submitted in the portable document format (PDF) that is not editable.

  - PI Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."

- Key Personnel Biographical Sketches (five-page limit each): Upload as "Biosketch_LastName.pdf."

  - If applying under the Partnering PI Option, include biographical sketches for the Partnering PI.

- Key Personnel Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."

  - If applying under the Partnering PI Option, include previous/current/pending support for the Partnering PI.

Research & Related Budget: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.4, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.3, for detailed information.

Budget Justification (no page limit): Upload as "BudgetJustification.pdf." The budget justification for the entire period of performance must be uploaded to the Research & Related Budget after completion of the budget for Period 1.

Partnering PI Option: ***Initiating and Partnering PIs must each submit a budget and justification specific to their own portion of the efforts as part of their separate application packages. The Initiating PI should not include budget information for the Partnering PI, even if they are located within the same organization. The anticipated combined direct costs budgeted for the entire period of performance for the Initiating and Partnering PIs' applications will not exceed $1.5M. Collaborating organizations should budget associated indirect costs in accordance with each organization's negotiated rate. The combined budgeted direct costs approved by the Government will not exceed $1.5M or use an indirect rate exceeding each organization's negotiated rate. Refer to Section II.D.5, Funding Restrictions, for detailed information.***

Project/Performance Site Location(s) Form: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.5, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.4, for detailed information.

- **Extramural Applications Only –**

  R&R Subaward Budget Attachment(s) Form (if applicable): Refer to the General Application Instructions, Section III.A.6, for detailed information.

  - **Extramural Subaward:** Complete the Research & Related Subaward Budget Form through Grants.gov. (Refer to the General Application Instructions, Section III.A.6, for detailed information.)

  - **Intramural DoD Collaborator(s):** Complete the DoD Military Budget Form and upload to Grants.gov as Attachment 9. (Refer to the General Application Instructions,

Section III.A.7, for detailed information.) Intramural DoD Collaborator(s) costs per year should be included on the Grants.gov Research and Related Budget form under subaward costs. **DoD Military Budget Form:** A military facility collaborating in the performance of the project (but not participating as a Partnering PI) should be treated as a subaward for budget purposes. However, do not complete the Grants.Gov R&R Subaward Budget Attachment Form; instead, complete the DoD Military Budget Form (Attachment 9) to show all direct and indirect costs. The costs per year should be included on the Grants.gov Research & Related Budget Form under subaward costs. Refer to the General Application Instructions, Section III.A.7, for detailed information.

Application Components for the Partnering PI, if applying under the Partnering PI Option

The Partnering PI MUST follow the link in the email from eBRAP and, if not registered in eBRAP, complete the registration process prior to the application submission deadline in order to associate his/her full application package with that of the Initiating PI.

For the Partnering PI, the Initiating PI must identify if the Partnering PI will be submitting an extramural or intramural application (in accordance with the guidelines in Section II.C.1.a, Organization) and the appropriate mode of submission (Grants.gov for extramural and eBRAP for intramural). The Partnering PI must verify his/her contact information and mode of submission within eBRAP to ensure proper submission of his/her application.

The application submission process for the Partnering PI uses an abbreviated full application package that includes:

- **Extramural and Intramural Applications –**

  Attachments:

  - **Attachment 5: Statement of Work (SOW) (three-page limit): Upload as "SOW.pdf."** Refer to the General Application Instructions, Section III.A.2, for detailed information on completing the SOW. The Initiating PI and Partnering PI must submit an identical copy of a jointly created SOW. The contributions of the Initiating PI and Partnering PI should be noted for each task.

  - **Attachment 9: DoD Military Budget Form (if applicable): Upload as "MilBudget.pdf."** Refer to the General Application Instructions, Section III.A.7, for detailed information. The costs per year should be included on the Grants.Gov Research and Related Budget form under subaward costs.

  Research & Related Budget: For extramural submissions, refer to the General Application Instructions, Section III.A.4, and for intramural submissions refer to the General Application Instructions, Section IV.A.3, for detailed information.

Budget Justification (no page limit): Upload as "BudgetJustification.pdf."

Initiating and Partnering PIs must each submit a budget and justification specific to their own portion of the efforts as part of their separate Grants.gov application packages. The Research & Related Budget for the Partnering PI should not include budget information for the Initiating PI, even if they are located within the same organization.

Project/Performance Site Location(s) Form. For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.5, and for intramural submissions (via eBRAP), refer to General Application Instructions, Section IV.A.4, for detailed information.

- **Extramural Applications Only –**

  R&R Subaward Budget Attachment(s) Form:

  - **Extramural Subaward:** Complete the Research & Related Subaward Budget Form through Grants.gov. (Refer to the General Application Instructions, Section III.A.6, for detailed information.)

  - **Intramural DoD Collaborator(s):** Complete the DoD Military Budget Form and upload to Grants.gov as Attachment 9. (Refer to the General Application Instructions, Section IV.A.3, for detailed information.)

II.D.3. Dun and Bradstreet Universal Numbering System (DUNS) Number and System for Award Management (SAM)

Applicant organizations and all subrecipient organizations must have a DUNS number to submit applications to Grants.gov. The applicant organization must also be registered in the Entity Management functional area of the SAM with an "Active" status to submit applications through the Grants.gov portal. Verify the status of the applicant's organization's Entity registration in SAM well in advance of the application submission deadline. Allow 3 to 4 weeks to complete the entire SAM registration process. If an applicant has not fully complied with the requirements by the time the Federal awarding agency is ready to make a Federal award, the Federal awarding agency may determine that the applicant is not qualified to receive a Federal award and use that determination as a basis for making a Federal award to another applicant. Refer to the General Application Instructions, Section III, for further information regarding Grants.gov requirements.

II.D.4. Submission Dates and Times

All submission dates and times are indicated in Section I, Overview of the Funding Opportunity. Pre-application and application submissions are required. The pre-application and application submission process should be started early to avoid missing deadlines. There are no grace periods. Failure to meet either of these deadlines will result in submission rejection.

Applicant Verification of Full Application Submission in eBRAP

Prior to the end of the application verification period, PIs and organizational representatives can review and modify in eBRAP certain components of a submitted application. Following retrieval and processing of the full application, eBRAP will notify the organizational representatives and PI by email to log into eBRAP to review, modify, and verify the full application submission. eBRAP will validate retrieved files against the specific Program Announcement requirements and discrepancies will be noted in both the email and in the Full Application Files tab in eBRAP. eBRAP does not confirm the accuracy of file content. It is the applicant's responsibility to review all application components and ensure proper ordering as specified in the Program Announcement. ***If either the Project Narrative or the budget fails eBRAP validation or needs to be modified, an updated full application package must be submitted prior to the application submission deadline.*** The Project Narrative and Budget Form cannot be changed after the application submission deadline.

II.D.5. Funding Restrictions

The maximum period of performance is **3** years.

Application submissions with a single PI:

The anticipated direct costs budgeted for the entire period of performance will not exceed **$1.2 million (M)**. If indirect cost rates have been negotiated, indirect costs are to be budgeted in accordance with the organization's negotiated rate. No budget will be approved by the Government exceeding **$1.2M** direct costs or using an indirect cost rate exceeding the organization's negotiated rate.

All direct and indirect costs of any subaward or contract must be included in the total direct costs of the primary award.

The applicant may request the entire maximum funding amount for a project that may have a period of performance less than the maximum **3** years.

Application submissions including the Partnering PI Option: The combined direct costs budgeted for the entire period of performance for the Initiating and Partnering PIs' applications will not exceed **$1.5M**. If indirect cost rates have been negotiated, indirect costs are to be budgeted in accordance with the organization's negotiated rate. Collaborating organizations should budget associated indirect costs in accordance with each organization's negotiated rate. The combined budgeted direct costs approved by the Government will not exceed **$1.5M** or use an indirect cost rate exceeding each organization's negotiated rate.

A separate award will be made to each PI's organization.

The Initiating PI and the Partnering PI are expected to be equal partners in the research, and direct cost funding should be divided accordingly, unless otherwise warranted and clearly justified. Each partner may request no more than 75% (or no less than 25%) of the proposed direct costs.

For this award mechanism, direct costs may be requested for (not all-inclusive):

- Salary
- Research supplies
- Clinical research costs *(clinical trials are not allowed)*
- Support for multidisciplinary collaborations, including travel
- Travel costs for the PI(s) to disseminate project results at one DoD-sponsored meeting to be specified by the program office during award negotiations
- Travel costs for one investigator to travel to one scientific/technical meeting per year in addition to the DoD-sponsored meeting described above.

Extramural (non-Federal) awards will consist solely of assistance agreements (Cooperative Agreements and Grants). For extramural awards with an intragovernmental component, direct transfer of funds from an extramural award recipient to a DoD or other Federal agency is not allowed except under very limited circumstances. Funding to intramural DoD and other Federal agencies will be managed through a direct fund transfer. Intragovernmental only funding to intramural DoD and other Federal agencies will be managed through a direct fund transfer. Intramural applicants are responsible for coordinating through their agency's procedures the use of contractual or assistance funding awards or other appropriate agreements to support extramural collaborators. Application packages from associated extramural partners will be funded through assistance agreements.

Refer to the General Application Instructions, Section III.A.4, for budget regulations and instructions for the Research & Related Budget. ***For Federal agencies or organizations collaborating with Federal agencies, budget restrictions apply as are noted in the General Application Instructions, Section III.A.4***.

The CDMRP expects to allot approximately $90M of the $300M FY17 appropriation to fund approximately 47 Investigator-Initiated Research Award applications, depending on the quality and number of applications received. Funding of applications received in response to this Program Announcement is contingent upon the availability of Federal funds for this program.

II.D.6. Other Submission Requirements

Refer to the General Application Instructions, Appendix 4, for detailed formatting guidelines.

II.E. Application Review Information

II.E.1. Criteria

II.E.1.a. Peer Review

To determine technical merit, all applications will be evaluated according to the following scored criteria, which are listed in decreasing order of importance:

- **Research Strategy and Feasibility**
    - How well the scientific rationale supports the project and its feasibility, as demonstrated by a critical review and analysis of the literature, supporting data, and logical reasoning.
    - How well the hypothesis, specific aims, and objective(s) are developed.
    - How well the experimental design, methods, data collection procedures, and analyses are developed and support completion of the aims.
    - If applicable, to what degree the statistical plan and power analysis are appropriate for the proposed project.
    - If animal studies are included, how well they are designed to achieve reproducible and rigorous results, including the choice of model and the endpoints/outcomes to be measured.
    - If applicable, whether data will be appropriately reported and documented to support a regulatory filing with the FDA.
    - If applicable, how well the application provides evidence of availability of and access to the necessary study populations and/or resources.
    - How well potential problems are identified and alternative approaches are addressed.
    - Whether the research can be completed within the proposed period of performance.
- **Impact**
    - To what extent the project impacts a central critical problem or question in at least one FY17 PRMRP Topic Area.
    - If applicable, how well the proposed research project addresses one or more FY17 PRMRP Areas of Encouragement.
    - How the proposed research project, if successful, will make important scientific advances in the relevant field of research.
    - To what degree the proposed project could, if successful, make a significant impact on the lives of relevant patient populations in the short term and/or long term.

- **Personnel**
    - How the background and expertise of the PI(s) and other key personnel demonstrate their ability to perform the proposed work.
    - How the levels of effort by the PI(s) and other key personnel are appropriate to ensure the successful conduct of the project.
    - How each PI's record(s) of accomplishment demonstrates his/her ability to accomplish the proposed work.

For applications submitted under the Partnering PI Option:

- **Partnership**
    - How well the research project is supported by the nature of the collaboration.
    - To what extent the proposed partnership will advance research beyond what would be possible through individual efforts.
    - How well the skills and perspectives of the Initiating and Partnering PIs complement each other.
    - How well the application reflects the requirement that the partners have equal intellectual input into the design of the project.

In addition, the following unscored criteria will also contribute to the overall evaluation of the application:

- **Environment**
    - How the scientific environment is appropriate for the proposed research.
    - How the research requirements are supported by the availability of and accessibility to facilities and resources (including collaborative arrangements).
    - How the quality and extent of organizational support are appropriate for the proposed research.
    - If applicable, to what degree the intellectual and material property plan is appropriate.
- **Budget**
    - Whether the budget is appropriate for the proposed research and within the limitations of this Program Announcement.

- **Application Presentation**
    - To what extent the writing, clarity, and presentation of the application components influence the review.

II.E.1.b. Programmatic Review

To make funding recommendations and select the application(s) that, individually or collectively, will best achieve the program objectives, the following criteria are used by programmatic reviewers:

- Ratings and evaluations of the peer reviewers
- Relevance to the mission of the DHP and FY17 PRMRP, as evidenced by the following:
    - Adherence to the intent of the award mechanism
    - Program portfolio composition
    - Military relevance
    - Relative impact

II.E.2. Application Review and Selection Process

All applications are evaluated by scientists, clinicians, and consumers in a two-tier review process. The first tier is peer review of applications against established criteria for determining technical merit. Each application is evaluated for its own merit, independent of other applications. The second tier is a programmatic review that makes recommendations for funding to the Commanding General, USAMRMC, on behalf of the DHA and the OASD(HA), based on technical merit, the relevance to the mission of the DHP and PRMRP, the specific intent of the award mechanism, and to other specified evaluation criteria in the Program Announcement. Programmatic review is a comparison-based process in which applications with scientific and technical merit compete in a common pool. ***The highest-scoring applications from the first tier of review are not automatically recommended for funding. Funding recommendations depend on various factors as described in Section II.E.1.b, Programmatic Review.*** Additional information about the two-tier process used by the CDMRP can be found at http://cdmrp.army.mil/about/fundingprocess.

All CDMRP review processes are conducted confidentially to maintain the integrity of the merit-based selection process. Panel members sign a statement that application and evaluation information will not be disclosed outside the panel. Violations of confidentiality can result in the dissolving of a panel(s) and other corrective actions. In addition, personnel at the applicant or collaborating organizations are prohibited from contacting persons involved in the review process to gain protected evaluation information or to influence the evaluation process. Violations of these prohibitions will result in the administrative withdrawal of the organization's application. Violations by panel members or applicants that compromise the confidentiality of the review process may also result in suspension or debarment from Federal awards.

Furthermore, the unauthorized disclosure of confidential information of one party to another third party is a crime in accordance with 18 USC 1905.

II.E.3. Integrity and Performance Information

Prior to making an assistance agreement award where the Federal share is expected to exceed the simplified acquisition threshold (currently $150,000) over the period of performance, the Federal awarding agency is required to review and consider any information about the applicant that is available in the Federal Awardee Performance and Integrity Information System (FAPIIS).

An applicant, at its option, may review FAPIIS, accessible through SAM, and submit comments to FAPIIS on any information about itself that a Federal awarding agency previously entered and is currently available in FAPIIS.

The Federal awarding agency will consider any comments by the applicant, in addition to other information in the designated integrity and performance system, in making a judgment about the applicant's integrity, business ethics and record of performance under Federal awards when determining a recipient's qualification prior to award, according to the qualification standards of the Department of Defense Grant and Agreement Regulations (DoDGAR), Section 22.415.

II.E.4. Anticipated Announcement and Federal Award Dates

All application review dates and times are indicated in Section I, Overview of the Funding Opportunity.

Each PI and organization will receive email notification of posting of the funding recommendation in eBRAP. Each PI will receive a peer review summary statement on the strengths and weaknesses of the application.

II.F. Federal Award Administration Information

II.F.1. Federal Award Notices

Awards will be made no later than September 30, 2018. Refer to the General Application Instructions, Appendix 2, for additional award administration information.

Awards are made to organizations, not to individual PIs. The types of awards made under the Program Announcement will be assistance agreements (grants or cooperative agreements). The level of involvement on the part of DoD during project performance is the key factor in determining whether to award a grant or cooperative agreement.

Extramural Organizations: An assistance agreement (grant or cooperative agreement) is appropriate when the Federal Government transfers a "thing of value," to a "state, local government," or "other recipient," to carry out a public purpose of support or stimulation authorized by a law of the United States, instead of acquiring property or service for the direct benefit and use of the U.S. Government. An assistance agreement can take the form of a grant or cooperative agreement. If "no substantial involvement" on the part of the funding agency is

anticipated, a grant award will be made (31 USC 6304). Conversely, if substantial involvement on the part of the funding agency is anticipated, a cooperative agreement will be made (31 USC 6305). Substantial involvement may include collaboration, participation, or intervention in the research to be performed under the award. The award type, along with the start date, will be determined during the negotiation process.

After email notification of application review results through the eBRAP, and if selected for funding, a representative from the USAMRAA will contact the business official authorized to negotiate on behalf of the PI's organization.

Only an appointed USAMRAA Grants Officer may obligate the Government to the expenditure of funds. No commitment on the part of the Government should be inferred from discussions with any other individual. The award document signed by the Grants Officer is the official authorizing documents.

Intramural Organizations: Awards to Federal Government organizations (to include intramural DoD organizations) will be executed through the Military Interdepartmental Purchase Request (MIPR) or Funding Authorization Document (FAD) process. Transfer of funds is contingent upon appropriate safety and administrative approvals. Intramural applicants and collaborators are reminded to coordinate receipt and commitment of funds through their respective resource managers (RM).

After email notification of application review results through the eBRAP, and if selected for funding, a representative from the CDMRP will contact the business official authorized to negotiate on behalf of the PI's organization.

II.F.1.a. Award Transfers

Changes in PI and an organizational transfer of an award supporting the Initiating or Partnering PI are discouraged and will be evaluated on a case-by-case basis and only allowed at the discretion of the Grants Officer. An organizational transfer of an award will not be allowed in the last year of the (original) period of performance or any extension thereof.

Refer to the General Application Instructions, Appendix 2, Section B, for general information on organization or PI changes.

II.F.2. Administrative and National Policy Requirements

Applicable requirements in the DoDGAR found in 32 CFR, Chapter 1, Subchapter C, and 2 CFR, Chapter XI, apply to grants and cooperative agreements resulting from this Program Announcement.

Refer to the General Application Instructions, Appendix 2, for general information regarding administrative requirements.

Refer to the General Application Instructions, Appendix 5, for general information regarding national policy requirements.

Refer to full text of the USAMRAA General Research Terms and Conditions for Institutions of Higher Education, Hospitals, and Non-Profit Organizations and the USAMRAA General Research Terms and Conditions with For-Profit Organizations for further information.

II.F.3. Reporting

Refer to the General Application Instructions, Appendix 2, Section A, for general information on reporting requirements. Annual progress reports as well as a final progress report will be required.

Quarterly technical progress reports may be required.

Award Chart: Complete the Award Chart template, a one-page PowerPoint file that must be downloaded from the CDMRP eBRAP System at https://ebrap.org/eBRAP/public/Program.htm, and submit to eBRAP at the time of award.

Award Expiration Transition Plan: Complete the Transition Plan template, a one-page Word document that must be submitted with the Final Progress Report. The Transition Plan must outline if and how the research supported by this award will progress, and include source(s) of funding, either known or pending.

Awards resulting from this Program Announcement will incorporate additional reporting requirements related to recipient integrity and performance matters. Recipient organizations that have Federal contract, grant, and cooperative agreement awards with a cumulative total value greater than $10,000,000 are required to provide information to FAPIIS about certain civil, criminal, and administrative proceedings that reached final disposition within the most recent 5-year period and that were connected with performance of a Federal award. Recipients are required to disclose semiannually information about criminal, civil, and administrative proceedings as specified in the applicable Terms and Conditions. The applicable Terms and Conditions for institutions of higher education, hospitals, and nonprofit organizations are available in OAR Article I, Section B, in the July 2016 R&D General Terms and Conditions. The applicable Terms and Conditions for for-profit organizations are available in Section 34 of the February 2017 USAMRAA General Research Terms and Conditions with For-Profit Organizations.

II.G. Federal Awarding Agency Contacts

II.G.1. CDMRP Help Desk

Questions related to Program Announcement content or submission requirements as well as questions related to the pre-application or intramural application submission through eBRAP should be directed to the CDMRP Help Desk, which is available Monday through Friday from 8:00 a.m. to 5:00 p.m. ET. Response times may vary depending upon the volume of inquiries.

Phone: 301-682-5507

Email: help@eBRAP.org

II.G.2. Grants.gov Contact Center

Questions related to extramural application submission through Grants.gov portal should be directed to the Grants.gov Contact Center, which is available 24 hours a day, 7 days a week (closed on U.S. Federal holidays). Note that the CDMRP Help Desk is unable to provide technical assistance with Grants.gov submission.

Phone: 800-518-4726; International 1-606-545-5035

Email: support@grants.gov

Sign up on Grants.gov for "send me change notification emails" by following the link on the Synopsis page for the Program Announcement or by responding to the prompt provided by Grants.gov when first downloading the Grants.gov application package. If the Grants.gov application package is updated or changed, the original version of the application package may not be accepted by Grants.gov.

II.H. Other Information

II.H.1. Program Announcement and General Application Instructions Versions

Questions related to this Program Announcement should refer to the Program name, the Program Announcement name, and the Program Announcement version code 20170516b. The Program Announcement numeric version code will match the General Applications Instructions version code 20170516.

II.H.2. Administrative Actions

After receipt of pre-applications or applications, the following administrative actions may occur:

II.H.2.a. Rejection

The following will result in administrative rejection of the pre-application:

- Preproposal Narrative is missing.

The following will result in administrative rejection of the application:

- Submission of an application for which a letter of invitation was not received.
- Project Narrative exceeds page limit.
- Project Narrative is missing.
- Budget is missing.

- Submission of the same research project to different Funding Opportunities within the same program and fiscal year. Refer to Section II.D., Application and Submission Information, for exceptions.

- **Partnering PI Option:** Both associated (Initiating and Partnering PI) applications are not submitted by the deadline.

II.H.2.b. Modification

- Pages exceeding the specific limits will be removed prior to review for all documents other than the Project Narrative.

- Documents not requested will be removed.

II.H.2.c. Withdrawal

The following may result in administrative withdrawal of the pre-application or application:

- An FY17 PRMRP Programmatic Panel member is named as being involved in the research proposed or is found to have assisted in the pre-application or application processes including, but not limited to, concept design, application development, budget preparation, and the development of any supporting documentation. ***A list of the FY17 PRMRP Programmatic Panel members can be found at http://cdmrp.army.mil/prmrp/panels/panels17***.

- The application fails to conform to this Program Announcement description to the extent that appropriate review cannot be conducted.

- Inclusion of URLs, with the exception of links in References Cited and Publication and/or Patent Abstract sections.

- Page size is larger than 8.5 inches x 11.0 inches (approximately 21.59 cm x 27.94 cm).

- To preserve the integrity of its peer and programmatic review processes, the CDMRP discourages inclusion of any employee of its review contractors having any role in the preparation, research or other duties for submitted applications. For FY17, the identities of the peer review contractor and the programmatic review contractor may be found at the CDMRP website (http://cdmrp.army.mil/about/2tierRevProcess). Applications that include names of personnel from either of these companies will be administratively withdrawn unless plans to manage COIs are provided and deemed appropriate by the Grants Officer. Refer to the General Application Instructions, Appendix 3, for detailed information.

- Personnel from applicant or collaborating organizations are found to have contacted persons involved in the review process to gain protected evaluation information or to influence the evaluation process.

- Applications from extramural organizations, including non-DoD Federal agencies, received through eBRAP may be withdrawn.

- Applications submitted by an intramural DoD organization may be withdrawn if the intramural organization cannot coordinate the use of contractual, assistance, or other appropriate agreements to provide funds to extramural collaborators.

- The proposed research project does not address at least one of the Congressionally directed FY17 PRMRP Topic Areas.

- If a clinical trial is proposed, the application will be withdrawn.

- An application submitted by a PI who does not meet the eligibility criteria will be withdrawn.

II.H.2.d. Withhold

Applications that appear to involve research misconduct will be administratively withheld from further consideration pending organizational investigation. The organization will be required to provide the findings of the investigation to the USAMRAA Grants Officer for a determination of the final disposition of the application.

II.H.3. Application Submission Checklist

Application Components	Action	Initiating PI Completed	Partnering PI Completed
SF424 (R&R) Application for Federal Assistance **(Extramural submissions only)**	Complete form as instructed.		
Summary (Tab 1) and Application Contacts (Tab 2) **(Intramural submissions only)**	Complete these tabs as instructed.		
Attachments	Project Narrative: Upload as Attachment 1 with file name "ProjectNarrative.pdf."		
	Supporting Documentation: Upload as Attachment 2 with file name "Support.pdf."		
	Technical Abstract: Upload as Attachment 3 with file name "TechAbs.pdf."		
	Lay Abstract: Upload as Attachment 4 with file name "LayAbs.pdf."		
	Statement of Work: Upload as Attachment 5 with file name "SOW.pdf."		
	Impact Statement: Upload as Attachment 6 with file name "Impact.pdf."		
	Military Relevance Statement: Upload as Attachment 7 with file name "MilRel.pdf."		
	Partnership Statement: Upload as Attachment 8 with file name "Partnership.pdf," if applicable.		
	DoD Military Budget Form(s): Upload as Attachment 9 with file name "MFBudget.pdf," if applicable.		
Research & Related Senior/Key Person Profile (Expanded)	Attach PI Biographical Sketch (Biosketch_LastName.pdf) to the appropriate field.		
	Attach PI Previous/Current/Pending Support (Support_LastName.pdf) to the appropriate field.		
	Attach Biographical Sketch (Biosketch_LastName.pdf) for each senior/key person to the appropriate field.		
	Attach Previous/Current/Pending (Support_LastName.pdf) for each senior/key person to the appropriate field.		

Application Components	Action	Initiating PI Completed	Partnering PI Completed
Research & Related Budget **(Extramural submissions only)**	Complete as instructed. Attach Budget Justification (BudgetJustification.pdf) to the appropriate field.		
Budget **(Intramural submissions only)**	Complete the DoD Military Budget Form and Justification.		
Project/Performance Site Location(s) Form	Complete form as instructed.		
R&R Subaward Budget Attachment(s) Form	Complete form as instructed.		

APPENDIX 1
ACRONYM LIST

ACURO	Animal Care and Use Review Office
CDMRP	Congressionally Directed Medical Research Programs
CFR	Code of Federal Regulations
DHA	Defense Health Agency
DHP	Defense Health Program
DoD	Department of Defense
DoDGAR	Department of Defense Grant and Agreement Regulations
DUNS	Data Universal Numbering System
eBRAP	Electronic Biomedical Research Application Portal
EC	Ethics Committee
ET	Eastern Time
FAPIIS	Federal Awardee Performance and Integrity Information System
FDA	U.S. Food and Drug Administration
FY	Fiscal Year
HRPO	Human Research Protection Office
IACUC	Institutional Animal Care and Use Committee
IRB	Institutional Review Board
M	Million
OASD(HA)	Office of the Assistant Secretary of Defense for Health Affairs
OMB	Office of Management and Budget
ORP	Office of Research Protections
PI	Principal Investigator
PRMRP	Peer Reviewed Medical Research Program
RDT&E	Research, Development, Test, and Evaluation
RM	Resource Manager
SAM	System for Award Management
SOW	Statement of Work
USAMRAA	U.S. Army Medical Research Acquisition Activity
USAMRMC	U.S. Army Medical Research and Materiel Command
USC	United States Code
VA	Department of Veterans Affairs

APPENDIX 2
AREAS OF ENCOURAGEMENT

Applications addressing any of the FY17 PRMRP Topic Areas are of interest to the program. ***Any aspect of research relevant to one or more FY17 PRMRP Topic Areas may be considered for funding.*** Areas of encouragement related to the FY17 PRMRP Topic Areas have been identified by the DoD, VA, and other relevant stakeholders. Applicants are urged to read and consider these areas of encouragement before preparing their applications. ***The information provided is not exhaustive, and applicants are not restricted to submitting applications that address an area of encouragement in this list.***

Acute Lung Injury

- Research on the etiology and prevention of acute respiratory distress syndrome caused by the host's or immune system's responses to infectious disease or septic shock.
- Preventive techniques, novel and/or innovative detection technologies, and therapeutics to reduce the incidence and/or severity of acute respiratory distress syndrome and/or other lung injury secondary to trauma, transfusion, burns, hemorrhagic shock, inhalation, and/or oxygen exposure.
- Development of a clinical laboratory-based device for manufacturing/amplification of stem cells to treat acute lung injury/acute respiratory distress syndrome due to inhalation injury, trauma, or other causes.
- Clinical studies to test cellular therapies in the treatment of acute lung injury/acute respiratory distress syndrome due to inhalation injury, trauma, or other causes.
- Metrics to associate the health outcomes of acute lung injury with physiological and physical performance, as well as the psychological health of military Service members.
- Strategies to stabilize and support the safe transport of patients with lung injury in order to optimize therapeutic interventions.
- Studies to identify the prevalence and associated morbidity and mortality of blast overpressure lung injury in forward operating environments.

Antimicrobial Resistance

- Identification and evaluation of novel and/or innovative antifungals against resistant fungal infections, particularly for use as topical therapies for wounds and for surgical and post-surgical therapies.
- Identification of novel and/or innovative targets for multi-drug resistant pathogens.
- Development of novel and/or innovative interventions, diagnostics, and treatment for multi-drug-resistant pathogens.
- Development of novel and/or innovative therapies that interrupt biofilm production in wound infections and infected hardware models.
- Development and testing of treatment protocols and/or diagnostic tests to limit prescribing antibiotics for conditions that are commonly viral in nature or conditions that would resolve themselves without antibiotic treatment (e.g., sinusitis, bronchitis, viral upper respiratory infections, and acute gastroenteritis).

- Development of rapid, rugged, sensitive, and specific diagnostics for antimicrobial resistance in remote environments.
- Research to improve understanding of the impact of travelers' diarrhea, as well as antibiotic treatment for the colonization of multi-drug-resistant organisms.

Arthritis

- Studies that define the risk of contracting septic arthritis; reactive arthritis; arthritis caused by a gastrointestinal, genitourinary, or viral infection (e.g., Lyme disease, Campylobacter, chikungunya); and other arthritis types in active duty military personnel.
- Research toward the development of clinical practice guidelines to prevent, identify, and treat arthritis, particularly that resulting from traumatic injury, surgery, and/or infectious diseases.
- Research to establish activity recommendations for maximal joint life following joint repair, particularly in young patient populations.
- Strategies for repairing focal cartilage defects using cell-based therapies.
- Research quantifying the impacts of obesity, weight loss, physical fitness (all components—cardiovascular, strength, flexibility, balance), and dietary factors on the development of or prevention/risk reduction of arthritis.
- Identification and/or validation of biomarkers for early psoriatic arthritis.
- Basic and translational research to identify treatments to mitigate and/or reverse osteoarthritis, particularly in the knee, hip, ankle, and shoulder.
- Studies to examine using existing regenerative medicine techniques and therapies to treat osteoarthritis, including dose response information and the frequency and timing of application.
- Development of novel and/or innovative treatments or combination therapies for psoriatic arthritis.

Burn Pit Exposure

- Research on the etiology and treatment of adverse health events related to military deployment to Iraq and Afghanistan that are associated with exposure to airborne hazards and open pit burning of solid waste and other materials.
- Toxicological studies to ascertain the toxicity of natural dust, burn pit combustion products and interactions between pollutants, as well as their mechanisms of action.
- Characterization of emissions from open air burns, burn boxes, and incinerators.
- Development and validation of instruments for assessing levels of exposure to airborne hazards for use in research and for occupational and environmental exposure monitoring.

Chronic Migraine and Post-Traumatic Headache

- Research to investigate, develop, and validate biomarkers that are useful in diagnosing and monitoring traumatic brain injury patients with chronic migraine or post-traumatic headache.
- Epidemiological/natural history studies to characterize specific types of post-traumatic headache, the pathobiology of these headaches (such as the role of cortical spreading

depression acutely after injury as a risk factor for chronic headaches of a migrainous type), and the risk factors that might predispose people to certain types of post-traumatic headache.

- Double-blind, placebo-controlled trials in the post-traumatic headache population in order to determine whether similar phenotypes in primary headache disorders and post-traumatic headache will respond similarly to treatment.
- Research on the optimal approaches to effective management of co-occurring psychological health disorders and acute and chronic pain management for chronic migraine and post-traumatic headache, with a focus on assessing and eliminating adverse outcomes and decreasing polypharmacy.
- Research on the utility of the Patient-Centered Medical Home model of care for patients with chronic migraine or post-traumatic headache.
- Research on the treatment and prevention of acute flares of chronic migraine and post-traumatic headaches with an emphasis on non-opioid medications.
- Evaluation of the use of mechanical stimulation and/or other non-pharmaceutical treatments to reduce acute and chronic migraines and headaches.
- Precision medicine research to investigate, develop, and validate biomarkers that are not only useful in diagnosing and monitoring traumatic brain injury patients with chronic migraine or post-traumatic headache, but can also identify individual responses to treatment.
- Evaluation of the differences in etiology, diagnosis, treatment plan, and prevention of migraine headaches between men and women.

Congenital Heart Disease

- Population-based and outcomes-based research projects to assess the health outcomes of individuals with congenital heart disease across their life spans.
- Research on tissue engineering approaches to patches, grafts, and transplantation that provide structural support, restore native activity, allow for tissue growth, and prevent the need for reoperation.
- Research to improve understanding of the causes of congenital heart defects, including genomic, proteomic, and metabolomic profiling.
- Research on transition of the care of congenital heart disease patients from pediatric to adult providers.
- Research both on the risk of neurologic injury and on enhanced neuroprotection before, during, and after surgery for congenital heart disease.
- Research to design and implement disease-in-a-dish and/or microfluidic models with an established phenotype to increase the efficacy of finding novel and/or innovative drug targets, screen existing drugs, perform cardiotoxicity testing, and/or uncover pathogenesis.

Constrictive Bronchiolitis

- Clinical assessments to determine the prevalence and severity of constrictive bronchiolitis and related respiratory diseases in previously deployed military Service members and/or Veterans.

- Development and testing of less invasive and non-invasive approaches for diagnosing constrictive bronchiolitis.
- Research to develop novel and/or innovative therapeutics to slow or reverse the progression of constrictive bronchiolitis.
- Development and/or validation of animal models for constrictive bronchiolitis.
- Research to understand the role of environmental exposures, including mineralized dust and other particulates, in the etiology of constrictive bronchiolitis.

Diabetes

- Development of biomarkers to identify and monitor at-risk individuals and assess treatment response.
- Research on interventions to prevent diabetes complications, including diabetic retinopathy and diabetic neuropathy.
- Research to better understand the heterogeneity of diabetes.
- Research on the transplantation of allogenic or autologous pancreatic islet cells for long-term natural insulin production, including current good manufacturing practices for cell line development.
- Identification and/or evaluation of interventions to reduce the development of diabetes among individuals meeting the clinical criteria for pre-diabetes.
- Development and evaluation of an inpatient glycemic management program with strategies to improve in-hospital dysglycemia, improve short-term and long-term morbidity/mortality, reduce the cost of health care, and promote a seamless transition of care between inpatient and outpatient arenas.
- Research to design and implement disease-in-a-dish and/or microfluidic models to model pancreatic islets to uncover pathogenesis and improve the efficiency of drug discovery.

Diarrheal Diseases

- Development and testing of vaccines, including multi-pathogen combination approaches, against the most common causes of bacterial and viral travelers' diarrhea, including *Campylobacter jejuni, Shigella,* and Enterotoxigenic *E. coli*.
- Development and evaluation of antibiotic and non-antibiotic therapies to treat all-cause travelers' diarrhea to improve the time to clinical cure, minimize resistance acquisition, and mitigate the chronic health consequences of these infections.
- Research to improve understanding of host responses and develop therapies to treat acute bacterial and viral infections that are associated with travel and may lead to chronic functional bowel and rheumatologic disorders.
- Development of evidence necessary to demonstrate the need, impact, and potential market value of a therapeutic for post-infectious functional bowel disorders to include preventive, treatment, or vaccine technology strategies.
- Development and evaluation of point-of-care diagnostics for acute diarrheal illness that distinguishes between bacterial, viral, protozoal, and toxin-mediated etiologies and leads to actionable public health or individual management interventions.

- Investigations into the relationship between existing gastrointestinal flora (microbiome) and the susceptibility, duration, and severity of diarrheal disease, as well as the impacts of dietary modification and/or probiotic use.
- Research to identify, reduce, or eliminate sources of infection causing diarrheal diseases.

Dystonia

- Research on the risk, incidence, and etiology of generalized dystonia, focal dystonia, multifocal dystonia, segmental dystonia, and/or hemidystonia.
- Research on interventions to prevent, slow the progression of, or treat dystonia.
- Research to improve identification of delayed onset dystonia following traumatic brain injury.

Early Trauma Thermal Regulation

- Research into regional cooling for different environments, including prolonged care, as a therapeutic for secondary ischemic injury (e.g., head with traumatic brain injury, limb with tourniquet).
- Development of a tunable support mechanism for thermal homeostasis for use in prolonged field care, extended evacuation, or unmanned evacuation.
- Development and evaluation of a novel and/or innovative technology and techniques to reduce the impact of hypothermia.
- Research to determine the benefits of invasive warming/cooling during patient movement.
- Research to validate the attenuating effects of (early) external head cooling on concussive brain injuries.
- Research into therapeutic cooling or targeted temperature management as a neuroprotective treatment for traumatic brain injury.

Eating Disorders

- Investigations into the prevalence, diagnosis, and treatment patterns of eating disorders in Service members and their families, including potential relationships with military-unique behaviors or conditions.
- Assessment of patterns of comorbidity between eating disorders and other mental health conditions, including an examination of whether eating disorders are more likely to precede or follow the development of other mental health conditions.
- Studies to identify the most effective treatments (and order of treatment) for patients with an eating disorder and a comorbid disorder.
- Research to advance the understanding of the biological, genetic, and environmental factors that influence eating disorders.
- Studies to elucidate risk factors for the onset or recurrence of eating disorders.
- Research to develop screening tools and valid biomarkers that accurately identify individuals with binge eating disorder versus healthy controls in order to monitor at-risk populations and tailor treatments for disordered eating.

- Research to better understand subclinical disordered eating and other specified feeding and eating disorders and the potential for these to develop into full-blown eating disorders.

Emerging Infectious Diseases

- Methods to minimize risk from arboviruses among military Service members and their families deployed to/living in high-risk areas, including personal protective equipment and environmental controls.
- Investigation of revaccination strategies and identification of correlates of protection for currently available vaccines (especially mumps).
- Targeted vector management of *Aedes aegypti, Anopheline sp.* and related mosquito species, including focus areas on novel and/or innovative adult surveillance devices, pathogen identification, spatial repellents, and barrier treatments.
- Research toward understanding the potential influence of dietary intake as a potential repellent to mosquito vectors and vector behavior.
- Evaluation of non-vaccine prophylactics or therapeutics to prevent/treat dengue or Zika virus disease.
- Characterization of health emergency relevant infectious disease threats in West Africa.
- Development of biomarker-guided clinical management strategies for severe infectious disease in the austere African setting.
- Validation of airborne and droplet precaution recommendations in the medical transport environment.
- Development of new testing methods for emerging pathogens and pathogen reduction technologies for blood-borne pathogens, including Zika, malaria, dengue, and others, in blood products.
- Modeling of epidemics and development of strategies to counter risks in emerging infectious diseases.
- Rapid, rugged, sensitive, and specific screening and diagnostics, including biomarkers for infectious threats such as Zika and other clinically relevant bacterial, viral, or protozoal pathogens in clinical samples or blood products that can be transmitted sexually or by blood transmission.
- Development of broad-acting detection systems to identify emerging members of a known viral family (for example, a broad Coronavirus panel that can detect Middle Eastern Respiratory Syndrome).
- Determination of the human health effects, host preference, vector competence, and co-infection status of the dengue, chikungunya, Zika, and Mayaro viruses in *Aedes aegypti* and related mosquito species.

Epidermolysis Bullosa

- Research to provide further insight into those cellular pathways that might be corrected or bypassed that otherwise promote the development of squamous cell carcinomas in recessive dystrophic and junctional epidermolysis bullosa.

- Research, including randomized controlled clinical trials, focused on systemic drugs that prevent, delay the onset, or modify the aggressiveness of squamous cell carcinoma in patients with recessive and junctional epidermolysis bullosa.
- Research, including clinical trials, focused on medications (topical or systemic) or dressings that might enhance wound healing in inherited epidermolysis bullosa.

Focal Segmental Glomerulosclerosis

- Development of a curative therapy or treatments to delay or halt the progression of focal segmental glomerulosclerosis and/or prevent post-transplantation recurrence.
- Research to determine the efficacy of medications used off-label (outside the FDA-approved indication) to treat focal segmental glomerulosclerosis.
- Research to improve understanding of the causes of primary and secondary focal segmental glomerulosclerosis, especially genetic mutations.
- Development of non-invasive methods to diagnose focal segmental glomerulosclerosis and its variants.
- Research on the impact of dietary modifications on the progression of renal disease.

Fragile X

- Research to advance the understanding of the pathophysiology of fragile X syndrome.
- Identification and validation of functional measures of the manifestations of fragile X syndrome across the life span.
- Identification and testing of novel and/or innovative targets for fragile X syndrome therapeutics.
- Development and evaluation of novel, innovative, or existing therapeutics for the treatment of fragile X syndrome.

Guillain-Barré Syndrome

- Research to elucidate the characteristics of various exposures (e.g., viruses, bacteria, vaccinations, surgery) associated with Guillain-Barré syndrome and their effects on the immune system.
- Research on the immune system cell types and molecular mechanisms responsible for the disease pathology of Guillain-Barré syndrome.
- Research to prevent or reduce the effects of residual weakness, relapse of muscle weakness, and other neurological symptoms of Guillain-Barré syndrome to improve patients' quality of life and increase their independence.
- Development of new treatments and refinement of existing treatments for Guillain-Barré syndrome.

Hepatitis B and C

- Research on vaccination options for those persons unable to mount immunity to the hepatitis B virus.

- Development of strategies for reliable, non-invasive, early detection of hepatitis-related liver disease and hepatocellular carcinoma.
- Research on strategies to promote reversal of liver fibrosis and/or assess the associated clinical and pathological outcomes.
- Natural history or other studies to establish risk indicators of progression from asymptomatic or inactive chronic hepatitis infection to symptomatic disease and parameters for the initiation and duration of disease treatment.
- Clinical studies to evaluate combination or curative therapies for treatment of hepatitis infection.
- Identification and reduction of hepatitis in blood products for transfusion.
- Research on strategies to reduce vertical (mother-to-child) transmission of hepatitis B virus and hepatitis C virus.
- Development of a vaccine against hepatitis C.
- Development of treatment protocols for patients with hepatitis B and C co-infections.

Hereditary Angioedema

- Research to improve early diagnosis of hereditary angioedema.
- Evaluation of existing, innovative, or novel therapeutics in pediatric hereditary angioedema patients.
- Research toward development of a cure for hereditary angioedema.
- Development and/or validation of novel and/or innovative therapeutic strategies for the treatment and/or prevention of hereditary angioedema attacks.

Hydrocephalus

- Research on the etiology, prevention, diagnosis, and treatment of post-traumatic hydrocephalus.
- Discovery or validation of novel and/or innovative therapies and therapeutic targets for the treatment of hydrocephalus and its sequelae, including therapies directed at myelin regeneration and repair.
- Research on approaches to lessening the impact of brain damage caused by hydrocephalus.
- Development or validation of biomarkers and imaging techniques, particularly multimodal approaches, to aid in diagnosis, prognosis, and monitoring of therapeutic efficacy.
- Research on the prevention of shunt failure.
- Studies to better understand the causes and/or pathogenesis of hydrocephalus.
- Development or validation of improved hydrocephalus model systems.

Immunomonitoring of Intestinal Transplants

- Studies to elucidate the role of the mucosal immune system, other host-derived factors, or gut microbiota-derived factors in maintaining intestinal transplant viability.

- Development and evaluation of intestinal implant strategies that circumvent the induction of immunity against the transplant through the use of autologous stem cells or organoids derived from intestinal stem cells.
- Development and evaluation of evidence-based approaches that focus on dampening the regional immune response against intestinal transplants without inducing global immune suppression.
- Development and evaluation of implant-associated materials (e.g., scaffolds) with anti-inflammatory properties that protect the intestinal transplant from immune attack.
- Development and evaluation of strategies for inducing and maintaining populations of anti-inflammatory regulatory immune cell populations at the transplant site.
- Studies to improve immunomonitoring of recipient immune responses after intestinal transplantation, with a focus on prospective leukocyte profiling, to aid in diagnosis and treatment of immunological and immunosuppression-related complications.
- Development and/or validation of precise, real-time implanted monitoring devices to improve individualized patient outcomes after intestinal transplantation.

Inflammatory Bowel Diseases

- Clinical studies in human subjects directed toward understanding how acute enteric infections may trigger chronic bowel diseases with acute and sub-acute inflammatory bowel disease, including systems biology approaches.
- Mechanistic studies in animal models designed to understand how enteric infection may trigger inflammatory bowel disease, including genomic, microbiomic, and immune mechanisms.
- Epidemiological studies of post-infectious acute and sub-acute inflammatory bowel diseases that define risk and provide estimates of illness-associated disability, health care costs, and symptom duration from a military health system and societal perspective.
- Studies to understand the interaction between acute/chronic stress and infection and the development of inflammatory bowel disease.
- Research to explore whether the travel patterns of active duty personnel increase the risk of developing inflammatory bowel disease by means of exposure to changes in intestinal microflora.
- Research to better characterize the association between the use of drugs, such as isotretinoin and long-term doxycycline, and the development of inflammatory bowel disease.
- Research on the role of diet in the development and progression of inflammatory bowel disease.
- Research on the influence of the microbiome on inflammatory bowel disease.
- Research on hyperbaric oxygen treatment of inflammatory bowel disease.
- Research on treatment strategies for patients whose inflammatory bowel disease is refractory to standard care medications or who positively respond to standard care medications, but become nonresponsive over time.

Influenza

- Development of a diagnostic test for influenza drug resistance.
- Development and testing of a universal influenza vaccine.
- Development and evaluation of novel, innovative, and/or combination influenza therapies.
- Research to improve the understanding of host responses to influenza infection and the mechanisms of drug resistance.
- Research on the factors that contribute to and/or reduce the risk of the emergence of zoonotic and pandemic influenza viruses.

Integrative Medicine

- Research on the use of integrative medicine strategies, such as meditation, tai chi, acupuncture, and/or herbal medicine/nutritional supplementation, to improve the psychological health and quality of life in cancer patients and survivors.
- Research on the use of integrative medicine in treatment and management of chronic pain disorders, including comparative efficacy studies relative to standard of care.
- Rigorous longitudinal studies of integrative medicine approaches for enhancing resilience and treating psychological health issues and co-occurring disorders.
- Precision medicine research to investigate, develop, and validate biomarkers that can help determine an individual's response to integrative medicine treatments for pain.
- Development of outcomes tools and measures to evaluate the effectiveness of integrative medicine pain management approaches.
- Definitive studies to determine the effectiveness of self-care, meditation-based treatments, courses, and training programs in improving resiliency to stress in a military environment; in austere operational platforms such as submarines and small-deck ships; or in combat-fatigued military health care providers.

Interstitial Cystitis

- Studies that define the risk, prevalence, and operational impact of interstitial cystitis among active duty personnel.
- Identification of biological markers for making a definitive diagnosis of interstitial cystitis.
- Evaluation and assessment of novel and/or innovative treatment options for interstitial cystitis patients, including intravesical therapy.
- Research on the etiology of interstitial cystitis to inform targeted therapy development.

Malaria

- Investigation of mechanisms of drug resistance in malaria, to include host and pathogen, against drugs used for treatment and prophylaxis.
- Development of a vaccine that induces high levels of long-lived, broadly protective immunity against *Plasmodium falciparum* and/or *P. vivax*.
- Operational research to optimize application of existing malaria drugs to reduce impact of artemisinin resistant *P. falciparum* in military forces.

- Operational research to optimize deployment of pharmacovigilance systems to ensure capture of adverse events related to malaria chemoprophylaxis and treatment.
- Operational research to assess efficacy and spread of artemisinin combination therapies across the Greater Mekong Subregion's military forces.
- Development of malaria prophylactic regimens that encourage higher compliance and methods to monitor compliance in deployed Service members.
- Identification of novel and/or innovative malaria drug targets for blood and liver stage malaria parasites.
- Development of new, or improvement of existing, strategies for culturing relapsing malaria parasites, with emphasis on *Plasmodium vivax, P. ovale,* and *P. cynomolgi* in vitro.
- Development, testing, and evaluation of spatial repellents to provide protection against Anopheline vectors.
- Identification of a correlate of immunity against malaria and development of a surrogate test of protection.
- Structurally optimize validated malaria antigens by engineering protective epitopes to elicit specific and enhanced immune responses.

Metals Toxicology

- Identification and validation of biomarkers to evaluate military Service members' acute exposure to toxic metals in an operational environment and predict potential consequent health risks and associated health outcomes.
- Retrospective studies to evaluate risk and exposure among workers at industrial facilities.
- Research on the toxicity of metal combinations and the interaction between different metal components.
- Research on the additive effects of multiple exposures to metal(s).
- Research on underlying diet quality and micronutrient sufficiency and the risk for metals toxicity.
- Research on the toxicity of metal-based engineered nanomaterials.

Mitochondrial Disease

- Research on the basic biology and physiology of mitochondria to better understand the pathology of primary mitochondrial diseases.
- Development of improved tools and animal models to study primary mitochondrial disease and evaluate therapeutics.
- Identification and testing of non-invasive techniques and biomarkers to monitor mitochondrial function, aid in diagnosis, and/or evaluate therapeutic efficacy.
- Research on novel and/or innovative treatments to alleviate symptoms or slow down the progression of mitochondrial diseases.

Musculoskeletal Disorders

- Research to increase understanding, diagnosis, prevention, and/or treatments of chronic overuse musculoskeletal disorders.
- Research on measures (e.g., clinical biomarkers, novel/innovative interventions, therapeutics) to diagnose, predict, reduce the incidence of, or optimize health outcomes in military training- and Service-related musculoskeletal disorders.
- Research on the validation and/or optimization of rehabilitation strategies for Service-related musculoskeletal disorders.
- Research to prevent, control, and/or optimize musculoskeletal health outcomes for work-related musculoskeletal disorders.
- Research on lower back pain strategies to prevent surgery and recurrence of symptoms, identify factors that predict optimal treatment response for different patients, and encourage self-management as a treatment.
- Research to determine whether tailored psychosocial education interventions are effective in improving psychological resilience to musculoskeletal pain.
- Research on standardized approaches for early identification and prevention of chronic musculoskeletal pain.

Nanomaterials for Bone Regeneration

- Research on nanomaterials-based methods to facilitate recruitment of endogenous cell populations for enhanced bone regeneration and osseointegration.
- Technologies addressing segmental/large bone defects in craniomaxillofacial and/or load-bearing regions.
- Development of controlled release/extended release of growth factors for bone regeneration.
- Development of technologies that repair the soft tissue envelope to enhance bone regeneration.

Non-Opioid Pain Management

- Research on pain management strategies for patients with limited access to skilled providers and resources, including battlefield, prolonged field, transport, and other resource-limited environments.
- Development of population-based outcomes, tools, and measures to evaluate the effectiveness of pain management approaches across time and environments.
- Research on treatments for chronic pain management, particularly in complex patients (i.e., chronic, high-utilization, polypharmacy patients).
- Research to increase understanding of and preventative treatments for conversion of acute to chronic pain.
- Research that provides evidence of which pain management strategies work for whom and under what conditions.
- Research to identify and address biopsychosocial aspects of pain to reduce or eliminate the use of opioid pain medication(s).

- Comparative studies evaluating the efficacy of different pain management strategies, including complementary and alternative medicine approaches.
- Identification and development of non-opioid pain management techniques that promote positive psychological health-related outcomes.
- Development of non-opioid pain medicine that can be given via inhalation or intramuscularly, submucosally, or intravenously on the battlefield to provide adequate relief of pain without affecting the cardiorespiratory systems.
- Research on case management strategies designed to optimize the effectiveness of pain clinics, support use of non-opioid pain therapies, and reduce dependence on opioid therapies.
- Research that will directly obtain an FDA analgesic indication for oral transmucosal or intramuscular ketamine.

Pancreatitis

- Retrospective studies to determine the risk and incidence of pancreatitis among former and current active duty personnel.
- Development and testing of novel and/or innovative therapeutics for acute and/or chronic pancreatitis.
- Research on the basic biology and physiology of the pancreas to better understand the etiology and pathology of pancreatitis.

Pathogen-Inactivated Dried Cryoprecipitate

- Development or validation of technology to produce pathogen-reduced dried cryoprecipitate in military or civilian donor centers that is acceptable for FDA licensure in support of combat or contingency operations.

Polycystic Kidney Disease

- Development of improved treatment strategies for polycystic kidney disease, including approaches to identify and monitor patients at higher risk for progressing to end-stage renal disease.
- Research on the underlying pathobiology and molecular mechanisms of polycystic kidney disease, including studies of genetic factors, cyst formation and growth, the role of cilia, and factors that modify disease progression and/or severity.
- Research on the lifestyle factors that may modify the progression of polycystic kidney disease.

Post-Traumatic Osteoarthritis

- Research to establish activity recommendations for maximal joint life following joint repair, particularly in young patient populations.
- Research into cell-based approaches for treatment or prevention of post-traumatic osteoarthritis.
- Studies to evaluate and develop best practices for multidisciplinary team approaches and treatment algorithms for post-traumatic osteoarthritis.

- Development or validation of novel and/or innovative approaches to restoring joint stability after injury.
- Sustained release, intra-articular injectable steroidal, non-steroidal, or disease-modifying therapies that offer two or more months of symptomatic relief of pain and/or inflammation in a single injection.
- Research on therapies that target multiple phases of the cellular response pathways that are implicated in the development of post-traumatic osteoarthritis, including cell death, inflammation, matrix changes, and changes in catabolic and anabolic responses.
- Development and/or validation of predictive models for early onset post-traumatic osteoarthritis in extremity-injured patients for the purpose of tailoring rehabilitation programs toward prevention, delay, or mitigation of onset.

Pulmonary Fibrosis

- Retrospective studies to determine the risk and incidence of pulmonary fibrosis among military Service members, including Veterans.
- Identification of biomarkers of pulmonary injury or early predictors of interstitial lung disease.
- Research into the pathobiology and molecular mechanisms underlying the development of pulmonary fibrosis.
- Development and/or testing of novel and/or innovative treatments to delay or modify the progression of pulmonary fibrosis.
- Development and/or validation of improved tools and animal models to study pulmonary fibrosis and evaluate therapeutics.

Respiratory Health (excludes lung cancer and mesothelioma)

- Research on the causes, treatment, and prevention of chronic obstructive pulmonary disease (COPD), including identification and validation of biomarkers and disease phenotypes, as well as employing personalized medicine in clinical research and disease management.
- Research investigating the treatment of patients with chronic mild hypoxia.
- Research on the cause, treatment, and prevention of respiratory symptoms and ailments possibly associated with deployed and redeployed military personnel, including acute eosinophilic pneumonia, asthma, allergies, and other chronic lung diseases and breathing problems.
- Research to evaluate the impact of deployment on the prevalence and severity of respiratory disease in military Service members and Veterans.
- Development and/or validation of methods to detect volatile organic hydrocarbons from the breath of individuals exposed to toxic chemicals or other agents that can cause lung injury, which can be utilized for diagnosis and prognosis of lung injury or disease.
- Identification of biochemical, physiological, or combined biomarkers for evaluating risk or extent of injury from either acute or long-term toxic occupational or environmental exposures.

- Research investigating exposure rates, detection, and treatment of diseases related to inhalation of mold and fungi, such as coccidioidomycosis.

Rett Syndrome

- Identification and/or validation of novel and/or innovative biological targets for the treatment of Rett syndrome.
- Development and testing of interventions to improve the neurological symptoms of Rett syndrome.
- Research to understand the relationship between genetic mutations, physical traits, and symptoms in individuals with Rett syndrome.
- Research on the pathobiology of the MeCP2 gene and protein.
- Research to understand Rett syndrome's commonalities with and differences from classic autism and regressive autism.

Rheumatoid Arthritis

- Research to better understand the relationship between genetic risk and environmental triggers, such as infection or smoking, in developing rheumatoid arthritis.
- Studies that identify or validate biomarkers or personalized medicine strategies that allow for individualized medication choice based on the patient's underlying biology or disease state.
- Research on the long-term use of immunosuppressants in patients with rheumatoid arthritis and the likelihood of developing infections.
- Research to establish activity recommendations following joint replacement for maximal joint life.

Scleroderma

- Research on the molecular mechanisms and pathogenesis of scleroderma.
- Development and/or testing of novel and/or innovative therapies and identification of novel and/or innovative therapeutic targets in scleroderma.
- Research on biomarkers and other approaches to diagnose scleroderma, monitor disease progression, and/or assess response to treatment.
- Epidemiologic studies investigating the impact of localized scleroderma (morphea) on duty performance, use of personal protective equipment, and deployability.
- Research on the efficacy of newer immunosuppressive agents, including biologics and small molecule inhibitors, in the treatment of morphea and scleroderma (systemic sclerosis).
- Research on the efficacy of UVA1 for topical treatment in localized scleroderma and systemic sclerosis.

Sleep Disorders

- Research on how the disruption of normal sleep and circadian biological rhythms adversely affects the physical and psychological health, safety, performance, and productivity of military and civilian populations.
- Studies to assess sleep disturbance and clinical sleep disorders in female military personnel.

- Research on the association between sleep disorders and post-traumatic stress disorder, traumatic brain injury, depression, and/or anxiety and suicidal behaviors.
- Research to assess clinical algorithms that improve adherence to continuous positive airway pressure (CPAP).
- Investigations into non-CPAP-based treatment regimens that enhance readiness and deployability in active duty military personnel.
- Research focused on investigating how psychiatric disorders (post-traumatic stress disorder in particular), combined with the disruption/degradation of sleep quality, impact long-term physical health through changes in hormonal physiology.
- Development and/or testing of non-pharmacological treatments for sleep disorders.
- Research on the impact of sleep deprivation on the efficacy of psychological treatments for post-traumatic stress disorder, depression, and other mood disorders.
- Research on the prevention and/or mitigation of sleep disorders that are associated with long aeromedical evacuation flights for both clinical team members and patients.
- Development of tools and technologies that allow rapid and objective measurement of Service member performance in the face of fragmented sleep and/or sleep maintenance difficulty.
- Research examining the broader effects of Warfighters' sleep disorders on the sleep, behavioral health, and productivity of their family members, as well as the readiness of their military unit.

Spinal Muscular Atrophy

- Research into phenotyping the spinal muscular atrophy disease state.
- Research into which molecular deficiency imposed by a deficiency of SMN, a motor neuron protein, causes motor neuron deficits.
- Exploration of the form and function of SMN-depleted neuromuscular junctions at ultrastructural (e.g., dysregulation of endocytosis), transcriptomic, and proteomic levels, particularly the mildest SMN reduction that leads to consistent quantifiable motor neuron loss.
- Research to find non-SMN-altering spinal muscular atrophy modifying genes in cellular, small animal, and human models that may lead to identification of novel and/or innovative therapeutic targets.
- Research to further understand SMN gene regulation and post-transcriptional mechanisms leading to synergistic SMN-repleting approaches, as well as to determine whether boosting SMN induction maximizes efficacy.
- Research to determine whether SMN can be repleted to physiologic levels in all cells in the spinal muscular atrophy model immediately post-natally and whether there is a limit to the therapeutic response.
- Clinical research to determine what clinical findings persist even with treatment; whether these findings are related to drug timing, dosage, biodistribution, or a combination of factors; and whether these factors are modifiable.

- Research involving dietary/nutritional studies in spinal muscular atrophy to identify the optimal anabolic diet.
- Research to determine mitochondrial involvement and astrocytic and other non-neuronal contributions to motor neuron vulnerability.

Sustained-Release Drug Delivery

- Development of technology that can provide a sustained-release delivery of drugs for a minimum of 1 week or more. Potential applications of this technology could include long-acting therapies for post-traumatic stress, opiate dependence, low-dose pain control, allergies, attention deficit/hyperactivity disorder, chemoprophylaxis, and other conditions.
- Research into the use of printable, sustained-release scaffolds for predictable delivery of prescription and non-prescription medications.
- Research into novel and/or innovative approaches to developing bioavailable and sustained-release oral formulations of intravenous broad-spectrum fungicidal and anti-microbial medications.

Tinnitus

- Research to understand the mechanisms of tinnitus, its relationship to noise-induced hearing loss, and progression to chronic tinnitus, with the focus on developing interventions for tinnitus.
- Research to increase knowledge of the prevalence, incidence, natural history, and occupational and sex-related differences of tinnitus and its possible relation to individual blast/noise exposures.
- Research to understand the operational readiness impacts of tinnitus on the military.
- Development and validation of objective tools/methods to diagnose and characterize tinnitus (e.g., imaging techniques to identify functional and structural changes in the brain, biomarkers of resiliency, and susceptibility to tinnitus).
- Identification of novel and/or innovative therapies for interventions to prevent, manage, and treat tinnitus, including behavioral approaches, new uses for existing drugs, nutritional and pharmaceutical strategies, and acoustic, electrical, and other stimulation technologies.

Tuberculosis

- Development of a diagnostic assay that can be used at the point of care to rapidly and accurately diagnose tuberculosis.
- Development of novel and/or innovative tuberculosis vaccines or optimization of current tuberculosis vaccines.
- Identification and/or validation of biomarkers that can be used to assess vaccine efficacy and protection against tuberculosis disease.
- Research to understand, diagnose, or treat multi-drug-resistant tuberculosis or extensively drug-resistant tuberculosis.
- Research to determine the appropriate precautions to use for transport of active tuberculosis patients.

- A sustained release formulation of anti-tuberculosis drugs that would facilitate long-term treatment and reduce the emergence of resistance due to poor compliance.

Vaccine Development for Infectious Disease

- Development of a therapeutic vaccine and/or other strategies to achieve HIV remission.
- Evaluation of passive immunization strategies to use in conjunction with dengue and Zika vaccinations.
- Research leading to a better understanding of the immune mechanisms involved in clearance of the dengue and Zika viruses and the mechanisms of immune enhancement that lead to more severe clinical disease.
- Development of flexible vaccine technologies that can be used to rapidly respond to emerging and re-emerging infectious diseases threats.
- Evaluation of humoral and cellular immune responses after vaccination compared to natural infection.
- Development of a safe, effective dengue human challenge model for clinical trials.
- Development of a non-human primate model to study Zika virus-induced microcephaly/fetal neuropathology as a tool to measure the safety and efficacy of vaccines and antivirals.
- Development and fielding of vaccines to prevent U.S. Service members from becoming ill from endemic disease exposure during operational deployments. This includes, but is not limited to, arthropod-borne diseases (Zika virus, dengue, chikungunya, hantavirus hemorrhagic fever with renal/pulmonary syndrome, rickettsioses, and trypanosomiasis), leptospirosis, HIV, norovirus, Middle East Respiratory Syndrome coronavirus, schistosomiasis, leishmaniasis, Nipah virus, Lassa fever, and West Nile fever.

Vascular Malformations

- Studies into the natural history, genetics, and pathogenesis of vascular malformations.
- Research to discover or develop novel and/or innovative therapeutic targets and treatments to regress or prevent vascular malformations.
- Research to improve methods to diagnose and manage vascular malformations.
- Development of non-invasive or minimally invasive technologies or approaches for the control of internal bleeding associated with vascular malformations.
- Studies to identify risk and/or establish standard practices for the treatment of hemorrhage from brain arteriovenous malformations.
- Research on the prevention or treatment of complications associated with vascular malformations such as pain, infection, seizures, tissue breakdown, tissue overgrowth, or airway obstruction.
- Studies to elucidate the progression of vascular malformations in disease-in-a-dish/microfluidic models for the purpose of identifying novel and/or innovative drug targets, screening existing drugs, and/or elucidating the pathogenesis of the disease.

Women's Heart Disease

- Retrospective studies to determine the risk and incidence of heart disease among former and current female active duty personnel, including Reservists and National Guard members.
- Research focused on elucidating the potential relationship between post-traumatic stress disorder and women's heart disease.
- Identification of sex-specific approaches to increase the effectiveness of diagnosis, treatment, and/or cardiac rehabilitation programs.
- Research on factors to predict and prevent the long-term impacts of gestational diabetes, gestational hypertension, and preeclampsia on the cardiovascular health of women.
- Research on trauma-induced cardiac arrest secondary to hemorrhage and polytrauma in the female population.

APPENDIX 3
DOD AND VA WEBSITES

PIs are encouraged to integrate and/or align their research projects with DoD and/or VA research laboratories and programs. Collaboration with DoD or VA investigators is also encouraged. Below is a list of websites that may be useful in identifying additional information about DoD and VA areas of research interest, ongoing research or potential opportunities for collaboration within the FY17 PRMRP Topic Areas.

Air Force Office of Scientific Research
http://www.wpafb.af.mil/afrl/afosr/

Air Force Research Laboratory
http://www.wpafb.af.mil/afrl

Armed Forces Radiobiology Research Institute
http://www.usuhs.edu/afrri/

Clinical and Rehabilitative Medicine Research Program
https://crmrp.amedd.army.mil

Combat Casualty Care Research Program
https://ccc.amedd.army.mil

Congressionally Directed Medical Research Programs
http://cdmrp.army.mil

Defense Advanced Research Projects Agency
http://www.darpa.mil/

Defense Technical Information Center
http://www.dtic.mil

Defense Threat Reduction Agency
http://www.dtra.mil/

Military Health System Research Symposium
https://mhsrs.amedd.army.mil/SitePages/Home.aspx

Military Infectious Diseases Research Program
https://midrp.amedd.army.mil

Military Operational Medicine Research Program
https://momrp.amedd.army.mil

Naval Health Research Center
http://www.med.navy.mil/sites/nhrc

Navy and Marine Corps Public Health Center
http://www.nmcphc.med.navy.mil/

Office of Naval Research
http://www.med.navy.mil/

Office of the Under Secretary of Defense for Acquisition, Technology and Logistics
http://www.acq.osd.mil/

Telemedicine and Advanced Technology Research Center
http://www.tatrc.org/

Uniformed Services University of the Health Sciences
http://www.usuhs.edu/research

U.S. Army Institute of Surgical Research
http://www.usaisr.amedd.army.mil/

U.S. Army Research Institute of Environmental Medicine
http://www.usariem.army.mil/

U.S. Army Medical Research Institute of Infectious Diseases
http://www.usamriid.army.mil/

U.S. Army Medical Research and Materiel Command
http://mrmc.amedd.army.mil

U.S. Army Research Laboratory
http://www.arl.army.mil

U.S. Department of Defense Blast Injury Research Program
https://blastinjuryresearch.amedd.army.mil/

U.S. Department of Veterans Affairs, Office of Research and Development
http://www.research.va.gov

U.S. Naval Research Laboratory
http://www.nrl.navy.mil

Walter Reed Army Institute of Research
http://www.wrair.army.mil/

INFECTIOUS DISEASE

Zika virus activates de novo and cross-reactive memory B cell responses in dengue-experienced donors

Copyright © 2017 The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original U.S. Government Works

Thomas F. Rogers[1*], Eileen C. Goodwin[2*], Bryan Briney[1], Devin Sok[1], Nathan Beutler[1], Alexander Strubel[1], Rebecca Nedellec[1], Khoa Le[1], Michael E. Brown[2], Dennis R. Burton[1,3†], Laura M. Walker[2†]

Zika virus (ZIKV) shares a high degree of homology with dengue virus (DENV), suggesting that preexisting immunity to DENV could affect immune responses to ZIKV. We have tracked the evolution of ZIKV-induced B cell responses in three DENV-experienced donors. The acute antibody (plasmablast) responses were characterized by relatively high somatic hypermutation and a bias toward DENV binding and neutralization, implying the early activation of DENV clones. A DENV-naïve donor in contrast showed a classical primary plasmablast response. Five months after infection, the DENV-experienced donors developed potent type-specific ZIKV neutralizing antibody responses in addition to DENV cross-reactive responses. Because cross-reactive responses were poorly neutralizing and associated with enhanced ZIKV infection in vitro, preexisting DENV immunity could negatively affect protective antibody responses to ZIKV. The observed effects are epitope-dependent, suggesting that a ZIKV vaccine should be carefully designed for DENV-seropositive populations.

INTRODUCTION

Zika virus (ZIKV) is a mosquito-borne flavivirus that has been linked to microcephaly and severe neurological complications, such as Guillain-Barré syndrome (*1*, *2*). The virus is closely related to the four serotypes of dengue virus (DENV) (DENV1, 2, 3, and 4), as well as other circulating flaviviruses including West Nile virus (WNV), resulting in substantial immunological cross-reactivity (*3*–*7*). Although neutralizing antibodies (nAbs) play an important role in protection against flavivirus infection, they can also contribute to severe disease through a phenomenon termed antibody-dependent enhancement (ADE) (*8*–*10*). In the case of DENV, subneutralizing concentrations of preexisting heterotypic nAbs have been implicated in promoting viral replication by facilitating the interaction of the virus with Fc receptor–bearing target cells (*8*). Enhancement of ZIKV infection by cross-reactive DENV-specific antibodies and vice versa has been demonstrated in vitro, and ADE of ZIKV pathogenesis by preexisting anti-flavivirus immunity has been observed in mouse models (*5*–*7*, *11*–*14*). Because ZIKV is currently circulating in regions that are highly endemic for DENV, an understanding of how previous DENV exposure affects the B cell response to ZIKV will be critical for the design of vaccines and therapies intended for DENV-immune populations.

Previous studies have shown that nonstructural protein 1 (NS1), envelope (E), and precursor membrane (prM) proteins are dominant targets for the human B cell response to flaviviruses. NS1 is secreted by infected cells and functions in pathogenesis and immune evasion (*15*), the surface E protein mediates viral entry and is the primary target for nAbs (*7*, *16*, *17*), and prM is a 166–amino acid protein that is associated with E on immature and partially mature virions (*18*). The E protein consists of three domains: domain I (DI), which participates in conformational changes required for viral entry; domain II (DII), which contains the conserved fusion loop (FL); and domain III (DIII), which is the putative receptor binding domain (*19*). Previous studies have established that monoclonal antibodies (mAbs)-targeting epitopes within DIII are typically type-specific and potently neutralizing, whereas mAbs targeting the conserved FL are cross-reactive and poorly neutralizing (*7*, *16*, *17*, *20*).

In this study, we have longitudinally tracked the ZIKV-specific B cell response in three DENV-experienced donors using single B cell cloning and large-scale antibody isolation. The acute-phase (plasmablast) response was dominated by somatically mutated clones that showed preferential binding and neutralization of DENV, providing evidence for original antigenic sin (OAS) during the early B cell response to ZIKV in DENV-experienced donors. However, by 5 months after infection, the memory B cell responses were composed of both OAS-phenotype antibodies that were broadly cross-reactive and poorly neutralizing and de novo generated antibodies that were ZIKV-specific and potently neutralizing. Collectively, the results have implications for the development of ZIKV vaccines intended for DENV-immune populations and provide insight into the role that preexisting B cell memory plays in modulating the antibody specificities induced by antigenically variable viruses.

RESULTS

Potent plasmablast induction in ZIKV-infected, DENV-experienced donors

To study the early ZIKV-induced B cell response in DENV-experienced individuals, three ZIKV-infected donors (donors 2, 3, and 4) living in a highly DENV endemic region of Colombia were sampled during acute infection (within 8 days after symptom onset) (table S1). A single donor (donor 1) from the United States who was infected with ZIKV while overseas was also sampled for comparison (table S1). As expected, the acute sera from donors 2, 3, and 4 showed strong immunoglobulin G (IgG) binding reactivity to whole DENV1–4 particles and recombinant DENV NS1 proteins (fig. S1). In contrast, the acute serum from donor 1 showed little to no such binding (fig. S1). Although the acute serum reactivity to DENV particles and NS1 proteins could, in principle, be due to cross-reactive ZIKV-induced antibodies, previous studies have shown that NS1-specific IgG antibodies are not present in the serum during the first week

[1]Department of Immunology and Microbiology, the Scripps Research Institute, La Jolla, CA 92037, USA. [2]Adimab LLC, Lebanon, NH 03766, USA. [3]Ragon Institute of MGH, MIT and Harvard, Cambridge, MA 02139, USA.
*These authors contributed equally to this work.
†Corresponding author. Email: laura.walker@adimab.com (L.M.W.); burton@scripps.edu (D.R.B.)

Downloaded from http://immunology.sciencemag.org/ on August 24, 2017

after the onset of symptoms of ZIKV infection (22, 23), implying that the acute serum reactivity to DENV NS1 observed for donors 2, 3, and 4 is largely mediated by preexisting antibodies that were induced by previous DENV exposure. Furthermore, the acute serum from these three donors showed similar or more potent neutralizing activity against DENV compared with ZIKV, whereas the acute serum from donor 1 showed little to no neutralizing activity against DENV1–4 and relatively weak neutralization of ZIKV. These serological and epidemiological data provide convincing evidence that donors 2, 3, and 4 were previously exposed to DENV, whereas donor 1 was DENV-naïve at the time of infection.

The appearance of antigen-specific plasmablasts in peripheral blood during acute viral infection is typically the first indication of a B cell response (24–26). Therefore, we first measured the plasmablast frequency in peripheral blood about 1 week after symptom onset (table S1). In all three DENV-experienced donors, a massive plasmablast population ($CD3^-CD20^{-/lo}CD19^+CD27^{hi}CD38^{hi}$) that accounted for 39 to 63% of peripheral B cells was observed (Fig. 1A and fig. S2). In contrast, the plasmablast population in the ZIKV-infected, DENV-naïve donor was relatively small, accounting for only 2.5% of peripheral B cells (Fig. 1A). To deconstruct the acute ZIKV-induced B cell response, we single cell–sorted between 100 and 300 plasmablasts from each donor sample and rescued the antibody variable heavy (V_H) and variable light (V_L) chain sequences by single-cell polymerase chain reaction (PCR). Between 42 and 119 cognate V_H and V_L pairs from each donor were cloned and expressed as full-length IgGs. Sequence analysis revealed that the plasmablasts sorted from the three DENV-experienced donors were somatically mutated—with per-donor averages ranging between 15 and 20 nucleotide substitutions in V_H—and clonally expanded (Fig. 1, B and C). This level of somatic hypermutation (SHM) is comparable with that observed in typical IgG memory B cells as well as in plasmablasts induced by influenza vaccination (25), supporting a memory B cell origin for these acutely induced cells. In contrast, most of the plasmablasts isolated from the DENV-naïve donor sample showed little to no SHM and somewhat more limited clonal expansion (Fig. 1, B and C), supporting their emergence via activation of naïve B cells. We conclude that ZIKV infection induces a rapid and robust plasmablast response in DENV-experienced donors and that the large majority of these acutely induced cells appear to originate from the memory B cell compartment.

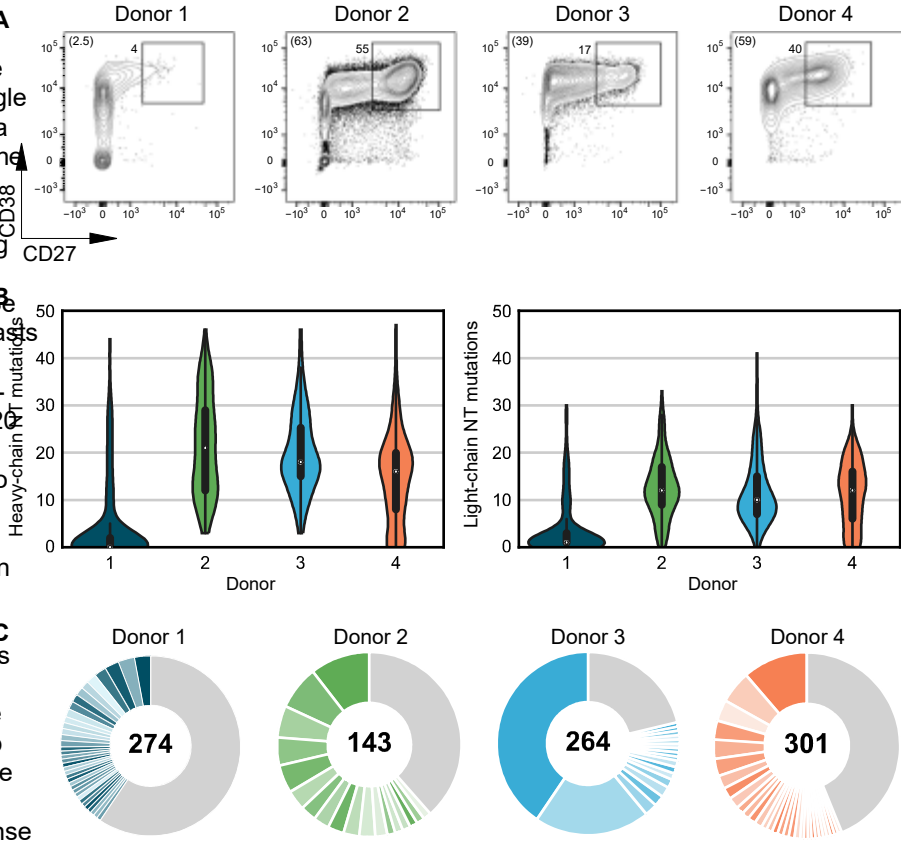


Fig. 1. Analysis of plasmablast responses in one naïve and three DENV-experienced donors. (**A**) Flow cytometric analysis of plasmablast responses in one naïve and three DENV-experienced donors during ongoing ZIKV infection. Plasmablasts are defined herein as $CD3^-CD20^{-/lo}CD38^{hi}CD27^{hi}$ cells. Plots shown are gated for $CD3^-CD20^{-/lo}$ cells, with the $CD27^{hi}CD38^{hi}$ plasmablasts marked by a box. The frequency of plasmablasts within the $CD3^-CD20^{-/lo}$ population is shown next to the gate. The numbers in parentheses show the percentage of peripheral B cells that are plasmablasts. Flow cytometry data were analyzed using FlowJo software (version 10.0.8). (**B**) Load of somatic mutations [expressed as the number of nucleotide (NT) substitutions] in V_H and V_L in plasmablast-derived mAbs isolated from a DENV-naïve (donor 1) and three DENV-experienced donors (donors 2 to 4). All V_H and V_L chains are shown, regardless of whether the paired antibody chain was recovered. (**C**) Clonal lineage analysis. Heavy-chain sequences were assigned to lineages using CHAIN (52). Each lineage is represented as a segment proportional to the lineage size. The total number of recovered heavy chains is shown in the center of each pie. All lineages that contain only a single sequence are combined and shown as a single gray segment.

A large proportion of plasmablast-derived mAbs isolated from ZIKV-infected, DENV-experienced donors bind the ZIKV E protein

Enzyme-linked immunosorbent assay (ELISA) binding studies showed that between 50 and 75% of the mAbs isolated from the DENV-experienced donors bound to whole ZIKV (fig. S3), which is similar to the percentage of antigen-specific mAbs recovered from plasmablasts induced by influenza vaccination and DENV infection (25, 27). In contrast, only 13% of the plasmablast-derived mAbs isolated from the DENV-naïve donor bound with detectable affinity to whole ZIKV particles. To further map the antigenic specificities of the 303 plasmablast-derived mAbs, we performed biolayer interferometry (BLI) binding experiments with recombinant ZIKV NS1 and E proteins. The percentage of mAbs isolated from the three DENV-experienced donors that showed reactivity with ZIKV NS1 was low at 8 to 12% and higher for recombinant E at 15 to 30% (Fig. 2A and table S2). In contrast, 17% of the mAbs isolated from the DENV-naïve donor bound to recombinant NS1, whereas only a single mAb bound with detectable affinity to recombinant E (Fig. 2A and table S2). The remaining ZIKV-specific mAbs, which accounted for 18 to 32% of the corresponding donor responses, bound to epitopes expressed on whole virus but not on recombinant E. Seventy percent of the plasmablast mAbs isolated from donor 1 and about 20 to 50% of the

Downloaded from http://immunology.sciencemag.org/ by guest on August 24, 2017

plasmablast mAbs isolated from donors 2, 3, and 4 could not be assigned to ZIKV in this assay (fig. S4). Hence, most of these mAbs likely bind to E-specific epitopes expressed only on intact virions. Similar ated with any ZIKV reactivity (Fig. 2A). Because anti-prM antibodies have been shown to dominate the antibody response during secondary specificities have been previously described for both DENV and ZIKV DENV infection (*28*), we next investigated the specificities of the whole (*4*, *7*, *27*, *29*).

virus–specific mAbs isolated from the DENV-experienced donors by To further define the epitopes targeted by the recombinant E– Western blot. None of the mAbs recognized prM, 30% showed reactive reactive mAbs isolated from the DENV-experienced donors, we (i) ity with E protein from viral lysates, and the remaining mAbs failed to compared the apparent binding affinities of the mAbs for recombin

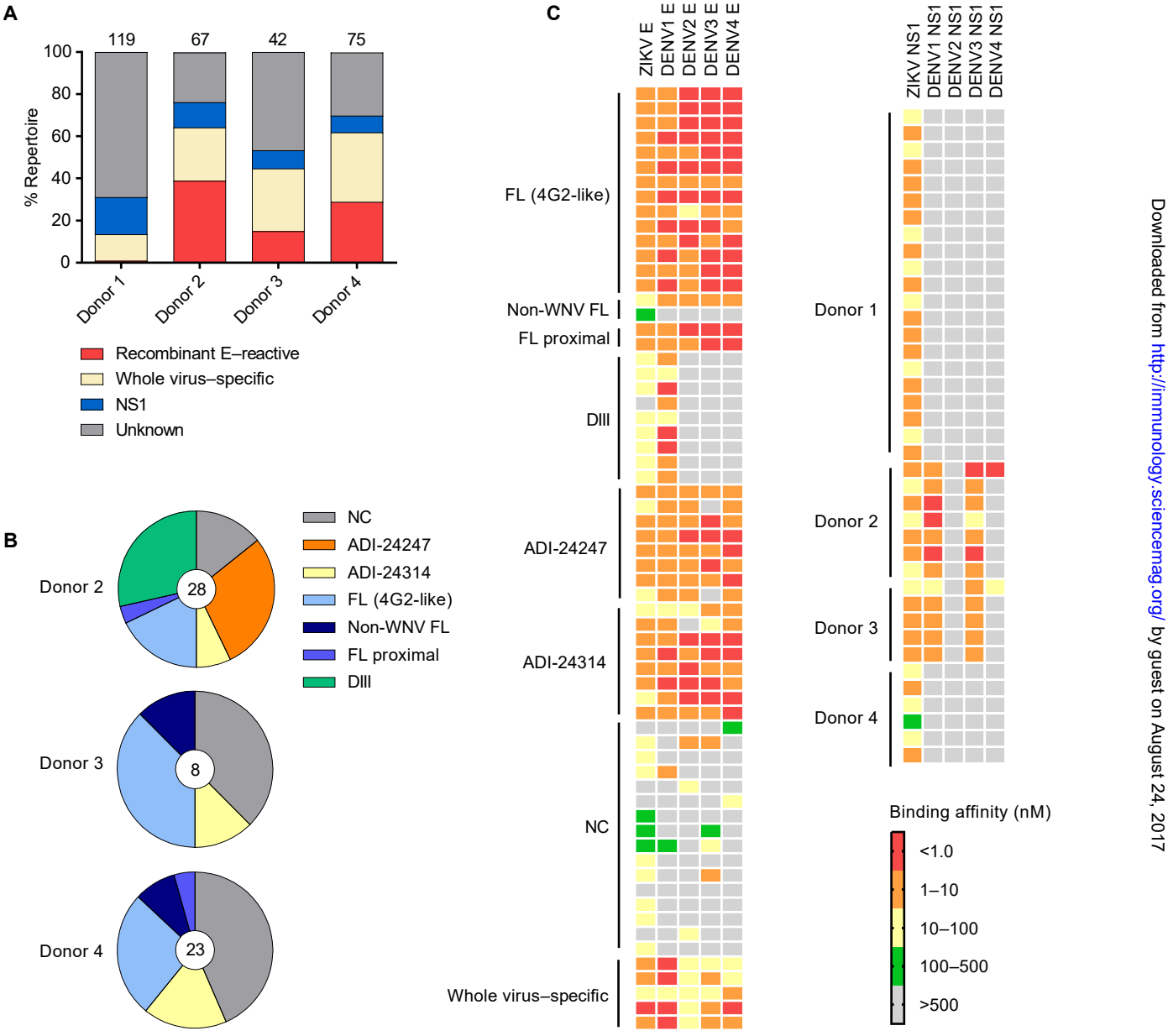


Fig. 2. Binding characteristics of mAbs isolated from plasmablasts during acute ZIKV infection. (**A**) Specificities of the mAbs isolated from one DENV naïve donor and three DENV-experienced donors. Distribution of mAbs that bind to NS1, recombinant E, epitopes expressed only on whole ZIKV, and those for which no specificity could be assigned are shown. The number at the top of each bar represents the total number of mAbs cloned from each donor. (**B**) Epitope mapping of recombinant E–specific antibodies isolated from three DENV-experienced donors. Pie charts show the distribution of antibodies that bind to epitopes within DIII, within or proximal to the FL on DII, and defined by mAbs ADI-24314 and ADI-24247. 4G2-like FL: Competition with 4G2, WNV E–reactive, and E-FL–sensitive; non-WNV FL: 4G2-compet E-FL–sensitive, and WNV E–nonreactive; FL proximal: 4G2 competitive, E-FL–reactive, and WNV E–nonreactive; DIII: Recombinant DIII binding; ADI-24314 competitor: 4G2-noncompetitive, ADI-24314–competitive, and E-FL–reactive; ADI-24247 competitor: 4G2-noncompetitive, ADI-24247–competitive, and E-FL–reactive. NC, not cha acterized because of low binding affinity. (**C**) Heat map of mAb binding reactivity to ZIKV and DENV1–4 E proteins or whole viral particles (left) and ZIKV and DENV1–4 NS1 proteins (right). The E-specific mAbs shown were isolated from DENV-experienced donors. Apparent binding affinities for recombinant E and NS1 proteins were de-termined by BLI measurements, and apparent binding affinities for whole virus were determined by ELISA.

Downloaded from http://immunology.sciencemag.org/ by guest on August 24, 2017

DIII, WNV E, and a previously described ZIKV E protein (E-FL) that contains substitutions within and proximal to the FL that abolish binding by most FL-specific antibodies (*30*) (fig. S5) and (ii) performed competitive binding experiments using two previously characterized mAbs, 4G2 and ZV-67, that bind to the FL on DII and the lateral ridge on DIII, respectively (table S2) (*31*, *32*). Because the FL-specific mAb 4G2 reacts with WNV E and fails to react with E-FL (table S3), we classified mAbs that competed with 4G2, bound to WNV E, and failed to bind to E-FL as "4G2-like FL binders." 4G2 competitor mAbs that failed to bind to E-FL but did not cross-react with WNV E were classified as "non-WNV FL binders," and 4G2 competitor mAbs that did not cross-react with WNV E but did bind to E-FL were classified as "FL proximal binders." Together, mAbs that bound within or proximal to the FL comprised up to 50% of the recombinant E–reactive responses (Fig. 2B and table S2). DIII-specific mAbs were absent from the donor 3 and donor 4 responses but comprised about 30% of the donor 2 response (Fig. 2B and table S2). Six of eight DIII-reactive mAbs competed with ZV-67, suggesting that they likely recognize epitopes within or proximal to the lateral ridge on DIII (table S2). This class of antibodies showed preferential VH3 germline gene usage and contained convergent sequence signatures in both CDRH3 and CDRL3 (table S4), suggesting that these mAbs may share a common mode of antigen recognition. To estimate the number of different antigenic sites recognized by the remaining E-specific mAbs, we performed competitive binding experiments with two broadly cross-reactive mAbs from the panel (ADI-24247 and ADI-24314) that did not compete with 4G2, ZV-67, or each other (tables S2 and S5). Between 12 and 35% of the E-specific donor responses bound to epitopes overlapping that of ADI-24247 or ADI-24314 (Fig. 2B). Overall, we conclude that substantial fractions of the acute ZIKV-induced B cell response in DENV-experienced donors are directed against quaternary epitopes expressed only on whole virus, and those that are reactive with recombinant E are largely directed to the FL and other highly conserved epitopes.

The ZIKV-induced plasmablast response in DENV-experienced donors is dominated by DENV cross-reactive clones

The E proteins of ZIKV and DENV share more than 50% sequence identity, resulting in substantial immunological cross-reactivity (*3*, *6*, *7*). To determine the degree to which mAbs generated from plasmablasts during acute ZIKV infection cross-reacted with DENV, we measured the apparent binding affinities of the mAbs for recombinant DENV1–4 E and NS1 proteins. Most of the E-specific mAbs from the three DENV-experienced donors bound with higher affinity to at least one of the four DENV E proteins than to ZIKV E, consistent with a recall response dominated by reactivated DENV-induced memory B cells (Fig. 2C, table S2, and fig. S6). Selected mAbs isolated from donors 2, 3, and 4 that targeted epitopes expressed only on whole ZIKV showed similar DENV-biased binding profiles (fig. S7). In contrast, the whole virus–specific mAbs isolated from donor 4 bound exclusively to ZIKV (fig. S7).

About 50% of the ZIKV E–reactive mAbs isolated from the DENV-experienced donors cross-reacted with all four DENV serotypes (Fig. 2C and table S2). Most of these broadly cross-reactive mAbs targeted epitopes within or proximal to the FL or unknown epitopes defined by ADI-24247 and ADI-24314 (Fig. 2C and table S2). In contrast, the DIII-specific mAbs only showed cross-reactivity with DENV1 E (Fig. 2C and table S2). One hundred percent of the NS1-specific mAbs isolated from donors 2 and 3 showed cross-reactivity with DENV1 and DENV3 NS1, whereas the NS1-specific mAbs from donor 4 were exclusively ZIKV-specific (Fig. 2C and table S6). Sequence analysis revealed that the ZIKV NS1-specific mAbs from donor 4 lacked SHM, supporting a naïve B cell origin (fig. S8). The reasons for this are unclear but may be due to the lack of preexisting cross-reactive NS1-specific memory B cells in this donor. As expected, all of the NS1-specific mAbs isolated from donor 1 were ZIKV-specific and lacked SHM (Fig. 2C). The affinities of these mAbs were comparable with the affinities of the NS1-specific mAbs isolated from the DENV-experienced donors (Fig. 2C and table S6), which is perhaps because the plasmablast-derived antibodies from the DENV-experienced donors had not undergone affinity maturation toward ZIKV. Together, the sequencing and binding results provide evidence for OAS during the acute-phase B cell response to ZIKV in DENV-experienced donors.

Most of the plasmablast-derived mAbs isolated from DENV-experienced donors are poorly neutralizing and potently enhancing

To assess the functional properties of the plasmablast-derived mAbs isolated from the DENV-experienced donors, we next performed neutralization and ADE assays. Because of the large number of mAbs, initial neutralization screening was performed using a single concentration of purified IgG. At a concentration of 1 mg/ml, about 20% of mAbs from each donor reduced ZIKV-Paraiba (Para) infectivity by ≥50%, and all of these nAbs cross-neutralized at least one of the four DENV serotypes (Fig. 3A). Additionally, the majority of mAbs (53 of 63 or 84%) showed more potent neutralizing activity against one of the four DENV serotypes than ZIKV-Para (Fig. 3A). We next performed neutralization titration experiments on a subset of mAbs to evaluate neutralization potency. Consistent with the binding and single-point neutralization results, a large proportion of mAbs showed preferential neutralization of DENV, further supporting a DENV-induced memory B cell origin for these recently activated cells (Fig. 3B, table S7, and fig. S9). Of significance, most of the plasmablast-derived mAbs showed poorly neutralizing activity against ZIKV-Para (Fig. 3, B and C). The notable exceptions were the four DIII-specific mAbs, which showed highly potent neutralizing activity against both ZIKV-Para and DENV1 (Fig. 3, B and C, and table S7). These nAbs displayed IC_{50} (half-maximal inhibitory concentrations) between 0.9 and 2.7 ng/ml against ZIKV-Para and between 0.2 and 27 ng/ml against DENV1 (Fig. 3, B and C, and table S7). Most of the mAbs that bound to quaternary epitopes on the E protein showed little to no neutralizing activity against ZIKV. Previous studies have described two classes of quaternary mAbs (EDE1 and EDE2), which are defined on the basis of differing sensitivity to removal of the N-glycan at Asp 153 (*4*). Given that mAbs directed against EDE2 have been shown to be substantially less potent against ZIKV than mAbs targeting EDE1, it is likely that the quaternary mAbs described here recognize the EDE2 epitope.

We next tested the abilities of selected mAbs to enhance ZIKV infection using nonpermissive K562 cells. Consistent with previous studies, all of the mAbs enhanced ZIKV infection, but peak enhancement was observed at lower concentrations for the potently neutralizing DIII-specific mAbs compared with the poorly neutralizing cross-reactive mAbs (fig. S10) (*16*, *32*). Additionally, there was a linear correlation between neutralization potency and the sum of the enhancement activity over the range of mAb concentrations tested,

Downloaded from http://immunology.sciencemag.org/ on August 24, 2017

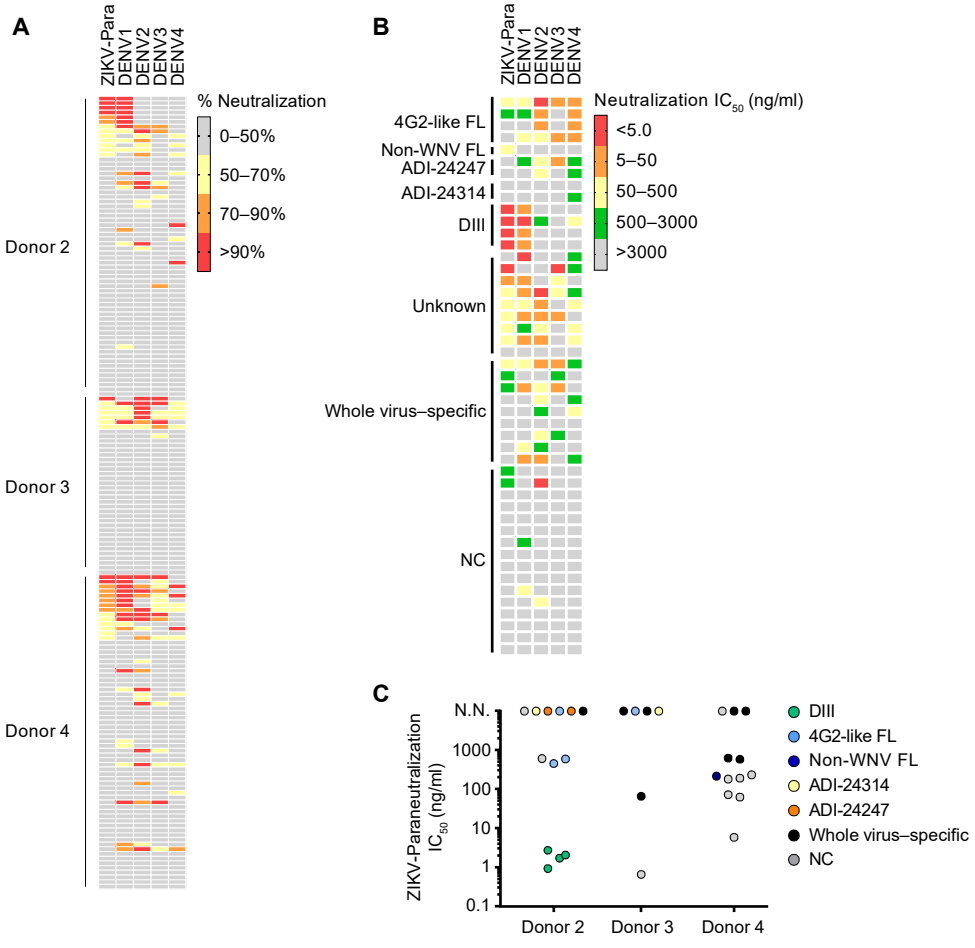


Fig. 3. Neutralizing activity of mAbs isolated from plasmablasts during acute ZIKV infection in DENV-experienced donors. (**A**) Heat map showing mAb neutralization of ZIKV-Para, DENV1, DENV2, DENV3, and DENV4 at a concentration of 1.0 mg/ml. (**B**) Heat map showing neutralization IC_{50} for selected mAbs against ZIKV-Para, DENV1, DENV2, DENV3, and DENV4. Epitope assignments are indicated on the left. (**C**) Neutralization IC_{50} for selected mAbs against ZIKV-Para. IC_{50} values represent the concentration of IgG required to reduce viral infectivity by 50%. Neutralization assays were performed using a live virus plaque reduction assay. N.N., non-neutralizing.

as defined by the area under the curve (fig. S10). Together, the results show that the acute B cell response to ZIKV in DENV-immune donors is dominated by cross-reactive antibodies that show poorly neutralizing activity and a propensity to enhance ZIKV infection in vitro.

Isolation of E-specific mAbs from longitudinal samples obtained from the DENV-experienced donors

To track the evolution of the B cell response to ZIKV in DENV-experienced donors, we collected blood samples from donors 2, 3, and 4 about 5 months after infection (table S1). To assess the magnitude of the E-specific memory B cell response at this time point, we stained peripheral B cells with a fluorescently labeled ZIKV-E protein and analyzed them by flow cytometry (Fig. 4A and fig. S11). Robust E-specific memory B cell responses were observed in donors 3 and 4, whereas E-specific memory B cells were undetectable in donor 2 (Fig. 4A). The reasons for this are unclear, but given that donor 2 showed the highest frequency of plasmablasts during acute infection, one explanation is that the virus was rapidly cleared by the plasmablast-derived antibodies, which prevented the formation of a germinal center (GC) response. Four hundred ZIKV E–specific memory B cells were single cell–sorted from donors 3 and 4, and about 200 V_H and V_L pairs were cloned and expressed as full-length IgGs for further characterization. To assess the extent of affinity maturation that occurred over this time period, the apparent binding affinities of the E-specific memory B cell–derived mAbs were measured and compared with the affinities of the E-specific plasmablast-derived mAbs. As expected, the average apparent affinity for ZIKV E was significantly higher in the memory B cell subset, suggesting that the ZIKV-induced B cells underwent affinity maturation over time (Fig. 4B). Clonal expansions were observed in both donor response repertoires (Fig. 4C), but there were few to no clonal lineages shared between the plasmablast and memory B cell–derived subsets (table S9 and fig. S12). Although the sampling size is too small to determine the degree of overlap between the two B cell populations, these results suggest that the most dominant clonal lineages likely differ between the two compartments.

ZIKV infection induces de novo and cross-reactive memory B cell responses in DENV-experienced donors

We next assessed the degree of cross-reactivity with the E proteins from DENV1–4. Unexpectedly, 60 and 30% of memory B cell–derived mAbs from donors 3 and 4, respectively, bound exclusively to ZIKV E, suggesting that these antibodies either arose from preexisting memory B cells that uniformly lost DENV reactivity during the process of affinity maturation or originated from activated naïve B cells that had entered GCs and undergone affinity maturation (Fig. 4D and table S8). In support of the latter hypothesis, sequence analysis revealed no overall increase in SHM in the ZIKV-specific memory B cell–derived mAbs compared with the plasmablast-derived mAbs (Fig. 4E). Epitope mapping experiments showed that about 10 to 20% of the ZIKV-specific memory B cell–derived mAbs bound to epitopes within DIII, 0 to 15% bound to epitopes within or proximal to the FL, and 0 to 2% bound to epitopes overlapping that of ADI-24314 (fig. S13 and table S8). To estimate the number of different antigenic sites recognized by the remaining ZIKV-specific mAbs, we performed competitive binding assays with two mAbs from the panel (ADI-30056 and ADI-30023) that did not compete with ZV-67, 4G2, ADI-24314, ADI-24247, or each other (table S8). On the basis of these results, we found that 50 to 60% of the ZIKV-specific mAbs targeted unknown sites defined by ADI-30056 or ADI-30023 and 10 to

Downloaded from http://immunology.sciencemag.org/ by guest on August 24, 2017

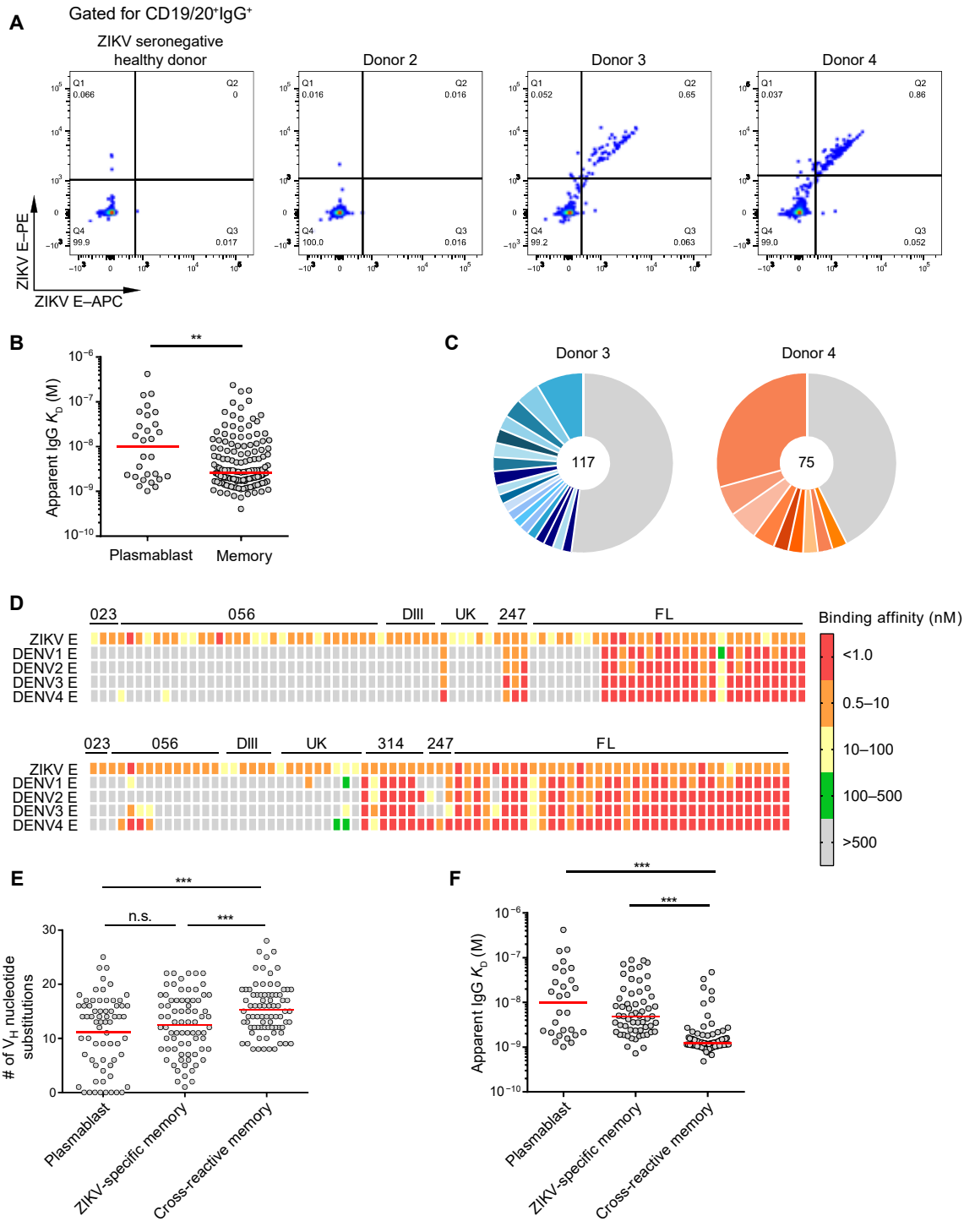


Fig. 4. Longitudinal analysis of ZIKV E–specific memory B cell responses in the DENV-experienced donors. (**A**) ZIKV E–specific memory B cell sorting. Fluorescence-activated cell sorting (FACS) plots show ZIKV E reactivity of IgG$^+$ B cells from the three convalescent DENV-experienced donors and a ZIKV seronegative healthy donor control. B cells in quadrant 2 (Q2) were single cell–sorted for mAb cloning. ZIKV E was labeled with two different colors to reduce background binding. (**B**) Apparent binding affinities of plasmablast and memory B cell–derived mAbs to recombinant ZIKV E. Red bars indicate the median IC_{50}s. (**C**) Clonal lineage analysis of memory B cell–derived mAbs. Heavy-chain sequences were assigned to lineages using Clonify (*52*). Each lineage is represented as a segment proportional to the lineage size. The total number of recovered heavy chains is shown in the center of each pie. All lineages that contain only a single sequence are combined and shown as a single gray segment. (**D**) Heat map showing apparent binding affinities of the memory B cell–derived mAbs to recombinant ZIKV E and DENV1–4 E proteins. Top: mAbs cloned from donor 3. Bottom: mAbs cloned from donor 4. 023, ADI-30023 competitor; 056, ADI-30056 competitor; 314, ADI-24314 competitor; 247, ADI-24247 competitor; FL, 4G2 competitor; DIII, recombinant DIII binder; UK, unknown. (**E**) Load of somatic mutations (expressed as the number of nucleotide substitutions in the V_H) in mAbs isolated from plasmablasts and memory B cells in DENV-experienced donors. Each point represents an individual mAb. Red bars indicate the median number of nucleotide substitutions. (**F**) Binding affinities of plasmablast and memory B cell–derived mAbs to recombinant ZIKV E. Apparent binding affinities are shown in the plot. Red bars indicate the apparent IC_{50}s. Statistical comparisons were made using Mann-Whitney test (***$P < 0.001$, **$P < 0.01$; n.s., not significant).

30% of mAbs did not compete with any of the control mAbs (fig. S13 and table S8).

The majority (84%) of DENV cross-reactive mAbs showed reactivity with all four DENV serotypes (Fig. 4D and table S8). Most of these broadly cross-reactive mAbs showed DENV-biased binding profiles and overall higher levels of SHM compared with the ZIKV-specific mAbs, suggesting that they likely arose from preexisting DENV-induced memory B cells that formed secondary GCs (Fig.

Downloaded from http://immunology.sciencemag.org/ by guest on August 24, 2017

D and E, and table S8). The latter result also provides additional evidence that the ZIKV-specific mAbs originated from naïve B cells that underwent affinity maturation rather than from preexisting memory B cells that lost DENV cross-reactivity. In addition, the average apparent affinity of the cross-reactive mAbs was significantly higher than those of the plasmablast-derived mAbs and the ZIKV-specific memory B cell–derived mAbs, providing further evidence for GC reentry by memory B cells (Fig. 4F). This result is consistent with recent studies in mice demonstrating that memory B cells can rediversify their B cell receptors within secondary GCs (*33*–*35*). As anticipated based on their broad cross-reactivity, most of these mAbs targeted epitopes within or proximal to the FL on DII (Fig. 4D and table S8).

We next tested the memory B cell–derived mAbs for neutralizing activity. Only a small minority of the cross-reactive mAbs (4 of 80 or 5%) showed neutralizing activity against ZIKV-Para at a concentration of 1 μg/ml, whereas the overwhelming majority (52 of 73 or 71%) of the ZIKV-specific mAbs showed such neutralizing activity (Fig. 5A and table S8). Neutralization titration experiments revealed that most potent ZIKV-specific mAbs displayed IC_{50} between 1.0 and 10 ng/ml and targeted epitopes overlapping that of ADI-30056 (Fig. 5B and table S10). About 90% of the cross-reactive mAbs tested showed weak potent neutralizing activity against at least one of the four DENV serotypes than ZIKV-Para, further suggesting that these mAbs originated from preexisting memory B cells that reentered GCs (Fig. 5B and table S10). We conclude that the ZIKV-induced memory B cell response is composed of both OAS-phenotype antibodies that display broad cross-reactivity and poorly neutralizing activity, and de novo generated ZIKV-specific antibodies that show potent but type-specific neutralizing activity.

DISCUSSION

Here, we have used single B cell cloning and large-scale antibody characterization to track the evolution of ZIKV-induced B cell responses in three DENV-experienced donors. In all three donors, massive plasmablast populations were observed during acute ZIKV infection, which were similar in magnitude to the plasmablast responses previously observed in individuals experiencing secondary DENV infection (*24*). The plasmablast population in a ZIKV-infected, DENV-naïve donor was relatively small, which could be due to multiple factors, such as differences in age, viral load, or immune history. However, the immune history explanation is consistent with previous studies that have shown that peripheral blood plasmablast responses during primary infection are delayed and substantially lower in magnitude than during secondary infection (*36*, *37*). The plasmablast responses in the three DENV-experienced donors were clonally expanded and showed relatively high levels of SHM—consistent with a recall response dominated by reactivated memory B cells—whereas the plasmablasts from the ZIKV-infected, DENV-naïve donor showed substantially lower levels of SHM and somewhat more limited clonal expansion, suggesting emergence via activation of naïve B cells. Over half of the plasmablast-derived mAbs isolated from the DENV-experienced donors showed reactivity with whole ZIKV particles or recombinant ZIKV NS1, whereas a substantially smaller fraction of the plasmablast-derived mAbs from the DENV-naïve donor showed such binding. This result could be either due to the low binding affinity of mAbs isolated from activated naïve B cells or that the plasmablast response in this donor was largely directed against viral antigens that were not tested in this study.

Binding and functional characterization of the plasmablast-derived mAbs from donors 2, 3, and 4 revealed that the acute B cell response to ZIKV is dominated by reactivated DENV clones, thus providing evidence of OAS during the acute-phase B cell response to ZIKV in DENV-experienced donors. Most of these antibodies, although broadly cross-reactive, displayed poorly neutralizing activity and enhanced ZIKV infection in vitro, suggesting that preexisting immunity to DENV virus may negatively affect early protective B cell responses to ZIKV. None of the mAbs isolated from the ZIKV-infected DENV-naïve donor showed cross-reactivity with any of the four serotypes of DENV, suggesting that highly conserved epitopes are not immunodominant in the context of primary ZIKV infection. Although ADE has been proposed to be primarily driven by

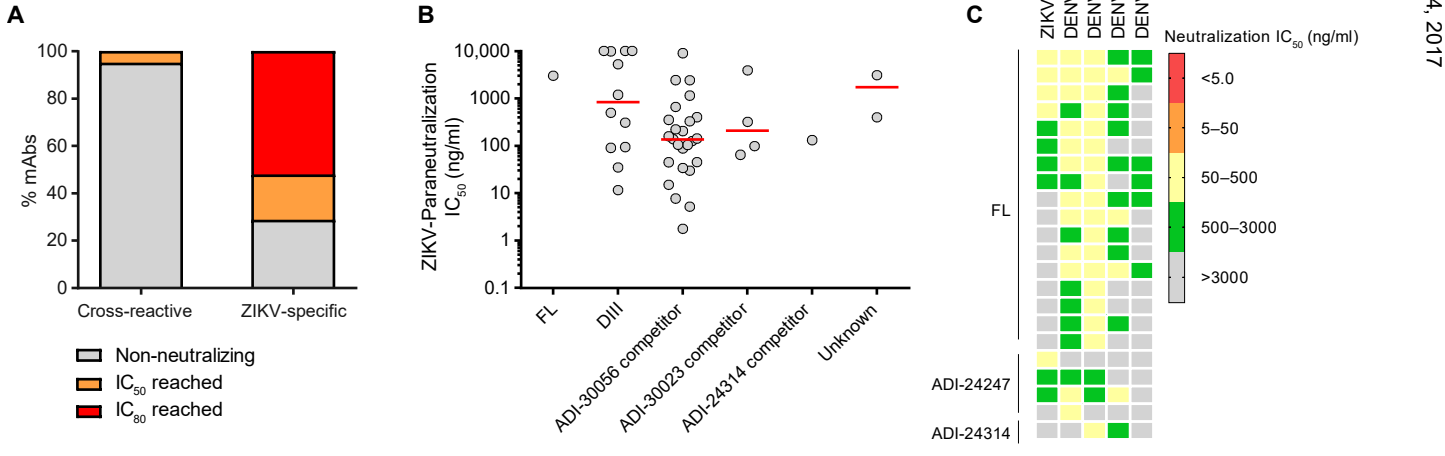


Fig. 5. Neutralizing activity of memory B cell–derived mAbs. (**A**) Percentage of cross-reactive and ZIKV-specific mAbs that reached neutralization IC_{50} or IC_{80} at a concentration of 1.0 μg/ml. (**B**) Neutralization IC_{50} values of selected mAbs from each competition group. IC_{50} values represent the concentration of IgG required to reduce viral infectivity by 50%. Red bars indicate median IC_{50}. (**C**) Heat map showing neutralization IC_{50} for selected cross-reactive mAbs against ZIKV-Para, DENV1, DENV2, DENV3, and DENV4. Neutralization assays were performed using a live virus plaque reduction assay.

Downloaded from http://immunology.sciencemag.org/ by guest on August 24, 2017

preexisting serum antibody, plasmablasts secrete substantial amounts of antibody during acute infection. It is therefore possible that boosting of poorly neutralizing responses during the early stages of infection could contribute to ADE. However, it is important to emphasize that although animal studies have suggested a role for ADE in vivo (*14*), the relevance of ADE in determining the severity and/or higher risk for ZIKV infection remains yet to be elucidated in humans.

Although most of the plasmablast-derived mAbs isolated from the three DENV-experienced donors were poorly neutralizing, a subset of mAbs from donor 2 showed highly potent neutralizing activity against both ZIKV and DENV1. These mAbs bound to epitopes within the lateral ridge of DIII and showed convergent sequence signatures in both the heavy and light chains. Similar antibodies were recently isolated from several DENV-immune donors that were selected on the basis of high ZIKV neutralizing titers (*38*). The identification of recurrent potent nAbs in multiple different donors suggests that this antigenic region may be a particularly attractive target for vaccines intended for DENV-immune populations. Despite the identification of potent nAbs from the donor 2 plasmablast population, the acute serum from this donor showed weaker neutralizing activity than the acute sera from donors 3 and 4. One possible explanation for this result is that the donor 3 and 4 sera contain overall higher titers of anti-ZIKV antibodies compared with the donor 2 serum. In support of this hypothesis, the donor 2 acute serum showed substantially weaker binding to whole DENV and ZIKV particles compared with the acute serum from donors 3 and 4.

The phenomenon of OAS has been documented in the context of repeated influenza virus exposure as well as during secondary DENV infections (*17*, *39*–*43*), and these studies have suggested that memory responses to the primary infecting strain may prevent the induction of effective de novo responses. Our results show that the ZIKV-induced memory B cell response in DENV-experienced donors is composed of both poorly neutralizing OAS-phenotype antibodies—which appear to have originated from preexisting DENV-induced memory B cells that reentered GCs—and potently neutralizing ZIKV-specific antibodies that likely originated from naïve B cells that entered primary GCs. Hence, our results suggest that effective de novo memory B cell responses to ZIKV can be induced in the presence of preexisting cross-reactive DENV clones. However, only about 50% of the mAbs isolated from E-specific memory B cells were ZIKV-specific, the remaining mAbs were broadly cross-reactive, poorly neutralizing, and potently enhancing. The relative contribution of these two different classes of antibodies to protection, virulence, and immunopathology of ZIKV infection remains to be elucidated.

One limitation of our study is the small number of donors studied. As a result, intradonor comparisons can be made with high certainty, but interdonor comparisons are less robust. However, we found evidence of OAS in all three DENV-experienced donors, suggesting that this phenomenon likely occurs in most ZIKV-infected donors that have been previously exposed to DENV. Another limitation of our study was the use of recombinant ZIKV E for memory B cell sorting, which prevented the isolation of memory B cell–derived mAbs that recognize quaternary epitopes. Last, because we were not able to obtain detailed medical histories for these donors, it was not possible to determine the number and frequency of previous DENV infections and the number of DENV serotypes to which they had been exposed. However, the serological data strongly suggest that donors 2, 3, and 4 had experienced at least one DENV infection before ZIKV infection, which we believe is sufficient for the interpretation of the results of this study.

Although our study was performed using naturally infected donors, vaccines expressing full-length ZIKV E—which include multiple recently described plasmid DNA, purified inactivated, protein subunit, adenovirus vector, and mRNA vaccines (*44*–*49*)—would be expected to induce similar responses in DENV-experienced individuals. Such vaccines, which will need to be deployed in areas that are highly endemic for DENV, will likely induce a substantial OAS response in these individuals. Given this possibility, the clinical development of ZIKV vaccines should also consider the recruitment and immunization of DENV-immune donors. Ongoing phase 1 studies on ZIKV vaccine candidates are currently being performed in individuals living in areas that are not endemic for DENV, and therefore, most of these individuals are likely DENV-naïve. Together, the results of this study provide rationale for the design of vaccines specifically focused on the DIII lateral ridge or other ZIKV-specific neutralizing epitopes for use in DENV-immune populations.

MATERIALS AND METHODS

Study design

We initiated this study to gain an in-depth understanding of the antibody response to ZIKV in DENV-experienced donors. To study these responses, we obtained peripheral blood mononuclear cells (PBMCs) from three ZIKV-infected donors that had serological evidence or previous DENV exposure and one ZIKV-infected naïve donor for comparison. Four donors were included in this study because this was the largest number of donors for which large numbers of antibodies could be practically cloned and characterized. At least two independent experiments were performed for affinity measurements, neutralization assays, enhancement assays, and antibody competition assays, and the results shown are derived from single representative experiments. All samples for this study were collected with informed consent of volunteers. This study was unblinded and not randomized.

PBMC and plasma isolation

Blood and serum samples were obtained through Antibody Systems Inc. under the Scripps Research Institute IRB-15-6683. PBMCs were isolated as previously described (*50*). Briefly, whole blood was diluted 1:2 with phosphate-buffered saline (PBS) and underwent ultracentrifugation over layered Lymphoprep (STEMCELL Technologies). The PBMC monolayer was collected, resuspended, and stored in liquid nitrogen.

Generation of ZIKV and DENV viruses

Vero cells were cultured in minimal essential medium (MEM) (Corning Cellgro) containing 5% heat-inactivated fetal bovine serum (Gibco-Invitrogen). Viruses were supplied by D. Watkins and included Zika-Paraiba 2015, DENV1 Western Pacific U88535.1, DENV2 NGC AF038403.1, DENV3 Selman/78 AY648961, and DENV4 AF326825.1. Vero cells were infected with each virus and maintained until 50% cytopathic reduction in viability was observed, after which supernatant was collected, filtered, and stored at −80°C.

Antibody sequencing

Reverse transcription and subsequent PCR amplification of IgV$_H$ and IgV$_L$ genes were performed using SuperScript III (Life Technologies) according to published protocols (*51*). All PCRs were performed in

Downloaded from http://immunology.sciencemag.org/ by guest on August 24, 2017

25-ml volume with 2.5 ml of complementary DNA (cDNA) transcript using HotStarTaq DNA polymerase master mix (Qiagen) and mixtures of previously described primers (*51*). Second-round nested PCRs were performed using Phusion proofreading polymerase (New England Biolabs). Two additional rounds of PCR were performed using primers with bar codes specific to the plate number and well location as well as adapters appropriate for sequencing on an Illumina MiSeq. This reaction was performed in a 25-ml volume with HotStarTaq DNA polymerase master mix (Qiagen). Amplified IgG V_H and V_L genes were sequenced on an Illumina MiSeq (600-base v3 reagent kit; Illumina), and reads corresponding to the same plate per well location were combined into consensus sequences. Germline assignment was performed with AbStar (https://github.com/briney/abstar), and clonal lineages were assigned with Clonify (*52*).

Western blots

Whole-virus ZIKV lysate, whole-virus DENV lysate, and recombinant E protein (catalog no. R01635, Meridian Life Science) were separated by SDS–polyacrylamide gel electrophoresis under reducing conditions in 4 to 20% gels (Bio-Rad) to polyvinylidene difluoride membranes (Thermo Fisher Scientific). Membranes were blocked overnight at 4°C in 5% nonfat dry milk in tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T). Subsequently, membranes were incubated with 1:2000 dilution of mAb in 10 ml of TBS-T containing 5% milk for 1 hour and washed three times with TBS-T. The secondary antibody used to detect 4G2 was a horseradish peroxidase (HRP)–conjugated goat polyclonal secondary antibody to mouse IgG (Abcam), and the secondary antibody used to detect human mAbs was an HRP-conjugated goat polyclonal secondary antibody to human IgG (Abcam). Secondary antibodies were diluted 1:2000 in 10 ml of TBS-T with 5% milk and applied to the membrane for 1 hour, and then the membrane was washed three times with TBS-T and developed with HRP development solution (Bio-Rad).

Focus reduction neutralization test

Virus-specific nAb responses were titrated essentially as previously described (*53*). Briefly, antibody was diluted serially in MEM (Corning Cellgro) containing 5% heat-inactivated fetal bovine serum (Gibco-Invitrogen) and incubated for 1 hour at 37°C with virus. The initial dilution tested for all mAbs was 10 mg/ml, and threefold dilutions were performed. After incubation, the antibody-virus mixture was added in triplicate to 96-well plates containing 80% confluent monolayers of Vero E6 cells. Plates were incubated for 1.5 hours at 37°C. After the incubation, wells were overlaid with 1% methylcellulose in supplemented MEM with 2% heat-inactivated fetal bovine serum (Gibco-Invitrogen) and 1:100 Hepes. Plates were incubated at 37°C and 5% CO_2 for 40 hours, after which cells were fixed and permeabilized with Perm/Wash buffer (BD Biosciences) for 5 min. After permeabilization, cells were incubated with 1:2000 dilution of anti-flavivirus antibody (MAB10216) for 2 hours and then washed with PBS (EMD Millipore). After washing, the cells were incubated with anti-mouse HRP-conjugated secondary antibody (Sigma) in Perm/Wash buffer for 2 hours. After washing of cells with PBS, plates were developed with peroxidase substrate (KPL). All viruses were titered to yield 100 plaques per well in the absence of neutralization and fixed at 40 hours to prevent plaque spread or overlap. Experiments were performed in triplicate. Neutralization IC_{50}s were determined using GraphPad Prism software. One site - Fit IC_{50} was used with the following model: $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{X(-\text{Log}IC_{50})})$. The top and bottom of the curves were constrained to values of 100 and 0. The IC_{50} was then defined from the best-fit curve for each antibody as defined by the concentration that resulted in 50% reduction in foci number.

Serum ELISAs

For NS1 ELISAs, plates were coated with NS1 protein (5 mg/ml) diluted in PBS and incubated overnight at 4°C. Wells were washed and then blocked with 3% bovine serum albumin (BSA) in PBS for 1 hour at 37°C. Wells were washed, and serial dilutions of human plasma diluted in 1% BSA and 0.05% Tween 20 were added and incubated for 1 hour at 37°C. Wells were washed, and alkaline phosphatase (AP)–conjugated goat anti-human IgG (Jackson ImmunoResearch) secondary antibody was added and incubated for 1 hour at 37°C. Wells were washed twice and developed with *para*-nitrophenylphosphate in 1× diethanolamine. Plates were read on a SpectraMax microplate reader (Molecular Devices) at 405-nm wavelength. For virus binding, ELISA plates were coated with 4G2 (2 mg/ml) (Millipore, MAB10216) diluted in PBS and incubated overnight at 4°C. After washing, wells were blocked with 3% BSA in PBS for 1 hour at 37°C. After removal of the blocking solution, whole ZIKV or DENV particles diluted in 1% BSA and 0.05% Tween 20 were applied to the plates and incubated at 37°C for 1 hour. After washing, serum was titrated and incubated at 37°C for 1 hour. The wells were washed, and AP-conjugated goat anti-human IgG (Jackson ImmunoResearch) secondary antibody was added and incubated for 1 hour at 37°C. Wells were washed twice and developed with *para*-nitrophenylphosphate in 1× diethanolamine. Plates were read on a SpectraMax microplate reader (Molecular Devices) at 405-nm wavelength.

mAb binding ELISAs

ELISA plates were coated with 4G2 (2 mg/ml) (Millipore MAB10216) diluted in PBS and incubated overnight at 4°C. After washing, wells were blocked with 3% BSA in PBS for 1 hour at 37°C. After removal of the blocking solution, 10^4 plaque-forming units of unpurified ZIKV or DENV particles diluted in 1% BSA and 0.05% Tween 20 was added to each well of the ELISA plate and incubated at 37°C for 1 hour. After washing, mAbs were either titrated or added at a concentration of 10 mg/ml and incubated at 37°C for 1 hour. Wells were washed, and AP-conjugated goat anti-human IgG (Jackson ImmunoResearch) secondary antibody was added and incubated for 1 hour at 37°C. Wells were washed twice and developed with *para*-nitrophenylphosphate in 1× diethanolamine. Plates were read on a SpectraMax microplate reader (Molecular Devices) at 405-nm wavelength.

Single B cell sorting

For plasmablast sorting, PBMCs were stained using anti-human CD38 (fluorescein isothiocyanate), CD27 [phycoerythrin (PE)–Cy7], CD20 (BUV737), CD3 [allophycocyanin (APC)–Cy7], CD8 (APC-Cy7), and CD14 (APC-Cy7). Plasmablasts were defined as $CD3^-CD8^-CD20^{-/lo}CD27^{+}CD38^{hi}$. For memory B cell sorting, purified B cells were stained using anti-human IgG [Brilliant Violet (BV) 605], CD27 (BV421), CD8 [peridinin chlorophyll protein (PerCP)–Cy5.5], CD14 (PerCP-Cy5.5), CD19 (PE-Cy7), CD20 (PE-Cy7), and a mixture of dual-labeled ZIKV E tetramers (50 nM each). Single cells were sorted on a BD FACSAria II into 96-well PCR plates (Bio-Rad) containing 20 ml of lysis buffer per well [5 ml of 5× first-strand cDNA buffer (Invitrogen), 0.25 ml of RNaseOUT (Invitrogen), 1.25 ml of dithiothreitol (Invitrogen), 0.625 ml of NP-40 (New England Biolabs), and 12.5 ml of deionized H_2O].

Downloaded from http://immunology.sciencemag.org/ by guest on August 24, 2017

Plates were immediately frozen on dry ice before storage at −80°C. Flow cytometry data were analyzed using FlowJo software.

Amplification and cloning of antibody variable genes

Single B cell PCR was performed essentially as previously described (*51*). Briefly, IgH, Igλ, and Igκ variable gene transcripts were amplified by reverse transcription PCR and nested PCRs using cocktails of primers specific for IgG (*51*). The primers used in the second round PCR contained 40 base pairs' and 5′ homology to the cut expression vectors to allow for cloning by homologous recombination into *Saccharomyces cerevisiae* (*54*). PCR products were cloned into *S. cerevisiae* using the lithium acetate method for chemical transformation (*55*). Each transformation reaction contained 20 μl of unpurified heavy-chain and light-chain PCR product and 200 ng of cut heavy-chain and light-chain plasmids. Individual yeast colonies were picked for sequencing and downstream characterization.

Expression and purification of antibodies

Antibodies used for binding experiments, competition assays, neutralization assays, and structural studies were expressed in *S. cerevisiae* cultures grown in 24-well plates (*54*). After 6 days of growth, the yeast cell culture supernatant was harvested by centrifugation and subject to purification. Cell supernatants were purified by passing over Protein A agarose (MabSelect SuRe from GE Healthcare Life Sciences). The bound antibodies were washed with PBS, eluted with 200 mM acetic acid/50 mM NaCl (pH 3.5) into one-eighth volume of 2 M Hepes (pH 8.0), and buffer-exchanged into PBS (pH 7.0). Fab fragments were generated by digesting the IgGs with papain for 2 hours at 30°C. The digestion was terminated by the addition of iodoacetamide, and the Fab and Fc mixtures were passed over Protein A agarose to remove Fc fragments and undigested IgG. The flowthrough of the Protein A resin was then passed over CaptureSelect IgG-CH1 affinity resin (ThermoFisher Scientific) and eluted with 200 mM acetic acid/50 mM NaCl (pH 3.5) into one-eighth volume of 2 M Hepes (pH 8.0). Fab fragments then were buffer-exchanged into PBS (pH 7.0).

BLI binding analysis

IgG binding affinities were determined by BLI measurements using a FortéBio Octet HTX instrument (Pall Life Sciences). For high-throughput binding affinity screening, IgGs were immobilized on AHQ sensors (Pall Life Sciences) and exposed to 100 nM ZIKV E (Meridian Life Science Inc.), DENV1–4 E (Native Antigen Company), or NS1 proteins (Native Antigen Company) in PBS containing 0.1% BSA (PBSF) for an association step, followed by a dissociation step in PBSF buffer. Data were analyzed using the FortéBio Data Analysis Software 7. The data were fit to a 1:1 binding model to calculate an association and dissociation rate, and binding K_Ds (equilibrium dissociation constant) were calculated using the ratio k_d (dissociation constant)/k_a (association constant).

Antibody competition assays

Antibody competition assays were performed as previously described (*54*). Antibody competition was measured by the ability of a control anti-ZIKV E Fab to inhibit binding of yeast surface–expressed anti-ZIKV E IgGs to ZIKV E (Meridian Life Science Inc.). Biotinylated ZIKV E (50 nM) was preincubated with 1 μM competitor Fab for 30 min at room temperature and then added to a suspension of yeast-expressed anti-ZIKV E IgG. After 5-min incubation, unbound antigen was removed by washing with PBSF. After washing, bound antigen was detected using streptavidin–Alexa Fluor 633 (Life Technologies) at a 1:500 dilution and analyzed by flow cytometry using a BD-FACS Canto II. Results are expressed as the fold reduction in antigen binding in the presence of competitor Fab relative to an antigen-only control.

Statistical analyses

All statistical analyses were performed using GraphPad Prism. Statistical significance was determined using an unpaired nonparametric Mann-Whitney test or Kruskal-Wallis test. *P* values less than 0.05 were considered significant.

SUPPLEMENTARY MATERIALS

immunology.sciencemag.org/cgi/content/full/2/14/eaan6809/DC1

Fig. S1. Binding of acute serum to ZIKV and DENV1–4 viral particles and NS1 proteins.
Fig. S2. Plasmablast sorting.
Fig. S3. Binding of plasmablast-derived mAbs to ZIKV-Para by ELISA.
Fig. S4. Western blot analysis of mAb binding to ZIKV lysate.
Fig. S5. Heat map of mAb binding reactivity to recombinant ZIKV E, WNV E, DIII, and E-FL.
Fig. S6. mAb binding to recombinant ZIKV and DENV E proteins.
Fig. S7. ELISA binding of whole virus–specific clones to ZIKV and DENV.
Fig. S8. SHM of NS1-specific mAbs.
Fig. S9. Representative neutralization curves.
Fig. S10. Enhancement of ZIKV infection by plasmablast-derived mAbs isolated from DENV-experienced donors.
Fig. S11. Memory B cell sorting.
Fig. S12. Distribution of clonal lineage members across plasmablast and memory B cell subsets.
Fig. S13. Epitope mapping of ZIKV-specific mAbs derived from memory B cells.
Table S1. Clinical characteristics of ZIKV-infected donors.
Table S2. Binding properties of ZIKV E–specific mAbs isolated from plasmablasts.
Table S3. Binding characteristics of mAb 4G2.
Table S4. Sequences of ZV-67 competitor mAbs.
Table S5. Competition of ADI-24247 and ADI-24314 with each other and with control mAbs.
Table S6. Binding properties of NS1-specific mAbs isolated from plasmablasts.
Table S7. Neutralizing activity of selected plasmablast-derived mAbs.
Table S8. Binding properties of ZIKV E–specific mAbs isolated from memory B cells.
Table S9. Clonal lineages shared between plasmablast and memory B cell–derived antibodies.
Table S10. Neutralizing activity of selected memory B cell–derived mAbs.

REFERENCES AND NOTES

1. J. Mlakar, M. Korva, N. Tul, M. Popović, M. Poljšak-Prijatelj, J. Mraz, M. Kolenc, K. Resman Rus, T. Vesnaver Vipotnik, V. Fabjan Vodušek, A. Vizjak, J. Pižem, M. Petrovec, T. Avšič Županc, Zika virus associated with microcephaly. *N. Engl. J. Med.* **374**, 951–958 (2016).
2. V.-M. Cao-Lormeau, A. Blake, S. Mons, S. Lastère, C. Roche, J. Vanhomwegen, T. Dub, L. Baudouin, A. Teissier, P. Larre, A.-L. Vial, C. Decam, V. Choumet, S. K. Halstead, J. J. Willison, L. Musset, J.-C. Manuguerra, P. Despres, E. Fournier, H.-P. Mallet, D. Musso, A. Fontanet, J. Neil, F. Ghawché, Guillain-Barré Syndrome outbreak associated with Zika virus infection in French Polynesia: A case-control study. *Lancet* **387**, 1531–1539 (2016).
3. D. Sirohi, Z. Chen, L. Sun, T. Klose, T. C. Pierson, M. G. Rossmann, R. J. Kuhn, The 3.8 Å resolution cryo-EM structure of Zika virus. *Science* **352**, 467–470 (2016).
4. G. Barba-Spaeth, W. Dejnirattisai, A. Rouvinski, M.-C. Vaney, I. Medits, A. Sharma, E. Simon-Lorière, A. Sakuntabhai, V.-M. Cao-Lormeau, A. Haouz, P. England, K. Stiasny, J. Mongkolsapaya, F. X. Heinz, G. R. Screaton, F. A. Rey, Structural basis of potent Zika–dengue virus antibody cross-neutralization. *Nature* **536**, 48–53 (2016).
5. W. Dejnirattisai, P. Supasa, W. Wongwiwat, A. Rouvinski, G. Barba-Spaeth, T. Duangchinda, A. Sakuntabhai, V.-M. Cao-Lormeau, P. Malasit, F. A. Rey, J. Mongkolsapaya, G. R. Screaton, Dengue virus sero-cross-reactivity drives antibody-dependent enhancement of infection with zika virus. *Nat. Immunol.* **17**, 1102–1108 (2016).
6. L. Priyamvada, K. M. Quicke, W. H. Hudson, N. Onlamoon, J. Sewatanon, S. Edupuganti, K. Pattanapanyasat, K. Chokephaibulkit, M. J. Mulligan, P. C. Wilson, R. Ahmed, M. S. Suthar, J. Wrammert, Human antibody responses after dengue virus infection are highly cross-reactive to Zika virus. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 7852–7857 (2016).
7. K. Stettler, M. Beltramello, D. A. Espinosa, V. Graham, A. Cassotta, S. Bianchi, F. Vanzetta, A. Minola, S. Jaconi, F. Mele, M. Foglierini, M. Pedotti, L. Simonelli, S. Dowall, B. Atkinson, E. Percivalle, C. P. Simmons, L. Varani, J. Blum, F. Baldanti, E. Cameroni, R. Hewson, E. Harris, A. Lanzavecchia, F. Sallusto, D. Corti, Specificity, cross-reactivity, and function of antibodies elicited by Zika virus infection. *Science* **353**, 823–826 (2016).

Downloaded from http://immunology.sciencemag.org/ by guest on August 24, 2017

8. S. B. Halstead, Neutralization and antibody-dependent enhancement of dengue viruses. *Adv. Virus Res.* **60**, 421–467 (2003).
9. S. C. Kliks, S. Nimmanitya, A. Nisalak, D. S. Burke, Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. *Am. J. Trop. Med. Hyg.* **38**, 411–419 (1988).
10. C. P. Simmons, T. N. B. Chau, T. T. Thuy, N. M. Tuan, D. M. Hoang, N. T. Thien, L. B. Lien, N. T. Quy, N. T. Hieu, T. T. Hien, C. McElnea, P. Young, S. Whitehead, N. T. Hung, J. Farrar, Maternal antibody and viral factors in the pathogenesis of dengue virus in infants. *J. Infect. Dis.* **196**, 416–424 (2007).
11. A. B. Kawiecki, R. C. Christofferson, Zika virus-induced antibody response enhances dengue virus serotype 2 replication in vitro. *J. Infect. Dis.* **214**, 1357–1360 (2016).
12. P. M. S. Castanha, E. L. M. Nascimento, C. Braga, M. T. Cordeiro, O. V. de Carvalho, L. R. de Mendonça, E. A. N. Azevedo, R. F. O. França, R. Dhalia, E. T. A. Marques Jr., Dengue virus (DENV)-specific antibodies enhance Brazilian Zika virus (ZIKV) infection. *J. Infect. Dis.* **2016**, 1–5 (2016).
13. A. P. Durbin, Dengue antibody and Zika: Friend or foe? *Trends Immunol.* **37**, 635–636 (2016).
14. S. V. Bardina, P. Bunduc, S. Tripathi, J. Duehr, J. J. Frere, J. A. Brown, R. Nachbagauer, G. A. Foster, D. Krysztof, D. Tortorella, S. L. Stramer, A. García-Sastre, F. Krammer, J. K. Lim, Enhancement of Zika virus pathogenesis by preexisting antiflavivirus immunity. *Science* **356**, 175–180 (2017).
15. D. A. Muller, P. R. Young, The flavivirus NS1 protein: Molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker. *Antiviral Res.* **98**, 192–208 (2013).
16. M. Beltramello, K. L. Williams, C. P. Simmons, A. Macagno, L. Simonelli, N. T. H. Quyen, S. Sukupolvi-Petty, E. Navarro-Sanchez, P. R. Young, A. M. de Silva, F. A. Rey, L. Varani, S. S. Whitehead, M. S. Diamond, E. Harris, A. Lanzavecchia, F. Sallusto, The human immune response to Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity. *Cell Host Microbe* **8**, 271–283 (2010).
17. L. Priyamvada, A. Cho, N. Onlamoon, N.-Y. Zheng, M. Huang, Y. Kovalenkov, K. Chokephaibulkit, N. Angkasekwinai, K. Pattanapanyasat, F. Ahmed, P. C. Wilson, J. Wrammert, B cell responses during secondary dengue virus infection are dominated by highly cross-reactive, memory-derived plasmablasts. *J. Virol.* **90**, 5574–5585 (2016).
18. L. Li, S.-M. Lok, I.-M. Yu, Y. Zhang, R. J. Kuhn, J. Chen, M. G. Rossmann, The flavivirus precursor membrane-envelope protein complex: Structure and maturation. *Science* **319**, 1830–1834 (2008).
19. Y. Modis, S. Ogata, D. Clements, S. C. Harrison, Structure of the dengue virus envelope protein after membrane fusion. *Nature* **427**, 313–319 (2004).
20. G. Sapparapu, E. Fernandez, N. Kose, B. Cao, J. M. Fox, R. G. Bombardi, H. Zhao, C. A. Nelson, A. L. Bryan, T. Barnes, E. Davidson, I. U. Mysorekar, D. H. Fremont, B. J. Doranz, M. S. Diamond, J. E. Crowe, Neutralizing human antibodies prevent Zika virus replication and fetal disease in mice. *Nature* **540**, 443–447 (2016).
21. S. Bhatt, P. W. Gething, O. J. Brady, J. P. Messina, A. W. Farlow, C. L. Moyes, J. M. Drake, J. S. Brownstein, A. G. Hoen, O. Sankoh, M. F. Myers, D. B. George, T. Jaenisch, G. R. W. Wint, C. P. Simmons, T. W. Scott, J. J. Farrar, S. I. Hay, The global distribution and burden of dengue. *Nature* **496**, 504–507 (2013).
22. Y. Lustig, H. Zelena, G. Venturi, M. Van Esbroeck, G. Rothe, C. Perret, R. Koren, S. Katz-Likvornik, E. Mendelson, E. Schwartz, Sensitivity and kinetics of an NS1-based Zika virus enzyme-linked immunosorbent assay in Zika virus-infected travelers from Israel, the Czech Republic, Italy, Belgium, Germany, and Chile. *J. Clin. Microbiol.* **55**, 1894–1901 (2017).
23. D. Huzly, I. Hanselmann, J. Schmidt-Chanasit, M. Panning, High specificity of a novel Zika virus ELISA in European patients after exposure to different flaviviruses. *Euro Surveill.* **21**, 30203 (2016).
24. J. Wrammert, N. Onlamoon, R. S. Akondy, G. C. Perng, K. Polsrila, A. Chandele, M. Kwissa, B. Pulendran, P. C. Wilson, O. Wittawatmongkol, S. Yoksan, N. Angkasekwinai, K. Pattanapanyasat, K. Chokephaibulkit, R. Ahmed, Rapid and massive virus-specific plasmablast responses during acute dengue virus infection in humans. *J. Virol.* **86**, 2911–2918 (2012).
25. J. Wrammert, K. Smith, J. Miller, W. A. Langley, K. Kokko, C. Larsen, N.-Y. Zheng, I. Mays, L. Garman, C. Helms, J. James, G. M. Air, J. D. Capra, R. Ahmed, P. C. Wilson, Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. *Nature* **453**, 667–671 (2008).
26. F. E.-H. Lee, J. L. Halliley, E. E. Walsh, A. P. Moscatiello, B. L. Kmush, A. R. Falsey, T. D. Randall, D. A. Kaminiski, R. K. Miller, I. Sanz, Circulating human antibody-secreting cells during vaccinations and respiratory viral infections are characterized by high specificity and lack of bystander effect. *J. Immunol.* **186**, 5514–5521 (2011).
27. W. Dejnirattisai, W. Wongwiwat, S. Supasa, X. Zhang, X. Dai, A. Rouvinski, A. Jumnainsong, C. Edwards, N. T. H. Quyen, T. Duangchinda, J. M. Grimes, W.-Y. Tsai, C.-Y. Lai, W.-K. Wang, P. Malasit, J. Farrar, C. P. Simmons, Z. H. Zhou, F. A. Rey, J. Mongkolsapaya, G. R. Screaton, A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with dengue virus. *Nat. Immunol.* **16**, 170–177 (2015).
28. W. Dejnirattisai, A. Jumnainsong, N. Onsirisakul, P. Fitton, S. Vasanawathana, W. Limpitikul, C. Puttikhunt, C. Edwards, T. Duangchinda, S. Supasa, K. Chawansuntati, P. Malasit, J. Mongkolsapaya, G. Screaton, Cross-reacting antibodies enhance dengue virus infection in humans. *Science* **328**, 745–748 (2010).
29. A. Rouvinski, P. Guardado-Calvo, G. Barba-Spaeth, S. Duquerroy, M.-C. Vaney, C. M. Kikuti, M. E. Navarro Sanchez, W. Dejnirattisai, W. Wongwiwat, A. Haouz, C. Girard-Blanc, S. Petres, W. E. Shepard, P. Desprès, F. Arenzana-Seisdedos, E. Dussart, J. Mongkolsapaya, G. R. Screaton, F. A. Rey, Recognition determinants of broadly neutralizing human antibodies against dengue viruses. *Nature* **520**, 109–113 (2015).
30. H. Zhao, E. Fernandez, K. A. Dowd, S. D. Speer, D. J. Platt, M. J. Gorman, J. Govero, C. A. Nelson, T. C. Pierson, M. S. Diamond, D. H. Fremont, Structural basis of Zika virus-specific antibody protection. *Cell* **166**, 1016–1027 (2016).
31. M. K. Gentry, E. A. Henchal, J. M. McCown, W. E. Brandt, J. M. Dalrymple, Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. *Am. J. Trop. Med. Hyg.* **31**, 548–555 (1982).
32. T. Oliphant, G. E. Nybakken, M. Engle, Q. Xu, C. A. Nelson, S. Sukupolvi-Petty, A. Marri, B.-E. Lachmi, U. Olshevsky, D. H. Fremont, T. C. Pierson, M. S. Diamond, Antibody recognition and neutralization determinants on domains I and II of West Nile virus envelope protein. *J. Virol.* **80**, 12149–12159 (2006).
33. L. J. McHeyzer-Williams, P. J. Milpied, S. L. Okitsu, M. G. McHeyzer-Williams, Class-switched memory B cells remodel BCRs within secondary germinal centers. *Nat. Immunol.* **16**, 296–305 (2015).
34. G. V. Zuccarino-Catania, S. Sadanand, F. J. Weisel, M. M. Tomayko, H. Meng, S. H. Kleinstein, K. L. Good-Jacobson, M. J. Shlomchik, CD80 and PD-L2 define functionally distinct memory B cell subsets that are independent of antibody isotype. *Nat. Immunol.* **15**, 631–637 (2014).
35. K. A. Pape, J. J. Taylor, R. W. Maul, P. J. Gearhart, M. K. Jenkins, Different B cell populations mediate early and late memory during an endogenous immune response. *Science* **331**, 1203–1207 (2011).
36. T. M. Garcia-Bates, M. T. Cordeiro, E. J. M. Nascimento, A. P. Smith, K. M. Soares de Melo, S. P. McBurney, J. D. Evans, E. T. A. Marques Jr., S. M. Barratt-Boyes, Association between magnitude of the virus-specific plasmablast response and disease severity in dengue patients. *J. Immunol.* **190**, 80–87 (2013).
37. G. Blanchard-Rohner, A. S. Pulickal, C. M. Jol-van der Zijde, M. D. Snape, A. J. Pollard, Appearance of peripheral blood plasma cells and memory B cells in a primary and secondary immune response in humans. *Blood* **114**, 4998–5002 (2009).
38. D. F. Robbiani, L. Bozzacco, J. R. Keeffe, R. Khouri, P. C. Olsen, A. Gazumyan, D. Schaefer-Babajew, S. Avila-Rios, L. Nogueira, R. Patel, S. A. Azzopardi, L. F. K. Uhl, M. Saeed, E. E. Sevilla-Reyes, M. Agudelo, K.-H. Yao, J. Golijanin, H. B. Gristick, Y. E. Lee, A. Hurley, M. Caskey, J. Pai, T. Oliveira, E. A. Wunder Jr., G. Sacramento, N. Nery Jr., C. Orge, F. Costa, M. G. Reis, N. M. Thomas, T. Eisenreich, D. M. Weinberger, A. R. P. de Almeida, A. P. West Jr., C. M. Rice, P. J. Bjorkman, G. Reyes-Teran, A. Ko, M. R. MacDonald, M. C. Nussenzweig, Recurrent potent human neutralizing antibodies to Zika virus in Brazil and Mexico. *Cell* **169**, 597–609.e11 (2017).
39. J. H. Kim, I. Skountzou, R. Compans, J. Jacob, Original antigenic sin responses to influenza viruses. *J. Immunol.* **183**, 3294–3301 (2009).
40. S. Fazekas de St. Groth, R. G. Webster, Disquisitions of original antigenic Sin. I. Evidence in man. *J. Exp. Med.* **124**, 331–345 (1966).
41. Y. S. Choi, Y. H. Baek, W. Kang, S. J. Nam, J. Lee, S. You, D.-Y. Chang, J.-C. Youn, Y. K. Choi, E.-C. Shin, Reduced antibody responses to the pandemic (H1N1) 2009 vaccine after recent seasonal influenza vaccination. *Clin. Vaccine Immunol.* **18**, 1519–1523 (2011).
42. C. M. Midgley, M. Bajwa-Joseph, S. Vasanawathana, W. Limpitikul, B. Wills, A. Flanagan, E. Waiyaiya, H. B. Tran, A. E. Cowper, P. Chotiyarnwong, J. M. Grimes, S. Yoksan, P. Malasit, C. P. Simmons, J. Mongkolsapaya, G. R. Screaton, An in-depth analysis of original antigenic sin in dengue virus infection. *J. Virol.* **85**, 410–421 (2011).
43. S. B. Halstead, S. Rojanasuphot, N. Sangkawibha, Original antigenic sin in dengue. *Am. J. Trop. Med. Hyg.* **32**, 154–156 (1983).
44. P. Abbink, R. A. Larocca, R. A. De La Barrera, C. A. Bricault, E. T. Moseley, M. Boyd, M. Kirilova, Z. Li, D. Ng'ang'a, O. Nanayakkara, R. Nityanandam, N. B. Mercado, E. N. Borducchi, A. Agarwal, A. L. Brinkman, C. Cabral, A. Chandrashekar, P. B. Giglio, D. Jetton, J. Jimenez, B. C. Lee, S. Mojta, K. Molloy, M. Shetty, G. H. Neubauer, K. E. Stephenson, J. P. S. Peron, P. M. d. A. Zanotto, J. Misamore, B. Finneyfrock, M. G. Lewis, G. Alter, K. Modjarrad, R. G. Jarman, K. H. Eckels, N. L. Michael, S. J. Thomas, D. H. Barouch, Protective efficacy of multiple vaccine platforms against Zika virus challenge in rhesus monkeys. *Science* **353**, 1129–1132 (2016).
45. K. A. Dowd, S.-Y. Ko, K. M. Morabito, E. S. Yang, R. S. Pelc, C. R. DeMaso, L. R. Castilho, P. Abbink, M. Boyd, R. Nityanandam, D. N. Gordon, J. R. Gallagher, X. Chen, J.-P. Todd, Y. Tsybovsky, A. Harris, Y.-J. S. Huang, S. Higgs, D. L. Vanlandingham, H. Andersen, M. G. Lewis, R. De La Barrera, K. H. Eckels, R. G. Jarman, M. C. Nason, D. H. Barouch, M. Roederer, W.-P. Kong, J. R. Mascola, T. C. Pierson, B. S. Graham, Rapid development of a DNA vaccine for Zika virus. *Science* **354**, 237–240 (2016).
46. R. A. Larocca, P. Abbink, J. P. S. Peron, P. M. d. A. Zanotto, M. J. Iampietro, A. Badamchi-Zadeh, M. Boyd, D. Ng'ang'a, M. Kirilova, R. Nityanandam, N. B. Mercado, Z. Li, E. T. Moseley, C. A. Bricault, E. N. Borducchi, P. B. Giglio, D. Jetton, G. Neubauer, J. P. Nkolola, L. F. Maxfield,

Downloaded from http://immunology.sciencemag.org/ by guest on August 24, 2017

R. A. De La Barrera, R. G. Jarman, K. H. Eckels, N. L. Michael, S. J. Thomas, D. H. Barouch, Vaccine protection against Zika virus from Brazil. *Nature* **536**, 474–478 (2016).
47. E. Kim, G. Erdos, S. Huang, T. Kenniston, L. D. Falo Jr., A. Gambotto, Preventative vaccines for Zika virus outbreak: Preliminary evaluation. *EBioMedicine* **13**, 315–320 (2016).
48. N. Pardi, M. J. Hogan, R. S. Pelc, H. Muramatsu, H. Andersen, C. R. DeMaso, K. A. Dowd, L. L. Sutherland, R. M. Scearce, R. Parks, W. Wagner, A. Granados, J. Greenhouse, M. Walker, E. Willis, J.-S. Yu, C. E. McGee, G. D. Sempowski, B. L. Mui, Y. K. Tam, Y.-J. Huang, D. Vanlandingham, V. M. Holmes, H. Balachandran, S. Sahu, M. Lifton, S. Higgs, S. E. Hensley, T. D. Madden, M. J. Hope, K. Karikó, S. Santra, B. S. Graham, M. G. Lewis, T. C. Pierson, B. F. Haynes, D. Weissman, Zika virus protection by a single low-dose nucleoside-modified mRNA vaccination. *Nature* **543**, 248–251 (2017).
49. J. M. Richner, S. Himansu, K. A. Dowd, S. L. Butler, V. Salazar, J. M. Fox, J. G. Julander, W. W. Tang, S. Shresta, T. C. Pierson, G. Ciaramella, M. S. Diamond, Modified mRNA vaccines protect against Zika virus infection. *Cell* **169**, 176 (2017).
50. I. J. Fuss, M. E. Kanof, P. D. Smith, H. Zola, Isolation of whole mononuclear cells from peripheral blood and cord blood. *Curr. Protoc. Immunol.* **Chapter 7**, Unit7.1 (2009).
51. T. Tiller, E. Meffre, S. Yurasov, M. Tsuiji, M. C. Nussenzweig, H. Wardemann, Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J. Immunol. Methods* **329**, 112–124 (2008).
52. B. Briney, K. Le, J. Zhu, D. R. Burton, Clonify: Unseeded antibody lineage assignment from next-generation sequencing data. *Sci. Rep.* **6**, 23901 (2016).
53. J. D. Brien, H. M. Lazear, M. S. Diamond, Propagation, quantification, detection, and storage of West Nile virus. *Curr. Protoc. Microbiol.* **31**, 15D.3.1–15D.3.18 (2013).
54. Z. A. Bornholdt, H. L. Turner, C. D. Murin, W. Li, D. Sok, C. A. Souders, A. E. Piper, A. Goff, J. D. Shamblin, S. E. Wollen, T. R. Sprague, M. L. Fusco, K. B. J. Pommert, L. A. Cavacini, H. L. Smith, M. Klempner, K. A. Reimann, E. Krauland, T. U. Gerngross, K. D. Wittrup, E. O. Saphire, D. R. Burton, P. J. Glass, A. B. Ward, L. M. Walker, Isolation of potent neutralizing antibodies from a survivor of the 2014 Ebola virus outbreak. *Science* **351**, 1078–1083 (2016).
55. R. D. Gietz, R. H. Schiestl, High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.* **2**, 31–34 (2007).

Acknowledgments: We thank C. Williams and S. M. Eagol for assistance with figure preparation. We thank D. Watkins and D. Magnani for supplying viral stocks for these studies and T. Fredeking for assistance in sample identification. We thank D. Fremont and H. Zhao for DIII, WNV, and the E-FL protein. **Funding:** This work was supported by a grant from the Defense Advanced Research Projects Agency (DARPA) Autonomous Diagnostics to Enable Prevention and Therapeutics: Prophylactic Options to Environmental and Contagious Threats (ADEPT-PROTECT) program (W31P4Q-13-1-0011). **Author contributions:** L.M.W. wrote the manuscript. T.F.R., E.C.G., D.S., and D.R.B. edited the manuscript. T.F.R., E.C.G., B.B., D.S., N.B., A.S., R.N., K.L., and M.E.B. designed and performed experiments and analyzed results. L.M.W., T.F.R., B.B., D.S., and D.R.B. provided intellectual oversight. L.M.W. and T.F.R. performed the statistical analyses. **Competing interests:** L.M.W., M.E.B., and E.C.G. have an equity position in Adimab LLC. The other authors declare that they have no competing interests.

Submitted 17 May 2017
Resubmitted 2 June 2017
Accepted 24 July 2017
Published 18 August 2017
10.1126/sciimmunol.aan6809

Citation: T. F. Rogers, E. C. Goodwin, B. Briney, D. Sok, N. Beutler, A. Strubel, R. Nedellec, K. Le, M. E. Brown, D. R. Burton, L. M. Walker, Zika virus activates de novo and cross-reactive memory B cell responses in dengue-experienced donors. *Sci. Immunol.* **2**, eaan6809 (2017).

Downloaded from http://immunology.sciencemag.org/ by guest on August 24, 2017

Science Immunology

Zika virus activates de novo and cross-reactive memory B cell responses in dengue-experienced donors

Thomas F. Rogers, Eileen C. Goodwin, Bryan Briney, Devin Sok, Nathan Beutler, Alexander Strubel, Rebecca Nedellec, Khoa Le, Michael E. Brown, Dennis R. Burton and Laura M. Walker

Sci. Immunol. **2**, eaan6809.
DOI: 10.1126/sciimmunol.aan6809

For zika virus, experience counts

For the immune system, practice makes perfect. Previous exposure to an infection elicits a stronger, faster memory response. But how does the immune system respond to a similar but not identical infection? Rogers *et al*. tracked the neutralizing antibody response to zika virus infection in individuals with and without previous exposure to the closely related dengue viruses. They found that zika virus infection primed the preexisting dengue virus response, but that this cross-reactive response was poorly neutralizing. In contrast, de novo zika virus responses were potently neutralizing. These data suggest that zika virus vaccines should target epitopes not present in dengue virus subtypes.

ARTICLE TOOLS http://immunology.sciencemag.org/content/2/14/eaan6809

SUPPLEMENTARY MATERIALS http://immunology.sciencemag.org/content/suppl/2017/08/15/2.14.eaan6809.DC1

REFERENCES This article cites 55 articles, 24 of which you can access for free
http://immunology.sciencemag.org/content/2/14/eaan6809#BIBL

PERMISSIONS http://www.sciencemag.org/help/reprints-and-permissions

Use of this article is subject to the Terms of Service

Science Immunology (ISSN 2470-9468) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. 2017 © The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original U.S. Government Works. The title *Science Immunology* is a registered trademark of AAAS.

Downloaded from http://immunology.sciencemag.org/ by guest on August 24, 2017

I. OVERVIEW OF THE FUNDING OPPORTUNITY

Program Announcement for the Department of Defense

Defense Health Program

Congressionally Directed Medical Research Programs

Peer Reviewed Medical Research Program

Focused Program Award

Announcement Type: Initial

Funding Opportunity Number: W81XWH-17-PRMRP-FPA

Catalog of Federal Domestic Assistance Number: 12.420 Military Medical Research and Development

SUBMISSION AND REVIEW DATES AND TIMES

- **Pre-Application Submission Deadline:** 5:00 p.m. Eastern time (ET), July 20, 2017
- **Invitation to Submit an Application:** August 2017
- **Application Submission Deadline:** 11:59 p.m. ET, October 26, 2017
- **End of Application Verification Period:** 5:00 p.m. ET, October 31, 2017
- **Peer Review:** December 2017
- **Programmatic Review:** February 2018

This Program Announcement must be read in conjunction with the General Application Instructions, version 20170516. The General Applications Instructions document is available for downloading from the Grants.gov funding opportunity announcement by selecting the "Package" tab, clicking "Preview," and then selecting "Download Instructions."

TABLE OF CONTENTS

I. OVERVIEW OF THE FUNDING OPPORTUNITY 1

II. DETAILED INFORMATION ABOUT THE FUNDING OPPORTUNITY 4

II.A. Program Description 4

II.A.1. FY17 PRMRP Topic Areas 4

II.B. Award Information 6

II.C. Eligibility Information 9

II.C.1. Eligible Applicants 9

II.C.2. Cost Sharing 10

II.C.3. Other 10

II.D. Application and Submission Information 10

II.D.1. Address to Request Application Package 11

II.D.2. Content and Form of the Application Submission 11

II.D.3. Dun and Bradstreet Universal Numbering System (DUNS) Number and System for Award Management (SAM) 29

II.D.4. Submission Dates and Times 29

II.D.5. Funding Restrictions 30

II.D.6. Other Submission Requirements 31

II.E. Application Review Information 31

II.E.1. Criteria 31

II.E.2. Application Review and Selection Process 36

II.E.3. Integrity and Performance Information 37

II.E.4. Anticipated Announcement and Federal Award Dates 37

II.F. Federal Award Administration Information 37

II.F.1. Federal Award Notices 37

II.F.2. Administrative and National Policy Requirements 39

II.F.3. Reporting 39

II.G. Federal Awarding Agency Contacts 40

II.G.1. CDMRP Help Desk 40

II.G.2. Grants.gov Contact Center 40

II.H. Other Information 40

II.H.1. Program Announcement and General Application Instructions Versions 40

II.H.2. Administrative Actions 40

II.H.3. Application Submission Checklist 43

APPENDIX 1 ACRONYM LIST 45
APPENDIX 2 AREAS OF ENCOURAGEMENT 46
APPENDIX 3 DOD AND VA WEBSITES 65

II. DETAILED INFORMATION ABOUT THE FUNDING OPPORTUNITY

II.A. Program Description

Applications to the Fiscal Year 2017 (FY17) Peer Reviewed Medical Research Program (PRMRP) are being solicited for the Defense Health Agency (DHA) J9, Research and Development Directorate, by the U.S. Army Medical Research Acquisition Activity (USAMRAA) using delegated authority provided by United States Code, Title 10, Section 2358 (10 USC 2358). As directed by the Office of the Assistant Secretary of Defense for Health Affairs (OASD[HA]), the DHA manages the Defense Health Program (DHP) Research, Development, Test, and Evaluation (RDT&E) appropriation. The execution management agent for this Program Announcement is the Congressionally Directed Medical Research Programs (CDMRP). The PRMRP was initiated in 1999 to provide support for military health-related research of exceptional scientific merit. Appropriations for the PRMRP from FY99 through FY16 totaled $1.371 billion. The FY17 appropriation is $300 million (M).

The vision of the FY17 PRMRP is to improve the health and well-being of all military Service members, Veterans, and beneficiaries. The PRMRP challenges the scientific and clinical communities to address at least one of the FY17 Topic Areas with original ideas that foster new directions along the entire spectrum of research and clinical care. The program seeks applications in laboratory, clinical, behavioral, epidemiologic, and other areas of research to advance knowledge in disease etiology, improve prevention, detection, diagnosis, treatment, and quality of life for those affected by a relevant disease or condition, and to develop and validate clinical care or public health guidelines.

II.A.1. FY17 PRMRP Topic Areas

All applications for PRMRP funding must specifically address at least one of the Topic Areas as directed by Congress and must be directly relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries. If the proposed research does not specifically address at least one of the FY17 PRMRP Topic Areas, the Government will administratively withdraw the application. The Government reserves the right to reassign the application's Topic Area if submitted under an inappropriate Topic Area. The FY17 PRMRP Topic Areas are listed below.

- Acute Lung Injury
- Antimicrobial Resistance
- Arthritis
- Burn Pit Exposure
- Chronic Migraine and Post-Traumatic Headache
- Congenital Heart Disease
- Constrictive Bronchiolitis
- Diabetes
- Diarrheal Diseases
- Dystonia
- Early Trauma Thermal Regulation
- Eating Disorders

- Emerging Infectious Diseases
- Epidermolysis Bullosa
- Focal Segmental Glomerulosclerosis
- Fragile X
- Guillain-Barré Syndrome
- Hepatitis B and C
- Hereditary Angioedema
- Hydrocephalus
- Immunomonitoring of Intestinal Transplants
- Inflammatory Bowel Diseases
- Influenza
- Integrative Medicine
- Interstitial Cystitis
- Malaria
- Metals Toxicology
- Mitochondrial Disease
- Musculoskeletal Disorders
- Nanomaterials for Bone Regeneration
- Non-Opioid Pain Management
- Pancreatitis
- Pathogen-Inactivated Dried Cryoprecipitate
- Polycystic Kidney Disease
- Post-Traumatic Osteoarthritis
- Pulmonary Fibrosis
- Respiratory Health
- Rett Syndrome
- Rheumatoid Arthritis
- Scleroderma
- Sleep Disorders
- Spinal Muscular Atrophy
- Sustained-Release Drug Delivery
- Tinnitus
- Tuberculosis
- Vaccine Development for Infectious Disease
- Vascular Malformations
- Women's Heart Disease

Research relevant to one or more FY17 PRMRP Topic Areas may be considered for funding. ***Applicants should select the FY17 PRMRP Program Announcement most appropriate to the stage of the proposed research.*** Areas of Encouragement related to the FY17 PRMRP Topic Areas have been identified by the Department of Defense (DoD), the Department of Veterans Affairs (VA), and other relevant stakeholders (Appendix 2). Applicants are urged to read and consider these Areas of Encouragement before preparing their applications. ***The information provided is not exhaustive, and applicants are not restricted to submitting applications that address an Area of Encouragement on this list.***

II.B. Award Information

The PRMRP Focused Program Award mechanism is intended to optimize research and accelerate the solution for a critical question related to at least one of the Congressionally directed FY17 PRMRP Topic Areas through a synergistic, multidisciplinary research program. The anticipated total costs budgeted for the entire period of performance for an FY17 PRMRP Focused Program Award will not exceed **$10M**. Refer to Section II.D.5, Funding Restrictions, for detailed funding information.

Key aspects of this award include:

Overarching Challenge: Focused Program Award applications must describe a unifying, overarching challenge that will be addressed by a set of research projects. The overarching challenge must be relevant to a critical problem or question in the field of research and/or patient care in at least one of the FY17 PRMRP Topic Areas.

Research Projects: Applications shall include multiple, distinct research projects led by individual project leaders that address complementary aspects of the overarching challenge. Applicants are strongly encouraged to submit a minimum of four research projects; additional studies are allowed. While individual projects must be capable of standing on their own high scientific merits, they must also be interrelated and synergistic with the other proposed projects and advance a solution beyond what would be possible through individual efforts. The exploration of multiple hypotheses or viewpoints of the same line of questioning is encouraged. This award mechanism is not intended to support a series of research projects that are dependent on the success of any other project. Each project should propose a unique approach to addressing the overarching challenge and be capable of producing research findings with potential to impact the field and/or patient care. Individual research projects may range from exploratory, hypothesis-developing studies through small-scale clinical trials (i.e., up to and including Phase II or equivalent). There should be a clear intent to progress toward translational/ clinical work over the course of the effort.

Implementation: The research strategy to address the overarching challenge must be supported by a detailed plan that identifies critical milestones; outlines the knowledge, resources, and technical innovations that will be utilized to achieve the milestones; and explains how the outcomes will be translated to patients. A robust statistical plan and statistical expertise should be included where applicable. A plan for assessing individual project performance and progress toward addressing the overarching challenge must be included in the implementation plan. Plans to include an External Advisory Board are encouraged; however, applicants must be careful to avoid potential conflicts of interest (COIs) during review of the application by ensuring no contact with, recruiting of, or naming of specific members in the application. For multi-institutional collaborations, plans for communication and data transfer among the collaborating institutions, as well as how data, specimens, and/or imaging products obtained during the study will be handled, must be included. An intellectual and material property plan agreed to by participating organizations is required in the application's supporting documentation.

Research Team: The overall effort will be led by a Principal Investigator (PI) with demonstrated success in leading large, focused projects. The PI is required to devote a minimum

of 20% effort to this award. The PI should create an environment that fosters and supports collaboration and innovation in a way that engages all members of the team in all aspects of the research plan. The research team assembled by the PI should be highly qualified and multidisciplinary, with identified project leaders for each of the complementary and synergistic research projects. The resources and expertise brought to the team by each project leader should combine to create a robust, synergistic collaboration. The PRMRP Science Officer assigned to a resulting award must be invited to participate in periodic research team meetings. The plan for such meetings should be noted in the application.

Milestone Meeting: The PI will be required to present an update on progress toward accomplishing the goals of the award at a Milestone Meeting to be held in the National Capital Region after the conclusion of Year 2 of the period of performance. The PI may bring up to three additional members of the research team to the meeting. The Milestone Meeting will be attended by members of the PRMRP Programmatic Panel, CDMRP staff, and the USAMRAA Grants Officer.

Military Relevance: Relevance to the healthcare needs of military Service members, Veterans, and beneficiaries is a key feature of this award. Investigators are encouraged to consider the following characteristics as examples of how a project may demonstrate military relevance:

- Explanation of how the project addresses an aspect of the target disease/condition that has direct relevance to military Service members, Veterans, or other military health system beneficiaries

- Description of how the knowledge, information, products, or technologies gained from the proposed research could be implemented in a dual-use capacity to benefit the civilian population and also address a military need

- Use of military or Veteran populations or datasets in the proposed research, if appropriate to the proposed research project

- Involvement of military consultants (Army, Air Force) or specialty leaders (Navy, Marine Corps) to the Surgeons General in a relevant specialty area

PIs are strongly encouraged to integrate and/or align their research projects with DoD and/or VA research laboratories and programs. Collaboration with the DoD or VA is also encouraged. A list of websites that may be useful in identifying additional information about ongoing DoD and VA areas of research interest or potential opportunities for collaboration within the FY17 PRMRP Topic Areas can be found in Appendix 3.

Use of Active Duty Military and VA Populations: If the proposed research plan involves access to active duty military and/or VA patient populations or resources, the PI is responsible for establishing such access. If possible, access to target active duty military and/or VA patient populations/resources should be confirmed at the time of application submission by inclusion of a letter of support, signed by the lowest ranking person with approval authority, for studies involving active duty military Service members, Veterans, military- and/or VA-controlled study materials, and military and/or VA databases. If access cannot be confirmed at the time of application submission, the Government reserves the right to withhold or revoke funding until

the PI has demonstrated support for and access to the relevant population(s) and/or resources. Note that access to a Veteran population for clinical studies may only be obtained by either collaboration with a VA investigator where the VA investigator has a substantial role in the research or by advertising to the general public.

Research Involving Human Anatomical Substances, Human Subjects, or Human Cadavers: All DoD-funded research involving new and ongoing research with human anatomical substances, human subjects, or human cadavers must be reviewed and approved by the U.S. Army Medical Research and Materiel Command (USAMRMC) Office of Research Protections (ORP), Human Research Protection Office (HRPO) prior to research implementation. This administrative review requirement is in addition to the local Institutional Review Board (IRB) or Ethics Committee (EC) review. Local IRB/EC approval at the time of submission is ***not*** required. The HRPO is mandated to comply with specific laws and requirements governing all research involving human anatomical substances, human subjects, or human cadavers that is supported by the DoD. These laws and requirements will necessitate information in addition to that supplied to the IRB/EC. ***Allow a minimum of 2 to 3 months for HRPO regulatory review and approval processes***. When possible, protocols should be written for research with human subjects and/or human anatomical substances that are specific to the DoD-supported effort outlined in the submitted application. Submission to HRPO of protocols covering more than the scope of work in the DoD-funded award will require HRPO review of the entire protocol as DoD-supported research and may include extensive modifications to meet DoD human subjects protection requirements. Refer to the General Application Instructions, Appendix 1, and the Human Subject Resource Document available on the electronic Biomedical Research Application Portal (eBRAP) "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) for additional information.

A clinical trial is defined as a prospective accrual of patients (human subjects) in whom an intervention (e.g., device, drug, biologic, surgical procedure, rehabilitative modality, behavioral intervention, or other) is tested for a measurable outcome with respect to safety, effectiveness, and/or efficacy. This outcome represents a direct effect on the subject of that intervention or interaction.

Research Involving Animals: All projects should adhere to a core set of standards for rigorous study design and reporting to maximize the reproducibility and translational potential of preclinical research. The standards are described in Landis, S.C., et al. A call for transparent reporting to optimize the predictive value of preclinical research. *Nature* 2012, 490:187-191 (www.nature.com/nature/journal/v490/n7419/full/nature11556.html). While these standards are written for preclinical studies, the basic principles of randomization, blinding, sample-size estimation, and data handling derive from well-established best practices in clinical studies. Applicants should consult the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines to ensure relevant aspects of rigorous animal research are adequately planned for and, ultimately, reported. The ARRIVE guidelines can be found at http://www.elsevier.com/__data/promis_misc/622936arrive_guidelines.pdf

All DoD-funded research involving new and ongoing research with animals must be reviewed and approved by the USAMRMC ORP Animal Care and Use Review Office (ACURO), in addition to the local Institutional Animal Care and Use Committee (IACUC) of record. IACUC

approval at the time of submission is ***not*** required. Specific documents relating to the use of animals in the proposed research will be requested **if the application is selected for funding**. The ACURO must review and approve all animal use prior to the start of working with animals, including amendments to ongoing projects. PIs must submit the institutional animal use protocol, IACUC approval of that protocol, and a version of the animal use appendix titled "Research Involving Animals." ***Allow at least 2 to 3 months for ACURO regulatory review and approval processes for animal studies.*** Refer to the General Application Instructions, Appendix 1, for additional information.

The CDMRP intends that information, data, and research resources generated under awards funded by this Program Announcement be made available to the research community (which includes both scientific and consumer advocacy communities) and to the public at large. For additional guidance, refer to the General Application Instructions, Appendix 2, Section K.

Awards will be made no later than September 30, 2018. For additional information refer to Section II.F.1, Federal Award Notices.

II.C. Eligibility Information

II.C.1. Eligible Applicants

II.C.1.a. Organization: All organizations, including international organizations, are eligible to apply.

Government Agencies within the United States: Local, state, and Federal Government agencies are eligible to the extent that applications do not overlap with their fully funded internal programs. Such agencies are required to explain how their applications do not overlap with their internal programs.

As applications for this Program Announcement may be submitted by extramural and intramural organizations, these terms are defined below.

Extramural Organization: An eligible non-DoD organization. Examples of extramural organizations include academia, biotechnology companies, foundations, Government, and research institutes. *Extramural Submission: Application submitted by a non-DoD organization to Grants.gov.*

Intramural DoD Organization: A DoD laboratory, DoD military treatment facility, and/or DoD activity embedded within a civilian medical center. *Intramural Submission: Application submitted by a DoD organization for an intramural investigator who is a DoD military or civilian employee working within a DoD laboratory or military treatment facility or in a DoD activity embedded within a civilian medical center.*

Note: Applications from an intramural organization or from an extramural non-DoD Federal organization may be submitted through a research foundation.

The USAMRAA makes awards to eligible organizations, not to individuals.

II.C.1.b. Principal Investigator:

- The PI must be an independent investigator at or above the level of Full Professor (or equivalent).
    - Project leaders for each of the complementary and synergistic research projects must be at or above the level of Assistant Professor (or equivalent).

An eligible PI, regardless of ethnicity, nationality, or citizenship status, must be employed by, or affiliated with, an eligible organization.

The CDMRP encourages all PIs to participate in a digital identifier initiative through Open Researcher and Contributor ID, Inc. (ORCID). Registration for a unique ORCID identifier can be done online at http://orcid.org/.

II.C.2. Cost Sharing

Cost sharing/matching is not an eligibility requirement.

II.C.3. Other

Extramural organizations must be able to access **.gov** and **.mil** websites in order to fulfill the financial and technical deliverable requirements of the award and submit invoices for payment.

There are no limitations on the number of applications for which an investigator may be named as a PI.

For general information on required qualifications for award recipients, refer to the General Application Instructions, Appendix 3.

Refer to Section II.H.2, Administrative Actions, for a list of administrative actions that may be taken if a pre-application or application does not meet the administrative, eligibility, or ethical requirements defined in this Program Announcement.

II.D. Application and Submission Information

Submission of applications that are essentially identical or propose essentially the same research project to different funding opportunities within the same program and fiscal year is prohibited and will result in administrative withdrawal of the duplicative application(s). ***As an exception, applicants may submit a research project described in their FY17 PRMRP Focused Program Award to the Discovery Award (Funding Opportunity Number: W81XWH-17-PRMRP-DA), Investigator-Initiated Research Award (Funding Opportunity Number: W81XWH-17-PRMRP-IIRA), and/or Clinical Trial Award (Funding Opportunity Number: W81XWH-17-PRMRP-CTA); however, accepting multiple awards to support the same project will not be allowed.***

Extramural Submission is defined as an application submitted by a non-DoD organization to Grants.gov.

Intramural Submission is defined as an application submission by a DoD organization for an intramural investigator, who is a DoD military or civilian employee working within a DoD laboratory or military treatment facility, or working in a DoD activity embedded within a civilian medical center.

II.D.1. Address to Request Application Package

Submitting Extramural and Intramural Organizations: Pre-application content and forms can be accessed at eBRAP (https://eBRAP.org).

Submitting Extramural Organizations: Full application packages can be accessed at Grants.gov.

Submitting Intramural DoD Organizations: Full application packages can be accessed at eBRAP.org.

Contact information for the CDMRP Help Desk and the Grants.gov Contact Center can be found in Section II.G, Federal Awarding Agency Contacts.

II.D.2. Content and Form of the Application Submission

Submission is a two-step process requiring both ***pre-application*** and ***full application*** as indicated below. The submission process should be started early to avoid missing deadlines. There are no grace periods.

Pre-application Submission: All pre-applications for both extramural and intramural organizations must be submitted through eBRAP (https://eBRAP.org/).

eBRAP is a multifunctional web-based system that allows PIs to submit their pre-applications electronically through a secure connection, to view and edit the content of their pre-applications and full applications, to receive communications from the CDMRP, and to submit documentation during award negotiations and period of performance.

Full Application Submission: Full applications must be submitted through the online portals as described below.

***Submitting Extramural Organizations*:** Full applications from extramural organizations must be submitted through Grants.gov. Applications submitted by extramural organizations (e.g., research foundations) on behalf of intramural DoD or other Federal organizations or investigators will be considered extramural submissions.

Submitting Intramural DoD Organizations: Intramural DoD organizations may submit full applications to either eBRAP or Grants.gov. Intramural DoD organizations that are unable to submit to Grants.gov should submit through eBRAP. Intramural DoD organizations with the capability to submit through Grants.gov may submit following the instructions for extramural submissions through Grants.Gov or may submit to eBRAP. Applications from extramural organizations, including non-DoD Federal organizations, received through eBRAP will be withdrawn. See definitions in Section II.C.1, Eligible Applicants.

For both Extramural and Intramural applicants: A key feature of eBRAP is the ability of an organization's representatives and PIs to view and modify the full application submissions associated with them. eBRAP will validate full application files against the specific Program Announcement requirements and discrepancies will be noted in an email to the PI and in the Full Application Files tab in eBRAP. It is the applicant's responsibility to review all application components for accuracy as well as ensure proper ordering as specified in this Program Announcement.

The application title, eBRAP log number, and all information for the PI, Business Official(s), performing organization, and contracting organization must be consistent throughout the entire pre-application and application submission process. Inconsistencies may delay application processing and limit or negate the ability to view, modify, and verify the application in eBRAP. If any changes need to be made, the applicant should contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507 prior to the application deadline.

II.D.2.a. Step 1: Pre-Application Submission Content

During the pre-application process, each submission is assigned a unique log number by eBRAP. This unique eBRAP log number will be needed during the full application submission process.

To begin the pre-application process, first select whether the submitting organization is extramural or intramural, then confirm your selection or cancel. **Incorrect selection of extramural or intramural submission type may result in delays in processing.**

If an error has been made in the selection of extramural versus intramural and the pre-application submission deadline has passed, the PI or Business Official must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

All pre-application components must be submitted by the PI through eBRAP (https://eBRAP.org/). Because the invitation to submit an application is based on the contents of the pre-application, investigators should not change the title or research objectives after the pre-application is submitted.

PIs and organizations identified in the pre-application should be the same as those intended for the subsequent application submission. If any changes are necessary after submission of the pre-application, the PI must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

The pre-application consists of the following components, which are organized in eBRAP by separate tabs (refer to the General Application Instructions, Section II.B, for additional information on pre-application submission):

- **Tab 1 – Application Information**

  Select the FY17 PRMRP Topic Area addressed by the proposed research. If the proposed research project is aligned with more than one FY17 PRMRP Topic Area, select the Topic Area of highest relevance as the required first choice.

- **Tab 2 – Application Contacts**

  Enter contact information for the PI. Enter the organization's Business Official responsible for sponsored program administration (the "person to be contacted on matters involving this application" in Block 5 of the Grants.gov SF424 (R&R) Form for extramural submissions). The Business Official must be either selected from the eBRAP list or invited in order for the pre-application to be submitted.

  Select the performing organization (site at which the PI will perform the proposed work) and the contracting organization (organization submitting on behalf of the PI, which corresponds to Block 5 on the Grants.gov SF424 (R&R) Form for extramural submissions), and click on "*Add Organizations to this Pre-application.*" The organizations must be either selected from the eBRAP drop-down list or invited in order for the pre-application to be submitted.

  It is recommended that PIs identify an Alternate Submitter in the event that assistance with pre-application submission is needed.

- **Tab 3 – Collaborators and Key Personnel**

  Enter the name, organization, and role of all collaborators and key personnel associated with the application.

  FY17 PRMRP Programmatic Panel members should not be involved in any pre-application or application. For questions related to Panel members and pre-applications or applications, refer to Section II.H.2.c, Withdrawal, or contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

  To preserve the integrity of its peer and programmatic review processes, the CDMRP discourages inclusion of any employee of its review contractors having any role in pre-application or application preparation, research, or other duties for submitted pre-applications or applications. For FY17, the identities of the peer review contractor and the programmatic review contractor may be found at the CDMRP website (http://cdmrp.army.mil/about/2tierRevProcess). Pre-applications or applications that include names of personnel from either of these companies will be administratively withdrawn unless plans to manage COIs are provided and deemed appropriate by the Grants Officer. Refer to the General Application Instructions, Appendix 3, for detailed information.

- **Tab 4 – Conflicts of Interest**

  List all individuals other than collaborators and key personnel who may have a COI in the review of the application (including those with whom the PI has a personal or professional relationship). Refer to the General Application Instructions, Appendix 3, Section C, for further information regarding COIs.

- **Tab 5 – Pre-Application Files**

  Note: Upload documents as individual PDF files unless otherwise noted. eBRAP will not allow a file to be uploaded if the number of pages exceeds the limit specified below.

- **Preproposal Narrative (five-page limit):** The Preproposal Narrative page limit applies to text and non-text elements (e.g., figures, tables, graphs, photographs, diagrams, chemical structures, drawings) used to describe the project. Inclusion of URLs that provide additional information to expand the Preproposal Narrative and could confer an unfair competitive advantage is prohibited and may result in administrative withdrawal of the pre-application.

  The Preproposal Narrative should include the following:

  - **Topic Area:** Describe how the proposed program relates to at least one of the FY17 PRMRP Topic Areas. If applicable, describe how the proposed research program addresses an FY17 PRMRP Area of Encouragement (Appendix 2).

  - **Overarching Challenge:** Describe the unifying challenge or question to be addressed and how it is relevant to a critical problem or question in the field of research and/or patient care in at least one of the FY17 PRMRP Topic Area(s). Clearly articulate the rationale for the overarching challenge; include relevant preliminary data and literature citations.

  - **Research Strategy:** The FY17 PRMRP Focused Program Award strongly encourages a minimum of four individual but complementary research projects addressing the overarching challenge. For each proposed project, state the hypothesis to be tested, the specific aims, and the objectives to be reached. Briefly describe the experimental approach. Describe how the projects are interrelated to and synergistic with each other and align with the overarching challenge.

  - **Impact:** Describe the potential short-term and long-term impact of the proposed research on at least one of the FY17 PRMRP Topic Areas and its related research field(s), and patient population(s). Explain how the effort is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

  - **Research Team:** Briefly describe the composition, expertise, and organization of the research team. Identify the project leaders and describe each team member's role in the projects, with additional emphasis on the leadership role of the PI. Briefly describe how these features will facilitate the success of the key aspects of the projects.

  - **Clinical Trial (if applicable):** If the proposed research includes a clinical trial(s), briefly state the clinical intervention(s), subject population(s), and the type and phase of the clinical trial(s). Describe the objectives of the clinical trial(s), how it addresses the overarching challenge, and how it complements the other proposed projects. ***Only small-scale (i.e., up to and including Phase II or equivalent) clinical trials are allowed.***

- **Pre-Application Supporting Documentation:** The items to be included as supporting documentation for the pre-application ***must be uploaded as individual files*** and are limited to the following:

- References Cited (one-page limit): List the references cited (including URLs if available) in the Preproposal Narrative using a standard reference format that includes the full citation (i.e., author[s], year published, reference title, and reference source, including volume, chapter, page numbers, and publisher, as appropriate).

- List of Abbreviations, Acronyms, and Symbols: Provide a list of abbreviations, acronyms, and symbols used in the Preproposal Narrative.

- Key Personnel Biographical Sketches (five-page limit per individual). ***All biographical sketches should be uploaded as a single combined file.*** Biographical sketches should be used to demonstrate background and expertise through education, positions, publications, and previous work accomplished.

- **Tab 6 – Submit Pre-Application**

  This tab must be completed for the pre-application to be accepted and processed.

Pre-Application Screening

Pre-Application Screening Criteria

To determine the technical merits of the pre-application and the relevance to the mission of the DHP and the PRMRP, pre-applications will be screened based on the following criteria:

- **Overarching Challenge:** How well the unifying challenge or question addresses a critical problem or question in the field of research and/or patient care of one or more FY17 PRMRP Topic Area(s). How well the rationale supports the overarching challenge.

- **Research Strategy:** How well a hypothesis and specific aims are defined for each proposed project and to what extent each project's approach will address them. How well the proposed projects complement each other and synergistically address the overarching challenge to advance a solution beyond what would be possible through individual efforts.

- **Impact:** Whether the potential immediate and long-range outcome(s)/product(s) (intellectual and/or material) of the proposed research, if successful, will impact a central critical problem or question in the field of research and/or patient care in the FY17 PRMRP Topic Area(s) addressed. To what degree the project is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

- **Research Team:** To what degree the background and expertise of the PI, project leaders, and key personnel are appropriate with respect to their abilities to successfully complete the projects and the extent to which the PI is well prepared and committed to lead the research team and proposed projects.

Notification of Pre-Application Screening Results

Following the pre-application screening, PIs will be notified as to whether or not they are invited to submit applications; however, they will not receive feedback (e.g., a critique of strengths and

weaknesses) on their pre-application. The estimated time frame for notification of invitation to submit an application is indicated in Section I, Overview of the Funding Opportunity. Invitations to submit a full application are based on the Pre-Application Screening Criteria listed above.

II.D.2.b. Step 2: Full Application Submission Content

Applications will not be accepted unless the PI has received notification of invitation.

All contributors and administrators to the application must use matching compatible versions of Adobe software when editing and preparing application components. The use of different software versions will result in corruption of the submitted file. Refer to the General Application Instructions, Section III, for details on compatible Adobe software.

The CDMRP cannot make allowances/exceptions to its policies for submission problems encountered by the applicant organization using system-to-system interfaces with Grants.gov.

Each application submission must include the completed full application package for this Program Announcement. The full application package is submitted by the Authorized Organizational Representative through Grants.gov (http://www.grants.gov/) for extramural organizations or through eBRAP (https://ebrap.org/) for intramural organizations. See Table 1 below for more specific guidelines.

II.D.2.b.i. Full Application Guidelines

Extramural organizations, including non-DoD Federal agencies, must submit full applications through Grants.gov. Submissions of extramural applications through eBRAP may be withdrawn.

Table 1. Full Application Submission Guidelines

Extramural Submissions	Intramural DoD Submissions
Application Package Location	
Download application package components for W81XWH-17-PRMRP-FPA from Grants.gov (http://www.grants.gov).	Download application package components for W81XWH-17-PRMRP-FPA from eBRAP (https://ebrap.org).
Full Application Package Components	
SF424 (R&R) Application for Federal Assistance Form: Refer to the General Application Instructions, Section III.A.1, for detailed information.	**Tab 1 – Summary:** Provide a summary of the application information. **Tab 2 – Application Contacts:** This tab will be pre-populated by eBRAP; add Authorized Organizational Representative.

Extramural Submissions	Intramural DoD Submissions
Descriptions of each required file can be found under Full Application Submission Components: • Attachments • Research & Related Senior/Key Person Profile (Expanded) • Research & Related Budget • Project/Performance Site Location(s) Form • R&R Subaward Budget Attachment(s) Form (if applicable)	**Tab 3 – Full Application Files:** Upload files under each Application Component in eBRAP. Descriptions of each required file can be found under Full Application Submission Components: • Attachments • Key Personnel • Budget • Performance Sites **Tab 4 – Application and Budget Data:** Review and edit proposed project start date, proposed end date, and budget data pre-populated from the Budget Form.
Application Package Submission	
Submit package components to Grants.gov (https://www.grants.gov/). If either the Project Narrative or the budget fails eBRAP validation or if the Project Narrative or the budget need to be modified, an updated Grants.gov application package must be submitted via Grants.gov as a "Changed/Corrected Application" with the previous Grants.gov Tracking ID prior to the application submission deadline.	**Submit package components to eBRAP (https://ebrap.org).** **Tab 5 – Submit/Request Approval Full Application:** After all components are uploaded and prior to the full application submission deadline, enter your password in the space provided "Enter Your Password Here" and press the "Submit Full Application" button. eBRAP will notify your Resource Manager/Comptroller or equivalent Business Official by email to log into eBRAP to review and to approve prior to the application submission deadline.
Application Verification Period	
The full application package submitted to Grants.gov may be viewed and modified in eBRAP until the end of the application verification period. During the application verification period, the full application package, ***with the exception of the Project Narrative and Budget Form,*** may be modified.	After eBRAP has processed the full application, the organizational Resource Manager/ Comptroller or equivalent Business Official and PI(s) will receive an email notification of this status and will be able to view and modify application components in eBRAP. During the application verification period, the full application package, ***with the exception of the Project Narrative and Budget Form,*** may be modified.
Further Information	
Refer to the General Application Instructions, Section III, for further information regarding Grants.gov requirements.	Refer to the General Application Instructions, Section IV, for further information regarding eBRAP requirements.

The organization's Business Official or Authorized Organization Representative (or Resource Manager/Comptroller) should approve/verify the full application submission prior to the application verification deadline.

Application viewing, modification, and verification in eBRAP are strongly recommended, but not required. ***The Project Narrative and Budget cannot be changed after the application submission deadline.*** Prior to the full application deadline, a corrected or modified full application package may be submitted. Other application components may be changed until the end of the application verification period. After the end of the application verification period, the full application cannot be modified.

Material submitted after the end of the application verification period, unless specifically requested by the Government, will not be forwarded for processing.

The full application package must be submitted using the unique eBRAP log number to avoid delays in application processing.

II.D.2.b.ii. Full Application Submission Components:

- **Extramural Applications Only –**

  SF424 (R&R) Application for Federal Assistance Form: Refer to the General Application Instructions, Section III.A.1, for detailed information.

- **Extramural and Intramural Applications –**

  Attachments:

  Each attachment to the full application components must be uploaded as an individual file in the format specified and in accordance with the formatting guidelines listed in the General Application Instructions, Appendix 4.

  For all attachments, ensure that the file names are consistent with the guidance. Attachments will be rejected if the file names are longer than 50 characters or incorrect file names that contain characters other than the following: A-Z, a-z, 0-9, underscore, hyphen, space, and period. In addition, there are file size limits that may apply in some circumstances. Individual attachments may not exceed 20 MB and the file size for the entire full application package may not exceed 200 MB.

  - **Attachment 1: Project Narrative (40-page limit):** Upload as "ProjectNarrative.pdf." The page limit of the Project Narrative applies to text and non-text elements (e.g., figures, tables, graphs, photographs, diagrams, chemical structures, drawings) used to describe the project. Inclusion of URLs that provide additional information to expand the Project Narrative and could confer an unfair competitive advantage is prohibited and may result in administrative withdrawal of the application.

    Describe the proposed project in detail using the outline below.

Overall Program: Provide a description of the comprehensive effort using the following outline. Applicants are strongly encouraged to submit a minimum of four research projects; additional studies are allowed. Emphasize areas of synergy throughout the narrative.

- **Overarching Challenge:** Describe the unifying, overarching challenge or question to be addressed and how it is relevant to a critical problem or question in the field of research and/or patient care in one or more FY17 PRMRP Topic Areas. Clearly articulate the rationale for the overarching challenge; include relevant literature citations. Clearly describe how the proposed research projects are interrelated and synergistic and will advance toward a solution through a multidisciplinary research program. Describe how each project will address the overarching challenge in a unique but complementary way and how the combined efforts of the projects will address the overarching challenge more effectively than if the projects were conducted independently.

- **Leadership:** Describe how the PI's research experience, leadership skills, and commitment to making an impact in his/her field of research and/or patient care demonstrate substantial qualifications to coordinate this collaborative effort. Describe the PI's demonstrated success in leading large, focused projects and outline the PI's responsibilities during the conduct of the award. Discuss the qualifications of the research team being brought together by the PI and how the assembled expertise will create a robust, synergistic collaboration necessary to address the overarching challenge and enable the success of the proposed research.

- **Implementation Plan and Environment:** Provide an overall strategic plan for completing the proposed projects that identifies critical milestones. Outline the knowledge, expertise, and technical innovations that the investigative team will utilize to make decisions, allocate resources, and accomplish the milestones. Describe and/or provide evidence that the research can be initiated without delay once the award is made. Present an overall management plan to facilitate consistent and intensive interactions by all team members, including aspects such as adherence to regulatory requirements, administrative support, and oversight to accelerate translation of the projects' outcomes to patients and/or for clinical use. Describe the research environment(s) and how the facilities and resources will support the research requirements and the collaboration. Outline shared resources and/or cores that will be created and/or leveraged through the award. Describe plans for communication, data transfer among the collaborating institutions, and how data, specimens, and/or imaging products obtained during the study will be handled. If applicable, describe how Standard Operating Procedures will be created, reviewed, implemented, and modified during the course of the award. Describe how individual project performance will be assessed during the course of the award, including progression toward defined milestones, realization of study objectives, and addressing the overarching challenge. If an External Advisory Board (EAB) is to be utilized, describe the role of the board and the expertise to be sought in its members. To avoid potential COIs in the review of the application, potential candidates for an EAB should not be contacted, recruited, or named during the application process.

Research Plan: Provide the following details for each proposed research project, organizing each project clearly and separately. Start each project on a separate page:

- **Title:** Provide a title for each project.

- **Project Leader:** Identify the project leader and any key personnel, as appropriate, describing each person's qualifications and specific contributions to the project.

- **Background:** Briefly describe the ideas and reasoning on which the proposed work is based. Provide sufficient preliminary data to support the feasibility of work proposed. If the project is exploratory/hypothesis-developing, preliminary data are not required. For each project, the leader must demonstrate logical reasoning and provide a sound scientific rationale for the proposed project as established through a critical review and analysis of published literature. If proposing translational or clinical research, it is important to describe the project showing proof of concept and, if applicable, efficacy in an in-vivo system(s) to support the translational feasibility and promise of the approach.

- **Hypothesis/Objective:** State the hypothesis to be tested and/or the objective(s) to be reached.

- **Specific Aims:** Concisely explain each project's specific aims. The specific aims should align with the overall goal of the program and associated tasks described in the Statement of Work.

- **Research Strategy:** Describe the experimental design, methods, and analyses, in sufficient detail for analysis. Provide a description of how the study will be controlled and how the study variables will be measured. If the project is a clinical trial, define the primary and secondary endpoints and how they will be assessed. Explain how the research strategy will address the overarching challenge and meet appropriate milestones. Address potential problem areas and present alternative methods and approaches. Clearly describe the statistical plan and the rationale for the statistical methodology as well as an appropriate power analysis, if applicable. If animal studies are proposed, describe how they will be conducted in accordance with the ARRIVE guidelines (http://www.elsevier.com/__data/promis_misc/622936arrive_guidelines.pdf). Justify how the model system or human subjects/samples are appropriate to the proposed research project. If human subjects or human biological samples will be used, describe the study population and include a detailed plan for the recruitment of human subjects or the acquisition of samples. Describe the availability of the proposed study population and past successes in recruiting similar populations. If active duty military, military families, and/or Veteran population(s) or datasets will be used in the proposed research project, describe how access to the population(s)/dataset(s) will be obtained. If applicable, describe how data will be reported and how it will be assured that the documentation will support a regulatory filing with the U.S. Food and Drug Administration (FDA).

- **Statistical Plan:** Clearly describe a statistical plan appropriate to the type of study; provide the rationale for the statistical methodology. Define the number of samples and/or subjects (animal and/or human) to be used, and include a power analysis to demonstrate that the sample size is appropriate to meet the objectives of the study.

- **Impact:** Describe the anticipated outcome(s)/product(s) (intellectual and/or material) that will be directly attributed to the results of the proposed research. Explain the anticipated long-term gains from this research. Compare to the information known/products currently available, if applicable.

- **Clinical Trial (if applicable):** ***Only small-scale (e.g., up to and including Phase II or equivalent) clinical trials are allowed.*** Provide detailed plans for initiating and conducting the clinical trial during the course of this award. As appropriate, outline a plan for applying for and obtaining Investigational New Drug/Investigational Device Exemption (IND/IDE) status (or other FDA approvals). Describe the type of clinical trial to be performed (e.g., prospective, randomized, controlled) and indicate the phase of trial and/or class of device, as appropriate. Outline the regulatory strategy. Provide preclinical and/or clinical evidence to support the safety of the intervention.

    - Identify the intervention to be tested and describe the projected outcomes. Describe how the proposed intervention compares with currently available interventions and/or standards of care. Include a discussion of any current clinical use of the intervention under investigation, and/or details of its study in clinical trials for other indications (as appropriate).

    - Describe the methods that will be used to recruit a sample of human subjects from the accessible population (e.g., convenience, simple random, stratified random). Provide information on and justification for the inclusion and exclusion criteria.

    - Describe the process for obtaining informed consent and any screening procedures required to determine eligibility for study participation.

    - Describe the human subject-to-group assignment process (e.g., randomization, block randomization, stratified randomization, age-matched controls, alternating group, or other procedures), if applicable. Explain the specific actions to accomplish the group assignment (e.g., computer assignment, use of table of random numbers). If multiple site studies are involved, state the approximate number of subjects to be enrolled at each site.

    - Provide evidence to document the availability of and access to all critical reagents, including the intervention itself, if applicable, for the duration of the proposed trial.

    - Describe how quality control will be addressed. Describe how compliance with Good Laboratory Practices (GLP), Good Manufacturing Practices (GMP), and Good Clinical Practices (GCP) guidelines will be established, monitored, and maintained, as applicable.

- Describe the composition of the clinical trial team. Provide details on how the team (including investigator(s), study coordinator, and statistician) possesses the appropriate expertise in conducting clinical trials.

- **Attachment 2: Supporting Documentation:** Combine and upload as a single file named "Support.pdf." Start each document on a new page. If documents are scanned to PDF, the lowest resolution (100 to 150 dpi) should be used. The Supporting Documentation attachment should not include additional information such as figures, tables, graphs, photographs, diagrams, chemical structures, or drawings. These items should be included in the Project Narrative. Any additional material viewed as an extension of the Project Narrative will be removed or may result in administrative withdrawal of the application.

  There are no page limits for any of these components unless otherwise noted. Include only those components described below; inclusion of items not requested will result in the removal of those items or may result in administrative withdrawal of the application.

  - References Cited: List the references cited (including URLs, if available) in the Project Narrative using a standard reference format that includes the full citation (i.e., author[s], year published, title of reference, source of reference, volume, chapter, page numbers, and publisher, as appropriate).

  - List of Abbreviations, Acronyms, and Symbols: Provide a list of abbreviations, acronyms, and symbols.

  - Facilities, Existing Equipment, and Other Resources: Describe the facilities and equipment available for performance of the proposed project and any additional facilities or equipment proposed for acquisition at no cost to the award. Indicate whether or not Government-furnished facilities or equipment are proposed for use. If so, reference should be made to the original or present Government award under which the facilities or equipment items are now accountable. There is no form for this information.

  - Publications and/or Patents: Include a list of relevant publication URLs and/or patent abstracts. If publications are not publicly available, then copies of up to eight published manuscripts may be included in Attachment 2. Extra items will not be reviewed.

  - Letters of Organizational Support: Provide a letter (or letters, if applicable), signed by the Department Chair or appropriate organization official, confirming the laboratory space, equipment, and other resources available for the project. Letters of support not requested in the Program Announcement, such as those from members of Congress, do not impact application review or funding decisions.

  - Letters of Collaboration: Provide a signed letter from each collaborating individual or organization that will demonstrate that the PI has the support or resources necessary for the proposed work. If an investigator at an intramural organization is

named as a collaborator on an application submitted through an extramural organization, the application must include a letter from the collaborator's Commander or Commanding Officer at the intramural organization that authorizes the collaborator's involvement.

- Letters of Commitment (if applicable): If the proposed study involves use of a commercially produced investigational drug, device, or biologic, provide a letter of commitment from the commercial entity indicating availability of the product for the duration of the study, support for the proposed phase of research, and support for the indication to be tested.

- Letters Confirming Access to Military or VA Patient Populations or Resources (if applicable): If the proposed research plan involves access to active duty military and/or VA patient populations or resources, include a letter of support, signed by the lowest ranking person with approval authority, confirming such access. If access cannot be confirmed at the time of application submission, the Government reserves the right to withhold or revoke funding until the PI has demonstrated support for and access to the relevant population(s) and/or resources.

- Intellectual Property: Information can be found in Code of Federal Regulations, Title 2, Part 200.315 (2 CFR 200.315), "Intangible Property."

  - Intellectual and Material Property Plan (if applicable): Provide a plan for resolving intellectual and material property issues among participating organizations.

- Data and Research Resources Sharing Plan: Describe how data and resources generated during the performance of the project will be shared with the research community. Refer to the General Application Instructions, Appendix 2, Section K, for more information about the CDMRP expectations for making data and research resources publicly available.

- **Attachment 3: Technical Abstract (one-page limit):** Upload as "TechAbs.pdf." The technical abstract is used by all reviewers. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information.*** Use only characters available on a standard QWERTY keyboard. Spell out all Greek letters, other non-English letters, and symbols. Graphics are not allowed.

  Clarity and completeness within the space limits of the technical abstract are highly important. Describe the proposed research effort including the following elements:

  - **Overarching Challenge:** Identify the unifying, overarching challenge or question that will be addressed by the research plan and describe how it relates to a critical problem or question in one or more of the FY17 PRMRP Topic Areas.

  - **Background:** Briefly articulate the rationale for the overarching challenge and the proposed research.

- **Research Plan:** Provide a brief description of the studies proposed, including hypotheses, objectives, and scientific approach.

- **Impact:** Briefly describe the potential short-term and long-term impact of the results of the proposed research on at least one of the FY17 PRMRP Topic Areas and its related research field(s) and patient population(s).

- **Military Relevance:** Explain how the effort is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

- **Attachment 4: Lay Abstract (one-page limit):** Upload as "LayAbs.pdf." The lay abstract is used by all reviewers. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information***. Use only characters available on a standard QWERTY keyboard. Spell out all Greek letters, other non-English letters, and symbols. Graphics are not allowed.

  State the FY17 PRMRP Topic Area(s) addressed by the proposed research program. Include a comprehensive overview of the effort that can be ***readily understood by readers without a background in science or medicine.*** Clearly describe the central critical problem or question to be addressed and the ultimate applicability and impact of the research. Do not duplicate the technical abstract.

- **Attachment 5: Statement of Work (SOW) (eight-page limit):** Upload as "SOW.pdf." The suggested SOW format and examples specific to different types of research projects are available on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm). For the Focused Program Award mechanism, use the SOW format example titled "SOW for Collaborative Projects ." The SOW must be in PDF format prior to attaching.

  The SOW should include a list of major tasks that support the proposed specific aims, followed by a series of subtasks outlined related to the major tasks and milestones within the period of performance. The SOW should describe only the work for which funding is being requested by this application and, as applicable, should also:

  Include the name(s) of the key personnel and contact information for each study site/ subaward site.

  Indicate the number (and type, if applicable) of research subjects (animal or human) and/or human anatomical samples projected or required for each task and at each site. Refer to the General Application Instructions, Appendix 1, for additional information regarding regulatory requirements.

  Briefly state the methods to be used.

  For studies with prospective accrual of human subjects, indicate quarterly enrollment targets.

Identify cell line(s) and commercial or organizational source(s) to be used. If human anatomical substances (including cell lines) will be used, specify whether or not identifiable information is accessible to the research team by any means.

If applicable, indicate timelines required for regulatory approvals relevant to human subjects research (e.g., IND/IDE applications) by the FDA or other Government agency.

- **Attachment 6: Impact Statement (two-page limit): Upload as "Impact.pdf."**

  - Explain why the proposed program is important and relevant to understanding the cause or progression of the disease or condition, and/or to developing improvements in prevention, detection, diagnosis, treatment, or quality of life in the FY17 PRMRP Topic Area(s) addressed. Describe how the overarching challenge addresses a central critical problem or question in the relevant Topic Area(s). If applicable, describe how the project addresses an FY17 PRMRP Area of Encouragement (Appendix 2). In addition to articulating the potential impact of the overall program, address the potential impact of each individual project. Explain how the various projects' outcomes will ultimately be translated to patients.

  - ***Describe the short-term impact:*** Detail the anticipated outcome(s)/product(s) (intellectual and/or material) that will be directly attributed to the results of the proposed research.

  - ***Describe the long-term impact:*** Explain the anticipated long-term gains from this research. Compare to the information known/products currently available, if applicable. Explain the long-range vision for how the research will impact the field of study and/or patient care.

- **Attachment 7: Military Relevance Statement (one-page limit): Upload as "MilRel.pdf."**

  - Describe how the proposed effort is responsive to the healthcare needs of military Service members, Veterans, and/or beneficiaries. Provide information about the incidence and/or prevalence of the disease or condition to be studied in the general population as well as in military Service members, Veterans, and/or beneficiaries.

  - If active duty military, military families, and/or Veteran population(s) or dataset(s) will be used in the proposed research project, describe the population(s)/dataset(s) and the appropriateness of the population(s)/dataset(s) for the proposed study. If a non-military population will be used for the proposed research project, explain how the population simulates the targeted population (i.e., military Service members, Veterans, and/or beneficiaries).

  - If applicable, show how the proposed research project aligns with DoD and/or VA areas of research interest and/or patient care. Provide a description of how the knowledge, information, products, or technologies gained from the research could be implemented in a dual-use capacity to benefit the civilian population and address a military need, as appropriate.

- **Attachment 8: Transition Plan and Regulatory Strategy (three-page limit): Upload as "Transition.pdf."**

  - Provide information on the methods and strategies proposed to move the product or knowledge outcomes of the program to the next phases of development and to eventual clinical use. Articulate this information for the overall effort as well as the individual projects. Applicants are encouraged to work with their organization's Technology Transfer Office (or equivalent) to develop the transition plan. The transition plan should include the components listed below, as appropriate:

  - A description of the outcomes expected upon completion of the proposed research efforts. Outcomes should be specific, measurable, and should include the intended end user.

  - Details of the funding strategy that will be used to bring the outcomes to the next phase of development and/or delivery to market or incorporation into patient care (e.g., specific potential industry partners, specific funding opportunities to be applied for).

  - Details of the development plan and FDA regulatory strategy, to include considerations for compliance with GMP, GLP, and GCP guidelines (if appropriate).

  - For knowledge outcomes, a description of how the knowledge will be further developed, disseminated, and incorporated into clinical/patient care.

  - A description of collaborations and other resources that will be used to provide continuity of development.

  - A brief schedule and milestones for bringing the outcomes to the next phase of development (e.g., further research, clinical trials, transition to industry, delivery to the market, incorporation into clinical practice, approval by the FDA).

  - If applicable, ownership rights and/or access to the intellectual property necessary for the development and/or commercialization of products or technologies supported with this award and the Government's ability to access such products or technologies in the future.

- **Attachment 9: Data and Research Resource Sharing Plan (one-page limit): Upload as "Sharing.pdf."**

  - Describe how data and resources generated during the performance of the proposed research projects will be shared with the research community. This includes cases where pre-existing data or research resources will be utilized and/or modified during the course of the proposed projects. Specifically describe a plan to make animal models, tissue samples, and other resources developed as part of the proposed research projects available to the scientific community. If there are limitations associated with a pre-existing agreement for the original data or research resources that preclude subsequent sharing, the applicant should explain this in the data- and/or

research resource-sharing plan. Refer to the General Application Instructions, Appendix 2, Section K, for more information about the CDMRP expectations for making data and research resources publicly available.

- In preparing requested budgets, applicants may include anticipated costs associated with data- and research resource-sharing (i.e., making a large dataset available to the public or developing an important resource for the scientific community).

- **Attachment 10: IND/IDE Documentation:** ***Only applicable for applications that include a clinical trial(s).*** If submitting multiple documents, start each document on a new page. **Combine and upload as a single file named "IND-IDE.pdf."** The IND/IDE Documentation Form located on the eBRAP website may ***not*** be used in place of this information:

  - State whether the trial(s) requires regulation by the FDA. If FDA regulation is required, describe the planned indication for the proposed product and whether an IND/IDE is necessary.

  - If an IND or IDE application is required, indicate when it was submitted to the FDA and provide an explanation of the status of the IND or IDE application (e.g., past the critical 30-day period, pending response to questions raised by the Agency, on clinical hold). If the IND or IDE application has not been submitted to the FDA yet, provide a detailed timeline with appropriate milestones for application preparation and submission. Identify any consultants or experts who will assist in the regulatory application, if applicable, and include a copy of any curricula vitae or biographical sketches in the Key Personnel Biographical Sketches section of the application. Provide a summary of previous meetings with the FDA on development of this product, if appropriate. A copy of the Agency meeting minutes should be included if available. Provide copies of communications from the FDA relevant to the most recent status of the IND or IDE application.

  - If an IND or IDE is not required for the proposed trial(s), or if it qualifies for an abbreviated IDE, provide evidence in the form of communication from the FDA or the IRB of record to that effect. Devices qualifying for an abbreviated IDE must comply with the abbreviated IDE requirements but do not require the submission of an IDE application to the FDA.

- **Attachment 11: DoD Military Budget Form(s), if applicable: Upload as "MFBudget.pdf."** If a military facility (military health system facility, research laboratory, treatment facility, dental treatment facility, or a DoD activity embedded with a civilian medical center) will be a collaborator in performance of the project, complete the DoD Military Budget Form, available for download on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm), including a budget justification, for each military facility as instructed. The costs per year should be included on the Grants.gov Research and Related Budget form under subaward costs. Refer to the General Application Instructions, Section III.A.7, for detailed information.

- **Extramural and Intramural Applications –**

  Research & Related Senior/Key Person Profile (Expanded): For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.3, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.2, for detailed information.

  - PI Biographical Sketch (five-page limit): Upload as "Biosketch_LastName.pdf." The suggested biographical sketch format is available on the "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) in eBRAP. The National Institutes of Health Biographical Sketch may also be used. All biographical sketches should be submitted in the portable document format (PDF) that is not editable.

  - PI Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."

  - Key Personnel Biographical Sketches (five-page limit each): Upload as "Biosketch_LastName.pdf."

  - Key Personnel Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."

  Research & Related Budget: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.4, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.3, for detailed information.

  Budget Justification (no page limit): Upload as "BudgetJustification.pdf." The budget justification for the entire period of performance must be uploaded to the Research & Related Budget after completion of the budget for Period 1.

  Project/Performance Site Location(s) Form: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.5, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.4, for detailed information.

- **Extramural Applications Only –**

  R&R Subaward Budget Attachment(s) Form (if applicable): Refer to the General Application Instructions, Section III.A.6, for detailed information.

  - **Extramural Subaward:** Complete the Research & Related Subaward Budget Form through Grants.gov. (Refer to the General Application Instructions, Section III.A.6, for detailed information.)

  - **Intramural DoD Collaborator(s):** Complete the DoD Military Budget Form and upload to Grants.gov as Attachment 11. (Refer to the General Application Instructions, Section III.A.7, for detailed information.) Intramural DoD Collaborator(s) costs per year

should be included on the Grants.gov Research and Related Budget form under subaward costs. **DoD Military Budget Form:** A military facility collaborating in the performance of the project should be treated as a subaward for budget purposes. However, do not complete the Grants.Gov R&R Subaward Budget Attachment Form; instead, complete the DoD Military Budget Form (Attachment 11) to show all direct and indirect costs. The costs per year should be included on the Grants.gov Research & Related Budget Form under subaward costs. Refer to the General Application Instructions, Section III.A.7, for detailed information.

II.D.3. Dun and Bradstreet Universal Numbering System (DUNS) Number and System for Award Management (SAM)

Applicant organizations and all subrecipient organizations must have a DUNS number to submit applications to Grants.gov. The applicant organization must also be registered in the Entity Management functional area of the SAM with an "Active" status to submit applications through the Grants.gov portal. Verify the status of the applicant's organization's Entity registration in SAM well in advance of the application submission deadline. Allow 3 to 4 weeks to complete the entire SAM registration process. If an applicant has not fully complied with the requirements by the time the Federal awarding agency is ready to make a Federal award, the Federal awarding agency may determine that the applicant is not qualified to receive a Federal award and use that determination as a basis for making a Federal award to another applicant. Refer to the General Application Instructions, Section III, for further information regarding Grants.gov requirements.

II.D.4. Submission Dates and Times

All submission dates and times are indicated in Section I, Overview of the Funding Opportunity. Pre-application and application submissions are required. The pre-application and application submission process should be started early to avoid missing deadlines. There are no grace periods. Failure to meet either of these deadlines will result in submission rejection.

Applicant Verification of Full Application Submission in eBRAP

Prior to the end of the application verification period, PIs and organizational representatives can review and modify in eBRAP certain components of a submitted application. Following retrieval and processing of the full application, eBRAP will notify the organizational representatives and PI by email to log into eBRAP to review, modify, and verify the full application submission. eBRAP will validate retrieved files against the specific Program Announcement requirements and discrepancies will be noted in both the email and in the Full Application Files tab in eBRAP. eBRAP does not confirm the accuracy of file content. It is the applicant's responsibility to review all application components and ensure proper ordering as specified in the Program Announcement. ***If either the Project Narrative or the budget fails eBRAP validation or needs to be modified, an updated full application package must be submitted prior to the application submission deadline.*** The Project Narrative and Budget Form cannot be changed after the application submission deadline.

II.D.5. Funding Restrictions

The maximum period of performance is **4** years.

The anticipated total costs budgeted for the entire period of performance will not exceed **$10M**. If indirect cost rates have been negotiated, indirect costs are to be budgeted in accordance with the organization's negotiated rate. No budget will be approved by the Government exceeding **$10M** total costs or using an indirect cost rate exceeding the organization's negotiated rate.

All direct and indirect costs of any subaward or contract must be included in the total direct costs of the primary award.

The applicant may request the entire maximum funding amount for a project that may have a period of performance less than the maximum **4** years.

For this award mechanism, direct costs must be requested for:

Travel costs for the PI and up to three additional members of the research team to attend a 1-day Milestone Meeting to be held in the National Capital Area during the award period of performance. This meeting will be held to provide a presentation on progress. Costs associated with travel to this meeting should be included in Year 3 of the budget. These travel costs are in addition to those allowed for annual scientific/technical meetings.

May be requested for (not all-inclusive):

- Salary
- Research supplies
- Equipment
- Research-related subject costs
- Clinical research costs
- Support for multidisciplinary collaborations, including travel
- Travel costs for up to four investigators to travel to one scientific/technical meeting per year in addition to the required meeting described above

Extramural (non-Federal) awards will consist solely of assistance agreements (Cooperative Agreements and Grants). For extramural awards with an intragovernmental component, direct transfer of funds from an extramural award recipient to a DoD or other Federal agency is not allowed except under very limited circumstances. Funding to intramural DoD and other Federal agencies will be managed through a direct fund transfer. Intragovernmental only funding to intramural DoD and other Federal agencies will be managed through a direct fund transfer. Intramural applicants are responsible for coordinating through their agency's procedures the use

of contractual or assistance funding awards or other appropriate agreements to support extramural collaborators.

Refer to the General Application Instructions, Section III.A.4, for budget regulations and instructions for the Research & Related Budget. ***For Federal agencies or organizations collaborating with Federal agencies, budget restrictions apply as are noted in the General Application Instructions, Section III.A.4***.

The CDMRP expects to allot approximately $60M of the $300M FY17 appropriation to fund approximately six Focused Program Award applications, depending on the quality and number of applications received. Funding of applications received in response to this Program Announcement is contingent upon the availability of Federal funds for this program.

II.D.6. Other Submission Requirements

Refer to the General Application Instructions, Appendix 4, for detailed formatting guidelines.

II.E. Application Review Information

II.E.1. Criteria

II.E.1.a. Peer Review

To determine technical merit, all applications will be evaluated according to the following scored criteria, which are of equal importance:

Scored Review Criteria for the Overall Program:

- **Overall Impact**
    - To what extent the overarching challenge impacts a critical problem or question in the designated FY17 PRMRP Topic Area(s).
    - To what degree the proposed program could, if successful, make a significant impact on the lives of relevant patient populations in the short term or long term.
    - How well the research projects are integrated, complement each other, and provide a synergistic, multidisciplinary approach to solving a critical problem.
    - How well the research program will, if successful:
        - Make important scientific advances in the relevant field of research
        - Promote greater understanding of the causes and progression of the relevant disease(s)/condition(s), or
        - Promote the development of improvements in prevention, detection, diagnosis, treatment, or quality of life

- **Implementation Plan**
    - How the proposed projects are supported by a detailed plan that identifies critical milestones and explains how these milestones will be achieved.
    - How well resources and/or cores that will be created or leveraged will be utilized and shared.
    - To what extent the plans to assess individual project performance during the course of the award are appropriate.
    - How well the overall management plan will facilitate consistent and intensive interactions by all team members in the projects.
    - How the proposed plans for communication, data and specimen collection, data transfer, and periodic meetings are appropriate and robust.
    - To what extent the plans for creating, reviewing, implementing, and modifying Standard Operating Procedures are appropriate, if applicable.
- **Leadership and Environment**
    - To what degree the PI is experienced in successfully leading large, focused projects and is therefore well-positioned to lead the research team in achieving the overarching goal of the proposed effort.
    - How well the PI demonstrates experience, leadership skills, and commitment to making an impact in the relevant field of research and/or patient care.
    - Whether the PI will devote a minimum of 20% effort to this award.
    - To what degree the scientific environment(s) is appropriate for the proposed research.
    - How well the research requirements are supported by the availability of and accessibility to facilities and resources (including patient populations, samples, and collaborative arrangements).
    - To what degree the quality and extent of organizational support are appropriate for the proposed research.
- **Transition Plan and Regulatory Strategy**
    - The degree to which the strategy proposed to bring the outcomes to the next level of development, including funding, milestones, and schedule, is realistic.
    - Whether appropriate collaborations and other resources for providing continuity of development are established and/or well described.
    - How the regulatory strategy and development plan are appropriate and well described.

- How well the application identifies intellectual property ownership, and whether there is sufficient evidence of a plan to resolve intellectual and material property issues, if applicable.

- Whether the applicant has demonstrated that they have access to all intellectual property rights necessary for development and commercialization and evidence that the Government has the ability to access such products or technologies, if applicable.

- If applicable, whether data will be appropriately reported and documented to support a regulatory filing with the FDA.

Scored Review Criteria for Individual Research Projects <u>without</u> a clinical trial:

- **Impact**

  - To what extent the individual project impacts the overarching challenge.

  - To what degree the individual project could, if successful, make a significant impact on the lives of relevant patient populations in the short term or long term.

  - How well the individual project will, if successful, make important scientific advances in the relevant field of research.

- **Research Strategy and Feasibility**

  - How well the scientific rationale supports the research and its feasibility, as demonstrated by a critical review and analysis of the literature, the presentation of preliminary data (where applicable), and logical reasoning.

  - How well the hypothesis, objectives, and aims are developed.

  - To what degree the experimental approach, methods, endpoints, and analyses support completion of the aims and are designed to achieve rigorous and reproducible results.

  - How well the choice of model (animal, cell line, or other) is justified and whether it is appropriate.

  - If applicable, to what degree the statistical plan and power analysis, including sample size projections, are appropriate for the proposed project.

  - Whether there is sufficient evidence to support availability and accessibility of the populations, samples, or other resources required for the study, if applicable.

  - How well potential problems are acknowledged and alternative approaches are addressed.

- **Personnel**

  - To what degree the project team's background and expertise are appropriate with respect to its ability to perform the proposed work, including whether there is evidence of

sufficient expertise for all aspects of the work and whether there is evidence of strong commitment to the projects.

- To what degree the levels of effort are appropriate for successful conduct of the proposed work.

Scored Review Criteria for Individual Research Projects <u>with</u> clinical trials:

- **Impact**
  - To what extent the individual project impacts the overarching challenge.
  - How well the individual project will, if successful, make important scientific advances in the relevant field of research.
  - How well the sample population represents the targeted patient population that might benefit from the proposed intervention.
  - How the potential outcomes of the proposed clinical trial will provide/improve short-term benefits for individuals.
  - How significantly the long-term benefits for implementation of the intervention may impact patient care and/or quality of life.
- **Research Strategy and Feasibility**
  - How well the scientific rationale supports the research and its feasibility, as demonstrated by a critical review and analysis of the literature, the presentation of preliminary data, and logical reasoning.
  - How well the hypothesis and/or objectives and specific aims are developed.
  - To what degree the experimental approach, methods, endpoints, and analyses support completion of the aims and are designed to achieve rigorous and reproducible results.
  - To what degree the statistical plan and power analysis, including sample size projections, are appropriate for the proposed trial and any proposed correlative studies.
  - How well the application demonstrates the availability of and access to the appropriate patient population(s), as well as the ability to accrue a sufficient number of subjects.
  - How well potential problems and delays (e.g., slow accrual, attrition) are acknowledged and alternative approaches are addressed.
- **Clinical Strategy**
  - How the intervention compares with currently available interventions and/or standards of care.

- Whether there is evidence of support, indicating availability of the intervention from its source, for the duration of the proposed clinical trial (if applicable).
- To what degree the PI has provided preclinical and/or clinical evidence to support the safety of the intervention.
- How well the inclusion and exclusion criteria meet the needs of the proposed clinical trial.
- The degree to which the informed consent, screening, and retention processes for human subjects will meet the needs of the proposed clinical trial.
- Whether a member of the study team holds the IND/IDE for the indication proposed or whether the timeline proposed for obtaining the IND/IDE is appropriate (if applicable).
- For investigator-sponsored INDs, whether there is evidence of appropriate institutional support, including capabilities to ensure monitoring as required by the FDA.
- Whether plans to comply with GMP, GLP, and GCP guidelines are appropriate.
- Whether measures are described to ensure the consistency of dosing of active ingredients for nutritional supplements (if applicable).

- **Personnel**
  - To what degree the project team's background and expertise are appropriate with respect to its ability to perform the proposed work, including whether there is evidence of sufficient expertise for all aspects of the work and whether there is evidence of strong commitment to the projects.
  - How well the project leader has assembled an appropriate and robust clinical team with the combined backgrounds and expertise needed to enable successful conduct of the clinical trial.
  - To what degree the levels of effort are appropriate for successful conduct of the proposed work.

In addition, the following unscored criteria will also contribute to the overall evaluation of the application:

- **Data and Resource Sharing**
  - To what degree the plan for sharing of project data and research resources is appropriate and reasonable to facilitate use by the wider research community.
- **Budget**
  - Whether the budget is appropriate for the proposed research and within the limitations of this Program Announcement.

- **Environment**
    - If applicable, to what degree the intellectual and material property plan is appropriate.
- **Application Presentation**
    - To what extent the writing, clarity, and presentation of the application components influence the review.

II.E.1.b. Programmatic Review

To make funding recommendations and select the application(s) that, individually or collectively, will best achieve the program objectives, the following criteria are used by programmatic reviewers:

- Ratings and evaluations of the peer reviewers
- Relevance to the mission of the DHP and FY17 PRMRP, as evidenced by the following:
    - Adherence to the intent of the award mechanism
    - Military relevance
    - Program portfolio composition
    - Relative impact

II.E.2. Application Review and Selection Process

All applications are evaluated by scientists, clinicians, and consumers in a two-tier review process. The first tier is peer review of applications against established criteria for determining technical merit. Each application is evaluated for its own merit, independent of other applications. The second tier is a programmatic review that makes recommendations for funding to the Commanding General, USAMRMC, on behalf of the DHA and the OASD(HA), based on technical merit, the relevance to the mission of the DHP and PRMRP, the specific intent of the award mechanism, and to other specified evaluation criteria in the Program Announcement. Programmatic review is a comparison-based process in which applications with scientific and technical merit compete in a common pool. ***The highest-scoring applications from the first tier of review are not automatically recommended for funding. Funding recommendations depend on various factors as described in Section II.E.1.b, Programmatic Review.*** Additional information about the two-tier process used by the CDMRP can be found at http://cdmrp.army.mil/about/fundingprocess.

All CDMRP review processes are conducted confidentially to maintain the integrity of the merit-based selection process. Panel members sign a statement that application and evaluation information will not be disclosed outside the panel. Violations of confidentiality can result in the dissolving of a panel(s) and other corrective actions. In addition, personnel at the applicant or collaborating organizations are prohibited from contacting persons involved in the review

process to gain protected evaluation information or to influence the evaluation process. Violations of these prohibitions will result in the administrative withdrawal of the organization's application. Violations by panel members or applicants that compromise the confidentiality of the review process may also result in suspension or debarment from Federal awards. Furthermore, the unauthorized disclosure of confidential information of one party to another third party is a crime in accordance with 18 USC 1905.

II.E.3. Integrity and Performance Information

Prior to making an assistance agreement award where the Federal share is expected to exceed the simplified acquisition threshold (currently $150,000) over the period of performance, the Federal awarding agency is required to review and consider any information about the applicant that is available in the Federal Awardee Performance and Integrity Information System (FAPIIS).

An applicant, at its option, may review FAPIIS, accessible through SAM, and submit comments to FAPIIS on any information about itself that a Federal awarding agency previously entered and is currently available in FAPIIS.

The Federal awarding agency will consider any comments by the applicant, in addition to other information in the designated integrity and performance system, in making a judgment about the applicant's integrity, business ethics and record of performance under Federal awards when determining a recipient's qualification prior to award, according to the qualification standards of the Department of Defense Grant and Agreement Regulations (DoDGAR), Section 22.415.

II.E.4. Anticipated Announcement and Federal Award Dates

All application review dates and times are indicated in Section I, Overview of the Funding Opportunity.

Each PI and organization will receive email notification of posting of the funding recommendation in eBRAP. Each PI will receive a peer review summary statement on the strengths and weaknesses of the application.

II.F. Federal Award Administration Information

II.F.1. Federal Award Notices

Awards will be made no later than September 30, 2018. Refer to the General Application Instructions, Appendix 2, for additional award administration information.

Awards are made to organizations, not to individual PIs. The types of awards made under the Program Announcement will be assistance agreements (grants or cooperative agreements). The level of involvement on the part of DoD during project performance is the key factor in determining whether to award a grant or cooperative agreement.

Extramural Organizations: An assistance agreement (grant or cooperative agreement) is appropriate when the Federal Government transfers a "thing of value," to a "state, local

government," or "other recipient," to carry out a public purpose of support or stimulation authorized by a law of the United States, instead of acquiring property or service for the direct benefit and use of the U.S. Government. An assistance agreement can take the form of a grant or cooperative agreement. If "no substantial involvement" on the part of the funding agency is anticipated, a grant award will be made (31 USC 6304). Conversely, if substantial involvement on the part of the funding agency is anticipated, a cooperative agreement will be made (31 USC 6305). Substantial involvement may include collaboration, participation, or intervention in the research to be performed under the award. The award type, along with the start date, will be determined during the negotiation process.

After email notification of application review results through the eBRAP, and if selected for funding, a representative from the USAMRAA will contact the business official authorized to negotiate on behalf of the PI's organization.

Only an appointed USAMRAA Grants Officer may obligate the Government to the expenditure of funds. No commitment on the part of the Government should be inferred from discussions with any other individual. The award document signed by the Grants Officer is the official authorizing documents.

Intramural Organizations: Awards to Federal Government organizations (to include intramural DoD organizations) will be executed through the Military Interdepartmental Purchase Request (MIPR) or Funding Authorization Document (FAD) process. Transfer of funds is contingent upon appropriate safety and administrative approvals. Intramural applicants and collaborators are reminded to coordinate receipt and commitment of funds through their respective resource managers (RM).

After email notification of application review results through the eBRAP, and if selected for funding, a representative from the CDMRP will contact the business official authorized to negotiate on behalf of the PI's organization.

II.F.1.a. Award Transfers

Changes in PI are not allowed, except under extenuating circumstances that will be evaluated on a case-by-case basis and at the discretion of the Grants Officer. An organizational transfer of an award will not be allowed in the last year of the (original) period of performance or any extension thereof.

The organizational transfer of an award supporting a clinical trial is strongly discouraged and in most cases will not be allowed. Approval of a transfer request will be on a case-by-case basis at the discretion of the Grants Officer.

Refer to the General Application Instructions, Appendix 2, Section B, for general information on organization or PI changes.

II.F.2. Administrative and National Policy Requirements

Applicable requirements in the DoDGAR found in 32 CFR, Chapter 1, Subchapter C, and 2 CFR, Chapter XI, apply to grants and cooperative agreements resulting from this Program Announcement.

Refer to the General Application Instructions, Appendix 2, for general information regarding administrative requirements.

Refer to the General Application Instructions, Appendix 5, for general information regarding national policy requirements.

Refer to full text of the USAMRAA General Research Terms and Conditions for Institutions of Higher Education, Hospitals, and Non-Profit Organizations and the USAMRAA General Research Terms and Conditions with For-Profit Organizations for further information.

II.F.3. Reporting

Refer to the General Application Instructions, Appendix 2, Section A, for general information on reporting requirements. Annual progress reports as well as a final progress report will be required.

Quarterly technical progress reports will be required. In addition to written progress reports, in-person presentations may be requested.

Award Chart: Complete the Award Chart template, a one-page PowerPoint file that must be downloaded from the CDMRP eBRAP System at https://ebrap.org/eBRAP/public/Program.htm, and submit to eBRAP at the time of award.

Award Expiration Transition Plan: Complete the Transition Plan template, a one-page Word document that must be submitted with the Final Progress Report. The Transition Plan must outline if and how the research supported by this award will progress, and include source(s) of funding, either known or pending.

Awards resulting from this Program Announcement will incorporate additional reporting requirements related to recipient integrity and performance matters. Recipient organizations that have Federal contract, grant, and cooperative agreement awards with a cumulative total value greater than $10,000,000 are required to provide information to FAPIIS about certain civil, criminal, and administrative proceedings that reached final disposition within the most recent 5-year period and that were connected with performance of a Federal award. Recipients are required to disclose semiannually information about criminal, civil, and administrative proceedings as specified in the applicable Terms and Conditions. The applicable Terms and Conditions for institutions of higher education, hospitals, and nonprofit organizations are available in OAR Article I, Section B, in July 2016 R&D General Terms and Conditions. The applicable Terms and Conditions for for-profit organizations are available in Section 34 of the February 2017 USAMRAA General Research Terms and Conditions with For-Profit Organizations.

II.G. Federal Awarding Agency Contacts

II.G.1. CDMRP Help Desk

Questions related to Program Announcement content or submission requirements as well as questions related to the pre-application or intramural application submission through eBRAP should be directed to the CDMRP Help Desk, which is available Monday through Friday from 8:00 a.m. to 5:00 p.m. ET. Response times may vary depending upon the volume of inquiries.

Phone: 301-682-5507

Email: help@eBRAP.org

II.G.2. Grants.gov Contact Center

Questions related to extramural application submission through Grants.gov portal should be directed to the Grants.gov Contact Center, which is available 24 hours a day, 7 days a week (closed on U.S. Federal holidays). Note that the CDMRP Help Desk is unable to provide technical assistance with Grants.gov submission.

Phone: 800-518-4726; International 1-606-545-5035

Email: support@grants.gov

Sign up on Grants.gov for "send me change notification emails" by following the link on the Synopsis page for the Program Announcement or by responding to the prompt provided by Grants.gov when first downloading the Grants.gov application package. If the Grants.gov application package is updated or changed, the original version of the application package may not be accepted by Grants.gov.

II.H. Other Information

II.H.1. Program Announcement and General Application Instructions Versions

Questions related to this Program Announcement should refer to the Program name, the Program Announcement name, and the Program Announcement version code 20170516b. The Program Announcement numeric version code will match the General Applications Instructions version code 20170516.

II.H.2. Administrative Actions

After receipt of pre-applications or applications, the following administrative actions may occur:

II.H.2.a. Rejection

The following will result in administrative rejection of the pre-application:

- Preproposal Narrative is missing.

The following will result in administrative rejection of the application:

- Submission of an application for which a letter of invitation was not received.
- Project Narrative exceeds page limit.
- Project Narrative is missing.
- Budget is missing.
- Submission of the same research project to different Funding Opportunities within the same program and fiscal year. Refer to Section II.D, Application and Submission Information, for exceptions.

II.H.2.b. Modification

- Pages exceeding the specific limits will be removed prior to review for all documents other than the Project Narrative.
- Documents not requested will be removed.

II.H.2.c. Withdrawal

The following may result in administrative withdrawal of the pre-application or application:

- An FY17 PRMRP Programmatic Panel member is named as being involved in the research proposed or is found to have assisted in the pre-application or application processes including, but not limited to, concept design, application development, budget preparation, and the development of any supporting documentation. ***A list of the FY17 PRMRP Programmatic Panel members can be found at http://cdmrp.army.mil/prmrp/panels/panels17.shtml***.
- The application fails to conform to this Program Announcement description to the extent that appropriate review cannot be conducted.
- Inclusion of URLs, with the exception of links in References Cited and Publication and/or Patent Abstract sections.
- Page size is larger than 8.5 inches x 11.0 inches (approximately 21.59 cm x 27.94 cm).
- To preserve the integrity of its peer and programmatic review processes, the CDMRP discourages inclusion of any employee of its review contractors having any role in the preparation, research or other duties for submitted applications. For FY17, the identities of the peer review contractor and the programmatic review contractor may be found at the

CDMRP website (http://cdmrp.army.mil/about/2tierRevProcess). Applications that include names of personnel from either of these companies will be administratively withdrawn unless plans to manage COIs are provided and deemed appropriate by the Grants Officer. Refer to the General Application Instructions, Appendix 3, for detailed information.

- Personnel from applicant or collaborating organizations are found to have contacted persons involved in the review process to gain protected evaluation information or to influence the evaluation process.
- Applications from extramural organizations, including non-DoD Federal agencies, received through eBRAP may be withdrawn.
- Applications submitted by an intramural DoD organization may be withdrawn if the intramural organization cannot coordinate the use of contractual, assistance, or other appropriate agreements to provide funds to extramural collaborators.
- The proposed research project does not address at least one of the Congressionally directed FY17 PRMRP Topic Areas.
- An application for which the PI does not meet the eligibility criteria will be withdrawn.

II.H.2.d. Withhold

Applications that appear to involve research misconduct will be administratively withheld from further consideration pending organizational investigation. The organization will be required to provide the findings of the investigation to the USAMRAA Grants Officer for a determination of the final disposition of the application.

II.H.3. Application Submission Checklist

Application Components	Action	Completed
SF424 (R&R) Application for Federal Assistance **(Extramural submissions only)**	Complete form as instructed.	
Summary (Tab 1) and Application Contacts (Tab 2) **(Intramural submissions only)**	Complete these tabs as instructed.	
Attachments	Project Narrative: Upload as Attachment 1 with file name "ProjectNarrative.pdf."	
	Supporting Documentation: Upload as Attachment 2 with file name "Support.pdf."	
	Technical Abstract: Upload as Attachment 3 with file name "TechAbs.pdf."	
	Lay Abstract: Upload as Attachment 4 with file name "LayAbs.pdf."	
	Statement of Work: Upload as Attachment 5 with file name "SOW.pdf."	
	Impact Statement: Upload as Attachment 6 with file name "Impact.pdf."	
	Military Relevance: Upload as Attachment 7 with file name "MilRel.pdf."	
	Transition Plan and Regulatory Strategy: Upload as Attachment 8 with file name "Transition.pdf."	
	Data and Research Resources Sharing Plan: Upload as Attachment 9 with file name "Sharing.pdf."	
	IND/IDE Documentation: Upload as Attachment 10 with file name "IND-IDE.pdf," if applicable.	
	DoD Military Budget Form(s): Upload as Attachment 11 with file name "MFBudget.pdf," if applicable.	
Research & Related Senior/Key Person Profile (Expanded)	Attach PI Biographical Sketch (Biosketch_LastName.pdf) to the appropriate field.	
	Attach PI Previous/Current/Pending Support (Support_LastName.pdf) to the appropriate field.	
	Attach Biographical Sketch (Biosketch_LastName.pdf) for each senior/key person to the appropriate field.	
	Attach Previous/Current/Pending (Support_LastName.pdf) for each senior/key person to the appropriate field.	

Application Components	Action	Completed
Research & Related Budget **(Extramural submissions only)**	Complete as instructed. Attach Budget Justification (BudgetJustification.pdf) to the appropriate field.	
Budget **(Intramural submissions only)**	Complete the DoD Military Budget Form and justification.	
Project/Performance Site Location(s) Form	Complete form as instructed.	
R&R Subaward Budget Attachment(s) Form, if applicable	Complete form as instructed.	
Additional Application Components	**Action**	**Completed**
Confidential Letters of Recommendation	Confirm upload to eBRAP.	

APPENDIX 1
ACRONYM LIST

ACURO	Animal Care and Use Review Office
CDMRP	Congressionally Directed Medical Research Programs
CFR	Code of Federal Regulations
DHA	Defense Health Agency
DHP	Defense Health Program
DoD	Department of Defense
DoDGAR	Department of Defense Grant and Agreement Regulations
DUNS	Data Universal Numbering System
eBRAP	Electronic Biomedical Research Application Portal
EC	Ethics Committee
ET	Eastern Time
FAPIIS	Federal Awardee Performance and Integrity Information System
FY	Fiscal Year
GCP	Good Clinical Practices
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
HRPO	Human Research Protection Office
IACUC	Institutional Animal Care and Use Committee
IRB	Institutional Review Board
M	Million
OASD(HA)	Office of the Assistant Secretary of Defense for Health Affairs
OMB	Office of Management and Budget
ORP	Office of Research Protections
PI	Principal Investigator
PRMRP	Peer Reviewed Medical Research Program
RDT&E	Research, Development, Test, and Evaluation
RM	Resource Manager
SAM	System for Award Management
SOW	Statement of Work
USAMRAA	U.S. Army Medical Research Acquisition Activity
USAMRMC	U.S. Army Medical Research and Materiel Command
USC	United States Code
VA	Department of Veterans Affairs

APPENDIX 2
AREAS OF ENCOURAGEMENT

Applications addressing any of the FY17 PRMRP Topic Areas are of interest to the program. ***Any aspect of research relevant to one or more FY17 PRMRP Topic Areas may be considered for funding.*** Areas of encouragement related to the FY17 PRMRP Topic Areas have been identified by the Department of Defense, Department of Veterans Affairs, and other relevant stakeholders. Applicants are urged to read and consider these areas of encouragement before preparing their applications. ***The information provided is not exhaustive, and applicants are not restricted to submitting applications that address an area of encouragement in this list.***

Acute Lung Injury

- Research on the etiology and prevention of acute respiratory distress syndrome caused by the host's or immune system's responses to infectious disease or septic shock.
- Preventive techniques, novel and/or innovative detection technologies, and therapeutics to reduce the incidence and/or severity of acute respiratory distress syndrome and/or other lung injury secondary to trauma, transfusion, burns, hemorrhagic shock, inhalation, and/or oxygen exposure.
- Development of a clinical laboratory-based device for manufacturing/amplification of stem cells to treat acute lung injury/acute respiratory distress syndrome due to inhalation injury, trauma, or other causes.
- Clinical studies to test cellular therapies in the treatment of acute lung injury/acute respiratory distress syndrome due to inhalation injury, trauma, or other causes.
- Metrics to associate the health outcomes of acute lung injury with physiological and physical performance, as well as the psychological health of military Service members.
- Strategies to stabilize and support the safe transport of patients with lung injury in order to optimize therapeutic interventions.
- Studies to identify the prevalence and associated morbidity and mortality of blast overpressure lung injury in forward operating environments.

Antimicrobial Resistance

- Identification and evaluation of novel and/or innovative antifungals against resistant fungal infections, particularly for use as topical therapies for wounds and for surgical and post-surgical therapies.
- Identification of novel and/or innovative targets for multi-drug resistant pathogens.
- Development of novel and/or innovative interventions, diagnostics, and treatment for multi-drug-resistant pathogens.
- Development of novel and/or innovative therapies that interrupt biofilm production in wound infections and infected hardware models.
- Development and testing of treatment protocols and/or diagnostic tests to limit prescribing antibiotics for conditions that are commonly viral in nature or conditions that would resolve themselves without antibiotic treatment (e.g., sinusitis, bronchitis, viral upper respiratory infections, and acute gastroenteritis).

- Development of rapid, rugged, sensitive, and specific diagnostics for antimicrobial resistance in remote environments.
- Research to improve understanding of the impact of travelers' diarrhea, as well as antibiotic treatment for the colonization of multi-drug-resistant organisms.

Arthritis

- Studies that define the risk of contracting septic arthritis; reactive arthritis; arthritis caused by a gastrointestinal, genitourinary, or viral infection (e.g., Lyme disease, Campylobacter, chikungunya); and other arthritis types in active duty military personnel.
- Research toward the development of clinical practice guidelines to prevent, identify, and treat arthritis, particularly that resulting from traumatic injury, surgery, and/or infectious diseases.
- Research to establish activity recommendations for maximal joint life following joint repair, particularly in young patient populations.
- Strategies for repairing focal cartilage defects using cell-based therapies.
- Research quantifying the impacts of obesity, weight loss, physical fitness (all components—cardiovascular, strength, flexibility, balance), and dietary factors on the development of or prevention/risk reduction of arthritis.
- Identification and/or validation of biomarkers for early psoriatic arthritis.
- Basic and translational research to identify treatments to mitigate and/or reverse osteoarthritis, particularly in the knee, hip, ankle, and shoulder.
- Studies to examine using existing regenerative medicine techniques and therapies to treat osteoarthritis, including dose response information and the frequency and timing of application.
- Development of novel and/or innovative treatments or combination therapies for psoriatic arthritis.

Burn Pit Exposure

- Research on the etiology and treatment of adverse health events related to military deployment to Iraq and Afghanistan that are associated with exposure to airborne hazards and open pit burning of solid waste and other materials.
- Toxicological studies to ascertain the toxicity of natural dust, burn pit combustion products and interactions between pollutants, as well as their mechanisms of action.
- Characterization of emissions from open air burns, burn boxes, and incinerators.
- Development and validation of instruments for assessing levels of exposure to airborne hazards for use in research and for occupational and environmental exposure monitoring.

Chronic Migraine and Post-Traumatic Headache

- Research to investigate, develop, and validate biomarkers that are useful in diagnosing and monitoring traumatic brain injury patients with chronic migraine or post-traumatic headache.
- Epidemiological/natural history studies to characterize specific types of post-traumatic headache, the pathobiology of these headaches (such as the role of cortical spreading

depression acutely after injury as a risk factor for chronic headaches of a migrainous type), and the risk factors that might predispose people to certain types of post-traumatic headache.

- Double-blind, placebo-controlled trials in the post-traumatic headache population in order to determine whether similar phenotypes in primary headache disorders and post-traumatic headache will respond similarly to treatment.
- Research on the optimal approaches to effective management of co-occurring psychological health disorders and acute and chronic pain management for chronic migraine and post-traumatic headache, with a focus on assessing and eliminating adverse outcomes and decreasing polypharmacy.
- Research on the utility of the Patient-Centered Medical Home model of care for patients with chronic migraine or post-traumatic headache.
- Research on the treatment and prevention of acute flares of chronic migraine and post-traumatic headaches with an emphasis on non-opioid medications.
- Evaluation of the use of mechanical stimulation and/or other non-pharmaceutical treatments to reduce acute and chronic migraines and headaches.
- Precision medicine research to investigate, develop, and validate biomarkers that are not only useful in diagnosing and monitoring traumatic brain injury patients with chronic migraine or post-traumatic headache, but can also identify individual responses to treatment.
- Evaluation of the differences in etiology, diagnosis, treatment plan, and prevention of migraine headaches between men and women.

Congenital Heart Disease

- Population-based and outcomes-based research projects to assess the health outcomes of individuals with congenital heart disease across their life spans.
- Research on tissue engineering approaches to patches, grafts, and transplantation that provide structural support, restore native activity, allow for tissue growth, and prevent the need for reoperation.
- Research to improve understanding of the causes of congenital heart defects, including genomic, proteomic, and metabolomic profiling.
- Research on transition of the care of congenital heart disease patients from pediatric to adult providers.
- Research both on the risk of neurologic injury and on enhanced neuroprotection before, during, and after surgery for congenital heart disease.
- Research to design and implement disease-in-a-dish and/or microfluidic models with an established phenotype to increase the efficacy of finding novel and/or innovative drug targets, screen existing drugs, perform cardiotoxicity testing, and/or uncover pathogenesis.

Constrictive Bronchiolitis

- Clinical assessments to determine the prevalence and severity of constrictive bronchiolitis and related respiratory diseases in previously deployed military Service members and/or Veterans.

- Development and testing of less invasive and non-invasive approaches for diagnosing constrictive bronchiolitis.
- Research to develop novel and/or innovative therapeutics to slow or reverse the progression of constrictive bronchiolitis.
- Development and/or validation of animal models for constrictive bronchiolitis.
- Research to understand the role of environmental exposures, including mineralized dust and other particulates, in the etiology of constrictive bronchiolitis.

Diabetes

- Development of biomarkers to identify and monitor at-risk individuals and assess treatment response.
- Research on interventions to prevent diabetes complications, including diabetic retinopathy and diabetic neuropathy.
- Research to better understand the heterogeneity of diabetes.
- Research on the transplantation of allogenic or autologous pancreatic islet cells for long-term natural insulin production, including current good manufacturing practices for cell line development.
- Identification and/or evaluation of interventions to reduce the development of diabetes among individuals meeting the clinical criteria for pre-diabetes.
- Development and evaluation of an inpatient glycemic management program with strategies to improve in-hospital dysglycemia, improve short-term and long-term morbidity/mortality, reduce the cost of health care, and promote a seamless transition of care between inpatient and outpatient arenas.
- Research to design and implement disease-in-a-dish and/or microfluidic models to model pancreatic islets to uncover pathogenesis and improve the efficiency of drug discovery.

Diarrheal Diseases

- Development and testing of vaccines, including multi-pathogen combination approaches, against the most common causes of bacterial and viral travelers' diarrhea, including *Campylobacter jejuni, Shigella,* and Enterotoxigenic *E. coli*.
- Development and evaluation of antibiotic and non-antibiotic therapies to treat all-cause travelers' diarrhea to improve the time to clinical cure, minimize resistance acquisition, and mitigate the chronic health consequences of these infections.
- Research to improve understanding of host responses and develop therapies to treat acute bacterial and viral infections that are associated with travel and may lead to chronic functional bowel and rheumatologic disorders.
- Development of evidence necessary to demonstrate the need, impact, and potential market value of a therapeutic for post-infectious functional bowel disorders to include preventive, treatment, or vaccine technology strategies.
- Development and evaluation of point-of-care diagnostics for acute diarrheal illness that distinguishes between bacterial, viral, protozoal, and toxin-mediated etiologies and leads to actionable public health or individual management interventions.

- Investigations into the relationship between existing gastrointestinal flora (microbiome) and the susceptibility, duration, and severity of diarrheal disease, as well as the impacts of dietary modification and/or probiotic use.
- Research to identify, reduce, or eliminate sources of infection causing diarrheal diseases.

Dystonia

- Research on the risk, incidence, and etiology of generalized dystonia, focal dystonia, multifocal dystonia, segmental dystonia, and/or hemidystonia.
- Research on interventions to prevent, slow the progression of, or treat dystonia.
- Research to improve identification of delayed onset dystonia following traumatic brain injury.

Early Trauma Thermal Regulation

- Research into regional cooling for different environments, including prolonged care, as a therapeutic for secondary ischemic injury (e.g., head with traumatic brain injury, limb with tourniquet).
- Development of a tunable support mechanism for thermal homeostasis for use in prolonged field care, extended evacuation, or unmanned evacuation.
- Development and evaluation of a novel and/or innovative technology and techniques to reduce the impact of hypothermia.
- Research to determine the benefits of invasive warming/cooling during patient movement.
- Research to validate the attenuating effects of (early) external head cooling on concussive brain injuries.
- Research into therapeutic cooling or targeted temperature management as a neuroprotective treatment for traumatic brain injury.

Eating Disorders

- Investigations into the prevalence, diagnosis, and treatment patterns of eating disorders in Service members and their families, including potential relationships with military-unique behaviors or conditions.
- Assessment of patterns of comorbidity between eating disorders and other mental health conditions, including an examination of whether eating disorders are more likely to precede or follow the development of other mental health conditions.
- Studies to identify the most effective treatments (and order of treatment) for patients with an eating disorder and a comorbid disorder.
- Research to advance the understanding of the biological, genetic, and environmental factors that influence eating disorders.
- Studies to elucidate risk factors for the onset or recurrence of eating disorders.
- Research to develop screening tools and valid biomarkers that accurately identify individuals with binge eating disorder versus healthy controls in order to monitor at-risk populations and tailor treatments for disordered eating.

- Research to better understand subclinical disordered eating and other specified feeding and eating disorders and the potential for these to develop into full-blown eating disorders.

Emerging Infectious Diseases

- Methods to minimize risk from arboviruses among military Service members and their families deployed to/living in high-risk areas, including personal protective equipment and environmental controls.
- Investigation of revaccination strategies and identification of correlates of protection for currently available vaccines (especially mumps).
- Targeted vector management of *Aedes aegypti, Anopheline sp.* and related mosquito species, including focus areas on novel and/or innovative adult surveillance devices, pathogen identification, spatial repellents, and barrier treatments.
- Research toward understanding the potential influence of dietary intake as a potential repellent to mosquito vectors and vector behavior.
- Evaluation of non-vaccine prophylactics or therapeutics to prevent/treat dengue or Zika virus disease.
- Characterization of health emergency relevant infectious disease threats in West Africa.
- Development of biomarker-guided clinical management strategies for severe infectious disease in the austere African setting.
- Validation of airborne and droplet precaution recommendations in the medical transport environment.
- Development of new testing methods for emerging pathogens and pathogen reduction technologies for blood-borne pathogens, including Zika, malaria, dengue, and others, in blood products.
- Modeling of epidemics and development of strategies to counter risks in emerging infectious diseases.
- Rapid, rugged, sensitive, and specific screening and diagnostics, including biomarkers for infectious threats such as Zika and other clinically relevant bacterial, viral, or protozoal pathogens in clinical samples or blood products that can be transmitted sexually or by blood transmission.
- Development of broad-acting detection systems to identify emerging members of a known viral family (for example, a broad Coronavirus panel that can detect Middle Eastern Respiratory Syndrome).
- Determination of the human health effects, host preference, vector competence, and co-infection status of the dengue, chikungunya, Zika, and Mayaro viruses in *Aedes aegypti* and related mosquito species.

Epidermolysis Bullosa

- Research to provide further insight into those cellular pathways that might be corrected or bypassed that otherwise promote the development of squamous cell carcinomas in recessive dystrophic and junctional epidermolysis bullosa.

- Research, including randomized controlled clinical trials, focused on systemic drugs that prevent, delay the onset, or modify the aggressiveness of squamous cell carcinoma in patients with recessive and junctional epidermolysis bullosa.
- Research, including clinical trials, focused on medications (topical or systemic) or dressings that might enhance wound healing in inherited epidermolysis bullosa.

Focal Segmental Glomerulosclerosis

- Development of a curative therapy or treatments to delay or halt the progression of focal segmental glomerulosclerosis and/or prevent post-transplantation recurrence.
- Research to determine the efficacy of medications used off-label (outside the FDA-approved indication) to treat focal segmental glomerulosclerosis.
- Research to improve understanding of the causes of primary and secondary focal segmental glomerulosclerosis, especially genetic mutations.
- Development of non-invasive methods to diagnose focal segmental glomerulosclerosis and its variants.
- Research on the impact of dietary modifications on the progression of renal disease.

Fragile X

- Research to advance the understanding of the pathophysiology of fragile X syndrome.
- Identification and validation of functional measures of the manifestations of fragile X syndrome across the life span.
- Identification and testing of novel and/or innovative targets for fragile X syndrome therapeutics.
- Development and evaluation of novel, innovative, or existing therapeutics for the treatment of fragile X syndrome.

Guillain-Barré Syndrome

- Research to elucidate the characteristics of various exposures (e.g., viruses, bacteria, vaccinations, surgery) associated with Guillain-Barré syndrome and their effects on the immune system.
- Research on the immune system cell types and molecular mechanisms responsible for the disease pathology of Guillain-Barré syndrome.
- Research to prevent or reduce the effects of residual weakness, relapse of muscle weakness, and other neurological symptoms of Guillain-Barré syndrome to improve patients' quality of life and increase their independence.
- Development of new treatments and refinement of existing treatments for Guillain-Barré syndrome.

Hepatitis B and C

- Research on vaccination options for those persons unable to mount immunity to the hepatitis B virus.

- Development of strategies for reliable, non-invasive, early detection of hepatitis-related liver disease and hepatocellular carcinoma.
- Research on strategies to promote reversal of liver fibrosis and/or assess the associated clinical and pathological outcomes.
- Natural history or other studies to establish risk indicators of progression from asymptomatic or inactive chronic hepatitis infection to symptomatic disease and parameters for the initiation and duration of disease treatment.
- Clinical studies to evaluate combination or curative therapies for treatment of hepatitis infection.
- Identification and reduction of hepatitis in blood products for transfusion.
- Research on strategies to reduce vertical (mother-to-child) transmission of hepatitis B virus and hepatitis C virus.
- Development of a vaccine against hepatitis C.
- Development of treatment protocols for patients with hepatitis B and C co-infections.

Hereditary Angioedema

- Research to improve early diagnosis of hereditary angioedema.
- Evaluation of existing, innovative, or novel therapeutics in pediatric hereditary angioedema patients.
- Research toward development of a cure for hereditary angioedema.
- Development and/or validation of novel and/or innovative therapeutic strategies for the treatment and/or prevention of hereditary angioedema attacks.

Hydrocephalus

- Research on the etiology, prevention, diagnosis, and treatment of post-traumatic hydrocephalus.
- Discovery or validation of novel and/or innovative therapies and therapeutic targets for the treatment of hydrocephalus and its sequelae, including therapies directed at myelin regeneration and repair.
- Research on approaches to lessening the impact of brain damage caused by hydrocephalus.
- Development or validation of biomarkers and imaging techniques, particularly multimodal approaches, to aid in diagnosis, prognosis, and monitoring of therapeutic efficacy.
- Research on the prevention of shunt failure.
- Studies to better understand the causes and/or pathogenesis of hydrocephalus.
- Development or validation of improved hydrocephalus model systems.

Immunomonitoring of Intestinal Transplants

- Studies to elucidate the role of the mucosal immune system, other host-derived factors, or gut microbiota-derived factors in maintaining intestinal transplant viability.

- Development and evaluation of intestinal implant strategies that circumvent the induction of immunity against the transplant through the use of autologous stem cells or organoids derived from intestinal stem cells.
- Development and evaluation of evidence-based approaches that focus on dampening the regional immune response against intestinal transplants without inducing global immune suppression.
- Development and evaluation of implant-associated materials (e.g., scaffolds) with anti-inflammatory properties that protect the intestinal transplant from immune attack.
- Development and evaluation of strategies for inducing and maintaining populations of anti-inflammatory regulatory immune cell populations at the transplant site.
- Studies to improve immunomonitoring of recipient immune responses after intestinal transplantation, with a focus on prospective leukocyte profiling, to aid in diagnosis and treatment of immunological and immunosuppression-related complications.
- Development and/or validation of precise, real-time implanted monitoring devices to improve individualized patient outcomes after intestinal transplantation.

Inflammatory Bowel Diseases

- Clinical studies in human subjects directed toward understanding how acute enteric infections may trigger chronic bowel diseases with acute and sub-acute inflammatory bowel disease, including systems biology approaches.
- Mechanistic studies in animal models designed to understand how enteric infection may trigger inflammatory bowel disease, including genomic, microbiomic, and immune mechanisms.
- Epidemiological studies of post-infectious acute and sub-acute inflammatory bowel diseases that define risk and provide estimates of illness-associated disability, health care costs, and symptom duration from a military health system and societal perspective.
- Studies to understand the interaction between acute/chronic stress and infection and the development of inflammatory bowel disease.
- Research to explore whether the travel patterns of active duty personnel increase the risk of developing inflammatory bowel disease by means of exposure to changes in intestinal microflora.
- Research to better characterize the association between the use of drugs, such as isotretinoin and long-term doxycycline, and the development of inflammatory bowel disease.
- Research on the role of diet in the development and progression of inflammatory bowel disease.
- Research on the influence of the microbiome on inflammatory bowel disease.
- Research on hyperbaric oxygen treatment of inflammatory bowel disease.
- Research on treatment strategies for patients whose inflammatory bowel disease is refractory to standard care medications or who positively respond to standard care medications, but become nonresponsive over time.

Influenza

- Development of a diagnostic test for influenza drug resistance.
- Development and testing of a universal influenza vaccine.
- Development and evaluation of novel, innovative, and/or combination influenza therapies.
- Research to improve the understanding of host responses to influenza infection and the mechanisms of drug resistance.
- Research on the factors that contribute to and/or reduce the risk of the emergence of zoonotic and pandemic influenza viruses.

Integrative Medicine

- Research on the use of integrative medicine strategies, such as meditation, tai chi, acupuncture, and/or herbal medicine/nutritional supplementation, to improve the psychological health and quality of life in cancer patients and survivors.
- Research on the use of integrative medicine in treatment and management of chronic pain disorders, including comparative efficacy studies relative to standard of care.
- Rigorous longitudinal studies of integrative medicine approaches for enhancing resilience and treating psychological health issues and co-occurring disorders.
- Precision medicine research to investigate, develop, and validate biomarkers that can help determine an individual's response to integrative medicine treatments for pain.
- Development of outcomes tools and measures to evaluate the effectiveness of integrative medicine pain management approaches.
- Definitive studies to determine the effectiveness of self-care, meditation-based treatments, courses, and training programs in improving resiliency to stress in a military environment; in austere operational platforms such as submarines and small-deck ships; or in combat-fatigued military health care providers.

Interstitial Cystitis

- Studies that define the risk, prevalence, and operational impact of interstitial cystitis among active duty personnel.
- Identification of biological markers for making a definitive diagnosis of interstitial cystitis.
- Evaluation and assessment of novel and/or innovative treatment options for interstitial cystitis patients, including intravesical therapy.
- Research on the etiology of interstitial cystitis to inform targeted therapy development.

Malaria

- Investigation of mechanisms of drug resistance in malaria, to include host and pathogen, against drugs used for treatment and prophylaxis.
- Development of a vaccine that induces high levels of long-lived, broadly protective immunity against *Plasmodium falciparum* and/or *P. vivax*.
- Operational research to optimize application of existing malaria drugs to reduce impact of artemisinin resistant *P. falciparum* in military forces.

- Operational research to optimize deployment of pharmacovigilance systems to ensure capture of adverse events related to malaria chemoprophylaxis and treatment.
- Operational research to assess efficacy and spread of artemisinin combination therapies across the Greater Mekong Subregion's military forces.
- Development of malaria prophylactic regimens that encourage higher compliance and methods to monitor compliance in deployed Service members.
- Identification of novel and/or innovative malaria drug targets for blood and liver stage malaria parasites.
- Development of new, or improvement of existing, strategies for culturing relapsing malaria parasites, with emphasis on *Plasmodium vivax, P. ovale,* and *P. cynomolgi* in vitro.
- Development, testing, and evaluation of spatial repellents to provide protection against Anopheline vectors.
- Identification of a correlate of immunity against malaria and development of a surrogate test of protection.
- Structurally optimize validated malaria antigens by engineering protective epitopes to elicit specific and enhanced immune responses.

Metals Toxicology

- Identification and validation of biomarkers to evaluate military Service members' acute exposure to toxic metals in an operational environment and predict potential consequent health risks and associated health outcomes.
- Retrospective studies to evaluate risk and exposure among workers at industrial facilities.
- Research on the toxicity of metal combinations and the interaction between different metal components.
- Research on the additive effects of multiple exposures to metal(s).
- Research on underlying diet quality and micronutrient sufficiency and the risk for metals toxicity.
- Research on the toxicity of metal-based engineered nanomaterials.

Mitochondrial Disease

- Research on the basic biology and physiology of mitochondria to better understand the pathology of primary mitochondrial diseases.
- Development of improved tools and animal models to study primary mitochondrial disease and evaluate therapeutics.
- Identification and testing of non-invasive techniques and biomarkers to monitor mitochondrial function, aid in diagnosis, and/or evaluate therapeutic efficacy.
- Research on novel and/or innovative treatments to alleviate symptoms or slow down the progression of mitochondrial diseases.

Musculoskeletal Disorders

- Research to increase understanding, diagnosis, prevention, and/or treatments of chronic overuse musculoskeletal disorders.
- Research on measures (e.g., clinical biomarkers, novel/innovative interventions, therapeutics) to diagnose, predict, reduce the incidence of, or optimize health outcomes in military training- and Service-related musculoskeletal disorders.
- Research on the validation and/or optimization of rehabilitation strategies for Service-related musculoskeletal disorders.
- Research to prevent, control, and/or optimize musculoskeletal health outcomes for work-related musculoskeletal disorders.
- Research on lower back pain strategies to prevent surgery and recurrence of symptoms, identify factors that predict optimal treatment response for different patients, and encourage self-management as a treatment.
- Research to determine whether tailored psychosocial education interventions are effective in improving psychological resilience to musculoskeletal pain.
- Research on standardized approaches for early identification and prevention of chronic musculoskeletal pain.

Nanomaterials for Bone Regeneration

- Research on nanomaterials-based methods to facilitate recruitment of endogenous cell populations for enhanced bone regeneration and osseointegration.
- Technologies addressing segmental/large bone defects in craniomaxillofacial and/or load-bearing regions.
- Development of controlled release/extended release of growth factors for bone regeneration.
- Development of technologies that repair the soft tissue envelope to enhance bone regeneration.

Non-Opioid Pain Management

- Research on pain management strategies for patients with limited access to skilled providers and resources, including battlefield, prolonged field, transport, and other resource-limited environments.
- Development of population-based outcomes, tools, and measures to evaluate the effectiveness of pain management approaches across time and environments.
- Research on treatments for chronic pain management, particularly in complex patients (i.e., chronic, high-utilization, polypharmacy patients).
- Research to increase understanding of and preventative treatments for conversion of acute to chronic pain.
- Research that provides evidence of which pain management strategies work for whom and under what conditions.
- Research to identify and address biopsychosocial aspects of pain to reduce or eliminate the use of opioid pain medication(s).

- Comparative studies evaluating the efficacy of different pain management strategies, including complementary and alternative medicine approaches.
- Identification and development of non-opioid pain management techniques that promote positive psychological health-related outcomes.
- Development of non-opioid pain medicine that can be given via inhalation or intramuscularly, submucosally, or intravenously on the battlefield to provide adequate relief of pain without affecting the cardiorespiratory systems.
- Research on case management strategies designed to optimize the effectiveness of pain clinics, support use of non-opioid pain therapies, and reduce dependence on opioid therapies.
- Research that will directly obtain an FDA analgesic indication for oral transmucosal or intramuscular ketamine.

Pancreatitis

- Retrospective studies to determine the risk and incidence of pancreatitis among former and current active duty personnel.
- Development and testing of novel and/or innovative therapeutics for acute and/or chronic pancreatitis.
- Research on the basic biology and physiology of the pancreas to better understand the etiology and pathology of pancreatitis.

Pathogen-Inactivated Dried Cryoprecipitate

- Development or validation of technology to produce pathogen-reduced dried cryoprecipitate in military or civilian donor centers that is acceptable for FDA licensure in support of combat or contingency operations.

Polycystic Kidney Disease

- Development of improved treatment strategies for polycystic kidney disease, including approaches to identify and monitor patients at higher risk for progressing to end-stage renal disease.
- Research on the underlying pathobiology and molecular mechanisms of polycystic kidney disease, including studies of genetic factors, cyst formation and growth, the role of cilia, and factors that modify disease progression and/or severity.
- Research on the lifestyle factors that may modify the progression of polycystic kidney disease.

Post-Traumatic Osteoarthritis

- Research to establish activity recommendations for maximal joint life following joint repair, particularly in young patient populations.
- Research into cell-based approaches for treatment or prevention of post-traumatic osteoarthritis.
- Studies to evaluate and develop best practices for multidisciplinary team approaches and treatment algorithms for post-traumatic osteoarthritis.

- Development or validation of novel and/or innovative approaches to restoring joint stability after injury.
- Sustained release, intra-articular injectable steroidal, non-steroidal, or disease-modifying therapies that offer two or more months of symptomatic relief of pain and/or inflammation in a single injection.
- Research on therapies that target multiple phases of the cellular response pathways that are implicated in the development of post-traumatic osteoarthritis, including cell death, inflammation, matrix changes, and changes in catabolic and anabolic responses.
- Development and/or validation of predictive models for early onset post-traumatic osteoarthritis in extremity-injured patients for the purpose of tailoring rehabilitation programs toward prevention, delay, or mitigation of onset.

Pulmonary Fibrosis

- Retrospective studies to determine the risk and incidence of pulmonary fibrosis among military Service members, including Veterans.
- Identification of biomarkers of pulmonary injury or early predictors of interstitial lung disease.
- Research into the pathobiology and molecular mechanisms underlying the development of pulmonary fibrosis.
- Development and/or testing of novel and/or innovative treatments to delay or modify the progression of pulmonary fibrosis.
- Development and/or validation of improved tools and animal models to study pulmonary fibrosis and evaluate therapeutics.

Respiratory Health (excludes lung cancer and mesothelioma)

- Research on the causes, treatment, and prevention of chronic obstructive pulmonary disease (COPD), including identification and validation of biomarkers and disease phenotypes, as well as employing personalized medicine in clinical research and disease management.
- Research investigating the treatment of patients with chronic mild hypoxia.
- Research on the cause, treatment, and prevention of respiratory symptoms and ailments possibly associated with deployed and redeployed military personnel, including acute eosinophilic pneumonia, asthma, allergies, and other chronic lung diseases and breathing problems.
- Research to evaluate the impact of deployment on the prevalence and severity of respiratory disease in military Service members and Veterans.
- Development and/or validation of methods to detect volatile organic hydrocarbons from the breath of individuals exposed to toxic chemicals or other agents that can cause lung injury, which can be utilized for diagnosis and prognosis of lung injury or disease.
- Identification of biochemical, physiological, or combined biomarkers for evaluating risk or extent of injury from either acute or long-term toxic occupational or environmental exposures.

- Research investigating exposure rates, detection, and treatment of diseases related to inhalation of mold and fungi, such as coccidioidomycosis.

Rett Syndrome

- Identification and/or validation of novel and/or innovative biological targets for the treatment of Rett syndrome.
- Development and testing of interventions to improve the neurological symptoms of Rett syndrome.
- Research to understand the relationship between genetic mutations, physical traits, and symptoms in individuals with Rett syndrome.
- Research on the pathobiology of the MeCP2 gene and protein.
- Research to understand Rett syndrome's commonalities with and differences from classic autism and regressive autism.

Rheumatoid Arthritis

- Research to better understand the relationship between genetic risk and environmental triggers, such as infection or smoking, in developing rheumatoid arthritis.
- Studies that identify or validate biomarkers or personalized medicine strategies that allow for individualized medication choice based on the patient's underlying biology or disease state.
- Research on the long-term use of immunosuppressants in patients with rheumatoid arthritis and the likelihood of developing infections.
- Research to establish activity recommendations following joint replacement for maximal joint life.

Scleroderma

- Research on the molecular mechanisms and pathogenesis of scleroderma.
- Development and/or testing of novel and/or innovative therapies and identification of novel and/or innoavtive therapeutic targets in scleroderma.
- Research on biomarkers and other approaches to diagnose scleroderma, monitor disease progression, and/or assess response to treatment.
- Epidemiologic studies investigating the impact of localized scleroderma (morphea) on duty performance, use of personal protective equipment, and deployability.
- Research on the efficacy of newer immunosuppressive agents, including biologics and small molecule inhibitors, in the treatment of morphea and scleroderma (systemic sclerosis).
- Research on the efficacy of UVA1 for topical treatment in localized scleroderma and systemic sclerosis.

Sleep Disorders

- Research on how the disruption of normal sleep and circadian biological rhythms adversely affects the physical and psychological health, safety, performance, and productivity of military and civilian populations.
- Studies to assess sleep disturbance and clinical sleep disorders in female military personnel.

- Research on the association between sleep disorders and post-traumatic stress disorder, traumatic brain injury, depression, and/or anxiety and suicidal behaviors.
- Research to assess clinical algorithms that improve adherence to continuous positive airway pressure (CPAP).
- Investigations into non-CPAP-based treatment regimens that enhance readiness and deployability in active duty military personnel.
- Research focused on investigating how psychiatric disorders (post-traumatic stress disorder in particular), combined with the disruption/degradation of sleep quality, impact long-term physical health through changes in hormonal physiology.
- Development and/or testing of non-pharmacological treatments for sleep disorders.
- Research on the impact of sleep deprivation on the efficacy of psychological treatments for post-traumatic stress disorder, depression, and other mood disorders.
- Research on the prevention and/or mitigation of sleep disorders that are associated with long aeromedical evacuation flights for both clinical team members and patients.
- Development of tools and technologies that allow rapid and objective measurement of Service member performance in the face of fragmented sleep and/or sleep maintenance difficulty.
- Research examining the broader effects of Warfighters' sleep disorders on the sleep, behavioral health, and productivity of their family members, as well as the readiness of their military unit.

Spinal Muscular Atrophy

- Research into phenotyping the spinal muscular atrophy disease state.
- Research into which molecular deficiency imposed by a deficiency of SMN, a motor neuron protein, causes motor neuron deficits.
- Exploration of the form and function of SMN-depleted neuromuscular junctions at ultrastructural (e.g., dysregulation of endocytosis), transcriptomic, and proteomic levels, particularly the mildest SMN reduction that leads to consistent quantifiable motor neuron loss.
- Research to find non-SMN-altering spinal muscular atrophy modifying genes in cellular, small animal, and human models that may lead to identification of novel and/or innovative therapeutic targets.
- Research to further understand SMN gene regulation and post-transcriptional mechanisms leading to synergistic SMN-repleting approaches, as well as to determine whether boosting SMN induction maximizes efficacy.
- Research to determine whether SMN can be repleted to physiologic levels in all cells in the spinal muscular atrophy model immediately post-natally and whether there is a limit to the therapeutic response.
- Clinical research to determine what clinical findings persist even with treatment; whether these findings are related to drug timing, dosage, biodistribution, or a combination of factors; and whether these factors are modifiable.

- Research involving dietary/nutritional studies in spinal muscular atrophy to identify the optimal anabolic diet.
- Research to determine mitochondrial involvement and astrocytic and other non-neuronal contributions to motor neuron vulnerability.

Sustained-Release Drug Delivery

- Development of technology that can provide a sustained-release delivery of drugs for a minimum of 1 week or more. Potential applications of this technology could include long-acting therapies for post-traumatic stress, opiate dependence, low-dose pain control, allergies, attention deficit/hyperactivity disorder, chemoprophylaxis, and other conditions.
- Research into the use of printable, sustained-release scaffolds for predictable delivery of prescription and non-prescription medications.
- Research into novel and/or innovative approaches to developing bioavailable and sustained-release oral formulations of intravenous broad-spectrum fungicidal and anti-microbial medications.

Tinnitus

- Research to understand the mechanisms of tinnitus, its relationship to noise-induced hearing loss, and progression to chronic tinnitus, with the focus on developing interventions for tinnitus.
- Research to increase knowledge of the prevalence, incidence, natural history, and occupational and sex-related differences of tinnitus and its possible relation to individual blast/noise exposures.
- Research to understand the operational readiness impacts of tinnitus on the military.
- Development and validation of objective tools/methods to diagnose and characterize tinnitus (e.g., imaging techniques to identify functional and structural changes in the brain, biomarkers of resiliency, and susceptibility to tinnitus).
- Identification of novel and/or innovative therapies for interventions to prevent, manage, and treat tinnitus, including behavioral approaches, new uses for existing drugs, nutritional and pharmaceutical strategies, and acoustic, electrical, and other stimulation technologies.

Tuberculosis

- Development of a diagnostic assay that can be used at the point of care to rapidly and accurately diagnose tuberculosis.
- Development of novel and/or innovative tuberculosis vaccines or optimization of current tuberculosis vaccines.
- Identification and/or validation of biomarkers that can be used to assess vaccine efficacy and protection against tuberculosis disease.
- Research to understand, diagnose, or treat multi-drug-resistant tuberculosis or extensively drug-resistant tuberculosis.
- Research to determine the appropriate precautions to use for transport of active tuberculosis patients.

- A sustained release formulation of anti-tuberculosis drugs that would facilitate long-term treatment and reduce the emergence of resistance due to poor compliance.

Vaccine Development for Infectious Disease

- Development of a therapeutic vaccine and/or other strategies to achieve HIV remission.
- Evaluation of passive immunization strategies to use in conjunction with dengue and Zika vaccinations.
- Research leading to a better understanding of the immune mechanisms involved in clearance of the dengue and Zika viruses and the mechanisms of immune enhancement that lead to more severe clinical disease.
- Development of flexible vaccine technologies that can be used to rapidly respond to emerging and re-emerging infectious diseases threats.
- Evaluation of humoral and cellular immune responses after vaccination compared to natural infection.
- Development of a safe, effective dengue human challenge model for clinical trials.
- Development of a non-human primate model to study Zika virus-induced microcephaly/fetal neuropathology as a tool to measure the safety and efficacy of vaccines and antivirals.
- Development and fielding of vaccines to prevent U.S. Service members from becoming ill from endemic disease exposure during operational deployments. This includes, but is not limited to, arthropod-borne diseases (Zika virus, dengue, chikungunya, hantavirus hemorrhagic fever with renal/pulmonary syndrome, rickettsioses, and trypanosomiasis), leptospirosis, HIV, norovirus, Middle East Respiratory Syndrome coronavirus, schistosomiasis, leishmaniasis, Nipah virus, Lassa fever, and West Nile fever.

Vascular Malformations

- Studies into the natural history, genetics, and pathogenesis of vascular malformations.
- Research to discover or develop novel and/or innovative therapeutic targets and treatments to regress or prevent vascular malformations.
- Research to improve methods to diagnose and manage vascular malformations.
- Development of non-invasive or minimally invasive technologies or approaches for the control of internal bleeding associated with vascular malformations.
- Studies to identify risk and/or establish standard practices for the treatment of hemorrhage from brain arteriovenous malformations.
- Research on the prevention or treatment of complications associated with vascular malformations such as pain, infection, seizures, tissue breakdown, tissue overgrowth, or airway obstruction.
- Studies to elucidate the progression of vascular malformations in disease-in-a-dish/microfluidic models for the purpose of identifying novel and/or innovative drug targets, screening existing drugs, and/or elucidating the pathogenesis of the disease.

Women's Heart Disease

- Retrospective studies to determine the risk and incidence of heart disease among former and current female active duty personnel, including Reservists and National Guard members.
- Research focused on elucidating the potential relationship between post-traumatic stress disorder and women's heart disease.
- Identification of sex-specific approaches to increase the effectiveness of diagnosis, treatment, and/or cardiac rehabilitation programs.
- Research on factors to predict and prevent the long-term impacts of gestational diabetes, gestational hypertension, and preeclampsia on the cardiovascular health of women.
- Research on trauma-induced cardiac arrest secondary to hemorrhage and polytrauma in the female population.

APPENDIX 3
DOD AND VA WEBSITES

PIs are encouraged to integrate and/or align their research projects with Department of Defense (DoD) and/or Department of Veterans Affairs (VA) research laboratories and programs. Collaboration with DoD or VA investigators is also encouraged. Below is a list of websites that may be useful in identifying additional information about DoD and VA areas of research interest, ongoing research or potential opportunities for collaboration within the FY17 PRMRP Topic Areas.

Air Force Office of Scientific Research
http://www.wpafb.af.mil/afrl/afosr/

Air Force Research Laboratory
http://www.wpafb.af.mil/afrl

Armed Forces Radiobiology Research Institute
http://www.usuhs.edu/afrri/

Clinical and Rehabilitative Medicine Research Program
https://crmrp.amedd.army.mil

Combat Casualty Care Research Program
https://ccc.amedd.army.mil

Congressionally Directed Medical Research Programs
http://cdmrp.army.mil

Defense Advanced Research Projects Agency
http://www.darpa.mil/

Defense Technical Information Center
http://www.dtic.mil

Defense Threat Reduction Agency
http://www.dtra.mil/

Military Health System Research Symposium
https://mhsrs.amedd.army.mil/SitePages/Home.aspx

Military Infectious Diseases Research Program
https://midrp.amedd.army.mil

Military Operational Medicine Research Program
https://momrp.amedd.army.mil

Naval Health Research Center
http://www.med.navy.mil/sites/nhrc

Navy and Marine Corps Public Health Center
http://www.nmcphc.med.navy.mil/

Office of Naval Research
http://www.med.navy.mil/

Office of the Under Secretary of Defense for Acquisition, Technology and Logistics
http://www.acq.osd.mil/

Telemedicine and Advanced Technology Research Center
http://www.tatrc.org/

Uniformed Services University of the Health Sciences
http://www.usuhs.edu/research

U.S. Army Institute of Surgical Research
http://www.usaisr.amedd.army.mil/

U.S. Army Research Institute of Environmental Medicine
http://www.usariem.army.mil/

U.S. Army Medical Research Institute of Infectious Diseases
http://www.usamriid.army.mil/

U.S. Army Medical Research and Materiel Command
http://mrmc.amedd.army.mil

U.S. Army Research Laboratory
http://www.arl.army.mil

U.S. Department of Defense Blast Injury Research Program
https://blastinjuryresearch.amedd.army.mil/

U.S. Department of Veterans Affairs, Office of Research and Development
http://www.research.va.gov

U.S. Naval Research Laboratory
http://www.nrl.navy.mil

Walter Reed Army Institute of Research
http://www.wrair.army.mil/

Sample Incoming UNC-CH Subcontract Commitment Letter

Instructions: Please use this letter as a template/guideline when UNC-CH is committing to a subcontract under another institution's prime award. Fill in the blank areas and those areas marked in ***BOLD ITALICS***.

UNC-CH Letterhead
UNC-CH Department/Center Name
UNC-CH Address

Date

Prime Awardee Name and Address

Reference: ***Response to solicitation/RFP/RFA number _____, entitled _____, dated _____***

Dear **_____,**

This letter confirms that the appropriate program and administrative personnel at The University of North Carolina at Chapel Hill (UNC-CH) have reviewed the above referenced Solicitation/RFP/RFA and are committed to enter into a subcontract with the ***Prime Awardee*** for the performance period of ____ to ____. The work to be performed by UNC-CH ___ does ___ does not include ___ animal and/or ____ human research subjects. UNC-CH maintains an active and enforced conflict of interest policy meeting the requirements of 42 CFR Part 50, Subpart F and 45CFR Part 94.

The ***Prime Awardee's*** Principal Investigator on this proposal is _____. The UNC-CH budget, budget justification and scope of work are provided as separate enclosures to this letter. The estimated cost of the proposed subcontract will not exceed $_____ and includes appropriate direct and indirect costs.

Furthermore, by submission of this commitment letter UNC-CH and its Principal Investigator (PI) certify (1) that the information submitted within the application is true, complete and accurate to the best of the UNC-CH's and PI's knowledge; (2) that any false, fictitious, or fraudulent statements or claims may subject the UNC-CH and PI to criminal, civil, or administrative penalties; and (3) that the PI agrees to accept responsibility for the scientific conduct of the project and to provide the required progress reports if an award is made as a result of UNC-CH's application.

If you have any questions, please contact the undersigned at **_____.**

Sincerely,

Signature of Authorized UNC-CH Official

Signature of UNC-CH Principal Investigator

Enclosed:

Budget
Budget Justification
Scope of Work

Journal of General Virology (1997), **78**, 2723–2733. Printed in Great Britain

Analysis of a recombinant dengue-2 virus–dengue-3 virus hybrid envelope protein expressed in a secretory baculovirus system

Helle Bielefeldt-Ohmann[1], David W. C. Beasley[1], David R. Fitzpatrick[2] and John G. Aaskov[1]

[1] Centre for Molecular Biotechnology, School of Life Science, Queensland University of Technology, GPO Box 2434, Brisbane, Australia
[2] Leukocyte Biology Unit, Queensland Institute of Medical Research, Herston, Queensland, Australia

In a step towards a tetravalent dengue virus subunit vaccine which is economical to produce, highly immunogenic and stable, a hybrid dengue virus envelope (E) protein molecule has been constructed. It consists of 36 amino acids from the membrane protein, the N-terminal 288 amino acids of the dengue-2 virus E protein plus amino acids 289–424 of the dengue-3 virus E protein. It has been engineered for secretory expression by fusion to a mellitin secretory signal sequence and truncation of the hydrophobic transmembrane segment. Using the baculovirus expression system and serum-free conditions, more than 95% of recombinant dengue-2 virus–dengue-3 virus hybrid E protein (rD2D3E) was secreted into the cell culture supernatant in a stable form with multiple features indicative of preserved conformation. The hybrid molecule reacted with a panel of dengue virus- and flavivirus-specific MAbs which recognize linear or conformational epitopes on dengue virions. Human dengue virus-specific antisera also reacted with the protein. The hybrid rD2D3E protein was able to inhibit the *in vitro* binding of dengue-2 and dengue-3 viruses to human myelomonocytic cells, suggesting that the receptor-binding epitope(s) was preserved. Adjuvant-free immunization with the hybrid protein induced an antibody response to both dengue-2 and dengue-3 virus in outbred mice, comparable in strength to that of individual rD2E and rD3E proteins. Notably, these antibody responses were primarily of the IgG2a and IgG2b isotype. A strong dengue virus cross-reactive T cell response was also induced in the mice, suggesting that dengue virus hybrid E proteins could form the basis of an efficacious multivalent dengue virus vaccine.

Introduction

Dengue viruses (serotypes 1–4) are now endemic in most tropical countries, and are a major cause of paediatric morbidity and mortality (Monath, 1994). Severe disease occurs most commonly, but not exclusively, following reinfection with virus serotypes not previously encountered (Morens, 1994). Epidemiological and laboratory data suggest that sub-neutralizing levels of antibody may enhance infection of Fc receptor-bearing cells, resulting in more severe disease (Morens, 1994). Other, not mutually exclusive, hypotheses suggest that it is particular combinations of antibody-independent host factors and virus pathogenic characteristics (strain characteristics) which result in a more severe inflammatory response, haemorrhagic manifestations and shock (Monath, 1994).

Author for correspondence: Helle Bielefeldt-Ohmann. Fax› 61 7 3864 1534. e-mail helle! biosci.uq.edu.au

The envelope (E) protein of dengue virus contains the majority of the determinants involved in attachment to, and penetration of, host cells as well as epitopes involved in neutralization (Rey *et al.*, 1995). Because of the similarity between the amino acid sequence (Chu, 1989; Deubel *et al.*, 1993) and predicted structure of the E protein of flaviviruses (Rey *et al.*, 1995), the possibility of preparing hybrid E proteins containing antigenic determinants from two or more dengue virus serotypes was considered. Epidemiological data suggest that clinical infections in humans are rare following exposure to a third or fourth dengue virus serotype (Morens, 1994; Monath, 1994), and Rhesus monkeys infected sequentially with three dengue virus serotypes did not develop a viraemia following quaternary challenge with any of the four dengue virus serotypes (Halstead *et al.*, 1973). Thus, broad-spectrum immunity would appear to be a desired and achievable goal for any dengue virus vaccine programme.

Intertypic chimaeric dengue viruses have been constructed in which all of the structural protein genes of dengue-4 virus

0001-4918 # 1997 SGM

CHCl

Downloaded from www.microbiologyresearch.org by
IP: 203.126.233.162
On: Tue, 05 Sep 2017 06:39:54

were replaced with those of dengue-1, dengue-2 or dengue-3 viruses, whilst the non-structural dengue-4 virus proteins were retained (Bray & Lai, 1991; Calain *et al.*, 1995). In the case of the dengue-2 virus–dengue-4 virus chimaera, retardation of virus replication relative to either parent virus type was observed, and it was speculated that this type of virus might be more amenable to attenuation (Bray & Lai, 1991). However, further attenuation and the safety of these hybrid viruses has not yet been reported.

Megret *et al.* (1992), employing recombinant fusion proteins, demonstrated that the maintenance of a topographical arrangement of discontinuous antigenic domains in the flavivirus E protein may be important for induction of neutralizing and protective antibodies. This interpretation was corroborated by the finding that most of the antigenic epitopes of the flavivirus E protein are located on ridges on the external surfaces of the molecule (Rey *et al.*, 1995). MAb neutralization escape mutants of flaviviruses have been used to delineate epitopes which might be involved in binding to and fusion of these viruses with host cell membranes. In the flaviviruses studied, regions surrounding amino acids 71–72, 128, 224–227, 270, 303–311, 333 and 402–403 appear to be important, directly or indirectly, for the binding and fusion of the virus to host cell membranes (Lobigs, 1987; Cecilia & Gould, 1991; Jiang *et al.*, 1993; Lin *et al.*, 1994). Our own studies suggest that some of these regions (adjacent to amino acids 128, 224–227 and 333) also contain epitopes commonly recognized by antibodies from human dengue patients (Aaskov *et al.*, 1989). The majority of these site(s) regions appear to localize in domains II and III of the crystallographic model of the flavivirus E protein (Rey *et al.*, 1995). It would therefore appear to be essential to ensure integrity of most or all of these regions in the design of any subunit vaccine.

The baculovirus expression system that utilizes *Autographa californica* nuclear polyhedrosis virus (AcNPV) has been used to obtain high-level expression of many foreign genes, including those that require proteolytic processing, glycosylation or secretion (Richardson, 1995). The system has also been employed to produce recombinant dengue virus E protein and NS-1, which, to varying degrees, induced protective immune responses in mice (Putnak *et al.*, 1991; Deubel *et al.*, 1991; Delenda *et al.*, 1994; Smucny *et al.*, 1995). Of these, only Delenda *et al.* (1994) described elicitation of dengue virus serotype cross-reactive antibody responses. Although an attenuated multivalent dengue virus vaccine is currently under evaluation (Bhamarapravati & Yoksan, 1989), a similar approach using multivalent recombinant E proteins has not been reported. This is perhaps not surprising considering the level of quality control required for a four-component vaccine. In this report, we describe the generation of a recombinant dengue-2 virus–dengue-3 virus hybrid E protein (rD2D3E), expressed in a secretory baculovirus expression system, which induced strong dengue virus serotype-specific antibody responses and dengue virus cross-reactive T cell responses in outbred mice.

Methods

Cells and viruses. C6.36 *Aedes albopictus* mosquito cells, used for growth of dengue virus stocks, were maintained in RPMI 1640 (Gibco) supplemented with 2–5 % heat-inactivated foetal bovine serum (FBS) at 30 °C. BHK cells were used for virus neutralization assays and grown in RPMI containing 5 % FBS at 37 °C. The human myelomonocytic cell line HL60 (Harris & Ralph, 1985) and the human non-Epstein–Barr virus-transformed B cell leukaemia cell line BM13674 (Baxter & Lavin, 1992) were maintained in RPMI containing 10 % FBS supplemented with 25 mM glutamine. *Spodoptera frugiperda* clone 21 (Sf21) cells were employed for growth of baculoviruses as described in detail below. The baculovirus (AcNPV) used throughout was strain AcBac6. Dengue-2 virus [prototype strain New Guinea C (NGC); GenBank accession no. D00346], dengue-1 virus (prototype Hawaii) and dengue-4 virus (prototype H241) were originally obtained from Yale Arbovirus Reference Center, and had been passaged in both sucking mice by intracerebral inoculation and in tissue culture in C6.36 cells. Dengue-3 virus (strain 1327, Tahiti-65; GenBank accession no. L11439) was generously provided by the Center for Disease Control, Atlanta, GA, USA, and upon receipt was passaged twice in C6.36 cells before use for RNA isolation (see below). For production of semi-purified dengue virus preparations, virus was grown in C6.36 cells under serum-free conditions. Following clarification of the culture supernatants (*g* 4300, 15 min), the virus was pelleted by centrifugation at 15 000 *g* for 16–18 h at 4 °C. The virus was resuspended in either TNE buffer (10 mM Tris–HCl pH 7·5; 100 mM NaCl; 1 mM EDTA) or RPMI and stored in 1 ml aliquots at −70 °C. Since dengue-3 virus strain 1327 is non-cytopathic, all viruses were quantified by haemagglutination assay, where the number of virus particles equals the number of red blood cells in the reaction mixture (Schlesinger, 1977).

Cloning of the hybrid rD2D3E protein and truncated rD2E and rD3E protein genes. Virus RNA was isolated from the supernatants of C6.36 cultures infected with dengue-2 virus and dengue-3 virus by the guanidinium isothiocyanate method, and reverse transcribed to cDNA using random hexamer primers and avian myeloblastosis virus reverse transcriptase (Amersham) (Bielefeldt-Ohmann *et al.*, 1994). The cDNA was amplified by PCR using primers which included unique endonuclease restriction sites for both the joining site of the two E protein genes (*Eco*RI) and for cloning into the baculovirus transfer vectors (*Xma*I at the 5′ end of the dengue-2 virus cDNA and a *Bgl*II site at the 3′ end of the dengue-3 virus cDNA, respectively), as well as a stop codon at the end of the dengue-3 virus gene (D2 sense, 5′ *ATCCCGGGC*CAGGCTTTACCATAATG 3′; D2 antisense, 5′ *GGAATTC*CATCCTCAGCCTGCAG 3′; D3 sense, 5′ *GGAATTC*CTGGAACTCAAGGGGATG 3′; D3 antisense, 5′ *GTAGATCT*CAACCCACTGACCCAAAG 3′). The *Eco*RI site was chosen to minimize alterations to the predicted secondary structure and hydrophilicity of the E protein in the joining region. The two PCR products were cloned individually into pGem-5Zf(+) shuttle T-vectors (Promega) and subsequently joined at the *Eco*RI site and subcloned into the baculovirus transfer vectors pMbac and pPbac (Stratagene), containing the mellitin and the human placental alkaline phosphatase secretory signals, respectively (Mroczkowski *et al.*, 1994). Truncated rD2E and rD3E protein genes, encompassing nucleotides 733–2074 and 672–2115, respectively, were cloned in a similar manner into pMbac, following initial cloning into the shuttle vector pGem by TA cloning of PCR products generated as above using the following primers: D2 sense, as above; D2 antisense, 5′ *GGAATTC*ACAGGACATCTCAAGTGC 3′; D3 sense, 5′ *ATCCCGGGT*GGATGTCGGCTGAAGGA 3′; D3 antisense, as above. The further truncation of the rD2E protein gene as

Downloaded from www.microbiologyresearch.org by
IP: 203.126.233.162
On: Tue, 05 Sep 2017 06:39:54

well as the inclusion of additional prM sequence in the rD3E construct were dictated by the constraints on PCR primer design using the OLIGO program. Construction integrity was verified by DNA sequencing using dye terminators, cycle sequencing reactions and automated sequence analysis (ABI Systems).

+ **Transfection and selection of recombinant viruses.** Using the pMbac-D2D3E, pBac-D2D3E, pMbac-D2E and pMbac-D3E plasmids, Sf21 cells were co-transfected with linearized AcPak6 DNA (ClonTech) using 25 ng baculovirus DNA and approximately 500 ng plasmid DNA per transfection. The DNAs were mixed with liposomes (ClonTech) and after a 15 min incubation were added to serum-free confluent monolayers of Sf21 cells in 10 cm# tissue culture plates. Following an overnight incubation in serum-free TC100 medium (Gibco), the cultures were supplemented with TC100 medium containing 5 % FBS and incubated for a further 48 h. The medium was then collected and replaced with fresh medium. The cultures were incubated for a further 24 h, after which the medium was collected and the cells fixed with X-Gal (0·3 %, v/v) to assess the level of recombinant virus replication. The two batches of culture media collected were employed separately for plaque purification of recombinant virus, which was performed according to published protocols (O'Reilly *et al.*, 1994) except that incubations were for 4–5 days. At this stage, X-Gal was applied to the cultures, and after a further overnight incubation, blue plaques were picked and resuspended in 500 µl TC100 medium containing 10 % serum. One hundred µl of this sample was adsorbed to 10^6 Sf21 cells in 5 cm# tissue culture wells, and the cell cultures were incubated for 4 days. The culture supernatant was then collected and subjected to a second round of plaque purification. Following each round of clone expansion, 80 µl of clarified culture medium was blotted onto nitrocellulose and the dot blots were immunolabelled with a flavivirus E protein-specific, conformation-dependent MAb, 4G2 (Gentry *et al.*, 1982), as described below, to confirm the presence of immunoreactive dengue virus E protein.

+ **Preparative production of recombinant proteins.** Confluent monolayers of Sf21 cells in 150# tissue culture flasks were inoculated with the recombinant or wild-type baculoviruses at an m.o.i. of 5 in less than 5 ml medium. Adsorption was allowed to proceed for 2 h, and was followed by addition of 40–50 ml serum-free medium [either ExCell 400 (JRH Biosciences) or Protein-Free Medium (Pharmingen)]. The conditioned medium was collected after 48 h (see Results) and the proteins were precipitated with a final concentration of 9 % sterile polyethylene glycol 6000 (PEG; BDH) by overnight incubation with stirring at 4 °C. The precipitates were pelleted by centrifugation (6 h at 14 300 *g*) and resuspended in sterile TNE buffer. The proteins were stored at −20 °C, without addition of proteinase inhibitors, until use.

+ **SDS–PAGE, electroblotting and immunodetection by enhanced chemiluminescence (ECL).** The recombinant dengue virus proteins and proteins from virions were separated on non-reducing 10 or 15 % SDS–PAGE gels (with 5 % stacking gels) and transferred to nitrocellulose membranes by electroblotting in methanol–Tris–glycine buffer, followed by blocking for 48 h with ECL blocking buffer (Staton *et al.*, 1995). The membranes were incubated with primary antibodies [either dengue virus-specific MAbs (see Table 1) or human or murine sera], diluted in Tris-buffered saline (TBS) (pH 7·4) containing 0·1 % Tween 20, 1 mg/ml BSA, 1 mg/ml non-fat skimmilk powder and 1 % FBS, for 2 h at room temperature, followed by 4×3 min washes in TBS containing 0·1 % Tween 20. This was followed by a 1 h incubation with horseradish peroxidase-conjugated rabbit anti-mouse or anti-human immunoglobulin antibody (Dakopatts), or in the case of isotyping studies, with isotype-specific goat anti-mouse IgM, IgG1, IgG2a, IgG2b or IgG3 (The Binding Site). Bound antibody was visualized by ECL (reagents and films from Amersham) as described by Staton *et al.* (1995). Unless otherwise indicated, the results shown are from the first 50–60 s exposure.

+ **Analysis of glycosylation of recombinant dengue virus E proteins.** The effect of endoglycosidase F and H (both from Boehringer Mannheim) on the recombinant E proteins was assessed as described by O'Reilly *et al.* (1994). Briefly, PEG-precipitated rD2D3E, rD2E or rD3E or the corresponding cell lysates were incubated with either endoglycosidase F in 20 mM sodium phosphate (pH 7) containing 2 % (v/v) NP40 and 10 mM EDTA, or endoglycosidase H in 100 mM sodium acetate (pH 5) (all final concentrations). Enzymes were omitted from the control samples. Following incubation at 37 °C for 20–24 h, the proteins were separated on 8, 10 or 15 % polyacrylamide gels, electroblotted, and immunolabelled with MAb 4G2 and visualized as described above. Alternatively, the lectin-binding properties of the recombinant E proteins, with or without prior endoglycosidase treatment, were examined by a protocol modified from Seto *et al.* (1995). Proteins transferred onto nitrocellulose membranes by electroblotting were reacted with different dilutions of biotinylated lectins (*Daturastramonium*, *Galanthus nivalis*, *Maackia amurensis* and concanavalin A; all from Vector Laboratories), followed by incubation with horseradish peroxidase-conjugated streptavidin (Boehringer Mannheim) and visualization of binding by ECL.

+ **ELISA.** Flexible PVP 96-well ELISA plates (Dynatech) were coated overnight at 4 °C with rD2D3E protein or dengue virus diluted in borate–saline buffer (pH 9·0). Following blocking for 60 min at 23 °C with 5 % KPL milk diluent (Kirkegaard & Perry Laboratories) and two washes in PBS containing 0·05 % Tween 20 (PBST), primary antibodies (MAbs or polyclonal sera) at appropriate dilutions in PBST containing 5 % KPL milk diluent were applied and incubation was continued for 45 min at 23 °C. After four washes in PBST, horseradish peroxidase-conjugated anti-mouse immunoglobulin (Dakopatts) diluted in PBST containing 5 % KPL milk diluent was added to the wells. Following incubation for 45 min at 23 °C, the plates were washed four times with PBST and four times in PBS, followed by application of *o*-phenylenediamine substrate (Sigma). After addition of 3 M H2SO4, the plates was read on a Dynatech MR5000 plate reader.

+ **Virus-binding blocking.** To demonstrate blocking of dengue virus binding to HL60 and BM cells by rD2D3E, washed and pre-cooled (4 °C) cells were pre-incubated for 2 h with either media control or PEG-precipitated proteins from wild-type baculovirus-infected cultures supernatant or the rD2D3E protein (10 µg per 10^6 cells). This was followed by incubation of the cells with semi-purified dengue-2 or dengue-3 virus, at a cell to particle ratio of 1 : 5×10^3 to 1 : 1×10^4, for 90 min. All incubations were at 4 °C. Following extensive washing of the cells, total RNA was extracted as described above, and the relative levels of virus binding were assessed by RT–PCR using primers for either the prM protein gene (D2 sense, 5′ ATGGCGTTCCATTTAACCACACGTA 3′; D2 antisense, 5′ CATTGAAGGAGCGACAGC 3′; D3 sense, 5′ ACTTGACTTCACGAGATGGAGA 3′; D3 antisense, 5′ CCACTCCCACGCATCTCATTGT 3′) or the transmembrane part (D2 sense, 5′ TAGAAGCAGAACCTCCAT 3′; D2 antisense, 5′ GAGCACCATAACTCCCAAAT 3′; D3 sense, 5′ TTGTGATTGGAACTGGAG 3′; D3 antisense, 5′ CACCACAGCTCCTAGAT 3′) of the dengue-2 and dengue-3 virus E protein genes, respectively. Using 28 cycles of amplification, approximately 5–8×10^3 virus particles in a reaction with 10^6 cells could be detected. Primers for human β-actin were included in the PCR reaction for sample equalization (Bielefeldt-Ohmann, 1994). This allowed semi-quantitative comparison of virus binding to target cells with or without pre-incubation with rD2D3E protein or controls following densitometric analysis using an ImageQuant densitometer and

Downloaded from www.microbiologyresearch.org by
IP: 203.126.233.162
On: Tue, 05 Sep 2017 06:39:54

Table 1. Antigenicity of rD2D3E protein assessed with MAbs and human primary dengue virus infection antibodies

MAb/serum	Specificity*	Conformation-dependent	Reactivity with rD2D3E in: Western blot‹	ELISA□
4G2´	Flavivirus E (domain III)	Yes	›››	›››
7E4a	D1, D2, D3, D4, MVE	No	›	› ›
4B5o	D1, D2, D3, D4, MVE	Yes	›	› ›
7E4o	D1, D2, D3, D4, MVE	Yes	f	i › ›
4D9d	D1, D2, D3 (domain I–II)	Yes	fi	›››
2H3p	D1, D2, D3, MVE	Yes	› } fi	›››
4D9k	D1, D2, D3 (domain II–III)	Yes	fi	›››
1B7p	D1, D3, MVE (domain III)	?	nd	›
1E10r	D1, D3, D4	No	›	›››
6D11f	D1, D3	No	›	› ›
3H5´	D2 (domain III)	Yes	fi	nd
128s	D2 (Eamino acids 403, 430)	No	››	››
316s	D2□	Yes	fi	›››
174s	D2 (Eamino acids 128, 303)	Yes	› } fi	›››
234s	D2 (Eamino acid 402)	No	›	›
6B9**	D2	Yes	›	› ›
4E6**	D2	Yes	fi	›››
Cas‹‹	D2		››	nd
Cal‹‹	D2		››	nd
Vic‹‹	D2		››	nd

* Determined by IFA and} or virus-neutralization assay. Unless otherwise indicated, the epitope reactivity of the MAbs has not been determined. Domains I–III refer to the structural regions of the tick-borne encephalitis virus E protein revealed by crystallography (Rey *et al.*, 1995). D1–D4, dengue virus serotypes 1–4; MVE, Murray Valley encephalitis virus.

‹ Non-reducing gel and} or dot blots of rD2D3E protein.

□ In the case of the first 10 MAbs listed, similar or slightly stronger reactivities were obtained with rD2E and rD3E (data not shown).

´ See reference by Trirawatanapong *et al.* (1992). This MAb reacts with rD2E in both test systems (H. Bielefeldt-Ohmann, unpublished data).

s Kindly provided by T. Maguire, Virus Research Unit, University of Otago, New Zealand. All four MAbs have been epitope-mapped, using neutralization-escape mutant virus. They are classified as specific for the dengue-2 virus E protein; however, their cross-reactivities have not been exhaustively examined (personal communication).

□ Specific for epitopes encompassing amino acids 45, 71, 126 and 494 of the E protein (T. Maguire, personal communication).

** See reference by Jianmin *et al.* (1995).

‹‹ Human post-primary dengue virus infection sera, collected in Queensland.

nd, Not done.

software (Molecular Dynamics) as described previously (Bielefeldt-Ohmann *et al.*, 1994).

+ **Immunization of mice.** Groups of four mice (female outbred Quackenbush Animal Resources Centre, Murdoch, Western Australia) were immunized by the intraperitoneal (i.p.) or intramuscular (i.m.) route with approximately 10 | g PEG-precipitated rD2D3E, rD2E or rD3E in TNE (the concentration was determined by comparison to purified histidine-tagged rD2E in a dot-blot titration; not shown), or one of the four dengue viruses (live in serum-free RPMI) in amounts equalized with respect to the E protein, as determined by 4G2 reactivity in a dot-blot titration and corresponding to approximately 4‹ 10& virus particles (infectious, non-infectious and incomplete; Setterquist, 1977). The mice received three injections, at days 0, 14 and 42, and were bled on days 28, 56 and 72 or 90. The sera recovered were subsequently tested by Western blotting and neutralization assays. All animal experiments were conducted with institutional ethics approval in accordance with the National Health and Medical Research Council Guidelines for Experiments in Animals.

+ **Virus neutralization assay.** Sera collected from immunized mice were assayed by a plaque reduction neutralization test (Aaskov *et al.*,

Downloaded from www.microbiologyresearch.org by
IP: 203.126.233.162
On: Tue, 05 Sep 2017 06:39:54

1988). Briefly, 1 : 20 dilutions of pools of heat-inactivated serum from groups of mice immunized in the same manner were mixed with 10-fold dilutions of dengue-2 virus (NGC) or dengue-3 virus (strain PRS225489) prior to addition of BHK cells. After 7 days incubation, the cells were stained with 0·5 % crystal violet and plaques were counted. Antibody titre was expressed as a neutralization index : $\log_{10}$ (p.f.u. virus alone) − (p.f.u. virus + antibody).

+ ***In vitro* lymphocyte stimulation and IL-3 assay.** Animals were terminated 4–8 days after the third vaccination and spleen cells were isolated as described by Bielefeldt-Ohmann *et al.* (1994). Triplicate cultures of 1×10^6 cells in RPMI containing 5 % FBS, in round-bottomed microculture wells (Nunc), were incubated with or without a dose range (approximately $0{\cdot}3–2{\cdot}3 \times 10^7$ virus particles per cell) of semi-pure dengue-2, dengue-3 or dengue-4 virus for 5 days at 37 °C in 7·5 % CO_2 in air. The culture supernatants were then collected and tested for IL-3 bioactivity using the FDC-P1 reporter cell line, as described by Fitzpatrick & Kelso (1995).

Results

Construction of a hybrid rD2D3E protein gene, expression in the baculovirus expression system, and biochemical features

Using the cloning strategy described in Methods and illustrated in Fig. 1, a hybrid molecule encoded by nucleotides 733–1709 from the dengue-2 virus genome and nucleotides 1710–2115 from the dengue-3 virus genome was generated. This approach introduced two amino acid changes at the joining site : from DK in both of the native dengue-2 virus and dengue-3 virus E proteins to EF in the hybrid protein (Fig. 1). This change did not alter the predicted secondary structure and hydrophilicity of the protein, as assessed by six algorithms using the GCG program suite (University of Wisconsin), and is within the range of inter- and intratypic amino acid variations seen in this region of the protein (Lanciotti *et al.*, 1994). Furthermore, a search of both GenBank and the SWISS-PROT databases failed to demonstrate homology of the newly created sequence with any known human protein sequences. The integrity of the hybrid gene was affirmed by DNA sequencing.

Spodoptera cells infected with recombinant baculovirus containing the mellitin secretory signal produced and secreted more recombinant protein more quickly than recombinant virus containing the alkaline phosphatase secretory signal. However, in both cases, maximum secretion, in excess of 250 µg recombinant protein per 10^7 cells, was obtained 48–72 h post-infection, with ≥ 95 % of the protein secreted during the first 48–56 h. After that time, increasing cell death correlated with intracellular retention of recombinant protein. The hybrid protein mainly occurred as monomers with an apparent molecular mass of 48–49 kDa on non-reducing gels. Low levels of rD2D3E dimerization could be detected by immunoblotting using MAb 4G2 following protein separation under the same gel conditions. Endoglycosidase F treatment of rD2D3E slightly increased the mobility of the protein compared to the undigested control, indicating glycosylation

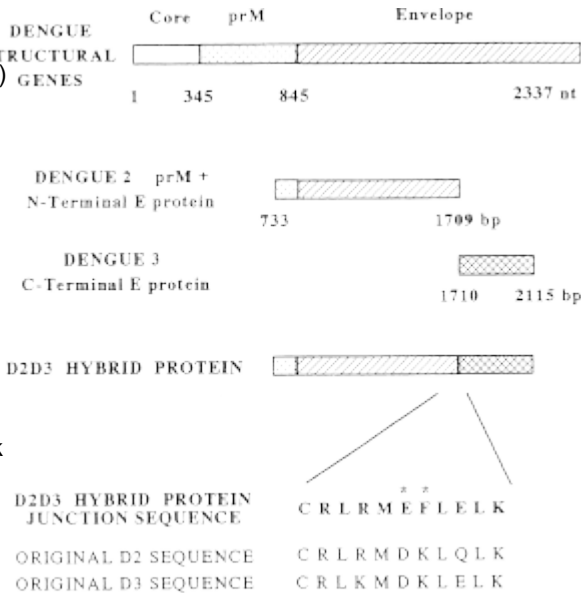


Fig. 1. Schematic depiction of the gene segments constituting the hybrid rD2D3E protein gene, and indication of the amino acid changes resulting from introduction of an *Eco*RI site in the hybrid gene.

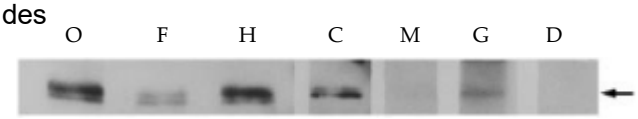


Fig. 2. Effect of endoglycosidases F and H on the mobility of rD2D3E in SDS–PAGE and lectin binding to the hybrid protein. Lanes : O, unmodified rD2D3E ; F, rD2D3E digested with endoglycosidase F ; H, rD2D3E digested with endoglycosidase H ; C, rD2D3E reacted with concanavalin A ; M, rD2D3E reacted with *Maackia amurensis* I (a very weak reaction discernable on the original blot) ; G, rD2D3E reacted with *Galanthus nivalis* ; D, rD2D3E reacted with *Datura stramonium*. Undigested and digested proteins were separated by SDS–PAGE (7·5 %) and electroblotted to nitrocellulose. Detection of protein in lanes O, F and H was performed using MAb 4G2. Visualization of antibody and lectin binding was by ECL. The arrowhead indicates a molecular mass of 48·3 kDa.

(Fig. 2). The molecular mass of the digested protein was approximately 47–48 kDa. These changes are comparable to those found for dengue-2 virus grown in insect cells (Johnson *et al.*, 1994) and for recombinant dengue virus proteins (Deubel *et al.*, 1991). Endoglycosidase H had no detectable effect on the mobility of either intracellular or secreted protein (Fig. 2 and data not shown). Concanavalin A, *Galanthus nivalis* and *Maackia amurensis* I, but not *Datura stramonium*, bound to the rD2D3E protein, suggesting the presence of mannose residues in high-mannose and / or hybrid oligosaccharide chains and sialic acid residues, but no complex or hybrid *N*-glycan structures (Fig. 2 and data not shown).

Antigenicity and functional activity of the hybrid rD2D3E protein

The antigenic properties of the hybrid rD2D3E proteins were analysed using MAbs raised against dengue virus

Downloaded from www.microbiologyresearch.org by
IP: 203.126.233.162
On: Tue, 05 Sep 2017 06:39:54

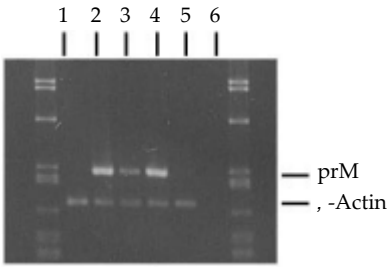


Fig. 3. Inhibition of binding of dengue-2 virus to HL60 cells by pre-incubation with the hybrid rD2D3E protein, assessed by semi-quantitative RT–PCR using the b-actin signal as reference. Lanes: 1, cells without virus or rD2D3E; 2, cells incubated with virus only; 3, cells pre-incubated with rD2D3E followed by virus binding; 4, cells incubated with a control protein preparation from wild-type baculovirus-infected cells followed by virus binding; 5, cells incubated with a control protein preparation from wild-type baculovirus-infected cells only; 6, PCR contamination control.

serotypes 1–4 and against other flaviviruses, and assessed by Western blotting and solid-phase ELISA. An abridged summary of the results is given in Table 1; the preservation of both conformation-dependent and cross-reactive determinants on the hybrid E protein is demonstrated. The lack of reactivity with MAb 3H5 is consistent with its specificity for an epitope in domain III of dengue-2 virus (Trirawatanapong *et al.*, 1992). This domain in the hybrid is derived from dengue-3 virus. In contrast, the interesting reactivity of MAbs 128 and 234, reportedly specific for dengue-2 virus, may be due to hitherto unknown cross-reactivities with the dengue-3 virus strain used here. Sera obtained from human dengue fever patients (primary infections) also reacted with the recombinant hybrid protein in Western blot experiments (Table 1).

Pre-incubation of the human myelomonocytic cell line HL60 or the human B cell line BM13674 with rD2D3E protein inhibited, in a specific manner, subsequent binding of both dengue-2 and dengue-3 viruses. Densitometric assessment, using equalization with respect to the b-actin signal, demonstrated a reduction of & 38 % of dengue-2 or dengue-3 virus binding following pre-incubation of the cells with rD2D3E (Fig. 3 and data not shown). This is a notable level of inhibition considering the primarily monomeric nature of the recombinant E protein and the highly multimeric occurrence of the E protein on virions (Rey *et al.*, 1995).

Immunogenicity of the rD2D3E protein

Groups of four outbred mice were immunized three times, by the i.p. or i.m. route, with approximately 10 l g adjuvant-free rD2D3E, rD2E or rD3E in TNE. The animals were bled 2 weeks after the second and third vaccinations, as well as at the termination of the experiment (day 90), and the sera were pooled and tested for reactivity with all four dengue virus serotypes in Western blot experiments (at final dilutions of 1:75), as well as for dengue-2 and dengue-3 virus neutralizing activity. A strong antibody response against both dengue-2 and dengue-3 virus E proteins was detected 2 weeks following the third vaccination, but no or only minimal cross-reactivity with dengue-1 and dengue-4 virus was detected (Fig. 4 and data not shown). Significant levels of antibody specific for dengue-3 virus were present after the second vaccination, with a further increase after the third vaccination, whereas notable levels of dengue-2 virus-specific antibodies were seen only after the third vaccination, and then only in the group receiving rD2D3E i.p. (Fig. 4 and data not shown). Significantly, however, the virus-specific antibodies were primarily of the IgG2a and IgG2b isotypes, with some additional anti-dengue-3 virus-specific activity of the IgM and IgG1 isotypes (Fig. 4).

In order to compare the immune response to the hybrid rD2D3E protein with that induced by live virus, groups of four Quackenbush mice were vaccinated with one of the three recombinant proteins (in the same amounts as in the previous experiment) or with semi-pure dengue-1, dengue-2, dengue-3

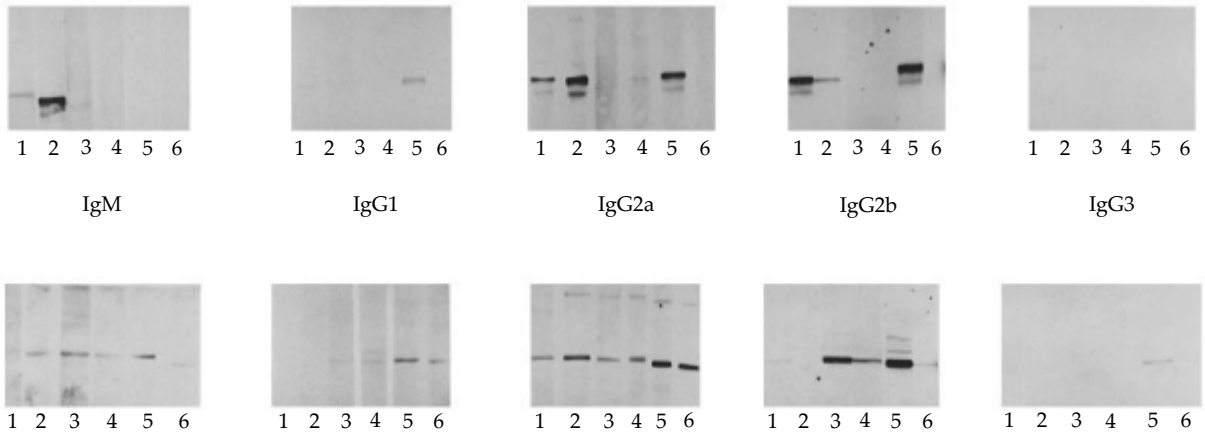


Fig. 4. Antibody class-and subclass-specific responses by mice to immunization with rD2E, rD3E or rD2D3E proteins, assessed on Western blots of dengue-2 virus (top panel) and dengue-3 virus (bottom panel) 2 weeks after the third vaccination. Lanes: 1, rD2E i.p.; 2, rD2E i.m.; 3, rD3E i.p.; 4, rD3E i.m.; 5, rD2D3E i.p.; 6, rD2D3E i.m.

Downloaded from www.microbiologyresearch.org by
IP: 203.126.233.162
On: Tue, 05 Sep 2017 06:39:54

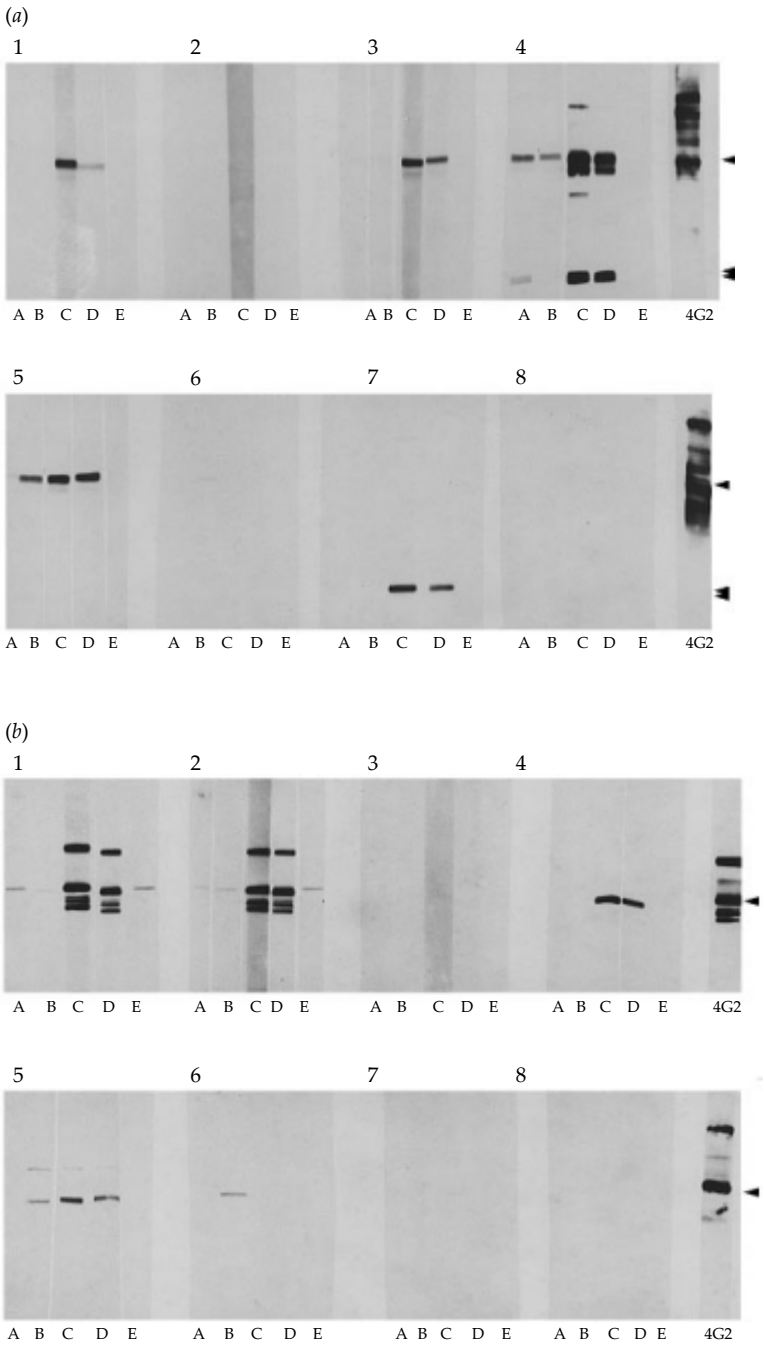


Fig. 5. Murine immunoglobulin subclass responses specific for (*a*) dengue-2 and (*b*) dengue-3 virus following adjuvant-free immunization with hybrid rD2D3E protein, rD2E protein or rD3E protein, or dengue-1, dengue-2, dengue-3 or dengue-4 virus. Pooled sera from each group bled 2 weeks after the third vaccination were tested at a final dilution of 1 : 75, and ECL films were exposed for 50–60 s. Groups : 1, rD2D3E ; 2, rD3E ; 3, rD2E ; 4, dengue-2 virus ; 5, dengue-3 virus ; 6, dengue-4 virus ; 7, dengue-1 virus ; 8, TNE buffer control. Lanes : A, IgM ; B, IgG1 ; C, IgG2a ; D, IgG2b ; E, IgG3. A weak IgG3 response could be detected in groups 1–5 upon longer exposure of the ECL films. Single arrowhead, virion E protein ; double arrowhead, virion M protein

or dengue-4 virus (the latter in amounts equalized to the recombinant proteins with respect to levels of native E protein). Vaccination was by the i.p. route, using the same vaccination schedule, and again no adjuvant was used. The results confirmed those of the previous experiment, i.e. strong antibody responses to dengue-2 and dengue-3 viruses, mainly of the IgG2a and IgG2b isotypes, were obtained with the hybrid rD2D3E protein, as well as with the rD2E and rD3E proteins, respectively (Fig. 5). Furthermore, the hybrid protein appeared to induce a response comparable to that of live dengue-2 and dengue-3 viruses, with low but detectable cross-reactivity to dengue-1 and dengue-4 viruses (data not shown).

A dose titration experiment in which groups of four mice received three i.p. adjuvant-free vaccinations with 10, 100 or 50 µg rD2D3E or TNE demonstrated that the antibody response reached a plateau at 10 µg per animal per dose.

In both the former experiments, 1 log or more of neutralizing activity was detected against dengue-2 but not dengue-3 virus in animals immunized with the hybrid protein by the i.p. route, as well as in animals immunized with rD2E

Downloaded from www.microbiologyresearch.org by
IP: 203.126.233.162
On: Tue, 05 Sep 2017 06:39:54

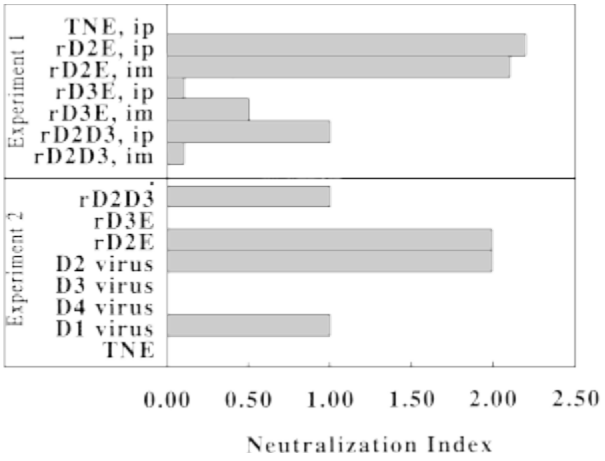


Fig. 6. Dengue-2 virus neutralizing antibody activity induced by adjuvant-free vaccination with hybrid rD2D3E protein, truncated rD2E protein or rD3E protein or with live dengue viruses. Pooled sera (four animals per group), obtained 2 weeks after the third vaccination, were tested at a final concentration of 1 : 20 in a plaque reduction assay (see Methods). Data are presented as neutralization indices, with & 1 significant. In experiment 2, all vaccinations were by the i.p. route.

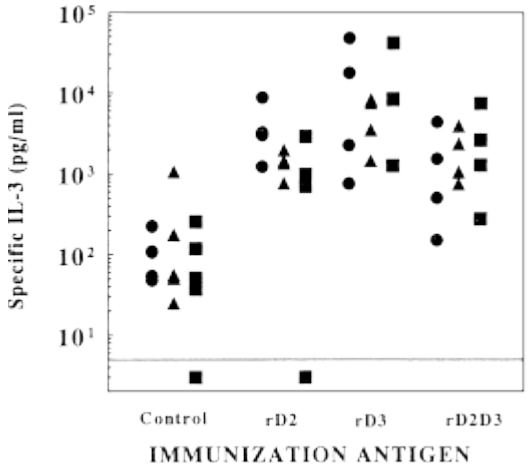


Fig. 7. Cross-reactive *in vitro* T lymphocyte responses (IL-3 production) induced by adjuvant-free immunization of mice with hybrid rD2D3E protein, rD2E protein or rD3E protein. Mice were terminated 6 weeks following the third i.p. vaccination (groups 1, 3 and 5 in Fig. 6, as well as the TNE buffer control group) and purified spleen cells were stimulated *in vitro* for 5 days with a dose range of semi-pure dengue-2 (E), dengue-3 (_) or dengue-4 (+) virus, followed by bioassay for IL-3 activity. Each data-point represents values obtained for individual mice with the optimal virus concentration (in this experiment approx. 10 haemagglutination units per 10^5 cells at the start of culture).

protein or dengue-2 virus (Fig. 6), whereas neither the hybrid molecule, rD3E nor dengue-3 virus induced antibodies with detectable neutralizing activity under the test conditions employed here (data not shown). Furthermore, the hybrid rD2D3E protein induced a strong dengue virus cross-reactive T cell response as evidenced by IL-3 production in response to *in vitro* dengue virus stimulation, with levels in immunized mice ranging from 10- to 1000-fold those seen in control mice (Fig. 7).

Discussion

This study demonstrates that a hybrid molecule consisting of the N-terminal two-thirds of the dengue-2 virus E protein and a truncated C-terminal one-third of dengue-3 virus, corresponding to domain I–II and domain III, respectively, of the tick-borne encephalitis virus E protein model (Rey *et al.*, 1995), can be secreted from baculovirus-infected insect cells in an antigenic form with high conformational integrity. The site of the junction between the dengue-2 virus and the dengue-3 virus portions of this E protein was chosen so as not to disrupt disulfide bridges (Lin *et al.*, 1994) or change the predicted hydrophilic characteristics of the native proteins. Both dengue-2 and dengue-3 virus-specific epitopes as well as dengue virus and flavivirus cross-reactive epitopes were preserved on the hybrid molecule (Table 1). Although the majority of the hybrid molecules occurred as monomers, the rD2D3E protein could still interfere with the binding to (Fig. 3), and subsequent infection of (unpublished data), several cell types by dengue-2 and dengue-3 viruses, suggesting that functional epitopes are preserved.

The hybrid protein induced a strong antibody response in outbred mice, with the majority of the anti-dengue-2 and anti-dengue-3 virus-specific antibodies being of the IgG2a and IgG2b isotype. This latter finding is significant in light of a recent report by Smucny *et al.* (1995) that recombinant baculovirus-derived dengue-2 virus E protein preferentially induced an IgG1 response in contrast to the dengue-2 virus, which preferentially induced IgG2a. The reason for the discrepancy between the results of Smucny *et al.* (1995) and those of the present study is not entirely clear, but three possibilities could be considered. Firstly, the strain of mice used and secondly the age of the animals are both factors known to influence the murine antibody response due to biased T helper cell stimulation (Barrios *et al.*, 1996; Hocart *et al.*, 1989). Thirdly, there is increasing evidence to suggest that the dose of antigen, perhaps more than any other single factor, influences the cytokine profile of both naive (primary response) and differentiated (secondary response) T cells (Bretscher 1992; Constant *et al.*, 1995; Hosken *et al.*, 1995), and thus, through cognate B cell–T cell interactions, the regulation of immunoglobulin isotype synthesis, including levels and specificities (Coffman *et al.*, 1993). Since most currently available reports on recombinant dengue virus E protein vaccines lack exact data on doses used, this aspect will have to await more definite comparisons. In our preliminary assessments of the T lymphocyte response in animals vaccinated with the rD2D3E protein, a strong dengue virus cross-reactive secondary *in vitro* IL-3 response was induced, suggesting preservation of T cell epitopes on the rD2D3E protein (Roehrig *et al.*, 1994; Kulkarni *et al.*, 1992). It is notable that IL-3 production is closely correlated with IFN-γ production (Maraskovsky *et al.*, 1992), a finding corroborated by testing a selected set of samples for IFN-γ from the experiment reported

Downloaded from www.microbiologyresearch.org by
IP: 203.126.233.162
On: Tue, 05 Sep 2017 06:39:54

here (H. Bielefeldt-Ohmann, unpublished data). IFN-γ is considered to be the main factor regulating the isotype switch from IgM to IgG2a production in mice (Coffman *et al.*, 1993).

The present results suggest that induction of IgG2a production is in itself not necessarily commensurate with virus neutralization, but the lack of *in vitro* neutralizing antibody activity does not preclude *in vivo* protection against virus challenge (Levine *et al.*, 1991). Unfortunately, the current murine model used for dengue virus protection studies, i.e. challenge of very young mice by the intracerebral route, may be inappropriate for revealing the true potential of this and previously described subunit dengue virus vaccines (Aaskov *et al.*, 1988; Delenda *et al.*, 1994). Susceptibility of mice to intracerebral dengue virus challenge is age-dependent, and immunization must therefore be initiated at a time when the immune system is still immature (Sarzotti *et al.*, 1996; Barrios *et al.*, 1996). Furthermore, the effector mechanisms required for protection of brain tissue may have little relevance to the effector mechanisms required for protection of humans against a mosquito-borne infection. Finally, challenge studies following immunization with the candidate rD2D3E protein vaccine have not been conducted due to the ethical problems associated with the intracerebral challenge model.

Although not yet studied in detail, the results depicted in Table 1 and Figs 4 and 5 suggest that the anti-dengue-2 and anti-dengue-3 virus antibody responses may be directed against different epitopes on the E protein, and also that neither part of the hybrid molecule significantly interferes with the response to the other part. These may thus constitute important factors in the overall safety of a human vaccine based on hybrid E proteins, in light of (i) the limited antibody cross-reactivity to dengue-1 and dengue-4 viruses induced by rD2D3E, (ii) the fact that the human antibody response to dengue viruses is directed towards multiple disparate epitopes on the E protein (Aaskov *et al.*, 1989; Innis *et al.*, 1989) and (iii) antibody-mediated infection enhancement of Fc receptor-bearing cells is mainly or solely due to flavivirus cross-reactive determinants (Brandt *et al.*, 1982; Morens, 1994).

The approach taken in this study offers several advantages over most systems so far reported for baculovirus-produced dengue virus E proteins: (i) greater stability of the secreted protein compared to protein accumulating intracellularly (unpublished data), (ii) easier purification, and thus (iii) relatively higher yields (amounts exceeding 250 μg per 10^9 cells) (Staropoli *et al.*, 1996; Delenda *et al.*, 1994; Richardson, 1995). In contrast to the original report on the baculovirus transfer vectors used in this study (Mroczkowski, 1994), higher yields of recombinant protein were consistently obtained with the mellitin construct than with the alkaline phosphatase construct. The present study has demonstrated that it is possible to construct a hybrid E protein which not only retains native tertiary structure and functional qualities in the form of putative receptor binding, but is also a strong immunogen, inducing B cell and T cell responses to the two dengue viruses constituting the hybrid and, at the T helper cell level, to the dengue virus serotypes not included in the construct. This suggests that a tetravalent dengue virus vaccine could be based on two components, a rD2D3E and a rD1D4E protein, thereby reducing the amount of safety and quality control, as well as preliminary assessment required, compared to a vaccine constituting four individually produced molecules.

Thanks are due to Dr T. Maguire (New Zealand) for making dengue virus-specific MAbs and pre-publication results available, to Professor A. Boyd (QIMR, Brisbane) for providing the HL60 cells, to Mr T. Forster (QUT) for providing the BM13674 cells, and to Miss Ina Serafin for performing neutralization assays. The animal attendants at QUT Animal Holding Facility are thanked for their caring for the experimental animals. A preliminary report of this work was presented at the Xth International Congress of Virology, Jerusalem, Israel, August 11–16, 1996. This work was supported by grants from the National Health and Medical Research Council (NHMRC) of Australia (grants nos 941446 and 961080).

References

Aaskov, J. G., Williams, L., Fletcher, J. & Hay, R. (1988). Failure of a dengue-1 subunit vaccine to protect mice against a lethal dengue virus infection. *American Journal of Tropical Medicine and Hygiene* **39**, 514–518.

Aaskov, J. G., Geysen, H. M. & Mason, T. J. (1989). Serologically defined linear epitopes in the envelope protein of dengue 2 (Jamaica strain 1409) *Archives of Virology* **105**, 209–221.

Barrios, C., Brawand, P., Berney, M., Brandt, C., Lambert, P. H. & Siegrist, C. A. (1996). Neonatal and early life immune responses to various forms of vaccine antigens qualitatively differ from adult responses: predominance of a Th2-biased pattern which persist after adult boosting. *European Journal of Immunology* **26**, 1489–1496.

Baxter, G. D. & Lavin, M. F. (1992). Specific protein dephosphorylation in apoptosis induced by ionizing radiation and heat shock in human lymphoid tumor lines. *Journal of Immunology* **148**, 1949–1954.

Bhamarapravati, N. & Yoksan, S. (1989). Study of bivalent dengue vaccine in volunteers. *Lancet* **i**, 1077.

Bielefeldt-Ohmann, H., Marzo, A., Fitzpatrick, D. R., Jarnicki, A., Davis, M., Manning, L. S. & Robinson, B. W. S. (1994). Immunobiology of murine malignant mesothelioma (MM): characterisation of tumour infiltrating leukocytes and cytokine production in a murine model of MM. *Cancer Immunology Immunotherapy* **39**, 347–359.

Brandt, W. E., McCown, J. M., Gentry, M. K. & Russell, P. K. (1982). Infection enhancement of dengue type 2 virus in the U-937 human monocyte cell line by antibodies to flavivirus cross-reactive determinants. *Infection and Immunity* **36**, 1036–1041.

Bray, M. & Lai, C. J. (1991). Construction of chimeric dengue viruses by substitution of structural protein genes. *Proceedings of the National Academy of Sciences, USA* **88**, 10342–10346.

Bretscher, P. A., Wei, G. J., Menon, J. N. & Bielefeldt-Ohmann, H. (1992). Establishment of stable, cell-mediated immunity that makes susceptible mice resistant to *Leishmania major*. *Science* **257**, 539–542.

Cecilia, D. & Gould, E. A. (1991). Nucleotide changes responsible for loss of neuroinvasiveness in Japanese encephalitis virus neutralization-resistant mutants. *Virology* **181**, 70–77.

Chen, W., Kawano, H., Men, R., Clark, D. & Lai, C. J. (1995). Construction of intertypic chimeric dengue viruses exhibiting type 3 antigenicity and neurovirulence for mice. *Journal of Virology* **69**, 5186–5190.

Downloaded from www.microbiologyresearch.org by
IP: 203.126.233.162
On: Tue, 05 Sep 2017 06:39:54

Chu, M. C., O'Rourke, E. J. & Trent, D. W. (1989). Genetic relatedness among structural protein genes of dengue 1 virus strains. *Journal of General Virology* **70**, 1701–1712.

Coffman, R. L., Lebman, D. A. & Rothman, R. (1993). Mechanisms and regulation of immunoglobulin isotype switching. *Advances in Immunology* **54**, 229–270.

Constant, S., Pfeiffer, C., Woodard, A., Pasqualini, T. & Bottomley, K. (1995). Extent of T cell receptor ligation can determine the functional differentiation of naive CD4+ T cells. *Journal of Experimental Medicine* **182**, 1591–1596.

Delenda, C., Staropoli, I., Frenkiel, M. P., Cabanie, L. & Deubel, V. (1994). Analysis of C-terminally truncated dengue 2 and dengue 3 virus envelope glycoproteins processing in insect cells and immunogenic properties in mice. *Journal of General Virology* **75**, 1569–1578.

Deubel, V., Bordier, M., Megret, F., Gentry, M. K., Schlesinger, J. J. & Girard, M. (1991). Processing, secretion, and immunoreactivity of carboxy terminally truncated dengue-2 virus envelope proteins expressed in insect cells by recombinant baculoviruses. *Virology* **180**, 442–447.

Deubel, V., Nogueira, R. M., Drouet, M. T., Zeller, H., Reynes, J. M. & Ha, D. Q. (1993). Direct sequencing of genomic cDNA fragments amplified by PCR for molecular epidemiology of dengue-2 viruses. *Archives of Virology* **129**, 197–210.

Fitzpatrick, D. R. & Kelso, A. (1995). Dissociated expression of granulocyte-macrophage CSF and IL-3 in short-term T cell clones from normal mice. *Journal of Immunology* **155**, 5140–5150.

Gentry, M. K., Henchal, E. A., McCown, J. M., Brandt, W. E. & Dalrymple, J. M. (1982). Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. *American Journal of Tropical Medicine and Hygiene* **31**, 548–553.

Halstead, S. B., Shotwell, H. & Casals, J. (1973). Studies of the pathogenesis of dengue infection in monkeys. II. Clinical laboratory responses to heterologous infection. *Journal of Infectious Diseases* **129**, 15–22.

Harris, P. & Ralph, P. (1985). Human leukemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. *Journal of Leukocyte Biology* **37**, 407–422.

Hocart, M. J., MacKenzie, J. S. & Stewart, G. A. (1989). The immunoglobulin G subclass responses of mice to influenza A virus: the effect of mouse strain, and the neutralizing abilities of individual protein A-purified subclass antibodies. *Journal of General Virology* **70**, 2439–2448.

Hosken, N. A., Shibuya, K., Heath, A. W., Murphey, K. M. & O'Garra, A. (1995). The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-αβ-transgenic model. *Journal of Experimental Medicine* **182**, 1579–1584.

Innis, B. L., Thirawuth, V. & Hemachudha, C. (1989). Identification of continuous epitopes of the envelope glycoprotein of dengue type 2 virus. *American Journal of Tropical Medicine and Hygiene* **40**, 676–687.

Jiang, W. R., Lowe, A., Higgs, S., Reid, H. & Gould, E. A. (1993). Single amino acid codon changes detected in Louping III virus antibody-resistant mutants with reduced neurovirulence. *Journal of General Virology* **74**, 931–935.

Jianmin, Z., Linn, M. L., Bilich, R., Gentry, M. K. & Aaskov, J. G. (1995). Analysis of functional epitopes on the dengue 2 E protein using monoclonal IgM antibodies. *Archives of Virology* **140**, 899–913.

Johnson, A. J., Guirakhoo, F. & Roehrig, J. T. (1994). The envelope glycoproteins of dengue 1 and dengue 2 viruses grown in mosquito cells differ in their utilization of potential glycosylation sites. *Virology* **203**, 241–249.

Kulkarni, A. B., Mullbacher, A., Parrish, C. R., Westaway, E. G., Coia, G. & Blanden, R. V. (1992). Analysis of murine major histocompatibility complex class II-restricted T-cell responses to the flavivirus Kunjin by using vaccinia virus expression. *Journal of Virology* **66**, 3583–3592.

Lanciotti, R. S., Lewis, J. G., Gubler, D. J. & Trent, D. W. (1994). Molecular evolution and epidemiology of dengue-3 viruses. *Journal of General Virology* **75**, 65–75.

Levine, B., Hardwick, J. M., Trapp, B. D., Crawford, T. O., Bollinger, R. C. & Griffin, D. E. (1991). Antibody-mediated clearance of alphavirus infection from neurons. *Science* **254**, 856–860.

Lin, B., Parrish, C. R., Murray, J. M. & Wright, P. J. (1994). Localization of a neutralizing epitope on the envelope protein of dengue virus type 2. *Virology* **202**, 885–890.

Lobigs, M., Dalgarno, L., Schlesinger, J. J. & Weir, R. C. (1987). Location of a neutralization determinant in the E protein of yellow fever virus. *Virology* **161**, 474–478.

Maraskovsky, E., Troutt, A. B. & Kelso, A. (1992). Co-engagement of CD3 with LFA-1 or ICAM-1 adhesion molecules enhances the frequency of activation of single murine CD4+ and CD8+ T cells and induces synthesis of IL-3 and IFN- but not IL-4 or IL-6. *International Immunology* **4**, 475–485.

Megret, F., Hugnoy, J. P., Falconer, A., Gentry, M. K., Morens, D. M., Murray, J. M., Schlesinger, J. J., Wright, P. J., Young, P., Van Regenmortel, H. V. & Deubel, V. (1992). Use of recombinant fusion proteins and monoclonal antibodies to define linear and discontinuous antigenic sites on the dengue virus envelope glycoprotein. *Virology* **187**, 480–491.

Monath, T. P. (1994). Yellow fever and dengue – the interactions of virus, vector and host in the re-emergence of epidemic disease. *Seminars in Virology* **5**, 133–145.

Morens, D. M. (1994). Antibody-dependent enhancement of infection and the pathogenesis of viral disease. *Clinical Infectious Diseases* **19**, 500–512.

Mroczkowski, B. S., Huvar, A., Lernhardt, W., Misono, K., Nielson, K. & Scott, B. (1994). Secretion of thermostable DNA polymerase using a novel baculovirus vector. *Journal of Biological Chemistry* **269**, 13522–13528.

O'Reilly, D. R., Miller, L. K. & Luckow, V. A. (1994). *Baculovirus Expression Vectors: A Laboratory Manual*. New York: Oxford University Press.

Putnak, R., Feighny, R., Burrous, J., Cochran, M., Hackett, C., Smith, G. & Hoke, C. (1991). Dengue-1 virus envelope glycoprotein gene expressed in recombinant baculovirus elicits virus-neutralizing antibody in mice and protects them from virus challenge. *American Journal of Tropical Medicine and Hygiene* **45**, 159–167.

Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C. & Harrison, S. C. (1995). The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature* **375**, 291–298.

Richardson, C. D. (1995). Baculovirus expression protocols. *Methods in Molecular Biology*, vol. 39.

Roehrig, J. T., Ris, P. A., Brubaker, J. R., Hunt, A. R., Beaty, B. J., Trent, D. W. & Mathews, J. H. (1994). T-helper cell epitopes on the E-glycoprotein of dengue 2 Jamaica virus. *Virology* **198**, 31–38.

Sarzotti, M., Robbins, D. S. & Hoffman, P. M. (1996). Induction of protective CTL responses in newborn mice by a murine retrovirus. *Science* **271**, 1726–1728.

Schlesinger, R. W. (1977). *Dengue Viruses*. New York: Springer-Verlag.

Seto, N. O., Ou, D. & Gillam, S. (1995). Expression and characterization of secreted forms of rubella virus E2 glycoprotein in insect cells. *Virology* **206**, 736–741.

Downloaded from www.microbiologyresearch.org by
IP: 203.126.233.162
On: Tue, 05 Sep 2017 06:39:54

Smucny, J.J., Kelly, E.P., Macarthy, P.O. & King, A.D. (1995). Murine immunoglobulin G subclass responses following immunization with dengue virus or a recombinant dengue envelope protein. *American Journal of Tropical Medicine and Hygiene* **53**, 432–437.

Staropoli, I., Clement, J.M., Frenkiel, M.P., Hofnung, M. & Deubel, V. (1996). Dengue virus envelope glycoprotein can be secreted from insect cells as fusion with the maltose-binding protein. *Journal of Virological Methods* **56**, 179–189.

Staton, J.M., Dench, J.E., Currie, B., Fitzpatrick, D.R., Himbeck, R.P., Allen, R., Bruce, J., Robinson, B. & Bielefeldt-Ohmann, H. (1995). Tissue-expression and immune recognition of stress proteins in sarcoidosis and other chronic lung diseases. *Immunology and Cell Biology* **73**, 23–32.

Trirawatanapong, T., Chandran, B., Putnak, R. & Padmanabhan, R. (1992). Mapping of a region of dengue virus type-2 glycoprotein required for binding by a neutralizing monoclonal antibody. *Gene* **116**, 139–150.

Received 2 May 1997; Accepted 3 July 1997

CHDI

Downloaded from www.microbiologyresearch.org by
IP: 203.126.233.162
On: Tue, 05 Sep 2017 06:39:54

Available online at www.sciencedirect.com
ScienceDirect
Virology 353 (2006) 166–173
VIROLOGY
www.elsevier.com/locate/yviro

A chimeric tetravalent dengue DNA vaccine elicits neutralizing antibody to all four virus serotypes in rhesus macaques

Kanakatte Raviprakash[a,*], Doris Apt[b], Alice Brinkman[b], Craig Skinner[b], Shumin Yang[b], Glenn Dawes[b], Dan Ewing[a], Shuenn-Jue Wu[a], Steve Bass[b], Juha Punnonen[b], Kevin Porter[a]

[a] Viral Diseases Department, Naval Medical Research Center, 503 Robert Grant Avenue, Silver Spring, MD 20910, USA
[b] Maxygen Inc., 515 Galveston Drive, Redwood City, CA 94063, USA

Received 15 March 2006; returned to author for revision 10 April 2006; accepted 1 May 2006
Available online 30 June 2006

Abstract

DNA shuffling and screening technologies were used to produce chimeric DNA constructs expressing antigens that shared epitopes from all four dengue serotypes. Three shuffled constructs (sA, sB and sC) were evaluated in the rhesus macaque model. Constructs sA and sC expressed pre-membrane and envelope genes, whereas construct sB expressed only the ectodomain of envelope protein. Five of six, and four of six animals vaccinated with sA and sC, respectively, developed antibodies that neutralized all 4 dengue serotypes in vitro. Four of six animals vaccinated with construct sB developed neutralizing antibodies against 3 serotypes (den-1, -2 and -3). When challenged with live dengue-1 or dengue-2 virus, partial protection against dengue-1 was observed. These results demonstrate the utility of DNA shuffling as an attractive tool to create tetravalent chimeric dengue DNA vaccine constructs, as well as a need to find ways to improve the immune responses elicited by DNA vaccines in general.
Published by Elsevier Inc.

Keywords: Dengue vaccines; Chimeric vaccines; DNA vaccines

Introduction

Dengue is one of the most important arboviral diseases of humans. An estimated 100 million dengue infections are caused each year by four antigenically distinct serotypes of dengue viruses (dengue-1 to dengue-4), resulting in widespread dengue fever (DF) and at least 250,000 cases of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (Halstead, 1988). These viruses are transmitted primarily by the mosquito Aedes aegypti and are endemic throughout the tropical and subtropical regions of the world. A major expansion of dengue transmission has occurred throughout the tropical Americas accompanied by a large increase in the number of DHF cases. Despite the public health importance of DF and DHF, vector control, which has proven difficult and costly to sustain over time, is the only available control measure (Gubler, 1994). Vaccines are available for two related flaviviruses, yellow fever (YF) virus and Japanese encephalitis (JE) virus (Barrett, 1997a, 1997b). However, decades of effort have not produced a successful dengue virus vaccine. A major obstacle to dengue vaccine development is the epidemiologic abbservation that a dengue virus infection only produces protective immunity against the infecting serotype, and that a secondary infection by a heterologous serotype increases the risk for DHF/DSS. It is postulated that this increased risk for severe dengue disease during secondary infections is due to antibody mediated enhancement (ADE) of infection (Halstead and O'Rourke, 1977), in which virus–antibody complex formed due to pre-existing antibodies from the primary infection gain greater access to target cells via the Fc-receptors. It is therefore imperative that a dengue vaccine produces robust protective immune responses to all 4 dengue serotypes. Conventional methodologies such as live attenuated virus vaccines (Edelman et al., 1994), inactivated dengue viruses (Schlesinger, 1977) and subunit vaccines (Deubel et al., 1988; Eckels et al., 1994) have not yet produced a vaccine. More recently, considerable advances have been made using dengue-yellow fever virus chimeras as vaccine candidates (Guirakhoo et al., 2001, 2002, 2004). However, these methodologies have relied on developing 4

* Corresponding author. Fax: +1 301 319 7451.
E-mail address: raviprakashk@nmrc.navy.mil (K. Raviprakash).

0042-6822/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2006.05.005

individual vaccines, one for each serotype, and mixing them to produce the final tetravalent formulation. This approach presents challenges related to commercial scale manufacture and formulation of a human dengue vaccine.

DNA vaccines offer an alternative strategy for developing effective dengue virus vaccines. This approach, in which plasmid DNA expressing an antigen of interest is used as a vaccine, has been shown to induce immune responses in animal models against a number of different viruses, including several flaviviruses (Colombage et al., 1998; Konishi et al., 1998; Phillpotts et al., 1996; Schmaljohn et al., 1997). A West Nile virus (also a flavivirus) DNA vaccine has recently been approved for use in horses (http://www.aphis.usda.gov/lpa/issues/issues_archive/wnv/wnv_vaccine.html), making it the first DNA vaccine to be commercially marketed. We have previously demonstrated the feasibility of DNA vaccines for dengue viruses in a number of studies (Kochel et al., 1997, 2000; Porter et al., 1998; Raviprakash et al., 1995, 2000a, 2000b, 2001, 2003).

In an effort to produce a single DNA vaccine capable of eliciting immune responses to all 4 serotypes, we employed directed molecular evolution by DNA shuffling and screening to generate chimeric vaccine constructs encoding antigens comprising epitopes from all four dengue serotypes. DNA shuffling and screening technologies have previously been used in evolving interferon α, IL-12, co-stimulatory molecules and viruses (Chang et al., 1999; Lazetic et al., 2002; Leong et al., 2003; Patten et al., 1997; Soong et al., 2000). Using this approach, we generated a number of chimeric DNA vaccines that were shown to contain sequences representative of all 4 dengue serotypes. A number of these chimeric DNA vaccines also produced multivalent neutralizing antibodies in mice and provided protection against intracerebral challenge by dengue-2 virus (Apt et al., 2005). In the present study, we show that 3 selected chimeric DNA vaccines produced by DNA shuffling and screening elicited multivalent dengue virus neutralizing antibodies in vaccinated rhesus macaques. When vaccinated animals were challenged with live virus, partial protection was observed.

Results

Shuffled vaccine candidates

The DNA shuffling and screening methods used to generate and select vaccine candidates that expressed antigens with combined attributes of all four dengue serotypes were recently described (Apt et al., 2005). Based on murine immunogenicity studies (Apt et al., 2005), 3 shuffled clones were selected for evaluation in a nonhuman primate model. The 3 shuffled clones, 6E12-D4, 18H6 and 2G11-D4 (referred to in this study as sA, sB and sC, respectively), all expressed antigens that reacted with all 4 dengue-specific antibodies and elicited antibodies in vaccinated mice that neutralized all 4 dengue serotypes in vitro (Apt et al., 2005). The distribution of amino acid sequences from all 4 dengue serotypes on the hydrophilic surface of each of the 3 shuffled E (envelope) antigens (sA, sB and sC) in a modeled dimer formation (Modis et al., 2003) is shown in Fig. 1.

Immunogenicity in rhesus macaques

Animals were vaccinated by intramuscular injection of 5 mg empty vector DNA , shuffled DNA vaccines sA, sB or sC, a mixture of 1.25 mg each of the 4 wild-type parental DNA (wt-mix) or a mixture of 1.65 mg each of sA, sB and sC DNA (sABC) on days 0, 28 (week 4) and 84 (week 12). Treatment groups and the immunization regimen are shown in Fig. 2. Sera obtained 4 weeks after the first vaccination were diluted 100-fold and dengue-specific IgG antibody was measured by ELISA. All animals (6/6) vaccinated with the wild-type vaccine mixture (wt-mix) had seroconverted after the first dose and elicited antibodies that reacted with all 4 dengue antigens (Fig. 3A). Similarly, a majority of animals vaccinated with sA (4/6), sC (3/6) or sABC (4/6) had seroconverted after first dose producing antibodies reacting to all 4 dengue antigens; remaining animals produced antibodies that reacted with 2 or 3 dengue antigens. Vaccines sB produced the lowest antibody levels after the initial dose, and the antibodies reacted primarily

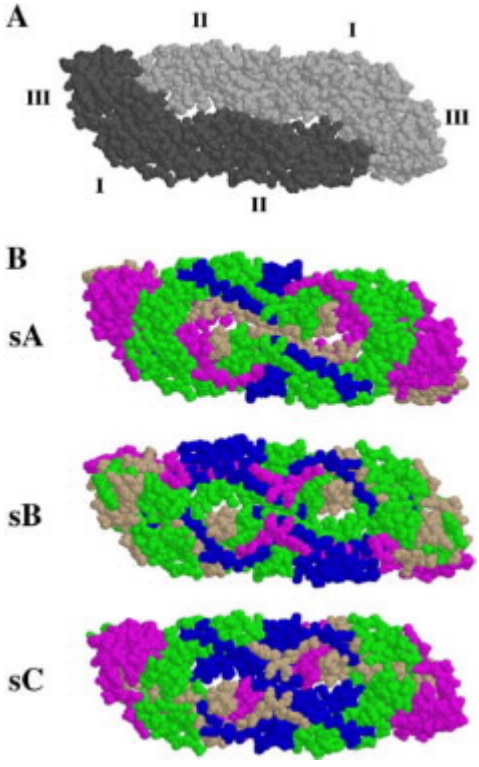


Fig. 1. Space-filling models depicting the chimeric nature of the deduced amino acid sequence expressed from shuffled DNA vaccines sA, sB and sC. (A) The hydrophilic face of the soluble dimeric ectodomain from dengue type 2 is shown. The dimer is formed by two anti-parallel E monomers, shown in light gray and dark gray. The 3 domains (I, II and III) of the ectoprotein are outlined. (B) The hydrophilic face of the shuffled E antigens, where amino acid residues from dengue-1, dengue-2, dengue-3 and dengue-4 are shown in magenta, tan, green and blue, respectively. This figure was created with protein explorer (Martz, 2002) using the atomic coordinates 1OAN (Modis et al., 2003) from the protein data bank.

Study Design

A. Vaccine and Challenge Groups

Group	Vaccine (n)	Challenge (n)
1	Vector DNA (5)	Den-1 (2); Den-2 (3)
2	Den1-Den4 Mix (wt-mix) (6)	Den-1 (3); Den-2 (3)
3	sA (6)	Den-1 (3); Den-2 (3)
4	sB (6)	Den-1 (3); Den-2 (3)
5	sC (6)	Den-1 (3); Den-2 (3)
6	sA+sB+sC (6)	Den-1 (3); Den-2 (3)

B. Vaccination and Challenge Schedule

Fig. 2. Study design. (A) Distribution of animals for vaccination and challenge. n = number of animals. (B) Vaccination and challenge schedule.

with dengue-1 and dengue-3 antigens. However, after 3 vaccinations, all animals except for the control group produced antibodies reacting with all 4 dengue antigens. Fig. 3B shows mean ELISA optical densities for each group for each dengue antigen, 4 weeks after the last immunization (week 16), measured at 1:1000 serum dilution.

The ability of these antibodies to neutralize virus in vitro was measured by plaque reduction neutralization test (PRNT). All vaccines, except sB, elicited antibodies in a subset of animals (4/6, 5/6, 4/6, 6/6 for wt-mix, sA, sC and sABC, respectively) that neutralized all 4 dengue serotypes in vitro. The geometric mean titers (GMT) for 50% neutralization ($PRNT_{50}$) for all vaccines (day 112 sera) against all 4 dengue serotypes were significantly higher compared to those for the vector control group ($P < 0.05$), with the exception of vaccine sB which elicited antibodies that only neutralized dengue-1, -2 and -3 (Fig. 4). An examination of the neutralization titers also revealed that among the 3 individual shuffled vaccines, vaccine sA performed the best, eliciting mean titers of 27, 23, 58 and 30 against dengue-1, -2, -3 and -4, respectively. However, by day 224 (day of virus challenge), the GMTs for all groups against all dengue serotypes had declined by 50% or more (Fig. 4).

Live virus challenge

Three animals from each group were challenged with live virus by subcutaneous inoculation of 10^5 plaque forming units (PFU) of dengue-1 (strain western pacific 74) or dengue-2 (strain OBS-8041) virus. The vector control group for dengue-1 challenge had 2 animals (1 animal died before the beginning of the protocol due to study unrelated cause). Viremia was assessed by detection of virus in the serum samples of daily bleeds following virus challenge. Table 1 shows a summary of viremia data, as well as neutralizing antibody titers at the time of challenge. Results suggest a partial protection against dengue-1 viremia in vaccinated groups compared to the control group. The mean days of viremia were reduced from 4 (control group) to 1.3, 2.3, 1.3, 2.3 and 0.6 for wt-mix, sA, sB, sC and sABC vaccinated groups, respectively. The reduction in the days of viremia for the sABC group was statistically significant ($P < 0.05$). However, none of the vaccinated groups showed any reduction in dengue-2 viremia. The neutralizing antibody levels at the time of challenge had declined from their observed peak levels at day 112 (Table 1 and Fig. 4), and a clear correlation between neutralizing antibody titers and protection from viremia could not be established.

To determine if the vaccination had primed the animals sufficiently to mount a secondary antibody response to virus challenge, IgM and IgG antibodies were measured by ELISA during the challenge phase (Fig. 5). For dengue-1, the vector control group exhibited a typical primary antibody response in which there was an initial IgM response by day 6 followed by an increasing IgG response by day 10 after challenge. The wt-mix, sB and sC vaccinated animals also showed an IgM response, although their IgGs were boosted at the same time. sA and

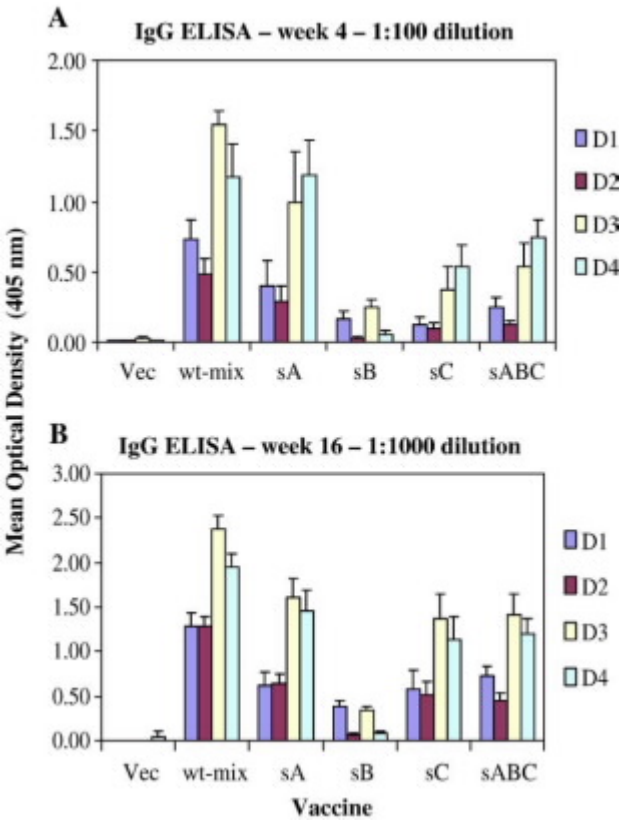


Fig. 3. ELISA reactive antibodies after 1st and 3rd dose of vaccine. Sera were diluted 1:100 (A) or 1:1000 (B) and dengue-specific IgG reacting with each serotype antigen was measured by ELISA (week 4 sera were barely positive at 1:1000 dilution). Mean optical density (405 nm) and standard error for each group against each antigen is shown. All animals were included in the analysis.

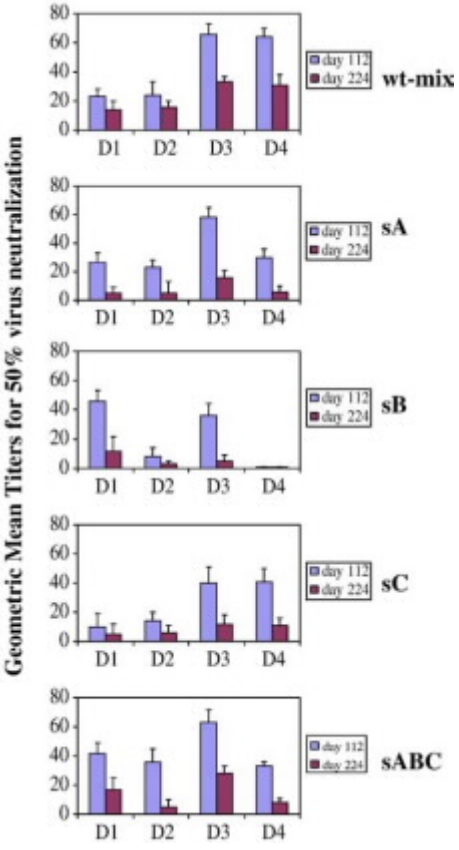


Fig. 4. Virus neutralizing antibodies. Sera from each animal obtained on day 112 (4 weeks after 3rd dose) and on day 224 (day of virus challenge) were used to measure neutralization of dengue-1, -2, -3 and -4 virus by PRNT. A pre-immune serum pool of all the animals was used as the baseline. GMT of ($PRNT_{50}$) for each vaccine group for each dengue serotype is shown.

sABC vaccinated animals demonstrated a typical secondary antibody response in which their IgG antibodies increased soon after challenge without significant accumulation of IgM antibodies. Response to dengue-2 challenge was slower; by day 10, IgM antibody was still increasing with little accumulation of IgG antibody in the vector control group. Anti-dengue2 IgM antibody was still rising on day 10 for sB vaccinated animals as well, but unlike vector control group, dengue2-IgG increased simultaneously. Although dengue2-IgG levels at the time of challenge were substantially lower compared to dengue-1 IgG, there was a rapid increase in IgG levels by day 6 without IgM accumulation in wt-mix, sA, sC and sABC vaccinated animals. Measurement of neutralizing antibodies on day 10 after challenge clearly showed a slow primary response for both dengue-1 and dengue-2 challenge in control animals whereas all vaccinated animals exhibited significantly high titers indicating an anamnestic antibody response (Table 2).

Discussion

Development of a dengue vaccine has been complicated by the need to raise protective immune responses against all 4 dengue serotypes. Traditional approaches that depend on using 4 individual dengue virus vaccines mixed to produce a final vaccine have been plagued by constraints of commercial scale manufacture and formulation. In this study, we have taken advantage of the DNA shuffling and screening technology to produce single DNA vaccine candidates that express antigens containing immunogenic epitopes from all 4 dengue serotypes. DNA shuffling and screening is a powerful technology that allows for the selection of clones based on functional attributes in the absence of precise information on the location or structure of the key epitopes. Three shuffled vaccine constructs were chosen for this study based on previous in vitro characterization and in vivo data in a murine model (Apt et al., 2005). All 3 chimeras elicited antibodies in mice that neutralized all 4 dengue serotypes in vitro.

This study demonstrated that single shuffled DNA vaccines sA and sC are capable of producing multiple serotype reactive antibodies in rhesus macaques. The mixture of 4 parental monovalent vaccine constructs and the mixture of the shuffled chimeras also produced antibodies for all serotypes. A tendency for higher antibody response for dengue-3 and dengue-4 compared to dengue-1 and dengue-2 in all vaccinated groups including the wt-mix (Figs. 3 and 4) may indicate inherent differences in the immunogenicity of these E proteins. Shuffled vaccines sA and sC induced neutralizing antibody titers that were comparable to those induced by the vaccine mixtures and caused a reduction in dengue-1 viremia indicating the feasibility of generating multivalent immune responses by single chimeric antigens in macaques. However, the best protection against dengue-1 viremia was demonstrated by the mixture of shuffled vaccines (sABC) and the mixture of parental DNA vaccines (wt-mix). No reduction in viremia was observed in vaccinated animals challenged with dengue-2 virus. It should be noted that the dengue-1 and dengue-2 sequences used for shuffling were derived from published envelope protein sequences of strains AHF 82-80 and D2-04, respectively (Apt et al., 2005). A comparison of E protein sequences of AHF 82-80 and Western Pacific 74 (dengue-1 challenge strain) revealed 7 amino acid substitutions of which 4 were conservative changes. Similarly, 10 amino acid substitutions, of which 4 were conservative changes, were found between D2-04 and OBS-8041 (dengue-2 challenge strain). It is not clear if these substitutions affected the level of protection. It is also possible that shuffling altered the folding of the chimeric envelope proteins.

Virus neutralizing antibodies are of paramount importance in protection against dengue virus (Kliks et al., 1988) and epidemiological data suggest that neutralizing antibodies are sufficient to provide protection against dengue infection. Our measurement of virus neutralizing antibodies showed that shuffled vaccines sA and sC produced virus neutralizing antibodies in macaques to all 4 dengue serotypes, just like the mixture of 4 parental vaccines did. The antibody titers were comparable to those reported for a single dose of recombinant tetravalent live attenuated virus vaccine formulation ($PRNT_{50}$ of 54, 16, 18 and 126 for dengue-1 to dengue-4, respectively) in rhesus macaques (Blaney et al., 2005). These animals were protected against dengue-1, -3 and -4 challenge but not dengue-2. In the study reported

Table 1
Viremia by virus isolation in vero or c6/36 cells

Vaccine	Challenge virus	Animal ID	Day post-challenge								Total days viremia	Mean days viremia	Den-1 PRNT-50 (day 0)	Den-2 PRNT-50 (day 0)
			1	2	3	4	5	6	7	8				
Vector DNA	D1	250										4.0		
		211	+	+	+	+	-	-	-	-	4.0		0	0
		234	+	+	+	+	-	-	-	-	4.0		0	0
	D2	212	+	+	+	+	+	-	-	-	5.0	4.6	0	0
		233	+	+	+	+	+	-	-	-	5.0		0	0
		213	+	+	+	-	+	-	-	-	4.0		0	0
wt Mix	D1	252	-	-	-	-	-	-	-	-	0.0	1.3	12	14
		247	-	-	+	-	-	-	-	-	1.0		0	0
		249	+	-	+	-	+	-	-	-	3.0		33	23
	D2	206	+	+	+	+	+	-	-	-	5.0	5.3	27	16
		225	+	-	+	+	+	+	-	-	5.0		39	25
		235	+	+	+	+	+	+	-	-	6.0		22	32
sA	D1	251	+	+	+	+	-	-	-	-	4.0	2.3	0	10
		240	-	-	+	+	-	-	-	-	2.0		0	16
		236	-	-	+	-	-	-	-	-	1.0		29	51
	D2	210	+	+	+	+	-	-	-	-	4.0	4.6	14	0
		246	+	+	+	+	-	-	-	-	4.0		10	0
		255	+	+	+	+	+	+	-	-	6.0		0	0
sB	D1	227	+	+	+	+	-	-	-	-	4.0	1.3	34	0
		214	-	-	-	-	-	-	-	-	0.0		56	13
		253	-	-	-	-	-	-	-	-	0.0		39	0
	D2	219	+	+	+	+	-	-	-	-	4.0	5.0	0	0
		208	+	+	+	+	+	+	-	-	6.0		48	0
		239	+	+	+	+	+	-	-	-	5.0		0	0
sC	D1	205	-	-	-	-	-	-	-	-	0.0	2.3	33	36
		254	+	+	+	+	-	-	-	-	4.0		0	0
		230	+	-	+	+	-	-	-	-	3.0		0	0
	D2	203	+	+	+	+	+	-	-	-	5.0	5.0	34	10
		217	+	+	+	+	+	+	-	-	6.0		20	19
		228	+	+	+	-	+	-			4.0		0	0
sABC	D1	231	-	-	-	-	-	-	-	-	0.0	0.6	32	0
		229	-	-	+	-	-	-	-	-	1.0		0	0
		258	-	-	+	-	-	-	-	-	1.0		42	0
	D2	223	+	+	+	+	+	+	-	-	6.0	5.0	53	24
		201	+	+	+	+	+	-	-	-	5.0		22	27
		224	+	+	+	+	-	-	-	-	4.0		15	13

Presence of virus in the serum of virus challenged animals was determined for 8 days after challenge as described under Materials and methods. Dengue-1 and dengue-2 viremia were determined by infection of vero and C6/36 cells, respectively. Dengue-1 and dengue-2 challenge virus stocks were produced by propagation in vero and C6/36 cells, respectively, and using these cells for viremia determination increased assay sensitivity (unpublished). '+' and '- ' indicate the presence or absence of virus in the serum of each animal for each of the days post-challenge. Also shown for reference are the dengue-1 and dengue-2 virus neutralization titers at the time of challenge (day 0).

here, we have demonstrated a reduction in viremia for dengue-1 but not dengue-2 in animals immunized with shuffled vaccine constructs. The same authors (Blaney et al., 2005) also reported that when dengue-2 neutralizing antibody titer was raised ($PRNT_{50}$ = 174) after a second dose of vaccine, animals were protected from dengue-2 challenge. In another study using recombinant MVA (modified vaccinia, Ankara) expressing dengue-2 truncated E (monovalent vaccine) protein, Men et al. (2000) have reported $PRNT_{50}$ titers of 70 and higher as being protective. Bray et al. (1996) have shown that rhesus monkeys vaccinated with a dengue-2/dengue-4 chimera vaccine (dengue-2 structural genes in an attenuated dengue-4 background) produced dengue-2 virus neutralizing antibody titers of about 640 and were protected from a dengue-2 challenge. Data presented here are consistent with these studies indicating higher neutralizing antibody titers are necessary for protection against dengue-2 challenge. However, in a tetravalent dengue vaccine study using yellow fever virus-dengue chimeras, low antibody titers of 20–80 were also found protective against dengue-2 challenge (Guirakhoo et al., 2004). It is possible that immune responses other than antibody response may be contributing to protection in this case.

It is clear from our study that the animals immunized with DNA vaccines encoding single chimeric dengue antigens provided efficient priming against multiple dengue serotypes. Accumulation of IgM antibody following challenge in some vaccinated animals may be due to presentation of some epitopes by the virus not presented by the chimeric antigen. The chimeric vaccines also provided partial protection against dengue-1

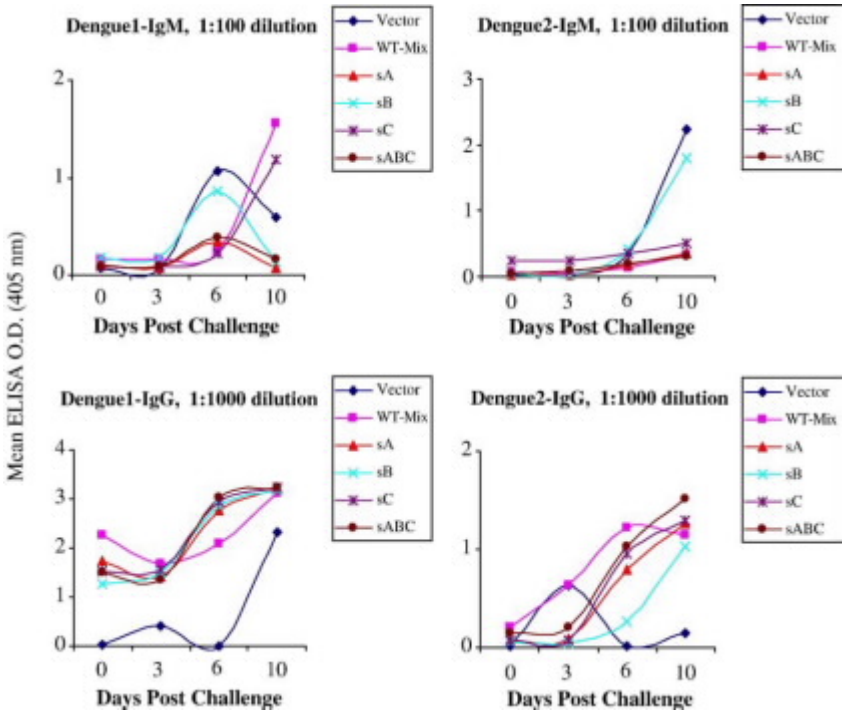


Fig. 5. IgM and IgG antibody responses following virus challenge. Sera obtained on days 0, 3, 6 and 10 (post-challenge) were diluted 1:100 (IgM) or 1:1000 (IgG) and dengue-1 or dengue-2-specific IgM and IgG were measured by ELISA as described in Materials and methods.

virus challenge. This is the first demonstration that the use of DNA shuffling technology can produce vaccine candidates that elicit immune responses against related multiple antigenic proteins in non-human primates. This is important especially to dengue vaccinology where epidemiological data mandate that a vaccine produces protective immune responses to all 4 dengue serotypes. We believe that further investigation to enhance immune responses to these and other shuffled vaccine candidates is warranted.

Table 2
Anamnestic neutralizing antibody response

Vaccine	Challenge virus	Challenge day 0 PRNT-50 (SE)		Challenge day 10 PRNT-50 (SE)	
		Den-1	Den-2	Den-1	Den-2
Vector	Den-1	1 (0)	1 (0)	182 (14)	1 (0)
	Den-2	1 (0)	1 (0)	1 (0)	56 (14)
wt-mix	Den-1	7 (9)	10 (5)	3965 (662)	1178 (616)
	Den-2	28 (5)	23 (4)	4140 (724)	2919 (359)
sA	Den-1	4 (9)	20 (12)	5339 (389)	2021 (969)
	Den-2	5 (3)	1 (0)	2611 (656)	1676 (388)
sB	Den-1	42 (6)	5 (3)	5708 (448)	472 (459)
	Den-2	3 (15)	2 (2)	706 (588)	1863 (335)
sC	Den-1	3 (10)	3 (11)	4369 (884)	1634 (418)
	Den-2	8 (9)	11 (3)	2090 (535)	2002 (587)
sABC	Den-1	11 (12)	1 (0)	4923 (713)	2560 (458)
	Den-2	26 (11)	20 (4)	3474 (767)	3434 (362)

Geometric mean of $PRNT_{50}$ titers are shown for each group at the time of challenge as well as 10 days post-challenge. Undetectable titers (<10) were assigned a value of 1 for purposes of GMT calculation. SE = standard error.

Materials and methods

Animals

Rhesus monkeys (Macaca mulatta), of either sex, age 3–8 years, weighing between 2 and 5 kg, were housed at the Naval Medical Research Center/Walter Reed Army Institute of Research animal facility in Silver Spring, MD. The monkeys were prescreened for the presence of dengue-specific antibody by enzyme linked immunosorbant assay (ELISA) and the plaque reduction neutralization test (PRNT). Only those animals that did not show evidence of previous dengue exposure were included in the study. Animals were excluded if the ELISA O.D. exceeded two or more standard deviations at a 1:100 dilution of their sera when tested against a cocktail of dengue antigens (PEG precipitated dengue-1, -2, -3 and -4 virions) compared to the O.D. obtained with a negative control antigen or if the sera (1:10 dilution) exhibited greater than 30% neutralization of input dengue virus (serotypes 1-4) in a standard PRNT.

Vaccines

Construction of the parent and shuffled DNA vaccines has been previously described (Apt et al., 2005). Four parental DNA constructs (D1, D2, D3 and D4) each expressed prM and E genes of dengue-1, -2, -3 and -4, respectively. Two shuffled DNA constructs, sA and sC (clones 6E12-d4 and 2G11-d4, respectively; Apt et al., 2005), expressed prM and E genes in which the N-terminal 90% of E gene was shuffled (contained

portions of sequence representing each of the 4 serotypes). A third shuffled DNA construct sB (clone 18H6; Apt et al., 2005) expressed a shuffled truncated E protein. The DNAs were produced free of endotoxin by Althea Corp., CA.

Immunizations

Groups of 6 animals were immunized by intramuscular injection of 5 mg empty vector DNA, shuffled vaccine A, B or C DNA (sA, sB or sC), a mixture of 1.25 mg each of the 4 wild-type parental DNA (wt-mix) or a mixture of 1.65 mg each of sA, sB and sC DNA (sABC). Immunizations were repeated at weeks 4 and 12. Three milliliters of DNA in PBS was administered at 4 sites (0.65 ml each), one in each of the deltoids and anterior tibialis muscles. Fig. 2 shows the study design indicating different vaccinations and challenges.

Antibody analyses

The monkeys were bled at 4-week intervals, and their sera were tested for dengue serotype-specific antibody by ELISA and/or PRNT. IgG antibody was detected by ELISA as previously described (Ansari et al., 1993) except that polyethylene glycol (PEG) precipitated dengue-1, -2, -3 or -4 virions were used as the antigen, and a peroxidase-labeled anti-human IgG was used as the conjugate. A similar procedure with peroxidase-labeled anti-human IgM was used for IgM determinations. PRNT was performed with vero cells as described (Russell et al., 1967), using two-fold serial dilutions of serum samples. A pool of the pre-immune sera of all the animals was used as the negative control for PRNT. Fifty percent PRNT titers ($PRNT_{50}$) were determined by probit analysis using Minitab software (Minitab Inc, State College, PA).

Virus challenge and viremia

At week 32 (20 weeks after the final dose of vaccine), 3 animals from each vaccine group were challenged with live dengue-1 or dengue-2 virus. Each monkey was bled prior to challenge, then inoculated subcutaneously in the upper left arm with 0.5 ml DMEM containing 10^5 plaque forming units (PFU) of dengue-1 (strain Western Pacific 74) or dengue-2 (strain OBS-8041) virus. The dengue-1 and dengue-2 challenge virus stocks were produced by propagation in vero or C6/36 cells, respectively. These virus doses had earlier been determined to produce uniform viremia of 3–5 days (unpublished). Monkeys were bled daily for 10 days and again on days 21 and 28 after challenge. Sera were used to measure viremia and antibody responses. Viremia was measured by inoculating 25 cm^2 flasks of confluent vero or C6/36 cells in duplicate with 0.2 ml of a 1:10 diluted serum sample. The inoculated cells were incubated for 10 days at 37 °C in a CO_2 incubator. The cells were then scraped off the flasks, washed with PBS and spotted in duplicate onto immunofluorescence slides. Dengue-specific antigen was detected by indirect immunofluorescence using mouse monoclonal antibody 7E11 and FITC-conjugated anti-mouse immunoglobulin. Monoclonal antibody 7E11 is specific to dengue NS-1 protein and is known to bind NS-1 protein of all 4 dengue serotypes (unpublished). Slides were examined under a fluorescent microscope with appropriate positive and negative controls.

Statistical analyses

Log transformed PRNT-50 titers for each of the 4 dengue serotypes were analyzed by one-way ANOVA for equality among different vaccine treatments. Animals that had titers of zero were assigned a value of 1 for log transformation purpose. Following one-way ANOVA, comparison of multiple means was performed using Fisher's LSD test. Because all the animals in the vector control group had titers of zero indicating no variability, thus nullifying the assumption of equal variances among groups, Fisher's LSD test was repeated excluding the vector control group from analysis. However, both analyses (with and without control group) produced identical results. Similarly, for virus challenge data, mean days of viremia for dengue-1 and dengue-2 were analyzed separately by one-way ANOVA and Fisher's LSD test.

Acknowledgments

We are grateful to Dr. Peter O'Hanley for his support and expert advice. This work was funded by a cooperative research and development agreement between the Naval Medical Research Center and Maxygen Inc., and by the Bill and Melinda Gates Foundation. The initial work to generate shuffled dengue antigens was supported by a grant from the Defense Advanced Research Projects Agency (DARPA). The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals", Institute of Laboratory Animals Resources, National Research Council, DHHS Publication No. (NIH) 86-23 (1985). The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position or the Department of the Navy, Department of Defense nor the U.S. Government.

References

Ansari, M.Z., Shope, R.E., Malik, S., 1993. Evaluation of vero cell lysate antigen for the ELISA of flaviviruses. J. Clin. Lab. Anal. 7 (4), 230–237.

Apt, D., Raviprakash, K., Brinkman, A., Semyonov, A., Yang, S., Skinner, C., Diehl, L., Lyons, R., Porter, K., Punnonen, J., 2005. Tetravalent neutralizing antibody response against four dengue serotypes by a single chimeric dengue envelope antigen. Vaccine, 335–344.

Barrett, A.D., 1997a. Japanese encephalitis and dengue vaccines. Biologicals 25 (1), 27–34.

Barrett, A.D., 1997b. Yellow fever vaccines. Biologicals 25 (1), 17–25.

Blaney Jr., J.E., Matro, J.M., Murphy, B.R., Whitehead, S.S., 2005. Recombinant, live-attenuated tetravalent dengue virus vaccine formulations induce a balanced, broad, and protective neutralizing antibody response against each of the four serotypes in rhesus monkeys. J. Virol. 79 (9), 5516–5528.

Bray, M., Men, R., Lai, C.J., 1996. Monkeys immunized with intertypic chimeric dengue viruses are protected against wild-type virus challenge. J. Virol. 70 (6), 4162–4166.

Chang, C.C., Chen, T.T., Cox, B.W., Dawes, G.N., Stemmer, W.P., Punnonen, J., Patten, P.A., 1999. Evolution of a cytokine using DNA family shuffling. Nat. Biotechnol. 17 (8), 793–797.

Colombage, G., Hall, R., Pavy, M., Lobigs, M., 1998. DNA-based and alphavirus-vectored immunisation with prM and E proteins elicits long-lived and protective immunity against the flavivirus, Murray Valley encephalitis virus. Virology 250 (1), 151–163.

Deubel, V., Kinney, R.M., Esposito, J.J., Cropp, C.B., Vorndam, A.V., Monath, T.P., Trent, D.W., 1988. Dengue 2 virus envelope protein expressed by a recombinant vaccinia virus fails to protect monkeys against dengue. J. Gen. Virol. 69 (Pt. 8), 1921–1929.

Eckels, K.H., Dubois, D.R., Summers, P.L., Schlesinger, J.J., Shelly, M., Cohen, S., Zhang, Y.M., Lai, C.J., Kurane, I., Rothman, A., et al., 1994. Immunization of monkeys with baculovirus-dengue type-4 recombinants containing envelope and nonstructural proteins: evidence of priming and partial protection. Am. J. Trop. Med. Hyg. 50 (4), 472–478.

Edelman, R., Tacket, C.O., Wasserman, S.S., Vaughn, D.W., Eckels, K.H., Dubois, D.R., Summers, P.L., Hoke, C.H., 1994. A live attenuated dengue-1 vaccine candidate (45AZ5) passaged in primary dog kidney cell culture is attenuated and immunogenic for humans. J. Infect. Dis. 170 (6), 1448–1455.

Gubler, D.J., 1994. Perspectives on the prevention and control of dengue hemorrhagic fever. Gaoxiong Yixue Kexue Zazhi 10, S15–S18 (Suppl.).

Guirakhoo, F., Arroyo, J., Pugachev, K.V., Miller, C., Zhang, Z.X., Weltzin, R., Georgakopoulos, K., Catalan, J., Ocran, S., Soike, K., Ratterree, M., Monath, T.P., 2001. Construction, safety, and immunogenicity in non-human primates of a chimeric yellow fever-dengue virus tetravalent vaccine. J. Virol. 75 (16), 7290–7304.

Guirakhoo, F., Pugachev, K., Arroyo, J., Miller, C., Zhang, Z.X., Weltzin, R., Georgakopoulos, K., Catalan, J., Ocran, S., Draper, K., Monath, T.P., 2002. Viremia and immunogenicity in nonhuman primates of a tetravalent yellow fever-dengue chimeric vaccine: genetic reconstructions, dose adjustment, and antibody responses against wild-type dengue virus isolates. Virology 298 (1), 146–159.

Guirakhoo, F., Pugachev, K., Zhang, Z., Myers, G., Levenbook, I., Draper, K., Lang, J., Ocran, S., Mitchell, F., Parsons, M., Brown, N., Brandler, S., Fournier, C., Barrere, B., Rizvi, F., Travassos, A., Nichols, R., Trent, D., Monath, T., 2004. Safety and efficacy of chimeric yellow fever-dengue virus tetravalent vaccine formulations in nonhuman primates. J. Virol. 78 (9), 4761–4775.

Halstead, S.B., 1988. Pathogenesis of dengue: challenges to molecular biology. Science 239 (4839), 476–481.

Halstead, S.B., O'Rourke, E.J., 1977. Antibody-enhanced dengue virus infection in primate leukocytes. Nature 265 (5596), 739–741.

Kliks, S.C., Nimmanitya, S., Nisalak, A., Burke, D.S., 1988. Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. Am. J. Trop. Med. Hyg. 38 (2), 411–419.

Kochel, T., Wu, S.J., Raviprakash, K., Hobart, P., Hoffman, S., Porter, K., Hayes, C., 1997. Inoculation of plasmids expressing the dengue-2 envelope gene elicit neutralizing antibodies in mice. Vaccine 15 (5), 547–552.

Kochel, T.J., Raviprakash, K., Hayes, C.G., Watts, D.M., Russell, K.L., Gozalo, A.S., Phillips, I.A., Ewing, D.F., Murphy, G.S., Porter, K.R., 2000. A dengue virus serotype-1 DNA vaccine induces virus neutralizing antibodies and provides protection from viral challenge in Aotus monkeys. Vaccine 18 (27), 3166–3173.

Konishi, E., Yamaoka, M., Khin Sane, W., Kurane, I., Mason, P.W., 1998. Induction of protective immunity against Japanese encephalitis in mice by immunization with a plasmid encoding Japanese encephalitis virus premembrane and envelope genes. J. Virol. 72 (6), 4925–4930.

Lazetic, S., Leong, S.R., Chang, J.C., Ong, R., Dawes, G., Punnonen, J., 2002. Chimeric co-stimulatory molecules that selectively act through CD28 or CTLA-4 on human T cells. J. Biol. Chem. 277 (41), 38660–38668.

Leong, S.R., Chang, J.C., Ong, R., Dawes, G., Stemmer, W.P., Punnonen, J., 2003. Optimized expression and specific activity of IL-12 by directed molecular evolution. Proc. Natl. Acad. Sci. U.S.A. 100 (3), 1163–1168.

Martz, E., 2002. Protein explorer: easy yet powerful macromolecular visualization. Trends Biochem. Sci. 27 (2), 107–109.

Men, R., Wyatt, L., Tokimatsu, I., Arakaki, S., Shameem, G., Elkins, R., Chanock, R., Moss, B., Lai, C.J., 2000. Immunization of rhesus monkeys with a recombinant of modified vaccinia virus Ankara expressing a truncated envelope glycoprotein of dengue type 2 virus induced resistance to dengue type 2 virus challenge. Vaccine 18 (27), 3113–3122.

Modis, Y., Ogata, S., Clements, D., Harrison, S.C., 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc. Natl. Acad. Sci. U.S.A. 100 (12), 6986–6991.

Patten P.A., Howard, R.J., Stemmer W.P., 1997. Applications of DNA shuffling to pharmaceuticals and vaccines. Curr. Opin. Biotechnol. 8 (6), 724–733.

Phillpotts, R.J., Venugopal, K., Brooks, T., 1996. Immunisation with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus. Arch. Virol. 141 (3–4), 743–749.

Porter, K.R., Kochel, T.J., Wu, S.J., Raviprakash, K., Phillips, I., Hayes, C.G., 1998. Protective efficacy of a dengue 2 DNA vaccine in mice and the effect of CpG immuno-stimulatory motifs on antibody responses. Arch. Virol. 143 (5), 997–1003.

Raviprakash, K., Liu, K., Matteucci, M., Wagner, R., Riffenburgh, R., Carl, M., 1995. Inhibition of dengue virus by novel, modified antisense oligonucleotides. J. Virol. 69 (1), 69–74.

Raviprakash, K., Kochel, T.J., Ewing, D., Simmons, M., Phillips, I., Hayes, C.G., Porter, K.R., 2000a. Immunogenicity of dengue virus type 1 DNA vaccines expressing truncated and full length envelope protein. Vaccine 18 (22), 2426–2434.

Raviprakash, K., Porter, K.R., Kochel, T.J., Ewing, D., Simmons, M., Phillips, I., Murphy, G.S., Weiss, W.R., Hayes, C.G., 2000b. Dengue virus type 1 DNA vaccine induces protective immune responses in rhesus macaques. J. Gen. Virol. 81 (Pt. 7), 1659–1667.

Raviprakash, K., Marques, E., Ewing, D., Lu, Y., Phillips, I., Porter, K.R., Kochel, T.J., August, T.J., Hayes, C.G., Murphy, G.S., 2001. Synergistic neutralizing antibody response to a dengue virus type 2 DNA vaccine by incorporation of lysosome-associated membrane protein sequence and use of plasmid expressing GM-CSF. Virology 290 (1), 74–82.

Raviprakash, K., Ewing, D., Simmons, M., Porter, K.R., Jones, T.R., Hayes, C.G., Stout, R., Murphy, G.S., 2003. Needle-free Biojector injection of a dengue virus type 1 DNA vaccine with human immunostimulatory sequences and the GM-CSF gene increases immunogenicity and protection from virus challenge in Aotus monkeys. Virology 315 (2), 345–352.

Russell, P.K., Nisalak, A., Sukhavachana, P., Vivona, S., 1967. A plaque reduction test for dengue virus neutralizing antibodies. J. Immunol. 99 (2), 285–290.

Schlesinger, R.W., 1977. Dengue viruses. Virol. Monogr. (16), 1–132.

Schmaljohn, C., Vanderzanden, L., Bray, M., Custer, D., Meyer, B., Li, D., Rossi, C., Fuller, D., Fuller, J., Haynes, J., Huggins, J., 1997. Naked DNA vaccines expressing the prM and E genes of Russian spring summer encephalitis virus and Central European encephalitis virus protect mice from homologous and heterologous challenge. J. Virol. 71 (12), 9563–9569.

Soong, N.W., Nomura, L., Pekrun, K., Reed, M., Sheppard, L., Dawes, G., Stemmer, W.P., 2000. Molecular breeding of viruses. Nat. Genet. 25 (4), 436–439.

Protein Engineering, Design & Selection vol. 26 no. 6 pp. 389–399, 2013
Published online March 10, 2013 doi:10.1093/protein/gzt010

Thermodynamic stability of domain III from the envelope protein of flaviviruses and its improvement by molecular design

Nora Zidane[1,2], Philippe Dussart[3], Laetitia Bremand[3], Maria Elena Villani[1,2,4] and Hugues Bedouelle[1,2,5]

[1]Department of Infection and Epidemiology, Unit of Molecular Prevention and Therapy of Human Diseases, Institut Pasteur, rue du Dr. Roux, F-75015 Paris, France, [2]CNRS, URA3012, rue du Dr. Roux, F-75015 Paris, France, [3]Laboratory of Virology, National Reference Center for Arboviruses, Institut Pasteur de la Guyane, Cayenne, French Guiana and [4]Permanent address: ENEA, UTBIORAD, Lab Biotecnol, CR Casaccia I-00123 Rome, Italy

[5]To whom correspondence should be addressed.
E-mail: hugues.bedouelle@pasteur.fr

Received February 5, 2013; revised February 5, 2013; accepted February 10, 2013

Edited by Alan Fersht

The Flavivirus genus includes widespread and severe human pathogens like the four serotypes of dengue virus (DENV1 to DENV4), yellow fever virus, Japanese encephalitis virus and West Nile virus. Domain III (ED3) of the viral envelope protein interacts with cell receptors and contains epitopes recognized by virus neutralizing antibodies. Its structural, antigenic and immunogenic properties have been thoroughly studied contrary to its physicochemical properties. Here, the ED3 domains of the above pathogenic flaviviruses were produced in the periplasm of Escherichia coli. Their thermodynamic stabilities were measured and compared in experiments of unfolding equilibriums, induced with chemicals or heat and monitored through protein fluorescence. A designed ED3 domain, with the consensus sequence of DENV strains from all serotypes, was highly stable. The low stability of the ED3 domain from DENV3 was increased by three changes of residues in the protein core without affecting its reactivity towards DENV-infected human serums. Additional changes showed that the stability of ED3 varied with the DENV3 genotype. The T_m of ED3 was higher than 69°C for all the tested viruses and reached 86°C for the consensus ED3. The latter, deprived of its disulfide bond by mutations, was predominantly unfolded at 20°C. These results will help better understand and design the properties of ED3 for its use as diagnostic, vaccine or therapeutic tools.

Keywords antigenicity/consensus sequence/envelope protein/flavivirus/protein stability

Introduction

The Flavivirus genus includes several human pathogens such as the yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV) and the four serotypes of dengue virus (DENV1 to DENV4). These viruses are widely distributed in the world, result in millions of human infections annually, and can cause deadly encephalitis and haemorrhagic fever. Here, we focus on these mosquito-borne flaviviruses but important studies have also been performed on tick-borne flaviviruses (Weaver and Reisen, 2010).

The flaviviruses are enveloped RNA viruses. The structures of the whole virus and of its envelope (E) protein have been solved by electron cryo-microscopy and X-ray crystallography for a number of them (Kuhn et al., 2002; Modis et al., 2003; 2005; Mukhopadhyay et al., 2003; Kanai et al., 2006; Nybakken et al., 2006; Luca et al., 2012). Ninety dimers of the E protein cover the surface of the virus. Each E protein monomer comprises three ectodomains, ED1–ED3, and a transmembrane segment. ED2 includes the dimerization interface, glycosylation sites and the peptide of fusion with the cellular membrane. ED3 is continuous and comprises residues 296–400 of the E protein (DENV1 numbering). Its fold is compact, immunoglobulin-like and stabilized by a disulfide bond between residues Cys302 and Cys333. The solution structures of recombinant ED3 domains have been solved by NMR methods for YFV, JEV, WNV, DENV2 and DENV4 (Wu et al., 2003a; Volk et al., 2004; Volk et al., 2007; Huang et al., 2008; Volk et al., 2009). The structure of the isolated ED3 domain is close to its structure in the crystals of the E protein.

The ED3 domain participates in the interaction between the virus and primary or secondary cell receptors, including heparan sulfates and ribosomal protein SA (Chen et al., 1996; Thullier et al., 2001; Hung et al., 2004; Lee et al., 2006; Pattnaik et al., 2007; Huerta et al., 2008; Hershkovitz et al., 2009; Kaufmann and Rossmann, 2011; Watterson et al., 2012; Zidane et al., 2013). Consistently, recombinant ED3 domains inhibit infectivity of the cognate virus for DENV1, DENV2, WNV and JEV and some authors have proposed to use ED3 as a basis for therapeutic molecules (Hung et al., 2004; Jaiswal et al., 2004; Chu et al., 2005; Chin et al., 2007; Chavez et al., 2010; Li et al., 2012). Mutations in the ED3 domains of YFV and DENV2 affect their cell tropism and virulence (Hahn et al., 1987; Jennings et al., 1994; Pryor et al., 2001).

In mice, the ED3 domain is highly antigenic and recognized by monoclonal antibodies (mAbs) that neutralize the virus, strongly block its adsorption to cells and protect against viral infection (Crill and Roehrig, 2001; Brien et al., 2010). ED3 contains type-, subcomplex- and complex-specific epitopes (Roehrig, 2003; Diamond et al., 2008; Chavez et al., 2010; Guzman et al., 2010). The crystal structures of several complexes between the ED3 domain and either the Fab or Fv fragment of neutralizing monoclonal antibodies have been solved to high resolution (Nybakken et al., 2005; Lok et al., 2008; Austin et al., 2012; Cockburn et al., 2012; Midgley et al., 2012).

In humans, only a small proportion of the IgG population in DENV2-, DENV3- or WNV-infected serums targets ED3,

© The Author 2013. Published by Oxford University Press All rights reserved.
For Permissions please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/peds/article-abstract/26/6/389/1541185/Thermodynamic-stability-of-domain-III-from-the
by White and Case LLP user
on 05 September 2017

it appears to play a minor role in the immune response to the infection, and it does not correlate with its clinical outcome (Oliphant et al., 2007; Crill et al., 2009; Wahala et al., 2009). However, one group has shown that the titer in IgG to ED3 in DENV2 infected serums correlates with the neutralization of DENV2 (Crill et al., 2009). Moreover, human mAbs (hmAb) to the ED3 of DENV potently neutralize DENV infection (Beltramello et al., 2010).

Several studies have shown that the ED3 domain is a useful antigen for flavivirus serologic diagnosis since antibodies to ED3 are less cross-reactive than those against ED1 or ED2 (Chavez et al., 2010). ED3 domains have been used as antigen in indirect IgM or IgG enzyme-linked immunosorbent assay (ELISA) to detect infection by DENV, WNV, JEV and several tick-borne flaviviruses (Simmons et al., 1998b; Beasley et al., 2004; Holbrook et al., 2004; Shukla et al., 2009; Batra et al., 2011). A single polypeptide that includes the ED3 domains of the four DENV serotypes has been used as an antigen for detecting infection by any of the four DENV serotypes (Batra et al., 2010b, 2011). An ED3 domain (ED3.DENVc) with the consensus sequence of ED3 domains from viral strains of the four DENV serotypes is recognized by immune ascitic fluids from mice infected by DENV1, DENV2 or DENV3 but not DENV4 (Danecek et al., 2010; Bowen et al., 2012).

ED3 has been considered as a potential immunogen for subunit vaccines against flaviviruses (Chavez et al., 2010; Guzman et al., 2010). Many studies have shown that the isolated ED3 domain is immunogenic in mice and elicits neutralizing and often protective antibodies, e.g. to JEV, WNV and the four DENV serotypes (Simmons et al., 1998a; Wu et al., 2003b; Alka et al., 2007; Chu et al., 2007; Zhang et al., 2007; Babu et al., 2008; Verma et al., 2009; Batra et al., 2010a; Guzman et al., 2010). The ED3 domain from DENV2 (ED3.DENV2) elicits neutralizing antibodies and partial protection in monkey against the cognate virus (Guzman et al., 2010). Multivalent ED3 domains, i.e. single polypeptides including the ED3 domains from several serotypes of DENV, elicit neutralizing and protective antibodies in mice, e.g. simultaneously to DENV2 and DENV4 or to the four DENV serotypes (Khanam et al., 2006; Chen et al., 2007; Etemad et al., 2008). A consensus ED3.DENV domain elicits protective antibodies to all four DENV serotypes (Leng et al., 2009; Chiang et al., 2011). Recombinant or synthetic genes coding for a single ED3 domain or several ED3.DENV domains in tandem have been inserted in the genome of infective non-pathogenic viruses and shown to elicit neutralizing antibodies in mice, e.g. using adenovirus or measle vaccine virus as vectors (Brandler et al., 2007, 2010; Khanam et al., 2009).

Thus, the ED3 domains have been thoroughly studied from the viewpoints of structure, function, antigenicity and immunogenicity. However, very few studies of their physicochemical properties have been reported (Yu et al., 2004). Here, we report the production of the isolated ED3 domains from several pathogenic flaviviruses in Escherichia coli. We measured and compared their thermodynamic stabilities in experiments of unfolding equilibriums, induced with chemical denaturants or heat, and monitored through the protein intrinsic fluorescence. In particular, we measured the stability of the ED3.DENVc domain that derived from the four serotypes of DENV by consensus (Danecek et al., 2010). We increased the stability of the least stable ED3 domain, ED3.DENV3, by molecular design without modifying its antigenicity and analyzed whether this stability varied with the genotype within the serotype. Finally, we abolished the disulfide bond of an ED3 domain by mutation and determined its folding state at 208C. We discuss our results in relation with the biology of the flaviviruses and the potential applications of the ED3 domains.

Materials and methods

Media, reagents and buffers

The culture medium 2-YT, NuPAGE Novex Bis-Tris 4–12% gradient gels, MES-SDS Running Buffer, sample buffer and See Blue Plus 2 Prestained Standards were purchased from Invitrogen; IPTG from Euromedex; phosphate-buffered saline (PBS), MES hydrate, Tween 20, 4-nitrophenyl phosphate (pNPP), polymyxin B sulfate, hen egg-white lysozyme and goat antibodies to human IgMs from Sigma-Aldrich; bovine serum albumin from Roche; low-fat milk powder from Regilait; Maxisorp ELISA plates from NUNC; the fast-flow Ni-NTA resin from Qiagen; the Quick change II site-directed mutagenesis kit from Agilent Technologies; and ultrapure urea and Guanidine-HCl (GuHCl) from MP Biochemicals. Ampicillin was used at 200 mg ml^{-1} and chloramphenicol at 34 mg ml^{-1}. The solutions of 10 M urea and 8 M GuHCl were prepared daily and their pH was adjusted as described (Pace, 1986). The concentrations of denaturant, urea or GuHCl, were measured with a refractometer and a precision of 0.01 M.

Buffer A contained 20 mM Tris-HCl, 500 mM NaCl; buffer B: 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0; buffer C: 20 mM Tris-HCl, 100 mM NaCl; buffer D: 50 mM Tris-HCl, 150 mM NaCl, pH 7.9; buffer E: 30 mM MES, 150 mM NaCl, pH 5.5; buffer F: 0.1% Tween 20 in PBS; buffer G: 5% (w/v) low-fat milk powder in buffer F; buffer H: 1% (w/v) low-fat milk powder in buffer F; and buffer J: 10% diethanolamine, pH 9.8, 10 mM $MgSO_4$, 20 mM $ZnCl_2$. The pH of buffers A and C was distant by 1 unit from the protein pI.

Bacterial, plasmid and viral strains

The E.coli strain BL21(DE3, pLysS), plasmid pET20b+ (Novagen) and the plasmids encoding the ED3-H6 and H6-ED3-PhoA hybrids (Brandler et al., 2007; Lisova et al., 2007; Zidane et al., 2013) have been described. The origin of the viral ED3 domains and the corresponding segments of the envelope protein are indicated in Table I.

Clinical samples

A first set of human serums (Group 1) was collected within the normal activity of the National Reference Center (NRC) for Arboviruses, Institut Pasteur de la Guyane, French Guiana. These serums were collected from patients who displayed clinical symptoms of dengue and whose infection by DENV was confirmed by laboratory methods. A second set of human serums (Group 2) was collected in the context of a clinical study (DENFRAME project) that was performed in French Guiana (Dussart et al., 2012). These serums were collected from patients who displayed clinical symptoms of dengue but were diagnosed as negative for DENV infection.

Downloaded from https://academic.oup.com/peds/article-abstract/26/6/389/1541185/Thermodynamic-stability-of-domain-III-from-the
by White and Case LLP user
on 05 September 2017

Table I. Viral origins of the ED3-H6 domains. The last column gives the residues of the viral E protein present in the ED3-H6 domains. The ED3 domains of WNV and JEV have three extra residues. The codons in the recombinant genes were synonymous but not necessarily identical with those in the original viral genomes.

Virus	Strain	Genbank no	E residues
DENV1	FGA/89	AF226687	296–400
DENV2	Jamaica/N.1409	M20558	296–400
DENV3	PaH881/88	AF349753	294–398
DENV4	ThD4–0113–76	AY618949	296–400
YFV	17D vaccine strain	X03700	294–398
YFV	Asibi	AY640589	294–398
WNV	IS-98-STD	AF481864	299–406
JEV	SA14	U14163	298–405

In the following, we designate these serums as DENV-uninfected serums. Both Group 1 and Group 2 serums consisted of paired blood samples collected during the acute and convalescent phases of infection. The samples were characterized by standard diagnostic methods, as described in details elsewhere, in particular, the DENV serotype that was responsible for the disease was identified by reverse transcriptase polymerase chain reaction (RT-PCR) (Dussartet al., 2012). All the serums of group 1 were positive for the presence of anti-DENV IgM in an IgM antibody capture ELISA (MAC-ELISA) that used mouse brain extracts as antigens. The constitution of the above human serum collections and their use for the present study were approved by the Institutional Review Board of Institut Pasteur and a regional ethical committee (Comité de Protection des Personnes, Ile-de-France 1).

Genetic constructions

The genes coding for the ED3.YFV(Asibi), ED3.DENVc and ED3.DENV3(st) domains were chemically synthesized by GenScript (USA) after optimization of the codon usage for E.coli. They were inserted between the NcoI and XhoI of plasmid pET20b+, under control of the pelB signal sequence as previously described (Lisova et al., 2007). Mutations were introduced by site-directed mutagenesis with synthetic oligonucleotides and the Quick change II kit. The changes of nucleotides that resulted in the change C302V of amino acid residue in ED3.DENVc, introduced a second NcoI site at codons 300–302, 5′ACC-ATG-GTT3′. The pelB signal sequence of the double mutant ED3.DENVc(C302V,C333A) was precisely deleted by inserting a synthetic double-stranded DNA cassette between the NdeI site, coding for the initiator Met, and the NcoI sites at codons 300–302. This cassette was formed by annealing two synthetic oligonucleotides: 5′TATGGGCATGAGCTATAC3′ and 5′CATGGTATA GCTCATGCCCA′. All the genetic constructions were verified by DNA sequencing.

Production and purification of the hybrids

The productions of the ED3-H6 and H6-ED3-PhoA hybrids in the periplasmic space of E.coli and their purification from periplasmic extracts through their His-tag were performed essentially as described (Brandler et al., 2007; Lisova et al., 2007; Zidane et al., 2013). The cysteine-less domain ED3.DENVc(C302V,C333A)-H6 was produced in the cytoplasm of strain BL21(DE3, pLysS). The producing bacteria were grown at 24–28°C in 2-YT broth, supplemented with ampicillin and chloramphenicol during a preculture and with ampicillin alone during the main culture. The expression of the Cys-less ED3-H6 domain was induced during the exponential phase of growth by the addition of 1.0 mM IPTG and the bacteria further grown overnight in the same conditions. The subsequent steps were performed at 4–8°C. One volume of culture was cooled on ice and then centrifuged to pellet the bacteria. The bacterial pellet was resuspended in 1/20th volume of 1 mg ml^{-1} lysozyme, 5 mM imidazole in buffer A and the cells were disrupted by sonication. The insoluble materials were pelleted by centrifugation, washed with 1/20th volume of 5 mM imidazole in buffer A and pelleted again. The subsequent steps were performed at room temperature to avoid crystallization of urea. The insolubles were resuspended in 1/20th volume of buffer B. The suspension was mildly agitated until it became translucent, and then centrifuged 20 min at 15000*g* to remove the cell debris. The supernatant contained the solubilized proteins.

The Cys-less ED3-H6 domain was then separated from the other proteins by affinity chromatography on a column of NiNTA resin (0.5 ml l^{-1} of culture), and refolded within the column before elution. The column was equilibrated with 6 volumes of buffer B, loaded with the preparation of solubilized proteins and then washed with 20 volumes of buffer C to remove urea and the unbound proteins. The subsequent steps were performed at 4–8°C. The outlet of the column was capped, one volume of buffer C was added and the reaction of refolding was left to run for several hours. The column was washed with 10 volumes of 5 mM imidazole in buffer A to remove the weakly bound proteins. The Cys-less ED3-H6 domain was then eluted by a step gradient of imidazole in buffer A. The fractions of purification were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in reducing conditions. The purest fractions were pooled, snap-frozen and kept at −20–80°C. They were homogeneous at ~98%. The yield of purified ED3.DENVc(C302V,C333A) domain was about 3 mg l^{-1} of bacterial culture.

Fluorescence measurements

The fluorescence spectra were recorded with a FP-6300 spectrofluorometer (Jasco) in a quartz cuvette of 10 × 2 mm^2 path lengths, between 310 and 370 nm with excitation at 278 nm in buffer D. The slit width was equal to 2.5 nm for the excitation light and 5 nm for the emission. The increment of wavelength was equal to 0.5 nm. The fluorescence signal for the protein was obtained by subtraction of the signal for the solvent alone.

Unfolding with urea or GuHCl

Unfolding with urea or GuHCl was performed as described (Pace, 1986). Each reaction mixture (1 ml) contained purified protein (10 mg ml^{-1}) and varying concentrations of urea (0–8 M) or GuHCl (0–6 M) in buffer D. Control reactions were prepared by replacing the protein with buffer. The mixtures were incubated for ~14 h at 20–28°C to enable the reaction of unfolding to reach equilibrium. To test the reversibility of the unfolding reaction, a protein sample was denatured in 8 M urea or 6 M GuHCl and buffer D for 4 h. The denatured protein was diluted with buffer D to reach varying concentrations of denaturant. The diluted mixtures were then incubated

Downloaded from https://academic.oup.com/peds/article-abstract/26/6/389/1541185/Thermodynamic-stability-of-domain-III-from-the
by White and Case LLP user
on 05 September 2017

for 14 h at 208C as above. The concentration of denaturant was measured in each reaction mixture after completion of the experiments. The equilibriums of unfolding were monitored with the intrinsic fluorescence of proteins as described in the previous paragraph.

Thermal unfolding

Unfolding with heat was performed with a Quantamaster spectrofluorometer (Photon Technology International), equipped with a computer-operated thermoelectric cuvette holder. The signal for the protein was obtained by subtraction of the signal for the solvent alone, recorded in a separate experiment. The purified protein (80 mg ml^{-1}) in buffer D or E was melted in a quartz cuvette of 10 10 mm^2 path-length with a constant heating rate of 0.258C min^{-1} between 45 and 908C. The intrinsic fluorescence was excited at 278 nm and the emission was recorded at 330 and 350 nm. The excitation and emission slit widths were equal to 1 and 10 nm, respectively.

Thermal precipitation

The periplasmic extracts were divided into identical portions, which were simultaneously incubated at 48C, 598C and 698C for 30 min. The samples were cooled for ten minutes on ice, the protein precipitates were eliminated by centrifugation and the supernatants were analyzed by SDS-PAGE in reducing conditions.

MAC-ELISA

The capture ELISAs were performed in 96-well flat-bottom microtiter plates with a volume of 100 ml/well. The plates were sensitized with antibodies to human IgMs as follows. A goat antibody to human IgMs (1.0 mg ml^{-1} in PBS) was loaded in wells of the plate. The plate was incubated overnight at 48C for the reaction of adsorption. The wells were washed with buffer F (three times), blocked with buffer G for 1 h at 378C and then washed as above. The serums and recombinant antigens were diluted in buffer H. The serums were diluted 100-fold. Wells were loaded with the diluted serums or with buffer H as a blank sample, and the plate was incubated for 1 h at 378C for the reaction of antibody capture. The wells were washed as above and then loaded with the solution of recombinant antigen. The plate was incubated for 1 h at 378C for the binding reaction. The wells were washed as above and the bound antigen, (ED3-PhoA)$_2$, was detected by addition of 5 mM pNPP in buffer J and measurement of A_{405nm} after 3 h at 258C.

Analysis of the sequences, structures and experimental data

The values of the theoretical molecular mass (MM_{th}) and extinction coefficient e_{280nm} of the proteins were computed from the amino acid sequences with the sub-program PepStat of the EMBOSS software suite (Rice et al., 2000). The atomic coordinates of the proteins were retrieved from the RCSB protein data bank (www.rcsb.org). The solvent accessible surface areas (ASA) were computed with the access routine of the What If software package and a probe radius of 1.4 Å (Vriend, 1990). The structures were drawn with RasMol version 2.7.3 (www.rasmol.org).

The equilibriums of unfolding were analyzed with a two-state system, exactly as described (Ould-Abeih et al., 2012). The curve fits were performed with Kaleidagraph (Synergy Software), which gives Pearson's coefficient of correlation, R_P. Semi-parametric receiver operating characteristics (ROC) curves were computed with the LABROC4 program (Metz et al., 1998) as implemented in the Web based calculator JLABROC4 by J. Eng (http://www.jrocfit.org). These curves plot the true positive fraction of samples as a function of their false-positive fraction when the value of the threshold varies. The area under the curve (AUC) is an unbiased measure of the assay accuracy. The standard error SE on a sum of two terms A and B was calculated from the equation of error propagation: $SE(A + B)^2 = SE(A)^2 + SE(B)^2$.

Results

Fluorescence properties of recombinant ED3 domains

The isolated ED3 domains from several pathogenic flaviviruses were produced in the periplasmic space of E. coli where the formation of their disulfide bond is enzymically catalyzed, and purified through their hexahistidine tag. Each ED3 domain includes a tryptophan residue, which is conserved in all flaviviruses, unique in the mosquito-borne flaviviruses (position 391 in the E protein of DENV1) and partially buried in the available crystal structures (see Introduction). The intrinsic fluorescence of the ED3 domains was excited at the wavelength of 278 nm. For all the ED3 domains that we studied here, the value of the fluorescence intensity F decreased and the wavelength l_{max} of its maximum increased when going from the native state (N) to the unfolded state (U) in 8 M urea or 6 M guanidinium-HCl (GuHCl) as previously reported for the ED3 domain from DENV1 (ED3.DENV1) (Monsellier and Bedouelle, 2005). More precisely, we found 340 nm, l_{max}, 343.3 nm for the N state and 347 nm, l_{max}, 350.5 nm for the U state at pH 7.9, 208C.

Stability of ED3 domains as measured with chemical denaturants

We measured the thermodynamic stability of ED3 domains by analyzing their unfolding equilibriums, induced with urea and monitored through their fluorescence intensity F. The signal was measured at the emission wavelength for which the difference between the F values for states N and U was maximal. The profiles of unfolding showed a single cooperative transition when the concentration of denaturant x varied (Fig. 1). We, therefore, modeled them by a two-state system, N and U. The fitting of this model to the experimental data allowed us to determine three characteristic parameters (only two of which are independent) the difference in free energy $DG(H_2O)$ between states N and U in the absence of denaturant, which is the protein stability; its coefficient of dependence m towards the concentration of denaturant, which measures the cooperativity of unfolding; and the concentration $x_{1/2}$ of denaturant for half-unfolding, which measures the protein resistance to unfolding (Table II).

The unfolding profile of ED3.DENV2, induced with urea, was incomplete because the cooperative transition happened at too high a value of x. We, therefore, induced its unfolding with GuHCl, a stronger denaturant. We performed both types of experiments, with urea and GuHCl, for ED3.DENV1 because we wanted to rank the stabilities of the different ED3 domains (Table II) and the $DG(H_2O)$ values are not

Downloaded from https://academic.oup.com/peds/article-abstract/26/6/389/1541185/Thermodynamic-stability-of-domain-III-from-the
by White and Case LLP user
on 05 September 2017

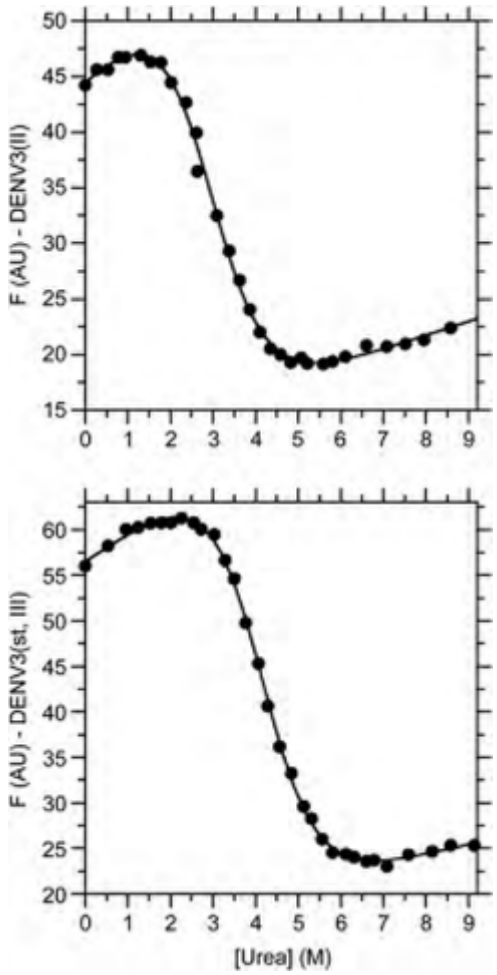


Fig. 1. Unfolding equilibria of ED3.DENV3 derivatives, induced with urea at 20°C in buffer D and monitored through their intrinsic fluorescence. The proteins were excited at 278 nm and their fluorescence intensity F recorded at 332.5 nm for ED3.DENV3(II) (top part) and 337 nm for ED3.DENV3(st, III) (bottom part), in arbitrary units (AU). The solid lines correspond to the fitting of a two-state model to the experimental data points (R_P = 0.99879 and 0.99967, respectively).

necessarily identical when obtained with urea or GuHCl (Monera et al., 1994). We checked that its unfolding equilibriums were reversible when induced with GuHCl, as they were with urea (Monsellier and Bedouelle, 2005).

We found the following decreasing order for the stabilities $\Delta G(H_2O)$ of the ED3 domains: WNV = DENV2 > DENV4 > DENV1 > JEV > YFV(17D) > YFV(Asibi) > DENV3. The values of $x_{1/2}$ gave a slightly different order because of the slight variations in the values of m and the relation $\Delta G(H_2O) = m x_{1/2}$. For proteins of identical lengths and close structures, the variations of m are usually due to differences in the residual interactions that exist in the unfolded state and are favored by the existence of a disulfide bond (Monsellier and Bedouelle, 2006).

Stability of ED3.DENV1 at pH 5.5

The envelope protein of flaviviruses undergoes a transition from a dimeric to a trimeric quaternary structure when it goes from a neutral to a low pH, as in the endosomes, and fuses with the cell membrane. The structure of the ED3 domain is maintained during this transition but its position relative to ED1 and ED2 is modified (Modis et al., 2004; Nayak et al., 2009). We compared the stabilities of the ED3.DENV1 domain at pH 7.9 and 5.5 to reveal a potential change in the endosomes. We found a significant difference in stability between the two pHs, $\Delta\Delta G(H_2O)$ = 0.9+ 0.2 kcal mol^{-1}, due to a smaller cooperativity of unfolding at pH 5.5, Δm = 0.19+ 0.03 kcal mol^{-1} M^{-1} (Table II).

Table II. Thermodynamic parameters for the unfolding equilibriums of ED3 domains, induced with chemical denaturants at 20°C and monitored through the intrinsic fluorescence intensity of the protein. The fluorescence was excited at 278 nm. Mean and SE value in at least three independent experiments.

ED3 origin	Denat.	$\Delta G(H_2O)$ (kcal mol^{-1})	m (kcal mol^{-1} M^{-1})	$x_{1/2}$ (M)
DENV1	Urea	5.21+ 0.10	1.04+ 0.01	5.02+ 0.03
DENV1 (pH5.5)	Urea	4.34+ 0.20	0.85+ 0.03	5.13+ 0.06
DENV3(II)	Urea	2.78+ 0.14	0.95+ 0.03	2.91+ 0.07
DENV4	Urea	5.54+ 0.08	1.32+ 0.02	4.20+ 0.01
YFV(Asibi)	Urea	3.92+ 0.24	1.21+ 0.06	3.23+ 0.06
YFV(17D)	Urea	4.86+ 0.15	1.17+ 0.03	4.17+ 0.03
WNV	Urea	6.05+ 0.30	1.47+ 0.07	4.12+ 0.02
JEV	Urea	5.10+ 0.23	1.39+ 0.06	3.65+ 0.04
DENV3(st, II)	Urea	3.73+ 0.12	1.00+ 0.03	3.75+ 0.03
DENV3(st, II, L303T)	Urea	5.28+ 0.47	1.24+ 0.12	4.28+ 0.08
DENV3(st, II, K385N)	Urea	3.72+ 0.27	0.97+ 0.06	3.83+ 0.06
DENV3(st, III)	Urea	4.37+ 0.21	1.05+ 0.05	4.17+ 0.03
DENV1	GuHCl	4.24+ 0.19	2.87+ 0.14	1.48+ 0.05
DENV2	GuHCl	5.07+ 0.16	2.69+ 0.09	1.89+ 0.01
DENVc	GuHCl	5.17+ 0.33	2.28+ 0.12	2.26+ 0.03

Denat, nature of the denaturant.

Stability of a consensus ED3 domain

We designed, optimized for E.coli, and chemically synthetized a recombinant gene that encoded the consensus sequence for the ED3 domains from viral strains of the four DENV serotypes (Daneceket al., 2010). We produced and purified this consensus domain, which we named ED3.DENVc, as described for the parental ED3 domains and measured its stability by monitoring its unfolding equilibriums induced with GuHCl. Its yield of production and purification was equal to 7 mg l^{-1} of culture in flask. Its stability was identical to that of ED3.DENV2, i.e. greater or equal to the stabilities of the individual ED3.DENV domains from the four serotypes. The resistance $x_{1/2}$ to denaturation of ED3.DENVc was significatively greater than those of ED3.DENV1 and ED3.DENV2, but its cooperativity m of unfolding was slightly lower (Table II).

Design of a stabilized ED3.DENV3 domain

The ED3.DENV3 domain was the least stable of all the flaviviral ED3 domains tested. This poor stability could be a disadvantage for its uses. We, therefore, sought to increase its stability without compromising its antigenicity. Our design consisted in changing buried residues of ED3.DENV3 into those of ED3.DENVc, while keeping unchanged its solvent exposed residues. Therefore, we considered only those residues of ED3.DENV3 whose side chains had solvent ASA lower than 10 Å^2 and which were different in ED3.DENVc. This search restricted the changes to three positions, 320,

Downloaded from https://academic.oup.com/peds/article-abstract/26/6/389/1541185/Thermodynamic-stability-of-domain-III-from-the
by White and Case LLP user
on 05 September 2017

322 and 339 (with the DENV1 numbering; Fig. 2). We, therefore, introduced three changes I320V, I322V and T339I in ED3.DENV3 at the genetic level. The resultant derivative, which we named ED3.DENV3(st), was more stable than the parental ED3.DENV3, with $\Delta\Delta G(H_2O) = 0.95 \pm 0.18$ kcal mol^{-1}. This stabilization was due to a greater resistance to denaturation, with $\Delta x_{1/2} = 0.84$ M urea, without any change in the cooperativity m of unfolding.

Variation of stability between genotypes within a DENV serotype

The sequence of our parental ED3.DENV3 domain was identical to that of the ED3 domain from the prototypic strain Philippines/H87/1956, which belongs to the Asian genotype II of DENV3. Therefore, we renamed ED3.DENV3(II) and ED3.DENV3(st, II) our parental ED3.DENV3 domain and its stabilized derivative to indicate their genotype. The ED3 domain from strain H87 is not recognized by human serums that were infected and sampled during the Havana (Cuba) epidemics of 2001–02 (Zulueta et al., 2006). We observed that the sequence of the ED3.DEN3 domains from strains of the Asian genotype II and from strains of the Latin-American genotype III differ only by two changes, L303T and K385N (DENV1 numbering), of residues whose side chains are exposed to the solvent and, for some of them, by an additional change, A331V (Fig. 2) (Modis et al., 2005; Wahala et al., 2010). These residues are, respectively, Thr303, Ala331 and Ser385 in ED3.DENVc. On the basis of the above, we constructed an additional derivative of ED3.DENV3(II) that derived from ED3.DENV3(st, II) by the two changes of residues L303T and K385N and that we named ED3.DENV3(st, III). We also constructed two intermediate domains that carried the single changes L303T or K385N and that we named ED3.DENV3(st,II, L303T) and ED3.DENV3(st,II, K385N).

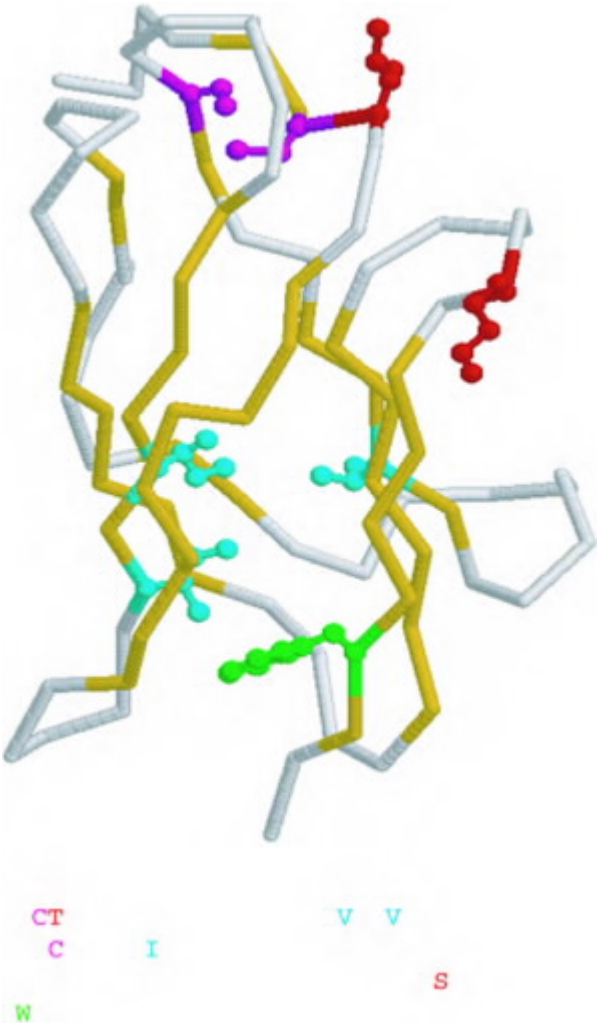


Fig. 2. Comparison between the structure of ED3.DENV3(II) and the sequence of ED3.DENVc. The structure of ED3.DENV3(II) is represented by the chain of its C_a atoms. The b-sheets are colored in yellow. Relevant amino acid side chains are in ball-and-stick representation. The residues are numbered according to the E protein of DENV3, as in the PDB file 1UZG. Add two to obtain the DENV1 numbering. The sequence of ED3.DENVc is numbered according to the E protein of DENV1. The color codes of the residues are identical in the structure and sequence.

The ED3.DENV3(st, III) domain was stabler than ED3.DENV3(st, II), with $\Delta\Delta G(H_2O) = 0.64 \pm 0.25$ kcal mol^{-1}. The main contribution to the difference in stability came again from the resistance to denaturation, with $\Delta x_{1/2} = 0.42 \pm 0.05$ M urea. Overall, ED3.DENV3(st, III) was 1.59 ± 0.26 kcal mol^{-1} more stable than ED3.DENV3(II) (Table II). ED3.DENV3(st, II, L303T) was 2.50 ± 0.49 kcal mol^{-1} more stable than ED3.DENV3(II) with $\Delta x_{1/2} = 1.37 \pm 0.11$ M urea. Interestingly, mutation K385N was neutral in the structural context of ED3.DENV3(st, II) and destabilizing in the context of ED3.DENV3(st, II, L303T). The effect of mutation K385N therefore depended on the side chain in position 303. Such a context effect is quite common (Monsellier and Bedouelle, 2006).

Unfolding with heat

The experiments of chemical unfolding, induced with urea or GuHCl, allowed us to determine and compare the stabilities of ED3 domains from various flaviviruses. However, heat rather than chemical unfolding is important for applications. Therefore, we analyzed the unfolding equilibriums of several ED3 domains, induced with heat and monitored through their fluorescence intensity.

The profiles of heat-induced unfolding showed a single cooperative transition for all the analyzed ED3 domains (Fig. 3). We checked that these equilibriums were reversible (not shown) and modeled them again with a two-state system, N and U. The model depended on three parameters: the temperature T_m at which the variation of free energy $\Delta G(T)$ between states N and U was nil; the variation of enthalpy ΔH_m between states N and U at temperature T_m, and the variation of caloric capacity ΔC_p between states N and U. We reduced this number to two characteristic parameters by empirically predicting $\Delta C_p = 1.678$ kcal mol^{-1} K^{-1} as described (Myers et al., 1995).

The results (Table III) showed that the ED3.DENV1 and ED3.DENVc domains had close values of ΔH_m, 113.33 and 118.49 kcal mol^{-1}, respectively, and therefore close cooperativities of unfolding. In contrast, T_m was greater by 7.8 ± 0.2°C for ED3.DENVc than for ED3.DENV1. The T_m value was remarkably high for these two proteins, 86.17°C for ED3.DENVc and 78.37°C for ED3.DENV1. The ED3.DENV3(II) domain had both T_m and ΔH_m values lower than those of ED3.DENV1, resulting in a lower stability. The T_m value of ED3.DENV3(II) was nevertheless quite high, 70.39°C. ED3.DENV3(II) and ED3.DENV3(st,III) had close

Downloaded from https://academic.oup.com/peds/article-abstract/26/6/389/1541185/Thermodynamic-stability-of-domain-III-from-the
by White and Case LLP user
on 05 September 2017

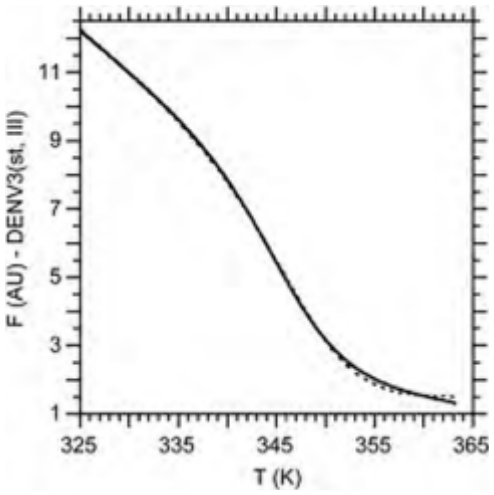


Fig. 3. Unfolding equilibria of the ED3.DENV3(st, III) domain, induced with heat in buffer D and monitored through its intrinsic fluorescence. The protein was excited at 278 nm and its fluorescence intensity F recorded at 330 nm in arbitrary units (AU). The solid line corresponds to the 3172 experimental data points and the dashed line to the fitting of a two-state model with DC_p ¼ 1.678 kcal mol^{-1} K^{-1} (R_P ¼ 0.99993).

Table III. Thermodynamic parameters for the unfolding equilibriums of ED3 domains, induced with heat and monitored through the intrinsic fluorescence intensity of the protein. The fluorescence was excited at 278 nm and its intensity recorded at 330 nm. SE in the curve fit.

ED3 origin	T_m (K)	DH_m (kcal mol^{-1})	R_P
DENV1	351.52 ± 0.17	113.3 ± 4.9	0.99966
DENV1 (pH5.5)	350.32 ± 0.04	74.1 ± 0.6	0.99996
DENV3(II)	343.54 ± 0.21	67.7 ± 1.6	0.99980
DENV3(st, III)	347.28 ± 0.03	61.0 ± 0.1	0.99993
DENVc	359.32 ± 0.18	118.5 ± 1.8	0.99985

values of DH_m, 67.7 and 61.0 kcal mol^{-1}, respectively. In contrast, T_m was greater by 3.7 °C for ED3.DENV3(st, III) than for ED3.DENV3(II). Finally, we found a slight destabilization of ED3.DENV1 at pH 5.5 relative to pH 7.9, mainly due to a lower cooperativity of unfolding, with heat as with urea (see above). It was not possible to calculate the values of $DG(H_2O)$ at 20 °C from the values of T_m and DH_m and to compare them with those obtained by unfolding with urea or GuHCl because of a too long extrapolation.

A consensus ED3 domain without disulfide-bond

We tested whether the ED3.DENVc domain, which had one of the highest thermodynamic stability ($DG(H_2O)$ ¼ 5.2 kcal mol^{-1} at 20 °C), could fold in a stable state in the absence of its disulfide bond. We constructed three double mutants of ED3.DENVc, carrying the couples of changes (C302A, C333V), (C302V, C333A) and (C302A, C333A), on the basis of published studies on immunoglobulin variable fragments (Proba et al., 1998). The three double mutants were produced in very small quantities in the periplasm of E.coli, in comparison with the parental ED3.DENVc domain. We further studied the double mutant ED3.DENVc(C302A, C333V), which was produced slightly better than the other two mutants. We constructed, at the genetic level, a derivative of ED3.DENVc(C302A, C333V) that was devoid of signal peptide for a cytoplasmic expression. An analysis of cellular extracts by SDS-PAGE showed that the double mutant was produced predominantly in the insoluble fraction and in very low quantities in the soluble fraction (not shown). The insoluble fraction was solubilized with 8 M urea and loaded on a nickel ion column. The ED3 domain was refolded within the column by washing out the urea, and then eluted with imidazole. The purified protein was highly homogeneous and its yield equal to several milligrams of protein per liter of culture in flask. An analysis of the unfolding equilibriums of the purified refolded ED3.DENVc(C302A, C333V) mutant, induced with urea at 20 °C and monitored through the ratio F_{330}/F_{350} of the intrinsic fluorescence intensities at 330 and 350 nm, showed a profile that could be approached by an exponential decay function, with a decay constant of 1.56 M^{-1}, and therefore a half-decay at 0.44 M urea (Fig. 4). These results showed that the ED3.DENVc(C302A, C333V) mutant, devoid of disulfide bond, was in equilibrium between a predominant fraction of unfolded state and a minority fraction of folded state even in the absence of urea. They were consistent with the presence of this domain as inclusion bodies in the cytoplasm and our observation that it slowly precipitated at 4 °C.

Thermoprecipitation of periplasmic extracts

The unfolding equilibriums, induced with heat, showed that the values of T_m were high for some ED3 domains. We tested whether it would be possible to obtain partially purified preparations of the ED3 domains by a differential heat-induced precipitation of the bulk of proteins in the periplasmic extracts. Identical aliquots of such extracts were treated at 48 °C, 59 °C and 69 °C for 30 min and cooled on ice. The protein precipitate was removed by centrifugation and the content of the supernatant analyzed by SDS-PAGE (not shown). We observed that the tested ED3 domains, from DENV1, DENVc and DENV3(st, III), resisted to the treatment at 69 °C and that the heat-treated periplasmic extracts were highly enriched in recombinant ED3 domain. The

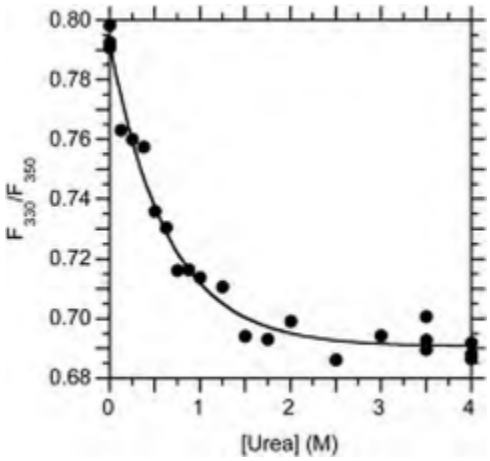


Fig. 4. Unfolding equilibria of the ED3.DENVc(C302V, C333A) domain, induced with urea at 20 °C in buffer D and monitored through its intrinsic fluorescence. The protein was excited at 278 nm and its fluorescence intensities F_{330} and F_{350} recorded at 330 and 350 nm. The urea concentration is given along the x axis and the ratio F_{330}/F_{350} along the y axis. The solid line corresponds to the fitting of an exponential decay function to the experimental data points (R_P ¼ 0.990).

Downloaded from https://academic.oup.com/peds/article-abstract/26/6/389/1541185/Thermodynamic-stability-of-domain-III-from-the
by White and Case LLP user
on 05 September 2017

results of these thermoprecipitation experiments confirmed the high resistance to heat of these three ED3 domains and could lead to simple methods of partial purification.

Antigenicity of the ED3.DENV3 derivatives

Since the engineering of the ED3.DENV3 domain aimed at increasing its stability without modifying its antigenicity, it was important to test this assumption. We constructed three dimeric hybrids ($H6\text{-}ED3\text{-}PhoA_2$) between ED3 and E.coli alkaline phosphatase to assay and compare the recognitions of the engineered ED3 domains by anti-DENV IgM in human serums. We assayed 6 serums of patients infected with DENV1, 5 with DENV2, 21 with DENV3, 5 with DENV4 and 6 non-infected serums by an IgM antibody capture ELISA (MAC-ELISA), using the dimeric hybrids as antigens. We focused our analysis on the IgMs because they are the first antibodies to appear after a flaviviral infection. The distribution of the MAC-ELISA signals were very similar for the antigens derived from DENV3(II), DENV3(st, II) and DENV3(st, III) (Fig. 5). We quantified the capacity of the three dimeric hybrids to distinguish either between DENV3-infected serums and non-infected serums or between DENV3-infected serums and the pool of serums that were non-infected or infected by DENV1, DENV2 or DENV4, by using ROC curves. AUC is a measure of the precision of the test. There were not significant differences between the precisions of the test when it was performed with any of the three dimeric hybrids, given the errors on the AUC values. Nevertheless, we observed that the signal was higher on average when the antigen was one of the stabilized ED3.DENV3 domains. In conclusion, we increased the stability of the ED3.DENV3 domain without modifying its antigenicity in a MAC-ELISA.

Discussion

A better knowledge of the biophysical properties of the ED3 domains is needed to clarify their role in antigenicity, immunogenicity and function, and to improve their applications. In particular, more data are needed on the relations between folding and either antigenicity or immunogenicity.

Periplasmic expression and folding state

Recombinant ED3 domains are generally produced in the cytoplasm of E.coli in the form of either insoluble aggregates or soluble fusion proteins, e.g. between ED3 and the maltose-binding protein. However, the disulfide bonds do not form in the reducing medium of the cytoplasm and therefore must be established in vitro. Here, we exported the isolated ED3 domains in the periplasmic space of E.coli where the formation of disulfide bonds is efficiently catalyzed in vivo by the Dsb system (Kadokura et al., 2003). In these conditions, we have previously shown that the experimental mass of the ED3.DENV1 domain is equal to the theoretical mass of a domain whose disulfide bond is correctly formed and whose N-terminal residue, resulting from the cleavage of the signal peptide, is Gly296 or Met-Gly296. An Ellman's test has shown that . 94% of the molecules of the ED3 domain have a disulfide bond, for DENV1, WNV, JEV and YFV(17D) (Lisova et al., 2007). The ED3.DENV1, ED3.DENV2 and ED3.DENV4 thus produced were crystallized in complex with the mouse neutralizing mAb 4E11, whose

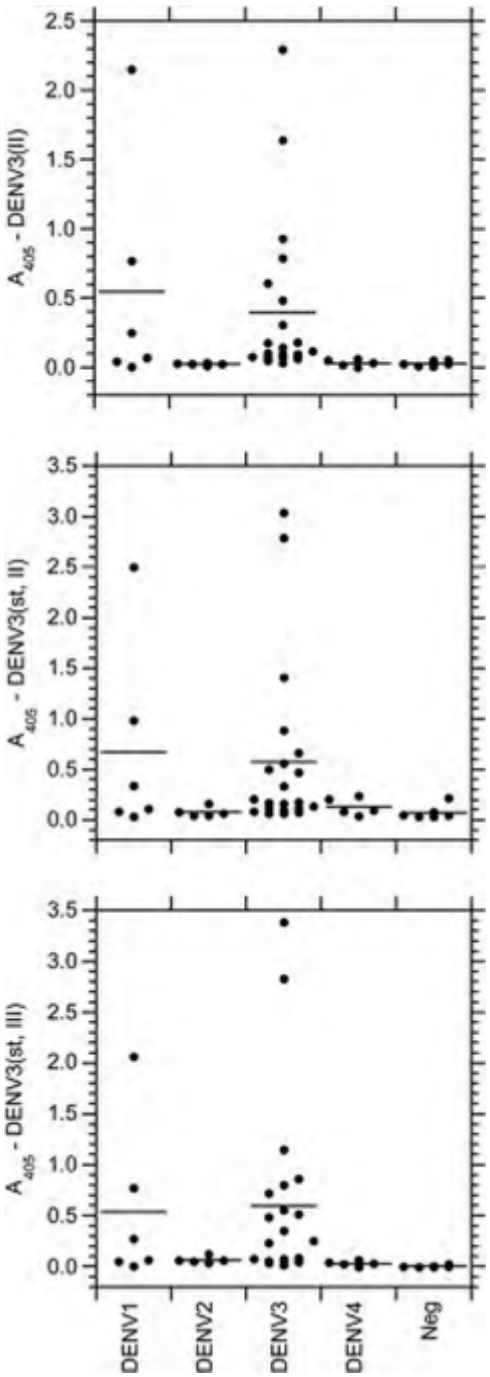


Fig. 5. MAC-ELISA performed with ($H6\text{-}ED3\text{-}PhoA_2$) dimers on five categories of human serums. The graphs give the distribution of the serum-specific signals along the y axis for each category of serum. The horizontal lines correspond to the mean value of the serum-specific signals for each category of serums. DENV1 to DENV4, human serums infected by the corresponding virus; Neg, DENV-uninfected serums. Top, middle and bottom parts, MAC-ELISA performed with ($H6\text{-}ED3\text{-}PhoA_2$) dimers from ED3.DENV3(II), ED3.DENV3(st,II) and ED3.DENV3(st,III), respectively.

epitope depends on the correct folding of the ED3 domain. The structures of the complexes at a resolution of 2 Å showed the presence of a disulfide bond and correct folding of the ED3 domain (Cockburn et al., 2012). All the ED3 domains that we studied here, except one, showed a cooperative transition of unfolding by denaturants, with a shift of their I_{max} of fluorescence emission from , 343.3nm to

Downloaded from https://academic.oup.com/peds/article-abstract/26/6/389/1541185/Thermodynamic-stability-of-domain-III-from-the
by White and Case LLP user
on 05 September 2017

. 347 nm, corresponding to an increased exposure of their unique Trp residue to solvent. The exception, ED3.DENVc (C302V, C333A), was devoid of disulfide bond, did not show any cooperative transition and was already more than half-unfolded in 0 M urea.

We tested the antigenicity of ED3.DENV3 and its various derivatives in the form of dimeric hybrids (ED3-PhoA)$_2$. The ED3 domain has one disulfide bond, the PhoA monomer has two disulfide bonds and PhoA is enzymically active only as a dimer. We have previously shown that the (ED3-PhoA)$_2$ dimers have both their ED3 and PhoA portions correctly folded and active when they are produced in the E.coli periplasm (Bedouelle et al., 2008). These dimeric hybrids have been used to probe the interaction between ED3 domains and cell receptors by using the reinforced interaction due to an avidity effect, e.g. with the NKp44 receptor of natural killer cells or the ribosomal protein SA, which is also known as the high-affinity laminin receptor 1 (Hershkovitz et al., 2009; Zidane et al., 2013). In conclusion, producing the ED3 domains, either isolated or in fusion with PhoA, in the periplasm ensures their proper folding, the formation of the disulfide bonds and thus the reproducibility of the biophysical and functional results.

Validity of a two-state model of unfolding

The recombinant ED3 domains are in a monomeric state at high concentration, as shown by structural methods (see Introduction). They can be refolded in vitro from a fully denatured state to their native state (Nybakken et al., 2005; Lok et al., 2008; Austin et al., 2012; Midgley et al., 2012). Previous studies have shown that the profiles of unfolding for the ED3 domains from several flaviviruses, monitored through circular dichroism, fluorescence or gel filtration, present a single transition in every case (Yu et al., 2004; Monsellier and Bedouelle, 2005). Here, we observed that the profiles of unfolding were cooperative, showed only one visible transition and were reversible. These profiles were approximated satisfactorily by functions that were derived from a two-state system of unfolding (Tables II and III). With regard to the experiments with chemical denaturants, the values of the cooperativity coefficient, m, were close to the values that could be predicted from their number of residues and disulfide bond, i.e. 1.218, m, 1.270 and 2.493, m, 2.593 for urea and GuHCl-induced unfolding, respectively (Myers et al., 1995). The $DG(H_2O)$ values were consistent with those reported for monomeric proteins having a similar molecular mass, and unfolding according to a two-state model (Kumar et al., 2006). With regard to the experiments with heat, the values of T_m and DH_m were consistent with those reported for similar proteins, although those for DENVc were among the highest reported for proteins from mesophilic organisms (Kumar et al., 2006). We concluded that our analysis of the unfolding profiles with a two-state model was pertinent.

Stability, structure and in vivo functions

The stability, disulfide bond and correct folding of the ED3 domain are important for its functions. For example, the epitopes of three neutralizing mouse mAbs in the E protein of DENV2 are inactivated by changing residue Cys333 into Ala at the genetic level and thus eliminating the disulfide bond between residues Cys302 and Cys333 (Roehrig et al., 2004). The epitope of another neutralizing mAb in a recombinant ED3.DENV2 domain is inactivated by the reduction and carbamidomethylation of the domain Cys residues. However, the binding of this ED3.DENV2 domain to its receptors in a cell microsomal fraction is unaffected by the chemical treatment (Huerta et al., 2008). Moreover, preparations of ED3 domains whose folding state is ill defined, can nevertheless detect antibodies to flaviviruses in infected human serums and elicit neutralizing antibodies in mice and monkeys (Simmons et al., 1998b; Beasley et al., 2004; Alka et al., 2007; Shukla et al., 2009; Wahala et al., 2009). Thus, the folding of the ED3 domain seems important only for some of its functions.

The folding of most mosquito-borne flaviviruses depends on the side chain of Tyr326, which belongs to a loop between two b-strands, is buried in the ED3 structure and forms hydrogen bonds with the main chain nitrogen atoms of residues 303 and 304 (DENV1 numbering). This structure, named 'tyrosine corner', does not exist in the viruses of the YFV group where Tyr326 is replaced by Val326 (Zhang et al., 2010). This structural difference could explain the lower stability of the ED3.YFV domains relative to those from other flaviviruses.

ED3.DENV3 was the least stable and least resistant to denaturation among the studied ED3 domains. This weak stability could explain the difficulties that we had to produce and crystallize this domain in complex with a monoclonal antibody (Cockburn et al., 2012). It could be related to the higher dynamics of this domain. Indeed, we observed that more residues are missing in the crystal structure of the isolated ED3 domain for DENV3 than for DENV1, DENV2, DENV4 or WNV (Nybakken et al., 2005; Lok et al., 2008; Austin et al., 2012; Cockburn et al., 2012; Midgley et al., 2012). This higher dynamics could be important for the interaction between the ED3 domain and cellular receptors.

Previously, differences in biophysical properties between various ED3 domains have been proposed to correlate with the higher tolerance of tick-borne flaviviruses to environmental conditions compared with mosquito-borne flaviviruses (Yu et al., 2004). Among the small sample of mosquito-borne flaviviruses that we analyzed here, a high virulence of the parental virus in primary infections was associated with a low stability of the ED3 domain, e.g. the wild-type strain YFV(Asibi) versus the vaccine strain YFV(17D); or DENV1 and DENV3 versus DENV2 and DENV4. Indeed, DENV1 and DENV3 are more pathogenic than DENV2 and DENV4 for patients who had had not an immune priming from other serotypes (Fried et al., 2010). Consistent with a proposed relation between lower stability and higher virulence, the interaction between ED3 and its cell receptors is strengthened at endosomal pH (Huerta et al., 2008) and we found that ED3.DENV1 was less stable in buffer E (pH 5.5) than buffer D (pH 7.9) (Tables II and III).

Applications

We found that some ED3 domains had strong resistances to chemical denaturant- and heat-induced unfolding. These properties are important for potential applications, e.g. use in diagnostic assays, vaccines or therapy. ED3.DENV3 was the least stable and least resistant to denaturation among the studied ED3 domains. This weak stability could bias the experimental data on antigenicity and immunogenicity obtained

Downloaded from https://academic.oup.com/peds/article-abstract/26/6/389/1541185/Thermodynamic-stability-of-domain-III-from-the
by White and Case LLP user
on 05 September 2017

with this domain. It could also be an impediment or a disadvantage for its applications. Here, we improved its stability without compromising its antigenicity. These results have potential applications for the diagnostic assays and recombinant vaccines that use ED3.DENV3 either alone or in fusion with the ED3 domains from other serotypes. It could be extended to protein domains from other pathogens. Previously, it has been reported that the binding and neutralization capacity of mouse mAbs to ED3.DENV3 are strongly influenced by naturally occuring mutations in DENV3 (Brien et al., 2010; Wahala et al., 2010). Here, the comparison between ED3.DENV3(st, II) and ED3.DENV3(st,III) showed that solvent-exposed residues could influence stability and that different genotypic strains of the same viral serotype may have different biophysical properties. These variabilities complicate the design of efficient diagnostic assays and vaccines against DENV.

As mentioned above, the ED3 domains of the four DENV serotypes are used both in diagnostic assays and vaccines. There is a clear interest in a unique antigen or immunogen that could detect an infection by any one of the four DENVs or vaccinate against all of them. Two types of solutions have been proposed to this problem. Some authors have created recombinant molecules that link the ED3 domains of the four serotypes in a single chimeric polypeptide (see Introduction). Others have created a unique ED3 domain that has the consensus sequence of the four serotypes. They have reported that such a consensus domain indeed raises protective antibodies against the four DENV serotypes and is recognized by murine antibodies to at least three of the serotypes (Leng et al., 2009; Danecek et al., 2010; Chiang et al., 2011; Bowen et al., 2012). The consensus ED3.DENVc domain has the advantage of being highly stable and having a defined fold relative to the four ED3.DENV domain linked in a single recombinant polypeptide. The greater stability of a consensus protein relative to the individual proteins of organisms that live in the same physiological conditions has been reported previously and form the basis of a general method for increasing the stability of proteins (Steipe et al., 1994; Steipe, 2004; Monsellier and Bedouelle, 2006). The present study shows that such an approach can be used to increase the stability not only of antibodies or enzymes, as previously reported, but also of antigens and immunogens provided that the solvent-accessible residues are kept unchanged.

In conclusion, our results could contribute to a better understanding of the flavivirus biology and immunology and should be useful for the potential applications of the ED3 domains in the fields of diagnostics, vaccines and therapeutic molecules against this important class of viral pathogens.

Funding

This work was supported by grants from Agence Nationale de la Recherche [grant number ANR 2010-INTB-1601–03 to H.B.] and Institut Pasteur [PTR 297].

References

Alka, Bharati,K., Malik,Y.P. and Vrati,S. (2007) Med. Microbiol. Immunol., 196, 227–231.
Austin,S.K., Dowd,K.A., Shrestha,B., Nelson,C.A., Edeling,M.A., Johnson,S., Pierson,T.C., Diamond,M.S. and Fremont,D.H. (2012) PLoS Pathog, 8, e1002930.
Babu,J.P., Pattnaik,P., Gupta,N., Shrivastava,A., Khan,M. and Rao,P.V. (2008) Vaccine, 26, 4655–4663.
Batra,G., Raut,R., Dahiya,S., Kamran,N., Swaminathan,S. and Khanna,N. (2010a) J. Virol. Methods, 167, 10–16.
Batra,G., Talha,S.M., Nemani,S.K., Dhar,N., Swaminathan,S. and Khanna,N. (2010b) Protein Expr. Purif., 74, 99–105.
Batra,G., Nemani,S.K., Tyagi,P., Swaminathan,S. and Khanna,N. (2011) BMC Infect. Dis., 11, 64.
Beasley,D.W., Holbrook,M.R., Travassos Da Rosa,A.P., et al. (2004) J. Clin. Microbiol., 42, 2759–2765.
Bedouelle,H., Brient-Litzler,E., Dussart,P., Despres,P. and Bremand,L. (2008) Patent publication Nos: EP2003144, WO2008152528, US2010291586, applicants: CNRS, Institut Pasteur.
Beltramello,M., Williams,K.L., Simmons,C.P., et al. (2010) Cell Host Microbe, 8, 271–283.
Bowen,D.M., Lewis,J.A., Lu,W. and Schein,C.H. (2012) Vaccine, 30, 6081–6087.
Brandler,S., Lucas-Hourani,M., Moris,A., Frenkiel,M.P., Combredet,C., Fevrier,M., Bedouelle,H., Schwartz,O., Despres,P. and Tangy,F. (2007) PLoS Negl. Trop. Dis., 1, e96.
Brandler,S., Ruffie,C., Najburg,V., Frenkiel,M.P., Bedouelle,H., Despres,P. and Tangy,F. (2010) Vaccine, 28, 6730–6739.
Brien,J.D., Austin,S.K., Sukupolvi-Petty,S., O'Brien,K.M., Johnson,S., Fremont,D.H. and Diamond,M.S. (2010) J. Virol. Methods, 84, 10630–10643.
Chavez,J.H., Silva,J.R., Amarilla,A.A. and Moraes Figueiredo,L.T. (2010) Biologicals, 38, 613–618.
Chen,Y., Maguire,T. and Marks,R.M. (1996) J. Virol., 70, 8765–8772.
Chen,S., Yu,M., Jiang,T., Deng,Y., Qin,C. and Qin,E. (2007) DNA Cell. Biol., 26, 361–367.
Chiang,C.Y., Liu,S.J., Tsai,J.P., Li,Y.S., Chen,M.Y., Liu,H.H., Chong,P., Leng,C.H. and Chen,H.W. (2011) PLoS One, 6, e23319.
Chin,J.F., Chu,J.J. and Ng,M.L. (2007) Microbes Infect., 9, 1–6.
Chu,J.J., Rajamanonmani,R., Li,J., Bhuvanakantham,R., Lescar,J. and Ng,M.L. (2005) J. Gen. Virol., 86, 405–412.
Chu,J.H., Chiang,C.C. and Ng,M.L. (2007) J. Immunol, 178, 2699–2705.
Cockburn,J.J., Navarro Sanchez,M.E., Fretes,N., Urvoas,A., Staropoli,I., Kikuti,C.M., Coffey,L.L., Arenzana Seisdedos,F., Bedouelle,H. and Rey,F.A. (2012) Structure, 20, 303–314.
Crill,W.D. and Roehrig,J.T. (2001) J. Virol., 75, 7769–7773.
Crill,W.D., Hughes,H.R., Delorey,M.J. and Chang,G.J. (2009) PLoS One, 4, e4991.
Danecek,P., Lu,W. and Schein,C.H. (2010) J. Mol. Biol., 396, 550–563.
Diamond,M.S., Pierson,T.C. and Fremont,D.H. (2008) Immunol. Rev., 225, 212–225.
Dussart,P., Baril,L., Petit,L., et al. (2012) PLoS Negl. Trop. Dis., 6, e1482.
Etemad,B., Batra,G., Raut,R., Dahiya,S., Khanam,S., Swaminathan,S. and Khanna,N. (2008) Am. J. Trop. Med. Hyg., 79, 353–363.
Fried,J.R., Gibbons,R.V., Kalayanarooj,S., Thomas,S.J., Srikiatkhachorn,A., Yoon,I.K., Jarman,R.G., Green,S., Rothman,A.L. and Cummings,D.A. (2010) PLoS Negl. Trop. Dis., 4, e617.
Guzman,M.G., Hermida,L., Bernardo,L., Ramirez,R. and Guillen,G. (2010) Expert Rev. Vaccines, 9, 137–147.
Hahn,C.S., Dalrymple,J.M., Strauss,J.H. and Rice,C.M. (1987) Proc. Natl Acad. Sci. USA, 84, 2019–2023.
Hershkovitz,O., Rosental,B., Rosenberg,L.A., et al. (2009) J. Immunol., 183, 2610–2621.
Holbrook,M.R., Shope,R.E. and Barrett,A.D. (2004) J. Clin. Microbiol., 42, 4101–4110.
Huang,K.C., Lee,M.C., Wu,C.W., Huang,K.J., Lei,H.Y. and Cheng,J.W. (2008) Proteins, 70, 1116–1119.
Huerta,V., Chinea,G., Fleitas,N., Sarria,M., Sanchez,J., Toledo,P. and Padron,G. (2008) Virus Res., 137, 225–234.
Hung,J.J., Hsieh,M.T., Young,M.J., Kao,C.L., King,C.C. and Chang,W. (2004) J. Virol., 78, 378–388.
Jaiswal,S., Khanna,N. and Swaminathan,S. (2004) Protein Expr. Purif., 33, 80–91.
Jennings,A.D., Gibson,C.A., Miller,B.R., et al. (1994) J. Infect. Dis., 169, 512–518.
Kadokura,H., Katzen,F. and Beckwith,J. (2003) Annu. Rev. Biochem., 72, 111–135.
Kanai,R., Kar,K., Anthony,K., Gould,L.H., Ledizet,M., Fikrig,E., Marasco,W.A., Koski,R.A. and Modis,Y. (2006) J. Virol., 80, 11000–11008.
Kaufmann,B. and Rossmann,M.G. (2011) Microbes Infect., 13, 1–9.
Khanam,S., Etemad,B., Khanna,N. and Swaminathan,S. (2006) Am. J. Trop. Med. Hyg., 74, 266–277.

Downloaded from https://academic.oup.com/peds/article-abstract/26/6/389/1541185/Thermodynamic-stability-of-domain-III-from-the
by White and Case LLP user
on 05 September 2017

Khanam,S., Pilankatta,R., Khanna,N. and Swaminathan,S (2009) Vaccine, 27, 6011–6021.
Kuhn,R.J., Zhang,W., Rossmann,M.G., et al. (2002) Cell, 108, 717–725.
Kumar,M.D., Bava,K.A., Gromiha,M.M., Prabakaran,P., Kitajima,K., Uedaira,H. and Sarai,A. (2006) Nucleic Acids Res., 34, D204–206.
Lee,J.W., Chu,J.J. and Ng,M.L. (2006) J. Biol. Chem., 281, 1352–1360.
Leng,C.H., Liu,S.J., Tsai,J.P., et al. (2009) Microbes Infect., 11, 288–295.
Li,C., Zhang,L.Y., Sun,M.X., Li,P.P., Huang,L., Wei,J.C., Yao,Y.L., Isahg,H., Chen,P.Y. and Mao,X. (2012) Antivir. Res., 94, 179–183.
Lisova,O., Hardy,F., Petit,V. and Bedouelle,H. (2007) J. Gen. Virol., 88, 2387–2397.
Lok,S.M., Kostyuchenko,V., Nybakken,G.E., et al. (2008) Nat. Struct. Mol. Biol., 15, 312–317.
Luca,V.C., AbiMansour,J., Nelson,C.A. and Fremont,D.H. (2012) J. Virol., 86, 2337–2346.
Metz,C.E., Herman,B.A. and Shen,J.H (1998) Stat. Med., 17, 1033–1053.
Midgley,C.M., Flanagan,A., Tran,H.B., et al. (2012) J. Immunol., 188, 4971–4979.
Modis,Y., Ogata,S., Clements,D. and Harrison,S.C. (2003) Proc. Natl Acad. Sci. USA, 100, 6986–6991.
Modis,Y., Ogata,S., Clements,D. and Harrison,S.C. (2004) Nature, 427, 313–319.
Modis,Y., Ogata,S., Clements,D. and Harrison,S.C. (2005) J. Virol., 79, 1223–1231.
Monera,O.D., Kay,C.M. and Hodges,R.S. (1994) Protein Sci., 3, 1984–1991.
Monsellier,E. and Bedouelle,H. (2005) Protein Eng. Des. Sel., 18, 445–456.
Monsellier,E. and Bedouelle,H. (2006) J. Mol. Biol., 362, 580–593.
Mukhopadhyay,S. Kim,B.S., Chipman,P.R., Rossmann,M.G and Kuhn,R.J. (2003) Science, 302, 248.
Myers,J.K., Pace,C.N and Scholtz,J.M. (1995) Protein Sci., 4, 2138–2148.
Nayak,V., Dessau,M., Kucera,K., Anthony,K., Ledizet,M. and Modis,Y. (2009) J. Virol., 83, 4338–4344.
Nybakken,G.E., Oliphant,T., Johnson,S., Burke,S., Diamond,M.S. and Fremont,D.H. (2005) Nature, 437, 764–769.
Nybakken,G.E., Nelson,C.A., Chen,B.R., Diamond,M.S. and Fremont,D.H. (2006) J. Virol., 80, 11467–11474.
Oliphant,T., Nybakken,G.E., Austin,S.K., Xu,Q., Bramson,J., Loeb,M., Throsby,M., Fremont,D.H., Pierson,T.C. and Diamond,M.S. (2007) J. Virol., 81, 11828–11839.
Ould-Abeih,M.B., Petit-Topin,I., Zidane,N., Baron,B. and Bedouelle,H. (2012) Biochemistry 51, 4807–4821.
Pace,C.N (1986) Methods Enzymol., 131, 266–280.
Pattnaik,P., Babu,J.P., Verma,S.K., Tak,V. and Rao,P.V. (2007) J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 846, 184–194.
Proba,K., Worn,A., Honegger,A. and Pluckthun,A. (1998) J. Mol. Biol., 275, 245–253.
Pryor,M.J., Carr,J.M., Hocking,H., Davidson,A.D., Li,P. and Wright,P.J. (2001) Am. J. Trop. Med. Hyg., 65, 427–434.
Rice,P., Longden,I. and Bleasby,A. (2000) Trends Genet., 16, 276–277.
Roehrig,J.T. (2003) Adv. Virus Res., 59, 141–175.
Roehrig,J.T., Volpe,K.E., Squires,J., Hunt,A.R., Davis,B.S. and Chang,G.J. (2004) J. Virol., 78, 2648–2652.
Shukla,J., Bhargava,R., Dash,P.K., Parida,M., Tripathi,N. and Rao,P.V. (2009) J. Virol. Methods, 158, 165–170.
Simmons,M., Nelson,W.M., Wu,S.J. and Hayes,C.G. (1998a) Am. J. Trop. Med. Hyg., 58, 655–662.
Simmons,M., Porter,K.R., Escamilla,J., Graham,R., Watts,D.M., Eckels,K.H. and Hayes,C.G (1998b) Am. J. Trop. Med. Hyg., 58, 144–151.
Steipe,B. (2004) Methods Enzymol., 388, 176–186.
Steipe,B., Schiller,B., Pluckthun,A. and Steinbacher,S (1994) J. Mol. Biol., 240, 188–192.
Thullier,P., Demangel,C., Bedouelle,H., Megret,F., Jouan,A., Deubel,V., Mazie,J.C. and Lafaye,P. (2001) J. Gen. Virol., 82, 1885–1892.
Verma,S.K., Kumar,S., Gupta,N., Vedi,S., Bhattacharya,S.M. and LakshmanaRao,P.V. (2009) Vaccine, 27, 6905–6909.
Volk,D.E., Beasley,D.W., Kallick,D.A., Holbrook,M.R., Barrett,A.D. and Gorenstein,D.G (2004) J. Biol. Chem., 279, 38755–38761.
Volk,D.E., Lee,Y.C., Li,X., Thiviyanathan,V., Gromowski,G.D., Li,L., Lamb,A.R., Beasley,D.W., Barrett,A.D. and Gorenstein,D.G. (2007) Virology, 364, 147–154.
Volk,D.E., May,F.J., Gandham,S.H., Anderson,A., Von Lindern,J.J., Beasley,D.W., Barrett,A.D. and Gorenstein,D.G. (2009) Virology, 394, 12–18.
Vriend,G. (1990) J. Mol. Graph, 8, 52–56, 29.
Wahala,W.M., Kraus,A.A., Haymore,L.B., Accavitti-Loper,M.A. and de Silva,A.M. (2009) Virology, 392, 103–113.
Wahala,W.M., Donaldson,E.F., de Alwis,R., Accavitti-Loper,M.A., Baric,R.S. and de Silva,A.M. (2010) PLoS Pathog, 6, e1000821.
Watterson,D., Kobe,B. and Young,P.R. (2012) J. Gen. Virol., 93, 72–82.
Weaver,S.C and Reisen,W.K. (2010) Antiviral Res., 85, 328–345.
Wu,K.P., Wu,C.W., Tsao,Y.P., Kuo,T.W., Lou,Y.C., Lin,C.W., Wu,S.C. and Cheng,J.W (2003a) J. Biol. Chem., 278, 46007–46013.
Wu,S.C., Yu,C.H., Lin,C.W. and Chu,I.M. (2003b) Vaccine, 21, 2516–2522.
Yu,S., Wuu,A., Basu,R., Holbrook,M.R., Barrett,A.D. and Lee,J.C. (2004) Biochemistry 43, 9168–9176.
Zhang,Z.S., Yan,Y.S., Weng,Y.W., Huang,H.L., Li,S.Q., He,S. and Zhang,J.M. (2007) J. Virol. Methods, 143, 125–131.
Zhang,S., Bovshik,E.I., Maillard,R., et al. (2010) Virology, 403, 85–91.
Zidane,N., Ould-Abeih,M.B., Petit-Topin,I. and Bedouelle,H. (2013) Biosci. Rep., 33(1), art:e00011.
Zulueta,A., Martin,J., Hermida,L., Alvarez,M., Valdes,I., Prado,I., Chinea,G., Rosario,D., Guillen,G. and Guzman,M.G. (2006) Virus Res., 121, 65–73.

Downloaded from https://academic.oup.com/peds/article-abstract/26/6/389/1541185/Thermodynamic-stability-of-domain-III-from-the
by White and Case LLP user
on 05 September 2017

Name
PROOF & WEB.joboptions

Comment	Size	Modified

Special Notice (SN) DARPA-SN-18-18: PREventing EMerging Pathogenic Threats (PREEMPT) Proposers Day

DATE: January 30, 2018
LOCATION: Arlington, VA
REGISTRATION DEADLINE: January 23, 2018 or when capacity is reached, whichever comes first. We will continue to accept webinar only registrations through January 26, 2018 or until webinar capacity is reached, whichever comes first.
REGISTRATION WEBSITE: https://events.sa-meetings.com/PREEMPTProposersDay
TECHNICAL POC: Dr. Jim Gimlett, DARPA/BTO
Email: DARPA-SN-18-18@darpa.mil

The Biological Technologies Office (BTO) of the Defense Advanced Research Projects Agency (DARPA) is hosting a Proposers Day for the potential proposer community in support of a planned Broad Agency Announcement (BAA) for the PREventing EMerging Pathogenic Threats (PREEMPT) program. The Proposers Day will be held on January 30, 2018 at the Executive Conference Center (ECC) in Arlington, VA. The event will be webcast for those who would like to participate remotely. Advance registration is required for both the physical meeting and the webcast. Note, all times listed in this announcement and on the registration website are Eastern Time.

The Proposers Day objectives are:

- To introduce the science and technology community (industry, academia, and government) to the PREventing EMerging Pathogenic Threats program vision and goals;
- To explain the general mechanics of a DARPA program and the specific objectives and milestones of this program; and
- To encourage and promote teaming arrangements among organizations with the expertise, research facilities, and capabilities needed to execute research and development responsive to PREEMPT program goals.

The Proposers Day will include overview presentations by government personnel, an information session to respond to questions from participants, and "lightning" talks (three minutes, one PowerPoint slide) for potential proposers to highlight technical capability or interest to promote teaming. Lightning talks must be scheduled via the registration website.

Attendance at this event is not a requirement for submission of a proposal or selection for funding. Information relayed during the Proposers Day will be made available on the BTO section of the DARPA Opportunities page: http://www.darpa.mil/work-with-us/opportunities.

PREEMPT Program Overview:

During US international operations, military forces are deployed to remote locations around the globe, often in areas where endemic and emerging diseases are prevalent. Most of these emerging and re-emerging diseases originate in animal reservoirs and then jump into humans. Despite biosurveillance efforts around the globe, viral outbreaks continue to outpace preparedness with new viral infectious diseases arising every year. Current approaches to biosurveillance focus on detection of known pathogens in human populations, human-to-human transmission, and modeling the trajectory of the epidemic following an outbreak event. Animal pathogens that have the potential to infect humans, but have not yet spilled over, are rarely considered. As a result, infectious agents are detected only after an outbreak, health responses are largely reactive, and often arrive late. The PREEMPT program represents a radical departure from current practice, aiming to target viral biothreats within animal reservoir to preempt their entry into human populations before an outbreak occurs.

To combat viruses that may emerge or re-emerge from animal populations or are transmitted by insect vectors, the PREEMPT program aims to produce and validate integrated multiscale models and experimental testbeds that quantify the probability a human virus pathogen will emerge from an animal reservoir and develop scalable approaches to suppress and prevent species jump from wild or domestic reservoirs and/or to impede insect-mediated viral transmission. Successful teams will address and integrate these program components to demonstrate proof-of-principle of a block-before-jump preemptive capability.

Attendee Presentations

Interested attendees are invited to present a brief **one-slide** PowerPoint summary of their interests and capabilities (**see Attachment 1**). These will be presented during 3-minute "lightning" presentations at Proposers Day. Presentations consisting of multiple slides will not be granted a time slot. Lightning talks will be accepted on a first-come, first-served basis until time constraints are reached. Lightning talk submissions are restricted to those attending the physical meeting. All presentations must be emailed to DARPA-SN-18-18@darpa.mil no later than 12:00 PM EDT on **January 23, 2018**.

Teaming

DARPA highly encourages teaming before proposal submission and, as such, will facilitate the formation of teams with the necessary expertise. Potential proposers may choose to be included on the (optional) Proposer Profile List by submitting a one-page profile consisting of their contact information (name, organization, email, telephone number, mailing address and, if applicable, organization website); a brief description of their technical competencies; and, if applicable, their desired expertise from other teams/organizations (**Attachment 2**).

All profiles must be emailed to DARPA-SN-18-18@darpa.mil no later than 4:00 PM ET on **January 23, 2018**. Following the deadline, the consolidated teaming profiles will be sent via

email to the interested parties who submitted a **valid profile (following the format provided and not exceeding the single page limit)**. Specific content, communications, networking, and team formation are the sole responsibility of the participants. Neither DARPA nor the DoD endorses the information and organizations contained in the consolidated teaming profile document, nor does DARPA or the DoD exercise any responsibility for improper dissemination of the teaming profiles.

Registration Information:

PLEASE NOTE: Registration for physical participation will close on January 23, 2018 at 12:00 PM EST or when capacity is reached and registration for webinar participation will close on January 26, 2018 at 12:00 PM EST or when webinar capacity is reached.

Registration is limited by the venue capacity, and early registration is strongly recommended. **There will be no on-site registration.**

Participants must register in advance through the registration website. There will an optional **$15** meal fee for the Proposers Day. Early registration is strongly recommended. Due to space limitations, attendance for the Proposers Day will be limited to the first **140** registrants. Remote participation via webcast is limited to **500** participants. Interested parties are encouraged to coordinate attendance internally within their organizations prior to registration. Physical attendance is limited to no more than two representatives per division/department. Individuals who are unable to register because the deadline has occurred or capacity has been reached, will be added to a waitlist. If slots remain open after registration closes or become available due to cancellations, the slots will be filled on a first-come, first-served basis from the waitlist.

An online registration form and various other meeting details can be found at the registration website, https://events.sa-meetings.com/PREEMPTProposersDay.

All attendees are required to present government-issued photo identification upon entry to the event.

Further administrative questions should be addressed to DARPA-SN-18-18@darpa.mil. Please refer to the PREventing EMerging Pathogenic Threats Proposers Day (DARPA-SN-18-18) in all correspondence. This announcement is not a request for proposals; any sent will be disregarded.

This SN is issued solely for information and potential new program planning purposes; the SN does not constitute a formal solicitation for proposals or proposal abstracts. In accordance with FAR 15.201(e), responses to this notice are not offers and cannot be accepted by the Government to form a binding contract. Submission is voluntary and is not required to propose to subsequent Broad Agency Announcements (if any) or research solicitations (if any) on this topic. DARPA will not provide reimbursement for costs incurred in responding to this SN. Respondents are advised that DARPA is under no obligation to acknowledge receipt of the information received,

or provide feedback to respondents with respect to any information submitted under this SN. NO CLASSIFIED INFORMATION SHOULD BE INCLUDED IN THE SN RESPONSE.

Broad Agency Announcement

PREventing EMerging Pathogenic Threats (PREEMPT)

BIOLOGICAL TECHNOLOGIES OFFICE

HR001118S0017

January 19, 2018

TABLE OF CONTENTS

PART I: OVERVIEW INFORMATION 3

PART II: FULL TEXT OF ANNOUNCEMENT 4

1. Funding Opportunity Description 4
1.1. Program Overview 4
1.2. Program Metrics 12
1.3. Ethical, Legal, and Societal Implications (ELSI) 15
1.4. Protection of Sensitive Information 15
2. Award Information 16
2.1. General award information 16
2.2. Fundamental Research 17
3. Eligibility Information 18
3.1. Eligible Applicants 18
3.2. Organizational Conflicts of Interest 19
3.3. Cost Sharing/Matching 20
4. Application and Submission Information 20
4.1. Address to Request Application Package 20
4.2. Content and Form of Application Submission 20
4.3. Funding Restrictions 31
4.4. Other Submission Requirements 31
5. Application Review Information 31
5.1. Evaluation Criteria 31
5.2. Review of Proposals 32
6. Award Administration Information 33
6.1. Selection Notices 33
6.2. Administrative and Policy Requirements 34
6.3. Reporting 35
6.4. Electronic Systems 35
7. Agency Contacts 35
8. Other Information 35
9. Appendix 1 – Volume II checklist 37

PART I: OVERVIEW INFORMATION

- **Federal Agency Name** – Defense Advanced Research Projects Agency (DARPA), Biological Technologies Office
- **Funding Opportunity Title –** PREventing EMerging Pathogenic Threats
- **Announcement Type –** Initial
- **Funding Opportunity Number –** HR001118S0017
- **Catalog of Federal Domestic Assistance Numbers (CFDA) – 12.910 Research and Technology Development**
- **Dates**
  - Posting Date – January 19, 2018
  - Proposal Abstract Due Date and Time – February 13, 2018 4:00 ET
  - Proposal Due Date and Time – March 27, 2018 4:00 ET
  - BAA Closing Date – March 27, 2018
  - Proposers' Day – January 30, 2018

  https://www.fbo.gov/spg/ODA/DARPA/CMO/DARPA-SN-18-18/listing.html

- **Concise description of the funding opportunity – DARPA is soliciting innovative proposals to develop novel and scalable approaches to preempt viral spillover and transmission from animals or vectors into humans.**

- **Anticipated individual awards - Multiple awards are anticipated.**
- **Types of instruments that may be awarded - Procurement contract, cooperative agreement or other transaction.**
- **Any cost sharing requirements -** Cost sharing may be required under applicable statutory regulations for other transactions for prototype projects awarded under the authority of 10 U.S.C. § 2371b.
- **Agency contact**
  - Points of Contact
    James Gimlett, Ph.D. Program Manager
    Biological Technologies Office

    The BAA Coordinator for this effort may be reached at:
    PREEMPT@darpa.mil
    DARPA/BTO
    ATTN: HR001118S0017
    675 North Randolph Street

PART II: FULL TEXT OF ANNOUNCEMENT

1. Funding Opportunity Description

This publication constitutes a Broad Agency Announcement (BAA) as contemplated in Federal Acquisition Regulation (FAR) 6.102(d)(2) and 35.016 and 2 CFR § 200.203. Any resultant award negotiations will follow all pertinent law and regulation, and any negotiations and/or awards for procurement contracts will use procedures under FAR 15.4, Contract Pricing, as specified in the BAA.

DARPA is soliciting innovative proposals for research to develop new tools and models to quantify the likelihood of a virus to jump from an animal host into humans, and to develop and validate new scalable technologies to target potential human-capable viral pathogens in wild reservoirs and/or mosquito vectors to prevent transmission to humans.

1.1. PROGRAM OVERVIEW

Introduction

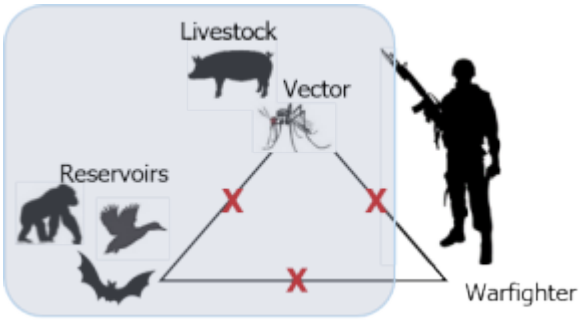
During U.S. international operations, military forces are deployed to remote locations around the globe, often in areas where endemic and emerging diseases are prevalent[1]. Most of these emerging and re-emerging diseases originate in animal reservoirs and then jump into humans. Numerous trends, including the increased interactions between human, animal and insect populations due to increased population densities, globalization, densification of livestock production, and rising human encroachment into animal habitats, have increased the risks of new viral outbreaks in those regions where Department of Defense (DoD) personnel are typically deployed. Often, DoD personnel are among the first responders in outbreak situations. Emerging infectious diseases, for which few medical countermeasures are available, represent a major threat to the warfighter and national security and could have devastating impacts on U.S. public health.

Despite biosurveillance efforts around the globe, new viral outbreaks continue to outpace preparedness efforts and show no signs of abating. During the first three quarters of 2017 outbreaks of avian influenza A (H7N9), Chikungunya, MERS coronavirus, Ebola, Seoul virus, Hepatitis E, Hepatitis A, Yellow Fever, Lassa, and Zika viruses were recorded[2]. While current biosurveillance strategies focus on detection of known pathogens within the human population following an infectious outbreak event, there is a dearth of research and surveillance on sentinel or reservoir animals[3]. Animal-specific viruses that have the potential to infect humans (*namely "*human-capable" pathogens), but have not yet spilled over into human populations, are rarely considered. As a result, infectious agents are detected only *after* an outbreak—that is, after an animal pathogen has adapted to become capable of infecting humans. Consequently, the outbreak response is largely reactive and not initiated until after an epidemic has already begun. The PREEMPT program represents a radical departure from current practice, aiming to target viral

[1] Halliday Jo E.B. et al. (2017). Driving improvements in emerging disease surveillance through locally relevant capacity strengthening. Science.
[2] World Health Organization (2017). http://www.who.int/csr/don/archive/year/2017/en/.
[3] Metcalf, J.E. and Lessler, J. (2017). Opportunities and challenges in modeling emerging infectious diseases. Science.

biothreats within the animal reservoirs where they originate and preempt their entry into human populations *before* an outbreak occurs.

Recently, the scientific community has advanced its understanding of host-pathogen genetics and mechanisms of adaptation across hosts[4,5], developed analytic tools to predict animal hosts of new and potential human-transmissible viruses, and learned how to identify "hot spot" geographic regions where an animal-to-human virus jump is imminent[6,7]. This understanding is empowered by new high-throughput data generation capabilities and sophisticated analytic and computational tools. Together, this new understanding and capability hold great promise for the development of advanced integrated models that can assess and likely provide guidance for action that prevents human virus emergence before the virus gains entry to the human population. The PREEMPT program aims to develop new tools and models to quantify the likelihood of a virus quasispecies (QS) to jump from an animal host into humans. In parallel, PREEMPT seeks to develop and validate new scalable technologies that prevent transmission of viral pathogens in wild reservoirs and/or mosquito vectors to humans or to bridge animals that serve as intermediary hosts prior to virus jump into humans.



Research Objectives

PREEMPT research objectives are structured along two Technical Areas (TAs). Both Technical Areas must be performed in parallel by vertically integrated, interdisciplinary teams. Proposers must present a plan to address both Technical Areas and meet key milestone decision points that occur at the end of year 2.

1) TA1: Develop and validate integrated, multiscale models that quantify the likelihood a human-capable virus will emerge from an animal reservoir residing in a "hot spot" geographic region.

2) TA2: Develop scalable approaches that target and suppress the animal virus in its reservoir(s) and/or vector(s), to reduce the likelihood of virus transmission into humans.

Technical Area 1 (TA1)

[4] Lloyd-Smith, J.O. (2010). Identifying genetic markers of adaptation for surveillance of viral host jumps. Nature Reviews Microbiology.
[5] Plowright, R.K. et al. (2017). Pathways to zoonotic spillover. Nature Reviews Microbiology.
[6] Olival, K.J. et al. (2017). Host and viral traits predict zoonotic spillover from mammals. Nature.
[7] Han, B.A. et al. (2016). Undiscovered Bat Hosts of Filoviruses. PLoS Negl Trop Dis.

Studies within TA1 must produce and validate models that: (a) quantify the likelihood of a virus to jump into a new animal species and/or humans, (b) identify opportunities for proactive intervention, and (c) determine likely efficacy, scalability, and sustainability of prevention strategies.

Proposers are expected to leverage high-throughput virus screening methods, metagenomics, ecological surveillance, and advanced modeling tools to generate risk models for species jump that will enable near real-time data analysis and identification of potential risks and risk factors. This far-forward biosurveillance system should also identify opportunities for preemptive intervention, assessing likely efficacy, scalability, safety, and sustainability of preemptive strategies to target viral threats in animal reservoirs and/or vectors before they enter the human population.

TA1 Components
Proposers should address, at minimum, the following aspects:

1) Selection of zoonotic or vector-borne viral pathogen(s) (multiple viruses within the same family may be addressed if they share a common animal reservoir and/or vector)
2) Field data collection
3) Multi-species field samples studied in a controlled laboratory setting
4) Data analysis, integration, and model development
5) Real-time data sharing and analysis
6) Model outputs
7) Experimental validation of model predictions in a controlled, environment-simulated laboratory setting

1. Selection of zoonotic or vector-borne viral pathogen

This BAA only will consider proposals focused on zoonotic and/or vector-borne viruses. Microorganisms other than viruses are not responsive to this announcement. A rationale for the viruses selected is required. Virus selection may be based on, but is not limited to, the following factors: high frequency of re-emergence (*e.g.* avian influenza virus), patterns of virus host range or host breadth (predicted zoonotic potential), potential for rapid spread due to vector-mediated transmissibility, severity of disease pathology, and likelihood of pandemic threat.

2. Field data collection

Proposers must identify and justify suitable geographic "hotspots" within which they will collect field data. Proposers must consider <u>all</u> of the following criteria when selecting geographic hot spots for field data collection:

1) Previous evidence of geographic distribution of zoonotic reservoirs and/or vectors for known or unknown human viruses; these maps may be based on epidemiological, phylogenetic, ecological, biogeographic, socio-economic data, or other;
2) Evidence of past species jump events in or near the selected geographic location;

3) Demonstrated capabilities and infrastructure to perform research in the selected geographic region and/or collaboration with an established DoD or Department of Health and Human Services (DHHS) partner (*e.g.*, a Naval Medical Research Unit site, Armed Forces Research Institute of Medical Sciences, or Centers for Disease Control), such that the performer can coordinate far-forward surveillance activities and access local lab and analytics capabilities;
4) Appropriate levels of in-country government approval, cooperation, infrastructure and logistical support where samples will be collected and analyzed; and
5) Rationale for reservoirs/species to be sampled.

Where applicable, proposers must consider seasonal distribution (*e.g.,* wet-dry seasons for mosquito), temporal ecological factors (*e.g.,* time of fruiting for fruit bats), and temporal behavioral traits (*e.g.,* sexual maturation) of zoonotic species for field sampling. Potential geographic areas may include, but are not limited to, endemic regions; those undergoing ecological shifts (thus increasing risk for spillover due to changes in animal-human interactions); those harboring host species with high zoonotic potential that are in proximity to human populations and "bridge" animal hosts (*e.g.,* human-bat-swine ecosystems); and prior sites of spillover events or outbreaks. The selection of geographic areas of common military deployment that also meet the above criteria is strongly encouraged. Proposers should describe feasible approaches to increase the probability of detecting viruses within animal reservoirs and/or vectors—residing in selected geographic areas—that have the potential to become human-capable. Proposers should describe sample collection methods in detail, being sure to include longitudinal sampling frequency. Development of novel and rapid sampling approaches for the real-time continuous screening of emerging or re-emerging pathogens at the human-animal interface is encouraged. Proposers are encouraged to identify field samples that were collected during past outbreak events, or field data already generated, that could be accessed for retrospective analyses. In such cases, proposers should describe how and where the data were collected, and establish quality control methods for data evaluation and use. Although human use research will not be funded by PREEMPT, the use of human samples or data from prior outbreaks obtained through other programs may be included in the research plan as long as samples are appropriately de-identified (see, for example, https://humansubjects.nih.gov/human-specimens-cell-lines-data).

3. Multi-species laboratory testing of field samples

Proposers should discuss protocols to determine and quantify the virus population QS diversity from the vector or reservoir at the time of sample collection (t=0) in a manner that minimizes QS alterations, which commonly result from cell line passaging. Proposers should assess the need for longitudinal collection of samples to understand viral QS temporal dynamics (temporal changes in sequence and fitness landscapes) in field virus populations. The initial viral QS isolated from a field sample (t=0) will be hereon annotated as "QS_0". Proposers must describe *in vitro* and/or *in vivo* experiments to assess jump potential of the QS_0 population to a relevant new host. Experimental approaches to monitor viral species jump may include, but are not limited to: changes in viral population QS during cell line passaging between relevant species; infection of appropriate animal models; infection of natural animal hosts; and controlled, multi-species laboratory ecosystems.

Lab testing should determine the key parameters influencing the probability of a viral QS_0 to jump and adapt to a new host species. Potential parameters across different host animals or vectors may include, but are not limited to:

1) QS diversity profiles
2) Rates of virus infection and amplification
3) Virus incubation period
4) Viremia and viral shedding
5) Transmission bottlenecks
6) Animal host evolutionary and immune pressures

The data generated should enable the development of genotype-to-phenotype maps and the determination of mutation(s) associated with virus jump to a new host.

4. Data analysis, integration, and model development

Proposers should identify the relevant data needed for developing integrated models of risk assessment. Proposers should discuss the development of probabilistic models of virus jump using advanced computational methods and tools, including both model-driven and data-driven approaches. Models should integrate multi-scale and cross-host species data, including but not limited to, field and experimental data (*e.g.,* QS dynamics), ecological data (*e.g.,* demographic, socio-economic, epidemiological, biogeographical, and other metadata), and other relevant data available, especially that generated from past spillover events. Models should consider all factors associated with pathogen emergence and transmission, particularly multi-host immunological landscapes. Models should also capture viral evolutionary trajectories, fitness landscapes in zoonotic and/or vector species, and quantify the transmission dynamics underlying species jump.

5. Real-time data sharing and analysis

The PREEMPT program is expected to generate significant amounts of data, primarily from next generation sequencing (NGS) of viral populations and analysis of host molecular signatures. Proposers should identify methods for near-real-time data sharing and analysis.

6. Model outputs

Proposers should explain how they will develop probabilistic models and machine learning techniques that integrate multi-scale and cross-species data (*e.g.,* molecular signatures, demographic, ecological, socio-economic, epidemiological, weather, climate, and other metadata) to quantify a pathogen's likelihood to cross species barriers and infect humans. Models should capture viral evolutionary trajectories and mutations that govern species jump. Models should quantify transmission dynamics, accounting for the diversity of viral QS. Models should identify key parameters of the pathogen, host species, vector dynamics, and ecological interactions contributing to species jump, and should inform a preemption strategy by identifying optimal pressure points (*e.g.,* jump-enabling mutations, stochastic transmission bottlenecks, and viral amplification requirements) that can be targeted to reduce the likelihood of species jump. For proposals addressing vector-borne viruses, proposers should describe methods to quantify

the likelihood of virus adaptation to a new vector and propose experimental methods to validate these predictions. Proposers should discuss metrics for grading model accuracy, sensitivity, and specificity. Models should be able to receive dynamic biosurveillance inputs and accommodate virus QS changes.

7. Experimental validation of model predictions

Proposers must describe in detail a plan to establish relevant *in vivo*, multi-species experimental approaches to validate model outputs. Experimental testing may closely resemble or recapitulate real-life settings (*e.g.,* climate, phylogenetically adjacent host species, and vector "biting" patterns) to enable the quantification of the probability of spillover and/or transmission events in a controlled manner. Approaches that closely recapitulate real-life ecosystems and natural hosts are strongly encouraged. To improve model accuracy, sensitivity, and specificity, performers must iterate both theoretical and empirical experiments.

TA1 Key Outputs
The key outputs for TA1 must include the following:

1) Integrated models that quantify likelihood of virus jump and can be easily adapted to receive dynamic surveillance and virus data input.
2) Stochastic models quantifying bottlenecks (e.g., transmission, cell entry, and infection rates) and mutational fitness maps (e.g., enabler mutations and their frequency).
3) Identification and assessment of potential preemptive intervention targets to preempt virus jump from the reservoir and/or vector.

Technical Area 2 (TA2)
Studies within this technical area aim to develop deployable and scalable methods to preempt viral jump across species.

TA2 Components
Technical Area 2 aims to develop deployable and scalable methods to preempt viral jump to other species. Proposers must address, at minimum, all of the following aspects:

1) Proof-of-concept preemption approaches;
2) Scalable delivery methods;
3) Analysis of long-term sustainability; and
4) Experimental validation.

1. Proof-of-concept preemption approaches

Proposers should describe how the output of TA1 *in silico* models will guide preventive method design, and how quantitative information of virus-host species barriers and transmission bottlenecks will be used to develop strategies to preempt emergence of human-capable viruses. Models should guide the selection of: host species to be treated (*e.g.*, wild animals, "bridge"

animals, vectors, and livestock); potential molecular targets (*e.g.,* key mutation(s) enabling receptor binding in a new host); targets associated with transmission cycle dynamics (*e.g.,* reduction of viral load within the reservoir and/or vector that would preclude transmission); and other relevant factors identified by the models. Proposers should describe the preemptive methods that address different model outputs. Examples of preemptive approaches include but are not limited to:

1) Specific disruption of jump-capable genes from virus QS in reservoirs and/or vectors using small interfering RNAs or CRISPR/Cas-based targeted deletions.
2) Suppression of virus jump to a new host through antibody-mediated virus neutralization.
3) Suppressed reservoir and/or vector viremia using virus defective interfering particles (DIPs) to outcompete virus replication.
4) Suppressed transmission among animal reservoirs through induced immunity (*e.g.,* vaccinate the animal).
5) Alternative methods informed by experimental and theoretical models. The development of novel preemptive approaches are strongly encouraged.

2. Scalable delivery methods

Proposers must describe scalable approaches to deliver the preemptive therapeutic to achieve animal and/or vector population-level control of the targeted virus, including strategies for reaching less accessible animal reservoirs (*e.g.,* rodents or non-human primates). Approaches that enable host-to-host therapeutic distribution (*i.e.,* do not require individual treatment) that are self-limiting, only activate when the viral pathogen target is present, and/or have a controllable "on/off-switch" are encouraged. Potential scalable methods of inoculation may include, but are not limited to:

1) Self-disseminating treatments or preventives (*e.g.,* transmissible recombinant vaccines, therapeutic interfering particles, or self-spreading antiviral therapies).
2) Bait vaccination or treatment of wild or domestic animals.
3) Spray-based methods.

Approaches that utilize genetic modifications of vectors (*e.g.,* engineered mitochondrial DNA) are acceptable. The proposed method of inoculation must be justified. The proposer must describe strategies for closely controlling preemptive delivery and spread.

3. Analysis of long-term safety and efficacy

Proposers must establish initial methods to assess the long-term safety and efficacy of preemptive approaches (*e.g*., determine the mechanism by which species specificity of a vaccine is maintained, and assess evolutionary stability and ecological safety).

4. Experimental validation

Proposers must describe approaches to validate preemptive methods of choice in controlled experimental models. Multi-species experimental platforms that closely recapitulate real-life ecosystems and use natural hosts are strongly encouraged.

TA2 Key Outputs
The key outputs of TA2 must include the validation of new "block-before-jump" preemption technologies for one of the following:

1) Validate suppression of virus jump from wild animal reservoir to humans and/or an intermediate animal carrier (*e.g.,* domestic livestock).
2) Validate suppression of virus jump or transmission from wild reservoir to vector, vector to a different vector species, and/or from vector to human.

Period of Performance
DARPA anticipates that the PREEMPT program will provide up to three and a half years of funding for research and development to be performed over Phase I (base) and II (option) periods of 24 and 18 months, respectively.

Timeline
PREEMPT spans a 42-month effort with a 24-month Phase I (base) and an 18-month Phase II (option). In general, Phase I should provide early validation of zoonosis risk models, and Phase II should establish efficacy and scalability of zoonosis prevention approaches.

Phase I (Base period)
Phase I efforts aim to develop experimental and mathematical models to quantify the likelihood a virus will jump from one host species to another, identify potential targets for spillover preemption, and develop scalable methods of preemption. During Phase I, performer teams will:

1) Identify the genetic adaptations that enable species jump.
2) Develop mathematical models to quantify the likelihood of species jump based on:
    a. Molecular data (*e.g.,* viral QS data from deep sequencing*)* and
    b. Ecological data *(e.g.,* immune state of the host population before pathogen emergence, species relatedness, etc.).
3) Identify bottlenecks for intervention (*e.g.. transmission, cell entry, viral amplification, infection rate, and other mechanisms associated with viral cross-species compatibility)*.
4) Develop initial scalable platforms that target viruses in reservoirs and/or vectors to prevent viral jump into other animals or humans.

By the end of year 1 (Phase I) performers will be expected to have:
1) Identified signatures of fitness and spillover potential of a pathogen between two species.
2) Quantified the genetic and transmission factors requirements of viral QS to jump to a new host (*e.g.,* develop genotype-to-phenotype maps, identify specific mutations, etc.) using far-forward biosurveillance data from selected high-risk regions.

By the end of year 2 (Phase I) performers will be expected to have:

1) Initially demonstrated that models can quantify the probability of human-capable virus pathogens to jump from one species to another species.
2) Demonstrated proof of concept methods for targeting human-capable virus pathogens in the reservoirs and/or vectors to reduce the probability of virus jump.
3) Provided initial strategies to scale up preemption methods.

Phase II (Option period)
Phase II efforts aim to develop probabilistic models for intra- and inter-species viral amplification and transmission dynamics, integrated models for risk assessment, and experimental validation of new approaches to preempt species jump. During Phase II, performer teams will extend Phase I modeling efforts to:

1) Quantify intra- and inter-species viral amplification dynamics and transmission.
2) Develop integrated models that quantify the probability of a virus QS to jump to bridge animal species or to humans.
3) Experimentally validate scalable methods for their ability to preempt zoonotic spillover.

By the end of year 3.5 (Phase II) performers will be expected to:

1) Demonstrate accuracy of risk assessment and preemption models in a relevant multi-species experimental setting.
2) Demonstrate the ability to suppress viral jump to a new species in controlled experimental settings.

It is recognized that appropriate milestones and metrics may depend upon the type of virus, the reservoir, the mechanisms of species jump, and the proposed preemption methods. Proposers must offer quantitative milestones and metrics (see Tables 1 and 2 below for notional metrics) for their proposed proof-of-principle use case. Proposers must demonstrate relevant research experience in the required technical areas. Proposals involving multiple teams and/or experimental approaches should be structured as unified efforts that address the program Technical Areas in parallel, in an integrated manner.

1.2. PROGRAM METRICS

In order for the Government to evaluate the effectiveness of a proposed solution in achieving the stated program objectives, proposers should note that the Government hereby promulgates the following program metrics that may serve as a guideline for assessing program progress, risk and impact. Although the following program metrics are provided, proposers should note that the Government has identified these goals with the intention of bounding the scope of effort while affording the maximum flexibility, creativity, and innovation in proposing solutions to the stated problem. Proposers should offer more appropriate and specific metrics for their particular use case and technical approach, including intermediate metrics (i.e. every 6 months, or sooner) to help further evaluate progress. Final metrics are to be negotiated at the time of contracting.

Table 1: Notional Milestones, Deliverables, and Program Metrics for TA1

Phase	Milestones and Deliverables	Program Metric
I	Collected field surveillance data: • Virus QS molecular data (*e.g.* from deep sequencing) and metadata from longitudinal samples (*e.g.* obtained from selected high-risk areas (*e.g.* bat cave) and/or from prior outbreak event • Host species immune molecular data	Quantitative measures of: • Longitudinal viral population QS ($QS_{t=0}$, $QS_{t=6\ months}$, $QS_{t=12\ months}$,..) diversity in selected high-risk areas (*e.g.* frequency of mutations, evolutionary trajectories) (**6 months**) • Viral QS diversity in samples obtained from animal, vector, and/or human from prior outbreak event (*e.g.* frequency of species-specific mutations) (**9 months**) • Immune molecular signatures from host reservoir or intermediate reservoir species (**12 months**)
	Multi-species lab test data: • Virus QS genotype-phenotype maps for at least 2 relevant host species	Quantitative measures of: • Cell entry and adaptation across species *in vitro* and/or *in vivo* (*e.g.* QS diversity during passage across species) (**18 months**)
	Initial mathematical models that assess risk of virus jump	Model capability to describe/predict: • Virus QS evolutionary trajectories between 2 relevant species (**9 months**) • Key molecular factors that could be targeted to prevent virus jump *in vitro* and/or *in vivo* (*e.g.* signatures of fitness of a pathogen between two relevant host species) (**18 months**) • Molecular targets for preemption (**24 months**)
	Established testbeds for validation of model predictions	Testbeds mimic natural environment as quantified by performer-defined parameters (**24 months**)
II	Multi-species lab test data • Quantify virus QS transmission factors between two species *in vivo*	Quantitative measures of: • Virus amplification and transmission dynamics (*e.g.* rate of infection vs. viremia, amplification rates, and incubation time) (**30 months**)
	Advanced mathematical models that assess risk of virus jump • Integration of molecular data and virus amplification/transmission dynamics • Integration of host immune evolutionary pressures and virus QS dynamics	Models predict: • Intra- and inter-species transmission dynamics (**36 months**) • Probability of spillover (risk assessment) (**42 months**) • Top 2 targets to reduce probability of transmission between two species to

Phase	Milestones and Deliverables	Program Metric
		inform TA2 (**42 months**)
	Further validation of model prediction in established testbeds	Validated model prediction accuracy in multispecies environment (**42 months**)

Table 2: Notional Milestones, Deliverables, and Program Metrics for TA2

Phase	Milestones and Deliverables	Program Metric
I	Proof-of-concept demonstration of preemptive approach that reduces either the probability of virus jump or the frequency of virus QS variants at high risk for species jump	Quantitative validation of preemptive approach as established by performer (**24 months**) Examples: • Frequency of high-risk mutation within virus QS in reservoir reduced >3X • Virus incubation period in vector extended >3X • Virus amplification rate in reservoir or vector reduced >3X • Viremia in host or vector reduced >5X
II	Demonstrated efficacy of preemption method	Reduced probability of transmission between two species by >5X *in vivo* for top 2 targets (**36 months**)
	Demonstrated scalability of preemption method	Quantitative scalability as established by performer (**42 months**)

Data Sharing

Proposers must ensure all technical data items (including experimental findings, processed data, methods of processing, research reports, and publications) and software (source code and executables) generated from PREEMPT program funding are made available to DARPA. Regularly submitted reports (*e.g.*, monthly or quarterly) should contain all relevant project data, including (but not limited to) raw and analyzed data and any necessary annotations and interpretation. Data and/or samples collected from de-identified human volunteers/patients from previous outbreak events must include associated anonymized metadata (*e.g.,* signs/symptoms, diagnostic test results, interventions, clinical observations, and outcomes). All raw data and metadata should be recorded according to approved experimental standards.

To gain enhanced scientific value from open collaboration in fundamental research, DARPA may seek permission to share some or all program-generated data with the broader research community as open data (including the possibility of accessing, reusing, and redistributing under appropriate licensing terms) to the extent permitted by applicable laws and regulations (*e.g.*, privacy, security, and export control).

DARPA anticipates that a large amount of data will be generated under this program by each performer and that the analyses and validation will be strengthened by compiling and integrating

information across all performers. Performers are strongly encouraged to establish the appropriate agreements to enable collaboration and data sharing. DARPA encourages sharing of pre-existing data, including those generated through funding by other sources, although this is not a requirement of the program.

As feasible, DARPA intends to share data within the PREEMPT performer community to promote program goals. To facilitate sharing and exchange of data items, performers will be required to enter an Associate Contractor Agreement (ACA); an ACA clause will be included in the contract or agreement awarded.

PREEMPT Transition Plan
Proposers must include a PREEMPT Technology Transition Plan. Proposers must indicate the types of partners (*e.g.*, government, private industry, non-profit) they plan to pursue and submit a timeline with incremental milestones toward successful engagement. Proposers should begin transition activities during the early stages of the program (Phase I). Awardees must include DARPA in the development of transition relationships. If the transition plan includes a start-up company, a business development strategy must be included as well. The extent by which the proposed intellectual property (IP) rights will impede the Government's ability to transition the technology will be considered in the proposal evaluation.

1.3. ETHICAL, LEGAL, AND SOCIETAL IMPLICATIONS (ELSI)

DARPA is committed to ensuring that efforts funded under this BAA adhere to ethical and legal regulations currently in place for federally and DoD-funded research. Program developments will be discussed with a panel of expert external advisors with expertise in bioethical and biosafety issues that may emerge as a consequence of advances in biomedical science and technology. Proposers to this BAA should address potential ethical, legal, and societal implications of the proposed technology.

1.4. PROTECTION OF SENSITIVE INFORMATION

PREEMPT is a 6.1 fundamental research program aimed at enhanced biosurveillance and novel approaches to preempt viral pathogens in animal reservoirs from jumping into human populations. DARPA follows current DoD policy for contracted fundamental research. DARPA recognizes, however, that PREEMPT program components aimed at understanding and quantifying mechanisms for viral zoonotic spillover could potentially generate sensitive information that could be misused. Since this is a fundamental research program, the risk of misuse currently cannot be reasonably evaluated. However, proposers are notified that during proposal evaluation and/or program performance, when such a risk reasonably can be evaluated, DARPA may determine that risk of misuse creates exceptional circumstances, compelling reasons, and/or national security reasons under current DoD policy for contracted fundamental research. DARPA therefore expects that proposers to this program understand and will comply with various government guidance regarding potential gain-of-function research of concern (GOFROC)[8] and dual use research of concern (DURC)[9,10,11,12,13]. See https://www.phe.gov/s3/dualuse/Pages/default.aspx for further information.

[8] Gain-of-Function Research (GOFROC) refers to studies with the potential to generate pathogens with pandemic potential exhibiting high transmissibility and high virulence.

DARPA requires that proposals include a Risk Mitigation Plan that will be incorporated into any resulting agreements or contracts and includes the following information:

1) An assessment of potential risks to public health, agriculture, plants, animals, the environment, and national security.
2) Proposed guidelines that the proposer will follow to ensure maximal biosafety and biosecurity during the course of the research.
3) A communication plan that addresses content, timing, and the extent of distribution of potentially sensitive dual-use information. The plan must also address how input from DARPA, other government, and community stakeholders will be taken into account in decisions regarding communication and publication of potentially sensitive dual-use information.

2. Award Information

2.1. GENERAL AWARD INFORMATION

Multiple awards are possible. The amount of resources made available under this BAA will depend on the quality of the proposals received and the availability of funds.

The Government reserves the right to select for negotiation all, some, one, or none of the proposals received in response to this solicitation and to make awards without discussions with proposers. The Government also reserves the right to conduct discussions if it is later determined to be necessary. If warranted, portions of resulting awards may be segregated into pre-priced options. Additionally, DARPA reserves the right to accept proposals in their entirety or to select only portions of proposals for award. In the event that DARPA desires to award only portions of a proposal, negotiations may be opened with that proposer. The Government reserves the right to fund proposals in phases with options for continued work, as applicable. The Government reserves the right to fund a Phase II option based on funding availability, an

---

[9] Dual Use Research of Concern (DURC) refers to life sciences research that can be reasonably anticipated to provide knowledge, information, products or technology that could be directly misapplied to pose a significant threat with broad potential consequences to public health and safety, agricultural crops and other plants, animals, the environment, materiel, or national security.
[10] Proposed framework for the oversight of dual use life sciences research: strategies for minimizing the potential misuse of research information, National Science Advisory Board for Biosecurity (NSABB). June 2007.
[11] Recommendations for the evaluation and oversight of proposed gain-of-function research by the National Science Advisory Board for Biosecurity (NSABB). May 2016.
[12] Tools for the Identification, Assessment, Management, and Responsible Communication of Dual Use Research of Concern: A Companion Guide to the United States Government Polices for Oversight of Life Sciences Dual Use Research of Concern. NIH. September 2014.
[13] United States Government Policy for Oversight of Life Sciences Dual Use Research of Concern. DURC Policy. March 2012.

assessment of Phase I research results, and a determination that awarding the option is in the best interests of the Government. The Government reserves the right to request any additional, necessary documentation once it makes the award instrument determination. Such additional information may include but is not limited to Representations and Certifications (see Section VI.B.2., "Representations and Certifications"). The Government reserves the right to remove proposers from award consideration should the parties fail to reach agreement on award terms, conditions, and/or cost/price within a reasonable time, and the proposer fails to timely provide requested additional information. Proposals identified for negotiation may result in a procurement contract, grant, cooperative agreement, or other transaction, depending upon the nature of the work proposed, the required degree of interaction between parties, whether or not the research is classified as Fundamental Research, and other factors.

Proposers looking for innovative, commercial-like contractual arrangements are encouraged to consider requesting Other Transactions. To understand the flexibility and options associated with Other Transactions, consult http://www.darpa.mil/work-with-us/contract-management#OtherTransactions.

In all cases, the Government contracting officer shall have sole discretion to select award instrument type, regardless of instrument type proposed, and to negotiate all instrument terms and conditions with selectees. DARPA will apply publication or other restrictions, as necessary, if it determines that the research resulting from the proposed effort will present a high likelihood of disclosing performance characteristics of military systems or manufacturing technologies that are unique and critical to defense. Any award resulting from such a determination will include a requirement for DARPA permission before publishing any information or results on the program. For more information on publication restrictions, see the section below on Fundamental Research.

2.2. FUNDAMENTAL RESEARCH

It is DoD policy that the publication of products of fundamental research will remain unrestricted to the maximum extent possible. National Security Decision Directive (NSDD) 189 defines fundamental research as follows:

> 'Fundamental research' means basic and applied research in science and engineering, the results of which ordinarily are published and shared broadly within the scientific community, as distinguished from proprietary research and from industrial development, design, production, and product utilization, the results of which ordinarily are restricted for proprietary or national security reasons.

As of the date of publication of this BAA, the Government expects that program goals as described herein may be met by proposers intending to perform fundamental research and proposers not intending to perform fundamental research or the proposed research may present a high likelihood of disclosing performance characteristics of military systems or manufacturing technologies that are unique and critical to defense. Based on the nature of the performer and the nature of the work, the Government anticipates that some awards will include restrictions on the resultant research that will require the awardee to seek DARPA permission before publishing any information or results relative to the program.

Proposers should indicate in their proposal whether they believe the scope of the research included in their proposal is fundamental or not. While proposers should clearly explain the intended results of their research, the Government shall have sole discretion to select award instrument type and to negotiate all instrument terms and conditions with selectees. Appropriate clauses will be included in resultant awards for non-fundamental research to prescribe publication requirements and other restrictions, as appropriate. This clause can be found at http://www.darpa.mil/work-with-us/additional-baa.

For certain research projects, it may be possible that although the research being performed by the awardee is restricted research, a subawardee may be conducting fundamental research. In those cases, it is the awardee's responsibility to explain in their proposal why its subawardee's effort is fundamental research

3. Eligibility Information

3.1. ELIGIBLE APPLICANTS

All responsible sources capable of satisfying the Government's needs may submit a proposal that shall be considered by DARPA.

3.1.1. Federally Funded Research and Development Centers (FFRDCs) and Government Entities

FFRDCs

FFRDCs are subject to applicable direct competition limitations and cannot propose to this BAA in any capacity unless they meet the following conditions: (1) FFRDCs must clearly demonstrate that the proposed work is not otherwise available from the private sector. (2) FFRDCs must provide a letter on official letterhead from their sponsoring organization citing the specific authority establishing their eligibility to propose to Government solicitations and compete with industry, and their compliance with the associated FFRDC sponsor agreement's terms and conditions. This information is required for FFRDCs proposing to be awardees or subawardees.

Government Entities

Government Entities (e.g., Government/National laboratories, military educational institutions, etc.) are subject to applicable direct competition limitations. Government entities must clearly demonstrate that the work is not otherwise available from the private sector and provide written documentation citing the specific statutory authority and contractual authority, if relevant, establishing their ability to propose to Government solicitations.

Authority and Eligibility

At the present time, DARPA does not consider 15 U.S.C. § 3710a to be sufficient legal authority to show eligibility. While 10 U.S.C.§ 2539b may be the appropriate statutory starting point for some entities, specific supporting regulatory guidance, together with evidence of agency approval, will still be required to fully establish eligibility. DARPA will consider FFRDC and

Government entity eligibility submissions on a case-by-case basis; however, the burden to prove eligibility for all team members rests solely with the proposer.

3.1.2. Non-U.S. Organizations

Non-U.S. organizations and/or individuals may participate to the extent that such participants comply with any necessary nondisclosure agreements, security regulations, export control laws, and other governing statutes applicable under the circumstances.

3.2. ORGANIZATIONAL CONFLICTS OF INTEREST

FAR 9.5 Requirements

In accordance with FAR 9.5, proposers are required to identify and disclose all facts relevant to potential OCIs involving the proposer's organization and *any* proposed team member (subawardee, consultant). Under this Section, the proposer is responsible for providing this disclosure with each proposal submitted to the BAA. The disclosure must include the proposer's, and as applicable, proposed team member's OCI mitigation plan. The OCI mitigation plan must include a description of the actions the proposer has taken, or intends to take, to prevent the existence of conflicting roles that might bias the proposer's judgment and to prevent the proposer from having unfair competitive advantage. The OCI mitigation plan will specifically discuss the disclosed OCI in the context of each of the OCI limitations outlined in FAR 9.505-1 through FAR 9.505-4.

Agency Supplemental OCI Policy

In addition, DARPA has a supplemental OCI policy that prohibits contractors/performers from concurrently providing Scientific Engineering Technical Assistance (SETA), Advisory and Assistance Services (A&AS) or similar support services and being a technical performer. Therefore, as part of the FAR 9.5 disclosure requirement above, a proposer must affirm whether the proposer or *any* proposed team member (subawardee, consultant) is providing SETA, A&AS, or similar support to any DARPA office(s) under: (a) a current award or subaward; or (b) a past award or subaward that ended within one calendar year prior to the proposal's submission date.

If SETA, A&AS, or similar support is being or was provided to any DARPA office(s), the proposal must include:

- The name of the DARPA office receiving the support;
- The prime contract number;
- Identification of proposed team member (subawardee, consultant) providing the support; and
- An OCI mitigation plan in accordance with FAR 9.5.

Government Procedures

In accordance with FAR 9.503, 9.504 and 9.506, the Government will evaluate OCI mitigation plans to avoid, neutralize or mitigate potential OCI issues before award and to determine whether it is in the Government's interest to grant a waiver. The Government will only evaluate OCI mitigation plans for proposals that are determined selectable under the BAA evaluation criteria and funding availability.

The Government may require proposers to provide additional information to assist the Government in evaluating the proposer's OCI mitigation plan.

If the Government determines that a proposer failed to fully disclose an OCI; or failed to provide the affirmation of DARPA support as described above; or failed to reasonably provide additional information requested by the Government to assist in evaluating the proposer's OCI mitigation plan, the Government may reject the proposal and withdraw it from consideration for award.

3.3. COST SHARING/MATCHING

Cost sharing is not required; however, it will be carefully considered where there is an applicable statutory condition relating to the selected funding instrument. Cost sharing is encouraged where there is a reasonable probability of a potential commercial application related to the proposed research and development effort.

For more information on potential cost sharing requirements for Other Transactions for Prototype, see http://www.darpa.mil/work-with-us/contract-management#OtherTransactions

4. Application and Submission Information

4.1. ADDRESS TO REQUEST APPLICATION PACKAGE

This announcement, any attachments, and any references to external websites herein constitute the total solicitation. If proposers cannot access the referenced material posted in the announcement found at http://www.darpa.mil, contact the administrative contact listed herein.

4.2. CONTENT AND FORM OF APPLICATION SUBMISSION

All submissions, including abstracts and proposals must be written in English with type not smaller than 12 point font. Smaller font may be used for figures, tables, and charts. Copies of all documents submitted must be clearly labeled with the DARPA BAA number, proposer organization, and proposal title/proposal short title.

4.2.1. Proposal Abstract Format

Proposers are strongly encouraged to submit an abstract in advance of a proposal to minimize effort and reduce the potential expense of preparing an out of scope proposal. The abstract is a concise version of the proposal comprising a **maximum of 8 pages** including all figures, tables, charts, and the Executive Summary slide. The (optional) submission letter is not included in the page count. All pages shall be formatted for printing on 8-1/2 by 11-inch paper with font size not smaller than 12 point. Smaller font sizes may be used for figures, tables, and charts.

Submissions must be written in English.

Abstracts must include the following components:

A. Cover Sheet (does not count towards page limit): Include the administrative and technical points of contact (name, address, phone, fax, email, lead organization). Also

include the BAA number, title of the proposed project, primary subcontractors, estimated cost, duration of the project, and the label "ABSTRACT."

B. Executive Summary Slide: Provide a one slide summary in PowerPoint that effectively and succinctly conveys the main objective, key innovations, expected impact, and other unique aspects of the proposed project. Proposers should use the slide template provided as Attachment 1 to the BAA posted at http://www.fbo.gov.

C. Goals and Impact: Clearly describe what is being proposed and what difference it will make (qualitatively and quantitatively), including brief answers to the following questions:

1. What is the proposed work attempting to accomplish or do?
2. How is it done today? And what are the limitations?
3. What is innovative in your approach and how does it compare to current practice and state-of-the-art (SOA)?
4. What are the key technical challenges in your approach and how do you plan to overcome these?
5. Who will care and what will the impact be if you are successful?
6. How much will it cost and how long will it take?

D. Technical Plan: Outline and address all technical challenges inherent in the approach and possible solutions for overcoming potential problems. This section should provide appropriate specific milestones (quantitative, if possible) at intermediate stages of the project to demonstrate progress and a brief plan for accomplishment of the milestones.

E. Capabilities: Provide a brief summary of expertise of the team, including subcontractors and key personnel. A principal investigator for the project must be identified, and a description of the team's organization. Include a description of the team's organization including roles and responsibilities. Describe the organizational experience in this area, existing intellectual property required to complete the project, and any specialized facilities to be used as part of the project. List Government-furnished materials or data assumed to be available. If desired, include a brief bibliography with links to relevant papers, reports, or resumes of key performers. Do not include more than two resumes as part of the abstract. Resumes count against the abstract page limit.

4.2.2. Proposal Format

All full proposals must be in the format given below. Proposals shall consist of two volumes: 1) **Volume I, Technical and Management Proposal**, and 2) **Volume II, Cost Proposal.** All pages shall be printed on 8-1/2 by 11-inch paper with type not smaller than 12 point. Smaller font may be used for figures, tables and charts. The page limitation for full proposals includes all figures, tables, and charts. Volume I, Technical and Management Proposal, may include an attached bibliography of relevant technical papers or research notes (published and unpublished) which document the technical ideas and approach upon which the proposal is based. Copies of not more than three (3) relevant papers may be included with the submission. The bibliography

and attached papers are not included in the page counts given below. The submission of other supporting materials along with the proposals is strongly discouraged and will not be considered for review. **The maximum page count for Volume 1 is 36 pages.** A submission letter is optional and is not included in the page count. Volume I should include the following components:

NOTE: Non-conforming submissions that do not follow the instructions herein may be rejected without further review.

a. Volume I, Technical and Management Proposal

Section I. Administrative

A. Cover Sheet (LABELED "PROPOSAL: VOLUME I"):

1. BAA number (HR001118S0017);
2. Lead organization submitting proposal (prime contractor);
3. Type of organization, selected from among the following categories: "LARGE BUSINESS," "SMALL DISADVANTAGED BUSINESS," "OTHER SMALL BUSINESS," "HBCU," "MI," "OTHER EDUCATIONAL," OR "OTHER NONPROFIT";
4. Proposer's reference number (if any);
5. Other team members (if applicable) and type of business for each;
6. Proposal title;
7. Technical point of contact (Program Manager or Principle Investigator) to include: salutation, last name, first name, street address, city, state, zip code, telephone, fax, e-mail;
8. Administrative point of contact (Contracting Officer or Grant Officer) to include: salutation, last name, first name, street address, city, state, zip code, telephone, fax, e-mail;
9. Award instrument requested: cost-plus-fixed-free (CPFF), cost-contract—no fee, firm-fixed-price, grant, cooperative agreement, other transaction, or other type (specify);
10. Place(s) and period(s) of performance ;
11. Proposal validity period;
12. Total funds requested from DARPA, and the amount of cost share (if any); AND
13. Date proposal was submitted.

Information on award instruments is available at http://www.darpa.mil/work-with-us/contract-management.

B. Official Transmittal Letter.

Section II. Detailed Proposal Information

A. Executive Summary: Provide a synopsis of the proposed project, including answers to the following questions:

- What is the proposed work attempting to accomplish or do?
- How is it done today, and what are the limitations?
- What is innovative in your approach?
- What are the key technical challenges in your approach and how do you plan to overcome these?
- Who or what will be affected and what will be the impact if the work is successful?
- How much will it cost, and how long will it take?

B. Executive Summary Slide: Provide a one slide summary in PowerPoint that effectively and succinctly conveys the main objective, key innovations, expected impact, and other unique aspects of the proposed project. Proposers should use the slide template provided as **Attachment 1** to the BAA posted at https://www.fbo.gov.

C. Goals and Impact: Clearly describe what the team is trying to achieve and the difference it will make (qualitatively and quantitatively) if successful. Describe the innovative aspects of the project in the context of existing capabilities and approaches, clearly delineating the uniqueness and benefits of this project in the context of the state of the art, alternative approaches, and other projects from the past and present. Describe how the proposed project is revolutionary and how it significantly rises above the current state of the art. Describe the deliverables associated with the proposed project and any plans to commercialize the technology, transition it to a customer, or further the work.

D. Technical Plan: Outline and address technical challenges inherent in the approach and possible solutions for overcoming potential problems. This section should provide appropriate measurable milestones (quantitative if possible) and program metrics (see Section 1.2) at intermediate stages of the program to demonstrate progress, and a plan for achieving the milestones. The technical plan should demonstrate a deep understanding of the technical challenges and present a credible (even if risky) plan to achieve the program goal. Discuss mitigation of technical risk. The technical plan should address the TA1 and TA2 proposal content requirements detailed in Section 1.1.

E. Management Plan: Provide a summary of expertise of the team, including any subcontractors, and key personnel who will be doing the work. Resumes count against the proposal page count. Identify a principal investigator for the project. Provide a clear description of the team's organization including an organization chart that includes, as applicable: the programmatic relationship of team members; the unique

capabilities of team members; the task responsibilities of team members, the teaming strategy among the team members; and key personnel with the amount of effort to be expended by each person during each year. Provide a detailed plan for coordination including explicit guidelines for interaction among collaborators/subcontractors of the proposed effort. Include risk management approaches. Describe any formal teaming agreements that are required to execute this program.

F. Capabilities: Describe organizational experience in relevant subject area(s), existing intellectual property, specialized facilities, and any Government-furnished materials or information. Discuss any work in closely related research areas and previous accomplishments.

G. Statement of Work (SOW): The SOW should provide a detailed task breakdown, citing specific tasks and their connection to the interim milestones and program metrics. Each phase of the program (Phase I base and Phase II option) should be separately defined in the SOW and each task should be identified by TA (1 or 2). The SOW must not include proprietary information.

For each task/subtask, provide:

- A detailed description of the approach to be taken to accomplish each defined task/subtask.
- Identification of the primary organization responsible for task execution (prime contractor, subcontractor(s), consultant(s), by name).
- A measurable milestone, i.e., a deliverable, demonstration, or other event/activity that marks task completion. Include quantitative metrics.
- A definition of all deliverables (e.g., data, reports, software) to be provided to the Government in support of the proposed tasks/subtasks.

H. Schedule and Milestones: Provide a detailed schedule showing tasks (task name, duration, work breakdown structure element as applicable, performing organization), milestones, and the interrelationships among tasks. The task structure must be consistent with that in the SOW. Measurable milestones should be clearly articulated and defined in time relative to the start of the project.

I. PREEMPT Transition Plan (see Section 1.2): Proposers must indicate the types of partners (e.g., government, private industry, non-profit) they plan to pursue and submit a timeline with incremental milestones toward successful engagement. Proposers should begin transition activities during the early stages of the program (Phase I). The plan should describe any potential DARPA roles. If the plan includes a start-up company, a business development strategy must be included as well.

J. PREEMPT Risk Mitigation Plan (see Section 1.4): Proposers must provide a risk mitigation plan that addresses the following:

- An assessment of potential risks to public health, agriculture, plants, animals, the environment, and national security.
- Proposed guidelines that the proposer will follow to ensure maximal biosafety and biosecurity during the course of the research.
- A communication plan that addresses content, timing, and the extent of distribution of potentially sensitive dual-use information. The plan must also address how input from DARPA, other government, and community stakeholders will be taken into account in decisions regarding communication and publication of potentially sensitive dual-use information.

K. Ethical, Legal, and Societal Implications (ELSI) (see Section 1.3): Proposers should address potential ethical, legal, and societal implications of the proposed technology.

Section III. Additional Information (Note: Does not count towards page limit)

A brief bibliography of relevant technical papers and research notes (published and unpublished) which document the technical ideas upon which the proposal is based. Copies of not more than three (3) relevant papers can be included in the submission.

a. Volume II, Cost Management Proposal

Cover Sheet (LABELED "PROPOSAL: VOLUME II"):

1. BAA number;
2. Lead Organization Submitting proposal;
3. Type of organization, selected among the following categories: "LARGE BUSINESS", "SMALL DISADVANTAGED BUSINESS", "OTHER SMALL BUSINESS", "HBCU", "MI", "OTHER EDUCATIONAL", OR "OTHER NONPROFIT";
4. Proposer's reference number (if any);
5. Other team members (if applicable), CAGE Code(s), and type of business for each;
6. Proposal title;
7. Technical point of contact (Program Manager or Principal Investigator) to include: salutation, last name, first name, street address, city, state, zip code, telephone, fax (if available), electronic mail (if available);
8. Administrative point of contact (Contracting Officer or Grant Officer) to include: salutation, last name, first name, street address, city, state, zip code, telephone, fax (if available), and electronic mail (if available);

9. Award instrument requested: cost-plus-fixed-free (CPFF), cost-contract—no fee, cost sharing contract – no fee, or other type of procurement contract (*specify*), grant, cooperative agreement, or other transaction;
10. Place(s) and period(s) of performance;
11. Total proposed cost separated by basic award and option(s) (if any);
12. Name, address, and telephone number of the proposer's cognizant Defense Contract Management Agency (DCMA) administration office (*if known)*;
13. Name, address, and telephone number of the proposer's cognizant Defense Contract Audit Agency (DCAA) audit office (*if known*);
14. Date proposal was prepared;
15. DUNS number (http://www.dnb.com/get-a-duns-number.html);
16. Taxpayer ID number (https://www.irs.gov/Individuals/International-Taxpayers/Taxpayer-Identification-Numbers-TIN);
17. CAGE code (https://www.dlis.dla.mil/bincs/FAQ.aspx);
18. Proposal validity period

Note that nonconforming proposals may be rejected without review.

Proposers that do not have a Cost Accounting Standards (CAS) complaint accounting system considered adequate for determining accurate costs that are negotiating a cost- type procurement contract must complete an SF 1408. For more information on CAS compliance, see http://www.dcaa.mil/cas.html. To facilitate this process, proposers should complete the SF 1408 found at http://www.gsa.gov/portal/forms/download/115778 and submit the completed form with the proposal. To complete the form, check the boxes on the second page, then provide a narrative explanation of your accounting system to supplement the checklist on page one. For more information, see (http://www.dcaa.mil/preaward_accounting_system_adequacy_checklist.html).

The Government strongly encourages that tables included in the cost proposal also be provided in an editable (e.g., MS Excel) format with calculation formulas intact to allow traceability of the cost proposal numbers across the prime and subcontractors.

The Government requires that the proposer provide a detailed cost breakdown to include:

(1) Total program cost broken down by Phase I (Base) and Phase II (Option) in Contractor Fiscal Year to include:
   i. Direct Labor – Including individual labor categories with associated labor hours and direct labor rates. If selected for award, be prepared to submit supporting documentation to justify labor rates. (i.e., screenshots of HR databases, comparison to NIH or other web-based salary database);
   ii. Consultants – If consultants are to be used, proposer must provide a copy of the consultant's proposed SOW as well as a signed consultant agreement or other document which verifies the proposed loaded daily / hourly rate, hours and any other proposed consultant costs (e.g., travel);

iii. Indirect Costs – Including Fringe Benefits, Overhead, General and Administrative Expense, Cost of Money, Fee, etc. (must show base amount and rate), if available, provide current Forward Pricing Rate Agreement or Forward Pricing Rate Proposal. If not available, provide 2 years historical data to include pool and expense costs used to generate the rates. For academia, provide DHHS or ONR negotiated rate package or, if calculated by other than a rate, provide University documentation identifying G&A and fringe costs by position;

iv. Travel – Provide the purpose of the trip, number of trips, number of days per trip, departure and arrival destinations, number of people, estimated rental car and airfare costs, and prevailing per diem rates as determined by gsa.gov, etc.; Quotes must be supported by screenshots from travel websites;

v. Other Direct Costs – Itemized with costs including tuition remission, animal per diem rates, health insurance/fee; back-up documentation is to be submitted to support proposed costs;

vi. Equipment Purchases – Itemization with individual and total costs, including quantities, unit prices, proposed vendors (if known), and the basis of estimate (e.g., quotes, prior purchases, catalog price lists, etc.); any item that exceeds $5,000 must be supported with back-up documentation such as a copy of catalog price lists or quotes prior to purchase (NOTE: For equipment purchases, include a letter stating why the proposer cannot provide the requested resources from its own funding), and;

vii. Materials – Itemization with costs, including quantities, unit prices, proposed vendors (if known), and the basis of estimate (e.g., quotes, prior purchases, catalog price lists, etc.); any item that exceeds $5,000 must be supported with back-up documentation such as a copy of catalog price lists or quotes prior to purchase.

(2) A summary of total program costs by major task;

(3) A summary of projected funding requirements by month;

(4) An itemization of any information technology (IT) purchase (including a letter stating why the proposer cannot provide the requested resources from its own funding), as defined in FAR Part 2.101;

(5) An itemization of Subcontracts. **All subcontractor cost proposal documentation must be prepared at the same level of detail as that required of the prime.** Subcontractor proposals should include Interdivisional Work Transfer Agreements (IWTA) or evidence of similar arrangements (an IWTA is an agreement between multiple divisions of the same organization);

(6) The source, nature, and amount of any industry cost-sharing. Where the effort consists of multiple portions which could reasonably be partitioned for purposes of funding, these should be identified as options with separate cost estimates for each;

(7) Identification of pricing assumptions of which may require incorporation into the resulting award instrument (e.g., use of Government Furnished Property/Facilities/Information, access to Government Subject Matter Expert/s, etc.);

(8) Any Forward Pricing Rate Agreement, DHHS rate agreement, other such approved rate information, or such documentation that may assist in expediting negotiations (if available); and

(9) Proposers with a Government acceptable accounting system who are proposing a cost-type contract must submit the DCAA document approving the cost accounting system.

4.2.3. Additional Proposal Information

Proprietary Markings

Proposers are responsible for clearly identifying proprietary information. Submissions containing proprietary information must have the cover page and each page containing such information clearly marked with a label such as "Proprietary" or "Company Proprietary." NOTE: "Confidential" is a classification marking used to control the dissemination of U.S. Government National Security Information as dictated in Executive Order 13526 and should not be used to identify proprietary business information.

Unclassified Submissions

DARPA anticipates that submissions received under this BAA will be unclassified. However, should a proposer wish to submit classified information, an *unclassified* email must be sent to the BAA mailbox requesting submission instructions from the Technical Office PSO. If a determination is made that the award instrument may result in access to classified information, a SCG and/or DD Form 254 will be issued by DARPA and attached as part of the award.

Human Research Subjects/Animal Use

Proposers that anticipate involving Human Research Subjects or Animal Use must comply with the approval procedures detailed at http://www.darpa.mil/work-with-us/additional-baa.

Small Business Subcontracting Plan

Pursuant to Section 8(d) of the Small Business Act (15 U.S.C. § 637(d)) and FAR 19.702(a)(1), each proposer who submits a contract proposal and includes subcontractors might be required to submit a subcontracting plan with their proposal. The plan format is outlined in FAR 19.704.

Section 508 of the Rehabilitation Act (29 U.S.C. § 749d)/FAR 39.2

All electronic and information technology acquired or created through this BAA must satisfy the accessibility requirements of Section 508 of the Rehabilitation Act (29 U.S.C. § 749d)/FAR 39.2.

Intellectual Property

All proposers must provide a good faith representation that the proposer either owns or possesses the appropriate licensing rights to all intellectual property that will be utilized under the proposed effort.

For Procurement Contracts

Proposers responding to this BAA requesting procurement contracts will need to complete the certifications at DFARS 252.227-7017. See http://www.darpa.mil/work-with-us/additional-baa for further information. If no restrictions are intended, the proposer should state "NONE."

The table below captures the requested information:

Technical Data	Summary of	Basis for	Asserted Rights	Name of Person

Computer Software To be Furnished With Restrictions	Intended Use in the Conduct of the Research	Assertion	Category	Asserting Restrictions
(LIST)	(NARRATIVE)	(LIST)	(LIST)	(LIST)

For All Non-Procurement Contracts

Proposers responding to this BAA requesting a Grant, Cooperative Agreement, Technology Investment Agreement, or Other Transaction for Prototypes shall follow the applicable rules and regulations governing these various award instruments, but, in all cases, should appropriately identify any potential restrictions on the Government's use of any Intellectual Property contemplated under the award instrument in question. This includes both Noncommercial Items and Commercial Items. Proposers are encouraged to use a format similar to that described in the section above. If no restrictions are intended, then the proposer should state "NONE."

System for Award Management (SAM) and Universal Identifier Requirements

All proposers must be registered in SAM unless exempt per FAR 4.1102. FAR 52.204-7, "System for Award Management" and FAR 52.204-13, "System for Award Management Maintenance" are incorporated into this BAA. See http://www.darpa.mil/work-with-us/additional-baa for further information.

4.2.4. Submission Information

DARPA will acknowledge receipt of all submissions and assign an identifying control number that should be used in all further correspondence regarding the submission. DARPA intends to use electronic mail correspondence regarding HR001118S0017. Submissions may not be submitted by fax or e-mail; any so sent will be disregarded.

Submissions will not be returned. An electronic copy of each submission received will be retained at DARPA and all other non-required copies destroyed. A certification of destruction may be requested, provided the formal request is received by DARPA within 5 days after notification that a proposal was not selected.

For (abstract and) proposal submission dates, see Part I., Overview Information. Submissions received after these dates and times may not be reviewed.

For Proposers Submitting Proposal Abstracts or Full Proposals as Hard Copies/On CD-ROM:

Proposers must submit an original hardcopy and one (1) electronic copy of the abstract or proposal in PDF (preferred) on a CD-ROM to the mailing address listed in Part I. Each copy must be clearly labeled with HR001118S0017, proposer organization, technical point of contact, and proposal title (short title recommended).

Please note that submitters via hardcopy/CD-ROM will still need to visit https://baa.darpa.mil to register their organization concurrently to ensure the BAA office can verify and finalize their submission.

For Proposers Submitting Proposal Abstracts or Full Proposals Requesting Procurement Contracts or OTs through DARPA's BAA Submission Portal:

Abstracts and Full Proposals sent in response to HR001118S0017 may be submitted via DARPA's BAA Website (https://baa.darpa.mil). Visit the website to complete the two-step registration process. Submitters will need to register for an Extranet account (via the form at the URL listed above) and wait for two separate e-mails containing a username and temporary password. After accessing the Extranet, submitters may then create an account for the DARPA BAA website (via the "Register your Organization" link along the left side of the homepage), view submission instructions, and upload/finalize the abstract. Proposers using the DARPA BAA Website may encounter heavy traffic on the submission deadline date; it is highly advised that submission process be started as early as possible.

All unclassified concepts submitted electronically through DARPA's BAA Website must be uploaded as zip files (.zip or .zipx extension). The final zip file should be no greater than 50 MB in size. Only one zip file will be accepted per submission. Classified submissions and proposals requesting assistance instruments (grants or cooperative agreements) should NOT be submitted through DARPA's BAA Website (https://baa.darpa.mil), though proposers will likely still need to visit https://baa.darpa.mil to register their organization (or verify an existing registration) to ensure the BAA office can verify and finalize their submission.

Technical support for BAA Website may be reached at BAAT_Support@darpa.mil, and is typically available during regular business hours, (9:00 AM- 5:00 PM EST Monday – Friday).

Proposers using the DARPA BAA Website may encounter heavy traffic on the submission deadline date; it is highly advised that submission process be started as early as possible.

For Full Proposals Requesting Cooperative Agreements:

Proposers requesting cooperative agreements may submit proposals through one of the following methods: (1) hard copy mailed directly to DARPA; or (2) electronic upload per the instructions at http://www.grants.gov/applicants/apply-for-grants.html. Cooperative agreement proposals may not be submitted through any other means. If proposers intend to use Grants.gov as their means of submission, then they must submit their entire proposal through Grants.gov; applications cannot be submitted in part to Grants.gov and in part as a hard-copy. Proposers using the Grants.gov do not submit paper proposals in addition to the Grants.gov electronic submission.

Grants.gov Submissions: Grants.gov requires proposers to complete a one-time registration process before a proposal can be electronically submitted. First time registration can take between three business days and four weeks. For more information about registering for Grants.gov, see http://www.darpa.mil/work-with-us/additional-baa.

Hard-copy Submissions: Proposers electing to submit grant or cooperative agreement proposals as hard copies must complete the SF 424 R&R form (Application for Federal Assistance,) available on the Grants.gov website http://aaply07.grants.gov/apply/forms/sample/RR_SF424_2_0-V2.0.pdf.

Failure to comply with the submission procedures may result in the submission not being evaluated. DARPA will acknowledge receipt of complete submissions via email and assign control numbers that should be used in all further correspondence regarding proposals.

4.2.5. Disclosure of Information and Compliance with Safeguarding Covered Defense Information Controls

The following provisions and clause apply to all solicitations and contracts; however, the definition of "controlled technical information" clearly exempts work considered fundamental research and therefore, even though included in the contract, will not apply if the work is fundamental research.

DFARS 252.204-7000, "Disclosure of Information"
DFARS 252.204-7008, "Compliance with Safeguarding Covered Defense Information Controls"
DFARS 252.204-7012, "Safeguarding Covered Defense Information and Cyber Incident Reporting"

The full text of the above solicitation provision and contract clauses can be found at http://www.darpa.mil/work-with-us/additional-baa#NPRPAC.

Compliance with the above requirements includes the mandate for proposers to implement the security requirements specified by National Institute of Standards and Technology (NIST) Special Publication (SP) 800-171, "Protecting Controlled Unclassified Information in Nonfederal Information Systems and Organizations" (see https://doi.org/10.6028/NIST.SP.800-171r1) that are in effect at the time the BAA is issued, or as authorized by the Contracting Officer, not later than December 31, 2017.

For awards where the work is considered fundamental research, the contractor will not have to implement the aforementioned requirements and safeguards; however, should the nature of the work change during performance of the award, work not considered fundamental research will be subject to these requirements.

4.3. FUNDING RESTRICTIONS

Not Applicable.

4.4. OTHER SUBMISSION REQUIREMENTS

Not Applicable.

5. Application Review Information

5.1. EVALUATION CRITERIA

Proposals will be evaluated using the following criteria, listed in descending order of importance: 5.1.1 Overall Scientific and Technical Merit; 5.1.2 Potential Contribution and Relevance to the DARPA Mission; and 5.1.3 Cost Realism.

5.1.1. Overall Scientific and Technical Merit

The proposed technical approach is innovative, feasible, achievable, and complete.

Task descriptions and associated technical elements provided are complete and in a logical sequence with all proposed deliverables clearly defined such that a final outcome that achieves the goal can be expected as a result of award. The proposal identifies major technical risks and planned mitigation efforts are clearly defined and feasible. The proposed PREEMPT Risk Mitigation Plan effectively provides the following: an assessment of potential risks; proposed guidelines to ensure maximal biosafety and biosecurity; a risk management plan for responsible communications; and a plan to address how input from the Government and community stakeholders will be considered regarding communication and publication of potentially sensitive dual-use information.

5.1.2. Potential Contribution and Relevance to the DARPA Mission

The potential contributions of the proposed effort are relevant to the national technology base. Specifically, DARPA's mission is to make pivotal early technology investments that create or prevent strategic surprise for U.S. National Security.

The proposer clearly demonstrates its capability to transition the technology to the research, industrial, and/or operational military communities in such a way as to enhance U.S. defense. In addition, the evaluation will take into consideration the extent to which the proposed intellectual property (IP) rights will potentially impact the Government's ability to transition the technology.

5.1.3. Cost Realism

The proposed costs are realistic for the technical and management approach and accurately reflect the technical goals and objectives of the solicitation. The proposed costs are consistent with the proposer's Statement of Work and reflect a sufficient understanding of the costs and level of effort needed to successfully accomplish the proposed technical approach. The costs for the prime proposer and proposed subawardees are substantiated by the details provided in the proposal (e.g., the type and number of labor hours proposed per task, the types and quantities of materials, equipment and fabrication costs, travel and any other applicable costs and the basis for the estimates).

It is expected that the effort will leverage all available relevant prior research in order to obtain the maximum benefit from the available funding. For efforts with a likelihood of commercial application, appropriate direct cost sharing may be a positive factor in the evaluation. DARPA recognizes that undue emphasis on cost may motivate proposers to offer low-risk ideas with minimum uncertainty and to staff the effort with junior personnel in order to be in a more competitive posture. DARPA discourages such cost strategies.

5.2. REVIEW OF PROPOSALS

Review Process

It is the policy of DARPA to ensure impartial, equitable, comprehensive proposal evaluations based on the evaluation criteria listed in Section V.A. and to select the source (or sources) whose offer meets the Government's technical, policy, and programmatic goals.

DARPA will conduct a scientific/technical review of each conforming proposal. Conforming proposals comply with all requirements detailed in this BAA; proposals that fail to do so may be deemed non-conforming and may be removed from consideration. Proposals will not be evaluated against each other since they are not submitted in accordance with a common work statement. DARPA's intent is to review proposals as soon as possible after they arrive; however, proposals may be reviewed periodically for administrative reasons

Award(s) will be made to proposers whose proposals are determined to be the most advantageous to the Government, consistent with instructions and evaluation criteria specified in the BAA herein, and availability of funding.

Handling of Source Selection Information

DARPA policy is to treat all submissions as source selection information (see FAR 2.101 and 3.104), and to disclose their contents only for the purpose of evaluation. Restrictive notices notwithstanding, during the evaluation process, submissions may be handled by support contractors for administrative purposes and/or to assist with technical evaluation. All DARPA support contractors performing this role are expressly prohibited from performing DARPA-sponsored technical research and are bound by appropriate nondisclosure agreements. Subject to the restrictions set forth in FAR 37.203(d), input on technical aspects of the proposals may be solicited by DARPA from non-Government consultants/experts who are strictly bound by the appropriate non-disclosure requirements.

Federal Awardee Performance and Integrity Information (FAPIIS)

Per 41 U.S.C. 2313, as implemented by FAR 9.103 and 2 CFR § 200.205, prior to making an award above the simplified acquisition threshold, DARPA is required to review and consider any information available through the designated integrity and performance system (currently FAPIIS). Awardees have the opportunity to comment on any information about themselves entered in the database, and DARPA will consider any comments, along with other information in FAPIIS or other systems prior to making an award.

6. Award Administration Information

6.1. SELECTION NOTICES

As soon as the evaluation of a proposal is complete, the proposers will be notified that 1) the proposal has been selected for funding pending contract negotiations, or 2) the proposal has not been selected. These official notifications will be sent via email to the Technical POC identified on the proposal coversheet.

6.1.1. Proposal Abstracts

DARPA will respond to abstracts with a statement as to whether DARPA is interested in the idea. If DARPA does not recommend the proposer submit a full proposal, DARPA will provide feedback to the proposer regarding the rationale for this decision. Regardless of DARPA's response to an abstract, proposers may submit a full proposal. DARPA will review all full proposals submitted using the published evaluation criteria and without regard to any comments resulting from the review of an abstract.

6.1.2. Full Proposals

As soon as the evaluation of a proposal is complete, the proposer will be notified that (1) the proposal has been selected for funding pending award negotiations, in whole or in part, or (2) the proposal has not been selected. These official notifications will be sent via e-mail to the Technical POC and/or Administrative POC identified on the proposal coversheet.

6.2. ADMINISTRATIVE AND POLICY REQUIREMENTS

6.2.1. Meeting and Travel Requirements

There will be a program kickoff meeting in the Arlington, VA vicinity and all key participants are required to attend. Performers should also anticipate regular program-wide PI meetings and periodic site visits at the Program Manager's discretion to the Arlington, VA vicinity. Proposers shall include within the content of their proposal details and costs of any travel or meetings they deem to be necessary throughout the course of the effort, to include periodic status reviews by the government.

6.2.1. FAR and DFARS Clauses

Solicitation clauses in the FAR and DFARS relevant to procurement contracts and FAR and DFARS clauses that may be included in any resultant procurement contracts are incorporated herein and can be found at http://www.darpa.mil/work-with-us/additional-baa.

6.2.2. Controlled Unclassified Information (CUI) on Non-DoD Information Systems

Further information on Controlled Unclassified Information on Non-DoD Information Systems is incorporated herein can be found at http://www.darpa.mil/work-with-us/additional-baa.

6.2.3. Representations and Certifications

If a procurement contract is contemplated, prospective awardees will need to be registered in the SAM database prior to award and complete electronic annual representations and certifications consistent with FAR guidance at 4.1102 and 4.1201; the representations and certifications can be found at www.sam.gov. Supplementary representations and certifications can be found at http://www.darpa.mil/work-with-us/additional-baa.

.

6.2.4. Terms and Conditions

A link to the DoD General Research Terms and Conditions for Grants and Cooperative Agreements and supplemental agency terms and conditions can be found at http://www.darpa.mil/work-with-us/contract-management#GrantsCooperativeAgreements.

6.3. REPORTING

The number and types of reports will be specified in the award document, but will include as a minimum monthly financial status reports and quarterly technical status reports. The reports shall be prepared and submitted in accordance with the procedures contained in the award document and mutually agreed on before award. Reports and briefing material will also be required as appropriate to document progress in accomplishing program metrics. A Final Report that summarizes the project and tasks will be required at the conclusion of the performance period for the award, notwithstanding the fact that the research may be continued under a follow-on vehicle.

6.4. ELECTRONIC SYSTEMS

6.4.1. Wide Area Work Flow (WAWF)

Performers will be required to submit invoices for payment directly to https://wawf.eb.mil, unless an exception applies. Performers must register in WAWF prior to any award under this BAA.

6.4.2. i-EDISON

The award document for each proposal selected for funding will contain a mandatory requirement for patent reports and notifications to be submitted electronically through i-Edison (http://public.era.nih.gov/iedison).

7. Agency Contacts

Communication via e-mail is preferred.

Points of Contact
The BAA Coordinator for this effort may be reached at:
PREEMPT@darpa.mil
DARPA/BTO
ATTN: HR001118S0017
675 North Randolph Street
Arlington, VA 22203-2114

For information concerning agency level protests see http://www.darpa.mil/work-with-us/additional-baa#NPRPAC.

8. Other Information

DARPA will host a Proposers Day in support of the PREEMPT program on **January 30, 2018**, at the Executive Conference Center in Arlington, VA. The purpose is to provide potential

proposers with information on the PREEMPT program, promote additional discussion on this topic, address questions, provide a forum to present their capabilities, and to encourage team formation.

Interested proposers are not required to attend to respond to the PREEMPT BAA, and relevant information and materials discussed at Proposers Day will be made available to all potential proposers in the form of a FAQ posted on the DARPA Opportunities Page. The event will be webcast for those who would like to participate remotely.

DARPA will not provide cost reimbursement for interested proposers in attendance.

An online registration form and various other meeting details can be found at the registration website, https://events.sa-meetings.com/PREEMPTProposersDay.

To encourage team formation, interested proposers are encouraged to submit information to be shared with all potential proposers through the Proposers Day website and the DARPA Opportunities Page. This information may include contact information, relevant publications, and a slide or poster to summarize the proposer's interests.

Participants are required to register no later than **January 23, 2018**, for physical attendance, and **January 26, 2018**, for the webcast. This event is not open to the Press. The Proposers Day will be open to members of the public who have registered in advance for the event; **there will be no onsite registration**.

All foreign nationals, including permanent residents, must complete and submit a DARPA Form 60 "Foreign National Visit Request," which will be provided in the registration confirmation email.

Proposers Day Point of Contact: DARPA-SN-18-18@darpa.mil.

9. Appendix 1 – Volume II checklist

Volume II, Cost Proposal
Checklist and Sample Templates

The following checklist and sample templates are provided to assist the proposer in developing a complete and responsive cost volume. Full instructions appear in Section 4.2.2 beginning on Page 25 of HR001118S0017. This worksheet must be included with the coversheet of the Cost Proposal.

1. Are all items from Section 4.2.2 (Volume II, Cost Proposal) of **HR001118S0017** included on your Cost Proposal cover sheet?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]
   If reply is "No", please explain:

2. Does your Cost Proposal include (1) a summary cost buildup by Phase, (2) a summary cost buildup by Year, and (3) a detailed cost buildup of for each Phase that breaks out each task and shows the cost per month?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

3. Does your cost proposal (detailed cost buildup #3 above in item 2) show a breakdown of the major cost items listed below:
   Direct Labor (Labor Categories, Hours, Rates)
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Indirect Costs/Rates (i.e., overhead charges, fringe benefits, G&A)
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Materials and/or Equipment
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Subcontracts/Consultants
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Other Direct Costs
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Travel
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

4. Have you provided documentation for proposed costs related to travel, to include purpose of trips, departure and arrival destinations and sample airfare?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

If reply is "No", please explain:

5. Does your cost proposal include a complete itemized list of all material and equipment items to be purchased (a priced bill-of-materials (BOM))?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

6. Does your cost proposal include vendor quotes or written engineering estimates (basis of estimate) for all material and equipment with a unit price exceeding $5000?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

7. Does your cost proposal include a clear justification for the cost of labor (written labor basis-of-estimate (BOE)) providing rationale for the labor categories and hours proposed for each task?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

8. Do you have subcontractors/consultants? If YES, continue to question 9. If NO, skip to question 13.
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

9. Does your cost proposal include copies of all subcontractor/consultant technical (to include Statement of Work) and cost proposals?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

10. Do all subcontract proposals include the required summary buildup, detailed cost buildup, and supporting documentation (SOW, Bill-of-Materials, Basis-of-Estimate, Vendor Quotes, etc.)?
    ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

    If reply is "No", please explain:

11. Does your cost proposal include copies of consultant agreements, if available?
    ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

    If reply is "No", please explain:

12. If requesting a FAR-based contract, does your cost proposal include a tech/cost analysis for all proposed subcontractors?
    ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

    If reply is "No", please explain:

13. Have all team members (prime and subcontractors) who are considered a Federally Funded Research & Development Center (FFRDC), included documentation that clearly demonstrates work is not otherwise available from the private sector AND provided a letter on letterhead from the sponsoring organization citing the specific authority establishing their eligibility to propose to government solicitations and compete with industry, and compliance with the associated FFRDC sponsor agreement and terms and conditions.

○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

If reply is "No", please explain:

14. Does your proposal include a response regarding Organizational Conflicts of Interest?

○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

If reply is "No", please explain:

15. Does your proposal include a completed Data Rights Assertions table/certification?

○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

If reply is "No", please explain:

Executive Summary: Proposal Title

Organization; PI Name

CONCEPT

Provide graphic.

APPROACH

Describe new ideas.

IMPACT

Describe need and problem being addressed.

Describe goal.

	Phase I	Phase II	Total
Proposed	$-	$-	$-

☐ Human Use/☐ Animal Use

CONTEXT

Describe existing approaches; compare to state of the art.

Slide 1 Notes

Goals and Impact

Clearly describe what is being proposed and what difference it will make (qualitatively and quantitatively), including brief answers to the following questions:

- What is the proposed work attempting to accomplish or do?
- How is it done today? And what are the limitations?
- What is innovative in your approach and how does it compare to current practice and state-of-the-art (SOA)?
- What are the key technical challenges in your approach and how do you plan to overcome these?
- Who will care and what will the impact be if you are successful?
- How much will it cost and how long will it take?

Technical Plan

Outline and address all technical challenges inherent in the approach and possible solutions for overcoming potential problems. This section should provide appropriate specific milestones (quantitative, if possible) at intermediate stages of the project to demonstrate progress and a brief plan for accomplishment of the milestones.

Capabilities

Provide a brief summary of expertise of the team, including subcontractors and key personnel. A principal investigator for the project must be identified. Include a description of the team's organization including roles and responsibilities. Describe the organizational experience in this area, existing intellectual property required to complete the project, and any specialized facilities to be used as part of the project. List Government-furnished materials or data assumed to be available. If desired, include a brief bibliography with links to relevant papers, reports, or resumes of key performers. Do not include more than two resumes as part of the abstract. Resumes count against the abstract page limit.

Broad Agency Announcement

Epigenetic CHaracterization and Observation

BIOLOGICAL TECHNOLOGIES OFFICE

HR001118S0023

February 12, 2018

TABLE OF CONTENTS

PART I: OVERVIEW INFORMATION 3

PART II: FULL TEXT OF ANNOUNCEMENT 4

1. Funding Opportunity Description 4
1.1. Program Overview 4
1.2. Technical Approach 5
1.3. Program Metrics and Schedule 9
1.4. Controlled Unclassified Information Statement 13
2. Award Information 14
2.1. General Award Information 14
2.2. Fundamental Research 15
3. Eligibility Information 16
3.1. Eligible Applicants 16
3.2. Organizational Conflicts of Interest 17
3.3. Cost Sharing/Matching 17
4. Application and Submission Information 18
4.1. Address to Request Application Package 18
4.2. Content and Form of Application Submission 18
4.3. Funding Restrictions 30
4.4. Other Submission Requirements 30
5. Application Review Information 30
5.1. Evaluation Criteria 30
5.2. Review of Proposals 32
6. Award Administration Information 33
6.1. Selection Notices 33
6.2. Administrative and National Policy Requirements 33
6.3. Reporting 34
6.4. Electronic Systems 34
7. Agency Contacts 34
8. Other Information 35
9. APPENDIX 1 – Volume II checklist 36

PART I: OVERVIEW INFORMATION

- **Federal Agency Name** – Defense Advanced Research Projects Agency (DARPA), Biological Technologies Office
- **Funding Opportunity Title –** Epigenetic CHaracterization and Observation
- **Announcement Type –** Initial Announcement
- **Funding Opportunity Number –** HR001118S0023
- **Catalog of Federal Domestic Assistance Numbers (CFDA) – 12.910 Research and Technology Development**
- **Dates**
  - Posting Date **– February 12, 2018**
  - Proposal Abstract Due Date and Time **– March 22, 2018, 4:00 PM ET**
  - Proposal Due Date and Time **– May 3, 2018, 4:00 PM ET**
  - BAA Closing Date **– May 3, 2018, 4:00 PM ET**
  - Proposers Day **– February 23rd, 2018**

https://www.fbo.gov/spg/ODA/DARPA/CMO/DARPA-SN-18-23/listing.html

- **Concise description of the funding opportunity:** The Epigenetic CHaracterization and Observation (ECHO) program will utilize an individual's epigenome to reveal their history of exposure to weapons of mass destruction (WMD) and WMD precursors. The program will build a field-deployable platform capable of using the epigenome to diagnose biothreat exposures and infections and support military forensics operations to counter-WMD proliferation.
- **Anticipated individual awards** - Multiple awards are anticipated.
- **Types of instruments that may be awarded** - Procurement contract, cooperative agreement or other transaction.
- **Agency contact**
  - Points of Contact
    The BAA Coordinator for this effort may be reached at:
    ECHO@darpa.mil
    DARPA/BTO
    ATTN: HR001118S0023
    675 North Randolph Street
    Arlington, VA 22203-2114

PART II: FULL TEXT OF ANNOUNCEMENT

1. Funding Opportunity Description

This publication constitutes a Broad Agency Announcement (BAA) as contemplated in Federal Acquisition Regulation (FAR) 6.102(d)(2) and 35.016 and 2 CFR § 200.203. Any resultant award negotiations will follow all pertinent law and regulation, and any negotiations and/or awards for procurement contracts will use procedures under FAR 15.4, Contract Pricing, as specified in the BAA.

The Defense Advanced Research Projects Agency (DARPA) is soliciting innovative proposals to provide a field-forward system that evaluates an individual's epigenome, revealing their history of exposure to threats, including weapons of mass destruction (WMD) and their molecular precursors. Success in this program will require groundbreaking approaches to characterize epigenetic signatures from militarily relevant exposure events, and new bioinformatics tools to perform forensic analysis and disease diagnostics with high sensitivity, specificity and temporal resolution. These novel signatures and associated analytics will integrate into a single, man-portable device that operates in an austere setting with an untrained user. Proposing teams should be multidisciplinary with expertise in WMD threat agents, chromatin biology, epigenetics, gene expression, bioinformatics, microfluidics, next-generation sequencing, forensics, circulating biomarker discovery, point-of-care diagnostic device development, and computational modeling. Proposals describing incremental research and device development will be excluded.

1.1. PROGRAM OVERVIEW

WMD production is dramatically proliferating among terror groups and non-state actors, severely impacting foreign policy and national security. To disrupt this growing threat, our military must identify and stop WMD development while also mitigating the increased risk of exposure to DoD personnel while deployed. Current practice uses residues associated with weapons manufacture or exposure which are often transient and in such low concentrations that samples are not available to collect. Even when remaining traces are collected, detection requires controlled laboratory environments that are not available in austere settings. Addressing the emerging WMD threat requires a capability that can move beyond the state of the art to forensically link an individual to WMD manufacture and diagnose WMD exposure, with high specificity and temporal resolution.

The Epigenetic CHaracterization and Observation (ECHO) program aims to reveal an individual's exposure history, recorded in their own epigenome after exposure events, with a focus on WMD and molecular precursors. ECHO will build a new forensic and diagnostic modality that provides an advantage over current tools, as the epigenome is persistent and detectable even when physical evidence has been erased. For this BAA, the epigenome is considered the combination of all genomic modifications that do not alter the DNA sequence but change gene activity. Exposure to environmental factors change these epigenetic features. Depending upon the exposure type, the epigenetic features can change within minutes, while also leaving a lasting 'mark' on the epigenome for decades. ECHO will build signatures that incorporate the pattern of unique epigenetic changes associated with a specific exposure to a single compound or agent (hereafter referred to as "*exposure-specific*"). The exposure-specific

signatures will contain information that denotes the specific exposure and time since exposure. The final epigenetic signature panel (ECHO Signature Panel - *ESP*) will consist of all identified exposure-specific signatures within the program, and integrate these into a point-of-need forensic and diagnostic system.

1.2. TECHNICAL APPROACH

The ECHO program will consist of two Technical Areas (TAs) with a total period of performance of 48 months. TA1 will focus on epigenetic signature identification, and TA2 will focus on the integrated device. ***Proposing teams will be required to address both TAs***, and thus will need multidisciplinary backgrounds. Teams are strongly encouraged to propose with industry partners that can develop commercial applications based on work performed in the ECHO program. The breadth and depth of a proposing technical team's capabilities and related experience will be considereded as part of the evaluation of proposals. The comprehensive team must accomplish the following goals for each technical area:

TA1 will sequentially:

1) Generate molecular epigenetic datasets associated with relevant WMD and/or precursor exposure
2) Characterize and define exposure-specific epigenetic signatures
3) Identify unknown exposures using the ESP

TA2 will build an integrated device to achieve:

1) Epigenetic molecular analysis integration
2) Onboard computational and bio-analytical capability

Teams will be required to produce epigenetic signatures from a ***minimum of 21*** total exposure-specific agents or compounds. ECHO teams must develop an exposure-specific signature for each of the ***4 required biological agents*** listed in Table 1. These required exposure-specific signatures will be created from Government-provided, de-identified human samples comprised of blood, nasal and buccal swabs, and sputum from infectious disease surveillance sites. Teams must source human samples to develop an ***additional 17*** exposure-specific signatures spanning across the 10 sub-categories listed in Table 1. Proposers are strongly encouraged to address militarily-relevant compounds or agents. As ECHO aims to maximize the forensic and diagnostic capability of the ESP on the final device, proposals will be evaluated on the diversity of the additional 17 compound or agents selected. Proposals will be evaluated on applicable team experience in the proposed exposure category. Teams may also propose animal models and human organ chip approaches to supplement the human samples for better time-resolution, dosing control, and creation of exposure scenarios involving highly dangerous pathogens and compounds.

Table 1. Classification of Exposures

Exposure Category	Exposure Sub-category	Minimum Required List of Exposure-Specific Agents/Compounds
Biological	1. Bacterial agents*	*Staphylococcus aureus* (MRSA) *Burkholderia pseudomallei* **(2 total)**
	2. Viral agents*:	Human immunodeficiency virus (HIV) Lassa fever virus **(2 total)**
	3. Other emerging infectious diseases	Proposers may select exposure-specific samples from any of the additional exposure categories. Teams are strongly encouraged to address militarily relevant compounds, precursors•, or agents from each of the 10 sub-categories **(17 total)**
Chemical	4. Chemical agent precursors‡	
	5. Conventional agents†	
	6. Toxic industrial compounds	
Radiological	7. Medical isotopes	
	8. Isotopes associated with weapons production	
Explosives	9. Explosives precursors	
	10. Explosives compounds#	

*https://www.selectagents.gov/selectagentsandtoxinslist.html
‡Precursors in schedules 1, 2, 3 of the OPCW CWC list: https://www.opcw.org/chemical-weapons-convention/annexes/annex-on-chemicals/schedule-1/
†Agents listed in schedules 1,2,3, of the OPCW CWC list: https://www.opcw.org/chemical-weapons-convention/annexes/annex-on-chemicals/schedule-1/
#https://www.atf.gov/file/97721/download
•Precursors should be selected based on their importance and uniqueness to a specific agent production process, while also considering the likelihood of acquiring human samples for a given precursor exposure.

Technical Area 1 (TA1):
The aim of TA1 is to build an ESP and associated bioinformatics algorithms to identify militarily relevant exposures. The signatures and algorithms will be iteratively refined throughout TA1 and transitioned to the device developed in TA2.

1) Generate molecular epigenetic datasets associated with relevant WMD and/or precursor exposure

To characterize the specific exposure event and time since exposure, teams will construct molecular epigenetic datasets from each of the 21 exposure-specific compounds/agents outlined above.
When building these datasets, teams must consider the following factors including, but not limited to:

- **Sample types** such as buccal, nasal, sputum or blood
- **Cell types** including, but not limited to T-cells, B-cells, and keratinocytes

- **The combination of molecular methods** used to reveal epigenetic marks. Epigenetic marks to consider include, but are not limited to, gene expression profiles, chromatin access/state, histone modifications, regulatory marks on cellular or cell-free DNA (cfDNA), circulating non-coding RNA, and cellular or circulating mitochondrial DNA (mtDNA).

Teams will generate the molecular epigenetic datasets by achieving a balance of the factors above, with the metric of maximizing the uniqueness of the epigenetic signature associated with a specific exposure. Of the factors above, proposers should aim to identify approaches that combine the minimum set of molecular methods that fully captures epigenetic events that correlate to a given exposure. Proposals should include a specific plan to develop molecular methods to maximize the uniqueness of the exposure-specific epigenetic signature. Table 2 describes an example set of epigenetic events and molecular methods that may be employed for generating molecular epigenetic datasets.

Table 2. Exemplar epigenetic events and molecular methods

Example Epigenetic Target	Example Epigenetic Event(s)	Example Method(s)
mRNA	Up or down regulation	RNA-SEQ
Chromatin State	Open, condensed	SONO-SEQ, ATAC-SEQ, DNase-SEQ, FAIRE-SEQ, MNase-SEQ
Histone Modifications	Activating, poised for activation, or repressing: For example, H3K4me2/3, H3K27me3	ChIP-SEQ, MINT-ChIP
Cellular or Circulating DNA (cfDNA) Modifications	Methylation, hydroxylation, or alkyklation: 5-mC, 5-hMC, 5-fC, 5-caC, and 3-mC	Meth-SEQ, WGBS, TAB-SEQ,
Cellular or Circulating Mitochondrial DNA (mtDNA)	Methylation	Meth-SEQ
Circulating Non-coding RNA	MicroRNA (miRNA), long non-coding RNAs (lncRNAs), and piwiRNAs (piRNA)	RNA-SEQ, ChIRP-SEQ
Chromosomal Interactions	Interaction of Genomic Loci	Hi-C, 3C, 4C, 5C, ChIA-PET

Teams will be required to share all molecular epigenetic datasets on a DARPA-specified database. This will provide other teams the opportunity to build exposure-specific signatures from samples to which they do not have access, increase the power of the bioinformatics analysis algorithms, as well as provide a means for verification and validation.

2) Characterize and define exposure-specific epigenetic signatures

The molecular epigenetic datasets discussed above will serve as the foundation to develop exposure-specific signatures. Teams must use quantitative approaches to justify which cell type(s), epigenetic changes, or combination of changes maximize specificity and temporal resolution of exposure-specific signatures.

Teams should additionally consider variability across individuals and other factors that can adversely affect signal-to-noise ratio. Algorithms should refine signature signal to noise ratio by segregating epigenetic components that are dynamic and static. These signatures will then populate the ESP.

3) Identify unknown exposures using the ESP

The developed algorithms will need to compare an unknown exposure against the ESP to identify the specific exposure, and when the individual was exposed. Therefore, algorithm developers will leverage a wide range of analytical approaches, which may include multifactorial, topographical, principal component, and other dimensional reduction methods.

Figure 1 summarizes a potential technical approach and factors to consider when building a signature and associated algorithms to identify unknown exposures against the ESP.

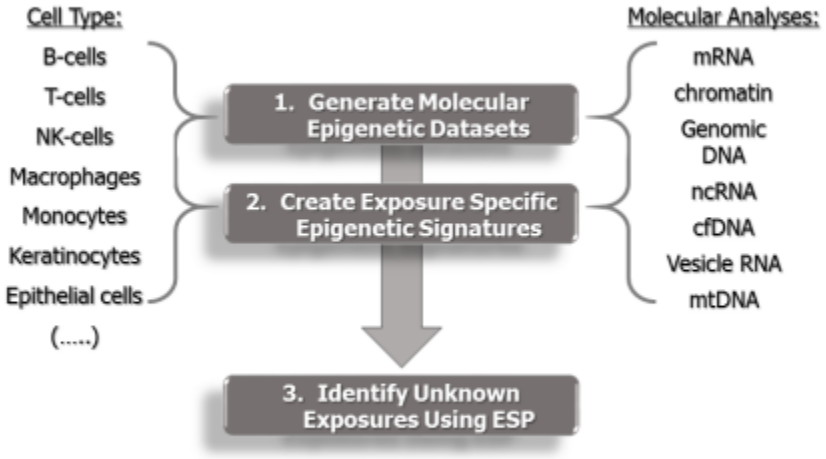


Figure 1. Possible pathway to generate signatures, include them in the ESP, and perform identification.

Technical Area 2 (TA2):

TA2 will require teams to integrate the down-selected molecular methods and bioinformatics tools necessary for exposure identification from TA1 into a single epigenetics analysis platform. The integrated platform should operate as man-portable device with a size, weight and power (SWaP) footprint equivalent to a point-of-care clinical diagnostic device, and should perform rapid analysis (< 30 minutes) in a field forward setting by minimally trained personnel.

1) Epigenetic molecular analysis integration

The TA2 system must ultimately produce an epigenetic profile from an unknown exposure for comparison to the ESP. The final epigenetic analysis system is expected to be an integrated sample-answer system capable of the following functions:

- Extract material for epigenetic analysis from blood, nasal and buccal swabs, and surface samples that may contain residual genetic material;
- Isolate nucleic acid and prepare it for epigenetic analysis; and
- Perform multiple epigenetic analyses via sequencing or other molecular analyses.

Fully automated execution of the above functions will require fluidics transfer, appropriately-timed activation of reaction and analysis modules, and data acquisition to occur through a built-in control system. Iterative progress towards a fully automated sample-to-answer device is expected. Teams may first demonstrate independent operation of subcomponents, with manual fluids transfer, before engineering the integration of all of the device components. Ultimately, by the end of the program, the system should operate with no human intervention between the time the sample is introduced and results pertinent to exposure and timing is provided.

2) Onboard computational and bio-analytical capability

Teams will translate the bioinformatics analysis capability from TA1 into purpose-built computing hardware, firmware, and software to support operations in a field-forward setting. Signature analyses must be performed onboard, rather than via cloud computing, to enable read assembly and identification in low Quality of Service (QoS) environments. This new informatics capability should rapidly identify an exposure type and time since exposure while being used by a minimally trained operator. This computational and analytical pipeline should operate seamlessly with the molecular analysis modules, on the same device.

1.3. PROGRAM METRICS AND SCHEDULE

Proposals should include a Gantt chart of the quantitative and qualitative milestones proposed to meet ECHO program metrics, listed by phase and technical area. Figure 2 lists the minimum DARPA-desired programmatic milestones and metrics. Although the following minimum milestones and metrics are specified, the Government identifies these to bound the effort while affording the maximum flexibility, creativity, and innovation in proposing solutions to the stated problems. Proposers are encouraged to add additional milestones or metrics based on the team's specific technical approach. Proposals must address all key milestones, technical metrics, and pressure tests described in this section. Proposers must clearly and uniquely itemize tasks needed to accomplish planned milestones, metrics and deliverables.

Phase I (Base) and Phase II (Option)

DARPA anticipates that the ECHO program will provide up to four years of funding for research and development to be performed over Phase I (base) and II (option). ECHO spans a 48-month effort with a 24-month Phase I base effort and 24-month Phase II option effort. In general, Phase I should provide seven exposure-specific signatures and functional small-scale epigenetic analysis modules, and, if awarded, Phase II should provide an additional 14 exposure-specific signatures and a final integrated man-portable epigenetic analysis device.

TA1 Phase I (Base)
Under TA1, teams will be required to generate two molecular epigenetic datasets (one virus and one bacteria) within six months of contract award. The samples for these datasets will be provided by the Government to support this early milestone, as indicated above. At 12 months after contract, teams will create exposure-specific signatures from the initial two biological epigenetic datasets. Also, at 12 months after contract award, teams will create five additional, non-biological exposure-specific signatures, over a range of compounds to include chemical, radiological, and explosive materials (per Table 1). At 18 months after contract, teams will be required to distinguish bacterial from viral signatures when presented with an unknown sample. By the end of Phase I, at 24 months after contract award, teams will have a multi-target test against an unknown sample to identify the specific exposure substance, organism, or biological agent, and when in time the exposure occurred (within +/- 1 year).

Teams are required to share the molecular epigenetic datasets with Independent Verification and Validation (IV&V) partners within 30 days of completing the molecular analyses for a specific exposure. IV&V partners will measure the quality of the datasets provided by each proposer team using method-specific parameters, such as read depth, error rates, and review of molecular analysis protocols. IV&V teams will identify discrepancies, which may lead to a request for teams to re-run analyses.

TA1 Phase II (Option)
By 36 months after contract award, teams will need to deliver seven additional exposure-specific signatures, with a demonstrated positive predictive value (PPV) of 65%. At 48 months after contract, teams must complete an additional seven finalized signatures with PPV increased to 85%. At program completion, 21 total exposure-specific signatures will be identified and incorporated onto the TA2 device by each team.

Table 3. ECHO Signature Development Timetable

Phase I (Base)	Phase II (Option)
TA1: Epigenetic Signature Identification	
6 mos. – Molecular epigenetic dataset creation from 1 virus and 1 bacterium.	36 mos. – 7 additional exposure-specific signatures per team.
12 mos. – Generation of 7 (1 virus, 1 bacterium, and 5 non-biological) exposure-specific signatures.	48 mos. – 7 additional exposure-specific signatures per team.
18 mos. – Determination of unknown sample. Distinguish between bacterial or viral.	
24 mos. – Multi-target test against an unknown sample. Identify the specific exposure substance, organism, or biological agent, and timing of exposure (within +/- 1 year).	

TA2 Phase I (Base)
In Phase I of TA2, the device will have to demonstrate capacity that will include human-in-the-loop fluid transfer, subsystem manual operation, and off-platform bioinformatics processing sample preparation, processing, and analysis components optimized independently. At 18

months after contract, a pressure test will evaluate the ability of the systems to operate as a complete unit for end-end epigenetic signature generation and analysis. This performance will be compared to traditional lab-based systems to ensure equivalent signature quality. QoS will initially be at 100% or the equivalent of 5 Mbps, but will be reduced to 85% by the end of Phase I to encourage a more local data analysis strategy (24 months after contract).

TA1 and TA2 Phase II (Option)

TA1 and TA2 will produce a fully integrated system with automatic fluid transfer, full subsystem automation, and onboard bioinformatics processing in Phase II. During Phase II, the QoS will continue to decrease gradually. At 30 months after contract, QoS will be 50% with intermittent coverage, and at 36 months after contract, QoS will be 25% with intermittent coverage. The final SWaP of the epigenetic deployable platform is expected to be equivalent to today's POC diagnostics systems: 1 ft^3, < 10 lbs., and < 20 W. The final system will have to pass a field forward system demonstration with military staff operating it, using < $5x10^4$ cells, with sample to answer under 30 min, 85% PPV, QoS 10% with intermittent coverage with minimally trained operators.

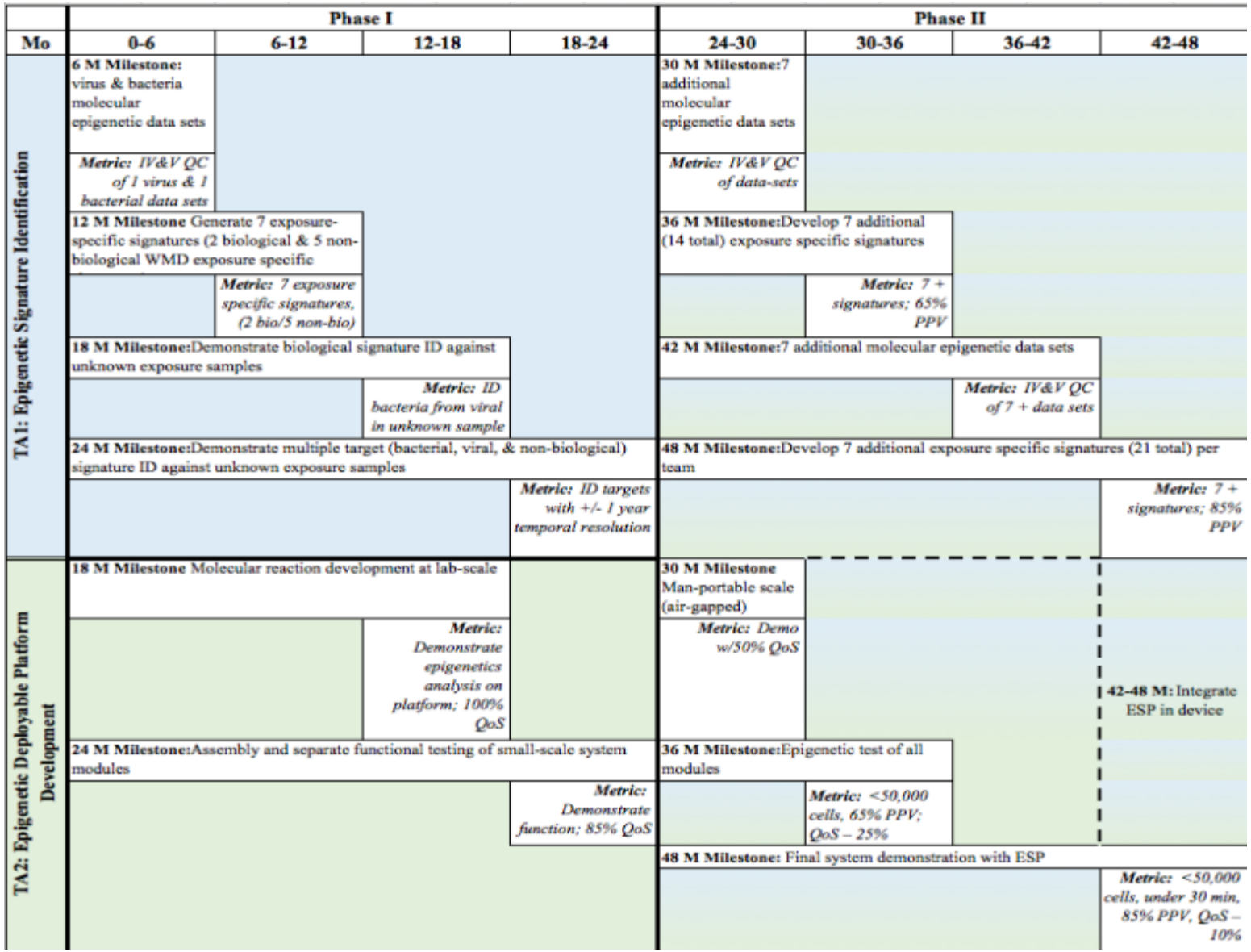


Figure 2. ECHO Milestones and Metrics

In addition to the pressure tests and system demonstration milestones, teams will be required to participate in program review meetings every six months. These meetings will include all ECHO team participants, allowing the researchers to present their latest results and update progress toward program goals. The meetings may include Government participation, program agents, defense laboratories, and the potential customers including IV&V partners. Government sidebars will be held to provide individual feedback to the performers and to ensure they are developing relevant technologies.

Deliverables
The following is the list of anticipated minimum deliverables for the Phase I base effort and Phase II option effort. Proposers may supplement this list with additional deliverables as appropriate.
Phase I Base:

- Monthly Technical/Financial Status Reports
- Epigenetic Datasets for seven compounds/agents (see table 1)
- Exposure-specific signatures for seven compounds/agents (see table 1)
- Demonstration (2) of signatures against unknown exposure samples at month 18 and 24
- Demonstration (2) of epigenetics analysis platform at month 18 and 24
- Final Report (if Phase II option is not awarded)

Phase II Option

- Monthly Technical/Financial Status Reports
- Epigenetic Datasets for an additional fourteen (see table 1)
- Exposure-specific signatures for additional fourteen (see table 1)
- Demonstration (3) of epigenetics analysis platform at months 30, 36, and 48
- ESP Prototype with operating instructions, collection cartridges, and software licenses (add description of all other items that may accompany delivery of the prototype)
- Final Report

Not all proposer teams selected for the Phase I base effort will be awarded the Phase II option effort. This decision will be based on funding availability and the performance against the metrics and milestones, and according to performance during the pressure tests described in this section.

1.4. CONTROLLED UNCLASSIFIED INFORMATION STATEMENT

To prevent the release of sensitive technical information, certain aspects of proposals may be considered Controlled Unclassified Information (CUI) and may require safeguarding or dissemination controls, pursuant to and consistent with applicable law, regulations, and Government-wide policies.

The following applied military technical information could be considered Controlled Technical Information (CTI) by DARPA:

- Potential procedure to evade ECHO Signature Panel identification: Any known techniques for rendering the exposure-specific signature undetectable.

- Epigenetic Signature Variances: Any known variances between populations that could lead to bias towards race, gender, geographic region, or inherited traits that cannot be controlled through careful clinical sampling design.

- DoD Collected Genetic or Epigenetic Data: Any genetic and epigenetic data collected from DoD efforts that has not been de-identified.

Proposals that produce any such information must deliver a detailed risk mitigation plan to DARPA (see 4.2.2. Proposal Format Section II: I). Proposers must partition potentially sensitive tasks from non-sensitive research efforts. All proposers (prime contractor and subcontractor) desiring public release of project information that may contain CTI, as defined above, must submit a request for public release to DARPA's Public Release Center (DARPA/PRC) in accordance with their contractual requirements.

2. Award Information

2.1. GENERAL AWARD INFORMATION

Multiple awards are possible. The amount of resources made available under this BAA will depend on the quality of the proposals received and the availability of funds.

The Government reserves the right to select for negotiation all, some, one, or none of the proposals received in response to this solicitation and to make awards without discussions with proposers. The Government also reserves the right to conduct discussions if it is later determined to be necessary. If warranted, portions of resulting awards may be segregated into pre-priced options. Additionally, DARPA reserves the right to accept proposals in their entirety or to select only portions of proposals for award. In the event that DARPA desires to award only portions of a proposal, negotiations may be opened with that proposer. The Government reserves the right to fund proposals in phases with options for continued work, as applicable. The Government reserves the right to fund a Phase II option based on funding availability, an assessment of Phase I research results, and a determination that awarding the option is in the best interest of the Government.

The Government reserves the right to request any additional, necessary documentation once it makes the award instrument determination. Such additional information may include but is not limited to Representations and Certifications (see Section VI.B.2., "Representations and Certifications"). The Government reserves the right to remove proposers from award consideration should the parties fail to reach agreement on award terms, conditions, and/or cost/price within a reasonable time, and the proposer fails to timely provide requested additional information. Proposals identified for negotiation may result in a procurement contract, grant, cooperative agreement, or other transaction, depending upon the nature of the work proposed, the

required degree of interaction between parties, whether or not the research is classified as Fundamental Research, and other factors.

Proposers looking for innovative, commercial-like contractual arrangements are encouraged to consider requesting Other Transactions. To understand the flexibility and options associated with Other Transactions, consult http://www.darpa.mil/work-with-us/contract-management#OtherTransactions.

In all cases, the Government contracting officer shall have sole discretion to select award instrument type, regardless of instrument type proposed, and to negotiate all instrument terms and conditions with selectees. DARPA will apply publication or other restrictions, as necessary, if it determines that the research resulting from the proposed effort will present a high likelihood of disclosing performance characteristics of military systems or manufacturing technologies that are unique and critical to defense. Any award resulting from such a determination will include a requirement for DARPA permission before publishing any information or results on the program. For more information on publication restrictions, see the section below on Fundamental Research.

2.2. FUNDAMENTAL RESEARCH

It is DoD policy that the publication of products of fundamental research will remain unrestricted to the maximum extent possible. National Security Decision Directive (NSDD) 189 defines fundamental research as follows:

> 'Fundamental research' means basic and applied research in science and engineering, the results of which ordinarily are published and shared broadly within the scientific community, as distinguished from proprietary research and from industrial development, design, production, and product utilization, the results of which ordinarily are restricted for proprietary or national security reasons.

As of the date of publication of this BAA, the Government expects that program goals as described herein may be met by proposers intending to perform fundamental research and proposers not intending to perform fundamental research or the proposed research may present a high likelihood of disclosing performance characteristics of military systems or manufacturing technologies that are unique and critical to defense. Based on the nature of the performer and the nature of the work, the Government anticipates that some awards will include restrictions on the resultant research that will require the awardee to seek DARPA permission before publishing any information or results relative to the program.

Proposers should indicate in their proposal whether they believe the scope of the research included in their proposal is fundamental or not. While proposers should clearly explain the intended results of their research, the Government shall have sole discretion to select award instrument type and to negotiate all instrument terms and conditions with selectees. Appropriate clauses will be included in resultant awards for non-fundamental research to prescribe publication requirements and other restrictions, as appropriate. This clause can be found at http://www.darpa.mil/work-with-us/additional-baa.

For certain research projects, it may be possible that although the research being performed by the awardee is restricted research, a subawardee may be conducting fundamental research. In those cases, it is the awardee's responsibility to explain in their proposal why its subawardee's effort is fundamental research

3. Eligibility Information

3.1. ELIGIBLE APPLICANTS

All responsible sources capable of satisfying the Government's needs may submit a proposal that shall be considered by DARPA.

3.1.1. Federally Funded Research and Development Centers (FFRDCs) and Government Entities

FFRDCs

FFRDCs are subject to applicable direct competition limitations and cannot propose to this BAA in any capacity unless they meet the following conditions: (1) FFRDCs must clearly demonstrate that the proposed work is not otherwise available from the private sector. (2) FFRDCs must provide a letter on official letterhead from their sponsoring organization citing the specific authority establishing their eligibility to propose to Government solicitations and compete with industry, and their compliance with the associated FFRDC sponsor agreement's terms and conditions. This information is required for FFRDCs proposing to be awardees or subawardees.

Government Entities

Government Entities (e.g., Government/National laboratories, military educational institutions, etc.) are subject to applicable direct competition limitations. Government entities must clearly demonstrate that the work is not otherwise available from the private sector and provide written documentation citing the specific statutory authority and contractual authority, if relevant, establishing their ability to propose to Government solicitations.

Authority and Eligibility

At the present time, DARPA does not consider 15 U.S.C. § 3710a to be sufficient legal authority to show eligibility. While 10 U.S.C.§ 2539b may be the appropriate statutory starting point for some entities, specific supporting regulatory guidance, together with evidence of agency approval, will still be required to fully establish eligibility. DARPA will consider FFRDC and Government entity eligibility submissions on a case-by-case basis; however, the burden to prove eligibility for all team members rests solely with the proposer.

3.1.2. Non-U.S. Organizations

Non-U.S. organizations and/or individuals may participate to the extent that such participants comply with any necessary nondisclosure agreements, security regulations, export control laws, and other governing statutes applicable under the circumstances.

3.2. ORGANIZATIONAL CONFLICTS OF INTEREST

FAR 9.5 Requirements

In accordance with FAR 9.5, proposers are required to identify and disclose all facts relevant to potential OCIs involving the proposer's organization and *any* proposed team member (subawardee, consultant). Under this Section, the proposer is responsible for providing this disclosure with each proposal submitted to the BAA. The disclosure must include the proposer's, and as applicable, proposed team member's OCI mitigation plan. The OCI mitigation plan must include a description of the actions the proposer has taken, or intends to take, to prevent the existence of conflicting roles that might bias the proposer's judgment and to prevent the proposer from having unfair competitive advantage. The OCI mitigation plan will specifically discuss the disclosed OCI in the context of each of the OCI limitations outlined in FAR 9.505-1 through FAR 9.505-4.

Agency Supplemental OCI Policy

In addition, DARPA has a supplemental OCI policy that prohibits contractors/performers from concurrently providing Scientific Engineering Technical Assistance (SETA), Advisory and Assistance Services (A&AS) or similar support services and being a technical performer. Therefore, as part of the FAR 9.5 disclosure requirement above, a proposer must affirm whether the proposer or *any* proposed team member (subawardee, consultant) is providing SETA, A&AS, or similar support to any DARPA office(s) under: (a) a current award or subaward; or (b) a past award or subaward that ended within one calendar year prior to the proposal's submission date.

If SETA, A&AS, or similar support is being or was provided to any DARPA office(s), the proposal must include:

- The name of the DARPA office receiving the support;
- The prime contract number;
- Identification of proposed team member (subawardee, consultant) providing the support; and
- An OCI mitigation plan in accordance with FAR 9.5.

Government Procedures

In accordance with FAR 9.503, 9.504 and 9.506, the Government will evaluate OCI mitigation plans to avoid, neutralize or mitigate potential OCI issues before award and to determine whether it is in the Government's interest to grant a waiver. The Government will only evaluate OCI mitigation plans for proposals that are determined selectable under the BAA evaluation criteria and funding availability.

The Government may require proposers to provide additional information to assist the Government in evaluating the proposer's OCI mitigation plan.

If the Government determines that a proposer failed to fully disclose an OCI; or failed to provide the affirmation of DARPA support as described above; or failed to reasonably provide additional information requested by the Government to assist in evaluating the proposer's OCI mitigation plan, the Government may reject the proposal and withdraw it from consideration for award.

3.3. COST SHARING/MATCHING

Cost sharing is not required; however, it will be carefully considered where there is an applicable statutory condition relating to the selected funding instrument. Cost sharing is encouraged where there is a reasonable probability of a potential commercial application related to the proposed research and development effort.

For more information on potential cost sharing requirements for Other Transactions for Prototype, see http://www.darpa.mil/work-with-us/contract-management#OtherTransactions

4. Application and Submission Information

4.1. ADDRESS TO REQUEST APPLICATION PACKAGE

This announcement, any attachments, and any references to external websites herein constitute the total solicitation. If proposers cannot access the referenced material posted in the announcement found at http://www.darpa.mil, contact the administrative contact listed herein.

4.2. CONTENT AND FORM OF APPLICATION SUBMISSION

All submissions, including abstracts and proposals must be written in English with type not smaller than 12 point font. Smaller font may be used for figures, tables, and charts. Copies of all documents submitted must be clearly labeled with the DARPA BAA number, proposer organization, and proposal title/proposal short title.

4.2.1. Proposal Abstract Format

Proposers are strongly encouraged to submit an abstract in advance of a proposal to minimize effort and reduce the potential expense of preparing an out of scope proposal. The abstract is a concise version of the proposal comprising a maximum of **6** pages including all figures, tables, and charts. The (optional) submission letter is not included in the page count. All pages shall be formatted for printing on 8-1/2 by 11 inch paper with font size not smaller than 12 point. Smaller font sizes (not smaller than 9 point) may be used for figures, tables, and charts.

Submissions must be written in English.

Abstracts must include the following components:

> A. Cover Sheet (does not count towards page limit): Include the administrative and technical points of contact (name, address, phone, fax, email, lead organization). Also include the BAA number, title of the proposed project, primary subcontractors, estimated cost, duration of the project, and the label "ABSTRACT."
>
> B. Goals and Impact (1 page): Clearly describe what is being proposed and what difference it will make (qualitatively and quantitatively), including brief answers to the following questions:
>
> 1. What is the proposed work attempting to accomplish or do?
> 2. How is it done today? And what are the limitations?
> 3. What is innovative in your approach and how does it compare to SOA?

4. What are the key technical challenges in your approach and how do you plan to overcome these?
5. Who will care and what will the impact be if you are successful?
6. How much will it cost and how long will it take?

C. Technical Plan (3-4 pages): Outline and address all technical challenges inherent in the approach and possible solutions for overcoming potential problems. The technical plan should address both TA1 and TA2. For TA1, the abstract should include a description of the epigenetic analysis methods used to generate ESP, the proposed exposure targets (i.e. chemical agents, biological agents, radiological, nuclear, and explosives); the proposed human sample type (buccal, nasal, or blood); and the proposed cell types (e.g., T-cells, B-cells, keratinocytes, etc.) your team plans to use. Any novel techniques or approaches that are unique to your organization should be highlighted. For TA2, the abstract should include your level of access and plan for incorporating the molecular and epigenetic analysis technologies in Phase I and a brief description of the components your final system will house. Where possible, please provide data and examples to support your plan. This section should provide appropriate specific milestones (quantitative, if possible) at intermediate stages of the project to demonstrate progress, and a brief plan for accomplishment of the milestones.

D. Capabilities (.5- 1 page): Provide a brief summary of expertise of the team, including subcontractors and key personnel. A principal investigator for the project must be identified, and a description of the team's organization. Include a description of the team's organization including roles and responsibilities. Describe the organizational experience in this area, existing intellectual property required to complete the project, any specialized facilities, and access to epigenetics and molecular analysis technologies to be used as part of the project. List Government-furnished materials or data assumed to be available. If desired, include a brief bibliography with links to relevant papers, reports, or resumes of key performers. Do not include more than two resumes as part of the abstract. Resumes count against the abstract page limit.

E. Budget (.5 page): Please provide a rough order of magnitude of the costs of accomplishing the goals of the ECHO program. Costs should be broken out by technical area and phase. Any anticipated Government furnished equipment should be identified.

4.2.2. Proposal Format

All full proposals must be in the format given below. Proposals shall consist of two volumes: 1) **Volume I, Technical and Management Proposal**, and 2) **Volume II, Cost Proposal.** All pages shall be printed on 8-1/2 by 11 inch paper with type not smaller than 12 point. Smaller font (not smaller than 9 point) may be used for figures, tables and charts. The page limitation for full proposals includes all figures, tables, and charts. Volume I, Technical and Management Proposal, may include an attached bibliography of relevant technical papers or research notes (published and unpublished), which document the technical ideas and approach upon which the proposal is based. Copies of not more than three (3) relevant papers may be included with the

submission. The bibliography and attached papers are not included in the page counts given below. The submission of other supporting materials along with the proposals is strongly discouraged and will not be considered for review. **The maximum page count for Volume 1 is 25 pages.** A submission letter is optional and is not included in the page count. Volume I should include the following components:

NOTE: Non-conforming submissions that do not follow the instructions herein may be rejected without further review.

a. Volume I, Technical and Management Proposal

Section I. Administrative

A. Cover Sheet (LABELED "PROPOSAL: VOLUME I"):

1. BAA number (HR001118S0023);
2. Technical area;
3. Lead organization submitting proposal (prime contractor);
4. Type of organization, selected from among the following categories: "LARGE BUSINESS," "SMALL DISADVANTAGED BUSINESS," "OTHER SMALL BUSINESS," "HBCU," "MI," "OTHER EDUCATIONAL," OR "OTHER NONPROFIT";
5. Proposer's reference number (if any);
6. Other team members (if applicable) and type of business for each;
7. Proposal title;
8. Technical point of contact (Program Manager or Principle Investigator) to include: salutation, last name, first name, street address, city, state, zip code, telephone, fax, e-mail;
9. Administrative point of contact (Contracting Officer or Grant Officer) to include: salutation, last name, first name, street address, city, state, zip code, telephone, fax, e-mail;
10. Award instrument requested: cost-plus-fixed-free (CPFF), cost-contract—no fee, firm-fixed-price, grant, cooperative agreement, other transaction, or other type (specify);
11. Place(s) and period(s) of performance ;
12. Proposal validity period;
13. Total funds requested from DARPA, and the amount of cost share (if any); AND
14. Date proposal was submitted.

Information on award instruments is available at http://www.darpa.mil/work-with-us/contract-management.

B. Official Transmittal Letter.

Section II. Detailed Proposal Information

A. Executive Summary (1-2 pages): Provide a synopsis of the proposed project, including answers to the following questions:

- What is the proposed work attempting to accomplish or do?
- How is it done today, and what are the limitations?
- What is innovative in your approach?
- What are the key technical challenges in your approach and how do you plan to overcome these?
- Who or what will be affected and what will be the impact if the work is successful?
- How much will it cost, and how long will it take?

B. Goals and Impact (1-2 pages): Clearly describe what the team is trying to achieve and the difference it will make (qualitatively and quantitatively) if successful. Describe the innovative aspects of the project in the context of existing capabilities and approaches, clearly delineating the uniqueness and benefits of this project in the context of the state of the art, alternative approaches, and other projects from the past and present. Describe how the proposed project is revolutionary and how it significantly rises above the current state of the art. Describe the deliverables associated with the proposed project and any plans to commercialize the technology, transition it to a customer, or further the work.

C. Technical Plan (7-10 pages): Outline and address technical challenges inherent in the approach and possible solutions for overcoming potential problems. This section should provide appropriate measurable milestones (qualitative and quantitative) and program metrics (see Section 1.3) at intermediate stages of the program to demonstrate progress, and a plan for achieving the milestones. The technical plan should demonstrate a deep understanding of the technical challenges and present a credible (even if risky) plan to achieve the program goal. Discuss mitigation of technical risk. The technical plan should address the TA1 and TA2 proposal content requirements detailed in Sections 1.2 and 1.3.

A complete technical plan must address the following:

- Proposed (7) candidate signatures for Phase I and (14) candidate signatures for Phase II.

- Proposed human sample types (buccal, nasal, or blood); and the proposed cell types (e.g., T-cells, B-cells, keratinocytes, etc.) your team plans to use. Any animal models and tissue chip technologies that are to be supplemented should be identified as well as the cohort or vender providing the samples.
- All molecular epigenetic analysis methods to be utilized in both TA1 and TA2 must be identified.
- Anticipated components or modules in the platform system and any anticipated off-the-shelf technologies
- Any data from other funded or internal efforts that support your approach.
- Any examples of past experience demonstrating capability of developing platform technologies

D. Management Plan (1-2 pages): Provide a summary of expertise of the team, including any subcontractors, and key personnel who will be doing the work. Resumes count against the proposal page count. Identify a principal investigator for the project. Provide a clear description of the team's organization including an organization chart that includes, as applicable: the programmatic relationship of team members; the unique capabilities of team members; the task responsibilities of team members, the teaming strategy among the team members; and key personnel with the amount of effort to be expended by each person during each year. Provide a detailed plan for coordination including explicit guidelines for interaction among collaborators/subcontractors of the proposed effort. Include risk management approaches. Describe any formal teaming agreements that are required to execute this program.

E. Capabilities (2-3 pages): Describe organizational experience in relevant subject area(s), existing intellectual property, specialized facilities, and any Government-furnished materials or information. Discuss any work in closely related research areas and previous accomplishments. Describe experience with the proposed exposure-specific agents or compounds.

F. Statement of Work (SOW) (2-3 pages): The SOW should provide a detailed task breakdown, citing specific tasks and their connection to the interim milestones and program metrics. Each phase of the program (Phase I base and Phase II option) should be separately defined in the SOW and each task should be identified by TA (1 or 2). The SOW must not include proprietary information.

For each task/subtask, provide:

- A detailed description of the approach to be taken to accomplish each defined task/subtask.

- Identification of the primary organization responsible for task execution (prime contractor, subcontractor(s), consultant(s), by name).
- A measurable milestone, i.e., a deliverable, demonstration, or other event/activity that marks task completion. Include quantitative metrics.
- A definition of all deliverables (e.g., data, reports, software) to be provided to the Government in support of the proposed tasks/subtasks.

G. Schedule and Milestones (1-2 pages): Provide a detailed schedule showing tasks (task name, duration, work breakdown structure element as applicable, performing organization), milestones, and the interrelationships among tasks. The task structure must be consistent with that in the SOW. Measurable milestones should be clearly articulated and defined in time relative to the start of the project.

H. Transition Plan (.5-1- page): As this program is expecting a prototype system at the end of 48 months; proposals must address plan to complete, beta-test, and market the deployable platform to the military and commercial partners. Proposals should include a rough order of magnitude of the prototype system and disposables cost. If off-the-shelf technologies were proposed, proposals should address IP licensing and associated risks.

I. CUI Risk Mitigation Plan (.5 page) (Required for proposers who anticipate generating work that may be considered CUI in accordance with Section 1.4 "Controlled Unclassified Information"): Provide a detailed plan for how the organization and its subcontractors will meet CUI safeguarding requirements. The plan should provide a detailed strategy to protect CUI without unnecessarily compartmentalizing information flow within or among performer teams. This plan must describe safeguard procedures for generating any sensitive program deliverables.

Section III. Additional Information (Note: Does not count towards page limit)

A brief bibliography of relevant technical papers and research notes (published and unpublished) which document the technical ideas upon which the proposal is based. Copies of not more than three (3) relevant papers can be included in the submission.

a. Volume II, Cost Management Proposal

Cover Sheet (LABELED "PROPOSAL: VOLUME II"):

1. BAA number;
2. Technical area;
3. Lead Organization Submitting proposal;

4. Type of organization, selected among the following categories: “LARGE BUSINESS”, “SMALL DISADVANTAGED BUSINESS”, “OTHER SMALL BUSINESS”, “HBCU”, “MI”, “OTHER EDUCATIONAL”, OR “OTHER NONPROFIT”;
5. Proposer’s reference number (if any);
6. Other team members (if applicable), CAGE Code(s), and type of business for each;
7. Proposal title;
8. Technical point of contact (Program Manager or Principal Investigator) to include: salutation, last name, first name, street address, city, state, zip code, telephone, fax (if available), electronic mail (if available);
9. Administrative point of contact (Contracting Officer or Grant Officer) to include: salutation, last name, first name, street address, city, state, zip code, telephone, fax (if available), and electronic mail (if available);
10. Award instrument requested: cost-plus-fixed-free (CPFF), cost-contract—no fee, cost sharing contract – no fee, or other type of procurement contract (*specify*), cooperative agreement, or other transaction;
11. Place(s) and period(s) of performance;
12. Total proposed cost separated by basic award and option(s) (if any);
13. Name, address, and telephone number of the proposer’s cognizant Defense Contract Management Agency (DCMA) administration office (*if known)*;
14. Name, address, and telephone number of the proposer’s cognizant Defense Contract Audit Agency (DCAA) audit office (*if known*);
15. Date proposal was prepared;
16. DUNS number (http://www.dnb.com/get-a-duns-number.html);
17. Taxpayer ID number (https://www.irs.gov/Individuals/International-Taxpayers/Taxpayer-Identification-Numbers-TIN);
18. CAGE code (https://cage.dla.mil/Home/UsageAgree);
19. Proposal validity period

Note that nonconforming proposals may be rejected without review.

Proposers that do not have a Cost Accounting Standards (CAS) complaint accounting system considered adequate for determining accurate costs that are negotiating a cost- type procurement contract must complete an SF 1408. For more information on CAS compliance, see http://www.dcaa.mil/cas.html. To facilitate this process, proposers should complete the SF 1408 found at http://www.gsa.gov/portal/forms/download/115778 and submit the completed form with the proposal. To complete the form, check the boxes on the second page, then provide a narrative explanation of your accounting system to supplement the checklist on page one. For more information, see (http://www.dcaa.mil/Home/Preaward).

The Government strongly encourages that tables included in the cost proposal also be provided in an editable (e.g., MS Excel) format with calculation formulas intact to allow traceability of the cost proposal numbers across the prime and subcontractors.

The Government strongly encourages that the proposer provide a detailed cost breakdown to include:

(1) Total program costs broken down by Phase I (Base) and Phase II (Option) in Contractor Fiscal Year to include:

i. Direct Labor – Including individual labor categories with associated labor hours and direct labor rates. If selected for award, be prepared to submit supporting documentation to justify labor rates. (i.e., screenshots of HR databases, comparison to NIH or other web-based salary database);

ii. Consultants – If consultants are to be used, proposer must provide a copy of the consultant's proposed SOW as well as a signed consultant agreement or other document which verifies the proposed loaded daily / hourly rate, hours and any other proposed consultant costs (e.g., travel);

iii. Indirect Costs – Including Fringe Benefits, Overhead, General and Administrative Expense, Cost of Money, Fee, etc. (must show base amount and rate), if available, provide current Forward Pricing Rate Agreement or Forward Pricing Rate Proposal. If not available, provide 2 years historical data to include pool and expense costs used to generate the rates. For academia, provide DHHS or ONR negotiated rate package or, if calculated by other than a rate, provide University documentation identifying G&A and fringe costs by position;

iv. Travel – Provide the purpose of the trip, number of trips, number of days per trip, departure and arrival destinations, number of people, estimated rental car and airfare costs, and prevailing per diem rates as determined by gsa.gov, etc.; Quotes must be supported by screenshots from travel websites;

v. Other Direct Costs – Itemized with costs including tuition remission, animal per diem rates, health insurance/fee; back-up documentation is to be submitted to support proposed costs;

vi. Equipment Purchases – Itemization with individual and total costs, including quantities, unit prices, proposed vendors (if known), and the basis of estimate (e.g., quotes, prior purchases, catalog price lists, etc.); any item that exceeds $5,000 must be supported with back-up documentation such as a copy of catalog price lists or quotes prior to purchase (NOTE: For equipment purchases, include a letter stating why the proposer cannot provide the requested resources from its own funding), and;

vii. Materials – Itemization with costs, including quantities, unit prices, proposed vendors (if known), and the basis of estimate (e.g., quotes, prior purchases, catalog price lists, etc.); any item that exceeds $5,000 must be supported with back-up documentation such as a copy of catalog price lists or quotes prior to purchase.

(2) A summary of total program costs by major TA1 and TA2 tasks;

(3) A summary of projected funding requirements by month;

(4) An itemization of any information technology (IT) purchase (including a letter stating why the proposer cannot provide the requested resources from its own funding), as defined in FAR Part 2.101;

(5) An itemization of Subcontracts. **All subcontractor cost proposal documentation must be prepared at the same level of detail as that required of the prime.** Subcontractor proposals should include Interdivisional Work Transfer Agreements (IWTA) or evidence

of similar arrangements (an IWTA is an agreement between multiple divisions of the same organization);
(6) The source, nature, and amount of any industry cost-sharing. Where the effort consists of multiple portions which could reasonably be partitioned for purposes of funding, these should be identified as options with separate cost estimates for each;
(7) Identification of pricing assumptions of which may require incorporation into the resulting award instrument (e.g., use of Government Furnished Property/Facilities/Information, access to Government Subject Matter Expert/s, etc.);
(8) Any Forward Pricing Rate Agreement, DHHS rate agreement, other such approved rate information, or such documentation that may assist in expediting negotiations (if available); and
(9) Proposers with a Government acceptable accounting system who are proposing a cost-type contract, must submit the DCAA document approving the cost accounting system.

4.2.3. Additional Proposal Information

Proprietary Markings

Proposers are responsible for clearly identifying proprietary information. Submissions containing proprietary information must have the cover page and each page containing such information clearly marked with a label such as "Proprietary" or "Company Proprietary." NOTE: "Confidential" is a classification marking used to control the dissemination of U.S. Government National Security Information as dictated in Executive Order 13526 and should not be used to identify proprietary business information.

Unclassified Submissions

DARPA anticipates that submissions received under this BAA will be unclassified. However, should a proposer wish to submit classified information, an *unclassified* email must be sent to the BAA mailbox requesting submission instructions from the Technical Office PSO. If a determination is made that the award instrument may result in access to classified information, a SCG and/or DD Form 254 will be issued by DARPA and attached as part of the award.

Human Research Subjects/Animal Use

Proposers that anticipate involving Human Research Subjects or Animal Use must comply with the approval procedures detailed at http://www.darpa.mil/work-with-us/additional-baa.

Approved Cost Accounting System Documentation

Proposers that do not have a Cost Accounting Standards (CAS) complaint accounting system considered adequate for determining accurate costs that are negotiating a cost- type procurement contract must complete an SF 1408. For more information on CAS compliance, see http://www.dcaa.mil/cas.html. To facilitate this process, proposers should complete the SF 1408 found at http://www.gsa.gov/portal/forms/download/115778 and submit the completed form with the proposal. To complete the form, check the boxes on the second page, then provide a narrative explanation of your accounting system to supplement the checklist on page one. For more information, see (http://www.dcaa.mil/preaward_accounting_system_adequacy_checklist.html).

Small Business Subcontracting Plan

Pursuant to Section 8(d) of the Small Business Act (15 U.S.C. § 637(d)) and FAR 19.702(a)(1), each proposer who submits a contract proposal and includes subcontractors might be required to submit a subcontracting plan with their proposal. The plan format is outlined in FAR 19.704.

Section 508 of the Rehabilitation Act (29 U.S.C. § 749d)/FAR 39.2

All electronic and information technology acquired or created through this BAA must satisfy the accessibility requirements of Section 508 of the Rehabilitation Act (29 U.S.C. § 749d)/FAR 39.2.

Intellectual Property

All proposers must provide a good faith representation that the proposer either owns or possesses the appropriate licensing rights to all intellectual property that will be utilized under the proposed effort.

For Procurement Contracts

Proposers responding to this BAA requesting procurement contracts will need to complete the certifications at DFARS 252.227-7017. See http://www.darpa.mil/work-with-us/additional-baa for further information. If no restrictions are intended, the proposer should state "none." The table below captures the requested information:

Technical Data Computer Software To be Furnished With Restrictions	Summary of Intended Use in the Conduct of the Research	Basis for Assertion	Asserted Rights Category	Name of Person Asserting Restrictions
(LIST)	(NARRATIVE)	(LIST)	(LIST)	(LIST)

For All Non-Procurement Contracts

Proposers responding to this BAA requesting a Grant, Cooperative Agreement, Technology Investment Agreement, or Other Transaction for Prototypes shall follow the applicable rules and regulations governing these various award instruments, but, in all cases, should appropriately identify any potential restrictions on the Government's use of any Intellectual Property contemplated under the award instrument in question. This includes both Noncommercial Items and Commercial Items. Proposers are encouraged to use a format similar to that described in the section above. If no restrictions are intended, then the proposer should state "NONE."

System for Award Management (SAM) and Universal Identifier Requirements

All proposers must be registered in SAM unless exempt per FAR 4.1102. FAR 52.204-7, "System for Award Management" and FAR 52.204-13, "System for Award Management Maintenance" are incorporated into this BAA. See http://www.darpa.mil/work-with-us/additional-baa for further information.

4.2.4. Submission Information

DARPA will acknowledge receipt of all submissions and assign an identifying control number that should be used in all further correspondence regarding the submission. DARPA intends to use electronic mail correspondence regarding HR001118S0023. Submissions may not be submitted by fax or e-mail; any so sent will be disregarded.

Submissions will not be returned. An electronic copy of each submission received will be retained at DARPA and all other non-required copies destroyed. A certification of destruction may be requested, provided the formal request is received by DARPA within 5 days after notification that a proposal was not selected.

For abstract and proposal submission dates, see Part I., Overview Information. Submissions received after these dates and times may not be reviewed.

For Proposers Submitting Proposal Abstracts or Full Proposals as Hard Copies/On CD-ROM:

Proposers must submit an original hardcopy and one (1) electronic copy of the abstract or proposal in PDF (preferred) on a CD-ROM to the mailing address listed in Part I. Each copy must be clearly labeled with HR001118S0023, proposer organization, technical point of contact, and proposal title (short title recommended).

Please note that submitters via hardcopy/CD-ROM will still need to visit https://baa.darpa.mil to register their organization concurrently to ensure the BAA office can verify and finalize their submission.

For Proposers Submitting Proposal Abstracts or Full Proposals Requesting Procurement Contracts or OTs through DARPA's BAA Submission Portal:

Abstracts and Full Proposals sent in response to HR001118S0023 may be submitted via DARPA's BAA Website (https://baa.darpa.mil). Visit the website to complete the two-step

registration process. Submitters will need to register for an Extranet account (via the form at the URL listed above) and wait for two separate e-mails containing a username and temporary password. After accessing the Extranet, submitters may then create an account for the DARPA BAA website (via the "Register your Organization" link along the left side of the homepage), view submission instructions, and upload/finalize the abstract. Proposers using the DARPA BAA Website may encounter heavy traffic on the submission deadline date; it is highly advised that submission process be started as early as possible.

All unclassified concepts submitted electronically through DARPA's BAA Website must be uploaded as zip files (.zip or .zipx extension). The final zip file should be no greater than 50 MB in size. Only one zip file will be accepted per submission. Classified submissions and proposals requesting assistance instruments (grants or cooperative agreements) should NOT be submitted through DARPA's BAA Website (https://baa.darpa.mil), though proposers will likely still need to visit https://baa.darpa.mil to register their organization (or verify an existing registration) to ensure the BAA office can verify and finalize their submission.

Technical support for BAA Website may be reached at BAAT_Support@darpa.mil, and is typically available during regular business hours, (9:00 AM- 5:00 PM EST Monday – Friday).

Proposers using the DARPA BAA Website may encounter heavy traffic on the submission deadline date; it is highly advised that submission process be started as early as possible.

For Full Proposals Requesting Cooperative Agreements:

Proposers requesting cooperative agreements may submit proposals through one of the following methods: (1) hard copy mailed directly to DARPA; or (2) electronic upload per the instructions at http://www.grants.gov/applicants/apply-for-grants.html. Cooperative agreement proposals may not be submitted through any other means. If proposers intend to use Grants.gov as their means of submission, then they must submit their entire proposal through Grants.gov; applications cannot be submitted in part to Grants.gov and in part as a hard-copy. Proposers using the Grants.gov do not submit paper proposals in addition to the Grants.gov electronic submission.

Grants.gov Submissions: Grants.gov requires proposers to complete a one-time registration process before a proposal can be electronically submitted. First time registration can take between three business days and four weeks. For more information about registering for Grants.gov, see http://www.darpa.mil/work-with-us/additional-baa.

Hard-copy Submissions: Proposers electing to submit grant or cooperative agreement proposals as hard copies must complete the SF 424 R&R form (Application for Federal Assistance,) available on the Grants.gov website
http://apply07.grants.gov/apply/forms/sample/RR_SF424_2_0-V2.0.pdf

Failure to comply with the submission procedures may result in the submission not being evaluated. DARPA will acknowledge receipt of complete submissions via email and assign control numbers that should be used in all further correspondence regarding proposals.

4.2.5. Disclosure of Information and Compliance with Safeguarding Covered Defense Information Controls

The following provisions and clause apply to all solicitations and contracts; however, the definition of "controlled technical information" clearly exempts work considered fundamental research and therefore, even though included in the contract, will not apply if the work is fundamental research.

DFARS 252.204-7000, "Disclosure of Information"
DFARS 252.204-7008, "Compliance with Safeguarding Covered Defense Information Controls"
DFARS 252.204-7012, "Safeguarding Covered Defense Information and Cyber Incident Reporting"

The full text of the above solicitation provision and contract clauses can be found at http://www.darpa.mil/work-with-us/additional-baa#NPRPAC.

Compliance with the above requirements includes the mandate for proposers to implement the security requirements specified by National Institute of Standards and Technology (NIST) Special Publication (SP) 800-171, "Protecting Controlled Unclassified Information in Nonfederal Information Systems and Organizations" (see https://doi.org/10.6028/NIST.SP.800-171r1) that are in effect at the time the BAA is issued, or as authorized by the Contracting Officer, not later than December 31, 2017.

For awards where the work is considered fundamental research, the contractor will not have to implement the aforementioned requirements and safeguards; however, should the nature of the work change during performance of the award, work not considered fundamental research will be subject to these requirements.

4.3. FUNDING RESTRICTIONS

Individual piece of equipment greater than $100,000.00. DARPA's selecting performers who have a proven track record with technologies specific to this solicitation. If equipment is limited due to high internal demand, leasing and external vendors are optional solutions.

4.4. OTHER SUBMISSION REQUIREMENTS

Not applicable.

5. Application Review Information

5.1. EVALUATION CRITERIA

Proposals will be evaluated using the following criteria, listed in descending order of importance:

5.1.1 Overall Scientific and Technical Merit; 5.1.2 Potential Contribution and Relevance to the DARPA Mission; 5.1.3 Cost Realism; 5.1.4 Realism of Proposed Schedule; 5.1.5 Proposer's Capabilities and/or Related Experience; and 5.1.6 Plans and Capabilities to Accomplish Technology Transition.

5.1.1. Overall Scientific and Technical Merit

The proposed technical approach is innovative, feasible, achievable, and complete. The proposed technical team has the expertise and experience to accomplish the proposed tasks. Task descriptions and associated technical elements provided are complete and in a logical sequence with all proposed deliverables clearly defined such that a final outcome that achieves the goal can be expected as a result of award. The proposal identifies major technical risks and planned mitigation efforts are clearly defined and feasible. The diversity of the proposed additional 17 exposure compounds and agents supports the program's goal to maximize the forensic and diagnostic capability of the ESP on the final device.

5.1.2. Potential Contribution and Relevance to the DARPA Mission

The potential contributions of the proposed effort are relevant to the national technology base. Specifically, DARPA's mission is to make pivotal early technology investments that create or prevent strategic surprise for U.S. National Security.

5.1.3. Cost Realism

The proposed costs are realistic for the technical and management approach and accurately reflect the technical goals and objectives of the solicitation. The proposed costs are consistent with the proposer's Statement of Work and reflect a sufficient understanding of the costs and level of effort needed to successfully accomplish the proposed technical approach. The costs for the prime proposer and proposed subawardees are substantiated by the details provided in the proposal (e.g., the type and number of labor hours proposed per task, the types and quantities of materials, equipment and fabrication costs, travel and any other applicable costs and the basis for the estimates).

It is expected that the effort will leverage all available relevant prior research in order to obtain the maximum benefit from the available funding. For efforts with a likelihood of commercial application, appropriate direct cost sharing may be a positive factor in the evaluation. DARPA recognizes that undue emphasis on cost may motivate proposers to offer low-risk ideas with minimum uncertainty and to staff the effort with junior personnel in order to be in a more competitive posture. DARPA discourages such cost strategies.

5.1.4. Realism of Proposed Schedule

The proposed schedule aggressively pursues performance metrics in the shortest timeframe and accurately accounts for that timeframe. The proposed schedule identifies and mitigates any potential schedule risk.

5.1.5. Proposer's Capabilities and/or Related Experience

The proposer's prior experience in similar efforts must clearly demonstrate an ability to deliver products that meet the proposed technical performance within the proposed budget and schedule. The proposed team has the expertise to manage the cost and schedule. Similar efforts

completed/ongoing by the proposer in this area are fully described including identification of other Government sponsors. The proposed team has experience with the proposed exposure categories.

5.1.6. Plans and Capability to Accomplish Technology Transition

The proposer clearly demonstrates its capability to transition the technology to the research, industrial, and/or operational military communities in such a way as to enhance U.S. defense. In addition, the evaluation will take into consideration the extent to which the proposed intellectual property (IP) rights will potentially impact the Government's ability to transition the technology.

5.2. REVIEW OF PROPOSALS

Review Process

It is the policy of DARPA to ensure impartial, equitable, comprehensive proposal evaluations based on the evaluation criteria listed in Section V.A. and to select the source (or sources) whose offer meets the Government's technical, policy, and programmatic goals.

DARPA will conduct a scientific/technical review of each conforming proposal. Conforming proposals comply with all requirements detailed in this BAA; proposals that fail to do so may be deemed non-conforming and may be removed from consideration. Proposals will not be evaluated against each other since they are not submitted in accordance with a common work statement. DARPA's intent is to review proposals as soon as possible after they arrive; however, proposals may be reviewed periodically for administrative reasons

Award(s) will be made to proposers whose proposals are determined to be the most advantageous to the Government, consistent with instructions and evaluation criteria specified in the BAA herein, and availability of funding.

Handling of Source Selection Information

DARPA policy is to treat all submissions as source selection information (see FAR 2.101 and 3.104), and to disclose their contents only for the purpose of evaluation. Restrictive notices notwithstanding, during the evaluation process, submissions may be handled by support contractors for administrative purposes and/or to assist with technical evaluation. All DARPA support contractors performing this role are expressly prohibited from performing DARPA-sponsored technical research and are bound by appropriate nondisclosure agreements. Subject to the restrictions set forth in FAR 37.203(d), input on technical aspects of the proposals may be solicited by DARPA from non-Government consultants/experts who are strictly bound by the appropriate non-disclosure requirements.

Federal Awardee Performance and Integrity Information (FAPIIS)

Per 41 U.S.C. 2313, as implemented by FAR 9.103 and 2 CFR § 200.205, prior to making an award above the simplified acquisition threshold, DARPA is required to review and consider any information available through the designated integrity and performance system (currently FAPIIS). Awardees have the opportunity to comment on any information about themselves

entered in the database, and DARPA will consider any comments, along with other information in FAPIIS or other systems prior to making an award.

6. Award Administration Information

6.1. SELECTION NOTICES

As soon as the evaluation of a proposal is complete, the proposers will be notified that 1) the proposal has been selected for funding pending contract negotiations, or 2) the proposal has not been selected. These official notifications will be sent via email to the Technical POC identified on the proposal coversheet.

6.1.1. Proposal Abstracts

DARPA will respond to abstracts with a statement as to whether DARPA is interested in the idea. If DARPA does not recommend the proposer submit a full proposal, DARPA will provide feedback to the proposer regarding the rationale for this decision. Regardless of DARPA's response to an abstract, proposers may submit a full proposal. DARPA will review all full proposals submitted using the published evaluation criteria and without regard to any comments resulting from the review of an abstract.

6.1.2. Full Proposals

As soon as the evaluation of a proposal is complete, the proposer will be notified that (1) the proposal has been selected for funding pending award negotiations, in whole or in part, or (2) the proposal has not been selected. These official notifications will be sent via e-mail to the Technical POC and/or Administrative POC identified on the proposal coversheet.

6.2. ADMINISTRATIVE AND NATIONAL POLICY REQUIREMENTS

6.2.1. Meeting and Travel Requirements

There will be a program kickoff meeting in the Arlington, VA vicinity and all key participants are required to attend. Performers should also anticipate 6 month program-wide PI meetings alternating between the East and West Coast, as well as periodic site visits at the Program Manager's discretion.

Proposers shall include within the content of their proposal details and costs of any travel or meetings they deem to be necessary throughout the course of the effort, to include periodic status reviews by the Government.

6.2.1. FAR and DFARS Clauses

(Full text or text selections from website may be used in addition to or in lieu of text below.) Solicitation clauses in the FAR and DFARS relevant to procurement contracts and FAR and DFARS clauses that may be included in any resultant procurement contracts are incorporated herein and can be found at http://www.darpa.mil/work-with-us/additional-baa.

6.2.2. Controlled Unclassified Information (CUI) on Non-DoD Information Systems

Further information on Controlled Unclassified Information on Non-DoD Information Systems is incorporated herein can be found at http://www.darpa.mil/work-with-us/additional-baa.

6.2.3. Representations and Certifications

If a procurement contract is contemplated, prospective awardees will need to be registered in the SAM database prior to award and complete electronic annual representations and certifications consistent with FAR guidance at 4.1102 and 4.1201; the representations and certifications can be found at www.sam.gov. Supplementary representations and certifications can be found at http://www.darpa.mil/work-with-us/additional-baa.

.

6.2.4. Terms and Conditions

A link to the DoD General Research Terms and Conditions for Grants and Cooperative Agreements and supplemental agency terms and conditions can be found at http://www.darpa.mil/work-with-us/contract-management#GrantsCooperativeAgreements.

6.3. REPORTING

The number and types of reports will be specified in the award document, but will include as a minimum monthly financial status reports and quarterly technical status reports. The reports shall be prepared and submitted in accordance with the procedures contained in the award document and mutually agreed on before award. Reports and briefing material will also be required as appropriate to document progress in accomplishing program metrics. A Final Report that summarizes the project and tasks will be required at the conclusion of the performance period for the award, notwithstanding the fact that the research may be continued under a follow-on vehicle.

6.4. ELECTRONIC SYSTEMS

6.4.1. Wide Area Work Flow (WAWF)

Performers will be required to submit invoices for payment directly to https://wawf.eb.mil, unless an exception applies. Performers must register in WAWF prior to any award under this BAA.

6.4.2. i-EDISON

The award document for each proposal selected for funding will contain a mandatory requirement for patent reports and notifications to be submitted electronically through i-Edison (http://public.era.nih.gov/iedison).

7. Agency Contacts

Administrative, technical or contractual questions should be sent via e-mail (preferred) to ECHO@darpa.mil.

Points of Contact
The BAA Coordinator for this effort may be reached at:
ECHO@darpa.mil
DARPA/BTO
ATTN: HR001118S0023
675 North Randolph Street

Arlington, VA 22203-2114

For information concerning agency level protests see http://www.darpa.mil/work-with-us/additional-baa#NPRPAC.

8. Other Information

DARPA will host a Proposers Day in support of the ECHO program on **February 23, 2018**, at the Executive Conference Center in Arlington, VA. The purpose is to provide potential proposers with information on the ECHO program, promote additional discussion on this topic, address questions, provide a forum to present their capabilities, and to encourage team formation.

Interested proposers are not required to attend to respond to the ECHO BAA, and relevant information and materials discussed at Proposers Day will be made available to all potential proposers in the form of a FAQ posted on the DARPA Opportunities Page.

DARPA will not provide cost reimbursement for interested proposers in attendance.

An online registration form and various other meeting details can be found at the registration website, https://events.sa-meetings.com/ECHOProposersDay.

To encourage team formation, interested proposers are encouraged to submit information to be shared with all potential proposers through the Proposers Day website and the DARPA Opportunities Page. This information may include contact information, a brief description of their technical capabilities, and the desired expertise from other teams, as applicable.

Participants are required to register no later than **February 16, 2018**, for the event. This event is not open to the Press. The Proposers Day will be open to members of the public who have registered in advance for the event; **there will be no onsite registration**.

All foreign nationals, including permanent residents, must complete and submit a DARPA Form 60 "Foreign National Visit Request," which will be provided in the registration confirmation email.

Proposers Day Point of Contact: DARPA-SN-18-23@darpa.mil.

9. APPENDIX 1 – Volume II checklist

Volume II, Cost Proposal
Checklist and Sample Templates

The following checklist and sample templates are provided to assist the proposer in developing a complete and responsive cost volume. Full instructions appear in Section 4.2.2 beginning on Page 23 of HR001118S0023. This worksheet must be included with the coversheet of the Cost Proposal.

1. Are all items from Section 4.2.2 (Volume II, Cost Proposal) of **HR001118S0023** included on your Cost Proposal cover sheet?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]
   If reply is "No", please explain:

2. Does your Cost Proposal include (1) a summary cost buildup by Phase, (2) a summary cost buildup by Year, and (3) a detailed cost buildup of for each Phase that breaks out each task and shows the cost per month?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

3. Does your cost proposal (detailed cost buildup #3 above in item 2) show a breakdown of the major cost items listed below:
   Direct Labor (Labor Categories, Hours, Rates)
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Indirect Costs/Rates (i.e., overhead charges, fringe benefits, G&A)
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Materials and/or Equipment
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Subcontracts/Consultants
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Other Direct Costs
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   Travel
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

4. Have you provided documentation for proposed costs related to travel, to include purpose of trips, departure and arrival destinations and sample airfare?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

If reply is "No", please explain:

5. Does your cost proposal include a complete itemized list of all material and equipment items to be purchased (a priced bill-of-materials (BOM))?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

6. Does your cost proposal include vendor quotes or written engineering estimates (basis of estimate) for all material and equipment with a unit price exceeding $5000?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

7. Does your cost proposal include a clear justification for the cost of labor (written labor basis-of-estimate (BOE)) providing rationale for the labor categories and hours proposed for each task?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

8. Do you have subcontractors/consultants? If YES, continue to question 9. If NO, skip to question 13.
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

9. Does your cost proposal include copies of all subcontractor/consultant technical (to include Statement of Work) and cost proposals?
   ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

   If reply is "No", please explain:

10. Do all subcontract proposals include the required summary buildup, detailed cost buildup, and supporting documentation (SOW, Bill-of-Materials, Basis-of-Estimate, Vendor Quotes, etc.)?
    ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

    If reply is "No", please explain:

11. Does your cost proposal include copies of consultant agreements, if available?
    ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

    If reply is "No", please explain:

12. If requesting a FAR-based contract, does your cost proposal include a tech/cost analysis for all proposed subcontractors?
    ○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

    If reply is "No", please explain:

13. Have all team members (prime and subcontractors) who are considered a Federally Funded Research & Development Center (FFRDC), included documentation that clearly demonstrates work is not otherwise available from the private sector AND provided a letter on letterhead from the sponsoring organization citing the specific authority establishing their eligibility to propose to government solicitations and compete with industry, and compliance with the associated FFRDC sponsor agreement and terms and conditions.

○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

If reply is "No", please explain:

14. Does your proposal include a response regarding Organizational Conflicts of Interest?

○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

If reply is "No", please explain:

15. Does your proposal include a completed Data Rights Assertions table/certification?

○ **YES** ○ **NO** **Appears on Page(s)** [Type text]

If reply is "No", please explain:

Risk Mitigation Plan- 'Gain of Function' Research

DARPA requires that proposals include a **Risk Mitigation Plan (Section J of the proposal)** that will be incorporated into any resulting agreements or contracts and includes the following information:

1) An assessment of potential risks to public health, agriculture, plants, animals, the environment, and national security.
2) Proposed guidelines that the proposer will follow to ensure maximal biosafety and biosecurity during the course of the research.
3) A communication plan that addresses content, timing, and the extent of distribution of potentially sensitive dual-use information. The plan must also address how input from DARPA, other government, and community stakeholders will be taken into account in decisions regarding communication and publication of potentially sensitive dual-use information.

1.4. PROTECTION OF SENSITIVE INFORMATION

PREEMPT is a 6.1 fundamental research program aimed at enhanced biosurveillance and novel approaches to preempt viral pathogens in animal reservoirs from jumping into human populations. DARPA follows current DoD policy for contracted fundamental research. DARPA recognizes, however, that PREEMPT program components aimed at understanding and quantifying mechanisms for viral zoonotic spillover could potentially generate sensitive information that could be misused. Since this is a fundamental research program, the risk of misuse currently cannot be reasonably evaluated. However, proposers are notified that during proposal evaluation and/or program performance, when such a risk reasonably can be evaluated, DARPA may determine that risk of misuse creates exceptional circumstances, compelling reasons, and/or national security reasons under current DoD policy for contracted fundamental research. DARPA therefore expects that proposers to this program understand and will comply with various government guidance regarding potential gain-of-function research of concern (GOFROC)[8] and dual use research of concern (DURC)[9,10,11,12,13].
See https://www.phe.gov/s3/dualuse/Pages/default.aspx for further information.

[8] Gain-of-Function Research (GOFROC) refers to studies with the potential to generate pathogens with pandemic potential exhibiting high transmissibility and high virulence.

2.2. FUNDAMENTAL RESEARCH

It is DoD policy that the publication of products of fundamental research will remain unrestricted to the maximum extent possible. National Security Decision Directive (NSDD) 189 defines fundamental research as follows:

> 'Fundamental research' means basic and applied research in science and engineering, the results of which ordinarily are published and shared broadly within the scientific community, as distinguished from proprietary research and from industrial development, design, production, and product utilization, the results of which ordinarily are restricted for proprietary or national security reasons.

As of the date of publication of this BAA, the Government expects that program goals as described herein may be met by proposers intending to perform fundamental research and proposers not intending to perform fundamental research or the proposed research may present a high likelihood of disclosing performance characteristics of military systems or manufacturing technologies that are unique and critical to defense. Based on the nature of the performer and the nature of the work, the Government anticipates that some awards will include restrictions on the resultant research that will require the awardee to seek DARPA permission before publishing any information or results relative to the program.

Proposers should indicate in their proposal whether they believe the scope of the research included in their proposal is fundamental or not. While proposers should clearly explain the intended results of their research, the Government shall have sole discretion to select award instrument type and to negotiate all instrument terms and conditions with selectees. Appropriate clauses will be included in resultant awards for non-fundamental research to prescribe publication requirements and other restrictions, as appropriate. This clause can be found at http://www.darpa.mil/work-with-us/additional-baa.

For certain research projects, it may be possible that although the research being performed by the awardee is restricted research, a subawardee may be conducting fundamental research. In those cases, it is the awardee's responsibility to explain in their proposal why its subawardee's effort is fundamental research

WORKSPACE FORM

1-800-518-4726
SUPPORT@GRANTS.GOV

This Workspace form is one of the forms you need to complete prior to submitting your Application Package. This form can be completed in its entirety offline using Adobe Reader. You can save your form by clicking the "Save" button and see any errors by clicking the “Check For Errors” button. In-progress and completed forms can be uploaded at any time to Grants.gov using the Workspace feature.

When you open a form, required fields are highlighted in yellow with a red border. Optional fields and completed fields are displayed in white. If you enter invalid or incomplete information in a field, you will receive an error message. Additional instructions and FAQs about the Application Package can be found in the Grants.gov Applicants tab.

OPPORTUNITY & PACKAGE DETAILS:

Opportunity Number:	HR001118S0017
Opportunity Title:	PREventing EMerging Pathogenic Threats
Opportunity Package ID:	PKG00237724
CFDA Number:	12.910
CFDA Description:	Research and Technology Development
Competition ID:	
Competition Title:	
Opening Date:	01/19/2018
Closing Date:	03/27/2018
Agency:	DARPA - Biological Technologies Office
Contact Information:	BAA Coordinator PREEMPT@darpa.mil

APPLICANT & WORKSPACE DETAILS:

Workspace ID:	WS00094394
Application Filing Name:	Project DEFUSE
DUNS:	0770900660000
Organization:	ECOHEALTH ALLIANCE INC.
Form Name:	R & R Subaward Budget 10 YR Subform
Form Version:	1.4
Subform Name:	UNC Medical School
Requirement:	Optional
Download Date/Time:	Mar 06, 2018 05:29:59 PM EST
Form State:	Error(s)

FORM ACTIONS:

RESEARCH & RELATED BUDGET - Budget Period 1

OMB Number: 4040-0001
Expiration Date: 10/31/2019

ORGANIZATIONAL DUNS: 6081952770000 **Enter name of Organization:** University of North Carolina School of Public Health

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 1** **Start Date:** 12/01/2018 **End Date:** 11/30/2019

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
Prof.	Ralph		Baric			1.20			28,123.00	7,137.00	35,260.00
Project Role:	Co-Investigator										
Dr.	Kristy		Ainslie			0.60			7,551.00	2,052.00	9,603.00
Project Role:	Investigator										
Dr.	Amy		Sims			3.00			30,590.00	8,593.00	39,183.00
Project Role:	Investigator										
Dr.	Timothy		Sheahan			3.00			22,385.00	6,681.00	29,066.00
Project Role:	Investigator										
Dr.	Eric		Bachelder			1.20			6,900.00	2,194.00	9,094.00
Project Role:	Investigator										

Additional Senior Key Persons: Add Attachment | Delete Attachment | View Attachment

Total Funds requested for all Senior Key Persons in the attached file

Total Senior/Key Person 122,206.00

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
2	Post Doctoral Associates	13.20			54,172.00	9,619.00	63,791.00
1	Graduate Students	4.80			12,600.00	2,690.00	15,290.00
	Undergraduate Students						
	Secretarial/Clerical						
2	Research Technicians	11.40			56,012.00	18,623.00	74,635.00

5	**Total Number Other Personnel**	**Total Other Personnel**	153,716.00
		Total Salary, Wages and Fringe Benefits (A+B)	275,922.00

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment item	Funds Requested ($)

Additional Equipment: Add Attachment | Delete Attachment | View Attachment

Total funds requested for all equipment listed in the attached file	
Total Equipment	

D. Travel

	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	10,591.00
2. Foreign Travel Costs	
Total Travel Cost	10,591.00

E. Participant/Trainee Support Costs

	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other	
Total Participant/Trainee Support Costs	

Number of Participants/Trainees

F. Other Direct Costs

		Funds Requested ($)
1.	Materials and Supplies	142,590.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	GRA Tuition	4,543.00
9.	Deep Sequencing RNA Seq and Microscopy	11,650.00
10.	Histology and Animal per diem	6,486.00
	Total Other Direct Costs	165,269.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	451,782.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
MTDC	55.50	447,239.00	248,218.00

Total Indirect Costs 248,218.00

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	700,000.00

J. Fee

Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	700,000.00

L. Budget Justification

(Only attach one file.) Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 2

OMB Number: 4040-0001
Expiration Date: 10/31/2019

ORGANIZATIONAL DUNS: 6081952770000 **Enter name of Organization:** University of North Carolina School of Public Health

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 2** **Start Date:** 12/1/2019 **End Date:** 11/30/2020

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
Prof.	Ralph		Baric			1.20			28,123.00	7,137.00	35,260.00
Project Role:	Co-Investigator										
Dr.	Kristy		Ainslie			0.60			7,551.00	2,052.00	9,603.00
Project Role:	Investigator										
Dr.	Amy		Sims			3.00			30,590.00	8,593.00	39,183.00
Project Role:	Investigator										
Dr.	Timothy		Sheahan			3.12			23,280.00	6,949.00	30,229.00
Project Role:	Investigator										
Dr.	Eric		Bachelder			1.20			6,900.00	2,194.00	9,094.00
Project Role:	Investigator										

Additional Senior Key Persons: Add Attachment | Delete Attachment | View Attachment

Total Funds requested for all Senior Key Persons in the attached file

Total Senior/Key Person 123,369.00

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
2	Post Doctoral Associates	13.68			56,158.00	9,971.00	66,129.00
1	Graduate Students	4.80			12,600.00	2,690.00	15,290.00
	Undergraduate Students						
	Secretarial/Clerical						
2	Research Technicians	11.40			56,012.00	18,623.00	74,635.00

5	**Total Number Other Personnel**	**Total Other Personnel**	156,054.00
		Total Salary, Wages and Fringe Benefits (A+B)	279,423.00

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment item	Funds Requested ($)

Additional Equipment: Add Attachment | Delete Attachment | View Attachment

Total funds requested for all equipment listed in the attached file	
Total Equipment	

D. Travel

	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	3,784.00
2. Foreign Travel Costs	3,174.00
Total Travel Cost	6,958.00

E. Participant/Trainee Support Costs

	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other	
Total Participant/Trainee Support Costs	

Number of Participants/Trainees

F. Other Direct Costs

		Funds Requested ($)
1.	Materials and Supplies	142,590.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	GRA Tuition	4,543.00
9.	Deep Sequencing RNA Seq and Microscopy	11,650.00
10.	Histology and Animal per diem	6,621.00
	Total Other Direct Costs	165,404.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	451,785.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
MTDC	55.50	447,235.00	248,215.00

Total Indirect Costs 248,215.00

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	700,000.00

J. Fee

Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	700,000.00

L. Budget Justification

(Only attach one file.) Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 3

OMB Number: 4040-0001
Expiration Date: 10/31/2019

ORGANIZATIONAL DUNS: 6081952770000 **Enter name of Organization:** University of North Carolina School of Public Health

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 3** **Start Date:** 12/1/2020 **End Date:** 11/30/2021

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
Prof.	Ralph		Baric			1.20			28,123.00	7,137.00	35,260.00
Project Role:	Co-Investigator										
Dr.	Kristy		Ainslie			0.60			7,551.00	2,052.00	9,603.00
Project Role:	Investigator										
Dr.	Amy		Sims			3.00			30,590.00	8,593.00	39,183.00
Project Role:	Investigator										
Dr.	Timothy		Sheahan			3.12			23,280.00	6,949.00	30,229.00
Project Role:	Investigator										
Dr.	Eric		Bachelder			1.20			6,900.00	2,194.00	9,094.00
Project Role:	Investigator										

Additional Senior Key Persons: Add Attachment | Delete Attachment | View Attachment

Total Funds requested for all Senior Key Persons in the attached file

Total Senior/Key Person 123,369.00

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
2	Post Doctoral Associates	13.20			54,172.00	9,619.00	63,791.00
1	Graduate Students	4.80			12,600.00	2,690.00	15,290.00
	Undergraduate Students						
	Secretarial/Clerical						
2	Research Technicians	11.40			56,012.00	18,623.00	74,635.00

5	**Total Number Other Personnel**	**Total Other Personnel**	153,716.00
		Total Salary, Wages and Fringe Benefits (A+B)	277,085.00

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment item	Funds Requested ($)

Additional Equipment: Add Attachment | Delete Attachment | View Attachment

Total funds requested for all equipment listed in the attached file	
Total Equipment	

D. Travel

	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	9,460.00
2. Foreign Travel Costs	
Total Travel Cost	9,460.00

E. Participant/Trainee Support Costs

	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other	
Total Participant/Trainee Support Costs	

Number of Participants/Trainees

F. Other Direct Costs

		Funds Requested ($)
1.	Materials and Supplies	142,590.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	GRA Tuition	4,543.00
9.	Deep Sequencing RNA Seq and Microscopy	11,650.00
10.	Histology and Animal per diem	6,454.00
	Total Other Direct Costs	165,237.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	451,782.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
MTDC	55.50	447,239.00	248,218.00

Total Indirect Costs 248,218.00

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	700,000.00

J. Fee

Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	700,000.00

L. Budget Justification

(Only attach one file.) Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 4

OMB Number: 4040-0001
Expiration Date: 10/31/2019

ORGANIZATIONAL DUNS: 6081952770000 **Enter name of Organization:** University of North Carolina School of Public Health

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 4** **Start Date:** 12/1/2021 **End Date:** 05/31/2022

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
Prof.	Ralph		Baric			0.60			14,061.00	3,569.00	17,630.00
Project Role:	Co-Investigator										
Dr.	Kristy		Ainslie			0.30			3,776.00	1,026.00	4,802.00
Project Role:	Investigator										
Dr.	Amy		Sims			1.50			15,295.00	4,296.00	19,591.00
Project Role:	Investigator										
Dr.	Timothy		Sheahan			1.56			11,640.00	3,474.00	15,114.00
Project Role:	Investigator										
Dr.	Eric		Bachelder			0.60			3,450.00	1,097.00	4,547.00
Project Role:	Investigator										

Additional Senior Key Persons: Add Attachment | Delete Attachment | View Attachment

Total Funds requested for all Senior Key Persons in the attached file

Total Senior/Key Person 61,684.00

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
2	Post Doctoral Associates	6.60			27,086.00	4,810.00	31,896.00
1	Graduate Students	2.40			6,300.00	1,345.00	7,645.00
	Undergraduate Students						
	Secretarial/Clerical						
2	Research Technicians	11.40			28,006.00	9,311.00	37,317.00

5	**Total Number Other Personnel**	**Total Other Personnel** 76,858.00
		Total Salary, Wages and Fringe Benefits (A+B) 138,542.00

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment item	Funds Requested ($)

Additional Equipment: Add Attachment | Delete Attachment | View Attachment

Total funds requested for all equipment listed in the attached file

Total Equipment

D. Travel

		Funds Requested ($)
1.	Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	4,730.00
2.	Foreign Travel Costs	
	Total Travel Cost	4,730.00

E. Participant/Trainee Support Costs

		Funds Requested ($)
1.	Tuition/Fees/Health Insurance	
2.	Stipends	
3.	Travel	
4.	Subsistence	
5.	Other	

Number of Participants/Trainees **Total Participant/Trainee Support Costs**

F. Other Direct Costs

		Funds Requested ($)
1.	Materials and Supplies	71,295.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	GRA Tuition	2,272.00
9.	Deep Sequencing RNA Seq and Microscopy	5,825.00
10.	Histology and Animal per diem	3,227.00
	Total Other Direct Costs	82,619.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	225,891.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
MTDC	55.50	223,619.00	124,109.00

Total Indirect Costs 124,109.00

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	350,000.00

J. Fee

Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	350,000.00

L. Budget Justification

(Only attach one file.) Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Cumulative Budget

		Totals ($)
Section A, Senior/Key Person		430,628.00
Section B, Other Personnel		540,344.00
Total Number Other Personnel	20	
Total Salary, Wages and Fringe Benefits (A+B)		970,972.00
Section C, Equipment		
Section D, Travel		31,739.00
1. Domestic	28,565.00	
2. Foreign	3,174.00	
Section E, Participant/Trainee Support Costs		
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other		
6. Number of Participants/Trainees		
Section F, Other Direct Costs		578,529.00
1. Materials and Supplies	499,065.00	
2. Publication Costs		
3. Consultant Services		
4. ADP/Computer Services		
5. Subawards/Consortium/Contractual Costs		
6. Equipment or Facility Rental/User Fees		
7. Alterations and Renovations		
8. Other 1	15,901.00	
9. Other 2	40,775.00	
10. Other 3	22,788.00	
Section G, Direct Costs (A thru F)		1,581,240.00
Section H, Indirect Costs		868,760.00
Section I, Total Direct and Indirect Costs (G + H)		2,450,000.00
Section J, Fee		
Section K, Total Costs and Fee (I + J)		2,450,000.00

Intellectual Property

All proposers must provide a good faith representation that the proposer either owns or possesses the appropriate licensing rights to all intellectual property that will be utilized under the proposed effort.

For All Non-Procurement Contracts

Proposers responding to this BAA requesting a Grant, Cooperative Agreement, Technology Investment Agreement, or Other Transaction for Prototypes shall follow the applicable rules and regulations governing these various award instruments, but, in all cases, **should appropriately identify any potential restrictions on the Government's use of any Intellectual Property contemplated under the award instrument in question**. This includes both Noncommercial Items and Commercial Items. Proposers are encouraged to use a format similar to that described in the section below. **If no restrictions are intended, then the proposer should state "NONE."**

Technical data computer software to be furnished with restrictions	Summary of intended use in the conduct of research	Basis for assertion	Asserted rights category	Name of person asserting rights
(LIST)	(NARRATIVE)	(LIST)	(LIST)	(LIST)

Middle East Respiratory Syndrome Coronavirus in Bats, Saudi Arabia

Ziad A. Memish, Nischay Mishra, Kevin J. Olival, Shamsudeen F. Fagbo, Vishal Kapoor, Jonathan H. Epstein, Rafat AlHakeem, Mushabab Al Asmari, Ariful Islam, Amit Kapoor, Thomas Briese, Peter Daszak, Abdullah A. Al Rabeeah, and W. Ian Lipkin

The source of human infection with Middle East respiratory syndrome coronavirus remains unknown. Molecular investigation indicated that bats in Saudi Arabia are infected with several alphacoronaviruses and betacoronaviruses. Virus from 1 bat showed 100% nucleotide identity to virus from the human index case-patient. Bats might play a role in human infection.

Since Middle East respiratory syndrome (MERS) was described in September 2012, over 90 cases have been reported worldwide, 70 from Saudi Arabia. The incidence of infection with the causative agent, a betacoronavirus (MERS CoV) (*1*), has not been determined; however, the mortality rate among those who received clinical care is ≈65% (*2*). Although instances of human-to-human transmission have been documented between case-patients and others in close contact (including hospital patients sharing rooms, family members, and medical personnel), the sources of infection for most patients remain unknown. Because of sequence similarities between β-CoVs identified in bats and those of MERS CoV isolated from humans, a bat reservoir has been posited (*3–5*). Although neither detection of MERS CoV in bats nor contact of human MERS patients with bats have been reported, a role for bats in human infection cannot be excluded because contact can be indirect (mediated through another animal vector or fomites).

Author affiliations: Ministry of Health, Riyadh, Saudi Arabia (Z.A. Memish, S.F. Fagbo, R. AlHakeem, A.A. Al Rabeeah); Columbia University, New York, New York, USA (N. Mishra, V. Kapoor, A. Kapoor, T. Briese, W.I. Lipkin); EcoHealth Alliance, New York (K.J. Olival, J.H. Epstein, P. Daszak); Ministry of Health, Bisha, Saudi Arabia (M. Al Asmari); and EcoHealth Alliance, Dhaka, Bangladesh (A. Islam)

DOI: http://dx.doi.org/10.3201/eid1911.131172

The Study

In October 2012 and April 2013, three agencies collected samples from bats in regions where MERS cases had been identified (Figure 1). The agencies are the Ministry of Health of Saudi Arabia, the Center for Infection and Immunity of Columbia University, and EcoHealth Alliance.

During the October investigation, the team interviewed the family of an index case-patient in Bisha and collected samples from bats <12 km from his home, in an abandoned date palm orchard, and <1 km from his place of employment, a hardware store that fronted a garden and date palm orchard. Although neither family members nor employees recalled seeing bats, the team observed roosting bats and guano in abandoned wells and ruins within 12 km of his home and insectivorous bats at dusk in the garden behind his store. Over 3 weeks, 96 bats representing 7 species (*Rhinopoma hardwickii*, *Rhinopoma microphyllum*, *Taphozous perforatus*, *Pipistrellus kuhlii*, *Eptesicus bottae*, *Eidolon helvum*, and *Rosettus aegyptiacus*) were captured in mist nets and harp traps, then released after visual speciation and collection of morphometric measurements; wing punch biopsy samples; blood; throat swab samples; and rectal swab samples or fecal pellets. Samples were collected into viral transport medium or lysis buffer.

During the 3-week April investigation, fecal samples were collected on tarps laid out at bat roosting sites in and around Bisha, Unaizah, and Riyadh. Representative animals at each roosting site were captured, identified morphologically, and released after wing punch biopsy samples were collected for speciation by DNA analysis. Samples were collected into cryovials.

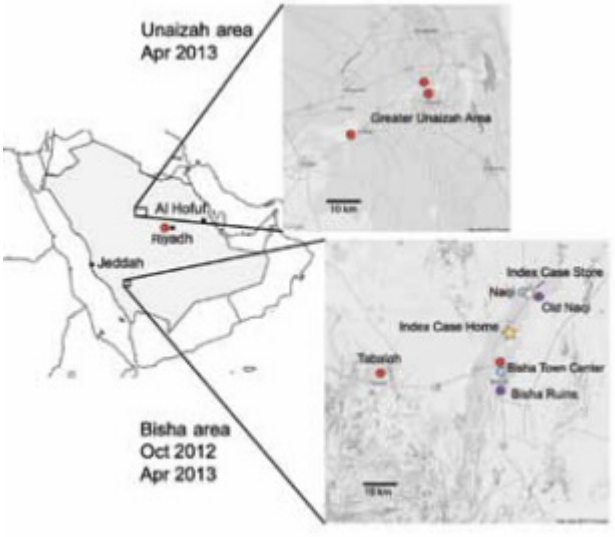


Figure 1. Bat sampling sites and locations of home and workplace of index case-patient with Middle East respiratory syndrome, Bisha, Saudi Arabia.

Table 1. PCRs and primers used in CoV detection*

PCRs (reference)	Primers, 5′→3′	Nested fragment size, region (primer locations on the reference genome)†	Type of CoV (no.)
Nested pan-CoV-I (*6*)	PLQ-F1, CGTTGGIACWAAYBTVCCWYTICARBTRGG PLQ-R1, GGTCATKATAGCRTCAVMASWWGCNACATG PLQ-F2, GGCWCCWCCHGGNGARCAATT PLQ-R2, GGWAWCCCCAYTGYTGWAYRTC	≈400 nt, RdRp (18310–187450)	α-CoV (8), β-CoV (1)
Nested pan-CoV-II (*7*)	WT-C0V-F1, GGTTGGGAYTAYCCHAARTGTGA WT-COV-R1, CCATCATCASWYRAATCATCATA WT-COV-F2, GAYTAYCCHAARTGTGAYAGAGC WT-COV-F3, GAYTAYCCHAARTGTGAUMGWGC	≈430 nt, RdRp (15260–15700)	α-CoV (5), β-CoV (2)
Hemi-nested RdRp-sequence assay (*9*)	EMC-SeqRdRP-Rev, GCATWGCNCWGTCACACTTAGG EMC-SeqRdRP-Fwd, TGCTATWAGTGCTAAGAATAGRGC EMC-SeqRdRP-Rnest, CACTTAGGRTARTCCCAWCCCA	≈230 nt, RdRp (15048–15290)	α-CoV (2), β-CoV (1)
Hemi-nested N-sequence assay (*9*)	EMC-SeqN-Fwd, CCTTCGGTACAGTGGAGCCA EMC-SeqN-Rev, GATGGGGTTGCCAAACACAAAC EMC-SeqN-Fnest, TGACCCAAAGAATCCCAACTAC	≈280 nt,N seq (29,549–29,860)	NA
Nested CII-pan-CoV-III	NM-CoV-2F1, ACWGTTCARGGICCWCCIGG NM-CoV-2F2, GTTCARGGGCCWCCGGGNAC NM-CoV-2R1, GGCAGCTGWGCWGGRTCICCNACRTA NM-CoV-2R2, AGCTGWGCWGGRTCGCCIACRTANAC	≈355 nt, helicase (17,060–17,410)	β-CoV (2)
Nested CII-MERS-RdRp	NM-HCOV-F1, GTGCTAAGAATAGAGCTCGCACT NM-HCOV-F2, AGAGCTCGCACTGTTGCAGGC NM-HCOV-F2, AGAGCTCGCACTGTTGCAGGC NM-HCOV-R1, ACCCATAAGATGCGGATTATCAAC NM-HCOV-R2, TGCGGATTATCAACATCTTTGTAC	≈190 nt, RdRp (15068–15249)	β-CoV (1, MERS CoV)
Hemi-nested CII-MERS N sequence	NM-NSeq-F-1, ACTTCCTTCGGTACAGTGGAGC NM-NSeq-R-1, GGCACTGTTCACTTGCAATC NM-NSeq-R-2, GGAGGTTCAGACATTTGGTCT	≈170 nt, N seq (29545–29713)	NA
upE and ORF1b real-time assays (*8*)	upE-Fwd: GCAACGCGCGATTCAGTT upE-Prb: FAM-CTCTTCACATAATCGCCCCGAGCTCG-TAMRA upE-Rev: GCCTCTACACGGGACCCATA ORF1b-Fwd: TTCGATGTTGAGGGTGCTCAT ORF1b-Prb: FAM-CCCGTAATGCATGTGGCACCAATGT-TAMRA ORF1b-Rev: TCACACCAGTTGAAAATCCTAATTG	Upstream of E gene and ORF 1b	NA

*CoV, coronavirus; MERS, Middle East respiratory syndrome; RdRp, RNA-dependent RNA polymerase; NA, not applicable; ORF, open reading frame.
†Primer locations are based on human β-CoV 2c EMC/2012, complete genome (GenBank accession no. JX869059).

All samples were stored in liquid nitrogen and conveyed to Riyadh for storage at −80°C before being transported to Columbia University in New York in dry nitrogen. The October 2012 shipment was inadvertently opened at customs in the United States and sat at room temperature for 48 hours before transfer to Columbia University; at arrival, all samples had thawed. The April 2013 samples arrived intact.

Total nucleic acid was extracted from samples by using the NucliSENS easyMAG system (bioMérieux, Durham, NC, USA) and subjected to 8 PCRs with primers and protocols designed to amplify regions within the helicase, RNA-dependent RNA polymerase (RdRp), and nucleocapsid or envelope proteins of CoVs (*6*–*9*). Products were sequenced and analyzed for similarity to GenBank database entries by using the BLASTn and BLASTx programs (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Primer sequences are shown in Table 1. The identity of bat species yielding specific viral products was determined by amplifying and sequencing a fragment of the cytochrome B gene (*10*). All visual classifications of species were confirmed except for that of *T. perforatus* bats. There is no reference sequence for *T. perforatus* bats in GenBank. However, because the closest reference sequence was from *T. nudiventris* bats, at 84% identity we presume that the product represents bona fide *T. perforatus* bat cytochrome B gene sequence. Representative cytochrome B sequences have been uploaded to GenBank (accession nos. KF498635–KF498641).

Table 1 indicates the CoV genera identified by using individual primer sets. As anticipated, pan-CoV assays detected α- and β-CoVs. One assay specific for MERS CoV (*9*) also detected α-CoVs. This finding reinforces the need for sequence confirmation of PCR products. Table 2 indicates the CoV species identified with respect to location, sample type, and bat species. CoV sequences were amplified from rectal swab samples or fecal pellets and from roost feces but not from serum, throat swab samples, or urine. α-CoV sequences were amplified more frequently than β-CoV sequences (223 vs. 4). Whereas α- and β-CoV sequences were amplified from CoVs from *T. perforatus*, *E. helvum,* and *R. hardwickii* bats, only alpha sequences were amplified from CoVs from *P. kuhlii* bat samples.

Table 2. CoVs detected in bats, Saudi Arabia*

Bat family, genus, species	Location	No. bats	No. samples tested (no. positive)					Total no. samples, n = 1,003	Total no. positive samples, n = 227 (closely related CoVs)†
			Throat swab	Fecal pellets	Urine	Serum	Roost feces		
October 2012									
Emballonuridae									
Taphozous perforatus	Bisha ruins	29				22	10 (1)	94	1 β-Cov (1 MERS novel CoV) and 2 α-CoVs (1 bovine respiratory CoV, 1 Kenya bat CoV BtKY86)
Pteropodidae									
Eidolon helvum	Bisha town center	25				19	NA	82	1 β-Cov (1 *Eidolon* bat CoV-HKU1) and 4 α-CoVs (4 Kenya bat CoV BtKY86)
Rousettus aegyptiacus	Bisha town center	3	3 (0)	3 (0)	1 (0)	2	NA	9	NA
Rhinopomatidae									
Rhinopoma hardwickii	Naqi and Old Naqi	36	36 (0)	35 (0)	4 (0)	NA	15 (0)	90	NA
Rh. microphyllum	Old Naqi	1	1 (0)	1 (0)	NA	NA	NA	2	NA
Vespertilionidae									
Eptesicus bottae	Bisha ruins	1	1 (0)	1 (0)	1 (0)	NA	32 (0)	35	*NA*
Pipistrellus kuhlii	Bisha ruins	1	1 (0)	1 (0)	NA	NA	NA	2	NA
April 2013									
Rhinopomatidae									
Rh. hardwickii	Greater Bisha area	NA	NA	NA	NA	NA	209 (93)	209	2 β-Covs (2 canine respiratory CoVs) and 91α-CoVs (5 canine CoVs, 2 *Miniopterus* bat CoVs, 84 *Chaerephon* bat CoV)
T. perforatus	Bisha ruins	NA	NA	NA	NA	NA	203 (0)	203	NA
Vespertilionidae									
P. kuhlii	Greater Unaizah area	9	9 (0)	NA	NA	NA	263 (126)	277	126 α-CoVs (69 alphaCoV P.kuh-Spain, 3 canine CoVs, 37 bat CoV P.pyg/Germany, 1 human CoV NL63, 2 *Rousettus* bat CoV HKU10, 11 porcine epidemic diarrhea virus, 2 *Cardioderma* bat CoVs, 1 *Hipposideros* bat CoV HKU10)
	Greater Riyadh area	5	5 (0)	NA	NA	NA	NA	NA	NA

*CoV, coronavirus; MERS, Middle East respiratory syndrome; NA, not applicable.
†Based on BLASTn (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

CoV sequences were amplified from 220 of 732 roost feces samples and 7 of 91 rectal swab samples or fecal pellets. A product obtained by PCR amplification of nucleic acid from a fecal pellet of a *T. perforatus* bat captured in October 2012 in Bisha showed 100% nt identity to the human β-CoV 2c EMC/2012 cloned from the index case-patient in Bisha. A phylogenetic analysis of CoVs obtained in this study is shown in Figure 2. CoV sequences have been uploaded in GenBank (accession nos. KF493884–KF493888).

Conclusions

A wide range of CoV species are circulating among bats in Saudi Arabia. Although the prevalence of CoVs was high (≈28% of fecal samples), MERS CoV was found in only 1 bat. A 3.5% MERS CoV infection rate (n = 29; 95% CI 0–20%) in *T. perforatus* bats is low compared with that for severe acute respiratory syndrome–like CoV in rhinolophid bats in China (10%–12.5%) but consistent with CoV prevalence among bats in Mexico (*4*). Furthermore, the sensitivity for viral nucleic acid detection in samples collected in October 2012 was probably reduced because of failure in cold chain transport. Whereas 219 (32%) of 675 of fecal pellets collected in April revealed a CoV sequence by PCR, only 8 (5%) of 148 of rectal swab samples or fecal pellets collected in October were positive by the same assays. We were unable to recover additional sequences beyond the 190-nt RdRp fragment represented in Figure 2 but are confident in the fidelity of the finding. First, although RdRp is a conserved portion of the CoV genome, there is no precedent for 100% identity of a bat sequence with a human MERS CoV sequence. Second, when this work began we did not have cultured MERS CoV, human MERS samples, or MERS CoV cDNA in the

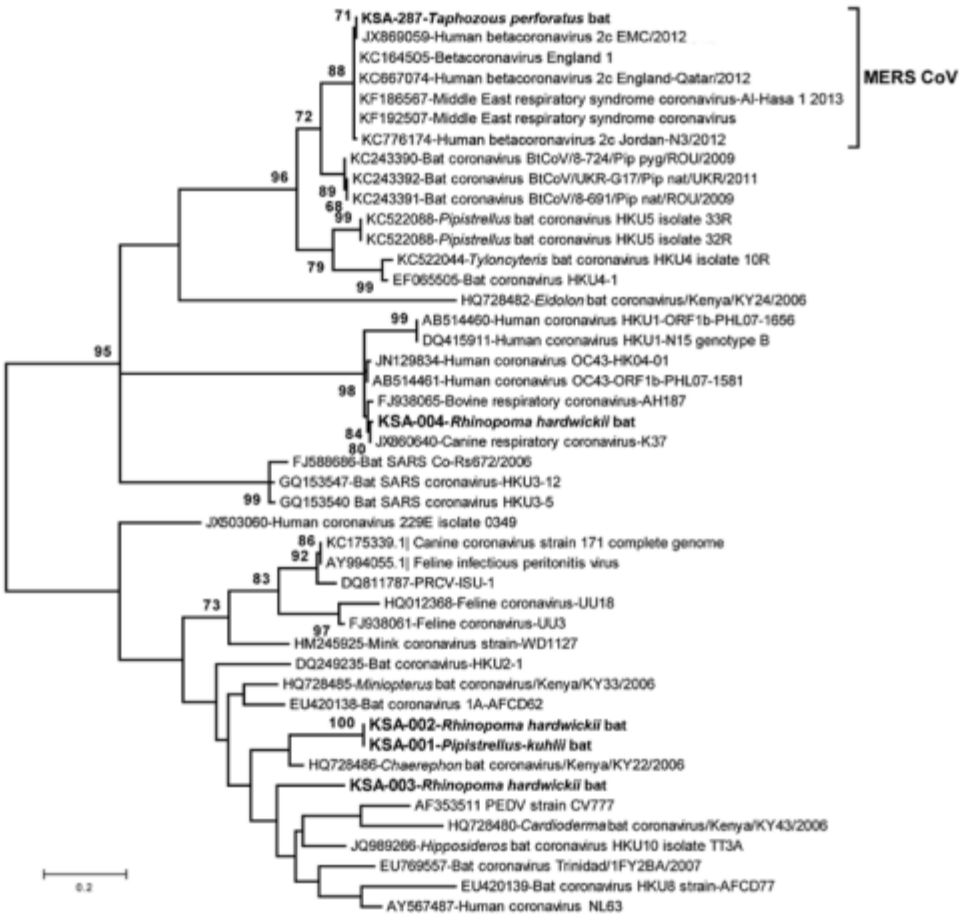


Figure 2. Phylogenetic tree showing genetic relatedness between coronaviruses identified in bat samples from Saudi Arabia (**boldface**), MERS coronaviruses, and other published coronavirus sequences available in GenBank. The maximum-likelihood tree of partial RNA-dependent RNA polymerase gene (nt position 15068–15249 of GenBank accession no. JX869059) was constructed by using the Tamura-Nei model with discrete gamma rate differences among sites as implemented in MEGA 5.2 (www.megasoftware.net). Each branch shows the GenBank accession number followed by a brief description of the sequence used. Scale bar indicates nucleotide substitutions per site. MERS, Middle East respiratory syndrome; CoV, coronavirus; SARS, severe acute respiratory syndrome; KSA, Kingdom of Saudi Arabia.

laboratory at Columbia University where samples were removed directly from the tubes in which they were collected in the field for nucleic acid extraction, PCR, and sequence analysis. Third, the only MERS-positive signal was obtained in PCR analysis of the *T. perforatus* bat captured in Bisha near the home and workplace of the MERS index case-patient used to generate the human β-CoV 2c EMC/2012 sequence.

Bats are reservoirs of several viruses that can cause human disease, including rabies, Hendra, Nipah, Marburg, severe acute respiratory syndrome CoV, and probably Ebola viruses (*11*–*14*). Cross-species transmission from bats to humans can be direct, through contact with infected bats or their excreta, or facilitated by intermediate hosts (*15*). Bat CoVs are typically host specific; however, MERS-related CoVs have reportedly been found in many bat families, including Vespertillionidae, Molosidae, Nyteridae, and now Emballonuridae (sheath-tailed bats) in Africa, the Americas, Asia, and Europe. We sampled only a small sample of bats in Saudi Arabia. Nonetheless, given the rarity of MERS CoV sequences detected by our survey and the broad distribution of MERS cases throughout the Middle East, we speculate that there are probably other hosts. Future work should investigate additional bat and other wildlife species and domestic animals for CoV infection and potential linkage to human disease.

Acknowledgments

We thank Rebecca Hersch and George Amato for cytochrome B gene sequencing, Lorenzo Uccellini and Steven Sameroff for assistance with nucleic acid extraction, Kawathar Muhammad for sample coordination, and Meera Bhat and Ellie Kahn for assistance with manuscript preparation.

Work in the Center for Infection and Immunity and EcoHealth Alliance was supported by the National Institute of Allergy and Infectious Diseases, the US Agency for International Development (PREDICT), and the Defense Threat Reduction Agency.

Dr Memish is deputy minister for public health, director of the WHO Collaborating Center for Mass Gathering Medicine in the Ministry of Health, and professor in the College of Medicine of Alfaisal University in Riyadh. His research interests include emerging infectious diseases, infection control, and preventive medicine.

References

1. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med. 2012;367:1814–20. http://dx.doi.org/10.1056/NEJMoa1211721
2. Assiri A, McGeer A, Perl TM, Price CS, Al Rabeeah AA, Cummings DA, et al. Hospital outbreak of Middle East respiratory syndrome coronavirus. N Engl J Med. 2013;369:407–16. http://dx.doi.org/10.1056/NEJMoa1306742
3. Annan A, Baldwin HJ, Corman VM, Klose SM, Owusu M, Nkrumah EE, et al. Human betacoronavirus 2c EMC/2012-related viruses in bats, Ghana and Europe. Emerg Infect Dis. 2013;19:456–9. http://dx.doi.org/10.3201/eid1903.121503
4. Anthony SJ, Ojeda-Flores R, Rico-Chavez O, Navarrete-Macias I, Zambrana-Torrelio CM, Rostal MK, et al. Coronaviruses in bats from Mexico. J Gen Virol. 2013;94:1028–38. http://dx.doi.org/10.1099/vir.0.049759-0
5. Ithlete NL, Stoffberg S, Corman VM, Cottontail VM, Richards LR, Schoeman MC, et al. Close relative of human Middle East respiratory syndrome coronavirus in bat, South Africa [letter]. Emerg Infect Dis. 2013:19 [cited 2013 Aug 19]. http://wwwnc.cdc.gov/eid/article/19/10/13-0946_article.htm
6. Quan PL, Firth C, Street C, Henriquez JA, Petrosov A, Tashmukhamedova A, et al. Identification of a severe acute respiratory syndrome coronavirus–like virus in a leaf-nosed bat in Nigeria. mBio. 2010;1:e00208–10. http://dx.doi.org/10.1128/mBio.00208-10
7. Watanabe S, Masangkay JS, Nagata N, Morikawa S, Mizutani T, Fukushi S, et al. Bat coronaviruses and experimental infection of bats, the Philippines. Emerg Infect Dis. 2010;16:1217–23. http://dx.doi.org/10.3201/eid1608.100208
8. Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, et al. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. Euro Surveill. 2012;17: pii 20288.
9. Corman VM, Muller MA, Costabel U, Timm J, Binger T, Meyer B, et al. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. Euro Surveill. 2012;17: pii 20334.
10. Teeling EC, Springer MS, Madsen O, Bates P, O'Brien SJ, Murphy WJ. A molecular phylogeny for bats illuminates biogeography and the fossil record. Science. 2005;307:580–4. http://dx.doi.org/10.1126/science.1105113
11. Smith I, Wang LF. Bats and their virome: an important source of emerging viruses capable of infecting humans. Curr Opin Virol. 2013;3:84–91.http://dx.doi.org/10.1016/j.coviro.2012.11.006
12. Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, et al. Severe acute respiratory syndrome coronavirus–like virus in Chinese horseshoe bats. Proc Natl Acad Sci U S A. 2005;102:14040–5. http://dx.doi.org/10.1073/pnas.0506735102
13. Poon LL, Chu DK, Chan KH, Wong OK, Ellis TM, Leung YH, et al. Identification of a novel coronavirus in bats. J Virol. 2005;79:2001–9. http://dx.doi.org/10.1128/JVI.79.4.2001-2009.2005
14. Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, et al. Bats are natural reservoirs of SARS-like coronaviruses. Science. 2005;310:676–9. http://dx.doi.org/10.1126/science.1118391
15. Khan SU, Gurley ES, Hossain MJ, Nahar N, Sharker MA, Luby SP. A randomized controlled trial of interventions to impede date palm sap contamination by bats to prevent Nipah virus transmission in Bangladesh. PLoS ONE. 2012;7:e42689. http://dx.doi.org/10.1371/journal.pone.0042689

Address for correspondence: W. Ian Lipkin, Center for Infection and Immunity, Columbia University, 722 West 168th St, New York, NY 10032, USA; email: wil2001@columbia.edu

EMERGING INFECTIOUS DISEASES®
Conference summaries and other reports available online only.

Full text available free online
www.cdc.gov/eid

GovDelivery
of customized content
Sign up to receive our table of contents
Online subscription: www.cdc.gov/ncidod/eid/subscribe.htm

SUMMARY OF RELATED ACTIVITIES

The following specific information must be provided by the offeror pertaining to the Project Director, Principal Investigator, and each of any other proposed key professional individuals designated for performance under any resulting contract.

a. Identify the total amount of all presently active federal contracts/cooperative agreements/grants and commercial agreements citing the committed levels of effort for those projects for each of the key individuals* in this proposal.

Professional's Name and Title/Position:

Identifying Number	Agency	Total Effort Committed

*If an individual has no obligation(s), so state.

b. Provide the total number of outstanding proposals, exclusive of the instant proposal, having been submitted by your organization, not presently accepted but in an anticipatory stage, which will commit levels of effort by the proposed professional individuals*.

Professional's Name and Title/Position:

Identifying Number	Agency	Total Effort Committed

*If no commitment of effort is intended, so state.

c. Provide a statement of the level of effort to be dedicated to any resultant contract awarded to your organization for those individuals designated and cited in this proposal.

Name	Title/Position	Total Proposed Effort

Documentation of Relationship to Candidate
(Required for external letters of recommendation only)

Please complete this form & submit with your letter of recommendation.

Candidate's Name: _____

Reviewer's Name: _____

Reviewer's Rank/Title: _____

Length of time you have known the candidate: ____ years

Check all that apply in each category:

1. Your relationship to the candidate and his/her work:

☐ Collaborator (worked with or co-authored papers)
☐ Present or past colleague, mentor or mentee, at same academic institution or hospital as a student, post-doctoral fellow, resident or faculty member
☐ Present or past colleague in professional activities (study sections, advisory boards, professional society activities, etc)
☐ None of the above (please provide brief description of your relationship:

2. Your knowledge of the candidate's work is based on (mark all that apply):

☐ CV and personal statement
☐ Publications
☐ Presentations
☐ Personal knowledge and discussions of his/her work
☐ Participation with candidate in professional activities (study sections, advisory boards, professional society activities, etc…)

3. Comments *(optional)*:

Signature:_____ **Date:**_____

I. OVERVIEW OF THE FUNDING OPPORTUNITY

Program Announcement for the Department of Defense

Defense Health Program

Congressionally Directed Medical Research Programs

Peer Reviewed Medical Research Program

Investigator-Initiated Research Award

Announcement Type: Initial

Funding Opportunity Number: W81XWH18PRMRPIIRA

Catalog of Federal Domestic Assistance Number: 12.420 Military Medical Research and Development

SUBMISSION AND REVIEW DATES AND TIMES

- **Pre-Application Submission Deadline:** 5:00 p.m. Eastern time (ET), June 12, 2018
- **Invitation to Submit an Application:** July 2018
- **Application Submission Deadline:** 11:59 p.m. ET, September 20, 2018
- **End of Application Verification Period:** 5:00 p.m. ET, September 24, 2018
- **Peer Review:** November 2018
- **Programmatic Review:** January 2019

This Program Announcement must be read in conjunction with the General Application Instructions, version 20180329. The General Applications Instructions document is available for downloading from the Grants.gov funding opportunity announcement by selecting the "Package" tab, clicking "Preview," and then selecting "Download Instructions."

TABLE OF CONTENTS

I. OVERVIEW OF THE FUNDING OPPORTUNITY 1
II. DETAILED INFORMATION ABOUT THE FUNDING OPPORTUNITY 3
II.A. Program Description 3
II.A.1. FY18 PRMRP Topic Areas 3
II.B. Award Information 5
II.C. Eligibility Information 9
II.C.1. Eligible Applicants 9
II.C.2. Cost Sharing 10
II.C.3. Other 10
II.D. Application and Submission Information 10
II.D.1. Address to Request Application Package 11
II.D.2. Content and Form of the Application Submission 11
II.D.3. Dun and Bradstreet Data Universal Numbering System (DUNS) Number and System for Award Management (SAM) 29
II.D.4. Submission Dates and Times 29
II.D.5. Funding Restrictions 30
II.D.6. Other Submission Requirements 32
II.E. Application Review Information 32
II.E.1. Criteria 32
II.E.2. Application Review and Selection Process 34
II.E.3. Integrity and Performance Information 35
II.E.4. Anticipated Announcement and Federal Award Dates 35
II.F. Federal Award Administration Information 35
II.F.1. Federal Award Notices 35
II.F.2. Administrative and National Policy Requirements 36
II.F.3. Reporting 36
II.G. Federal Awarding Agency Contacts 37
II.G.1. CDMRP Help Desk 37
II.G.2. Grants.gov Contact Center 37
II.H. Other Information 38
II.H.1. Program Announcement and General Application Instructions Versions 38
II.H.2. Administrative Actions 38
II.H.3. Application Submission Checklist 41
APPENDIX 1: ACRONYM LIST 43
APPENDIX 2: AREAS OF ENCOURAGEMENT 44
APPENDIX 3: DOD AND VA WEBSITES 61

II. DETAILED INFORMATION ABOUT THE FUNDING OPPORTUNITY

***New for 2018*: Application submission by extramural organizations through Grants.gov requires use of the Workspace interface, which separates the application package into individual forms. Applicants must create a Workspace in Grants.gov, complete the required forms, and submit their application Workspace package.**

II.A. Program Description

Applications to the Fiscal Year 2018 (FY18) Peer Reviewed Medical Research Program (PRMRP) are being solicited for the Defense Health Agency (DHA) J9, Research and Development Directorate, by the U.S. Army Medical Research Acquisition Activity (USAMRAA) using delegated authority provided by United States Code, Title 10, Section 2358 (10 USC 2358). As directed by the Office of the Assistant Secretary of Defense for Health Affairs (OASD[HA]), the DHA manages the Defense Health Program (DHP) Research, Development, Test, and Evaluation (RDT&E) appropriation. The execution management agent for this Program Announcement is the Congressionally Directed Medical Research Programs (CDMRP). The PRMRP was initiated in 1999 to provide support for military health-related research of exceptional scientific merit. Appropriations for the PRMRP from FY99 through FY17 totaled $1.7 billion. The FY18 appropriation is $330 million (M).

The vision of the FY18 PRMRP is to improve the health and well-being of all military Service members, Veterans, and beneficiaries. The PRMRP challenges the scientific and clinical communities to address at least one of the FY18 PRMRP Topic Areas with original ideas that foster new directions along the entire spectrum of research and clinical care. The program seeks applications in laboratory, clinical, behavioral, epidemiologic, and other areas of research to advance knowledge in disease etiology, improve prevention, detection, diagnosis, treatment, and quality of life for those affected by a relevant disease or condition, and to develop and validate clinical care or public health guidelines.

II.A.1. FY18 PRMRP Topic Areas

All applications for PRMRP funding must specifically address at least one of the Topic Areas as directed by Congress and must be directly relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries. If the proposed research does not specifically address at least one of the FY18 PRMRP Topic Areas, the Government will administratively withdraw the application. The Government reserves the right to reassign the application's Topic Area if submitted under an inappropriate Topic Area. The FY18 PRMRP Topic Areas are listed below.

- Acute Lung Injury
- Antimicrobial Resistance
- Arthritis
- Burn Pit Exposure
- Cardiomyopathy
- Cerebellar Ataxia

- Chronic Migraine and Post-Traumatic Headache
- Chronic Pain Management
- Congenital Heart Disease
- Constrictive Bronchiolitis
- Diabetes
- Dystonia
- Eating Disorders
- Emerging Infectious Diseases
- Endometriosis
- Epidermolysis Bullosa
- Focal Segmental Glomerulosclerosis
- Fragile X
- Frontotemporal Degeneration
- Guillain-Barré Syndrome
- Hepatitis B and C
- Hereditary Angioedema
- Hydrocephalus
- Immunomonitoring of Intestinal Transplants
- Inflammatory Bowel Diseases
- Interstitial Cystitis
- Lung Injury
- Malaria
- Metals Toxicology
- Mitochondrial Disease
- Musculoskeletal Disorders
- Myotonic Dystrophy
- Non-Opioid Pain Management
- Nutrition Optimization
- Pancreatitis
- Pathogen-Inactivated Blood Products
- Post-Traumatic Osteoarthritis
- Pressure Ulcers
- Pulmonary Fibrosis
- Respiratory Health
- Rett Syndrome
- Rheumatoid Arthritis
- Scleroderma
- Sleep Disorders
- Spinal Muscular Atrophy
- Sustained-Release Drug Delivery
- Tinnitus
- Tissue Regeneration
- Tuberculosis
- Vaccine Development for Infectious Diseases
- Vascular Malformations
- Women's Heart Disease

Research relevant to one or more of the FY18 PRMRP Topic Areas may be considered for funding. ***Applicants should select the FY18 Program Announcement most appropriate to the stage of the proposed research.*** Areas of Encouragement related to the FY18 PRMRP Topic Areas have been identified by the Department of Defense (DoD), the Department of Veterans Affairs (VA), and other relevant stakeholders (Appendix 2). Applicants are urged to read and consider these Areas of Encouragement before preparing their applications. ***The information provided is not exhaustive, and applicants are not restricted to submitting applications that address an Area of Encouragement on this list.***

II.B. Award Information

The PRMRP Investigator-Initiated Research Award (IIRA) is intended to support studies that will make an important contribution toward research and/or patient care for a disease or condition related to at least one of the FY18 PRMRP Topic Areas.

The rationale for a research idea may be derived from a laboratory discovery, population-based studies, a clinician's first-hand knowledge of patients, or anecdotal data. Applications must include relevant data that support the rationale for the proposed study. These data may be unpublished or from the published literature.

Impact: The Investigator-Initiated Research Award is designed to support research with the potential to yield highly impactful data that could lead to critical discoveries or major advancements. It is the responsibility of the Principal Investigator (PI) to clearly articulate the project's potential immediate and long-range outcome(s)/product(s) (knowledge and/or materiel) and how they will impact a central critical problem or question in the field of research and/or patient care in the FY18 PRMRP Topic Area(s) addressed.

Research projects may focus on any phase of research from basic laboratory research through translational research, including preclinical studies in animal models and human subjects, as well as correlative studies associated with an existing clinical trial. Research involving human subjects and human anatomical substances is permitted; ***however, this award may not be used to conduct clinical trials.*** A clinical trial is defined as a prospective accrual of patients (human subjects) in whom an intervention (e.g., device, drug, biologic, surgical procedure, rehabilitative modality, behavioral intervention, or other) is tested for a measurable outcome with respect to safety, effectiveness, and/or efficacy. This outcome represents a direct effect on the subject of that intervention or interaction. For more information on how to distinguish clinical research from clinical trials, see the Human Subject Resource Document at https://ebrap.org/eBRAP/public/Program.htm. PIs seeking funding for a clinical trial should apply to the FY18 PRMRP Clinical Trial Award mechanism (Funding Opportunity Number: W81XWH18PRMRPCTA).

The anticipated direct costs budgeted for the entire period of performance for a single PI FY18 PRMRP IIRA award will not exceed **$1.2M**. The anticipated direct costs budgeted for the entire period of performance for an FY18 PRMRP IIRA award with the Partnering PI Option will not exceed **$1.5M**. Refer to Section II.D.5, Funding Restrictions, for detailed funding information.

Partnering PI Option: As a method to bring new perspectives in addressing the critical problem or question and facilitate progress in the field through collaborative efforts, the FY18 PRMRP is offering a Partnering PI Option for this award mechanism. The results of this partnering project should significantly advance the research beyond what would be possible through individual efforts. The Partnering PI Option is structured so that two investigators, each of whom will be designated as a PI, work synergistically on a single project. Each PI should bring complementary skills and perspectives to the research project. Developing the research plan should involve a reciprocal flow of ideas and information between the partners. The application should clearly demonstrate that both PIs have equal intellectual input into the design of the project and will devote equivalent levels of effort to the conduct of the project. Each PI must demonstrate that they possess the research experience and resources to function as a PI and must also exhibit an appropriate level of authority and responsibility to direct the project supported by the awards. ***New and multi-institutional collaborative efforts are strongly encouraged.*** PIs should include plans for communication between investigators at different organizations, if applicable. Additionally, participating organizations must be willing to resolve potential intellectual and material property issues and to remove any barriers that might interfere with achieving high levels of cooperation to ensure successful completion of the proposed research project. As noted above, collaborations with DoD or VA researchers and clinicians are encouraged.

For the application process, one PI will be identified as the Initiating PI and will be responsible for the majority of the administrative tasks associated with application submission. The other PI will be identified as the Partnering PI. Initiating and Partnering PIs each have different submission requirements as described in Section II.D.2.b, Full Application Submission Content. If recommended for funding, each PI will receive their own award. ***A separate application submission is required for each partner, even if both PIs are at the same organization.*** Additional collaborators may be included in the application without being designated as PIs.

Applications submitted under the Partnering PI Option are allowed to request a higher direct cost budget than applications submitted with a single PI (see Section II.D.5, Funding Restrictions, for details). Costs should be divided as evenly as possible between both partners unless appropriately justified, but no more than 75% (or no less than 25%) of the proposed direct costs may be requested by a single partner. Submission of a Partnership Statement, Attachment 8, is required for applications submitted under the Partnering PI Option.

The proposed research must be relevant to active duty Service members, Veterans, military beneficiaries, and/or the American public.

Military Relevance: Relevance to the healthcare needs of military Service members, Veterans, and beneficiaries is a key feature of this award. Investigators are encouraged to consider the following characteristics as examples of how a project may demonstrate military relevance:

- Explanation of how the project addresses an aspect of the target disease/condition that has direct relevance to military Service members, Veterans, or other Military Health System beneficiaries

- Description of how the knowledge, information, products, or technologies gained from the proposed research could be implemented in a dual-use capacity to benefit the civilian population and also address a military need

- Use of military or Veteran populations or datasets in the proposed research, if appropriate to the proposed research project

- Involvement of military consultants (Army, Air Force) or specialty leaders (Navy, Marine Corps) to the Surgeons General in a relevant specialty area

PIs are encouraged to integrate and/or align their research projects with DoD and/or VA research laboratories and programs. Collaboration with DoD or VA investigators is also encouraged. A list of websites that may be useful in identifying additional information about ongoing DoD and VA areas of research interest or potential opportunities for collaboration within the FY18 PRMRP Topic Areas can be found in Appendix 3.

Use of DoD or VA Resources: If the proposed research involves access to active duty military patient populations and/or DoD resources or databases, the PI is responsible for demonstrating such access at the time of application submission and should develop a plan for maintaining access as needed throughout the proposed research. Access to target active duty military patient population(s) and/or DoD resource(s) or database(s) should be confirmed by including a letter of support, signed by the lowest-ranking person with approval authority.

If the proposed research involves access to VA patient populations, VA study resources and databases, and/or VA research space and equipment, VA PIs must have a plan for obtaining and maintaining access throughout the proposed research. Access to VA patients, resources, and/or VA research space should be confirmed by including a letter of support from the VA Facility Director(s) or individual designated by the VA Facility Director(s), such as the Associate Chief of Staff for Research and Development (ACOS/R&D) or Clinical Service Chief. If appropriate, the application should identify the VA-affiliated non-profit corporation (NPC) as the applicant institution for VA PIs. If the VA NPC is not identified as the applicant institution for administering the funds, the application should include a letter from the VA ACOS/R&D confirming this arrangement and identifying the institution that will administer the funds associated with the proposed research.

Access to certain DoD or VA patient populations, resources, or databases may only be obtained by collaboration with a DoD or VA investigator who has a substantial role in the research and may not be available to a non-DoD or non-VA investigator if the resource is restricted to DoD or VA personnel. Investigators should be aware of which resources are available to them if the proposed research involves a non-DoD or non-VA investigator collaborating with the DoD and/or VA. If access cannot be confirmed at the time of application submission, the Government reserves the right to withdraw or revoke funding until the PI has demonstrated support for and access to the relevant population(s) and/or resource(s). Refer to Section II.D.2.b.ii, Full Application Submission Components, for detailed information.

Research Involving Human Anatomical Substances, Human Subjects, or Human Cadavers: All DoD-funded research involving new and ongoing research with human

anatomical substances, human subjects, or human cadavers must be reviewed and approved by the U.S. Army Medical Research and Materiel Command (USAMRMC) Office of Research Protections (ORP), Human Research Protection Office (HRPO), prior to research implementation. This administrative review requirement is in addition to the local Institutional Review Board (IRB) or Ethics Committee (EC) review. Local IRB/EC approval at the time of submission is ***not*** required. The HRPO is mandated to comply with specific laws and requirements governing all research involving human anatomical substances, human subjects, or human cadavers that is supported by the DoD. These laws and requirements will necessitate information in addition to that supplied to the IRB/EC. ***Allow a minimum of 2 to 3 months for HRPO regulatory review and approval processes***. When possible, protocols should be written for research with human subjects and/or human anatomical substances that are specific to the DoD-supported effort outlined in the submitted application. Submission to HRPO of protocols covering more than the scope of work in the DoD-funded award will require HRPO review of the entire protocol as DoD-supported research and may include extensive modifications to meet DoD human subjects protection requirements. Refer to the General Application Instructions, Appendix 1, and the Human Subject Resource Document available on the electronic Biomedical Research Application Portal (eBRAP) "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) for additional information.

A clinical trial is defined as a prospective accrual of patients (human subjects) in whom an intervention (e.g., device, drug, biologic, surgical procedure, rehabilitative modality, behavioral intervention, or other) is tested for a measurable outcome with respect to safety, effectiveness, and/or efficacy. This outcome represents a direct effect on the subject of that intervention or interaction.

Research Involving Animals: All projects should adhere to a core set of standards for rigorous study design and reporting to maximize the reproducibility and translational potential of preclinical research. The standards are described in Landis, S.C., et al. A call for transparent reporting to optimize the predictive value of preclinical research. *Nature* 2012, 490:187-191 (www.nature.com/nature/journal/v490/n7419/full/nature11556.html). While these standards are written for preclinical studies, the basic principles of randomization, blinding, sample-size estimation, and data handling derive from well-established best practices in clinical studies. Applicants should consult the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines to ensure relevant aspects of rigorous animal research are adequately planned for and, ultimately, reported. The ARRIVE guidelines can be found at http://www.elsevier.com/__data/promis_misc/622936arrive_guidelines.pdf.

All DoD-funded research involving new and ongoing research with animals must be reviewed and approved by the USAMRMC ORP Animal Care and Use Review Office (ACURO), in addition to the local Institutional Animal Care and Use Committee (IACUC) of record. IACUC approval at the time of submission is ***not*** required. Specific documents relating to the use of animals in the proposed research will be requested **if the application is selected for funding**. The ACURO must review and approve all animal use prior to the start of working with animals, including amendments to ongoing projects. PIs must submit the institutional animal use protocol, IACUC approval of that protocol, and a version of the animal use appendix titled, "Research Involving Animals." ***Allow at least 2 to 3 months for ACURO regulatory review and***

approval processes for animal studies. Refer to the General Application Instructions, Appendix 1, for additional information.

The types of awards made under the Program Announcement will be assistance agreements (grants or cooperative agreements). The level of involvement on the part of the DoD during project performance is the key factor in determining whether to award a grant or cooperative agreement.

Extramural Organizations: An assistance agreement (grant or cooperative agreement) is appropriate when the Federal Government transfers a "thing of value" to a "state, local government," or "other recipient" to carry out a public purpose of support or stimulation authorized by a law of the United States, instead of acquiring property or service for the direct benefit and use of the U.S. Government. An assistance agreement can take the form of a grant or cooperative agreement. If "no substantial involvement" on the part of the funding agency is anticipated, a grant award will be made (31 USC 6304). Conversely, if substantial involvement on the part of the funding agency is anticipated, a cooperative agreement will be made (31 USC 6305) and the award will identify the specific substantial involvement. Substantial involvement may include collaboration, participation, or intervention in the research to be performed under the award. The award type, along with the start date, will be determined during the negotiation process.

The CDMRP intends that information, data, and research resources generated under awards funded by this Program Announcement be made available to the research community (which includes both scientific and consumer advocacy communities) and to the public at large. For additional guidance, refer to the General Application Instructions, Appendix 2, Section K.

Awards will be made no later than September 30, 2019. For additional information refer to Section II.F.1, Federal Award Notices.

II.C. Eligibility Information

II.C.1. Eligible Applicants

II.C.1.a. Organization: All organizations, including international organizations, are eligible to apply.

Government Agencies Within the United States: Local, state, and Federal Government agencies are eligible to the extent that applications do not overlap with their fully funded internal programs. Such agencies are required to explain how their applications do not overlap with their internal programs.

As applications for this Program Announcement may be submitted by extramural and intramural organizations, these terms are defined below.

Extramural Organization: An eligible non-DoD organization. Examples of extramural organizations include academic institutions, biotechnology companies, foundations, Government, and research institutes.

Intramural DoD Organization: A DoD laboratory, DoD military treatment facility, and/or DoD activity embedded within a civilian medical center.

Note: Applications from an intramural DoD organization or from an extramural Federal organization may be submitted through a research foundation.

The USAMRAA makes awards to eligible organizations, not to individuals.

II.C.1.b. Principal Investigator

- Investigators at or above the level of Assistant Professor (or equivalent) are eligible to submit applications.
    - **Partnering PI Option:** Investigators at or above the level of Assistant Professor (or equivalent) that bring complementary skills and perspectives to the research project.

An eligible PI, regardless of ethnicity, nationality, or citizenship status, must be employed by, or affiliated with, an eligible organization.

The CDMRP encourages all PIs to participate in a digital identifier initiative through Open Researcher and Contributor ID, Inc. (ORCID). Registration for a unique ORCID identifier can be done online at http://orcid.org/.

II.C.2. Cost Sharing

Cost sharing/matching is not an eligibility requirement.

II.C.3. Other

Organizations must be able to access **.gov** and **.mil** websites in order to fulfill the financial and technical deliverable requirements of the award and submit invoices for payment.

There are no limitations on the number of applications for which an investigator may be named as a PI or Partnering PI.

For general information on required qualifications for award recipients, refer to the General Application Instructions, Appendix 3.

Refer to Section II.H.2, Administrative Actions, for a list of administrative actions that may be taken if a pre-application or application does not meet the administrative, eligibility, or ethical requirements defined in this Program Announcement.

II.D. Application and Submission Information

Submission of applications that are essentially identical or propose essentially the same research project to different funding opportunities within the same program and fiscal year is prohibited and will result in administrative withdrawal of the duplicative application(s). As an exception, applicants may submit the research project described in their Investigator-Initiated

Research Award application as part of an application to the FY18 PRMRP Focused Program Award (Funding Opportunity Number: W81XWH18PRMRPFPA); however, accepting multiple awards to support the same project will not be allowed.

Extramural Submission is defined as an application submitted by an organization to Grants.gov.

Intramural DoD Submission is defined as an application submitted by a DoD organization to eBRAP.

II.D.1. Address to Request Application Package

eBRAP is a multifunctional web-based system that allows PIs to submit their pre-applications electronically through a secure connection, to view and edit the content of their pre-applications and full applications, to receive communications from the CDMRP, and to submit documentation during award negotiations and period of performance.

Extramural Submissions: Pre-application content and forms must be accessed and submitted at eBRAP.org. Full application packages must be accessed and submitted at Grants.gov.

Intramural DoD Submissions: Pre-application content and forms and full application packages must be accessed and submitted at eBRAP.org.

Contact information for the CDMRP Help Desk and the Grants.gov Contact Center can be found in Section II.G, Federal Awarding Agency Contacts.

II.D.2. Content and Form of the Application Submission

Submission is a two-step process requiring both ***pre-application*** and ***full application*** as indicated below. The submission process should be started early to avoid missing deadlines. There are no grace periods.

Pre-Application Submission: All pre-applications for both extramural and intramural organizations must be submitted through eBRAP (https://eBRAP.org/).

Full Application Submission: Full applications must be submitted through the online portals as described below.

Submitting Extramural Organizations: Full applications from extramural organizations must be submitted through a Grants.gov Workspace. Applications submitted by extramural organizations (e.g., research foundations) on behalf of intramural DoD or other Federal organizations or investigators will be considered extramural submissions. Applications from extramural organizations, including non-DoD Federal organizations, received through eBRAP will be withdrawn. See definitions in Section II.C.1, Eligible Applicants.

Submitting Intramural DoD Organizations: Intramural DoD organizations may submit full applications to either eBRAP or Grants.gov. Intramural DoD organizations that are unable to submit to Grants.gov should submit through eBRAP. Intramural DoD organizations with the

capability to submit through Grants.gov may submit following the instructions for extramural submissions through Grants.gov or may submit to eBRAP.

For Both Extramural and Intramural Applicants: A key feature of eBRAP is the ability of an organization's representatives and PIs to view and modify the full application submissions associated with them. eBRAP will validate full application files against the specific Program Announcement requirements, and discrepancies will be noted in an email to the PI and in the "Full Application Files" tab in eBRAP. It is the applicant's responsibility to review all application components for accuracy as well as ensure proper ordering as specified in this Program Announcement.

The application title, eBRAP log number, and all information for the PI, Business Official(s), performing organization, and contracting organization must be consistent throughout the entire pre-application and full application submission process. Inconsistencies may delay application processing and limit or negate the ability to view, modify, and verify the application in eBRAP. If any changes need to be made, the applicant should contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507 prior to the application submission deadline.

The Investigator-Initiated Research Award mechanism is structured to accommodate two PIs under the Partnering PI Option. One PI will be identified as the Initiating PI and will be responsible for the majority of the administrative tasks associated with application submission. The other PI will be identified as the Partnering PI. Initiating and Partnering PIs each have different submission requirements; however, both PIs should contribute significantly to the development of the proposed research project, including the Project Narrative, Statement of Work, and other required components.

The Initiating PI must complete the pre-application submission process and submit the contact information for each Partnering PI. The Partnering PI will then be notified of the pre-application submission separately by email. ***The Partnering PI must follow the link in the notification email in order to associate his/her full application package with that of the Initiating PI. After following the link, the Partnering PI must verify his/her contact information, organization, and designation as an extramural or intramural submission within eBRAP.*** If not previously registered, the Partnering PI must register in eBRAP. A new pre-application based on this research project should not be initiated by the Partnering PI. Do not delay completing these steps. If they are not completed, the Partnering PI will not be able to view and modify his/her application during the verification period in eBRAP. If these steps are not completed, an intramural partner will not be able to submit the Partnering PI required full application package components to eBRAP.

II.D.2.a. Step 1: Pre-Application Submission Content

During the pre-application process, each submission is assigned a unique log number by eBRAP. This unique eBRAP log number is required during the full application submission process.

To begin the pre-application process, first select whether the submitting organization is extramural or intramural, then confirm your selection or cancel. **Incorrect selection of extramural or intramural submission type will delay processing.**

If an error has been made in the selection of extramural versus intramural and the pre-application submission deadline has passed, the PI or Business Official must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507 to request a change in designation.

All pre-application components must be submitted by the PI (for single PI applicants) or Initiating PI (for applicants submitting under the Partnering PI Option) through eBRAP (https://eBRAP.org/). Because the invitation to submit an application is based on the contents of the pre-application, investigators should not change the title or research objectives after the pre-application is submitted.

PIs and organizations identified in the pre-application should be the same as those intended for the subsequent application submission. If any changes are necessary after submission of the pre-application, the PI must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

PIs with an ORCID identifier should enter that information in the appropriate field in the "My Profile" tab in the "Account Information" section of eBRAP.

The pre-application consists of the following components, which are organized in eBRAP by separate tabs (refer to the General Application Instructions, Section II.B, for additional information on pre-application submission):

- **Tab 1 – Application Information**

  Submission of application information includes assignment of primary and secondary research classification codes, which may be found at https://ebrap.org/eBRAP/public/Program.htm. Note that the codes have recently been revised. Applicants are strongly encouraged to review and confirm the codes prior to making their selection.

  Select the FY18 PRMRP Topic Area addressed by the proposed research. If the proposed research project is aligned with more than one FY18 PRMRP Topic Area, select the Topic Area of highest relevance as the required first choice.

- **Tab 2 – Application Contacts**

  Enter contact information for the PI. Enter the organization's Business Official responsible for sponsored program administration (the "person to be contacted on matters involving this application" in Block 5 of the Grants.gov SF424 (R&R) Form). The Business Official must

be either selected from the eBRAP list or invited in order for the pre-application to be submitted.

Select the performing organization (site at which the PI will perform the proposed work) and the contracting organization (organization submitting on behalf of the PI, which corresponds to Block 5 on the Grants.gov SF424 (R&R) Form), and click on "Add Organizations to this Pre-application." The organization(s) must be either selected from the eBRAP drop-down list or invited in order for the pre-application to be submitted.

It is recommended that PIs identify an Alternate Submitter in the event that assistance with pre-application submission is needed.

- **Tab 3 – Collaborators and Key Personnel**

  Enter the name, organization, and role of all collaborators and key personnel associated with the application. The Initiating PI must enter the contact information for the Partnering PI in the Partnering PI section.

  FY18 PRMRP Programmatic Panel members should not be involved in any pre-application or application. For questions related to panel members and pre-applications or applications, refer to Section II.H.2.c, Withdrawal, or contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

  To preserve the integrity of its peer and programmatic review processes, the CDMRP discourages inclusion of any employee of its review contractors having any role in pre-application or application preparation, research, or other duties for submitted pre-applications or applications. For FY18, the identities of the peer review contractor and the programmatic review contractor may be found at the CDMRP website (http://cdmrp.army.mil/about/2tierRevProcess). Pre-applications or applications that include names of personnel from either of these companies will be administratively withdrawn unless plans to manage conflicts of interest (COIs) are provided and deemed appropriate by the Grants Officer. Refer to the General Application Instructions, Appendix 3, for detailed information.

- **Tab 4 – Conflicts of Interest**

  List all individuals other than collaborators and key personnel who may have a COI in the review of the application (including those with whom the PI has a personal or professional relationship). Refer to the General Application Instructions, Appendix 3, Section C, for further information regarding COIs.

- **Tab 5 – Pre-Application Files**

  Note: Upload documents as individual PDF files unless otherwise noted. eBRAP will not allow a file to be uploaded if the number of pages exceeds the limit specified below.

  - **Preproposal Narrative (four-page limit):** The Preproposal Narrative page limit applies to text and non-text elements (e.g., figures, tables, graphs, photographs, diagrams, chemical structures, drawings) used to describe the project. Inclusion of URLs that

provide additional information to expand the Preproposal Narrative and could confer an unfair competitive advantage is prohibited and may result in administrative withdrawal of the pre-application.

The Preproposal Narrative should include the following:

- **Topic Area:** Describe how the proposed project relates to at least one of the FY18 PRMRP Topic Areas. If applicable, describe how the proposed research project addresses an FY18 PRMRP Area of Encouragement (Appendix 2).
- **Research Idea:** Clearly articulate the rationale for the project by presenting the ideas and reasoning behind the proposed research; include relevant literature citations.
- **Research Strategy:** State the hypothesis to be tested and the objective(s) to be reached. State the project's specific aims and briefly describe the experimental approach. If applying under the Partnering PI Option, describe how the collaborative effort will provide synergy and enhance the research.
- **Impact:** Describe the potential short-term and long-term impact of the results of the proposed study on at least one of the FY18 PRMRP Topic Areas, and its related research field(s) and/or patient population(s).
- **Military Relevance:** Explain how the project is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

- **Pre-Application Supporting Documentation:** The items to be included as supporting documentation for the pre-application ***must be uploaded as individual files*** and are limited to the following:
  - References Cited (one-page limit): List the references cited (including URLs if available) in the Preproposal Narrative using a standard reference format that includes the full citation (i.e., author[s], year published, reference title, and reference source, including volume, chapter, page numbers, and publisher, as appropriate).
  - List of Abbreviations, Acronyms, and Symbols: Provide a list of abbreviations, acronyms, and symbols used in the Preproposal Narrative.
  - Key Personnel Biographical Sketches (five-page limit per individual): ***All biographical sketches should be uploaded as a single combined file.*** Biographical sketches should be used to demonstrate background and expertise through education, positions, publications, and previous work accomplished.

- **Tab 6 – Submit Pre-Application**

  This tab must be completed for the pre-application to be accepted and processed.

Pre-Application Screening

- **Pre-Application Screening Criteria**

  To determine the technical merits of the pre-application and the relevance to the mission of the DHP and the PRMRP, pre-applications will be screened based on the following criteria:

  - **Research Idea:** How well the rationale for the research idea is supported by presented background information.

  - **Research Strategy:** How well the specific aims and proposed methodology support the research idea and objectives. If applying under the Partnering PI Option, how the partnership will advance the research beyond what would be possible through individual efforts.

  - **Impact:** Whether the potential immediate and long-range outcome(s)/product(s) (knowledge and/or materiel) of the proposed research, if successful, will impact a central critical problem or question in the field of research and/or patient care in the FY18 PRMRP Topic Area(s) addressed.

  - **Programmatic Relevance:** Whether the proposed research idea supports the objectives of the PRMRP. To what degree the project is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

- **Notification of Pre-Application Screening Results**

  Following the pre-application screening, PIs (or Initiating PIs, if applying under the Partnering PI Option) will be notified as to whether or not they are invited to submit applications; however, they will not receive feedback (e.g., a critique of strengths and weaknesses) on their pre-application. The estimated timeframe for notification of invitation to submit an application is indicated in Section I, Overview of the Funding Opportunity. Invitations to submit a full application are based on the Pre-Application Screening Criteria listed above.

II.D.2.b. Step 2: Full Application Submission Content

Applications will not be accepted unless the PI or Initiating PI has received notification of invitation.

The CDMRP cannot make allowances/exceptions to its policies for submission problems encountered by the applicant organization using system-to-system interfaces with Grants.gov.

Each application submission must include the completed full application package for this Program Announcement. The full application package is submitted by the Authorized Organizational Representative through Grants.gov (http://www.grants.gov/) for extramural organizations or through eBRAP (https://ebrap.org/) for intramural organizations. See Table 1 below for more specific guidelines.

II.D.2.b.i. Full Application Guidelines

Extramural organizations must submit full applications through Grants.gov. Applicants must create a Grants.gov Workspace for submission, which allows the application components to be completed online and routed through the applicant organization for review prior to submission. Applicants may choose to download and save individual PDF forms rather than filling out webforms in the Workspace. A compatible version of Adobe Reader **must** be used to view, complete, and submit an application package consisting of PDF forms. If more than one person is entering text into an application package, the ***same version*** of Adobe Reader software should be used by each person. Check the version number of the Adobe software on each user's computer to make sure the versions match. Using different versions of Adobe Reader may cause submission and/or save errors – even if each version is individually compatible with Grants.gov. Refer to the General Application Instructions, Section III, and the "Apply For Grants" page of Grants.gov (https://www.grants.gov/web/grants/applicants/apply-for-grants.html) for further information about the Grants.gov Workspace submission process. Submissions of extramural applications through eBRAP may be withdrawn.

Table 1. Full Application Submission Guidelines

Extramural Submissions	Intramural DoD Submissions
Application Package Location	
Download application package components for W81XWH18PRMRPIIRA from Grants.gov (http://www.grants.gov) and create a Grants.gov Workspace. The Workspace allows online completion of the application components and routing of the application package through the applicant organization for review prior to submission.	Download application package components for W81XWH18PRMRPIIRA from eBRAP (https://ebrap.org).
Full Application Package Components	
SF424 (R&R) Application for Federal Assistance Form: Refer to the General Application Instructions, Section III.A.1, for detailed information.	**Tab 1 – Summary:** Provide a summary of the application information. **Tab 2 – Application Contacts:** This tab will be pre-populated by eBRAP; add Authorized Organizational Representative.
Descriptions of each required file can be found under Full Application Submission Components: • Attachments • Research & Related Personal Data • Research & Related Senior/Key Person Profile (Expanded) • Research & Related Budget • Project/Performance Site Location(s) Form	**Tab 3 – Full Application Files:** Upload files under each Application Component in eBRAP. Descriptions of each required file can be found under Full Application Submission Components: • Attachments • Key Personnel • Budget • Performance Sites

Extramural Submissions	Intramural DoD Submissions
• R&R Subaward Budget Attachment(s) Form (if applicable)	**Tab 4 – Application and Budget Data:** Review and edit proposed project start date, proposed end date, and budget data pre-populated from the Budget Form.
Application Package Submission	
Create a Grants.gov Workspace. Add participants (investigators and Business Officials) to the Workspace, complete all required forms, and check for errors before submission. **Submit a Grants.gov Workspace Package.** An application may be submitted through Workspace by clicking the "Sign and Submit" button on the "Manage Workspace" page, under the "Forms" tab. Grants.gov recommends submission of the application package **at least 24-48 hours prior to the close date** to allow time to correct any potential technical issues that may disrupt the application submission. Note: If either the Project Narrative or the budget fails eBRAP validation or if the Project Narrative or the budget needs to be modified, an updated Grants.gov application package must be submitted via Grants.gov as a "Changed/Corrected Application" with the previous Grants.gov Tracking ID prior to the application submission deadline.	**Submit package components to eBRAP (https://ebrap.org).** **Tab 5 – Submit/Request Approval Full Application:** After all components are uploaded and prior to the full application submission deadline, enter your password in the space provided next to "Enter Your Password Here" and press the "Submit Full Application" button. eBRAP will notify your Resource Manager/Comptroller/Task Area Manager or equivalent Business Official by email.

Extramural Submissions	Intramural DoD Submissions
Application Verification Period	
The full application package submitted to Grants.gov may be viewed and modified in eBRAP until the end of the application verification period. During the application verification period, the full application package, ***with the exception of the Project Narrative and Budget Form,*** may be modified.	After eBRAP has processed the full application, the organizational Resource Manager/Comptroller/Task Area Manager or equivalent Business Official and PI(s) will receive email notification of this status and will be able to view and modify application components in eBRAP. During the application verification period, the full application package, ***with the exception of the Project Narrative and Budget Form,*** may be modified. Your Resource Manager/Comptroller/Task Area Manager or equivalent Business Official should log into eBRAP to review and to approve prior to the application verification deadline.
Further Information	
Tracking a Grants.gov Workspace Package. After successfully submitting a Workspace package, a Grants.gov Tracking Number is automatically assigned to the package. The number will be listed on the "Confirmation" page that is generated after submission. Refer to the General Application Instructions, Section III, for further information regarding Grants.gov requirements.	Refer to the General Application Instructions, Section IV, for further information regarding eBRAP requirements.

Application viewing, modification, and verification in eBRAP are strongly recommended, but not required. ***The Project Narrative and Budget cannot be changed after the application submission deadline.*** Prior to the full application deadline, a corrected or modified full application package may be submitted. Other application components may be changed until the end of the application verification period. Verify that subaward budget(s) and budget justification forms are present in eBRAP during the application verification period. If these components are missing, upload them to eBRAP before the end of the application verification period. After the end of the application verification period, the full application cannot be modified.

Material submitted after the end of the application verification period, unless specifically requested by the Government, will not be forwarded for processing.

Partnering PI Option: The CDMRP requires separate full application package submissions for the Initiating PI and the Partnering PI, even if the PIs are located within the same organization. Initiating and Partnering PIs will each be assigned a unique eBRAP log number. Each full

application package must be submitted using the unique eBRAP log number. ***Note: All associated applications (Initiating and Partnering PIs') must be submitted by the full application submission deadline.***

The full application package must be submitted using the unique eBRAP log number to avoid delays in application processing.

II.D.2.b.ii. Full Application Submission Components

- **Extramural Applications Only**

  SF424 (R&R) Application for Federal Assistance Form: Refer to the General Application Instructions, Section III.A.1, for detailed information.

- **Extramural and Intramural Applications**

  Attachments:

  Each attachment to the full application components must be uploaded as an individual file in the format specified and in accordance with the formatting guidelines listed in the General Application Instructions, Appendix 4.

  For all attachments, ensure that the file names are consistent with the guidance. Attachments will be rejected if the file names are longer than 50 characters or have incorrect file names that contain characters other than the following: A-Z, a-z, 0-9, underscore, hyphen, space, and period. In addition, there are file size limits that may apply in some circumstances. Individual attachments may not exceed 20 MB, and the file size for the entire full application package may not exceed 200 MB.

  - **Attachment 1: Project Narrative (15-page limit):** Upload as "ProjectNarrative.pdf." The page limit of the Project Narrative applies to text and non-text elements (e.g., figures, tables, graphs, photographs, diagrams, chemical structures, drawings) used to describe the project. Inclusion of URLs that provide additional information to expand the Project Narrative and could confer an unfair competitive advantage is prohibited and may result in administrative withdrawal of the application.

    Describe the proposed project in detail using the outline below.

    - **Background:** Describe how the proposed research project addresses one or more of the FY18 PRMRP Topic Areas. Present the ideas and reasoning behind the proposed work. Cite relevant literature. Describe previous experience most pertinent to the project. Include relevant preliminary data; these data may be unpublished or from the published literature.

    - **Hypothesis:** State the hypothesis to be tested.

    - **Specific Aims:** Concisely explain the project's specific aims and the objective(s) to be reached. These aims should agree with the primary aims and associated tasks

described in the Statement of Work. If the proposed work is part of a larger study, present only aims that this DoD award would fund.

- **Research Strategy:** Describe the experimental design, methods, and analyses, including appropriate controls, in sufficient detail for analysis. Address potential problem areas and present alternative methods and approaches. Clearly describe the statistical plan and the rationale for the statistical methodology as well as an appropriate power analysis, if applicable. If animal studies are proposed, describe how they will be conducted in accordance with the ARRIVE guidelines (http://www.elsevier.com/__data/promis_misc/622936arrive_guidelines.pdf).

- If human subjects or human biological samples will be used, describe the study population and include a detailed plan for the recruitment of human subjects or the acquisition of samples. Describe the availability of the proposed study population and past successes in recruiting similar populations. If active duty military, military families, and/or Veteran population(s) or datasets will be used in the proposed research project, describe the feasibility of accessing the population(s)/dataset(s). ***Clinical trials are not allowed under the Investigator-Initiated Research Award.***

- If applicable, describe how data will be reported and how it will be assured that the documentation will support a regulatory filing with the U.S. Food and Drug Administration (FDA).

- **Attachment 2: Supporting Documentation:** Combine and upload as a single file named "Support.pdf." Start each document on a new page. If documents are scanned to PDF, the lowest resolution (100 to 150 dpi) should be used. The Supporting Documentation attachment should not include additional information such as figures, tables, graphs, photographs, diagrams, chemical structures, or drawings. These items should be included in the Project Narrative.

  There are no page limits for any of these components unless otherwise noted. Include only those components described below; inclusion of items not requested or viewed as an extension of the Project Narrative will result in the removal of those items or may result in administrative withdrawal of the application.

  References Cited: List the references cited (including URLs, if available) in the Project Narrative using a standard reference format that includes the full citation (i.e., author[s], year published, title of reference, source of reference, volume, chapter, page numbers, and publisher, as appropriate).

  List of Abbreviations, Acronyms, and Symbols: Provide a list of abbreviations, acronyms, and symbols.

  Facilities, Existing Equipment, and Other Resources: Describe the facilities and equipment available for performance of the proposed project and any additional facilities or equipment proposed for acquisition at no cost to the award. Indicate whether or not Government-furnished facilities or equipment are proposed for use. If so, reference should be made to the original or present Government award under

which the facilities or equipment items are now accountable. There is no form for this information.

Publications and/or Patents: Include a list of relevant publication URLs and/or patent abstracts. If publications are not publicly available, then copies of up to five published manuscripts may be included in Attachment 2. Extra items will not be reviewed.

Letters of Organizational Support: Provide a letter (or letters, if applicable) signed by the Department Chair or appropriate organization official, confirming the laboratory space, equipment, and other resources available for the project. Letters of support not requested in the Program Announcement, such as those from members of Congress, do not impact application review or funding decisions.

Letters of Collaboration: Provide a signed letter from each collaborating individual or organization that will demonstrate that the PI has the support or resources necessary for the proposed work. If an investigator at an intramural organization is named as a collaborator on an application submitted through an extramural organization, the application must include a letter from the collaborator's Commander or Commanding Officer at the intramural organization that authorizes the collaborator's involvement.

Use of Military Resources (if applicable): If the proposed research plan involves access to active duty military patient populations or resources, the PI is responsible for demonstrating such access. Provide a letter of support signed by the lowest-ranking person with approval authority confirming access to active duty military patient populations and/or DoD resources or databases.

Use of Veterans Affairs Resources (if applicable): Provide a letter of support from the VA Facility Director(s) or individual designated by the VA Facility Director(s), such as the ACOS/R&D or Clinical Service Chief confirming access to VA patients, resources, and/or VA research space. For VA PIs, if the VA NPC is not identified as the applicant institution for administering the funds, include a letter from the VA ACOS/R&D confirming this arrangement and identifying the institution that will administer the funds associated with the proposed research.

Intellectual Property: Information can be found in Code of Federal Regulations, Title 2, Part 200.315 (2 CFR 200.315), "Intangible Property."

- Intellectual and Material Property Plan (if applicable): Provide a plan for resolving intellectual and material property issues among participating organizations.

- Commercialization Strategy (if applicable): Describe the commercialization plan. The plan should include intellectual property, market size, financial analysis, strengths and weaknesses, barriers to the market, competitors, and management team. Discuss the significance of this development effort, when it can be anticipated, and the potential commercial use for the technology being developed.

Data and Research Resources Sharing Plan: Describe how data and resources generated during the performance of the project will be shared with the research community. Refer to the General Application Instructions, Appendix 2, Section K, for more information about the CDMRP expectations for making data and research resources publicly available.

- **Attachment 3: Technical Abstract (one-page limit):** Upload as "TechAbs.pdf." The technical abstract is used by all reviewers. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information.*** Use only characters available on a standard QWERTY keyboard. Spell out all Greek letters, other non-English letters, and symbols. Graphics are not allowed.

  The technical abstract is used by all reviewers. Of particular importance, programmatic reviewers typically do not have access to the full application and therefore rely on the technical abstract for appropriate description of the project's key aspects. Therefore, clarity and completeness within the space limits of the technical abstract are highly important.

  Describe the proposed research project, including the following elements: background, rationale, hypothesis, specific aims and objectives, study design, long-term and short-term impact to the relevant research field and patient population(s), and the relevance of the project to at least one FY18 PRMRP Topic Area.

- **Attachment 4: Lay Abstract (one-page limit):** Upload as "LayAbs.pdf." The lay abstract is used by all reviewers. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information***. Use only characters available on a standard QWERTY keyboard. Spell out all Greek letters, other non-English letters, and symbols. Graphics are not allowed.

  Describe how the proposed research project addresses one or more of the FY18 PRMRP Topic Areas. Include a comprehensive overview of the proposed research project that will be ***readily understood by readers without a background in science or medicine.*** Clearly describe the central critical problem or question to be addressed and the ultimate applicability and impact of the research. ***Do not duplicate the technical abstract***.

- **Attachment 5: Statement of Work (SOW) (three-page limit):** Upload as "SOW.pdf." The suggested SOW format and examples specific to different types of research projects are available on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm). For the Investigator-Initiated Research Award mechanism, use the SOW format example titled, "SOW (Statement of Work) Generic Format." The SOW must be in PDF format prior to attaching.

  The SOW should include a list of major tasks that support the proposed specific aims, followed by a series of subtasks outlined related to the major tasks and milestones within the period of performance. The SOW should describe only the work for which funding is being requested by this application and, as applicable, should also:

Include the name(s) of the key personnel and contact information for each study site/ subaward site.

Indicate the number (and type, if applicable) of research subjects (animal or human) and/or human anatomical samples projected or required for each task and at each site. Refer to the General Application Instructions, Appendix 1, for additional information regarding regulatory requirements.

For studies with prospective accrual of human subjects, indicate quarterly enrollment targets.

Identify cell line(s) and commercial or organizational source(s) to be used. If human anatomical substances (including cell lines) will be used, specify whether or not identifiable information is accessible to the research team by any means.

If applicable, indicate timelines required for regulatory approvals relevant to human subjects research (e.g., Investigational New Drug and Investigational Device Exemption applications) by the FDA or other Government agency.

Partnering PI Option: The Initiating and Partnering PIs must each submit an identical copy of a jointly created SOW. The contributions of the Initiating PI and the Partnering PI should be noted for each task.

- **Attachment 6: Impact Statement (one-page limit):** Upload as "Impact.pdf."

  Explain why the proposed research project is important and relevant to understanding the cause or progression of the disease or condition, or to developing improvements in prevention, detection, diagnosis, treatment, patient care, or quality of life in the FY18 PRMRP Topic Area(s) addressed. Describe how the study will address a central critical problem or question in the relevant Topic Area(s). If applicable, describe how the project addresses an FY18 PRMRP Area of Encouragement (Appendix 2).

  - ***Describe the short-term impact:*** Detail the anticipated outcome(s)/product(s) (knowledge and/or materiel) that will be directly attributed to the results of the proposed research.

  - ***Describe the long-term impact:*** Explain the anticipated long-term gains from this research. Compare to the information known/products currently available, if applicable. Explain the long-range vision for how the research will impact the field of study and/or clinical care.

- **Attachment 7: Military Relevance Statement (one-page limit):** Upload as "MilRel.pdf."

  Describe how the proposed study is responsive to the healthcare needs of military Service members, Veterans, and/or beneficiaries. Provide information about the incidence and/or prevalence of the disease or condition in the general population as well as in military Service members, Veterans, and/or beneficiaries.

If active duty military, military families, and/or Veteran population(s) or datasets will be used in the proposed research project, describe the population(s)/dataset(s) and the appropriateness of the population(s)/dataset(s) for the proposed study. If a non-military population will be used for the proposed research project, explain how the population simulates the targeted population (i.e., military Service members, Veterans, and/or beneficiaries).

If applicable, show how the proposed research project aligns with DoD and/or VA areas of research interest. Provide a description of how the knowledge, information, products, or technologies gained from the research could be implemented in a dual-use capacity to benefit the civilian population and address a military need, as appropriate

- **Attachment 8: Partnership Statement (one-page limit):** Upload as "Partnership.pdf." ***This attachment is only required for applications submitted under the Partnering PI Option.***

  Describe the expertise of the Initiating and Partnering PIs and how each will bring different strengths to the proposed project. Describe how the combined effort will be synergistic and produce an outcome greater than what could be achieved by independent efforts. Outline the contribution and time commitment of each partner and how each will have equal intellectual input on the design, conduct, and analysis of the project. Describe how the PIs will manage the collaboration and workflow to optimize research efforts.

- **Attachment 9: Representations, if applicable (extramural submissions only):** Upload as "MandatoryReps.pdf." All extramural applicants must complete and submit the Required Representations template available on eBRAP (https://ebrap.org/eBRAP/public/Program.htm). For more information, see the General Application Instructions, Appendix 5, Section B, Representations.

- **Attachment 10: DoD Military Budget Form(s), if applicable:** Upload as "MFBudget.pdf." If a military facility (Military Health System facility, research laboratory, medical treatment facility, dental treatment facility, or a DoD activity embedded with a civilian medical center) will be a collaborator in performance of the project, complete the DoD Military Budget Form, available for download on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm), including a budget justification, for each military facility as instructed. The costs per year should be included on the Grants.gov Research and Related Budget form under subaward costs. Refer to the General Application Instructions, Section III.A.7, for detailed information.

- **Extramural and Intramural Applications**

  To evaluate compliance with Title IX of the Education Amendments of 1972 (20 USC A§1681 et seq.), the DoD is collecting certain demographic and career information to be able to assess the success rates of women who are proposed for key roles in applications in science, technology, engineering, or mathematics disciplines. To enable this assessment, each application must include the following forms completed as indicated.

Research & Related Personal Data: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.3, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.2, for detailed information.

Research & Related Senior/Key Person Profile (Expanded): For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.3, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.2, for detailed information.

- PI Biographical Sketch (five-page limit): Upload as "Biosketch_LastName.pdf." The suggested biographical sketch format is available on the "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) in eBRAP. The National Institutes of Health Biographical Sketch may also be used. All biographical sketches should be submitted in the PDF format that is not editable.

- PI Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."

- Key Personnel Biographical Sketches (five-page limit each): Upload as "Biosketch_LastName.pdf."

- Key Personnel Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."

Research & Related Budget: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.4, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.3, for detailed information.

Budget Justification (no page limit): Upload as "BudgetJustification.pdf." The budget justification for the entire period of performance must be uploaded to the Research & Related Budget after completion of the budget for Period 1.

Partnering PI Option: ***Initiating and Partnering PIs must each submit a budget and justification specific to their own portion of the effort as part of their separate Grants.gov or eBRAP application packages. The Initiating PI should not include budget information for the Partnering PI, even if they are located within the same organization. The anticipated combined direct costs budgeted for the entire period of performance for the Initiating and Partnering PIs' applications will not exceed $1.5M. Refer to Section II.D.5, Funding Restrictions, for detailed information.***

Project/Performance Site Location(s) Form: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.5, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.4, for detailed information.

- **Extramural Applications Only**

R&R Subaward Budget Attachment(s) Form (if applicable): Refer to the General Application Instructions, Section III.A.6, for detailed information.

- **Extramural Subaward:** Complete the Research & Related Subaward Budget Form through Grants.gov. (Refer to the General Application Instructions, Section III.A.6, for detailed information.) Verify subaward budget(s) and budget justification forms are present in eBRAP during the application verification period. If these components are missing, upload them to eBRAP before the end of the application verification period.

Intramural DoD Collaborator(s): Complete the DoD Military Budget Form and upload to Grants.gov attachment form as Attachment 10. (Refer to the General Application Instructions, Section III.A.7, for detailed information.) Intramural DoD Collaborator(s) costs per year should be included on the Grants.gov Research and Related Budget form under subaward costs.

DoD Military Budget Form: A military facility collaborating in the performance of the project (but not participating as a Partnering PI) should be treated as a subaward for budget purposes. However, do not complete the Grants.gov R&R Subaward Budget Attachment Form; instead, complete the DoD Military Budget Form (Attachment 10) to show all direct and indirect costs. The costs per year should be included on the Grants.gov Research & Related Budget Form under subaward costs. Refer to the General Application Instructions, Section III.A.7, for detailed information.

Application Components for the Partnering PI, if applying under the Partnering PI Option

The Partnering PI must follow the link in the email from eBRAP and, if not registered in eBRAP, complete the registration process prior to the application submission deadline in order to associate their full application package with that of the Initiating PI.

The Initiating PI must identify if that Partnering PI will be submitting an extramural or intramural application (in accordance with the guidelines in Section II.C.1.a, Organization) and the appropriate mode of submission (Grants.gov for extramural and eBRAP for intramural). The Partnering PI must verify his/her contact information and mode of submission within eBRAP to ensure proper submission of his/her application.

The application submission process for the Partnering PI uses an abbreviated full application package that includes:

- **Extramural and Intramural Applications**

  Attachments:

  - **Attachment 5: Statement of Work (three-page limit):** Upload as "SOW.pdf." Refer to the General Application Instructions, Section III.A.2, for detailed information on completing the SOW. Each PI must submit an identical copy of a jointly created SOW. The contributions of the Initiating PI and Partnering PI should be noted for each task.

- **Attachment 9: Representations, if applicable (extramural submissions only):** Upload as "MandatoryReps.pdf." All extramural applicants must complete and submit the Required Representations template available on eBRAP (https://ebrap.org/eBRAP/public/Program.htm). For more information, see the General Application Instructions, Appendix 5, Section B, Representations.

- **Attachment 10: DoD Military Budget Form:** Upload as "MFBudget.pdf." Refer to the General Application Instructions, Section III.A.7, for detailed information. The costs per year should be included on the Grants.gov Research and Related Budget form under subaward costs.

Research & Related Personal Data: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.3, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.2, for detailed information.

Research & Related Senior/Key Person Profile (Expanded): For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.3, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.2, for detailed information.

- PI Biographical Sketch (five-page limit): Upload as "Biosketch_LastName.pdf." The suggested biographical sketch format is available on the "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) in eBRAP. The National Institutes of Health Biographical Sketch may also be used. All biographical sketches should be submitted in the PDF format that is not editable.

- PI Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."

- Key Personnel Biographical Sketches (five-page limit each): Upload as "Biosketch_LastName.pdf."

- Key Personnel Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."

Research & Related Budget: For extramural submissions, refer to the General Application Instructions, Section III.A.4, and for intramural submissions, refer to the General Application Instructions, Section IV.A.3, for detailed information.

Budget Justification (no page limit): Upload as "BudgetJustification.pdf."

Initiating and Partnering PIs must each submit a budget and justification specific to their own portion of the efforts as part of their separate Grants.gov application packages. The Research & Related Budget for the Partnering PI should not include budget information for the Initiating PI, even if they are located within the same organization. Refer to Section II.D.5, Funding Restrictions, for detailed information.

Project/Performance Site Location(s) Form: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.5, and for intramural submissions (via eBRAP), refer to General Application Instructions, Section IV.A.4, for detailed information.

- **Extramural Applications Only**

  R&R Subaward Budget Attachment(s) Form:

  - **Extramural Subaward:** Complete the R&R Subaward Budget Form through Grants.gov. (Refer to the General Application Instructions, Section III.A.6, for detailed information.)

  - **Intramural DoD Collaborator(s):** Complete the DoD Military Budget Form and upload to Grants.gov attachment form as Attachment 10. (Refer to the General Application Instructions, Section IV.A.3, for detailed information.)

II.D.3. Dun and Bradstreet Data Universal Numbering System (DUNS) Number and System for Award Management (SAM)

Applicant organizations and all subrecipient organizations must have a DUNS number to submit applications to Grants.gov. The applicant organization must also be registered in the Entity Management functional area of the SAM with an "Active" status to submit applications through the Grants.gov portal. Verify the status of the applicant's organization's Entity registration in SAM well in advance of the application submission deadline. Allow 3 to 4 weeks to complete the entire SAM registration process. If an applicant has not fully complied with the requirements at the time the Federal awarding agency is ready to make a Federal award, the Federal awarding agency may determine that the applicant is not qualified to receive a Federal award and use that determination as a basis for making a Federal award to another applicant. Refer to the General Application Instructions, Section III, for further information regarding Grants.gov requirements.

II.D.4. Submission Dates and Times

All submission dates and times are indicated in Section I, Overview of the Funding Opportunity. Pre-application and application submissions are required. The pre-application and application submission process should be started early to avoid missing deadlines. There are no grace periods. Failure to meet either of these deadlines will result in submission rejection.

Applicant Verification of Full Application Submission in eBRAP

Following retrieval and processing of the full application, eBRAP will notify the organizational representatives and PI by email to log into eBRAP to review, modify, and verify the full application submission. eBRAP will validate retrieved files against the specific Program Announcement requirements and discrepancies will be noted in both the email and in the "Full Application Files" tab in eBRAP. eBRAP does not confirm the accuracy of file content. It is the applicant's responsibility to review all application components and ensure proper ordering as specified in the Program Announcement. ***If either the Project Narrative or the budget fails eBRAP validation or needs to be modified, an updated full application package must be***

submitted prior to the application submission deadline. The Project Narrative and Budget Form cannot be changed after the application submission deadline.

Extramural Submission: The full application package submitted to Grants.gov may be viewed and modified in eBRAP until the end of the application verification period. During the application verification period, the full application package, ***with the exception of the Project Narrative and Budget Form,*** may be modified.

Intramural DoD Submission: After eBRAP has processed the full application, the organizational Resource Manager/Comptroller/Task Area Manager or equivalent Business Official and PIs will receive email notification of the status and will be able to view and modify application components in eBRAP. During the application verification period, the full application package, ***with the exception of the Project Narrative and Budget Form***, may be modified. The Resource Manager/Comptroller/Task Area Manager or equivalent Business Official should log into eBRAP to review and to approve prior to the application verification deadline.

For All Submissions: Verify that subaward budget(s) with budget justification are present in eBRAP during the application verification period. If these components are missing, upload them to eBRAP before the end of the application verification period.

II.D.5. Funding Restrictions

The maximum period of performance is **3** years.

Application submissions with a single PI:

The anticipated direct costs budgeted for the entire period of performance will not exceed **$1.2M**. If indirect cost rates have been negotiated, indirect costs are to be budgeted in accordance with the organization's negotiated rate. No budget will be approved by the Government exceeding **$1.2M** direct costs or using an indirect cost rate exceeding the organization's negotiated rate.

All direct and indirect costs of any subaward or contract must be included in the total direct costs of the primary award.

The applicant may request the entire maximum funding amount for a project that may have a period of performance less than the maximum **3** years.

Application submissions, including the Partnering PI Option: The anticipated combined direct costs budgeted for the entire period of performance for the Initiating and Partnering PIs' applications will not exceed **$1.5M**. If indirect cost rates have been negotiated, indirect costs are to be budgeted in accordance with the organization's negotiated rate. Collaborating organizations should budget associated indirect costs in accordance with each organization's negotiated rate. The combined budgeted direct costs approved by the Government will not exceed **$1.5M** or use an indirect cost rate exceeding each organization's negotiated rate.

A separate award will be made to each PI's organization.

The Initiating and the Partnering PIs are expected to be partners in the research, and direct cost funding should be divided accordingly, unless otherwise warranted and clearly justified. Each partner may request no more than 75% (or no less than 25%) of the proposed direct costs.

For this award mechanism, direct costs may be requested for (not all-inclusive):

- Salary
- Research supplies
- Clinical research costs (clinical trials are not allowed)
- Support for multidisciplinary collaborations, including travel
- Travel costs for the PIs to disseminate project results at one DoD-supported meeting to be specified by the program office during award negotiations (i.e., the Military Health System Research Symposium)
- Travel costs for one investigator to travel to one scientific/technical meeting per year to present project outcomes or disseminate project results

Awards made to extramural organizations will consist solely of assistance agreements (Cooperative Agreements and Grants). For extramural awards with an intragovernmental component, direct transfer of funds from an extramural award recipient to a DoD or other Federal agency is not allowed except under very limited circumstances. Funding to intramural DoD and other Federal agencies will be managed through a direct fund transfer. Intramural applicants are responsible for coordinating through their agency's procedures the use of contractual or assistance funding awards or other appropriate agreements to support extramural collaborators. Application packages from associated extramural partners will be funded through assistance agreements.

Refer to the General Application Instructions, Section III.A.4, for budget regulations and instructions for the Research & Related Budget. ***For Federal agencies or organizations collaborating with Federal agencies, budget restrictions apply as are noted in the General Application Instructions, Section III.A.4***.

The CDMRP expects to allot approximately $95M of the $330M FY18 appropriation to fund approximately 47 Investigator-Initiated Research Award applications, depending on the quality and number of applications received. Funding of applications received in response to this Program Announcement is contingent upon the availability of Federal funds for this program.

Funds to be obligated on any award resulting from this funding opportunity will be available for use for a limited time period based on the fiscal year of the funds. The time is considered when establishing the award's period of performance. It is anticipated that awards made from this funding opportunity will be funded with FY18 funds, which will expire for use on September 30, 2024.

II.D.6. Other Submission Requirements

Refer to the General Application Instructions, Appendix 4, for detailed formatting guidelines.

II.E. Application Review Information

II.E.1. Criteria

II.E.1.a. Peer Review

To determine technical merit, all applications will be evaluated according to the following scored criteria, which are listed in decreasing order of importance:

- **Research Strategy and Feasibility**
    - How well the scientific rationale supports the project and its feasibility, as demonstrated by a critical review and analysis of the literature, supporting data, and logical reasoning.
    - How well the hypothesis, specific aims, and objective(s) are developed.
    - How well the experimental design, methods, data collection procedures, and analyses are developed and support completion of the aims.
    - If applicable, to what degree the statistical plan and power analysis are appropriate for the proposed project.
    - If animal studies are included, how well they are designed to achieve reproducible and rigorous results, including the choice of model and the endpoints/outcomes to be measured.
    - If applicable, whether data will be appropriately reported and documented to support a regulatory filing with the FDA.
    - If applicable, how well the application provides evidence of availability of and access to the necessary study populations and/or resources.
    - How well potential problems are identified and alternative approaches are addressed.
    - Whether the research can be completed within the proposed period of performance.
- **Impact**
    - To what extent the project impacts a central critical problem or question in at least one FY18 PRMRP Topic Area.
    - If applicable, how well the proposed research project addresses one or more FY18 PRMRP Areas of Encouragement.

- How the proposed research project, if successful, will make important scientific advances in the relevant field of research.

- To what degree the proposed project could, if successful, make a significant impact on the lives of relevant patient populations in the short term and/or long term.

- **Personnel**

  - How the background and expertise of the PIs and other key personnel demonstrate their ability to perform the proposed work.

  - How the levels of effort by the PIs and other key personnel are appropriate to ensure the successful conduct of the project.

  - How each PI's record(s) of accomplishment demonstrates their ability to accomplish the proposed work.

For applications submitted under the Partnering PI Option:

- **Partnership**

  - How well the research project is supported by the nature of the collaboration.

  - To what extent the proposed partnership will advance research beyond what would be possible through individual efforts.

  - How well the skills and perspectives of the Initiating and Partnering PIs complement each other.

  - How well the application reflects the requirement that the partners have equal intellectual input into the design of the project.

In addition, the following unscored criteria will also contribute to the overall evaluation of the application:

- **Environment**

  - How the scientific environment is appropriate for the proposed research.

  - How the research requirements are supported by the availability of and accessibility to facilities and resources (including collaborative arrangements).

  - How the quality and extent of organizational support are appropriate for the proposed research.

  - If applicable, to what degree the intellectual and material property plan is appropriate.

- **Budget**
    - Whether the budget is appropriate for the proposed research and within the limitations of this Funding Opportunity.
- **Application Presentation**
    - To what extent the writing, clarity, and presentation of the application components influence the review.

II.E.1.b. Programmatic Review

To make funding recommendations and select the application(s) that, individually or collectively, will best achieve the program objectives, the following criteria are used by programmatic reviewers:

- Ratings and evaluations of the peer reviewers
- Relevance to the mission of the DHP and FY18 PRMRP, as evidenced by the following:
    - Adherence to the intent of the award mechanism
    - Program portfolio composition
    - Military relevance
    - Relative impact

II.E.2. Application Review and Selection Process

All applications are evaluated by scientists, clinicians, and consumers in a two-tier review process. The first tier is peer review of applications against established criteria for determining technical merit. Each application is evaluated for its own merit, independent of other applications. The second tier is a programmatic review that makes recommendations for funding to the Commanding General, USAMRMC, on behalf of the DHA and the OASD(HA), based on technical merit, the relevance to the mission of the DHP and PRMRP, the specific intent of the award mechanism, and to other specified evaluation criteria in the Program Announcement. Programmatic review is a comparison-based process in which applications with scientific and technical merit compete in a common pool. ***The highest-scoring applications from the first tier of review are not automatically recommended for funding. Funding recommendations depend on various factors as described in Section II.E.1.b, Programmatic Review.*** Additional information about the two-tier process used by the CDMRP can be found at http://cdmrp.army.mil/about/fundingprocess.

All CDMRP review processes are conducted confidentially to maintain the integrity of the merit-based selection process. Panel members sign a statement that application and evaluation information will not be disclosed outside the panel. Violations of confidentiality can result in the dissolving of a panel(s) and other corrective actions. In addition, personnel at the applicant or

collaborating organizations are prohibited from contacting persons involved in the review and approval process to gain protected evaluation information or to influence the evaluation process. Violations of these prohibitions will result in the administrative withdrawal of the organization's application. Violations by panel members or applicants that compromise the confidentiality of the review and approval process may also result in suspension or debarment from Federal awards. Furthermore, the unauthorized disclosure of confidential information of one party to another third party is a crime in accordance with 18 USC 1905.

II.E.3. Integrity and Performance Information

Prior to making an assistance agreement award where the Federal share is expected to exceed the simplified acquisition threshold (currently $150,000) over the period of performance, the Federal awarding agency is required to review and consider any information about the applicant that is available in the Federal Awardee Performance and Integrity Information System (FAPIIS).

An applicant organization may review FAPIIS, accessible through SAM, and submit comments to FAPIIS on any information about the organization that a Federal awarding agency previously entered and is currently available in FAPIIS.

The Federal awarding agency will consider any comments by the applicant, in addition to other information in the designated integrity and performance system, in making a judgment about the applicant's integrity, business ethics, and record of performance under Federal awards when determining a recipient's qualification prior to award, according to the qualification standards of the Department of Defense Grant and Agreement Regulations (DoDGAR), Section 22.415.

II.E.4. Anticipated Announcement and Federal Award Dates

All application review dates and times are indicated in Section I, Overview of the Funding Opportunity.

Each PI and organization will receive email notification of posting of the funding recommendation in eBRAP. Each PI will receive a peer review summary statement on the strengths and weaknesses of the application.

II.F. Federal Award Administration Information

II.F.1. Federal Award Notices

Awards will be made no later than September 30, 2019. Refer to the General Application Instructions, Appendix 2, for additional award administration information.

After email notification of application review results through eBRAP, and if selected for funding, a representative from the USAMRAA will contact the business official authorized to negotiate on behalf of the PI's organization.

Only an appointed USAMRAA Grants Officer may obligate the Government to the expenditure of funds. No commitment on the part of the Government should be inferred from discussions

with any other individual. The award document signed by the Grants Officer is the official authorizing document.

Federal Organizations: Awards to Federal Government organizations (to include intramural DoD organizations) will be executed through the Military Interdepartmental Purchase Request (MIPR) or Funding Authorization Document (FAD) process. Transfer of funds is contingent upon appropriate safety and administrative approvals. Intramural applicants and collaborators are reminded to coordinate receipt and commitment of funds through their respective Resource Manager/Task Area Manager/Comptroller or equivalent Business Official.

After email notification of application review results through eBRAP, and if selected for funding, a representative from the CDMRP will contact the business official authorized to negotiate on behalf of the PI's organization.

II.F.1.a. PI Changes and Award Transfers

Changes in PI and an organizational transfer of an award supporting the Initiating or Partnering PI are discouraged and will be evaluated on a case-by-case basis and only allowed at the discretion of the Grants Officer. An organizational transfer of an award will not be allowed in the last year of the (original) period of performance or any extension thereof.

Refer to the General Application Instructions, Appendix 2, Section B, for general information on organization or PI changes.

II.F.2. Administrative and National Policy Requirements

Applicable requirements in the DoDGAR found in 32 CFR, Chapter 1, Subchapter C, and 2 CFR, Chapter XI, apply to grants and cooperative agreements resulting from this Program Announcement.

Refer to the General Application Instructions, Appendix 2, for general information regarding administrative requirements.

Refer to the General Application Instructions, Appendix 5, for general information regarding national policy requirements.

Refer to full text of the USAMRAA General Research Terms and Conditions with Institutions of Higher Education, Hospitals, and Non-Profit Organizations: Addendum to the DoD R&D Terms and Conditions and the USAMRAA General Research Terms and Conditions with For-Profit Organizations for further information.

II.F.3. Reporting

Refer to the General Application Instructions, Appendix 2, Section A, for general information on reporting requirements. ***If there are technical reporting requirement delinquencies for any existing USAMRAA-sponsored awards at the applicant organization, no new awards will be issued to the applicant organization until all delinquent reports have been submitted.***

Annual progress reports as well as a final progress report will be required.

Quarterly technical progress reports may be required.

Award Chart: Complete the Award Chart template, a one-page PowerPoint file that must be downloaded from the CDMRP eBRAP System at https://ebrap.org/eBRAP/public/Program.htm, and submit to eBRAP at the time of award.

Award Expiration Transition Plan: An Award Expiration Transition Plan must be submitted with the final progress report. Use the one-page template titled, "Award Expiration Transition Plan," available on the on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) under the "Progress Report Formats" section.

Awards resulting from this Program Announcement will incorporate additional reporting requirements related to recipient integrity and performance matters. Recipient organizations that have Federal contract, grant, and cooperative agreement awards with a cumulative total value greater than $10,000,000 are required to provide information to FAPIIS about certain civil, criminal, and administrative proceedings that reached final disposition within the most recent 5-year period and that were connected with performance of a Federal award. Recipients are required to disclose semiannually information about criminal, civil, and administrative proceedings as specified in the applicable Terms and Conditions (see General Application Instructions, Section III.A.4).

II.G. Federal Awarding Agency Contacts

II.G.1. CDMRP Help Desk

Questions related to Program Announcement content or submission requirements as well as questions related to the pre-application or intramural application submission through eBRAP should be directed to the CDMRP Help Desk, which is available Monday through Friday from 8:00 a.m. to 5:00 p.m. ET. Response times may vary depending upon the volume of inquiries.

Phone: 301-682-5507

Email: help@eBRAP.org

II.G.2. Grants.gov Contact Center

Questions related to extramural application submission through Grants.gov portal should be directed to the Grants.gov Contact Center, which is available 24 hours a day, 7 days a week (closed on U.S. Federal holidays). Note that the CDMRP Help Desk is unable to provide technical assistance with Grants.gov submission.

Phone: 800-518-4726; International 1-606-545-5035

Email: support@grants.gov

Sign up on Grants.gov for "send me change notification emails" by following the link on the "Synopsis" page for the Program Announcement or by responding to the prompt provided by Grants.gov when first downloading the Grants.gov application package. If the Grants.gov application package is updated or changed, the original version of the application package may not be accepted by Grants.gov.

II.H. Other Information

II.H.1. Program Announcement and General Application Instructions Versions

Questions related to this Program Announcement should refer to the Program name, the Program Announcement name, and the Program Announcement version code 20180329c. The Program Announcement numeric version code will match the General Applications Instructions version code 20180329.

II.H.2. Administrative Actions

After receipt of pre-applications or applications, the following administrative actions may occur:

II.H.2.a. Rejection

The following will result in administrative rejection of the pre-application:

- Preproposal Narrative is missing.

The following will result in administrative rejection of the application:

- Submission of an application for which a letter of invitation was not received.
- Project Narrative exceeds page limit.
- Project Narrative is missing.
- Budget is missing.
- Submission of the same research project to different Funding Opportunities within the same program and fiscal year. Refer to Section II.D, Application and Submission Information, for exceptions.
- **Partnering PI Option:** Failure to submit both associated (Initiating and Partnering PI) applications by the deadline.

II.H.2.b. Modification

- Pages exceeding the specific limits will be removed prior to review for all documents other than the Project Narrative.
- Documents not requested will be removed.

II.H.2.c. Withdrawal

The following may result in administrative withdrawal of the pre-application or application:

- An FY18 PRMRP Programmatic Panel member is named as being involved in the research proposed or is found to have assisted in the pre-application or application processes including, but not limited to, concept design, application development, budget preparation, and the development of any supporting documentation. ***A list of the FY18 PRMRP Programmatic Panel members can be found at http://cdmrp.army.mil/prmrp/panels/panels18***.

- The application fails to conform to this Program Announcement description to the extent that appropriate review cannot be conducted.

- Inclusion of URLs, with the exception of links in References Cited and Publication and/or Patent Abstract sections.

- Page size is larger than 8.5 inches x 11.0 inches (approximately 21.59 cm x 27.94 cm).

- To preserve the integrity of its peer and programmatic review processes, the CDMRP discourages inclusion of any employee of its review contractors having any role in the preparation, research or other duties for submitted applications. For FY18, the identities of the peer review contractor and the programmatic review contractor may be found at the CDMRP website (http://cdmrp.army.mil/about/2tierRevProcess). Applications that include names of personnel from either of these companies will be administratively withdrawn unless plans to manage COIs are provided and deemed appropriate by the Grants Officer. Refer to the General Application Instructions, Appendix 3, for detailed information.

- Personnel from applicant or collaborating organizations are found to have contacted persons involved in the review or approval process to gain protected evaluation information or to influence the evaluation process.

- Applications from extramural organizations, including non-DoD Federal agencies, received through eBRAP may be withdrawn.

- Applications submitted by an intramural DoD organization may be withdrawn if the intramural organization cannot coordinate the use of contractual, assistance, or other appropriate agreements to provide funds to extramural collaborators.

- The proposed research project does not address at least one of the Congressionally directed FY18 PRMRP Topic Areas.

- If a clinical trial is proposed, the application will be withdrawn.

- An application submitted by a PI who does not meet the eligibility criteria will be withdrawn.

II.H.2.d. Withhold

Applications that appear to involve research misconduct will be administratively withheld from further consideration pending organizational investigation. The organization will be required to provide the findings of the investigation to the USAMRAA Grants Officer for a determination of the final disposition of the application.

II.H.3. Application Submission Checklist

Application Components	Action	Initiating PI Completed	Partnering PI Completed
SF424 (R&R) Application for Federal Assistance **(Extramural submissions only)**	Complete form as instructed.		
Summary (Tab 1) and Application Contacts (Tab 2) **(Intramural submissions only)**	Complete these tabs as instructed.		
Attachments	Project Narrative: Upload as Attachment 1 with file name "ProjectNarrative.pdf."		
	Supporting Documentation: Upload as Attachment 2 with file name "Support.pdf."		
	Technical Abstract: Upload as Attachment 3 with file name "TechAbs.pdf."		
	Lay Abstract: Upload as Attachment 4 with file name "LayAbs.pdf."		
	Statement of Work: Upload as Attachment 5 with file name "SOW.pdf."		
	Impact Statement: Upload as Attachment 6 with file name "Impact.pdf."		
	Military Relevance Statement: Upload as Attachment 7 with file name "MilRel.pdf."		
	Partnership Statement: Upload as Attachment 8 with file name "Partnership.pdf," if applicable.		
	Representations (Extramural submissions only): Upload as Attachment 9 with file name "MandatoryReps.pdf," if applicable.		
	DoD Military Budget Form(s): Upload as Attachment 10 with file name "MFBudget.pdf," if applicable.		
Research & Related Personal Data	Complete form as instructed.		

Application Components	Action	Initiating PI Completed	Partnering PI Completed
Research & Related Senior/Key Person Profile (Expanded)	Attach PI Biographical Sketch (Biosketch_LastName.pdf) to the appropriate field.		
	Attach PI Previous/Current/Pending Support (Support_LastName.pdf) to the appropriate field.		
	Attach Biographical Sketch (Biosketch_LastName.pdf) for each senior/key person to the appropriate field.		
	Attach Previous/Current/Pending (Support_LastName.pdf) for each senior/key person to the appropriate field.		
Research & Related Budget **(Extramural submissions only)**	Complete as instructed. Attach Budget Justification (BudgetJustification.pdf) to the appropriate field.		
Budget **(Intramural submissions only)**	Complete the DoD Military Budget Form and Justification.		
Project/Performance Site Location(s) Form	Complete form as instructed.		
R&R Subaward Budget Attachment(s) Form	Complete form as instructed.		

APPENDIX 1: ACRONYM LIST

ACOS/R&D	Associate Chief of Staff for Research and Development
ACURO	Animal Care and Use Review Office
CDMRP	Congressionally Directed Medical Research Programs
CFR	Code of Federal Regulations
COI	Conflict of Interest
DHA	Defense Health Agency
DHP	Defense Health Program
DoD	Department of Defense
DoDGAR	Department of Defense Grant and Agreement Regulations
DUNS	Data Universal Numbering System
eBRAP	Electronic Biomedical Research Application Portal
EC	Ethics Committee
ET	Eastern Time
FAD	Funding Authorization Document
FAPIIS	Federal Awardee Performance and Integrity Information System
FDA	Food and Drug Administration
FY	Fiscal Year
HRPO	Human Research Protection Office
IACUC	Institutional Animal Care and Use Committee
IDE	Investigational Device Exemption
IND	Investigational New Drug
IRB	Institutional Review Board
M	Million
MIPR	Military Interdepartmental Purchase Request
OASD(HA)	Office of the Assistant Secretary of Defense for Health Affairs
OMB	Office of Management and Budget
ORCID	Open Researcher and Contributor ID, Inc.
ORP	Office of Research Protections
PI	Principal Investigator
PRMRP	Peer Reviewed Medical Research Program
RDT&E	Research, Development, Test, and Evaluation
SAM	System for Award Management
SOW	Statement of Work
USAMRAA	U.S. Army Medical Research Acquisition Activity
USAMRMC	U.S. Army Medical Research and Materiel Command
USC	United States Code
VA	Department of Veterans Affairs

APPENDIX 2: AREAS OF ENCOURAGEMENT

Applications addressing any of the FY18 PRMRP Topic Areas are of interest to the program. ***Any aspect of research relevant to one or more of the FY18 PRMRP Topic Areas may be considered for funding.*** Areas of Encouragement related to the FY18 PRMRP Topic Areas have been identified by the DoD, VA, and other relevant stakeholders. Applicants are urged to read and consider these Areas of Encouragement before preparing their applications. ***The information provided is not exhaustive, and applicants are not restricted to submitting applications that address an Area of Encouragement in this list.***

Acute Lung Injury

- Research on the etiology and prevention of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) caused by the host's (e.g., immune system's) responses to trauma, transfusion, burns, infection, hemorrhagic shock, inhalation, and/or oxygen exposure.
- Novel and/or innovative detection technologies or therapeutics to reduce the incidence and/or severity of ALI/ARDS and/or other lung injury secondary to trauma, transfusion, infection, burns, hemorrhagic shock, inhalation, and/or oxygen exposure.
- Strategies to stabilize and support the safe transport of patients with ALI/ARDS in order to optimize therapeutic interventions, particularly in operational scenarios requiring prolonged field care and/or longer transport times.
- Development of metrics to associate the long-term health outcomes of ALI/ARDS with physiological and physical performance.

Antimicrobial Resistance

- Development of novel and/or innovative interventions, diagnostics, and treatment for multidrug-resistant pathogens, especially those that can be used in austere settings.
- Development of novel and/or innovative therapies that prevent and/or interrupt biofilm production in wound infections and infected hardware models.
- Development and testing of treatment protocols and/or diagnostic tests to limit prescribing antibiotics for conditions that are commonly viral in nature or conditions that would resolve themselves without antibiotic treatment (e.g., sinusitis, bronchitis, viral upper respiratory infections, and acute gastroenteritis).
- Development and evaluation of therapies to treat travelers' diarrhea to improve the time to clinical cure and minimize resistance acquisition.
- Development of gene editing tools (e.g., designer nucleases) that optimize treatment and raise the threshold for resistance of anti-infective agents.

Arthritis (other than Rheumatoid Arthritis or Post-Traumatic Osteoarthritis)

- Research toward the development of clinical practice guidelines to prevent, identify, and treat arthritis.

- Research quantifying the impacts of obesity, weight loss, physical fitness (all components including cardiovascular, strength, flexibility, balance), and dietary factors on the development of or prevention/risk reduction of arthritis.
- Studies to examine using regenerative medicine techniques and therapies (including cell-based therapies) to prevent or treat osteoarthritis, including dose response information and the frequency and timing of application.
- Basic and translational research to identify treatments to mitigate and/or reverse osteoarthritis, particularly in the knee, hip, ankle, and shoulder.
- Identification and/or validation of biomarkers for early psoriatic arthritis.
- Research to establish activity recommendations for maximal joint life following joint repair, particularly in young patient populations.

Burn Pit Exposure

- Research on the etiology and treatment of adverse health events related to military deployment to Iraq and Afghanistan that are associated with exposure to airborne hazards and open pit burning of solid waste and other materials.
- Toxicological studies to characterize emissions from open air burns, burn boxes, and incinerators and ascertain the toxicity and mechanisms of action for injury from such chemicals and airborne environmental dust and mixtures, as well as interactions among pollutants and particulate materials.
- Identification and validation of biomarkers of both exposure to and health effects of burn pit combustion products, burning biomass and refuse, and geogenic dusts.
- Development and validation of instruments for assessing (including in real time) levels of exposure to airborne hazards for use in research and for occupational and environmental exposures monitoring.

Cardiomyopathy

- Improve understanding of the pathophysiology of cardiomyopathies.
- Strategies to identify risk factors associated with the development of cardiomyopathy (i.e., genetic, lifestyle, exposure) in the civilian and/or military populations.
- Improve diagnosis and treatment of primary and secondary cardiomyopathies.
- Assess the multiple etiologies of cardiomyopathy (e.g., hypertension, ischemia, hemochromatosis, sleep apnea, radiation therapy, medications, smallpox vaccine, infections) in military Service members.

Cerebellar Ataxia

- Research to improve understanding of all causes of cerebellar ataxia.
- Research to improve understanding of the association between nutrition and cerebellar ataxia.
- Research to better understand the role of physical rehabilitation/exercise in affecting postural disorders, balance, and coordination in cerebellar ataxia patients.

- Research to identify therapeutic targets and novel therapeutic modalities, including gene silencing/gene editing.

Chronic Migraine and Post-Traumatic Headache

- Precision medicine research to investigate, develop, and validate biomarkers that are not only useful in diagnosing and monitoring traumatic brain injury patients with chronic migraine or post-traumatic headache, but can also identify individual responses to treatment.
- Epidemiological/natural history studies to characterize specific types of post-traumatic headache, the pathobiology of these headaches (such as the role of acute cortical spreading depression after injury as a risk factor for chronic headaches of a migrainous type), and the risk factors that might predispose people to certain types of post-traumatic headache.
- Research on the optimal approaches to effective management of acute and chronic pain management, and co-occurring psychological health disorders, for chronic migraine and post-traumatic headache, with a focus on assessing and eliminating adverse outcomes and decreasing polypharmacy.
- Evaluation of the use of mechanical stimulation and/or other non-pharmaceutical treatments to reduce acute and chronic migraines and headaches.
- Evaluation of the differences in etiology, diagnosis, treatment, and prevention of migraine headaches between men and women.

Chronic Pain Management

- Develop and validate a user-friendly, objective pain measurement tool (e.g., instrument, questionnaire, scale) to assess the severity of pain and to inform pain management clinical decisions.
- Development of tools to objectively assess and/or prevent pain chronification.
- Research to advance understanding of the impact of psychosocial factors and comorbidities on pain chronification.
- Research on non-addictive and/or alternative methods to treat and manage chronic pain, particularly in complex patients (i.e., chronic, high-utilization, polypharmacy patients).
- Research to understand the mechanisms that sustain or resolve chronic pain.

Congenital Heart Disease

- Development of approaches, including tissue engineering, that provide structural support, restore native activity, allow for tissue growth, and prevent the need for reoperation.
- Research to improve understanding of the causes of congenital heart defects, including genomic, proteomic, and metabolomic profiling.
- Research to design and implement disease-in-a-dish and/or microfluidic models with an established phenotype to increase the efficacy of finding novel and/or innovative drug targets, screen exiting drugs, perform cardiotoxicity testing, and/or uncover pathogenesis.
- Research both on the risk of neurologic injury and on enhanced neuroprotection before, during, and after surgery for congenital heart disease.

- Population-based and outcomes-based research to assess the health outcomes of individuals with congenital heart disease across their life spans.

Constrictive Bronchiolitis

- Research to understand the role of occupational and environmental exposures, including military relevant volatile compounds, mineral and soil dusts, and other airborne particulates, in the etiology of constrictive bronchiolitis.
- Clinical assessments to determine the prevalence and severity of constrictive bronchiolitis and related respiratory diseases in previously deployed military Service members and/or Veterans.
- Development and testing of minimally invasive and non-invasive approaches for diagnosing constrictive bronchiolitis.
- Research to develop novel and/or innovative therapeutics to slow or reverse the progression of constrictive bronchiolitis.
- Development and/or validation of animal models for understanding mechanisms and etiology of constrictive bronchiolitis.

Diabetes

- Research to better understand the heterogeneity of diabetes, including the identification of novel biomarkers.
- Identification and/or evaluation of interventions to reduce the development of diabetes among individuals meeting the clinical criteria for prediabetes.
- Research on interventions to prevent or treat diabetes complications, including diabetic retinopathy, nephropathy, neuropathy, and impaired wound healing.
- Research on the transplantation of allogenic or autologous pancreatic islet cells for long-term natural insulin production, including current good laboratory/clinical/manufacturing practices (as needed) for cell line development.
- Research to design and implement disease-in-a-dish and/or microfluidic models to model pancreatic islets to uncover pathogenesis and improve the efficiency of drug discovery.

Dystonia

- Research to improve identification of delayed onset dystonia following traumatic brain injury.
- Research on the risk, incidence, and etiology of generalized dystonia, focal dystonia, multifocal dystonia, segmental dystonia, and/or hemidystonia.
- Research on interventions to prevent, slow the progression of, or treat dystonia.

Eating Disorders

- Investigations into the prevalence, diagnosis, and treatment patterns of eating disorders in Service members and their families, including potential relationships with military-unique behaviors or conditions.

- Assessment of patterns of comorbidity between eating disorders and other mental health conditions, including an examination of whether eating disorders are more likely to precede or follow the development of other mental health conditions.
- Studies to identify the most effective treatments (and order of treatment) for patients with an eating disorder and a comorbid disorder.
- Research to advance the understanding of the biological, genetic, and environmental factors that influence eating disorders.
- Studies to elucidate and/or mitigate risk factors for the onset or recurrence of eating disorders, including the influence of social media.

Emerging Infectious Diseases

- Development of biomarker-guided clinical management strategies for severe infectious diseases in austere settings.
- Development of rapid, specific, sensitive, and broadly applicable diagnostic methods for emerging pathogens including SARS, H1N1, Ebola, Zika, malaria, dengue, chikungunya, and others.
- Development of risk assessment for vector-borne diseases and novel strategies for vector control, including, but not limited to, novel insecticides, larvicide applications, and barrier methods. Target vectors include major disease dipteran (including but not limited to vectors such as mosquitoes and sand flies) and non-dipteran (including but not limited to reduviids and fleas) and other arthropods of military medical importance (e.g., ticks).
- Surveillance and modeling of epidemics and development of strategies to counter risks in emerging infectious diseases.
- Development of broad-acting detection systems to identify emerging members of a known viral family (e.g., a broad coronavirus panel that can detect Middle Eastern Respiratory Syndrome).

Endometriosis

- Discovery and identification of new and/or validation of existing endometriosis-associated biomarkers for accurate, minimally invasive diagnosis.
- Research to elucidate the pathogenesis, evolution, pathophysiology, infertility, and pelvic pain associated with endometriosis.
- Development of novel treatments, including non-opioid pain therapies, or alternative therapies to alleviate symptoms and reduce secondary effects of endometriosis.
- Studies assessing the environmental etiology of endometriosis.

Epidermolysis Bullosa

- Research to provide further insight into those cellular pathways that promote the development of squamous cell carcinomas in recessive dystrophic and junctional epidermolysis bullosa.

- Research, including clinical trials, focused on therapeutics (topical or systemic) or dressings that enhance wound healing in inherited epidermolysis bullosa.
- Research, including clinical trials, focused on systemic drugs that prevent, delay the onset, or modify the aggressiveness of squamous cell carcinoma in patients with recessive and junctional epidermolysis bullosa.
- Development of novel therapeutics to cure epidermolysis bullosa, reduce epidermolysis bullosa symptoms, or improve quality of life.

Focal Segmental Glomerulosclerosis

- Research to improve understanding of the causes of primary and/or secondary focal segmental glomerulosclerosis, especially genetic mutations.
- Development of non-invasive methods to diagnose focal segmental glomerulosclerosis and its variants.
- Development of a curative therapy or treatments to delay or halt the progression of focal segmental glomerulosclerosis and/or prevent post-transplantation recurrence.
- Research to determine the efficacy of medications used off-label (i.e., outside the FDA-approved indication) to treat focal segmental glomerulosclerosis.

Fragile X

- Development and evaluation of gene modification (e.g., gene editing or gene reactivation) therapeutics for the treatment of fragile X syndrome [including fragile X-associated tremor/ataxia syndrome (FXTAS) and fragile X-associated primary ovarian insufficiency (FXPOI)].
- Identification and validation of functional measures of the manifestations of fragile X syndrome (including FXTAS and FXPOI) across the life span.
- Research to advance the understanding of the pathophysiology/natural history or life course of fragile X syndrome (including FXTAS and FXPOI).
- Identification of novel targets and/or testing novel or existing therapeutics (e.g., repurposing drugs) for fragile X syndrome (including FXTAS and FXPOI).
- Research to establish the benefits of early diagnosis/early treatment of fragile X syndrome in patients and progeny.
- Development of a preclinical model that is representative of human fragile X syndrome.

Frontotemporal Degeneration

- Basic research to establish in vivo and in vitro models for disease pathology, behavioral/ cognitive symptoms, and motor dysfunction.
- Research to understand the neurological basis of deficits in social cognition and emotional regulation.
- Research to identify biomarkers and/or improve diagnostics for frontotemporal degeneration.

- Research to identify risk factors (e.g., gene networks, environmental factors).
- Development of evidence-based non-pharmacological and/or pharmacological treatments for behavioral, cognitive, speech, and/or motor dysfunctions of frontotemporal degeneration.

Guillain-Barré Syndrome

- Research on the immune system cell types and molecular mechanisms responsible for the pathology of Guillain-Barré syndrome.
- Research to elucidate the characteristics of various exposures (e.g., viruses, bacteria, vaccinations, surgery, trauma) associated with Guillain-Barré syndrome and their effects on the immune system.
- Research to prevent or reduce the effects of residual weakness, relapse of muscle weakness, and other neurological and psychological symptoms of Guillain-Barré syndrome to improve patients' quality of life and increase their independence.
- Development of new treatments and refinement of existing treatments for Guillain-Barré syndrome.

Hepatitis B and C

- Development of a vaccine against hepatitis C.
- Identification and reduction of hepatitis in blood products for transfusion.
- Research on strategies to reduce vertical (mother-to-child) transmission of hepatitis B virus and hepatitis C virus.
- Development of strategies for reliable, non-invasive, early detection of hepatitis-related liver disease and hepatocellular carcinoma.
- Research on strategies to promote reversal of liver fibrosis and/or assess the associated clinical and pathological outcomes.
- Clinical studies to evaluate combination or curative therapies for treatment of hepatitis B infection.

Hereditary Angioedema

- Research toward the development of a cure for hereditary angioedema.
- Development and/or validation of novel and/or innovative therapeutic strategies for the treatment and/or prevention of hereditary angioedema attacks.
- Research to improve early diagnosis of hereditary angioedema.
- Evaluation of existing or novel and/or innovative therapeutics in pediatric hereditary angioedema patients.

Hydrocephalus

- Research on the etiology, prevention, diagnosis, and treatment of post-traumatic hydrocephalus.

- Discovery or validation of novel and/or innovative therapies and therapeutic targets for the treatment of hydrocephalus and its sequelae, including therapies directed at myelin regeneration and repair.
- Development or validation of biomarkers and imaging techniques, particularly multimodal approaches, to aid in diagnosis, prognosis, and monitoring of therapeutic efficacy.
- Research on the prevention of shunt failure.
- Development or validation of improved hydrocephalus model systems.

Immunomonitoring of Intestinal Transplants

- Studies to elucidate the role of the mucosal immune system, humoral, innate, and adaptive cellular immunity, other host-derived factors, or gut microbiota-derived factors in maintaining intestinal transplant viability and improving outcomes.
- Development and evaluation of evidence-based intestinal transplant strategies that focus on dampening the regional immune response against intestinal transplants or circumvent the induction of immunity against the transplant.
- Development and evaluation of implant-associated materials (e.g., scaffolds) with anti-inflammatory properties that protect the intestinal transplant from immune attack.
- Development and evaluation of strategies for inducing and maintaining populations of anti-inflammatory regulatory immune cell populations at the transplant site.
- Studies to improve immunomonitoring of recipient immune responses after intestinal transplantation, with a focus on prospective leukocyte profiling, to aid in diagnosis and treatment of immunological and immunosuppression-related complications.
- Development and/or validation of precise, real-time implanted monitoring devices to improve individualized patient outcomes after intestinal transplantation.

Inflammatory Bowel Diseases

- Studies directed toward understanding how acute enteric infections may trigger chronic bowel diseases with acute and sub-acute inflammatory bowel disease, including genomic, microbiomic, immune mechanisms, and systems biology approaches.
- Studies to understand the interaction between acute/chronic stress and infection and the development of inflammatory bowel disease.
- Research to better characterize the association between the use of drugs, such as isotretinoin and long-term doxycycline, and the development of inflammatory bowel disease.
- Research on the role of diet in the development and progression of inflammatory bowel disease.
- Research on the influence of the microbiome on inflammatory bowel disease.
- Research on treatment strategies for patients with inflammatory bowel disease, including those who are refractory to standard care.

Interstitial Cystitis

- Studies that define the risk, prevalence, and operational impact of interstitial cystitis among active duty personnel.
- Identification of biological markers for making a definitive diagnosis of interstitial cystitis.
- Evaluation and assessment of novel and/or innovative treatment options for interstitial cystitis patients, including intravesical therapy.
- Research on the etiology of interstitial cystitis to inform targeted therapy development.

Lung Injury

- Studies to identify the prevalence and associated morbidity and mortality of blast overpressure lung injury.
- Development of improved methods for assessing lung injury due to chemical or physical (e.g., radiation) hazards and materials in occupational, operational, and training environments to improve surveillance, diagnosis, and prognosis.
- Development of improved methods for monitoring pulmonary exposure to chemical or physical agents that might cause lung injury.
- Identification of factors that predispose an individual's susceptibility to lung injury resulting from environmental exposures (i.e., genetic predisposition).
- Development of preventive techniques, novel and/or innovative detection technologies, and therapeutics to reduce the incidence and/or severity of lung injury.

Malaria

- Investigation of mechanisms of drug resistance in malaria, to include host, pathogen, and region-specific resistance against drugs used for treatment and prophylaxis.
- Development of passive immunization approaches for the management of malaria.
- Development of malaria prophylactic regimens that encourage higher compliance and methods to monitor compliance in deployed Service members.
- Identification of novel and/or innovative malaria drug targets for blood and liver stage malaria parasites.
- Development of a vaccine that induces high levels of long-lived, broadly protective immunity against *Plasmodium falciparum* and/or *Plasmodium vivax*.

Metals Toxicology

- Identification and validation of biomarkers to evaluate military Service members' acute exposure to toxic metals in an operational environment and predict potential consequent health risks and associated health outcomes.
- Retrospective studies to evaluate risks and exposure to military-relevant toxic metals among workers at industrial facilities.
- Research on the toxicity of metal combinations and the interaction between different metal components.

- Research on the toxicity of metal-based engineered nanomaterials, including those used in military applications.

Mitochondrial Disease

- Identification and testing of non-invasive techniques and biomarkers to monitor mitochondrial function, aid in clinical diagnosis, and/or evaluate therapeutic efficacy.
- Development of improved tools and animal models to study primary mitochondrial diseases and evaluate therapeutics.
- Research to better understand the pathology of primary mitochondrial diseases.
- Development of tools and methodologies to assess mitochondrial heteroplasmy at the cellular, tissue, and organ levels.
- Research on novel and/or innovative treatments to alleviate symptoms or slow down the progression of mitochondrial diseases.

Musculoskeletal Disorders

- Research to increase understanding and improve diagnosis, prevention, and/or treatments of chronic overuse musculoskeletal disorders.
- Research on measures (e.g., clinical biomarkers, novel/innovative interventions) to diagnose, predict, reduce the incidence of, or optimize health or return-to-duty outcomes in military training- and Service-related musculoskeletal disorders.
- Research on the validation and/or optimization of rehabilitation strategies for musculoskeletal disorders.
- Research to prevent, control, and/or optimize musculoskeletal health outcomes for work-related musculoskeletal disorders.
- Research on back pain treatment and/or management strategies to prevent surgery and recurrence of symptoms, identify factors that predict optimal treatment response for different patients, and encourage self-management.

Myotonic Dystrophy

- Research on the role of epigenetic factors in the onset, progression, and/or severity of myotonic dystrophy in relevant animal models or patients.
- Research into the mechanisms of expanded CTG or CCTG repeat instability in somatic or germline cells in myotonic dystrophy.
- Identification of biomarkers that can be detected through minimally invasive means to signal early changes in the progression of myotonic dystrophy.
- Development and/or testing of novel and/or innovative treatments, including those utilizing gene editing or silencing.
- Clinical research into the natural history of myotonic dystrophy, to understand disease progression and develop/validate clinical trial endpoint measures across the multiple organ systems involved in the disease.

Non-Opioid Pain Management

- Development of non-opioid pain management therapies, including complementary and alternative medicine approaches.
- Research to increase understanding and prevention of the progression from acute pain to chronic pain.
- Research to identify and address biopsychosocial aspects of pain to reduce or eliminate the use of opioid pain medication(s).
- Research on pain management strategies for patients with limited access to skilled providers and resources, including battlefield, prolonged field, transport, and other resource-limited environments.
- Development of non-opioid pain medicine that can be given via inhalation or intramuscularly, submucosally, or intravenously on the battlefield to provide adequate relief from pain without affecting the cardiorespiratory system.

Nutrition Optimization

- Development and/or validation of nutrition-based strategies that mitigate the consequences of operational stressors on Service member health, readiness, and performance.
- Research on the impact of dietary supplements (e.g., vitamin supplements, probiotics, protein powders) on the physical and/or cognitive performance, including the readiness of military Service members.
- Development and/or validation of improved nutrition strategies for physical and/or cognitive performance enhancement and sustainment in the operational environment.
- Development of tools or devices to monitor nutritional intake at an individual level to promote a culture of wellness.
- Strategies to apply metabolomics to optimize nutrition, including improving Warfighter performance in training and operational environments.
- Research to optimize nutrition in resource-limited settings.

Pancreatitis

- Development and testing of novel and/or innovative therapeutics for acute and/or chronic pancreatitis.
- Research on the basic biology and physiology of the pancreas to better understand the etiology and pathology of pancreatitis.
- Research to improve understanding and management of complications of pancreatitis.
- Retrospective studies to determine the risk and incidence of pancreatitis among former and current active duty personnel.

Pathogen-Inactivated Blood Products

- Research on lyophilization of pathogen reduced/inactivated blood products and derivatives (platelets, plasma, red cells, cryoprecipitate, coagulation factors, etc.).

- Development and advancement of technology to improve the safety of blood products to include pathogen reduction/inactivation in whole blood for military/civilian blood donor centers and blood banks that meets the requirements for FDA licensure in support of domestic and global contingency/combat operations.
- Expansion and validation of the library of blood-borne pathogens that are reduced/inactivated to include emerging pathogens, genetically modified pathogens, and pathogens designed for biological warfare.
- Advancement in pathogen reduction technology to further improve the log-killed reduction for blood-borne pathogen of common interest (e.g., hepatitis B, Korean hemorrhagic fever virus, Bunyavirus, human immunodeficiency virus [HIV], Rift Valley fever, malaria, Babesia, Ebola, West Nile virus, dengue, chikungunya, Zika virus).
- Research studies, including clinical trials, to further characterize the effects of pathogen reduction technology in blood products (e.g., whole blood, platelets, plasma, cryoprecipitate).
- Development and validation of next generation technology and/or devices to reduce the production time for pathogen reduction/inactivation in whole blood.

Post-Traumatic Osteoarthritis

- Research into cell-based approaches for treatment or prevention of post-traumatic osteoarthritis.
- Development or validation of novel and/or innovative approaches to restoring joint stability after injury.
- Studies to evaluate and develop best practices for multidisciplinary team approaches and treatment algorithms for post-traumatic osteoarthritis.
- Sustained release, intra-articular injectable steroidal, non-steroidal, or disease-modifying therapies that offer two or more months of symptomatic relief of pain and/or inflammation in a single injection.
- Research on therapies that target multiple phases of the cellular response pathways that are implicated in the development of post-traumatic osteoarthritis, including cell death, inflammation, matrix changes, and changes in catabolic and anabolic responses.

Pressure Ulcers

- Novel strategies for the treatment (including mitigation of the advancement of stages) of pressure ulcers.
- Novel strategies for the prevention or early detection of pressure ulcers.
- Strategies to prevent or reduce the formation of pressure ulcers during long-range transport/aeromedical evacuation.
- Research on novel techniques for synthetic production, delivery, and adhesion methodologies leading to permanent closing of pressure ulcers. This might encompass synthetic fibers, novel tissue culture methodologies, growth factors, dermal printing, artificial skin, skin graft substitutes, regenerative medicine, etc.

Pulmonary Fibrosis

- Identification of biomarkers of pulmonary injury or early predictors of interstitial lung disease.
- Development and/or validation of improved tools and animal models (excluding mice) to study pulmonary fibrosis and evaluate therapeutics.
- Research into the pathobiology and molecular mechanisms underlying the development and progression of pulmonary fibrosis.
- Retrospective studies to determine the risk and incidence of pulmonary fibrosis among military Service members and Veterans.
- Development and/or testing of novel and/or innovative treatments, to include precision medicine approaches, to delay or modify the progression of pulmonary fibrosis.

Respiratory Health (excluding lung cancer and mesothelioma)

- Research on the causes, treatment, and prevention of chronic obstructive pulmonary disease (COPD), including identification and validation of biomarkers and disease phenotypes, as well as employing personalized medicine approaches in clinical research and disease management.
- Research on the cause, treatment, and prevention of respiratory symptoms and ailments possibly associated with deployment and redeployment military personnel.
- Research to evaluate the impact of military service, primarily deployment, on the prevalence and severity of respiratory disease.
- Identification and/or validation of biochemical, physiological, or combined biomarkers for evaluating risk or extent of injury from either acute or long-term toxic occupational or environmental exposures.
- Research investigating exposure rates, detection, and treatment of diseases related to inhalation of mold and fungi, such as coccidioidomycosis, from both indoor and outdoor sources.

Rett Syndrome

- Identification and/or validation of novel and/or innovative biological targets for the treatment of Rett syndrome.
- Development and testing of interventions to improve the neurological symptoms of Rett syndrome.
- Research to understand the relationship between genetic mutations, physical traits, and symptoms in individuals with Rett syndrome.
- Research to understand Rett syndrome's commonalities with, and differences from, classic autism and regressive autism.
- Research on the pathobiology of MeCP2 and associated genes and proteins.

Rheumatoid Arthritis

- Research to better understand the relationship between genetic risk and environmental triggers, such as infection or smoking, in developing rheumatoid arthritis.
- Studies that identify or validate biomarkers or personalized medicine strategies that allow for individualized medication choice based on the patient's underlying biology or disease state.
- Research on the long-term use of immunosuppressive and other therapies in patients with rheumatoid arthritis.
- Research to better characterize and understand the preclinical disease stage of rheumatoid arthritis for early diagnosis and treatment.
- Research on management of comorbidities, including biopsychosocial outcomes, for patients with rheumatoid arthritis.
- Research to establish activity recommendations following joint replacement for maximal joint life.

Scleroderma

- Research on the molecular mechanisms and pathogenesis of scleroderma, including the identification of novel and/or innovative therapeutic targets.
- Development and/or validation of novel and/or innovative therapies for scleroderma.
- Identification and/or validation of biomarkers and other approaches for early diagnosis, monitoring disease progression, and/or assessment of treatment response.
- Epidemiologic studies investigating the impact of localized scleroderma (morphea) on duty performance, use of personal protective equipment, and deployability of military personnel.

Sleep Disorders

- Research on how the disruption of normal sleep and circadian rhythms adversely affects the physical and psychological health, safety, performance, and productivity of military personnel and civilian populations, including sex and gender differences.
- Identification and/or validation of non-Continuous Positive Airway Pressure (CPAP)-based treatment regimens that enhance compliance in military personnel and civilian populations.
- Research on the prevention and/or mitigation of sleep disorders that are associated with long aeromedical evacuation flights for both clinical team members and patients.
- Development and/or testing of non-pharmacological treatments for sleep disorders associated with long-term exposure to enclosed environments (e.g., aircraft, submarines, tanks).

Spinal Muscular Atrophy

- Research to conduct molecular and proteomic phenotyping of spinal muscular atrophy disease states.
- Research to determine mitochondrial involvement and astrocytic and other non-neuronal contributions to motor neuron vulnerability.

- Exploration of the form and function of SMN-depleted neuromuscular junctions at ultrastructural (e.g., dysregulation of endocytosis), transcriptomic, and proteomic levels, particularly the mildest SMN reduction that leads to consistent quantifiable motor neuron loss.
- Research to find non-SMN-altering spinal muscular atrophy modifying genes that may lead to identification of novel and/or innovative therapeutic targets or treatments.
- Research to further understand SMN gene regulation and post-transcriptional mechanisms leading to synergistic SMN-repleting approaches, as well as to determine whether boosting SMN induction maximizes efficacy.

Sustained-Release Drug Delivery

- Development of technology that can provide long-term (for up to one week or more) sustained-release delivery of oral drugs. Potential applications of this technology could include long-term delivery of agents for post-traumatic stress disorder, opiate dependence, low-dose pain control, allergies, attention deficit/hyperactivity disorder, chemoprophylaxis, and other conditions.
- Development of a delivery system (including novel GMP-grade biomaterials) that could accurately deliver prescription and non-prescription medications.
- Development of novel and/or innovative approaches for bioavailable and sustained-release oral formulations of existing broad-spectrum fungicidal, antimicrobial, antiparasitic, and antiviral medications.
- Development of a sustained release formulation of anti-tuberculosis drugs that would facilitate long-term treatment and reduce the emergence of resistance due to poor compliance.
- Research into techniques to provide sustained release of drugs in tissue repair applications, such as bone or nerve regeneration or vision restoration.

Tinnitus

- Development and validation of objective tools/methods to diagnose and characterize tinnitus (e.g., imaging techniques to identify functional and structural changes in the brain, biomarkers of resiliency, and susceptibility to tinnitus).
- Research to understand the mechanisms of acute tinnitus, its relationship to noise-induced hearing loss, and progression to chronic tinnitus, with the focus on developing interventions.
- Research to understand and mitigate the negative impact of tinnitus on operational readiness of the military.
- Identification of novel and/or innovative therapies and/or devices for interventions to prevent, manage, and treat tinnitus, including behavioral approaches, new uses for existing drugs, nutritional and pharmaceutical strategies, and acoustic, electrical, and other stimulation technologies.

Tissue Regeneration

- Development of novel therapies to repair neurosensory damage, maintain the distal end organ interface, or regenerate the neuromuscular junction for reinnervation of end organs during peripheral nerve regeneration.
- Development of novel therapies for regeneration of tendons and musculotendinous junctions.
- Development of novel therapies for regeneration of functional skeletal muscle, particularly stem cell-based approaches and treatments for volumetric muscle loss.
- Development and improvement of extended-time tissue preservation therapies and technologies for ischemia-reperfusion injury prevention and treatment.
- Research on novel approaches and therapies to understand mechanisms of immune rejection and obviate the need for chronic toxic immunosuppression in reconstructive transplantation and vascularized composite allotransplantation.
- Research into innovative methods for developing biocompatible scaffolds and stem cell therapies for manufacturing and production of tissues.

Tuberculosis

- Development of a diagnostic assay that can be used at the point of care to rapidly and accurately diagnose tuberculosis to include multidrug-resistant tuberculosis or extensively drug-resistant tuberculosis.
- Development of novel and/or innovative tuberculosis vaccines or optimization of current tuberculosis vaccines.
- Research to understand, diagnose, or treat multidrug-resistant tuberculosis or extensively drug-resistant tuberculosis.

Vaccine Development for Infectious Diseases

- Development of a therapeutic vaccine and/or other strategies for infectious diseases.
- Identification of correlate(s) of immunity against dengue, malaria, and other infectious agents and development of a surrogate test of protection.
- Development and fielding of vaccines to prevent Service members from becoming ill from endemic disease exposure during operational deployments. This includes, but is not limited to, Zika, dengue, chikungunya, hantavirus hemorrhagic fever with renal/pulmonary syndrome, rickettsioses, trypanosomiasis, leptospirosis, HIV, norovirus, Middle East Respiratory Syndrome, coronavirus, schistosomiasis, leishmaniasis, nipah, Lassa fever, and West Nile fever.
- Development of flexible vaccine technologies that can be used to rapidly respond to emerging and re-emerging infectious diseases threats.
- Evaluation of passive immunization strategies to use in conjunction with emerging infectious disease vaccinations.

Vascular Malformations

- Studies into the natural history, genetics, and pathogenesis of vascular malformations.

- Research to improve methods to diagnose and manage vascular malformations.
- Research to discover or develop novel and/or innovative therapeutic targets and treatments to regress or prevent vascular malformations.
- Development of non-invasive or minimally invasive technologies or approaches for the control of internal bleeding, including cerebral arteriovenous malformations, associated with vascular malformations.
- Development of in vivo or in vitro models of vascular malformations for the purpose of identifying novel and/or innovative drug targets, screening existing drugs, and/or elucidating the pathogenesis of the disease.

Women's Heart Disease

- Identification of gender-specific approaches to either develop novel diagnostics and treatments or increase the effectiveness of current practice to improve clinical care of women.
- Research on factors to predict and prevent the long-term impacts of gestational diabetes, gestational hypertension, and preeclampsia on the cardiovascular health of women.
- Research on trauma-induced cardiac arrest secondary to hemorrhage and polytrauma in the female population.
- Research focused on elucidating the potential relationship between post-traumatic stress disorder and women's heart disease.
- Studies to determine the risk and incidence of heart disease among current and/or former female Service members.

APPENDIX 3: DOD AND VA WEBSITES

PIs are encouraged to integrate and/or align their research projects with DoD and/or VA research laboratories and programs. Collaboration with DoD or VA investigators is also encouraged. Below is a list of websites that may be useful in identifying additional information about DoD and VA areas of research interest, ongoing research or potential opportunities for collaboration within the FY18 PRMRP Topic Areas.

Air Force Office of Scientific Research
http://www.wpafb.af.mil/afrl/afosr/

Air Force Research Laboratory
http://www.wpafb.af.mil/afrl

Armed Forces Radiobiology Research Institute
http://www.usuhs.edu/afrri/

Clinical and Rehabilitative Medicine Research Program
https://crmrp.amedd.army.mil

Combat Casualty Care Research Program
https://ccc.amedd.army.mil

Congressionally Directed Medical Research Programs
http://cdmrp.army.mil

Defense Advanced Research Projects Agency
http://www.darpa.mil/

Defense Technical Information Center
http://www.dtic.mil

Defense Threat Reduction Agency
http://www.dtra.mil/

Military Health System Research Symposium
https://mhsrs.amedd.army.mil/SitePages/Home.aspx

Military Infectious Diseases Research Program
https://midrp.amedd.army.mil

Military Operational Medicine Research Program
https://momrp.amedd.army.mil

Naval Health Research Center
http://www.med.navy.mil/sites/nhrc

Navy and Marine Corps Public Health Center
http://www.nmcphc.med.navy.mil/

Office of Naval Research
http://www.med.navy.mil/

Office of the Under Secretary of Defense for Acquisition, Technology and Logistics
http://www.acq.osd.mil/

Telemedicine and Advanced Technology Research Center
http://www.tatrc.org/

Uniformed Services University of the Health Sciences
http://www.usuhs.edu/research

U.S. Army Institute of Surgical Research
http://www.usaisr.amedd.army.mil/

U.S. Army Medical Materiel Development Activity
http://www.usammda.army.mil/

U.S. Army Medical Research and Materiel Command
http://mrmc.amedd.army.mil

U.S. Army Medical Research Institute of Infectious Diseases
http://www.usamriid.army.mil/

U.S. Army Research Institute of Environmental Medicine
http://www.usariem.army.mil/

U.S. Army Research Laboratory
http://www.arl.army.mil

U.S. Department of Defense Blast Injury Research Program
https://blastinjuryresearch.amedd.army.mil/

U.S. Department of Veterans Affairs, Office of Research and Development
http://www.research.va.gov

U.S. Naval Research Laboratory
http://www.nrl.navy.mil

Walter Reed Army Institute of Research
http://www.wrair.army.mil/

DEFENSE THREAT REDUCTION AGENCY
BROAD AGENCY ANNOUNCEMENT
HDTRA1-14-24-FRCWMD-BAA
Amendment 9
January 2019

Research and Development Directorate (RD)
Chief Scientist and Innovations Department (RD-ST)

Fundamental Research to Counter Weapons of Mass Destruction (C-WMD)

Original Posting Date: March 2015

TABLE OF CONTENTS

SECTION NO. **PAGE NO.**

OVERVIEW INFORMATION 3
1. FUNDING OPPORTUNITY DESCRIPTION 5
2. AWARD INFORMATION 10
3. ELIGIBILITY INFORMATION 12
4. APPLICATION AND SUBMISSION INFORMATION 13
5. APPLICATION REVIEW INFORMATION 29
6. AWARD ADMINISTRATION INFORMATION 32
7. AGENCY CONTACTS 38
8. OTHER INFORMATION 39

ATTACHMENT 1: SPECIFIC TOPICS 40
ATTACHMENT 2: INTELLECTUAL PROPERTY 56

OVERVIEW INFORMATION

Agency Name:

Defense Threat Reduction Agency (DTRA)
Research and Development (RD) Directorate
Chief Scientist and Innovation Department (ST)
8725 John J. Kingman Road, MS 6201
Fort Belvoir, VA 22060-6201

Funding Opportunity Title: Fundamental Research to Counter Weapons of Mass Destruction (FRCWMD) Broad Agency Announcement (BAA)

Announcement Type: This is an amended announcement of this funding opportunity. This BAA is in effect from March 2015 through September 2024. It is anticipated that a majority of the actions funded from this announcement will be in the form of grants; however, other instruments such as contracts, cooperative agreements (CAs) or other transactions agreements (OTAs) may also be awarded from this announcement. Submissions for this BAA may occur in two ways: 1) in response to the published topics detailed in Attachment 1 or 2) to a general thrust area as described in Section 1.5.

In general, all topic-specific and general thrust area submissions require pre-coordination in accordance with the guidelines in Section 1.5 and Section 4.2.1. DTRA reserves the right to waive the pre-coordination requirement for topics on a case-by-case basis; and will state the waiver applies within the individual topic description. If a pre-application white paper is received without prior coordination, DTRA may not review it. From the date of the disposition email the applicant has 63 days to submit the pre-application white paper. If the submission is not feasible within this 63-day window, the abstract must be coordinated again to ensure the interest in the white paper remains.

The evaluation of all submissions will be conducted in two phases. Phase I is for receipt and evaluation of pre-application white papers in direct response to a published topic or by invitation based on the assessment of the idea by the Technical POC. Phase II is for receipt and evaluation of invited proposal applications. Invitation to the Phase II, invited proposal submission, will be based on the evaluation results of the Phase I pre-application white paper.

Funding Opportunity Number: HDTRA1-14-24-FRCWMD-BAA

Catalog of Federal Domestic Assistance (CFDA) Number: 12.351

Dates: This BAA is open continuously from March 2015 through September 2024. Published topics will include instructions on any topic-specific opening and closing dates as well as any topic-specific limitations on award types, dollar values, and eligibility. Submissions to a general thrust area may occur at any time this BAA is in effect. Applicants should take care to note requirements for pre-coordination of an abstract.

ADDITIONAL OVERVIEW CONTENT

Research, educational program, or other effort proposals are sought from accredited degree-granting colleges and universities. Research, educational program, or other effort proposals are also sought from industrial, commercial (including small businesses), and not-for-profit research entities. DTRA strongly encourages and may give preference to pre-application white papers and proposals that demonstrate a significant contribution (significant is defined as a minimum of

30% of total value) by one (1) or more universities.

All submissions (pre-application white papers and invited proposals) must be made in accordance with the submission instructions in this BAA through www.grants.gov using the application packages linked with this BAA (under the "Package" tab) on www.grants.gov. Applicants are responsible for ensuring compliant and final submission of their pre-application white papers and proposal applications. Any submission that does not conform to the requirements outlined in the BAA and in the invitation for proposal may not be reviewed or considered further at the discretion of DTRA.

Pre-application white papers may be evaluated any time after receipt. Invitations for full proposal submission may occur any time after the pre-application white paper evaluation and will be limited to available program funds.

Efforts may be proposed for up to five (5) years. Awards may be for a base period of one (1) year with four (4) additional years as possible options, a base period of two (2) years with three (3) additional years as possible options, or a base period of three (3) years with two (2) additional years as possible options. Applicants should take care to propose the most logical mix of base and option years for the scope of work. Further, the base period should yield a logical completion point for the work. In cases where option years are proposed, decisions regarding exercising those options will be based on the evaluation of the work accomplished in the base period. Pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable; however, the Government reserves the right to invite option years for awards that originally only included a base period.

Grants may range from small dollar value (e.g., $25K) up to $1M annually (total, including both direct and indirect costs) depending on the nature and the scope of work. Payments on grants will be made in advance, subject to the conditions described in 2 CFR 200.305. Funding amounts for contracts, CAs, and other procurement instruments will be considered on a case-by-case basis. Thirty (30)-60 individual awards are anticipated each year.

Any assistance instrument awarded under this announcement will be governed by the award terms and conditions, which conform to DoD's implementation of OMB circulars applicable to financial assistance. This includes DoD implementation of OMB guidance in 2 CFR part 200, "Uniform Administrative Requirements, Cost Principles, and Audit Requirements for Federal Awards."

1. FUNDING OPPORTUNITY DESCRIPTION

1.1. DTRA safeguards America and its allies from weapons of mass destruction (WMD) and provides capabilities to reduce, eliminate, and counter the threat and effects from chemical, biological, radiological, nuclear, and high yield explosives. DTRA seeks to identify, adopt, and adapt emerging, existing and revolutionary sciences that may demonstrate high payoff potential to Counter-WMD (C-WMD) threats, including improvised threats. This BAA is an extramural endeavor that combines the fundamental research, educational program, or other effort needs appropriate for basic or applied research funding of DTRA and other DoD interests.

This announcement solicits ideas and topic-based pre-application white papers for long-term challenges that offer a significant contribution: to the current body of knowledge, to the understanding of phenomena and observable facts, to significantly advance revolutionary technology, to new concepts for technology application, or that may have impact on future C-WMD threat reduction, expertise, or capabilities.

A portion of this effort is expected to be devoted to awards for science, technology, engineering and mathematics education programs with a C-WMD focus; such as, but not limited to postdoctoral fellowships, stipends, degrees, visiting scientist programs, student exchange programs, and development of accredited C-WMD curricula.

1.2. Fundamental research means basic and applied research in science and engineering, the results of which ordinarily are published and shared broadly within the scientific community, as distinguished from proprietary research and from industrial development, design, production, and product utilization, the results of which ordinarily are restricted for proprietary or national security reasons.

Contracted Fundamental Research includes research performed under grants, contracts (awards), or OTAs that are (a) funded by budget Category 6.1 (Basic Research), whether performed by universities or industry or (b) funded by budget Category 6.2 (Applied Research) performed on-campus at a university. Fundamental research provides for science and technology (S&T) research and early applied development. It seeks to lower performance risk to a manageable level and facilitate transition and funding to capability end-state programs.

1.3. Technology Readiness Levels (TRLs) provide a systematic metric/measurement system that supports assessments of the maturity of a particular technology and the consistent comparison of maturity between different types of technology. Fundamental research may be defined within the first four (4) TRLs.

1.4. This BAA seeks optimum approaches to meet DTRA fundamental research objectives. The Government encourages pre-application white papers and proposals that span a wide spectrum of research to expand fundamental scientific knowledge in response to specific topics and to the more general thrust areas. The Government reserves the right to award any combination of approaches which offer the best overall value to the Government and to oversee any and all processes and approaches once ongoing.

1.5. Thrust Areas 1-7 are described below. When a specific set of topics has been identified, these detailed needs will be described in Attachment 1 along with any topic-specific submission instructions, deadlines, anticipated award structure, and funding requirements. Otherwise, pre-application white papers and proposals may be written against one of the general thrust area descriptions.

DTRA may not review any pre-application white papers without prior coordination of the idea with the appropriate thrust area- e-mail address (Section 7). Applicants should note that there is extremely limited funding available for many of the thrust areas. Pre-application white papers will only be accepted from the coordinated abstracts under limited circumstances.

1.5.1. **Thrust Area 1—*Science of WMD Sensing and Recognition:*** The science of WMD sensing and recognition investigates the fundamental understanding of materials that demonstrate measurable changes when stimulated by radiation or particles from WMD in the environment. This involves the exploration and exploitation of interactions between materials and various electromagnetic phenomena, molecules, nuclear radiation, and particles. Furthermore, these interactions and the specific form of recognition they offer are used for the subsequent generation of information, providing knowledge of the presence, identity, and quantity of material or energy in the environment that may be significant. The goal of this thrust area's portfolio is to advance the following capabilities: location, identification and characterization of radiological-nuclear (RN) materials; detection of RN materials at significant stand-off distances; and the reduction of the technical nuclear forensics timeline. Thrust Area 1 is currently not interested in research focusing on the sensing of explosives or the detection of Improvised Explosive Devices (IEDs).

1.5.2. **Thrust Area 2—*Network Sciences:*** The fundamental science of network sciences includes advancing knowledge of complex disparate but interdependent networks critical to military operations where WMD-related robustness, resiliency, recovery of, and informational and operational utility is required. It includes response, resilience, and recovery of interdependent, multi-layered physical networks after exposure from electromagnetic pulse and other nuclear weapons effects, rapid discovery and analyzing low-observable WMD-related information from large, disparate WMD-related data sets from multiple types of networks, and to develop theories and representations for low observable WMD-related radical ideation from social networks.

1.5.3. **Thrust Area 3—*Science for Protection:*** Fundamental science for protection involves advancing knowledge in physical, biological, and engineering sciences to protect personnel, sensitive electronic systems, and structural infrastructure from the effects of weapons of mass destruction. Protection includes both passive and active defense against threats. Approaches include advanced highly-ordered materials and nanomaterials to hardening infrastructure and facilities against blast, nuclear events, or other CBRNE effects; exploring new methods to experimentally and computationally simulate the effects of a nuclear event; investigations of the interaction of radiation with sensitive electronics and systems as well as development of novel materials and methods that are robust against radiation effects; novel methods to protect personnel from the physical, radiological, and nuclear effects of WMDs; and the study of biological systems, including intact structures, metabolic products, or discrete components and pathways, as applied to protection of U.S. Forces during operations in areas actually or potentially contaminated by radiation. For protection of personnel the areas of interest include development of radiation countermeasures to prevent biological damage associated with exposure to ionizing radiation and development of novel biologically-based or -produced detection systems for wide area surveillance to determine the nature, extent, and distribution of contamination.

1.5.4. **Thrust Area 4—*Science to Defeat WMD:*** Fundamental science for significantly improving energetic materials for use against WMD facilities and systems with minimal

collateral effects from post-blast WMD release, for deeper penetration to deny the adversary sanctuary of WMD, and for predictable modeling of counter-WMD munitions and simulation of in-theater scenarios with accurate lethality calculations.

1.5.5. **Thrust Area 5—*Science to Secure WMDs:*** Fundamental science to support securing WMD includes: revolutionary means to safely handle, transport, control access, or eliminate WMD components and weapons; new physical or other methods to monitor compliance to support future agreements or treaties; and, exploring phenomena and means that facilitate reduction of nuclear or non-nuclear WMD proliferation pathways. This includes focus on: science principles to assist tagging, tracking, location to secure WMD; novel means to mark and read objects in order to secure inventories; remote or unattended monitoring to understand the nature of objects (e.g., is it nuclear, biological, chemical or conventional?); monitoring to detect intrusion, diversion or substitution, tampering, and other adverse activity; and, understanding of both physical and life science environmental signatures as witnesses of WMD-related activity. The ability to secure WMD may impact either verification of treaties, or control of WMD outside treaty regimes.

1.5.6. **Thrust Area 6—*Cooperative Counter WMD Research with Global Partners:*** Cooperative fundamental research to reduce the global threat of WMD in collaboration with a broad range of global research partners. This thrust area involves exploratory basic and applied research that will address opportunities to reduce, eliminate, and counter WMD across the Chemical, Biological, Radiological, Nuclear, and High Explosive (CBRNE) spectrum. Efforts in this area will develop strong international relationships which will foster a smooth transition of program ownership to the partnering country. The goal is to improve international collaboration to detect, characterize, and report WMD, and to advance partner nation sustainment through a culture of long-term cooperation and scientific responsibility for such programs. Multi-disciplinary, multinational research in science, technology, engineering, and mathematics development will be conducted to promote transparency through quality research publications and continual dialogue between scientists/engineers and young researchers.

The Cooperative Biological Engagement Program (CBEP), a component of the DoD Cooperative Threat Reduction (CTR) Program, recognizes the danger to U.S. and global health security posed by the risk of outbreaks of dangerous infectious diseases, whether natural or manmade. Consistent with the national and departmental strategies, CBEP strives to address this risk by promoting best practices in biological safety and security, improving partner country capability to safely and rapidly detect and report dangerous diseases, and establish and enhance international research partnerships that focus on informing the disease surveillance network. The desired end state for CBEP engagements is the development of sustainable partner country capabilities to:

- Employ responsible bio-risk management best practices and principles,
- Conduct a modern and effective disease surveillance mission,
- Independently sustain engagement with, and effectively compete for funding within, the international scientific community,
- Comply with World Health Organization (WHO) International Health Regulations (IHR) and World Organization for Animal Health (OIE)/U.N. Food and Agriculture Organization (FAO) reporting guidelines, and

- Promote the One Health Concept.

The goals and objectives of CBEP international research partnerships are to:

- Goal 1: Support Biosurveillance, Biosafety and Biosecurity (BS&S) Capability Building Efforts
    - Objective 1: Inform and enhance operational biosurveillance strategies and implementation through improved understanding of endemic disease burden and pathogen biology.
    - Objective 2: Institutionalize responsible biorisk management best practices with partner country scientists.
- Goal 2: Engage Partner Country Scientists in Hypothesis-Driven Research
    - Objective 1: Support local, national, and regional priorities for understanding and mitigating human and animal disease risk (e.g., small, focused projects within individual countries linked by broad, integrating projects that include regional partners).
    - Objective 2: Improve international collaborations to survey, detect, characterize, and report disease.
- Goal 3: Promote One Health Initiative
    - Objective 1: Emphasize the nexus of human health, animal health, and the environment, and seek to further understand the mechanisms and factors involved in disease transmission.
    - Objective 2: Advance partner country sustainment of global health security initiatives.
- Goal 4: Foster an International Culture of Responsible and Ethical Conduct in Biological Research
    - Objective 1: Transition to a culture of responsibility and ethical conduct in biological research through thoughtful experimental design and good laboratory practices that result in high-quality data, and active participation in professional societies and the peer-review process.
    - Objective 2: Train partner country researchers to think critically in the pursuit of ethical research and to be competitive in soliciting funding from the international scientific community.

Ultimately, the techniques, procedures, and approaches must be sustainable for the partner country and linked with appropriate training in order to promote global health security, reinforce norms of safe and responsible conduct, obtain timely and accurate insight on current and emerging infectious disease risks, and transform the international dialogue on biological threats.

CBEP research projects are not determined by or limited to specific biological agents, but must be plausibly linked to pathogens of security concern and aimed at measurably supporting threat reduction objectives that:

- Enhance partner country's/region's capability to identify, consolidate, and secure collections of pathogens and diseases of security concern in order to prevent the sale, theft, diversion, or accidental release of such pathogens and diseases.

- Enhance partner country's/region's capability to rapidly and accurately survey, detect, diagnose, and report biological terrorism and outbreaks of pathogens and diseases of security concern in accordance with international reporting requirements.

Region-specific areas of interest are described in CBEP Regional Science Plans. Examples of general CBEP research areas of interest include: Epidemiology (e.g. studies measuring disease prevalence and incidence), Pathogen Biology, Pathogen Characterization, Assay Adaptation and Optimization, Microbial Ecology within a Public Health Context, and Preventative Strategies and Countermeasures. For clarification, medical countermeasure development (i.e., development of diagnostic tools, vaccines, therapeutics) is supported by many other U.S. Government or international agencies and is generally not supported by CBEP; however, research projects may inform medical countermeasure development and support validation and verification testing (e.g., as part of proficiency testing, pilot studies/testing, or exercises, etc.). Additionally, CBEP does ***not*** generally support research with common disease agents such as HIV/AIDS, malaria, and tuberculosis where other U.S. agencies have dedicated missions to do so; however, the program may choose to capitalize on opportunities to leverage research on these diseases to further CBEP goals, for example by testing samples collected under the auspices of other programs. CBEP also **will not** support research which poses risks to the overall threat reduction mission of CBEP, Dual-Use Research of Concern, or related activities (i.e., *in vivo* pathogenicity studies, virulence studies, animal passaging, etc.).

CBEP is interested in collaborative research engagements with foreign partners in any one of the following regions: Countries of the Former Soviet Union (FSU) (specifically, Armenia, Azerbaijan, Georgia, Kazakhstan, and Ukraine), Africa (including, but not limited to, Kenya, Tanzania Uganda, South Africa), Southeast Asia (including, but not limited to, Cambodia, Indonesia, Laos, Malaysia, Philippines, Thailand), and Middle Eastern /South Asian countries (including, but not limited to, Afghanistan, Iraq, India and Pakistan). CBEP encourages proposers to develop projects in conjunction with foreign institutions in CBEP-engaged countries.

1.5.7. Thrust Area 7—*Fundamental Science for Chemical and Biological Defense:*

Fundamental science for chemical and biological (CB) defense includes science and technology research that advances knowledge in physical and life sciences to defend and counter chemical and biological WMD that could be used against our Nation's warfighters. Fundamental research efforts enable capabilities such as development of improved detection devices for traditional and nontraditional chemical agents; development of diagnostics for existing and emerging infectious disease threats; increasing knowledge and improved capabilities for development of new or improved medical and material countermeasures to CB threats for both pre- and post-exposure scenarios; enhanced personal protection against, modeling of, prevention of, or decontamination of CB threats; and providing effective elimination strategies via non-kinetic approaches for threat agent destruction, neutralization and/or sequestration.

1.6. This BAA, in addition to any amendments issued in conjunction with this BAA, will be posted to the Grant Opportunities Website (www.grants.gov), the Federal Business Opportunities Website (www.fbo.gov), and the DTRA website (www.dtra.mil). The DTRA website is not the official sites; applicants are responsible for monitoring both www.fbo.gov and www.grants.gov. Posted amendments supersede all previous versions of the BAA. Note that topics will be listed in Attachment 1 and will be added/closed with Amendments to this BAA.

1.7. All administrative coordination and communication between applicants and the Government will be conducted using the e-mail address associated with this BAA, specified in Section 7. DTRA will not release employee personal contact information.

---

2. AWARD INFORMATION

2.1. Award Types. The full range of flexible procurement instruments available to DTRA are possible results from this announcement, including but not limited to contracts, grants, CAs, and OTAs; however, grants will likely be the predominant procurement instrument. Each of the applicable procurement instruments offer different advantages, liabilities and responsibilities for applicants and the Government.

Applicants must specify in their submittal their recommended approach (e.g. contract, grant, CA, or OTA); however, the Government reserves the right to negotiate and award the types of procurement instruments determined most appropriate under the circumstances. If warranted, portions of resulting awards may be segregated into pre-priced options.

Except for OTAs, the Government actions under this BAA shall adhere to the requirements of the Federal Acquisition Regulation (FAR), Defense Federal Acquisition Regulation Supplement (DFARS) and/or DoD Grant and Agreement Regulations (DoDGARS), as appropriate for the type of instrument. DoDGARs can be accessed online at http://www.ecfr.gov/cgi-bin/text-idx?SID=e5d686f6760f3274b3368f36723fbb7e&mc=true&tpl=/ecfrbrowse/Title32/32CIsubchapC.tpl . See also 32 Code of Federal Regulations (CFR) 22, which can be accessed online at http://www.ecfr.gov/cgi-bin/text-idx?rgn=div5;node=32%3A1.1.1.3.16 . Any assistance instrument awarded under this announcement will be governed by the award terms and conditions, which conform to DoD's implementation of OMB circulars applicable to financial assistance. The current version of the Terms and Conditions for grant awards may be found online in the Document & Template Library of the DTRA Basic and Fundamental Research Community Portal (www.dtrasubmission.net/portal).

On average, DTRA expects to award 30-60 individual awards each year. The predominance of awards will be grants. Payments on grants will be made in advance, subject to the conditions described in 2 CFR 200.305.

2.2. Scope of Awards. Awards may range from focused, exploratory projects with a high risk approach in innovative research in subjects with potential for high impact to C-WMD science to comprehensive programs of innovative research in an interdisciplinary area with potential for high impact.

Awards may have multiple Co-Principal Investigators (Co-PIs) and subawards. Authors of pre-application white papers and proposals should detail the contribution of all Co-PIs and any subawards to the C-WMD scientific impact.

Preference will be given to projects where undergraduate and/or graduate students, and/or postgraduate students are supported by the awards. Details regarding the participation of the students and the value of the research to the students as part of the pre-application white paper and full proposal are expected. Additional guidance regarding student and/or postgraduate student participation may be provided in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-

application white paper. Any specific guidance provided in a topic or to an applicant supersedes the information provided herein.

2.3. Subawards. Subawards (subgrants and/or subcontracts) are permitted. Subawards may be used to carry out a portion of the research or efforts. Awards may have multiple subawards. Awards will be made by a single award, e.g., grant or contract, to the lead organization. All subawards are the responsibility of the award recipient; exceptions will not be made.

For submissions made to Thrust Area 6 and associated topics, there is no limitation on subawards. DTRA will review and consider the proposed subawards for all pre-application white papers and proposals on a case-by-case basis. The prime awardee will be responsible for transferring funds to the subawardee. Applicants are reminded that priority is given to projects with the main locus of activity in the region-of-interest, so budgets should be allocated accordingly. Preference will be given to proposals where the subaward component to the region-of-interest partner(s) represents more than half of the award value (as measured in U.S. dollars).

2.4. Award Values. Grants resulting from submissions to Thrust Areas 1-7, including topics associated with these thrust areas, may range from small dollar value (e.g., $25K) up to $1M annually (total, including both direct and indirect costs) depending on the nature and the scope of work. Contracts, CAs, and OTAs will be considered on a case-by-case basis. All awards are subject to the availability of funds. Additional guidance regarding award values may be provided in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper. Any specific guidance provided in a topic or to an applicant supersedes the information provided herein. Funding for participation in this program is highly competitive and the cost of proposed research should strictly be maintained as detailed herein or as indicated in the invitation instructions.

2.5. Period of Performance and Award Structure. Efforts for Thrust Areas 1-7, including topics associated with these thrust areas, may be proposed for up to five (5) years. Awards may be for a base period of one (1) year with four (4) additional years as possible options, a base period of two (2) years with three (3) additional years as possible options, or a base period of three (3) years with two (2) additional years as possible options. Additional guidance regarding award structure may be provided in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper. Any specific guidance provided in a topic or to an applicant supersedes the information provided herein.

Applicants should take care to propose the most logical mix of base and option years for the scope of work. Further, the base period should yield a logical completion point for the work. In cases where option years are proposed, decisions regarding exercising those options will be based on the evaluation of the work accomplished in the base period.

DTRA is flexible on the award structure unless otherwise specified in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper. Applicants should take care to clearly label the tasks and anticipated outcomes for the base and option years in the pre-application white papers and the proposals. Pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable; however, the Government reserves the right to invite option years for awards that were originally awarded with only a base

period.

2.6. The Government Accountability Office, in its report GAO-16-14, WOMEN IN STEM RESEARCH: Better Data and Information Sharing Could Improve Oversight of Federal Grant-making and Title IX Compliance, December 3, 2015, recommended that the DoD collect certain demographic and career information to be able to assess the success rates of women who are proposed for key roles in applications in science, technology, engineering, or mathematics disciplines. To enable this assessment, DTRA will include with each Phase II application package the Research and Related Senior/Key Person Profile (Expanded) form and the Research and Related Personal Data form.

2.7. The Government does not anticipate the need to provide any hardware or software to execute the proposed research. However, DTRA will review and consider any hardware/software requests for all pre-application white papers and proposals on a case-by-case basis.

2.8. The Government reserves the right to fund all, some, or none of the proposals submitted; may elect to fund only part of any or all proposals; and may incrementally fund any or all awards under this BAA. The Government also reserves the right to request applicants make any changes necessary to submitted full proposals to increase the feasibility of making the proposal fundable. Applicants may decline to participate in any revisions to application packages requested by DTRA.

2.9. The Government may offer funding for any full proposals or portions of proposals at any time during the lifetime of this BAA.

---

3. ELIGIBILITY INFORMATION

3.1. Pre-application white papers and proposals submitted for this BAA will be considered from the following U.S. and Foreign Institutions as follows:

- Accredited degree-granting colleges, universities, and academic institutions.
- Industrial and commercial entities, including small businesses.
- Not-for-profit entities with a portfolio predominantly in research and foreign government laboratories. Proof of 501(c)(3) status from the Internal Revenue Service may be required. For foreign-based establishments entirely based outside the U.S. and/or its territories, proof of not-for-profit status may be required. Foreign based government laboratory equivalents include those residing in the Ministry of Defense, Ministry of Health, Ministry of Agriculture, Ministry of Education and Science and Food Safety Agencies.

DTRA strongly encourages and may give preference to pre-application white papers and proposals that demonstrate a significant contribution (significant is defined as a minimum of 30% of total value) by one (1) or more universities. Applicants should note that university participation may be mandatory for some published topics. Additional guidance regarding university participation may be provided in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper. Any specific guidance provided in a topic or to an applicant supersedes the information provided herein.

The following entities may not participate as prime awardees nor furnish Principal Investigators (PIs) in awards made under this BAA but may act as collaborators, including as Co-PIs, and/or subawardees:

- Federal Academic organizations (e.g., Naval Postgraduate School).
- Federal laboratories (including DoD and Department of Energy (DOE)).
- U.S. Government agencies.
- DoD-sponsored Federally Funded Research and Development Centers (FFRDCs) specified in the Defense Federal Acquisition Regulation Supplement (DFARS) 235.017-1 (http://farsite.hill.af.mil/VFDFARA.HTM) and click on 'DFARS Part 35'.
- DOE-sponsored FFRDCs.

Note: Federal laboratories (including DoD and DOE) and FFRDCs are eligible to submit abstracts (when required), pre-application white papers, and proposals in response to the Government Call issued by the DTRA Chief Scientist and Innovations Department. However, a FFRDC (other than the DoD FFRDCs specified in DFARS 235.017-1) must have authorization from its sponsoring agency in accordance with FAR 35.017-1. Eligibility requirements under the Call are subject to change. See http://www.dtrasubmission.net and after logging in, follow the link to the 'FY12-17 Fundamental Research to Counter Weapons of Mass Destruction (C-WMD) Government Call'.

3.2. Cost Sharing or Matching. In general, cost sharing or matching is not required for applications to this announcement. However, DTRA reserves the right to require cost sharing or matching on a case-by-case basis. Such instances will be specifically detailed in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper.

3.3. Other. DTRA uses the System for Award Management (SAM) to exclude recipients ineligible to receive Federal awards. SAM can be accessed online at http://sam.gov (Reference 2 CFR 1125).

---

4. APPLICATION AND SUBMISSION INFORMATION

4.1. Address to Request Application Package. This announcement contains all information required to submit a pre-application white paper and invited proposal. For convenience, Microsoft (MS) Word and MS PowerPoint templates for Phase II proposal submissions are provided in the Document & Template Library of the DTRA Basic and Fundamental Research Community Portal (www.dtrasubmission.net/portal) for applicant use. Applicants are encouraged to use the templates for preparing submissions; however, use of the templates is not required. Note: there is not a template available for the pre-application white paper.

4.1.1. The required application packages for the pre-application white papers and for the invited proposals are posted with this announcement. Note that each thrust area (as outlined in Section 1.6) and each topic (as outlined in Attachment 1) has a unique application package posted with this BAA. The application package corresponding to both: a.) the thrust area or topic of interest and b.) the phase, should be used for submission of pre-application white papers and invited full

proposals.

4.1.2. The application packages posted to www.grants.gov consist of the forms as detailed in Table 2.

Form Name	Phase I Pre-Application White Paper	Phase II Invited Proposal
SF-424 (R&R) Application for Federal Assistance Form	Required	Required
RR Budget Form	N/A	Required
R&R Subaward Budget Attachment(s) Form(s)	N/A	If Applicable
Research & Related Senior/Key Person Profile Form	N/A	Required
RR Personal Data	N/A	Required
Research & Related Other Project Information	N/A	Required
Disclosure of Lobbying Activities (SF-LLL)	N/A	If Applicable
Attachments Form	N/A	Required

Table 2: Forms. The instructions for completing each of these forms may be found online at the following web link: http://www.grants.gov/web/grants/form-instructions.html.

4.2. Content and Form of Application Submission. Submissions for this BAA will be conducted in two phases. Phase I is for receipt of pre-application white papers. Phase II is for receipt of invited proposal applications. Invitation to the Phase II proposal submission will be based on the evaluation results of the Phase I pre-application white paper.

4.2.1. The predominance of efforts, including all submissions to the thrust areas and most submissions to topics posted in Attachment 1, **must be** coordinated with the relevant technical point of contact (POC) for the appropriate thrust area prior to submission of a pre-application white paper; an e-mail for the DTRA technical POCs for Thrust Areas 1-7 are provided in Section 7. Coordination of research ideas and efforts must be accomplished via these email addresses, except in cases where a topic specifically states that pre-coordination is not required, and includes submission of an abstract (recommend less than 250 words) of the proposed project/effort or a paragraph description of the proposed project/effort to the email address in Section 7 and a reply email from the relevant email address in Section 7 with the disposition to the applicant. Pre-coordination may not be accomplished with email addresses other than those listed in Section 7. DTRA may not review white papers without prior coordination. Please note that attachments to e-mails may not be reviewed.

Applicants should note that there is extremely limited funding available for the general thrust areas. Pre-application white papers will only be accepted from the coordinated abstracts under very limited circumstances.

Topics may be posted in Attachment 1 of this announcement that may not require pre-coordination of an abstract. Please review the topics carefully.

4.2.2. Pre-application white papers and invited proposals **must be** submitted electronically using www.grants.gov and the corresponding application packages linked with this BAA on www.grants.gov (under the "Packages" tab). All applications, including all supporting documents, must be submitted in the English language.

Applicants are responsible for ensuring compliant and final submission of their Phase I pre-application white paper and Phase II invited proposal application. Note that this also applies to applicants using third party systems to submit application packages and attachments. Any submission that does not conform to the requirements outlined in the BAA and in the invitation for proposal may not be reviewed or considered further at the discretion of DTRA.

4.2.3. DTRA will not review any of the following:

- Pre-application white papers that are not pre-coordinated as required
- Pre-application white papers and proposals that are not submitted in the English language.
- Pre-application white papers that are submitted to topics that have been previously closed via an amendment to the BAA.
- Application packages and proposals for Phase II submissions that were not invited.

Exceptions WILL NOT be made under any circumstances.

4.2.4. Phase I Pre-Application White Paper Submission and Content.

Each pre-application white paper must address only one thrust area or topic. Each pre-application white paper must use the corresponding thrust area or topic application package.

Each Phase I application package contains the following forms:

Form	Attachment	Action
SF-424 (R&R) Application for Federal Assistance Form	Up to four (4) page white paper	Enter the appropriate information in data fields

Table 3: Phase I Pre-Application White Paper Package Chart.

Each Phase I application package contains the SF 424 (R&R) Application for Federal Assistance. To be considered a complete package, an up to four (4) page white paper is required to be uploaded as an attachment to the SF 424 (R&R).

DTRA-specific instructions for completing the SF 424 (R&R) Application for Federal Assistance are below, general application instructions can be found on www.grants.gov:

- Block 1 – Type of Submission. Applicants should indicate the Phase I submission is a "Pre-Application."
- Block 2.1 – Applicant Identifier. Not applicable.
- Block 3 – Date Received by State. Not applicable.
- Block 3.1 – State Application Identifier. Not applicable.
- Block 5 – Applicant Information. You must provide a Business Office Point of Contact (BPOC) with an e-mail address.
- Block 19 – Authorized Representative. The "signature of AOR" is not an actual signature

and is automatically completed upon submission of the electronic application package. Hard copies or email attachments of applications will not be accepted.

- Block 20 – Pre-application. Must be used to attach an up to four (4) page white paper. The white paper itself should provide sufficient information on the research being proposed (e.g., the hypothesis, theories, concepts, approaches, data measurements and analysis, etc.) to allow for an assessment by a technical expert.

Any pages submitted for the white paper that exceed the limit of four pages will not be read or evaluated. A page is defined as 8 ½ x 11 inches, single-spaced, with one-inch margins in type not smaller than 12 point Times New Roman font. The white paper must be provided in portrait layout.

At minimum, the white paper should address the following:

- A project abstract, which should be concise (less than 250 words), provide a summary of the proposed work, and demonstrate relevance to the topic being addressed. The abstract should not contain any proprietary data or markings.
- Potential scientific impact to provide greater knowledge or understanding of the fundamental aspects of phenomena and of observable facts, including how the research contributes to the C-WMD science needs outlined in the thrust area or topic.
- The impact of the research on C-WMD science must be clearly delineated.
- Cost estimate by year and total dollars required to accomplish the research as presented in the white paper (no details or breakout of costs is required).
- Potential team and management plan, including details on student involvement.
- Multidisciplinary white papers should carefully detail each of the institutions/departments involved and the contribution that will be made by each of the investigators.
- Do NOT include corporate or personnel qualifications, past experience, or any supplemental information with the white paper. References may be included within the 4-page limit at the discretion of the applicant; however, extensive references are not required.
- Thrust Area 6 pre-application white papers must also include a description of the extent and duration of the relationship/collaboration between the universities/institutes/entities and/or scientists.
- The thrust area or the topic should be included as a header on the white paper attachment and referenced in the text of the white paper.

4.2.5. Phase I Pre-Application White Paper Re-Submission and Content.

On a limited basis a second pre-application white paper may be submitted without pre-coordination of an abstract. These re-submissions will be based on the review of the original pre-application white paper and will be allowed when changes to the project scope, technical approach, and/or cost are envisioned for any potential full proposals. Revised pre-application white papers must conform to the standards for the pre-application white papers detailed in Section 4.2.4.

All submissions should be made with the appropriate Phase I application package which contains

the following form:

Form	Attachment	Action
SF-424 (R&R) Application for Federal Assistance Form	Up to four (4) page white paper	Enter the appropriate information in data fields

Table 4: Phase I Pre-Application White Paper Package Chart.

Each Phase I application package contains the SF 424 (R&R) Application for Federal Assistance. To be considered a complete package, an up to four (4) page white paper is required to be uploaded as an attachment to the SF 424 (R&R).

The DTRA-specific instructions for completing the SF 424 (R&R) Application for Federal Assistance are the same as for the original pre-application white paper submission except for the following:

- Block 1 – Type of Submission. Applicants should indicate the Phase I re-submission is a "Changed/Corrected Application."
- Block 4c – Previous Grants.gov Tracking ID. Enter the Phase I Grant ID for the original submission.

At minimum, the revised white paper should address the issues and questions detailed in the debrief summary.

4.2.6. Phase II - Invited Proposal Submission and Content.

Each proposal must address only the thrust area or topic for which it was invited. The application package corresponding to the thrust area or topic of interest should be used for submission of invited full proposals.

Each Phase II application package contains the following forms and attachments:

Form	Attachment	Action
SF-424 (R&R) Application for Federal Assistance Form	*N/A*	Enter the appropriate information in data fields
RR Budget Form	Budget Justification for entire performance period	Attach to Section K in budget period one
RR Subaward Budget Attachment(s) Form (*if applicable*)	Individual subaward budgets	Attach a separate budget with justification for each subaward
Research & Related Senior/Key Person Profile Form	PI Biographical Sketch	Attach to Biographical Sketch field
	PI Current/Pending Support	Attach to Current & Pending Support field
	Key Personnel Biographical Sketches	Attach to Biographical Sketch field for each senior/key person
	Key Personnel Current/Pending Support	Attach to Current & Pending Support field for each senior/key person
RR Personal Data Form	*N/A*	Enter the appropriate information in data fields
Research & Related Other Project Information Form	Publically Releasable Proposal Summary/ Abstract	Attach to Block 7 Project Summary/ Abstract
	Project Narrative/Technical Proposal	Attach to Block 8 Project Narrative
Disclosure of Lobbying Activities (SF-LLL) *(if applicable)*	*N/A*	Enter the appropriate information
Attachments Form	Attachment 1 – SOW	Upload as Attachment 1
	Attachment 2 – Quad Chart	Upload as Attachment 2
	Attachment 3 – Supporting Documentation (Thrust Area 6 submissions only)	Upload as Attachment 3

Table 5: Phase II Proposal Package Forms and Attachments.

DTRA reserves the right to consider incomplete application packages and required attachments and to request any missing information via email. Should the applicant fail to provide all the requested information either as part of the www.grants.gov submission or in response to email requests from DTRA, at their discretion, DTRA may not consider the proposal further.

SF 424 (R&R) Application for Federal Assistance: DTRA-specific instructions for completing the SF 424 (R&R) are below. General application instructions can be found on www.grants.gov:

Block 1 – Type of Submission. Applicants should indicate the Phase II submission is an "Application."

Block 2.1 – Applicant Identifier. Not applicable.

Block 3 – Date Received by State. Not applicable.

Block 3.1 – State Application Identifier. Not applicable.

Block 4b – Agency Routing Identifier. Enter the corresponding Phase I Grant ID. If

resubmissions were involved, enter the Grant ID for the last submission.

Block 5 – Applicant Information. You must provide a Business Office Point of Contact (BPOC) with an e-mail address.

Block 19 – Authorized Representative. The "signature of AOR" is not an actual signature and is automatically completed upon submission of the electronic application package.

RR Budget Form: The Research and Related Budget Form provided as part of the application package for the Phase II submission should be filled out in its entirety for each project year proposed. Applicants are responsible for ensuring appropriate, approved rates are used in their budget forms. When notified of selection applicants will be requested to provide their current rate agreement and the rate agreement of their subcontractor(s), if applicable. Applicants should note that in accordance with 32 CFR 22.205(b), grants shall not provide for the payment of fee or profit to the recipient.

Applicants should plan and budget for travel to accommodate the two meetings outlined below:

- National Conferences/Workshops/Symposia: Applicants are strongly encouraged to attend a nationally recognized conference, workshop, or symposium in the field of research each calendar year (1 at minimum). Research should be presented as soon as adequate data are available to support posters and presentations. Conferences/workshops/symposia should be attended by the PI and students supporting the research, as appropriate.
- Annual Technical Review: Applicants should plan to attend an annual technical program review meeting. For planning purposes the review will be for five days and will be held in Northern Virginia.

Budget Justification: Applicants are required to submit a budget justification. The budget justification should be prepared as outlined in the instructions for the Research and Related Budget and uploaded as an attachment to Section K "Budget Justification" of the Research and Related Budget Form. The budget justification does not have a page limit, but should include sufficiently detailed information for meaningful evaluation. In addition, the budget justification must specifically address subaward costs and type to include the portion of work to be subawarded with a supporting rationale. The budget justification should include a discussion of how the subawardee(s) cost was determined to be fair and reasonable.

RR Subaward Budget Attachment(s) Form (if applicable): Detailed cost estimates are required for each proposed subaward. The cost estimate for the subawards should include sufficiently detailed information for meaningful evaluation, including labor rates and indirect cost rates.

Research and Related Senior/Key Person Profile Form: The Research and Related Senior/Key Person Profile Form should be completed in its entirety for each of the PIs and Co-PIs on the project. The inclusion of additional personnel is at the discretion of the PI. The Degree Type and Degree Year fields will be used by DoD as the source for career information to assess the success rates of women. In addition to the required fields on the form, applicants should complete these two fields for all individuals that are identified as senior or key persons. For Thrust Area 6 submissions, the PI (and Co-PIs) in the region-of-interest should be included as key personnel.

A biographical sketch is required for each PI and Co-PI on the project. DTRA does not have a preference for the format of the biographical sketch; however, it should be limited to 1 page per

person. The biographical sketch should be uploaded as an attachment to the corresponding field on the Research and Related Senior/Key Person Profile Form.

Additionally, a statement of current and pending support must be provided for each PI and Co-PI on the project. This statement should include a summary of the current and pending support of related work and requires disclosure of all grants and contracts through which each PI and Co-PI is currently receiving or may potentially receive financial support.

RR Personal Data Form: This form will be used by DoD as the source of demographic information, such as gender, race, ethnicity, and disability information for the PI and Co-PI(s). Each application must include this form with the name fields of the PI and any Co-PI(s) completed; however, provision of the demographic information in the form is voluntary. The demographic information, if provided, will be used for statistical purposes only and will not be made available to merit reviewers. Applicants who do not wish to provide some or all of the information should check or select the "Do not wish to provide" option.

Research and Related Other Project Information Form:

Block 7 – Project Summary/Abstract. To fulfill the requirements of Section 8123 of the Defense Appropriations Act, which states: "The Secretary of Defense shall post grant awards on a public Web site in a searchable format," DTRA will collect and post via the Defense Technical Information Center (DTIC) basic information about all awards made under this BAA. The information posted will include the abstract submitted to Block 7 of this form.

The uploaded project abstract should be less than one page and provide a summary of the proposed work and demonstrate relevance to the topic being addressed. Most importantly, the abstract **must be** written such that the general public may easily understand the potential scientific contribution and the impact of the research. The header of this uploaded document must contain the following statement:

> *"This publically releasable abstract is provided to DTRA for use in fulfillment of Section 8123 of the Defense Appropriations Act and future versions of the same."*

The abstract absolutely must not contain any proprietary data or markings.

Block 8 – Project Narrative (Technical Proposal). The uploaded technical proposal must not exceed 20 pages (including references). If the proposal exceeds 20 pages, only the first 20 pages will be reviewed. A page is defined as 8 ½ x 11 inches, single-spaced, with one-inch margins in type not smaller than 12 point Times New Roman font. The technical proposal must be provided in portrait layout. A template for the technical proposal format may be found online in the Document & Template Library of the DTRA Basic and Fundamental Research Community Portal (www.dtrasubmission.net/portal) (MS Word format).

The project narrative (technical proposal) must include the following components:

- ***Abstract.*** Should be a technical project abstract that is distinct from the Project Summary/Abstract that is attached to Block 7.
- ***Scope.***
- ***Objective.*** A clear and concise objective of the proposed project.
- ***Background.*** Provide the necessary technical and scientific background to support the scientific and/or technical merit of the proposed project.

- ***Programmatics.*** Describe your organization's management plan for the proposed project; list supporting and collaborating centers, and the roles/responsibilities of each identified academic and/or industrial subcontractor supporting the project. Authors of multidisciplinary proposals must take great care to clearly outline the impact to C-WMD science that is to be gained from the investment and justify the scientific contribution from each investigator.

  Thrust Area 6 narratives must also describe of the extent and duration of the relationship/collaboration between the universities/institutes/entities and/or scientists. Teams with pre-existing collaborative research relationships and those which propose to establish new collaborations will be considered, provided teams can supply documentation to demonstrate that an operational framework exists to support the proposed work. Please see Attachment 3 below for information on the submission of this documentation.

- ***Relevance.*** Describe the relevance of the proposed project in terms of advancing the state of the science and the anticipated scientific impact on capabilities to potentially reduce, eliminate, counter, provide greater knowledge or understanding of the threat, and mitigate the effects of WMD fundamental aspects of phenomena and of observable facts.

- ***Credentials.*** Describe the PI's qualifications and the organization's qualifications to perform the proposed work. Summarize the credentials of the primary performing center, and supporting academic and industrial partners to perform the work. Describe specific examples of equipment and/or facilities available to perform the proposed work. Focus on information directly relevant to the proposed work.

- ***Work to be Performed.*** Provide details of the work to be performed by task and subtask. Tasks must be grouped by project year; base and option years should be clearly labeled. Additional details that are required include the following:

  - ***Sample Repository.*** Thrust Area 6 narratives must also clearly identify how the applicant plans to maintain samples collected during the proposed research effort, along with relevant metadata, for at least 12 months after the project end date. Note that annual sample repository information must be submitted using a DTRA-specified format that is available in the Document and Template Library online at the DTRA Basic and Fundamental Research Community Portal (www.dtrasubmission.net/portal)).

  - ***Protection of Human Subjects.*** For full discussion, see Section 6.2.2. If the proposed research does involve human subjects or materials, applicants are asked to: a) justify the use of human subjects, b) outline the human use, and c) include the source of the human subjects or materials involved in the research. Applicants shall submit written evidence, to include a provisional protocol number and Institutional Review Board (IRB) point of contact information, that a human use protocol has been submitted to, and is pending approval by, a qualified IRB. Further information may be required if the proposal is successful.

  - ***Animal Use.*** For full discussion, see Section 6.2.3. If the proposed research involves animal use, applicants are asked to justify the use of animals. Any proposals involving animal studies or animal work must include detailed information on the animal protocols to be used and verify the location where the studies will be conducted. Animal studies are subject to review and approval for safety and adherence to regulations. Applicants shall submit with the full proposal package written evidence, to include a provisional

protocol number and Institutional Animal Care and Use Committee (IACUC) point of contact information, that a vertebrate animal use protocol has been submitted to, and is pending approval by, a qualified IACUC. Further information may be required if the proposal is successful.

- ***Performance Schedule.*** Provide a table of tasks and sub-tasks and the duration of performance of each in a Gantt or other suitably formatted chart.
- ***References.*** List any relevant documents referenced.

Disclosure of Lobbying Activities (SF-LLL) Form: The Disclosure of Lobbying Activities Standard Form-LLL, if applicable, should be completed.

Attachments Form: The attachments form should be used to include the following three items with the application:

Attachment 1 – SOW. The SOW does not have a page limit, but should be approximately 3-5 pages in length for incorporation into an award document. The SOW should not contain any proprietary data or markings. Pages should be numbered and the initial page should have a date (document date) shown under the title (the title of the SOW should match that of the proposal). The SOW must be provided in portrait layout. A template for the SOW format may be found online in the Document & Template Library of the DTRA Basic and Fundamental Research Community Portal (www.dtrasubmission.net/portal) (MS Word format).

The proposed SOW must accurately describe the research to be performed. The proposed SOW must also contain a summary description of the technical methodology as well as the task description, but not in so much detail as to make the SOW inflexible. The SOW format/guidance is as follows:

- ***Objective:*** Brief overview of the specialty area. Describe why the research is being pursued and what knowledge is being sought.
- ***Scope:*** Include a statement of what the SOW covers including the research area to be investigated, objectives/goals, and major milestones and schedule for the effort.
- ***Background:*** The applicant must identify appropriate documents, including publications that are applicable to the research to be performed. This section includes any information, explanations, or constraints that are necessary in order to understand the hypothesis and scientific impact on capabilities needed to reduce, eliminate, and counter the threat, and also mitigate the effects of WMD. It may also include previously performed relevant research and preliminary data.
- ***Tasks/Scientific Goals:*** This section contains the detailed description of tasks which represent the research to be performed that are contractually binding. Thus, this portion of the SOW should be developed in an orderly progression and presented in sufficient detail to establish the methodology and feasibility of accomplishing the overall program goals. The work effort should be segregated by performance period for all tasks to be performed and anticipated milestones realized in that year (e.g., Year 1, Year 2, etc., should be detailed separately). Identify the major tasks in separately numbered sub-paragraphs. Each major task should delineate, by subtask, the research to be performed by year and number each task using the decimal system (e.g., 4.1, 4.1.1, 4.1.1.1, 4.2, etc.). The sequence of performance of tasks and achievement of milestones must be presented by project year and task in the same

sequence as in the Project Narrative/Technical Proposal. The SOW must contain every task to be accomplished to include a detailed schedule.

- The tasks must be definite, realistic, and clearly stated. Use "the awardee shall" whenever the work statement expresses a provision that is binding. Use "should" or "may" whenever it is necessary to express a declaration of purpose. Use active voice in describing work to be performed. Do not use acronyms or abbreviations without spelling out acronyms and abbreviations at the first use; place the abbreviation in parenthesis immediately following a spelled-out phrase. If presentations/meetings are identified in your schedule, include the following statement in your SOW: "Conduct presentations/meetings at times and places specified in the grant schedule."
- ***Deliverables:*** Thrust Area 6 **requires** several additional items be included in the SOW. These items are as follows:
    - Submission of annual sample repository information using a DTRA-specified format (for the template, please see the Document & Template Library of the DTRA Basic and Fundamental Research Community Portal (www.dtrasubmission.net/portal)).
    - Access to all samples collected and data generated during the course of the project, up to and including at least 12 months after the project end date.

Attachment 2 – Quad Chart. The quad chart must be presented on one (1) page. The quad chart must not contain any proprietary data or markings. The quad chart must be provided in landscape layout. A template for the quad chart format may be found online in the Document & Template Library of the DTRA Basic and Fundamental Research Community Portal (www.dtrasubmission.net/portal) (MS PowerPoint format). The inclusion of the DTRA logo is not required. The quad chart should be uploaded as "Attachment 2" of the Attachments Form.

Attachment 3 – Supporting Documentation. For Thrust Area 6 proposals ONLY. Thrust Area 6 narratives must also describe an operational framework to support the proposed work. This includes, but is not limited to the following: the extent and duration of the relationship/collaboration between the universities/institutes/entities and/or scientists. Teams with pre-existing collaborative research relationships and those which propose to establish new collaborations will be considered, provided teams can supply documentation to demonstrate that an operational framework exists to support the proposed work. Each of the following should be concatenated into a single document, in the order specified:

- Specific identification of foreign Principal Investigators (PIs) and number of/job title for other members of the foreign research team.
- Detailed description of the relationship between the proposed research project and current research efforts at the foreign institution.
- Description of facilities and any other evidence of suitability of foreign collaborators and sites. In the event that the foreign research component will involve human or other vertebrate animal use, appropriate facilities compliance and certifications documents must be provided. Refer to Section 6.2.2 and Section 6.2.3 for specific information on required approvals and documentation.
- Foreign PI letter of collaboration describing, at minimum, the suitability of the proposed work with respect to ongoing research efforts at the foreign institution, merit of the proposed

collaboration, and the expected mutual benefits.

Protocol Risk Assessment Tool (PRAT). For Thrust Area 6 proposals ONLY. Applicants **must** download the PRAT from the Document & Template Library of the DTRA Basic and Fundamental Research Community Portal (www.dtrasubmission.net/portal) and complete it in its entirety for **each** foreign institution participating in the project. Additional instructions for completing the PRAT may be found within the file. The completed PRAT file(s) should be emailed as a Portable Document File (PDF) format to HDTRA1-FRCWMD-A@mail.mil within two (2) weeks of the full proposal submission. **DO NOT** attempt to attach the PRAT(s) to the www.grants.gov submission.

4.2.7. Phase II - Additional Information Requests by DTRA. A revised proposal may be requested based on the review of the original proposal. Revised proposals will be requested when changes to the project scope, technical approach, and/or cost are required before the proposal could be further considered for an award. Applicants whose proposals are of interest to DTRA may be contacted to provide additional information or to make requested revisions prior to the final decision on funding. This request for further information may include revised budgets or budget explanations, revised SOWs, and other information, as applicable, to the proposed award. Applicants who are not responsive to Government requests for information in a timely manner, defined as meeting Government deadlines established and communicated with the request and not making satisfactory updates as requested, may be removed from award consideration. Applicants may also be removed from award consideration if the applicant and the Government fail to negotiate mutually agreeable terms within a reasonable period of time.

Re-submissions should be made with the appropriate Phase II application package for the thrust area or topic of interest and should be completed in accordance with the instructions provided in the notification email.

The DTRA-specific instructions for completing a proposal re-submission are the same as for the original submission, except the SF 424 (R&R) Application for Federal Assistance should be marked as follows:

- Block 1 – Type of Submission. Applicants should indicate the Phase II submission is a "Changed/Corrected Application."
- Block 4b – Agency Routing Identifier. Enter the corresponding Phase I Grant ID.
- Block 4c – Previous Grants.gov Tracking ID. Enter the Phase II Grant ID for the original Phase II submission.

4.2.8. File Format.

Documents should be uploaded as a Portable Document File (PDF) format. Perform a virus check before uploading any files to www.grants.gov as part of your application package. If a virus is detected, it may cause rejection of the file.

Do not lock or encrypt any files you upload to www.grants.gov as part of your application package. Movie and sound file attachments will not be accepted.

4.2.9. All submissions must be completely UNRESTRICTED and UNCLASSIFIED; submissions must not contain For Official Use Only (FOUO) or Official Use Only (OUO) information or be marked as such.

4.2.10. Confirmed Proposal Expiration Date. Applicants requesting contracts must provide written confirmation that holds the proposal, to include proposed costs, firm for 180 days after the submission due date, as included in the invitation to submit a full proposal. This information must be included in the text of the technical proposal.

4.2.11. Withdrawal of Proposals. Proposals may be withdrawn by written notice received at any time before award. Withdrawals are effective upon receipt of notice by the Grants/Contracting Officer via the administrative e-mail address listed in Section 7.

4.3. Submission Dates and Times.

Coordination of abstracts may be accomplished at any time that this BAA is in effect, unless otherwise stated as part of a specific topic. Once an applicant has been notified that a pre-application white paper is welcomed, the white paper should be submitted within 60 days. If the white paper is not submitted within 60 days, DTRA reserves the right to require the applicant to re-initiate the process with another abstract coordination.

Pre-application white papers may be submitted anytime that this BAA is in effect (as long as it occurs within the 60 day window following pre-coordination of the abstract), unless otherwise stated as part of a specific topic. Pre-application white papers may be evaluated at any time after submission and invitations for full proposal submission may occur any time after pre-application white paper evaluation. Note that proposal invitations may be limited to available program funds.

The due date for the Phase II invited proposal submissions will be provided in the letter of invitation. The applicant will not be allowed less than 45 days to prepare a full proposal submission; there is no penalty for early submissions. An extension for submission of the Phase II proposal submission may be requested by emailing the administrative email address in Section 7 prior to the deadline for the proposal submission. Full proposals may be evaluated at any time after submission.

Applicants are responsible for submitting all materials to www.grants.gov. When sending electronic files, the applicant should allow for potential delays in file transfer from the originator's computer server to the www.grants.gov website/computer server, as well as the delay associated with the www.grants.gov validation of applications, which may be up to 48 hours. Applicants are encouraged to submit their proposals early to avoid issues with file transfers, rejection of applications by www.grants.gov, and delays due to high website demand.

Acceptable evidence to establish the time of receipt at the Government office includes documentary and electronic evidence of receipt maintained by DTRA. Applicants should also print, and maintain for their records, the electronic receipt following submission of a proposal to www.grants.gov.

Applicants should note that DTRA uses a system that pulls applications from www.grants.gov en masse, but this system does not mark applications as "retrieved" on www.grants.gov. As a result, when applicants check the status on www.grants.gov the applications will always look like they have not been retrieved by DTRA. Should you require confirmation of receipt by the Agency, you may request such via the administrative email address provided in Section 7. Note that such requests will generally be treated with low priority by the Agency.

Please note 15 U.S.C. 260a establishes daylight saving time as the standard time during the daylight saving period.

If the application package and required attachments are submitted to www.grants.gov after the exact time and date specified in this announcement or in any written communications provided by DTRA, the application may be considered "late" and may not be reviewed.

If an emergency or unanticipated event interrupts normal Government processes so that proposals cannot be submitted to www.grants.gov by the exact time specified by DTRA correspondence, the time specified for receipt of applications will be deemed to be extended to the same time of day specified in the BAA or in the letter of invitation on the first work day on which normal Government processes resume.

4.4. Intergovernmental Review. Not Applicable.

4.5. Other Submission Requirements.

4.5.1. Registration with www.grants.gov. Applicants should note that each organization must complete a one-time registration in order to submit its pre-application white paper(s) and full proposal(s) through www.grants.gov. Please see the following web link on information about registering with www.grants.gov: http://www07.grants.gov/applicants/applicants.jsp. If your organization is already registered in www.grants.gov, no further action should be required.

The registration process may take up to **four (4) weeks** to complete depending on your organization and requires multiple steps, some of which are detailed below.

- Identifying the Data Universal Number Systems (DUNS) number or registering for one with Dun & Bradstreet at http://fedgov.dnb.com/webform/displayHomePage.do if your organization does not have a DUNS number.
- Registering with the System for Award Management (SAM) by calling the SAM Assistance Center at 1-866-606-8220, or you may register online at www.sam.gov. You will NOT be able to complete your SAM registration until SAM has confirmed your Employer Identification Number (EIN) or Taxpayer Identification Number (TIN) with the Internal Revenue Service (IRS).

4.5.2. Compliance with Appendix A to 32 CFR 28. All awards require certifications of compliance with Appendix A to 32 CFR 28 regarding lobbying. The full text of this certification is available in the Document & Template Library of the DTRA Basic and Fundamental Research Community Portal (www.dtrasubmission.net/portal). Proposers are certifying compliance with this regulation by submitting the invited proposal. It is not necessary to include the certification text with your invited proposal. If applicable, proposers should submit the Disclosure of Lobbying Activities (SF-LLL) Form in accordance with Section 4.2.6.

4.5.3. Marking Guidance for Pre-Application White Paper and Invited Proposal and Disclosure of Proprietary Information other than to the Government. The pre-application white papers and invited proposals submitted in response to this BAA may contain technical and other data that the applicant does not want disclosed to the public or used by the Government for any purpose other than application evaluation. Public release of information in any pre-application white paper and invited proposal submitted will be subject to existing statutory and regulatory requirements.

If proprietary information which constitutes a trade secret, proprietary commercial or financial information, confidential personal information, or data affecting national security, is provided by an applicant in a pre-application white paper and/or invited proposal, it will be treated in

confidence, to the extent permitted by law, provided that the following legend is included on the front page of the pre-application white paper and/or invited proposal:

"For any purpose other than to evaluate the pre-application white paper and/or proposal, this data shall not be disclosed outside the Government and shall not be duplicated, used, or disclosed in whole or in part, provided that if an award is made to the applicant as a result of or in connection with the submission of this data, the Government shall have the right to duplicate, use or disclose the data to the extent provided in the agreement. This restriction does not limit the right of the Government to use information contained in the data if it is obtained from another source without restriction. The data subject to this restriction is contained in page(s) _____ of this pre-application white paper and/or proposal."

Any other legend may be unacceptable to the Government and may constitute grounds for removing the pre-application white paper and/or invited proposal from further consideration without assuming any liability for inadvertent disclosure.

The Government will limit dissemination of properly marked information to within official channels. In addition, the pages indicated as restricted must be marked with the following legend:

> *"Use or disclosure of the pre-application white paper and/or proposal data on lines specifically identified by asterisk (*) are subject to the restriction on the front page of this pre-application white paper and/or proposal."*

The Government assumes no liability for disclosure or use of unmarked data and may use or disclose such data for any purpose.

In the event that properly marked data contained in a pre-application white paper and/or invited proposal submitted in response to this BAA is requested pursuant to the Freedom of Information Act (FOIA), 5 U.S.C. § 552, the applicant will be advised of such request and prior to such release of information, will be requested to expeditiously submit to DTRA a detailed listing of all information in the pre-application white paper and/or invited proposal which the applicant believes to be exempt from disclosure under the Act. Such action and cooperation on the part of the applicant will ensure that any information released by DTRA pursuant to the Act is properly identified.

By submission of a pre-application white paper and/or invited proposal, the applicant understands that proprietary information may be disclosed outside the Government for the sole purpose of technical evaluation. DTRA will obtain a non-disclosure agreement from the evaluator that proprietary information in the pre-application white paper and/or invited proposal will only be used for evaluation purposes and will not be further disclosed or utilized.

4.6. Applicants that Propose Use of Contracts or OTAs.

4.6.1. Recommended Procurement Instrument and Pricing Arrangement. Applicants that propose use of contracts or OTAs must provide a summary of their recommended procurement instrument and pricing arrangement as part of the Phase II proposal. However, the Government reserves the right to negotiate and award the types of instruments determined most appropriate under the circumstances. It is anticipated that most instruments will be grants.

4.6.2. Representations and Certifications. Representations and Certifications must be completed at the time of Phase II submission. The applicant must complete the annual

representations and certifications electronically via the System for Award Management (SAM) website at https://www.sam.gov/portal/SAM/#1#1. After reviewing their information, the applicant verifies by submission of the application that the representations and certifications currently posted electronically have been entered or updated within the last 12 months.

4.6.3. Organization Conflict of Interest Advisory. Certain post-employment restrictions on former federal officers and employees may exist, including special Government employees (including but not limited to 18 U.S.C § 207, the Procurement Integrity Act, 41 U.S.C. § 2101 *et.seq*). If a prospective applicant believes that a conflict of interest exists, the situation should be raised to the DTRA Contract/Grant Officer before time and effort are expended in preparing a proposal. All applicants and proposed sub-contractors must therefore affirmatively state whether they are providing scientific, engineering and technical assistance (SETA), advisory and assistance services (A&AS) or similar support, through an active contract or subcontract, to any DoD technical office to include, but not limited to, the Joint Program Executive Office (JPEO), the Office of the Assistant Secretary of Defense for Nuclear, Chemical, and Biological Defense Programs (ASD-NCB), or the Office of the Special Assistant for Chemical and Biological Defense and Chemical Demilitarization Programs (OSA (CBD&CDP)). This information must be included in Technical Proposal of the Phase II full submission. All affirmations must state which office(s) the applicant(s) supports, and identify the prime contract number. Affirmations must be furnished at the time of Phase II full proposal submission. All facts relevant to the existence or potential existence of organizational conflicts of interest, including but not limited to those arising out of activities with the above-referenced organizations, must be disclosed. The disclosure must include a description of the action the applicant has taken or proposes to take to avoid, neutralize, or mitigate such conflict.

4.6.4. Contracts with Subcontracts. Any applicant, other than small businesses, submitting a proposal that exceeds $650,000.00 must submit a subcontracting plan in accordance with FAR 19.704(a) (1) and (2). This information must be included in Technical Proposal of the Phase II full submission. The plan format is outlined in FAR 19.704. Pursuant to Section 8(d) of the Small Business Act (15 U.S.C. § 637(d)), it is the policy of the Government to enable small business and small disadvantaged business concerns to be considered fairly as subcontractors to contractors performing work or rendering services as prime contractors or subcontractors under Government contracts, and to assure that prime contractors and subcontractors carry out this policy.

4.6.5. Limitations on OTAs. Applicants are advised that an Other Transaction for Research Agreement (10 U.S. Code § 2371) or an Other Transaction for Prototype Agreement (10 U.S. Code § 2371b) will only be awarded if the use of a standard contract or CA is not feasible or appropriate. Applicants are advised that an OTA may only be awarded if there is:

a. At least one nontraditional defense contractor participating to a significant extent in the prototype project, or

b. All significant participants in the transaction other than the Federal Government are small businesses or nontraditional defense contractors; or

c. At least one-third of the total cost of the prototype project is to be paid out of funds provided by the parties to the transaction other than the Federal Government. The cost share should generally consist of labor, materials, equipment, and facilities costs (including allocable indirect costs).

d. Exceptional circumstances justify the use of a transaction that provides for innovative business arrangements or structures that would not be feasible or appropriate under a procurement contract.

e. Although use of one of these options is required to use an Other Transaction for Prototype agreement as the procurement vehicle, no single option is encouraged or desired over the others.

NOTE: For purposes of determining whether or not a participant may be classified as a nontraditional defense contractor or a small business and whether or not such participation is determined to be participating to a significant extent in the prototype project, the following definitions are applicable:

a. "Nontraditional defense contractor" means an entity that is not currently performing or has not performed, for at least the one-year period preceding this solicitation, any of the following for the Department of Defense: any contract or subcontract that is subject to full coverage under the cost accounting standards prescribed pursuant to section 26 of the Office of Federal Procurement Policy Act (41 USCS §§ 1501 et seq.) and the regulations implementing such section; or any other contract in excess of $500,000 under which the contractor is required to submit certified cost or pricing data under section 2306a of this title (10 USCS § 2306a).b. "Small business" means a small business concern as defined under Section 3 of the Small Business Act (15 U.S.C. § 632).

"Participating to a significant extent in the prototype project" means that the nontraditional defense contractor or small business is supplying a new key technology or product, is accomplishing a significant amount of the effort wherein the role played is more than a nominal or token role in the research effort, or in some other way plays a significant part in causing a material reduction in the cost or schedule of the effort or an increase in performance of the prototype in question. NOTE: Applicants are cautioned that if they are classified as a traditional defense contractor, and propose the use of an Other Transaction for Prototype Agreement, the Government will require submittal of both a cost proposal under the guidelines of the FAR/DFARS, and a cost proposal under the proposed Other Transaction for Prototype Agreement, so that an evaluation may be made with respect to the cost tradeoffs applicable under both situations. The Government reserves the right to negotiate either a FAR based procurement contract, or Other Transaction for Prototype Agreement as it deems is warranted under the circumstances.

---

5. APPLICATION REVIEW INFORMATION

5.1. Evaluation Criteria. The four evaluation criteria to be used for responses received to this BAA are as follows:

1. Scientific and Technical Merit. The objective of this criterion is to assess the extent to which the applicant presents ideas that are innovative and/or unique with the potential for high payoff in the science area and details a comprehensive technical approach based on sound scientific principles. Innovation will be judged contextually against the white

paper's/proposal's scope, goals, and setting. To the extent possible, the technical risks, including those of biosafety and security, to accomplish the research or project should be identified with appropriate mitigation/management details.

For Thrust Area 6 white papers/proposals, innovation will also be considered with respect to partner country capabilities.

2. Value to Mission Goals. The objective of this criterion is to assess the extent to which the applicant demonstrates an understanding of the C-WMD research or mission challenges and the contribution to the C-WMD research or mission needs of that thrust area/topic. White papers/proposals must detail research or a project that is responsive to the thrust area/topic as presented in this solicitation. This criterion also addresses the benefit of the proposed effort on enabling knowledge, technology, or capabilities over current methods and/or practices and on the transition potential that is appropriate to the proposed effort. Applicants must also demonstrate an impact of the proposed effort on the institution's ability to perform research relevant to reducing the global WMD threat; and/or to train, through the proposed effort, students and/or partner scientists in science, technology, engineering and/or mathematics.

   Thrust Area 6 white papers/proposals must demonstrate an understanding of the CBEP priorities and mission. As such, the degree to which the proposed collaborations may lead to long-term partner country self-sufficiency and sustainment of the jointly developed capabilities will be considered.

3. Capability of the Personnel and Facilities to Perform the Proposed Effort. The objective of this criterion is to assess the extent to which the applicant's team has the requisite expertise, skills and resources necessary to perform the proposed program. This includes an assessment of the team's management construct, key personnel, facilities and past technical experience in conducting similar efforts of the proposed scope. Applicants must demonstrate that their team has the necessary background and experience to perform this project. Facilities should be detailed with discussion of any unique capabilities pertinent to the research. Subcontractors may include Government facilities or Agencies; however the unique expertise or specialized facilities provided through their inclusion must be clearly presented and the validity of the proposer-Governmental relationship must be clearly documented.

4. Cost Realism Evaluation. The objective of this criterion is to establish that the proposed costs are reasonable, realistic, and justified for the technical approach offered and to assess the applicant's practical understanding of the scope of the proposed effort.

5.2. Review and Selection Process.

The pre-application white paper and proposal selection process will be conducted based upon a technical review as described in the DoDGARs (32 CFR 22.315(c)) and includes the use of non-Government peer-reviewers.

Each pre-application white paper and invited proposal submitted to a general TA will be reviewed on a rolling basis; topic-based submissions will be reviewed as a batch following receipt deadlines. All applications will be reviewed based on the merit and relevance of the specific pre-application white paper/proposal as it relates to the DTRA program, rather than against other pre-application white papers/proposals for research in the same general area.

Pre-application white paper (Phase I) evaluation will be based on the two (2) equally weighted criteria of (1) Technical/Scientific Merit and (2) Value to Mission Goals. The criteria will be

scored as Outstanding (O), Good (G), Acceptable (A), Marginal (M) or Unacceptable (U). Any criterion scored as "Unacceptable (U)" will render the pre-application white paper "Not Selectable," and the pre-application white paper will not be considered further.

The full proposal evaluation will be based on the four criteria listed above. Of these, the first two (2) criteria of (1) Technical/Scientific Merit and (2) Value to Mission Goals are equally weighted and more important than the third criterion of (3) Capability of the Personnel and Facilities to Perform the Proposed Effort. These first three criteria will be scored Outstanding (O), Good (G), Acceptable (A), Marginal (M) or Unacceptable (U). The fourth criterion of Cost Realism will be scored as either Acceptable (A) or Unacceptable (U). Any criterion scored as "Unacceptable (U)" will render the proposal "Not Selectable," and the proposal will not be considered further.

Other factors that may be considered are duplication with other research, program balance, past performance and budget limitations. Prior to award, the Government reserves the right to perform a review of past performance. Sources that may be used for past performance review may include the Past Performance Information Retrieval System (PPIRS) and the Federal Awardee Performance and Integrity Information System (FAPIIS). The Government will also evaluate the impact of any proposed limitations to the use of intellectual property (e.g. asserted technical data/computer software restrictions or patents) during the selection and/or negotiation process, and may request additional information from the applicant, as may be necessary, to evaluate the applicant's assertions. Accordingly, proposals may be selected for funding which are not reviewed as highly as others, which are of higher risk and/or which may be of a higher cost.

The Government reserves the right to select all, some, or none of the proposals, or any part of any proposal received in response to this BAA and to make awards without discussions with applicants; however, the Government reserves the right to conduct discussions if determined necessary.

5.3. DTRA anticipates that the total Federal share of awards made under this announcement will be greater than the simplified acquisition threshold over the period of performance (see §200.88 Simplified Acquisition Threshold). Therefore, in accordance with Appendix I to 2 CFR Part 200, Section E.3, this section serves to inform applicant:

i. That DTRA, prior to making a Federal award with a total amount of Federal share greater than the simplified acquisition threshold, is required to review and consider any information about the applicant that is in the designated integrity and performance system accessible through SAM (currently Federal Awardee Performance and Integrity Information System (FAPIIS)) (see 41 U.S.C. 2313);

ii. That an applicant, at its option, may review information in the designated integrity and performance systems accessible through SAM and comment on any information about itself that a Federal awarding agency previously entered and is currently in the designated integrity and performance system accessible through SAM;

iii. That DTRA will consider any comments by the applicant, in addition to the other information in the designated integrity and performance system, in making a judgment about the applicant's integrity, business ethics, and record of performance under Federal awards when completing the review of risk posed by applicants as described in §200.205

Federal awarding agency review of risk posed by applicants.

iv. For awards that exceed $500,000 over the period of performance, DTRA will employ the additional post-award reporting requirements reflected in Appendix XII—Award Term and Condition for Recipient Integrity and Performance Matters of 2 CFR 200.

5.4. Technical and Administrative Support by Non-Government Personnel.

It is the intent of DTRA to use both Government and non-Government personnel to assist with the review and administration of submittals for this BAA. All pre-application white papers and invited proposals may be reviewed by subject matter experts, including, but not limited to, peer reviewers from across the academic and industrial community, as applicable to the research proposed.

Further, participation in this BAA requires DTRA support contractors to have access to pre-application white paper and invited proposal information including information that may be considered proprietary or otherwise marked with restrictive legends. Existing DTRA contractors include but may not be limited to the following: Engility Corporation (Advisory & Assistance Services) and their subcontractors, Infinity Technology LLC, Quanterion Solutions Inc., Kforce Government Solutions Inc., KCK Inc., CACI,, , SBG Technology Solutions and their subcontractors, and Terremark Worldwide Inc. Each contract contains organizational conflict of interest provisions and/or includes contractual requirements for non-disclosure of proprietary contractor information or data/software marked with restrictive legends. The Offeror, by submitting a white paper or proposal, is deemed to have consented to the disclosure of its information to the aforementioned contractors under the conditions and limitations described herein.

All individuals—including subject matter experts and support contractors—having access to any proprietary data must certify that they will not disclose any information pertaining to this BAA including any submittal, the identity of any submitters, or any other information relevant to this BAA. All applicants to this BAA consent to the disclosure of their information under these conditions.

---

6. AWARD ADMINISTRATION INFORMATION

6.1. Award Notices. Applicants will be notified regarding the status of their applications (invitation/non-invitation for full proposals, re-submission of white papers, selection/non-selection for award, etc.) via e-mail to the BPOC listed in Block 5 of the SF-424 and the PI listed in Block 14 of the SF-424 provided at the time of submission. A debrief summary will be provided as part of all notification e-mails.

A notice of selection should not be construed as an obligation on the part of the Government; only duly authorized procurement personnel may commit resources, this will be done by issuing a grant or contract document to the selected applicant. Also, this notification must not be used as a basis for accruing costs to the Government prior to award. Selected applicants are not authorized to begin work, as any award is subject to successful negotiations (if determined necessary by DTRA) between the DTRA contracting division and the selected organization, and to the availability of funds.

All notifications will be made from notification@dtrasubmission.net. **E-mails to this e-mail**

address will not be answered or forwarded.

Applicants must be aware that it is their responsibility to ensure: (1) correct e-mail addresses are provided at the time of submission, (2) this e-mail notification reaches the intended recipient(s), and (3) the e-mail is not blocked by the use of 'spam blocker' software or other means that the recipient's Internet Service Provider may have implemented as a means to block the receipt of certain e-mail messages.

If for any reason there is a delivery failure of these e-mail notices, DTRA will not further attempt to contact the applicants.

6.2. Administrative and National Policy Requirements. The DTRA Grant Terms and Conditions may be found online in the Document & Template Library of the DTRA Basic and Fundamental Research Community Portal (www.dtrasubmission.net/portal). There are different versions for different recipients. As the Terms and Conditions are updated, they will be posted in the Document & Template Library of the DTRA Basic and Fundamental Research Community Portal (www.dtrasubmission.net/portal). All awards require certifications of compliance with national policy requirements. Statutes and Government-wide regulations require some certifications to be submitted at the time of proposal submission. See Section 4.5.2 and Section 4.6.2 for the certification(s) required at the time of submission.

This BAA focuses on fundamental research in a DoD contractual context, which was defined in Section 1.2 of this BAA. Per DoD policy[1], "…products of fundamental research are to remain unrestricted to the maximum extent possible." Furthermore, "The DoD will place no other restrictions on the conduct or reporting of unclassified fundamental research, except as otherwise required by statue [sic], regulation, or Executive Order." As such, fundamental research is normally exempt from controls under the International Traffic in Arms Regulation (ITAR) (22 CFR Parts 120-130) and/or the Department of Commerce regarding the Export Administration Regulations (15 CFR Parts 730-774), but the DoD rule recognizes that there are "rare" situations where export-controlled information or technology may be used in fundamental research that may require a license(s) or restrictions on products.

6.2.1. Export Control Notification. Applicants are responsible for ensuring compliance with any export control laws and regulations that may be applicable to the export of and foreign access to their proposed research. Applicants may consult with the Department of State with any questions regarding the International Traffic in Arms Regulation (ITAR) (22 CFR Parts 120-130) and/or the Department of Commerce regarding the Export Administration Regulations (15 CFR Parts 730-774). Please note that the prime awardee is responsible for monitoring ITAR compliance of all subawardees.

6.2.2. Protection of Human Subjects. If the proposed research involves human subjects or materials, applicants are asked to: a) justify the use of human subjects, b) outline the human use, and c) include the source of the human subjects or materials involved in the research. As a condition precedent to receipt of DTRA funding, applicants must ensure that the basic rights and welfare of human subjects are protected. Applicants shall submit with the full proposal package written evidence, to include a provisional protocol number and Institutional Review Board (IRB)

[1] Under Secretary of Defense for Acquisition, Technology and Logistics Memorandum, SUBJECT: Contracted Fundamental Research, dated 26 Jun 2008

point of contact information, that a human use protocol has been submitted to, and is pending approval by, a qualified IRB. Further information may be required if the proposal is successful.

All research under any award made under this BAA involving human subjects must be conducted in accordance with 32 CFR 219, 10 U.S.C. § 980, and DoD Instruction 3216.02, and, as applicable, 21 CFR parts 11, 50, 56, Good Clinical Practice, the ICH, as well as other applicable federal and state regulations. Awardees must be cognizant of and abide by the additional restrictions and limitations imposed on the DoD regarding research involving human subjects, specifically as regards vulnerable populations (32 CFR 219 modifications to subparts B-D of 45 CFR 46), recruitment of military research subjects (32 CFR 219), and surrogate consent (10 U.S.C. § 980).

DTRA Directive 3216.01 of June 9, 2010, modified March 18, 2015, established the DTRA Human Subjects Protection Program, set forth the policies, defined the applicable terms, and delineated the procedures necessary to ensure DTRA compliance with federal and DoD regulations and legislation governing human subject research. The regulations mandate that all DoD activities, components, and agencies protect the rights and welfare of human subjects of study in DoD supported research, development, test and evaluation, and related activities hereafter referred to as "research." The requirement to comply with the regulations applies to new starts and to continuing research.

The DTRA Directive requires that research using human subjects may not begin or continue until the DTRA Research Oversight Board (ROB) has reviewed and approved the proposed protocol. Contractors and subcontractors are required to submit a valid federal assurance for their organization (institution, laboratory, facility) that has been issued by either DoD or the Department of Health and Human Services, and documentation of review of proposed protocols by the local IRB to include consent forms for any planned research using human subjects to the DTRA ROB for its review through the contracting officer's representative (if assigned) or the contracting officer. The ROB review is separate from, and in addition to, local IRB review.

A study is considered to involve human research subjects if: 1) there is interaction with the subject (even simply talking to the subject qualifies; no needles are required); and 2) if the study involves collection and/or analysis of personal/private information about an individual, or if material used in the study contains links to such information.

Written approval to begin research or to subcontract for the use of human subjects under the proposed protocol will be provided in writing from the DTRA ROB, through the contracting officer. Both the contractor and the Government must maintain a copy of this approval. Any proposed modifications or amendments to the approved protocol or consent forms must be submitted to the local IRB and the DTRA ROB for review and approval. Examples of modifications/amendments to the protocol include, but are not limited to:

- a change of the Principal Investigator;
- changes in duration or intensity of exposure to some stimulus or agent;
- changes in the information requested of volunteers, or changes to the use of specimens or data collected; or
- changes in perceived or measured risks or benefits to volunteers that require changes to the study.

Research pursuant to such modifications or amendments must not be initiated without IRB and ROB approval except when necessary to eliminate apparent and immediate hazards to the subject(s).

Research projects lasting more than one year require IRB review at least annually, or more frequently as required by the responsible IRB. The contractor or subcontractor must provide documentation of continued IRB review of protocols for ROB review and approval in accordance with the Contract Data Requirements List. Research changes must be reviewed by the IRB and ROB in advance unless necessary to eliminate apparent and immediate hazards to the subject(s).

A clause regarding human subjects research will be included in all contracts involving human subjects research. Non-compliance with any provision of this clause may result in withholding of payments under the contract pursuant to the contract's payments clause(s) and/or contract termination pursuant to the contract's termination clause(s). The Government shall not be responsible for any costs incurred for research involving human subjects prior to protocol approval by the ROB.

6.2.3. Animal Use. If the proposed research involves the use of live nonhuman vertebrate animals, applicants are required to justify the use of animals by providing detailed information on the proposed animal use, to include the proposed species and number of animals planned, along with the location(s) where the animal study(ies) is planned. Additional information will be required if the proposal is selected for award subject to successful negotiations. The Animal Care and Use Review Office (ACURO), a component of the USAMRMC Office of Research Protections (ORP), must review and approve all animal use prior to the start of working with animals. Therefore, Principle Investigators will be required to complete and submit the animal use appendix titled "Research Involving Animals", after award of the procurement instrument, which is available on the ACURO website (http://mrmc.amedd.army.mil/index.cfm?pageid=research_protections.acuro). Allow 2 to 4 months for regulatory review and approval processes for animal studies. Applicants are to build this review time into their project schedules.

DoD Instruction 3216.01, dated September 13, 2010, provides policy and requirements for the use of animals in DoD-funded research based on Army Regulation (AR) 40-33. The DoD definition of animal is any live nonhuman vertebrate. All proposals that involve the use of animals must be in compliance with DoD Instruction 3216.01 and AR 40-33. DTRA requires that research using animals not begin or continue until the ACURO has reviewed and approved the proposed animal use. For animals, the provisions include rules on animal acquisition, transport, care, handling, and use in: (i) 9 CFR parts 1-4, Department of Agriculture rules that implement the Laboratory Animal Welfare Action of 1966 (U.S.C. 2131-2156); and (ii) the "Guide for the Care and Use of Laboratory Animals," National Institutes of Health Publication No. 86-23.

6.2.4. Biological Defense Research Program (BDRP) Requirements: BioSurety and Select Agent Use.

Proposals must specify what Select Agent work will be conducted at the applicant's facility and what Select Agent work will be performed in other facilities. Proposals also must provide the source of the Select Agent(s), any appropriate registration information for the facilities, and specify the Laboratory Bio-safety Level. All Select Agent work is subject to verification of

information and certifications. Further information may be required if the proposal is successful.

For those institutions in which PI's are conducting research with Bio-safety Levels 3 and 4 material, a Facility Safety Plan must be prepared and made available during the project award phase in accordance with 32 CFR 626.18. For grants awarded to foreign institutions, you must follow either local or U.S. laws (as stated above) depending on which laws provide stronger protection. (DTRA requires that research using Select Agents not begin or continue until DTRA has reviewed and approved the proposed agent use. See URL: https://www.gpo.gov/fdsys/pkg/CFR-2002-title32-vol3/pdf/CFR-2002-title32-vol3-sec626-18.pdf for a copy of 32 CFR 626.18, Biological Defense Safety Program.)

For projects that will employ the use of chemical agents, either neat agent or dilute agent, the offeror must provide approved Facility Standard Operating Procedures that conform to Federal, State and local regulations and address the storage, use and disposition of these chemical materials.

6.2.5. Dual-Use Potential. In accordance with National Science Advisory Board for Biosecurity (NSABB) recommendations, DTRA will not support research that, based on current understanding, can reasonably be anticipated to provide knowledge, information, products, or technologies that could be directly misapplied to pose a significant threat with broad potential consequences to public health and safety, agricultural crops and other plants, animals, the environment, materiel, or national security. Research involving select agents and toxins is within scope of the DTRA mission; however, the use of select agents and toxins in certain experimental categories is considered "dual-use research of concern" (DURC) according to U.S. policy. (http://www.phe.gov/s3/dualuse/Documents/us-policy-durc-032812.pdf) Proposals that contain DURC will not be funded. Dual-use potential will be assessed based on application of the following criteria:

- Use of select agents or toxins. This factor evaluates whether the proposed research involves use of one or more select agents or toxins [as identified by the Select Agent Program under Federal Law (7 C.F.R. part 331, 9 C.F.R. part 121, and 42 C.F.R. part 73)] which pose significant risk of deliberate misuse with potential for mass casualties or devastating effects to the economy, critical infrastructure, or public confidence.
- Scope of proposed experiments. This factor evaluates whether the proposed research involves experiments that will produce, aim to produce, or is reasonably anticipated to produce: (a) Enhanced harmful consequences of the agent or toxin; (b) Disruption of immunity or effectiveness of an immunization against the agent or toxin without clinical or agricultural justification; (c) Conferred resistance by the agent or toxin to clinically or agriculturally useful prophylactic or therapeutic interventions against the agent or toxin, or facilitated ability to evade detection methodologies; (d) Increased stability, transmissibility, or dissemination ability of the agent or toxin; (e) Altered host range or tropism of the agent or toxin; (f) Enhanced susceptibility of a host population to the agent or toxin; or (g) Eradicated or extinct select agents or toxins.

6.2.6. Military Recruiting. This is to notify potential applicants that each award under this announcement to an institution of higher education, with exception of any grants awarded to institutions of higher education entirely located outside the United States and/or its territories, must include the following term and condition: "As a condition for receipt of funds available to DoD under this award, the recipient agrees that it is not an institution of higher education (as

defined in 32 CFR 216) that has a policy of denying, and that it is not an institution of higher education that effectively prevents, the Secretary of Defense from obtaining the following for military recruiting purposes: (A) entry to campuses or access to students on campuses; or (B) access to directory information pertaining to students. If the recipient is determined, using procedures in 32 CFR 216 to be such an institution of higher education during the period of performance of this agreement, and therefore to be in breach of this clause, the Government will cease all payments of DoD funds under this agreement and all other DoD grants and CAs, and it may suspend or terminate such grants and agreements unilaterally for material failure to comply with the terms and conditions of award." 32 CFR 216 may be accessed electronically at http://www.ecfr.gov/cgi-bin/text-idx?SID=ee45add5e352854b7089ce420c7fd0a6&mc=true&tpl=/ecfrbrowse/Title32/32cfr216_main_02.tpl . If your institution has been identified under the procedures established by the Secretary of Defense to implement Section 558 of Public Law 103-337, then: (1) no funds available to DoD may be provided to your institution through any grant, including any existing grant; and (2) your institution is not eligible to receive a grant in response to this BAA. This is to notify potential applicants that each award under this announcement to an institution of higher education, with exception of any grants awarded to institutions of higher education entirely located outside the United States and/or its territories, must include the following clause: 32 CFR 22.520 (DoDGARS 22.520), Military Recruiting and Reserve Officer Training Corps Program Access to Institutions of Higher Education.

6.2.7. Combating Trafficking in Persons. The recipient agrees to comply with the trafficking in persons requirement in Section 106(g) of the Trafficking Victims Protection Act of 2000 (TVPA), as amended (22 U.S.C. 7104(g)).

6.2.8. Reporting Subawards and Executive Compensation. The recipient agrees to ensure they have the necessary processes and systems in place to comply with the reporting requirements of the Transparency Act, as defined at 2 CFR 170.320, unless they meet the exception under 2 CFR 170.110(b).

6.2.9. Representation Regarding the Prohibition on Using Funds under Grants and Cooperative Agreements with Entities that Require Certain Internal Confidentiality Agreements. By submission of its proposal or application, the applicant represents that it does not require any of its employees, contractors, or subrecipients seeking to report fraud, waste, or abuse to sign or comply with internal confidentiality agreements or statements prohibiting or otherwise restricting those employees, contractors, or subrecipients from lawfully reporting that waste, fraud, or abuse to a designated investigative or law enforcement representative of a Federal department or agency authorized to receive such information. Note that: (1) the basis for this representation is a prohibition in section 743 of the Financial Services and General Government Appropriations Act, 2015 (Division E of the Consolidated and Further Continuing Appropriations Act, 2015, Pub. L. 113-235) and any successor provision of law on making funds available through grants and cooperative agreements to entities with certain internal confidentiality agreements or statements; and (2) section 743 states that it does not contravene requirements applicable to Standard Form 312, Form 4414, or any other form issued by a Federal department or agency governing the nondisclosure of classified information.

6.3. Reporting. General requirements are provided below; however, each awardee should check the award agreement and its contract data requirements list (CDRLs) and/or terms and conditions to determine the requirements for that specific award.

6.3.1. Annual Reports. Annual Reports will be due no later than 1 July of each year. Awards effective after 31 January will not require an Annual Report until 1 July of the following year. The Annual Report is *not* a cumulative report.

6.3.2. Final Technical Reports. A comprehensive final technical report is required prior to the end of an effort, due on the date specified in CDRLs and/or the terms and conditions of the award document. The purpose of the Final Report is to document the results of the effort. The Final Report *is* a cumulative report.

The final report will always be sent to the Defense Technical Information Center (DTIC) and reports may be available to the public through the National Technical Information Service (NTIS).

6.3.3. Financial Reports. Federal Financial Reports (SF-425) are due no later than 1 July of each year. Grants effective after 31 January will not require a Federal Financial Report until 1 July of the following year.

6.3.4. Foreign Travel Reports. Within thirty (30) days after returning to the United States from foreign travel, the PI may be required to submit an acceptable trip report summarizing the highlights of the trip. For grants, contracts, or OTAs awarded to institutions entirely located outside the United States and/or its territories, this is not required.

6.4. After-the-Award Requirements for *Grants*. Closeout, subsequent adjustments, continuing responsibilities, and collection of amounts due are subject to requirements found in 32 CFR 32.71 – 73 (Institutions of Higher Education, Hospitals, and Other Non-Profit Organizations) and 32 CFR 34.61 – 63 (For-Profit Organizations).

---

7. AGENCY CONTACTS

Administrative Correspondence and Questions	HDTRA1-FRCWMD-A@mail.mil
Thrust Area 1: *Science of WMD Sensing and Recognition*	HDTRA1-FRCWMD-TA1@mail.mil
Thrust Area 2: *Network Sciences*	HDTRA1-FRCWMD-TA2@mail.mil
Thrust Area 3: *Science for Protection*	HDTRA1-FRCWMD-TA3@mail.mil
Thrust Area 4: *Science to Defeat WMD*	HDTRA1-FRCWMD-TA4@mail.mil
Thrust Area 5: *Science to Secure WMD*	HDTRA1-FRCWMD-TA5@mail.mil
Thrust Area 6: *Cooperative Counter WMD Research with Global Partners*	HDTRA1-FRCWMD-TA6@mail.mil
Thrust Area 7: *Fundamental Science for Chemical and Biological Defense*	HDTRA1-FRCWMD-TA7@mail.mil

Table 6: Agency Contacts.

7.1. Questions regarding administrative content of this BAA must be addressed to the administrative e-mail address listed above.

7.2. Questions regarding technical content of this BAA must be referred to the technical staff responsible for the relevant thrust areas.

DTRA will not release employee personal contact information.

---

8. OTHER INFORMATION

Topics from previous periods may or may not be repeated. DTRA will not provide additional information regarding the posting of future topics, including dates for posting, the potential for a topic to be repeated in out years, the potential for similar topics to be posted, and/or topic details in advance of issuance of an amended BAA.

ATTACHMENT 1: SPECIFIC TOPICS

Thrust Areas 1, 2, 3, 4, 5, and 6 have no specific topics at this time.

Thrust Area 7 has nine (9) topics —Topics I1-I9—detailed below. Submissions to the general thrust area descriptions for this thrust area in accordance with the requirements detailed in this BAA are also welcome.

Great care must be taken to use the appropriate application package on www.grants.gov, as the package selection dictates how each submission will be reviewed:

- If NOT submitting to one of the specific topic numbers detailed below, use one of the **Thrust Area N NO TOPIC** application packages
- If you ARE submitting to one of the specific topic numbers detailed below, use the applicable **Basic Research-Thrust Area 7-Topic I1 to I9** application package

*****BASIC RESEARCH TOPICS I1-I9*****

In accordance with Section 4.2.1, the requirement for abstract pre-coordination is waived for Topics I1-I9; this topic does NOT require pre-coordination of an abstract prior to the submission of pre-application white papers. All other pre-coordination requirements remain in effect.

The pre-application white paper deadline for Topics I1-I9 is 4 March 2019. **PRE-APPLICATION WHITE PAPERS FOR THESE TOPICS MUST BE SUBMITTED BY 11:59 PM (MIDNIGHT) EST ON 4 March 2019.** White papers submitted to Topics I1-I9 may not be considered if they are received after this deadline.

Responses to Topics I1-I9 must address ONLY basic research (Budget Category 6.1). Basic research is the systematic study directed toward greater knowledge or understanding of the fundamental aspects of phenomena and of observable facts without specific applications toward processes or products in mind. It includes all scientific study and experimentation directed toward increasing fundamental knowledge and understanding in those fields of the physical, engineering, environmental, and life sciences related to long-term national security needs. It is farsighted, high payoff research that provides the basis for technological programs.[2]

Topics I1-I9 are interested in research projects that span from those that focus on exploratory aspects of a unique problem or a high-risk approach to those that involve a comprehensive program with interdisciplinary areas. Consistent across all proposals should be the focus on innovative research with the potential for high impact to C-WMD science.

DTRA anticipates that the predominance of awards made under Topics I1-I9 will be grants. Pre-application white papers and proposals submitted to Topics I1-I9 must have a single lead organization and single submission for the pre-application white paper and the invited proposal. Awards will be made by a single award to the lead institution. Subawards, including all grants and/or contracts, are the responsibility of the award recipient; exceptions will not be made.

Thrust Area 7, Topic I1: In Search of the "lnc": Long Non-Coding Ribonucleic Acids (lncRNA) Role in Pathogenesis

Award Amounts for this topic are anticipated to be between $350,000 and $500,000 for year 1

and up to $1,000,000 per subsequent year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort.

The preferred award structure for this topic is a base period of two (2) years with one (1) additional year as possible option. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of three (3) years will not be considered.

Background: Infections caused by viral or bacterial pathogens are known to significantly up/down regulate various genes which can play a significant role in disease progression. Most of the transcriptomic studies currently sponsored by DTRA have focused on mRNA and their role in disease. Unfortunately, mRNAs represent a very small portion of the transcriptome since over 98% is represented by non-coding RNAs (ncRNAs). LncRNAs represent a class of ncRNAs that are longer than 200 nucleotides and are not well studied nor understood in relation to infectious diseases. LncRNAs are typically expressed at lower levels, but often are implicated to have greater tissue specificity related to biogenesis and epigenetic regulatory factors. LncRNAs are also known to interact with other molecules such as proteins, peptides, DNA, RNA, and even metal ions to form secondary and tertiary structures. By understanding the various lncRNAs interactions this knowledge can potentially be leveraged to understand their role in pathogenesis and to best design and develop novel diagnostic assays.

Impact: Studying lncRNAs potentially offer the ability to elucidate novel biothreat-relevant signatures for diagnosis and potential therapeutic application. It is believed that there is a great potential to best understand the role that lncRNAs play in infection from relevant pathogens to the Chemical and Biological Defense Program (CBDP) with current technologies, capabilities, and methods currently available. Learning the role that non-coding elements potentially play can greatly enhance the overall knowledge of pathogenesis and virulence that typically lead to adverse clinical symptoms and can impact the Warfighter's combat effectiveness and the greater mission.

Objective: The aim of this topic is to develop proof of concept to determine whether novel diagnostic markers can be discovered from lncRNA research. Specifically, the goal is to understand if lncRNAs play a significant role in pathogenesis and determine the appropriate mechanisms of action. For this basic research topic, the ideal focus is on lncRNAs, which are defined to be greater than 200 nucleotides in length, however, this topic is willing to entertain ncRNAs smaller than 200 nucleotides if their role, function, and mechanism of action can be accurately described. The primary goals and objectives of this basic research effort for the potential 2 year effort will be to:

a) Leverage the appropriate technologies to rapidly discover and identify lncRNAs
b) Determine lncRNA's role in infection and pathogenesis for relevant animal models and/or human infectious disease studies

Please note that only infectious disease studies that are directly relevant to the CBDP will be thoroughly evaluated and considered. These pathogen threats are typically considered to be high-containment microorganisms that are associated with extremely high rates of morbidity and

mortality as well as the high risk of transmissibility. Solicitations will be reviewed for experimental design and the necessary studies to elucidate and, if successful, conduct initial verification to showcase the role of lncRNAs in pathogenesis and disease progression.

References:

Hu, Guoku et al. "Molecular mechanisms of long noncoding RNAs and their role in disease pathogenesis" Oncotarget vol. 9,26 18648-18663. 1 Jan. 2018, doi:10.18632/oncotarget.24307

Thrust Area 7, Topic I2: Algorithm Development for Optimization of Biologic Medical Countermeasures

Award Amounts for this topic are anticipated to be up to $400,000 per year through the base period phase to establish proof of concept and, if successful, up to $800,000 per year thereafter (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation.

The preferred award structure for this topic is a base period of one (1) year with up to four (4) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Note: For this topic, awardees will be required to grant the US Government a licensing agreement to all software and or hardware in perpetuity for all Chemical and Biological Defense Program (CBDP) uses.

Background: Machine learning and artificial intelligence (AI) has advanced greatly in the past decade. Coupled with cheaper computer power, the technology is rapidly moving into diverse areas from natural language processing to autonomous vehicles. The basic underpinnings of the technology is in the ability to "train" the software to recognize, generate, and analyze patterns from vast amounts of heterogeneous data that may not be readily obvious to the human mind. For example, AI has been successfully used in the field of clinical oncology medicine to accurately read mammograms.[1] The next step is leveraging the potential of AI to drive the next generation of rational drug discovery.[2]

Relevance: The DoD has a unique set of challenges within the CBDP for which AI may be ideally suited to help close gaps in our basic understanding. It is envisioned that a DoD-specific AI drug discovery platform will support the CBDP Agile Medical Platform. Specifically, a DoD-specific AI platform could ultimately be developed that is focused on generating and analyzing biological data specific to monoclonal antibody based therapies, to aid in identifying optimal candidates for advanced development of biological medical countermeasures (MCM) to protect the Warfighter and Nation against biological threat agents. Methods developed that can optimize the antibody for any number or numbers of traits that would accelerate MCM development, by reducing the time and cost, while maximizing efficiency and effectiveness for the DoD are especially of interest. To this end, this topic seeks to solicit groups working in the field of machine learning and algorithm design to help determine if AI/machine learning

technology is advanced enough to help define materiel monoclonal antibody based medical countermeasure solutions to the DoD.

Impact: If successful, the work will support the CBDP through improved pathways for moving product through the regulatory process to provide MCMs for the warfighter. It would also support in laying the groundwork for the potential to develop a more encompassing AI drug discovery platform that could be applied across the spectrum of DoD medical areas within the CBDP.

Objective: As a proof of concept, the initial work will be directed towards the optimization of monoclonal antibody biologics. While discovery of antibodies with strong binding is fairly routine, the ability to develop those into feasible drug candidates remains a challenge. Basic understanding of which amino acids can be changed to optimize an antibody to maximize its protection against a biological threat agent of interest to DoD is still a relatively novel research area.

This topic aims to answer fundamental research questions related to addressing whether AI can be utilized to provide information to aid optimizations in rational antibody based drug design, and whether AI can help address issues relevant to the development of antibody based MCMs for biological agents of relevance to DoD:

- Plasma stability / half-life – can we routinely achieve 6 months or greater duration of protection?
- Route of administration – can we optimize for intramuscular injection?
- Shelf-life – can we routinely lyophilize and reconstitute?
- Greater avidity – can we develop countermeasures with increased binding and clearance?
- Aggregation—can we minimize the aggregation potential of monoclonal antibodies?

Other directed applications that optimize manufacturability will also be considered.

The DoD has made significant investments in the development of antibody-based MCMs, and as such, has access to a potential "learning set" of proteins that could aid in the development of a useful algorithm. While not required, there may be some benefits to partnering with groups that have been involved in the design of antibodies against pathogens of interest to the DoD. Further guidance on potential partnerships/collaborations may be provided with each full proposal invitation.

The end state for this work will be a validated algorithm capable of optimizing an existing antibody for specific biological properties and the accompanying laboratory data to prove its predictability.

Proposals for small molecules will not be considered.

References:

*Tejal A. Patel MD, Mamta Puppala MS, Richard O. Ogunti MBBS, Joe E. Ensor PhD, Tiancheng He PhD, Jitesh B. Shewale BDS, MPH, Donna P. Ankerst PhD, Virginia G. Kaklamani MD, DSc, Angel A. Rodriguez MD, Stephen T. C. Wong PhD, Jenny C. Chang MD, "Correlating mammographic and pathologic findings in clinical decision support using natural language processing and data mining methods," Cancer **123**, 114–121 (**2017**)*

See for example: https://endpts.com/ai-drug-discovery-success-inspires-a-machine-learning-startup-at-the-mayo-clinic/; https://www.reuters.com/article/us-pharmaceuticals-ai-gsk/big-pharma-turns-to-ai-to-speed-drug-

*discovery-gsk-signs-deal-idUSKBN19N003 and Eric Smalley, "AI-powered drug discovery captures pharma interest", Nature Biotech, **35**, 604–605 (**2017**) doi:10.1038/nbt0717-604*

*"From machine learning to deep learning: progress in machine intelligence for rational drug discovery", Zhang L, Tan J, Han D, Zhu H, Drug Discov Today. **2017** Sep 4. pii: S1359-6446(16)30436-6. doi: 10.1016/j.drudis.2017.08.010.*

*"druGAN: An Advanced Generative Adversarial Autoencoder Model for de Novo Generation of New Molecules with Desired Molecular Properties in Silico", Kadurin A, Nikolenko S, Khrabrov K, Aliper A, Zhavoronkov A., Mol Pharm. **2017** Sep 5;14(9):3098-3104. doi: 10.1021/acs.molpharmaceut.7b00346.*

*"Machine learning reveals a non-canonical mode of peptide binding to MHC class II molecules", Andreatta M, Jurtz VI, Kaever T, Sette A, Peters B, Nielsen M., Immunology. **2017** Oct;152(2):255-264. doi: 10.1111/imm.12763.*

*"Mathematical Modelling of Immune Parameters in the Evolution of Severe Dengue", Premaratne MK, Perera SS, Malavige GN, Jayasinghe S., Comput Math Methods Med. **2017** ;2017:2187390. doi: 10.1155/2017/2187390.*

*"Learning the Relationship between the Primary Structure of HIV Envelope Glycoproteins and Neutralization Activity of Particular Antibodies by Using Artificial Neural Networks", Buiu C, Putz MV, Avram S., Int J Mol Sci. **2016** Oct 11;17(10). pii: E1710.*

*"A computational method for designing diverse linear epitopes including citrullinated peptides with desired binding affinities to intravenous immunoglobulin", Patro R, Norel R, Prill RJ, Saez-Rodriguez J, Lorenz P, Steinbeck F, Ziems B, Luštrek M, Barbarini N, Tiengo A, Bellazzi R, Thiesen HJ, Stolovitzky G, Kingsford C., BMC Bioinformatics. **2016** Apr 8;17:155. doi: 10.1186/s12859-016-1008-7.*

Thrust Area 7, Topic I3: Identification of Common Molecular Pathways Associated Chemical Warfare Agent (CWA)-Induced Inflammation

Award Amounts for this topic are anticipated to be between $250,000 and $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation.

The preferred award structure for this topic is a base period of two (2) years with up to three (3) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: Chemical warfare agents (CWAs) are amongst the most brutal weapons of mass destruction (WMD) created by mankind. CWAs are extremely toxic synthetic chemicals that can be dispersed as a gas, liquid or aerosol or as agents adsorbed to particles to become a powder. These CWAs have either lethal or incapacitating effects on humans. Thousands of toxic substances are known, but only some of them are considered as CWAs based on their characteristics, high toxicity, imperceptibility to senses and rapidity of action after dissemination and persistency, and are listed as scheduled chemicals in the Chemical Weapons Convention. The CWAs possess different characteristics and belong to various classes of compounds with pronounced physicochemical, physiological and chemical properties. While CWAs are known to cause a multitude of physiological insults, a common feature among many CWAs is the ability to provoke inflammation. The goal of this topic is to discover common molecular pathways

and/or mechanisms of action for inflammation that results from exposure to a broad range of chemicals – including nerve agents, pulmonary agents, blister agents, and other CBDP-relevant compounds.

Impact: Improved understanding of CWA-induced inflammation may point to novel therapeutic targets for intervention. Furthermore, identification of common molecular pathways and /or mechanisms of action for inflammation may enable the design or repurposing of potential therapeutics with broad spectrum action across a range of CWAs. Identification of novel therapeutic targets and or interventions are critical activities for advancing treatments associated with CWA exposure.

Objective: The objective of this topic is to solicit proposals that aim to both:

a) Characterize molecular pathways and/or mechanisms of action for CWA-induced inflammation. Competitive proposals will focus on inflammation from a broad range of CWA, including; nerve agents, pulmonary agents, blister agents, and other CBDP-relevant compounds. Additionally, proposals are expected to consider inflammation across a variety of tissues (e.g. nervous, epithelial, etc.). Activities may also rely upon both established and novel techniques (e.g. in-silico, in-vitro, ex-vivo, and in-vitro).

b) Identify common molecular pathways and/or mechanisms of action for CWA-induced inflammation that may be suggestive of novel targets for therapeutic intervention. An expected emphasis is in the identification of common inflammation-specific molecular elements that span CWAs and tissue type.

Thrust Area 7, Topic I-4: Identification of Novel Methods for Improving the Pharmacokinetic Properties of Proteins

Award Amounts for this topic are anticipated to be between $280,000 and $450,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation.

The preferred award structure for this topic is a base period of two (2) years with up to three (3) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: Prophylactic and therapeutic medical countermeasures (MCMs) for chemical and biological agents currently in development have limited circulatory stability and may elicit an immune response during repeat dosing. Therefore, the goal of this topic is the identification of novel methods for improving the Absorption Distribution Metabolism Excretion (ADME) properties specific to Excretion. This approach can incorporate either humanizing non-human proteins to decrease immunogenicity as well as identifying novel methods for increasing the half-life of biologic based MCMs. The first interest is the stabilization and decreased immunogenicity of antibodies derived from non-human sources (e.g. – murine model). The

second area of interest is identification of novel mechanisms to decrease methods of clearance and elimination to improve half-life of biologic MCMs (e.g. – increasing affinity of Fc to FcRN). Prolonged stability of therapeutic antibodies directed against chemical warfare agents (CWAs) or biological warfare agents (BWAs) has the potential to advance both novel and previously developed MCMs.

Impact: This research would be to be able to provide the Warfighter with an improved prophylactic and therapeutic MCMs against CWAs and BWAs. Discovery of novel technologies to improve the ADME properties of biologic MCMs such as antibodies would greatly increase the Warfighters' capability to operate during a biological or chemical attack.

Objective: The objective of this topic is to solicit proposals that aim to both:

a) Identify novel methods for humanizing non-human proteins to decrease immunogenicity and increase stability.

b) Provide solutions to extend the half-life of prophylactic or therapeutic MCMs.

Thrust Area 7, Topic I5: Receptor Mapping Across Humans and Animal Models

Award Amounts for this topic are anticipated to be between $250,000 and $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation.

The preferred award structure for this topic is a base period of two (2) years with up to three (3) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: A proper understanding of how receptors and enzymes map across humans and laboratory animal models is critical for providing relevant, translatable data necessary to develop safe and efficacious medical countermeasures for the warfighter. Little data exists that comprehensively compares the distribution (blood, brain, lung, heart, liver and kidney, etc.), type, and function of receptors that interact with the various chemical or biological warfare agents (CWAs and BWAs) in humans to those in the various laboratory animal models (mice, rates, guinea pigs, rabbits, ferrets, swine, and non-human primates). Such data would help determine which animal models are suited for studying the various CWAs and BWAs for their signs, symptoms and medical countermeasures. Receptors should include standard chemical weapon target receptors (AChE, GABA, opioid, and other CBDP-relevant chemical classes), as well as off-target receptors that have relatively high interactions with the chemicals of interest. Therefore, the goal of this topic is to utilize receptor mapping to aid in identifying the appropriate choice of animal models for medical countermeasures development studies as well as reveal potential areas for novel prophylactic and/or therapeutic intervention.

Impact: Understanding how receptors targeted by CWAs map and function across humans and laboratory animal models will allow for more relevant animal model selection in medical

countermeasure development.

Objective: The objective of this topic is to solicit proposals that aim to:

a) Define the type and distribution of receptors across humans and laboratory animal models for mice, rats, guinea pigs, rabbits, ferrets, swine, and non-human primates (NHP) to allow for more relevant animal model selection in medical countermeasure development. Targeted tissues in each animal model should include blood, brain, lung, heart, liver and kidney, etc. Receptors should include AChE, GABA, and opioid receptors, as well as off-target receptors that have relatively high interactions with CBDP relevant chemical classes.

b) Characterize the structural and physiological homologies of receptors and enzymes across humans and laboratory animal models for mice, rats, guinea pigs, rabbits, ferrets, swine, and NHP to allow for more relevant animal model selection in medical countermeasure development. Targeted tissues in each animal model should include blood, brain, lung, heart, liver and kidney, etc. Receptor and enzyme families should include AChE, GABA, opioid receptors, as well as off-target receptors that have relatively high interactions with the chemicals or biologics interest.

c) Amongst receptors and enzymes that differ significantly in their homology relative to human (characterized in Objective b, above), describe their relative affinities for (or inhibition by) chemicals of interest to include cholinesterase inhibitors, GABA inhibitors, opioids, biological toxins and other CBDP relevant chemical agents.

Thrust Area 7, Topic I6: Molecular Cascades for Signaling of Chemical and Biological Warfare Agents

Award Amounts for this topic are anticipated to be between $350,000 and $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation.

The preferred award structure for this topic is a base period of three (3) years with up to two (2) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: DTRA seeks to understand the design, synthesis, and characterization of a signal cascade or amplification scheme that may be incorporated into a stimuli responsive system which reacts and responds to chemical and biological warfare agents (CWAs and BWAs). In recent years, it has been shown that materials can selectively bind to CWAs and BWAs or stimulate a response. For example, aptamers and single domain antibodies have been explored for various assay applications for the detection of BWAs [Turner 2017; U.S. Patent US9310357B2]. Polymers can be synthesized to change shape or depolymerize when exposed to chemical warfare simulants [Sha, 2017; Phillips, 2014], or enzyme systems can be manipulated to elicit a pH or colorimetric change when exposed to CWAs [FLIR, 2017]. In addition,

responsive materials have been engineered for detection applications, as in the case of *"molecular beacons" for fluorescent detection of bacterial cells or spores*, or fluorophores released from shape responsive small molecules in the presence of cyanide [Jo, 2013]. However, in all cases, the response generated from the binding event is typically stoichiometric, not amplified sufficiently for live observation, and not amenable for non-assay based applications. A reaction scheme involving materials that bind CWA and BWA and elicit a cascade reaction leading to an amplified response for real-time observation is highly desired.

Impact: Successful novel materials that can be designed and scaled to react to CWA and BWAs can greatly enhance the capabilities of current contamination mapping efforts by enhancing protection and guiding decontamination.

Objective: This research topic seeks to develop a cascade reaction scheme involving a rationally-designed material that can bind to CWA and BWAs, resulting in an amplified response. The research should propose to: 1) Identify chemical and biological stimuli (i.e., signatures) that are unique to specific CWA and BWAs; 2) Iteratively design and synthesize materials that can bind to CWAs and BWAs, resulting in a cascade reaction scheme that leads to a rapid amplified response; and 3) Characterize amplified responses that support the iterative process of design and synthesis to achieve a sensitive response to one unit of CWAs and BWAs. A material or a reaction scheme that produces a change in infra-red or an anti-stokes shift is of particular interest to the agency; however, other acceptable responses include, but are not limited to, colorimetric, fluorescence, and phosphorescence. The amplified signal, or the cascade scheme should be robust and not easily interfered with from environmental factors: temperature, presence or absence of water/humidity, and pH. For example, BWA-responsive materials should be able to bind to specific signatures of the endospore and the vegetative bacteria and agnostic to delivery media (aqueous, solvent, or combination thereof). Ideal BWA-responsive materials would respond to a single colony or plaque forming unit of the BWA, with abilities to differentiate BWA strains through the amplified response or binding event.

Research areas may include, but are not limited to:

a) Studies involving multiplexed approaches for developing stimuli-responsive materials or classes of materials that respond to two or more CWAs or BWAs in the same sample, with the potential to identify CWAs or BWAs by the type of response;

b) Molecular-level interrogation of the interactions between a stimuli-responsive material and specific CWAs and BWAs (to include environmental requirements, specificity, sensitivity, and interference potential);

c) Approaches involving oligonucleotide aptamers or single domain antibodies or derivatives thereof.

A single model CWA or BWA can be used to demonstrate the technique or technology developed under this program; however, it is desired for the technology to be amenable to bind and react with a wide variety of agents. Due to the large body of literature and existing projects supporting responsive materials for CWAs, proposals wishing to study CWA responsive materials must address gaps in existing technologies supporting CWA contamination mapping. Proposals or teams looking to build sensors or devices will not be selected. Strategies and

partnerships for live agent testing of designed system is highly encouraged.

References:

Turner, Kendrick, et al. "Pairing Alpaca and Llama-Derived Single Domain Antibodies to Enhance Immunoassays for Ricin." Antibodies, vol. 6, no. 1, Feb. 2017, p. 3., doi:10.3390/antib6010003.

U.S. Patent US9310357B2: Detection of chemical and biological agents using oligonucleotide aptamers, 2016-04-12. https://patents.google.com/patent/US9310357.

Sha, Sheng-Chun, et al. "Chemical Warfare Simulant-Responsive Polymer Nanocomposites: Synthesis and Evaluation." Journal of Polymer Science Part A: Polymer Chemistry, vol. 55, no. 18, 2017, pp. 3034–3040., doi:10.1002/pola.28580.

Phillips, Scott T., et al. "Amplified Responses in Materials Using Linear Polymers That Depolymerize from End-to-End When Exposed to Specific Stimuli." Journal of Applied Polymer Science, vol. 131, no. 19, Mar. 2014, doi:10.1002/app.40992.

FLIR Agentase C2. 2017. FLIR Systems, Inc. Retrieved Nov. 2, 2018 from https://www.flir.com/globalassets/imported-assets/document/flir-agentase-c2-datasheet-en.pdf.

Jo, Junyong, et al. "Interdigitated Hydrogen Bonds: Electrophile Activation for Covalent Capture and Fluorescence Turn-On Detection of Cyanide." Journal of the American Chemical Society, vol. 135, no. 9, 2013, pp. 3620–3632., doi:10.1021/ja312313f.

Thrust Area 7, Topic I7: An Every-Atom-Counts Approach to Designing Small Cluster Catalysts

Award Amounts for this topic are anticipated to be between $350,000 and $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation.

The preferred award structure for this topic is a base period of three (3) years with up to two (2) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: DTRA has invested considerable resources over the past two decades in the design and development of active materials for the capture and decomposition of chemical agents.

Relevance of Metal Oxide Research. In the 1990s and early 2000s much work was done in understanding properties of bulk metal oxides in supported/unsupported form to bind and decompose chemical agents at ambient and elevated temperatures. This period of work established an important benchmark as a historical departure from many decades of research on activated carbon chemistry. Insight into the role of the active nature of mixed metal oxides on porous activated carbons provided the motivation to explore these bulk oxides in a more rigorous manner. The results of this research showed important properties and limitations of certain metal systems to bind and decompose chemical agents. Many forms of the bulk oxides were shown to be limited in porosity which reduced accessibility of chemical adsorbates to a high number of

active centers. Bulk oxides demonstrated good hydrolytic chemistry at ambient temperatures due to the active nature of the proton on the metal-oxygen center (H-O-M) as well as binding with the Lewis acid metal. At high temperatures (350-900 ºC) these oxides showed increased activity for oxidation of chemical agents. The outcome of this body of work suggested that metal oxide systems of high porosity and accessibility to active centers (M-O-M) was critical to achieving high chemical adsorption and decomposition of chemical agents.

The emergence of reticulated metal organic frameworks in the middle of the last decade (~2005) provided new motivation to address the technical challenges as learned with bulk metal oxide systems. This era of periodic structures as presented by Metal Oxide Frameworks (MOF) and other related forms was driven by the rapidly emerging science of nanostructured materials in general, such as small metal cluster s (<10 nm) of Au, Ag and Pt in unsupported and supported form. For the past decade DTRA has invested heavily in understanding the structure and activity relationships of MOF. By design (in pristine form), MOFs are highly coordinated structures with high porosity making them attractive candidates for strong binding and active site utilization of adsorbed chemical agents. The chemistry of the MOF, like bulk metal oxides, demonstrate moderate to high hydrolytic chemistry for decomposition of chemical agents. Recently questions emerged regarding the role of defects in MOF structures and have provided additional motivation for understanding design rules regarding adsorption, diffusion and chemical decomposition. An important observation from all of the MOF work regarding chemical activity suggests that they are important platforms for inclusion of guest atoms and clusters that can be designed to enhance binding and decomposition beyond hydrolysis of chemical agents (such as oxidation and dealkylation).

Metal Atoms and Clusters. The next generation of active oxides centers on utilization of highly energetic forms of single atom and small clusters to promote stronger binding and molecular dissociation without the dependency on external energy sources (i.e., thermal, light). The ultimate small-size limit for metal particles is the single-atom catalyst (SAC), which contains isolated metal atoms singly dispersed on supports. SACs maximize the efficiency of metal atom use, which is particularly important for supported noble metal catalysts. Moreover, with well-defined and uniform single-atom dispersion, SACs offer great potential for achieving high activity and selectivity. Heterogeneous catalysis usually occurs at the surface of a solid catalyst, which ideally has a high surface area to volume ratio. For example, smaller metal particles have a higher fraction of surface atoms than do larger metal particles. This fraction not only has an impact on the fraction of metal atoms that are catalytically active (hereafter referred to as metal atom utilization), but also has a substantial effect on selectivity. The metal atom utilization in homogeneous molecular catalysts can reach 100% — a figure that may be orders of magnitude higher than that of heterogeneous catalysts. Heterogeneous catalysts might feature non-uniform aggregates of hundreds and/or thousands of metal atoms, only a small fraction of which are exposed to reactants. For example, the reactive, coordinately unsaturated metal atoms at apices, edges, steps and corners usually represent less than 20% of the total metal atoms. However, the surface free energy of metals increases significantly with decreasing particle size, promoting aggregation of small clusters. Using an appropriate support material that strongly interacts with the metal species prevents this aggregation, creating stable, finely dispersed metal clusters with a high catalytic activity, an approach industry has used for a long time.

Objective: To advance understanding on how to design new forms of highly active materials that exhibit new chemistries to bind, decompose and detoxify chemical agents of interest to

DTRA. This topic focuses on advancing the current knowledge in the design of active materials to provide tunable binding properties in order to promote highly efficient molecular dissociation (bond breaking) and detoxification of chemical agents. Specifically the work will concentrate on the design of very small metal oxide clusters (<2 nm) that possess a high surface concentration of undercoordinated metal centers to promote molecular dissociation through oxidation and dealkylation.

Research areas may include, but are not limited to:

a) Design of sub-nanometer clusters of metal atoms and molecules with strong size-dependent properties to promote active centers for binding and decomposition of chemical agents.

b) Employing theory and surface sensitive techniques to systematically investigate size-dependent properties of metal centers on binding and molecular dissociation of relevant systems (live agent/simulant).

c) Understanding the interactions of ambient chemicals on structure-activity of promising metal systems relevant to binding and dissociation of chemical adsorbates.

d) Investigating the dynamics of metal center aggregation and stabilization under ambient conditions, to include stabilizing substrates and environments to preserve activity of small metal clusters.

References:

Lichen Liu and Avelino Corma Metal Catalysts for Heterogeneous Catalysis: From Single Atoms to Nanoclusters and Nanoparticles, Chem. Rev. 2018, 118, 4981−5079.

Aiqin Wang, Jun Li and Tao Zhang, Heterogeneous single- atom catalysis, Nature Reviews Chemistry volume 2, 65-81, JUNE 2018.

Somorjai, G. A.; Carrazza, J. Structure Sensitivity of Catalytic Reactions. Ind. Eng. Chem. Fundam. 1986, 25, 63−69.

Che, M.; Bennett, C. O. The Influence of Particle Size on the Catalytic Properties of Supported Metals. Adv. Catal. 1989, 36, 55−172.

Ye, R., Hurlburt, T. J., Sabyrov, K., Alayoglu, S. & Somorjai, G. A. Molecular catalysis science: perspective on unifying the fields of catalysis. Proc. Natl Acad. Sci. USA 113, 5159–5166, 2016.

Qiao, B. et al. Single- atom catalysis of CO oxidation using Pt1/FeOx. Nat. Chem. 3, 634–641 2011.

Xiao-Feng Yang, Aiqin Wang, Botao Qiao, Jun Li, Jingyue Liu, And Tao Zhang Single-Atom Catalysts: A New Frontier, In Heterogeneous Catalysis, Accounts Of Chemical Research, 1740–1748, 2013 Vol. 46, No. 8.

Thrust Area 7, Topic I8: Design Rules for a Biomimetic Membrane with Selective Water Permeability

Award Amounts for this topic are anticipated to be between $350,000 and $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation.

The preferred award structure for this topic is a base period of three (3) years with up to two (2) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable.

Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: DTRA seeks to understand the design and synthesis of biomimetic membranes that are selectively water permeable for use as protective barriers to CWAs. Current CWA protective barrier membranes like coated PTFE and butyl rubber offer high protection against CWA, but have low to no moisture vapor transport rates (MVTR). By contrast, in nature biological cells have highly selective membrane channels (e.g., aquaporins, AQP), that can selectively transport water molecules across membranes at a rate of 3×10^9 s^{-1} per channel [Groot, 2001], and have been impregnated in membranes for filtration applications [McCutcheon, 2017]. There is also a large body of literature describing synthetic water channels that transport water at equal or greater rates than biological channels, but with a significant loss of selectivity [Song, 2018]. For example, researchers have demonstrated synthetic membranes derived from self-assembled block copolymers with high selectivity and high tenability [Mulveena, 2014; Qu, 2015]. Ideally, biomimetic membrane channels might be leveraged to maximize selectivity and MVTR across the membrane while maintaining protection from CWAs. However, a number of knowledge gaps in critical areas must be addressed prior to achieving this, including: 1) Modeling at all length scales to develop a coherent molecular understanding of synthetic channel and membrane properties, provide insight for future materials design, and predict selectivity for water transport (and not CWA); 2) Basic understanding of compatibility of water transport channels in CWA protective polymer and tolerance to various processing conditions; 3) Fundamental understanding of biomimetic channel and membrane structure optimization to control transport properties; 4) Laboratory evaluation of membranes is often conducted with highly idealized mixtures, so separation performance in real applications with complex mixtures needs to be demonstrated; and 5) Lack of systematic understanding of methodologies to incorporate channels and scale promising membranes from the few square centimeters needed for laboratory characterization to the thousands of square meters needed for large applications impedes membrane deployment. Nevertheless, opportunities for biomimetic water-permeable channels and membranes in both existing and emerging technologies, together with an expanding set of membrane materials, hold great promise for the technology to effectively address CWA protection needs.

Impact: Successful design and synthesis of biomimetic, selective, water-permeable channels and their incorporation into substrate membranes could potentially be transformative in bringing new barrier technologies that are breathable, stretchable, and protective against CWA.

Objective: This research topic seeks to develop rational design approaches for robust, predictable and cost effective construction of synthetic membranes containing biomimetic water channels (natural, synthetic, or combination thereof) that can perform selective/facilitated transport of water molecules and air, and ability to block chemical warfare agent. The research should propose an iterative (spiral) approach to understand the opportunities for advancing biomimetic, water-selective, CWA impermeable membranes, to include: 1) novel discoveries in chemistries that lend themselves to scaling in a stimuli responsive system; 2) higher permeability and selectivity of water in membrane applications, without sacrificing CWA protection; and 3) membrane systems that lend itself to scaling. The biomimetic water channels can be derived from ruggedizing existing natural AQP channels or producing *de novo* [Lu, 2018] water selective

transmembrane proteins or synthetic channels that can tolerate chemical processing techniques. The biomimetic water channels can also be from completely synthetic materials embedded in a membrane. However, all membranes must be protective against CWA, and therefore strategies and partnerships for simulant (DMMP, 2-CEES) liquid and vapor permeation testing are highly encouraged.

Proposals offering only theoretical approaches (e.g., modeling) will not be entertained.

Research areas may include, but are not limited to:

a) Ruggedizing natural AQP through de novo design for incorporating on protective substrates;

b) Artificial water channels in self-assembled membrane substrates and testing against CWA

c) Other biomimetic mechanisms that could increase MVTR and selectivity without sacrificing protection

References:

Groot, B. L. (2001). Water Permeation Across Biological Membranes: Mechanism and Dynamics of Aquaporin-1 and GlpF. Science, 294(5550), 2353-2357. doi:10.1126/science.1062459

Song, W., Lang, C., Shen, Y., & Kumar, M. (2018). Design Considerations for Artificial Water Channel–Based Membranes. Annual Review of Materials Research, 48(1), 57-82. doi:10.1146/annurev-matsci-070317-124544

Mulveena, R.A., Weidman, J.L.; Jing, B.; Pople, J.A.; Zhu, Y.; Boudouris, B.W.; Phillip, W.A. (2014). Tunable Nanoporous Membranes with Chemically-Tailored Pore Walls from Triblock Polymer Templates. J. Mem Sci, 470, 246-256. doi.org/10.1016/j.memsci.2014.07.021

Qu, S.; Dilenschineider, T.; Phillip, W.A. (2015). Preparation of Chemically Tailored Copolymer Membranes with Tunable Ion Transport Properties. ACS Appl. Mater. Interfaces, 7, 19746−19754. doi.org/10.1021/acsami.5b05592

Xia L., Andersen, M.F., Hélix-Nielsen C., McCutcheon J.R. (2017). Novel Commercial Aquaporin Flat-Sheet Membrane for Forward Osmosis. Industrial & Engineering Chemistry Research, 56 (41), 11919-11925 doi: 10.1021/acs.iecr.7b02368

Lu, P., Min, D., DiMaio, F., Wei, K.Y., Vahey, M.D., Boyken, S.E., Chen, Z., Fallas, J.A., Ueda, G., Sheffler, W., Mulligan, V.K., Xu, W., Bowie, J.U., Baker, D. (2018). Accurate computation design of multipass transmembrane proteins. Science, 6379 (359), 1042-1046 doi: 10.1126/science.aaq1739

Thrust Area 7, Topic I9: Design of Repellant Permanent Thin Films for Chemical and Biological Agent Resistance

Award Amounts for this topic are anticipated to be between $500,000 and $1,000,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation.

The preferred award structure for this topic is a base period of two (2) years with up to three (3) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: Current chemical and biological agent resistant coatings fail to provide desired levels of contamination prevention. This means warfighters are required to expend significant effort during field decontamination of equipment. Attempts to control penetration of liquids into surfaces often harness the lotus leaf effect. This involves the use of a textured surface providing air-liquid and air-solid interfaces. For these surfaces, increases in pressure, for example applied weight or pressure washing, can lead to liquid intrusion into the textured surface resulting in a defeat of the repellent characteristic. The surface features that produce this effect also tend to be fragile. Alternatives to the lotus leaf effect have been described, including slippery liquid-infused porous surfaces (SLIPS) and slippery omniphobic covalently attached liquids (SOCAL). The liquid-liquid interaction interfaces of these materials address some shortfalls in the lotus effect, but current technologies tend to be temporary, or to weather poorly.

Improved coatings and thin films for agent resistance would decrease the logistical burden associated with decontamination of equipment by warfighters. In order to develop these materials, a fundamental understanding of the surfaces and the physical and chemical properties that assist with resisting contamination is needed. Ideally the thin film would allow for decreased sliding and shedding angles for agents and other contaminants as well as simplified decontamination approaches.

Impact: The research explored in this topic seeks to develop a fundamental understanding of a permanent technology for repelling chemical agents and decreasing the logistics of decontamination. Ideally, chemical and biological warfare agents and other contamination would be shed from coated surfaces, minimizing the need for additional decontamination strategies.

Objective: Proposals are sought for work which seeks to understand the fundamental aspects of coating technologies (or thin films) and how those aspects control penetration and repellency of chemical and biological warfare agents. The technologies should be permanent with a minimum persistence of 18-24 months and be transparent or offer pathways to achieving transparency. The work will include synthesis of the technology and evaluation of wetting by, and retention of, simulants for chemical warfare agents.

Research areas may include, but are not limited to:

a) Determination (theoretical or experimental) of composition impact on chemical agent resistance
    a. Experimental synthesis and characterization of the technologies and measurements at the laboratory scale with chemical warfare agent simulants
    b. Technologies must adhere to a polyurethane base coating
    c. CB warfare agent resistance with no more than 0.4% agent (or simulant) retention by the technology
    d. Technology must be compatible with military decontamination procedures or, ideally, with a water rinse
    e. Must be amenable to use under varying environmental conditions low/high temperatures, low/high humidity, continuous UV exposure, water/mud, and mechanical and thermal stresses etc.

f. Determination of chemical agent resistance over time (degradation of the thin film) physical, chemical mechanical and environmental
    i. Material should perform at initial metrics for a minimum of 18-24 months aging
    ii. Similar repellant performance in low/high temperatures, low/high humidity, mechanical stress, thermal stress, UV exposure after 18-24 months

b) Evaluation of chemical warfare agent simulant retention and repellency
    a. Technologies must be amenable to varying environmental conditions and provide a minimum resistance to chemical agents
    b. Applied technology layers must be thin while providing desired performance
    c. Thin films must be durable to mechanical and thermal stresses
    d. Determination of agent (simulant) retention/penetration after rinsing with water

The focus of proposals should be on development of thin film or coating technology and its characterization. Proposals should show preliminary simulant retention or wetting data, or evidence that the technology is capable of providing repellency. Finally, simplicity in the synthetic approach and in coating application is paramount for potential follow-on manufacturing and scaling; successful technologies could be candidates for transition to a product. The potential for development of a one-step spray coating is highly desirable.

References:

L. Wang, T. McCarthy. Covalently Attached Liquids: Instant Omniphobic Surfaces with Unprecedented Repellency. Angewandte Chem.2016, 55, 244-248

C. Zhang, Y. Xia, H. Zhang, N. Zacharia, Surface Functionalization for a Nontextured Liquid-Infused Surface with Enhanced LifetimeACS Appl. Mater. Interfaces 2018, 10, 5892-5901.

J. Wang, K. Kato, A. Biois, T-S. Wong, Bioinspired Omniphobic Coatings with a Thermal Self-Repair Function on Industrial MaterialsACS App. Mater. Interfaces 2016, 8, 8265-8271.

J. Conti, J. De Coninck, M. Ghazzal. Design of water-repellant coating using dual scale size of hybrid silica nanoparticles on polymer surface Appl. Surf. Sci, 2018, 436, 234-241.

P. Irajizad, M. Hasnain, N. Farokhnia, S.M. Sajadi, H. Ghasemi. Magnetic slippery extreme icephobic surfaces. Nat. Commun. 2016, 7, 13395.

X. Feng, L. Jiang. Design and Creation of Superwetting/Antiwetting Surfaces. Adv. Mater. 2006, 18, 3063-3078.

ATTACHMENT 2: INTELLECTUAL PROPERTY

(Applies to FAR Contracts & OTAs)

Applicants must describe any limitations on the use of any intellectual property (patents, inventions, trade secrets, copyrights, trademarks, technical data or computer software) that will impact the offeror's performance of the contract or impact the Government's subsequent use of any deliverable under the contract. In particular, the applicant must describe the intellectual property in sufficient detail and describe the limitations on its use (potential patent licenses required by the Government, data assertions of the offeror or any subcontractor, etc.) and describe how the Government can accomplish the stated objectives of this BAA with the limitations described or proposed by the offeror.

Patents. Applicants must list any known patents, patent applications, or inventions which the offeror may be required to license in order to perform the work described in the Applicant's proposal, or which the Government may be required to license to make or use the deliverables of the contract should the Applicant's proposal be selected for award. For any patent, patent application or invention listed, the Offeror must provide the invention title, a summary of the invention, patent number, patent application publication number, or provisional patent application number, and indicate whether the offeror is the patent or invention owner. If a patent or invention is in-licensed by the offeror, identify the licensor.

If any listed patent, patent application or invention is owned or licensed by the applicant, the applicant must provide a statement, in writing, confirming that it either owns or possesses the appropriate licensing rights to patent, patent application or invention to perform the work described in the proposal and/or to grant the Government a license to make or use the deliverables for this program. If any listed patent, patent application or invention is not owned or licensed by the applicant, then the applicant must explain how it will obtain a license, how the Government may obtain a license and/or whether the offeror plans to obtain these rights on behalf of the Government.

Be advised that no patent, patent application, or invention disclosure will be accepted if identified in the Data Rights Assertion list. **The list of patents, patent applications, and inventions of this section must be a separate list from the Data Rights Assertion list.**

Government rights in any technology that might be invented or reduced to practice under the contract are addressed in the patent rights clause to be included in the contract.

Data Rights. Applications submitted in response to this BAA shall identify, to the extent known at the time an offer is submitted to the Government, the technical , the technical data, or computer software that the Offeror, its subcontractors or suppliers, or potential subcontractors or suppliers assert should be furnished to the Government with restrictions on use, release, or disclosure, in accordance with DFARS 252.227-7017, Identification and Assertion of Use, Release or Disclosure Restrictions, and DFARS 252.227-7028, Technical Data or Computer Software Previously Delivered to the Government. The applicant's assertions, including the assertions of its subcontractors or suppliers, or potential subcontractors or suppliers, shall be submitted in the following format, dated and signed by an official authorized to contractually obligate the applicant. If the applicant will deliver all technical data and computer software to the Government without restrictions, enter "NONE" in this table under the heading "Technical Data or Computer Software to be Furnished with Restrictions."

Identification and Assertion of Restrictions on the Government's Use, Release, or Disclosure of Technical Data or Computer Software.

The applicant asserts for itself, or the persons identified below, that the Government's rights to use, release, or disclose the following technical data or computer software should be restricted:

Technical Data or Computer Software to be Furnished with Restrictions*	Basis for Assertion**	Asserted Rights Category***	Name of Person Asserting Restrictions****
(LIST)*****	(LIST)	(LIST)	(LIST)

*For technical data (other than computer software documentation) pertaining to items, components, or processes developed at private expense, identify both the deliverable technical data and each such item, component, or process. For computer software or computer software documentation identify the software or documentation.

**Generally, development at private expense, either exclusively or partially, is the only basis for asserting restrictions. For technical data, other than computer software documentation, development refers to development of the item, component, or process to which the data pertain. The Government's rights in computer software documentation generally may not be restricted. For computer software, development refers to the software. Indicate whether development was accomplished exclusively or partially at private expense. If development was not accomplished at private expense, or for computer software documentation, enter the specific basis for asserting restrictions.

***Enter asserted rights category (e.g., government purpose license rights from a prior contract, rights in SBIR data generated under another contract, limited, restricted, or government purpose rights under this or a prior contract, or specially negotiated licenses).

****Corporation, individual, or other person, as appropriate.

*****Enter “none” when all data or software will be submitted without restrictions.

Date _____
Printed Name _____
Printed Title _____
Signature _____

Applicants responding to this BAA requesting an Other Transaction or Other Transaction for Prototype shall specifically identify any asserted restrictions on the Government’s use of intellectual property contemplated under those award instruments. For this purpose, offerors must propose specific Intellectual Property terms and conditions and a data deliverable list. Further, the offerors must explain why an Other Transaction is necessary and, in particular, how the intellectual property terms and conditions proposed will meet the objectives of this BAA.

William B. Karesh, D.V.M
Executive Vice President for Health and Policy

EcoHealth Alliance
460 West 34th Street - 17th Floor
New York, NY 10001 USA

+1.212.380.4463 (direct)
+1.212.380.4465 (fax)
www.ecohealthalliance.org

President, OIE Working Group on Wildlife

Co-chair, IUCN Species Survival Commission - Wildlife Health Specialist Group

EPT Partners Liaison, USAID Emerging Pandemic Threats - PREDICT-2 Program

EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that promote conservation and prevent pandemics.

I. OVERVIEW OF THE FUNDING OPPORTUNITY

Program Announcement for the Department of Defense

Defense Health Program

Congressionally Directed Medical Research Programs

Peer Reviewed Medical Research Program

Investigator-Initiated Research Award

Announcement Type: Initial

Funding Opportunity Number: W81XWH-19-PRMRP-IIRA

Catalog of Federal Domestic Assistance Number: 12.420 Military Medical Research and Development

SUBMISSION AND REVIEW DATES AND TIMES

- **Pre-Application Submission Deadline:** 5:00 p.m. Eastern time (ET), March 14, 2019
- **Invitation to Submit an Application:** May 2019
- **Application Submission Deadline:** 11:59 p.m. ET, July 11, 2019
- **End of Application Verification Period:** 5:00 p.m. ET, July 16, 2019
- **Peer Review:** August 2019
- **Programmatic Review:** October/November 2019

This Program Announcement must be read in conjunction with the General Application Instructions, version 20181120. The General Applications Instructions document is available for downloading from the Grants.gov funding opportunity announcement by selecting the "Package" tab, clicking "Preview," and then selecting "Download Instructions."

TABLE OF CONTENTS

I. OVERVIEW OF THE FUNDING OPPORTUNITY 1
II. DETAILED INFORMATION ABOUT THE FUNDING OPPORTUNITY 3
II.A. Program Description 3
II.A.1. FY19 PRMRP Topic Areas 3
II.B. Award Information 5
II.C. Eligibility Information 9
II.C.1. Eligible Applicants 9
II.C.2. Cost Sharing 10
II.C.3. Other 10
II.D. Application and Submission Information 10
II.D.1. Address to Request Application Package 11
II.D.2. Content and Form of the Application Submission 11
II.D.3. Dun and Bradstreet Data Universal Numbering System (DUNS) Number and System for Award Management (SAM) 28
II.D.4. Submission Dates and Times 28
II.D.5. Funding Restrictions 29
II.D.6. Other Submission Requirements 31
II.E. Application Review Information 31
II.E.1. Criteria 31
II.E.2. Application Review and Selection Process 33
II.E.3. Integrity and Performance Information 34
II.E.4. Anticipated Announcement and Federal Award Dates 34
II.F. Federal Award Administration Information 34
II.F.1. Federal Award Notices 34
II.F.2. Administrative and National Policy Requirements 35
II.F.3. Reporting 35
II.G. Federal Awarding Agency Contacts 36
II.G.1. CDMRP Help Desk 36
II.G.2. Grants.gov Contact Center 36
II.H. Other Information 37
II.H.1. Program Announcement and General Application Instructions Versions 37
II.H.2. Administrative Actions 37
II.H.3. Application Submission Checklist 40
APPENDIX 1: ACRONYM LIST 42
APPENDIX 2: AREAS OF ENCOURAGEMENT 43
APPENDIX 3: DOD AND VA WEBSITES 62

II. DETAILED INFORMATION ABOUT THE FUNDING OPPORTUNITY

II.A. Program Description

Applications to the Fiscal Year 2019 (FY19) Peer Reviewed Medical Research Program (PRMRP) are being solicited for the Defense Health Agency (DHA) J9, Research and Development Directorate, by the U.S. Army Medical Research Acquisition Activity (USAMRAA) using delegated authority provided by United States Code, Title 10, Section 2358 (10 USC 2358). As directed by the Office of the Assistant Secretary of Defense for Health Affairs (OASD[HA]), the DHA manages the Defense Health Program (DHP) Research, Development, Test, and Evaluation (RDT&E) appropriation. The execution management agent for this Program Announcement is the Congressionally Directed Medical Research Programs (CDMRP). The PRMRP was initiated in 1999 to provide support for military health-related research of exceptional scientific merit. Appropriations for the PRMRP from FY99 through FY18 totaled $2.0 billion (B). The FY19 appropriation is $350 million (M).

The vision of the FY19 PRMRP is to improve the health, care, and well-being of all military Service members, Veterans, and beneficiaries. The PRMRP challenges the scientific and clinical communities to address at least one of the FY19 PRMRP Topic Areas with original ideas that foster new directions along the entire spectrum of research and clinical care. The program seeks applications in laboratory, clinical, behavioral, epidemiologic, and other areas of research to advance knowledge in disease etiology, improve prevention, detection, diagnosis, treatment, and quality of life for those affected by a relevant disease or condition, and to develop and validate clinical care or public health guidelines.

II.A.1. FY19 PRMRP Topic Areas

All applications for PRMRP funding must specifically address at least one of the Topic Areas as directed by Congress and must be of clear scientific merit and direct relevance to military health. If the proposed research does not specifically address at least one of the FY19 PRMRP Topic Areas, the Government will administratively withdraw the application. The Government reserves the right to reassign the application's Topic Area if submitted under an inappropriate Topic Area. The FY19 PRMRP Topic Areas are listed below.

- Acute Lung Injury
- Antimicrobial Resistance
- Arthritis
- Burn Pit Exposure
- Cardiomyopathy
- Cerebellar Ataxia
- Chronic Migraine and Post-Traumatic Headache
- Congenital Heart Disease
- Constrictive Bronchiolitis
- Diabetes
- Dystonia
- Eating Disorders

- Emerging Infectious Diseases
- Epidermolysis Bullosa
- Focal Segmental Glomerulosclerosis
- Frontotemporal Degeneration
- Guillain-Barré Syndrome
- Hemorrhage Control
- Hepatitis B
- Hereditary Angioedema
- Hydrocephalus
- Immunomonitoring of Intestinal Transplants
- Inflammatory Bowel Diseases
- Interstitial Cystitis
- Lung Injury
- Metals Toxicology
- Mitochondrial Disease
- Musculoskeletal Disorders
- Myotonic Dystrophy
- Nanomaterials for Bone Regeneration
- Nutrition Optimization
- Pancreatitis
- Pathogen-Inactivated Blood Products
- Polycystic Kidney Disease
- Post-Traumatic Osteoarthritis
- Pressure Ulcers
- Pulmonary Fibrosis
- Resilience Training
- Respiratory Health
- Rett Syndrome
- Rheumatoid Arthritis
- Scleroderma
- Sleep Disorders
- Spinal Muscular Atrophy
- Tinnitus
- Tissue Regeneration
- Tuberculosis
- Vascular Malformations
- Women's Heart Disease

Applicants should select the FY19 Program Announcement most appropriate to the stage of the proposed research. Areas of Encouragement related to the FY19 PRMRP Topic Areas have been identified by the Department of Defense (DoD), the Department of Veterans Affairs (VA), and other relevant stakeholders (Appendix 2). Applicants are urged to read and consider these Areas of Encouragement before preparing their applications. ***The information provided is not exhaustive, and applicants are not restricted to submitting applications that address an Area of Encouragement on this list.***

II.B. Award Information

The PRMRP Investigator-Initiated Research Award (IIRA) is intended to support studies that will make an important contribution toward research and/or patient care for a disease or condition related to at least one of the FY19 PRMRP Topic Areas.

The rationale for a research idea may be derived from a laboratory discovery, population-based studies, a clinician's first-hand knowledge of patients, or anecdotal data. Applications must include relevant data that support the rationale for the proposed study. These data may be unpublished or from the published literature.

Impact: The Investigator-Initiated Research Award is designed to support research with the potential to yield highly impactful data that could lead to critical discoveries or major advancements. The application must clearly demonstrate the project's potential immediate and long-range outcome(s)/product(s) (knowledge and/or materiel) and how they will impact a central critical problem or question in the field of research and/or patient care in the FY19 PRMRP Topic Area(s) addressed.

Research projects may focus on any phase of research from basic laboratory research through translational research, including preclinical studies in animal models and human subjects, as well as correlative studies associated with an existing clinical trial. Research involving human subjects and human anatomical substances is permitted; ***however, this award may not be used to conduct clinical trials.*** A clinical trial is defined as a prospective accrual of patients (human subjects) in whom an intervention (e.g., device, drug, biologic, surgical procedure, rehabilitative modality, behavioral intervention, or other) is tested for a measurable outcome with respect to safety, effectiveness, and/or efficacy. This outcome represents a direct effect on the subject of that intervention or interaction. For more information on how to distinguish clinical research from clinical trials, see the Human Subject Resource Document at https://ebrap.org/eBRAP/public/Program.htm. Principal Investigators (PIs) seeking funding for a clinical trial should apply to the FY19 PRMRP Clinical Trial Award mechanism (Funding Opportunity Number: W81XWH-19-PRMRP-CTA).

The anticipated direct costs budgeted for the entire period of performance for a single PI FY19 PRMRP IIRA award will not exceed **$1.2M**. The anticipated direct costs budgeted for the entire period of performance for an FY19 PRMRP IIRA award with the Partnering PI Option will not exceed **$1.5M**. Refer to Section II.D.5, Funding Restrictions, for detailed funding information.

The CDMRP expects to allot approximately $79.6M to fund approximately 42 Investigator-Initiated Research Award applications. Funding of applications received is contingent upon the availability of Federal funds for this program as well as the number of applications received, the quality and merit of the applications as evaluated by scientific and programmatic review, and the requirements of the Government. Funds to be obligated on any award resulting from this funding opportunity will be available for use for a limited time period based on the fiscal year of the funds. It is anticipated that awards made from this FY19 funding opportunity will be funded with FY19 funds, which will expire for use on September 30, 2025.

Awards will be made no later than September 30, 2020. For additional information, refer to Section II.F.1, Federal Award Notices.

The types of awards made under the Program Announcement will be assistance agreements (grants or cooperative agreements). The level of involvement on the part of the DoD during project performance is the key factor in determining whether to award a grant or cooperative agreement.

An assistance agreement (grant or cooperative agreement) is appropriate when the Federal Government transfers a "thing of value" to a "state, local government," or "other recipient" to carry out a public purpose of support or stimulation authorized by a law of the United States, instead of acquiring property or service for the direct benefit and use of the U.S. Government. An assistance agreement can take the form of a grant or cooperative agreement. If "no substantial involvement" on the part of the funding agency is anticipated, a grant award will be made (31 USC 6304). Conversely, if substantial involvement on the part of the funding agency is anticipated, a cooperative agreement will be made (31 USC 6305) and the award will identify the specific substantial involvement. Substantial involvement may include collaboration, participation, or intervention in the research to be performed under the award. The award type, along with the start date, will be determined during the negotiation process.

Partnering PI Option: As a method to bring new perspectives in addressing the critical problem or question and facilitate progress in the field through collaborative efforts, the FY19 PRMRP is offering a Partnering PI Option for this award mechanism. The results of this partnering project should significantly advance the research beyond what would be possible through individual efforts. The Partnering PI Option is structured so that two investigators, each of whom will be designated as a PI, work synergistically on a single project. Each PI should bring complementary skills and perspectives to the research project. Developing the research plan should involve a reciprocal flow of ideas and information between the partners. The application should clearly demonstrate that both PIs have equal intellectual input into the design of the project and will devote equivalent levels of effort to the conduct of the project. Each PI must demonstrate that they possess the research experience and resources to function as a PI and must also exhibit an appropriate level of authority and responsibility to direct the project supported by the awards. ***New and multi-institutional collaborative efforts are strongly encouraged.*** PIs should include plans for communication between investigators at different organizations, if applicable. Additionally, participating organizations must be willing to resolve potential intellectual and material property issues and to remove any barriers that might interfere with achieving high levels of cooperation to ensure successful completion of the proposed research project.

For the application process, one PI will be identified as the Initiating PI and will be responsible for the majority of the administrative tasks associated with application submission. The other PI will be identified as the Partnering PI. Initiating and Partnering PIs each have different submission requirements, as described in Section II.D.2, Content and Form of the Application Submission; however, both PIs should contribute significantly to the development of the proposed research project, including the Project Narrative, Statement of Work, and other required components. If recommended for funding, each PI will receive their own award. ***A separate application submission is required for each partner, even if both PIs are at the same***

organization. Additional collaborators may be included in the application without being designated as PIs.

Applications submitted under the Partnering PI Option are allowed to request a higher direct cost budget than applications submitted with a single PI (see Section II.D.5, Funding Restrictions, for details). Costs should be divided as evenly as possible between both partners unless appropriately justified, but no more than 75% (or no less than 25%) of the proposed direct costs may be requested by a single partner. Submission of a Partnership Statement, Attachment 8, is required for applications submitted under the Partnering PI Option.

Relevance to Military Health: Relevance to the healthcare needs of military Service members, Veterans, military beneficiaries, and/or the American public is a key feature of this award. Investigators are encouraged to consider the following characteristics as examples of how a project may demonstrate relevance to military health:

- Explanation of how the project addresses an aspect of the target disease/condition/technology that has direct relevance or is unique to the health of military Service members, Veterans, or beneficiaries

- Explanation of how the project addresses an aspect of the target disease/condition/technology that has relevance or is unique to the military or family readiness of Service members

- Description of how the knowledge, information, products, or technologies gained from the proposed research could be implemented in a dual-use capacity to benefit the civilian population and also address a military need

- Use of military or Veteran populations or datasets, if appropriate to the proposed research

PIs are encouraged to integrate and/or align their research projects with DoD and/or VA research laboratories and programs. Collaboration with DoD or VA investigators is also encouraged. A list of websites that may be useful in identifying additional information about ongoing DoD and VA areas of research interest or potential opportunities for collaboration within the FY19 PRMRP Topic Areas can be found in Appendix 2.

Use of DoD or VA Resources: If the proposed research involves access to active duty military patient populations and/or DoD resources or databases, the application must describe the access at the time of submission and include a plan for maintaining access as needed throughout the proposed research. Access to target active duty military patient population(s) and/or DoD resource(s) or database(s) should be confirmed by including a letter of support, signed by the lowest-ranking person with approval authority.

If the proposed research involves access to VA patient populations, VA study resources and databases, and/or VA research space and equipment, VA PIs/Co-PIs must have a plan for obtaining and maintaining access throughout the proposed research. Access to VA patients, resources, and/or VA research space should be confirmed by including a letter of support from the VA Facility Director(s) or individual designated by the VA Facility Director(s), such as the Associate Chief of Staff for Research and Development (ACOS/R&D) or Clinical Service Chief. If appropriate, the application should identify the VA-affiliated non-profit corporation (NPC) as

the applicant institution for VA PIs. If the VA NPC is not identified as the applicant institution for administering the funds, the application should include a letter from the VA ACOS/R&D confirming this arrangement and identifying the institution that will administer the funds associated with the proposed research.

Access to certain DoD or VA patient populations, resources, or databases may only be obtained by collaboration with a DoD or VA investigator who has a substantial role in the research and may not be available to a non-DoD or non-VA investigator if the resource is restricted to DoD or VA personnel. Investigators should be aware of which resources are available to them if the proposed research involves a non-DoD or non-VA investigator collaborating with the DoD and/or VA. If access cannot be confirmed at the time of application submission, the Government reserves the right to withdraw or revoke funding until the PI has demonstrated support for and access to the relevant population(s) and/or resource(s). Refer to Section II.D.2.b.ii, Full Application Submission Components, for detailed information.

Research Involving Human Anatomical Substances, Human Subjects, or Human Cadavers: All DoD-funded research involving new and ongoing research with human anatomical substances, human subjects, or human cadavers must be reviewed and approved by the U.S. Army Medical Research and Materiel Command (USAMRMC) Office of Research Protections (ORP), Human Research Protection Office (HRPO), prior to research implementation. This administrative review requirement is in addition to the local Institutional Review Board (IRB) or Ethics Committee (EC) review. Local IRB/EC approval at the time of submission is ***not*** required. The HRPO is mandated to comply with specific laws and requirements governing all research involving human anatomical substances, human subjects, or human cadavers that is supported by the DoD. These laws and requirements will necessitate information in addition to that supplied to the IRB/EC. ***Allow a minimum of 2 to 3 months for HRPO regulatory review and approval processes***. Additional time for regulatory reviews may be needed for clinical studies taking place in international settings. When possible, protocols should be written for research with human subjects and/or human anatomical substances that are specific to the DoD-supported effort outlined in the submitted application as a stand-alone study. Submission to HRPO of protocols involving more than the scope of work in the DoD-funded award will require HRPO review of the entire protocol (DoD and non-DoD funded). DoD human subjects protection requirements may be applied to non-DoD funded work and necessitate extensive revisions to the protocol. Applications that involve recruitment of human subjects must indicate the quarterly enrollment targets across all sites in Attachment 5: Statement of Work (SOW). Successful applicants will work with USAMRAA to establish milestones for human subjects recruitment. Continued support for the project will be based upon satisfactory progress in meeting the established milestones. Refer to the General Application Instructions, Appendix 1, and the Human Subject Resource Document available on the electronic Biomedical Research Application Portal (eBRAP) "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) for additional information.

Research Involving Animals: All projects should adhere to a core set of standards for rigorous study design and reporting to maximize the reproducibility and translational potential of preclinical research. The standards are described in Landis, S.C., et al. A call for transparent reporting to optimize the predictive value of preclinical research, *Nature* 2012, 490:187-191 (www.nature.com/nature/journal/v490/n7419/full/nature11556.html). While these standards are

written for preclinical studies, the basic principles of randomization, blinding, sample-size estimation, and data handling derive from well-established best practices in clinical studies. Applicants should consult the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines to ensure relevant aspects of rigorous animal research are adequately planned for and, ultimately, reported. The ARRIVE guidelines can be found at http://www.elsevier.com/__data/promis_misc/622936arrive_guidelines.pdf.

All DoD-funded research involving new and ongoing research with animals must be reviewed and approved by the USAMRMC ORP Animal Care and Use Review Office (ACURO), in addition to the local Institutional Animal Care and Use Committee (IACUC) of record. IACUC approval at the time of submission is ***not*** required. Specific documents relating to the use of animals in the proposed research will be requested **if the application is selected for funding**. The ACURO must review and approve all animal use prior to the start of working with animals, including amendments to ongoing projects. PIs must submit the institutional animal use protocol, IACUC approval of that protocol, and a version of the animal use appendix titled, "Research Involving Animals." ***Allow at least 2 to 3 months for ACURO regulatory review and approval processes for animal studies.*** Refer to the General Application Instructions, Appendix 1, for additional information.

The CDMRP intends that information, data, and research resources generated under awards funded by this Program Announcement be made available to the research community (which includes both scientific and consumer advocacy communities) and to the public at large. For additional guidance, refer to the General Application Instructions, Appendix 2, Section K.

II.C. Eligibility Information

II.C.1. Eligible Applicants

II.C.1.a. Organization: All organizations, including international organizations, are eligible to apply.

Government Agencies Within the United States: Local, state, and Federal Government agencies are eligible to the extent that applications do not overlap with their fully funded internal programs. Such agencies are required to explain how their applications do not overlap with their internal programs.

As applications for this Program Announcement may be submitted by extramural and intramural organizations, these terms are defined below.

Extramural Organization: An eligible non-DoD organization. Examples of extramural organizations include academic institutions, biotechnology companies, foundations, other Federal Government organization other than the DoD, and research institutes.

Intramural DoD Organization: A DoD laboratory, DoD military treatment facility, and/or DoD activity embedded within a civilian medical center.

Note: Applications from an intramural DoD organization or from an extramural Federal Government organization may be submitted to Grants.gov through a research foundation.

The USAMRAA makes awards to eligible organizations, not to individuals.

II.C.1.b. Principal Investigator

- Investigators at or above the level of Assistant Professor (or equivalent) may be named by the organization as the PI on the application.
    - **Partnering PI Option:** Investigators at or above the level of Assistant Professor (or equivalent) that bring complementary skills and perspectives to the research project.

An eligible PI, regardless of ethnicity, nationality, or citizenship status, must be employed by, or affiliated with, an eligible organization.

The CDMRP encourages all PIs to participate in a digital identifier initiative through Open Researcher and Contributor ID, Inc. (ORCID). Registration for a unique ORCID identifier can be done online at https://orcid.org/.

II.C.2. Cost Sharing

Cost sharing/matching is not an eligibility requirement.

II.C.3. Other

Organizations must be able to access **.gov** and **.mil** websites in order to fulfill the financial and technical deliverable requirements of the award and submit invoices for payment.

There are no limitations on the number of applications for which an investigator may be named as a PI or Partnering PI.

For general information on required qualifications for award recipients, refer to the General Application Instructions, Appendix 3.

Refer to Section II.H.2, Administrative Actions, for a list of administrative actions that may be taken if a pre-application or application does not meet the administrative, eligibility, or ethical requirements defined in this Program Announcement.

II.D. Application and Submission Information

Submission of applications that are essentially identical or propose essentially the same research project to different funding opportunities within the FY19 PRMRP is prohibited and will result in administrative withdrawal of the duplicative application(s). As an exception, applicants may submit the research project described in their Investigator-Initiated Research Award application as part of an application to the FY19 PRMRP Focused Program Award (Funding Opportunity Number: W81XWH-19-PRMRP-FPA); however, accepting multiple awards to support the same project will not be allowed.

Extramural Submission: An application submitted by an organization to Grants.gov.

Intramural DoD Submission: An application submitted by a DoD organization to eBRAP.

II.D.1. Address to Request Application Package

eBRAP is a multifunctional web-based system that allows PIs to submit their pre-applications electronically through a secure connection, to view and edit the content of their pre-applications and full applications, to receive communications from the CDMRP, and to submit documentation during award negotiations and period of performance.

Extramural Submissions:

- Pre-application content and forms must be accessed and submitted at eBRAP.org.
- Full application packages must be accessed and submitted at Grants.gov.

Intramural DoD Submissions:

- Pre-application content and forms must be accessed and submitted at eBRAP.org.
- Full application packages must be accessed and submitted at eBRAP.org.

Contact information for the CDMRP Help Desk and the Grants.gov Contact Center can be found in Section II.G, Federal Awarding Agency Contacts.

II.D.2. Content and Form of the Application Submission

Submission is a two-step process requiring both ***pre-application*** and ***full application*** as indicated below. The submission process should be started early to avoid missing deadlines. There are no grace periods.

Pre-Application Submission: All pre-applications for both extramural and intramural organizations must be submitted through eBRAP (https://eBRAP.org/).

Full Application Submission: Full applications must be submitted through the online portals as described below.

Extramural Organization Submissions: Full applications from extramural organizations must be submitted through Grants.gov Workspace. Applications submitted by extramural organizations (e.g., research foundations) on behalf of intramural DoD or other Federal organizations or investigators will be considered extramural submissions. Applications from extramural organizations, including non-DoD Federal organizations, received through eBRAP will be withdrawn. See definitions in Section II.C.1, Eligible Applicants.

Intramural DoD Organization Submissions: Intramural DoD organizations may submit full applications to either eBRAP or Grants.gov. Intramural DoD organizations that are unable to submit to Grants.gov should submit through eBRAP. Intramural DoD organizations with the

capability to submit through Grants.gov may submit following the instructions for extramural submissions through Grants.gov or may submit to eBRAP.

For Both Extramural and Intramural Applicants: eBRAP allows an organization's representatives and PIs to view and modify the full application submissions associated with them. eBRAP will validate full application files against the specific Program Announcement requirements, and discrepancies will be noted in an email to the PI and in the "Full Application Files" tab in eBRAP. It is the applicant's responsibility to review all application components for accuracy as well as ensure proper ordering as specified in this Program Announcement.

The application title, eBRAP log number, and all information for the PI, Business Official(s), performing organization, and contracting organization must be consistent throughout the entire pre-application and full application submission process. Inconsistencies may delay application processing and limit or negate the ability to view, modify, and verify the application in eBRAP. If any changes need to be made, the applicant should contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507 prior to the application submission deadline.

The Investigator-Initiated Research Award mechanism is structured to accommodate two PIs under the Partnering PI Option. One PI will be identified as the Initiating PI and will be responsible for the majority of the administrative tasks associated with application submission. The other PI will be identified as the Partnering PI. Initiating and Partnering PIs each have different submission requirements; however, both PIs should contribute significantly to the development of the proposed research project, including the Project Narrative, Statement of Work, and other required components.

The Initiating PI must complete the pre-application submission process and submit the contact information for the Partnering PI. The Partnering PI will then be notified of the pre-application submission separately by email. ***The Partnering PI must follow the link in the notification email in order to associate his/her full application package with that of the Initiating PI. After following the link, the Partnering PI must verify his/her contact information, organization, and designation as an extramural or intramural submission within eBRAP.*** If not previously registered, the Partnering PI must register in eBRAP. A new pre-application based on this research project should not be initiated by the Partnering PI. Do not delay completing these steps. If they are not completed, the Partnering PI will not be able to view and modify his/her application during the verification period in eBRAP. If these steps are not completed, an intramural partner will not be able to submit the Partnering PI required full application package components to eBRAP.

II.D.2.a. Step 1: Pre-Application Submission Content

During the pre-application process, eBRAP assigns each submission a unique log number. This unique eBRAP log number is required during the full application submission process.

To begin the pre-application process, first select whether the submitting organization is extramural or intramural, then confirm your selection or cancel. **Incorrect selection of extramural or intramural submission type will delay processing.**

If an error has been made in the selection of extramural versus intramural and the pre-application submission deadline has passed, the PI or Business Official must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507 to request a change in designation.

All pre-application components must be submitted by the PI (for single PI applicants) or Initiating PI (for applicants submitting under the Partnering PI Option) through eBRAP (https://eBRAP.org/). Because the invitation to submit an application is based on the contents of the pre-application, investigators should not change the title or research objectives after the pre-application is submitted.

The applicant organization and associated PIs identified in the pre-application should be the same as those intended for the subsequent application submission. If any changes are necessary after submission of the pre-application, the PI must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

PIs with an ORCID identifier should enter that information in the appropriate field in the "My Profile" tab in the "Account Information" section of eBRAP.

The pre-application consists of the following components, which are organized in eBRAP by separate tabs (refer to the General Application Instructions, Section II.B, for additional information on pre-application submission):

- **Tab 1 – Application Information**

  Submission of application information includes assignment of primary and secondary research classification codes, which may be found at https://ebrap.org/eBRAP/public/Program.htm. Note that the codes have recently been revised. Applicants are strongly encouraged to review and confirm the codes prior to making their selection.

  Select the FY19 PRMRP Topic Area addressed by the proposed research. If the proposed research project is aligned with more than one FY19 PRMRP Topic Area, select the Topic Area of highest relevance as the required first choice.

- **Tab 2 – Application Contacts**

  Enter contact information for the PI. Enter the organization's Business Official responsible for sponsored program administration (the "person to be contacted on matters involving this application" in Block 5 of the Grants.gov SF424 Research & Related Form). The Business Official must be either selected from the eBRAP list or invited in order for the pre-application to be submitted.

  Select the performing organization (site at which the PI will perform the proposed work) and the contracting organization (organization submitting on behalf of the PI, which corresponds to Block 5 on the Grants.gov SF424 Research & Related Form), and click on "Add Organizations to this Pre-application." The organization(s) must be either selected from the eBRAP drop-down list or invited in order for the pre-application to be submitted.

It is recommended that PIs identify an Alternate Submitter in the event that assistance with pre-application submission is needed.

- **Tab 3 – Collaborators and Key Personnel**

  Enter the name, organization, and role of all collaborators and key personnel associated with the application. The Initiating PI must enter the contact information for the Partnering PI in the Partnering PI section.

  FY19 PRMRP Programmatic Panel members should not be involved in any pre-application or application. For questions related to panel members and pre-applications or applications, refer to Section II.H.2.c, Withdrawal, or contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

- **Tab 4 – Conflicts of Interest**

  List all individuals other than collaborators and key personnel who may have a COI in the review of the application (including those with whom the PI has a personal or professional relationship).

- **Tab 5 – Pre-Application Files**

  Note: Upload documents as individual PDF files unless otherwise noted. eBRAP will not allow a file to be uploaded if the number of pages exceeds the limit specified below.

  - **Preproposal Narrative (four-page limit):** The Preproposal Narrative page limit applies to text and non-text elements (e.g., figures, tables, graphs, photographs, diagrams, chemical structures, drawings) used to describe the project. Inclusion of URLs that provide additional information to expand the Preproposal Narrative and could confer an unfair competitive advantage is prohibited and may result in administrative withdrawal of the pre-application.

    The Preproposal Narrative should include the following:

    - **Topic Area:** Describe how the proposed project relates to at least one of the FY19 PRMRP Topic Areas. If applicable, describe how the proposed research project addresses an FY19 PRMRP Area of Encouragement (Appendix 2).

    - **Research Idea:** Clearly articulate the rationale for the project by presenting the ideas and reasoning behind the proposed research; include relevant literature citations.

    - **Research Strategy:** State the hypothesis to be tested and the objective(s) to be reached. State the project's specific aims and briefly describe the experimental approach. If applying under the Partnering PI Option, describe how the collaborative effort will provide synergy and enhance the research.

- **Impact:** Describe the potential short-term and long-term impact of the results of the proposed study on at least one of the FY19 PRMRP Topic Areas, and its related research field(s) and/or patient population(s).

- **Relevance to Military Health:** Explain how the project is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

  - **Pre-Application Supporting Documentation:** The items to be included as supporting documentation for the pre-application ***must be uploaded as individual files*** and are limited to the following:

    - References Cited (one-page limit): List the references cited (including URLs if available) in the Preproposal Narrative using a standard reference format that includes the full citation (i.e., author[s], year published, reference title, and reference source, including volume, chapter, page numbers, and publisher, as appropriate).

    - List of Abbreviations, Acronyms, and Symbols: Provide a list of abbreviations, acronyms, and symbols used in the Preproposal Narrative.

    - Key Personnel Biographical Sketches (five-page limit per individual): ***All biographical sketches should be uploaded as a single combined file.*** Biographical sketches should be used to demonstrate background and expertise through education, positions, publications, and previous work accomplished.

- **Tab 6 – Submit Pre-Application**

  This tab must be completed for the pre-application to be accepted and processed.

Pre-Application Screening

- **Pre-Application Screening Criteria**

  To determine the technical merits of the pre-application and the relevance to the mission of the DHP and the PRMRP, pre-applications will be screened based on the following criteria:

  - **Research Idea:** How well the rationale for the research idea is supported by presented background information.

  - **Research Strategy:** How well the specific aims and proposed methodology support the research idea and objectives. If applying under the Partnering PI Option, how the partnership will advance the research beyond what would be possible through individual efforts.

  - **Impact:** Whether the potential immediate and long-range outcome(s)/product(s) (knowledge and/or materiel) of the proposed research, if successful, will impact a central critical problem or question in the field of research and/or patient care in the FY19 PRMRP Topic Area(s) addressed.

- **Programmatic Relevance:** Whether the proposed research idea supports the objectives of the PRMRP. To what degree the project is relevant to the healthcare needs of military Service members, Veterans, and/or beneficiaries.

- **Notification of Pre-Application Screening Results**

  Following the pre-application screening, PIs (or Initiating PIs, if applying under the Partnering PI Option) will be notified as to whether or not they are invited to submit applications; however, they will not receive feedback (e.g., a critique of strengths and weaknesses) on their pre-application. The estimated timeframe for notification of invitation to submit an application is indicated in Section I, Overview of the Funding Opportunity. Invitations to submit a full application are based on the Pre-Application Screening Criteria listed above.

II.D.2.b. Step 2: Full Application Submission Content

Applications will not be accepted unless notification of invitation has been received by the PI or Initiating PI.

The CDMRP cannot make allowances/exceptions to its policies for submission problems encountered by the applicant organization using system-to-system interfaces with Grants.gov.

Each application submission must include the completed full application package for this Program Announcement. The full application package is submitted by the Authorized Organizational Representative through Grants.gov (https://www.grants.gov/) for extramural organizations or through eBRAP (https://ebrap.org/) for intramural organizations. See Table 1 below for more specific guidelines.

II.D.2.b.i. Full Application Guidelines

Extramural organizations must submit full applications through Grants.gov. Applicants must create a Grants.gov Workspace for submission, which allows the application components to be completed online and routed through the applicant organization for review prior to submission. Applicants may choose to download and save individual PDF forms rather than filling out webforms in Workspace. A compatible version of Adobe Reader **must** be used to view, complete, and submit an application package consisting of PDF forms. If more than one person is entering text into an application package, the ***same version*** of Adobe Reader software should be used by each person. Check the version number of the Adobe software on each user's computer to make sure the versions match. Using different versions of Adobe Reader may cause submission and/or save errors – even if each version is individually compatible with Grants.gov. Refer to the General Application Instructions, Section III, and the "Apply For Grants" page of Grants.gov (https://www.grants.gov/web/grants/applicants/apply-for-grants.html) for further information about the Grants.gov Workspace submission process. Submissions of extramural applications through eBRAP may be withdrawn.

Table 1. Full Application Submission Guidelines

Extramural Submissions	Intramural DoD Submissions
Application Package Location	
Download application package components for W81XWH-19-PRMRP-IIRA from Grants.gov (https://www.grants.gov) and create a Grants.gov Workspace. Workspace allows online completion of the application components and routing of the application package through the applicant organization for review prior to submission.	Download application package components for W81XWH-19-PRMRP-IIRA from eBRAP (https://ebrap.org).
Full Application Package Components	
SF424 Research & Related Application for Federal Assistance Form: Refer to the General Application Instructions, Section III.A.1, for detailed information.	**Tab 1 – Summary:** Provide a summary of the application information. **Tab 2 – Application Contacts:** This tab will be pre-populated by eBRAP; add Authorized Organizational Representative.
Descriptions of each required file can be found under Full Application Submission Components: • Attachments • Research & Related Personal Data • Research & Related Senior/Key Person Profile (Expanded) • Research & Related Budget • Project/Performance Site Location(s) Form • Research & Related Subaward Budget Attachment(s) Form (if applicable)	**Tab 3 – Full Application Files:** Upload files under each Application Component in eBRAP. Descriptions of each required file can be found under Full Application Submission Components: • Attachments • Key Personnel • Budget • Performance Sites **Tab 4 – Application and Budget Data:** Review and edit proposed project start date, proposed end date, and budget data pre-populated from the Budget Form.
Application Package Submission	
Create a Grants.gov Workspace. Add participants (investigators and Business Officials) to Workspace, complete all required forms, and check for errors before submission. **Submit a Grants.gov Workspace Package.** An application may be submitted through Workspace by clicking the "Sign and Submit" button on the "Manage Workspace" page, under the "Forms" tab. Grants.gov recommends	**Submit package components to eBRAP (https://ebrap.org).** **Tab 5 – Submit/Request Approval Full Application:** After all components are uploaded and prior to the full application submission deadline, enter your password in the space provided next to "Enter Your Password Here" and press the "Submit Full Application" button. eBRAP will notify your

Extramural Submissions	Intramural DoD Submissions
submission of the application package **at least 24-48 hours prior to the close date** to allow time to correct any potential technical issues that may disrupt the application submission. **Note:** If either the Project Narrative or the budget fails eBRAP validation or if the Project Narrative or the budget needs to be modified, an updated Grants.gov application package must be submitted via Grants.gov as a "Changed/Corrected Application" with the previous Grants.gov Tracking ID ***prior to*** the application submission deadline.	Resource Manager/Comptroller/Task Area Manager or equivalent Business Official by email.
Application Verification Period	
The full application package submitted to Grants.gov may be viewed and modified in eBRAP until the end of the application verification period. During the application verification period, the full application package may be modified ***with the exception of the Project Narrative and Research & Related Budget Form***.	After eBRAP has processed the full application, the organizational Resource Manager/Comptroller/Task Area Manager or equivalent Business Official and PI will receive email notification of this status and will be able to view and modify application components in eBRAP. During the application verification period, the full application package may be modified ***with the exception of the Project Narrative and Research & Related Budget Form***. Your Resource Manager/Comptroller/Task Area Manager or equivalent Business Official should log into eBRAP to review and to approve prior to the application verification deadline.
Further Information	
Tracking a Grants.gov Workspace Package. After successfully submitting a Workspace package, a Grants.gov Tracking Number is automatically assigned to the package. The number will be listed on the "Confirmation" page that is generated after submission. Refer to the General Application Instructions, Section III, for further information regarding Grants.gov requirements.	Refer to the General Application Instructions, Section IV, for further information regarding eBRAP requirements.

Both Extramural and Intramural Organizations: Application viewing, modification, and verification in eBRAP are strongly recommended, but not required. ***The Project Narrative and***

Research & Related Budget form cannot be changed after the application submission deadline. Prior to the full application deadline, a corrected or modified full application package may be submitted. Other application components may be changed until the end of the application verification period. Verify that subaward budget(s) and budget justification forms are present in eBRAP during the application verification period. If these components are missing, upload them to eBRAP before the end of the application verification period. After the end of the application verification period, the full application cannot be modified.

Partnering PI Option: The CDMRP requires separate full application package submissions for the Initiating PI and the Partnering PI, even if the PIs are located within the same organization. Initiating and Partnering PIs will each be assigned a unique eBRAP log number. Each full application package must be submitted using the unique eBRAP log number. ***Note: All associated applications (Initiating PI's and the Partnering PI's) must be submitted by the full application submission deadline.***

The full application package must be submitted using the unique eBRAP log number to avoid delays in application processing.

II.D.2.b.ii. Full Application Submission Components

- **Extramural Applications Only**

  SF424 Research & Related Application for Federal Assistance Form: Refer to the General Application Instructions, Section III.A.1, for detailed information.

- **Extramural and Intramural Applications**

  Attachments:

  Each attachment to the full application components must be uploaded as an individual file in the format specified and in accordance with the formatting guidelines listed in the General Application Instructions, Appendix 4.

  For all attachments, ensure that the file names are consistent with the guidance. Attachments will be rejected if the file names are longer than 50 characters or have incorrect file names that contain characters other than the following: A-Z, a-z, 0-9, underscore, hyphen, space, and period. In addition, there are file size limits that may apply in some circumstances. Individual attachments may not exceed 20 MB, and the file size for the entire full application package may not exceed 200 MB.

  - **Attachment 1: Project Narrative (15-page limit): Upload as "ProjectNarrative.pdf".** The page limit of the Project Narrative applies to text and non-text elements (e.g., figures, tables, graphs, photographs, diagrams, chemical structures, drawings) used to describe the project. Inclusion of URLs that provide additional information to expand the Project Narrative and could confer an unfair competitive advantage is prohibited and may result in administrative withdrawal of the application.

Describe the proposed project in detail using the outline below.

- **Background:** Describe how the proposed research project addresses one or more of the FY19 PRMRP Topic Areas. Present the ideas and reasoning behind the proposed work. Cite relevant literature. Describe previous experience most pertinent to the project. Include relevant preliminary data; these data may be unpublished or from the published literature.

- **Hypothesis:** State the hypothesis to be tested.

- **Specific Aims:** Concisely explain the project's specific aims and the objective(s) to be reached. These aims should agree with the primary aims and associated tasks described in the Statement of Work. If the proposed work is part of a larger study, present only aims that this DoD award would fund.

- **Research Strategy:** Describe the experimental design, methods, and analyses, including appropriate controls, in sufficient detail for analysis. Address potential problem areas and present alternative methods and approaches. Clearly describe the statistical plan and the rationale for the statistical methodology as well as an appropriate power analysis, if applicable. If animal studies are proposed, describe how they will be conducted in accordance with the ARRIVE guidelines (http://www.elsevier.com/__data/promis_misc/622936arrive_guidelines.pdf).

- If human subjects or human biological samples will be used, describe the study population and include a detailed plan for the recruitment of human subjects or the acquisition of samples. Describe the availability of the proposed study population and past successes in recruiting similar populations. If active duty military, military families, and/or Veteran population(s) or datasets will be used in the proposed research project, describe the feasibility of accessing the population(s)/dataset(s). ***Clinical trials are not allowed under the Investigator-Initiated Research Award.***

- If applicable, describe how data will be reported and how it will be assured that the documentation will support a regulatory filing with the U.S. Food and Drug Administration (FDA).

- **Attachment 2: Supporting Documentation: Combine and upload as a single file named "Support.pdf".** Start each document on a new page. If documents are scanned to PDF, the lowest resolution (100 to 150 dpi) should be used. The Supporting Documentation attachment should not include additional information such as figures, tables, graphs, photographs, diagrams, chemical structures, or drawings. These items should be included in the Project Narrative.

  There are no page limits for any of these components unless otherwise noted. Include only those components described below; inclusion of items not requested or viewed as an extension of the Project Narrative will result in the removal of those items or may result in administrative withdrawal of the application.

- References Cited: List the references cited (including URLs, if available) in the Project Narrative using a standard reference format that includes the full citation (i.e., author[s], year published, title of reference, source of reference, volume, chapter, page numbers, and publisher, as appropriate).

- List of Abbreviations, Acronyms, and Symbols: Provide a list of abbreviations, acronyms, and symbols.

- Facilities, Existing Equipment, and Other Resources: Describe the facilities and equipment available for performance of the proposed project and any additional facilities or equipment proposed for acquisition at no cost to the award. Indicate whether or not Government-furnished facilities or equipment are proposed for use. If so, reference should be made to the original or present Government award under which the facilities or equipment items are now accountable. There is no form for this information.

- Publications and/or Patents: Include a list of relevant publication URLs and/or patent abstracts. If articles are not publicly available, then copies of up to five published manuscripts may be included in Attachment 2. Extra items will not be reviewed.

- Letters of Organizational Support: Provide a letter (or letters, if applicable), signed by the Department Chair or appropriate organization official, confirming the laboratory space, equipment, and other resources available for the project. Letters of support not requested in the Program Announcement, such as those from members of Congress, do not impact application review or funding decisions.

- Letters of Collaboration: Provide a signed letter from each collaborating individual or organization that will demonstrate that the PI has the support or resources necessary for the proposed work. If an investigator at an intramural organization is named as a collaborator on an application submitted through an extramural organization, the application must include a letter from the collaborator's Commander or Commanding Officer at the intramural organization that authorizes the collaborator's involvement.

- Intellectual Property: Information can be found in Code of Federal Regulations, Title 2, Part 200.315 (2 CFR 200.315), "Intangible Property."

    - Intellectual and Material Property Plan (if applicable): Provide a plan for resolving intellectual and material property issues among participating organizations.

- Data and Research Resources Sharing Plan: Describe how data and resources generated during the performance of the project will be shared with the research community. Refer to the General Application Instructions, Appendix 2, Section K, for more information about the CDMRP expectations for making data and research resources publicly available.

- Use of DoD Resources (if applicable): If the proposed research plan involves access to active duty military patient populations or resources, the PI is responsible for demonstrating such access. Provide a letter of support signed by the lowest-ranking person with approval authority confirming access to active duty military populations and/or DoD resources or databases.

- Use of VA Resources (if applicable): Provide a letter of support from the VA Facility Director(s) or individual designated by the VA Facility Director(s), such as the ACOS/R&D or Clinical Service Chief confirming access to VA patients, resources, and/or VA research space. For VA PIs, if the VA NPC is not identified as the applicant institution for administering the funds, include a letter from the VA ACOS/R&D confirming this arrangement and identifying the institution that will administer the funds associated with the proposed research.

- **Attachment 3: Technical Abstract (one-page limit): Upload as "TechAbs.pdf".** The technical abstract is used by all reviewers. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information.*** Use only characters available on a standard QWERTY keyboard. Spell out all Greek letters, other non-English letters, and symbols. Graphics are not allowed.

  Programmatic reviewers typically do not have access to the full application and therefore rely on the technical abstract for appropriate description of the project's key aspects. Therefore, clarity and completeness within the space limits of the technical abstract are highly important.

  Describe the proposed research project, including the following elements: background, rationale, hypothesis, specific aims and objectives, study design, long-term and short-term impact to the relevant research field and patient population(s), and the relevance of the project to at least one FY19 PRMRP Topic Area.

- **Attachment 4: Lay Abstract (one-page limit): Upload as "LayAbs.pdf".** The lay abstract is used by all reviewers. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information***. Use only characters available on a standard QWERTY keyboard. Spell out all Greek letters, other non-English letters, and symbols. Graphics are not allowed.

  Describe how the proposed research project addresses one or more of the FY19 PRMRP Topic Areas. Include a comprehensive overview of the proposed research project that can be ***readily understood by readers without a background in science or medicine.*** Clearly describe the central critical problem or question to be addressed and the ultimate applicability and impact of the research. ***Do not duplicate the technical abstract***.

- **Attachment 5: Statement of Work (three-page limit): Upload as "SOW.pdf".** The suggested SOW format and examples specific to different types of research projects are available on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm). For the Investigator-Initiated Research

Award mechanism, use the SOW format example titled "SOW (Statement of Work) Generic Format." The SOW must be in PDF format prior to attaching.

The SOW should include a list of major tasks that support the proposed specific aims, followed by a series of subtasks outlined related to the major tasks and milestones within the period of performance. The SOW should describe only the work for which funding is being requested by this application and, as applicable, should also:

- Include the name(s) of the key personnel and contact information for each study site/ subaward site.

- Indicate the number (and type, if applicable) of research subjects (animal or human) and/or human anatomical samples projected or required for each task and at each site. Refer to the General Application Instructions, Appendix 1, for additional information regarding regulatory requirements.

- If applicable, indicate timelines required for regulatory approvals relevant to human or animal subjects research (e.g., IRB, IACUC, ORP, Investigational New Drug and Investigational Device Exemption applications) by the FDA or other Government agency.

Partnering PI Option: The Initiating and Partnering PIs must each submit an identical copy of a jointly created SOW. The contributions of the Initiating PI and the Partnering PI should be noted for each task.

- **Attachment 6: Impact Statement (one-page limit): Upload as "Impact.pdf".**

  Explain why the proposed research project is important and relevant to understanding the cause or progression of the disease or condition, or to developing improvements in prevention, detection, diagnosis, treatment, patient care, or quality of life in the FY19 PRMRP Topic Area(s) addressed. Describe how the study will address a central critical problem or question in the relevant Topic Area(s). If applicable, describe how the project addresses an FY19 PRMRP Area of Encouragement (Appendix 2).

  - ***Describe the short-term impact:*** Detail the anticipated outcome(s)/product(s) (knowledge and/or materiel) that will be directly attributed to the results of the proposed research.

  - ***Describe the long-term impact:*** Explain the anticipated long-term gains from this research. Compare to the information known/products currently available, if applicable. Explain the long-range vision for how the research will impact the field of study and/or clinical care.

- **Attachment 7: Relevance to Military Health Statement (one-page limit): Upload as "MilRel.pdf".**

  Describe how the proposed study is responsive to the healthcare needs of military Service members, Veterans, and/or beneficiaries. Provide information about the incidence and/or

prevalence of the disease or condition in the general population as well as in military Service members, Veterans, and/or beneficiaries.

If active duty military, military families, and/or Veteran population(s) or datasets will be used in the proposed research project, describe the population(s)/dataset(s) and the appropriateness of the population(s)/dataset(s) for the proposed study. If a non-military population will be used for the proposed research project, explain how the population simulates the targeted population (i.e., military Service members, Veterans, and/or beneficiaries).

If applicable, show how the proposed research project aligns with DoD and/or VA areas of research interest. Provide a description of how the knowledge, information, products, or technologies gained from the research could be implemented in a dual-use capacity to benefit the civilian population and address a military need, as appropriate

- **Attachment 8: Partnership Statement (one-page limit): Upload as "Partnership.pdf".** ***This attachment is only required for applications submitted under the Partnering PI Option.***

  Describe the expertise of the Initiating and Partnering PIs and how each will bring different strengths to the proposed project. Describe how the combined effort will be synergistic and produce an outcome greater than what could be achieved by independent efforts. Outline the contribution and time commitment of each partner and how each will have equal intellectual input on the design, conduct, and analysis of the project. Describe how the PIs will manage the collaboration and workflow to optimize research efforts.

- **Attachment 9: Representations, if applicable (extramural submissions only): Upload as "RequiredReps.pdf".** All extramural applicants must complete and submit the Required Representations template available on eBRAP (https://ebrap.org/eBRAP/public/Program.htm). For more information, see the General Application Instructions, Appendix 5, Section B, Representations.

- **Attachment 10: DoD Military Budget Form(s), if applicable: Upload as "MFBudget.pdf".** If a military facility (Military Health System facility, research laboratory, medical treatment facility, dental treatment facility, or a DoD activity embedded with a civilian medical center) will be a collaborator in performance of the project, complete the DoD Military Budget Form, available for download on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm), including a budget justification, for each military facility as instructed. The costs per year should be included on the Grants.gov Research & Related Budget form under subaward costs. Refer to the General Application Instructions, Section III.A.8, for detailed information.

- **Extramural and Intramural Applications**

  To evaluate compliance with Title IX of the Education Amendments of 1972 (20 USC A§1681 et seq.), the DoD is collecting certain demographic and career information to be able to assess the success rates of women who are proposed for key roles in applications in

science, technology, engineering, and/or mathematics (STEM) disciplines. To enable this assessment, each application must include the following forms completed as indicated.

Research & Related Personal Data: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.3, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.2, for detailed information.

Research & Related Senior/Key Person Profile (Expanded): For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.4, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.3, for detailed information.

- PI Biographical Sketch (five-page limit): Upload as "Biosketch_LastName.pdf". The suggested biographical sketch format is available on the "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) in eBRAP. The National Institutes of Health (NIH) Biographical Sketch may also be used. All biographical sketches should be submitted in uneditable PDF format.

- PI Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf".

- Key Personnel Biographical Sketches (five-page limit each): Upload as "Biosketch_LastName.pdf".

- Key Personnel Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf".

Research & Related Budget: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.5, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.4, for detailed information.

Budget Justification (no page limit): Upload as "BudgetJustification.pdf". The budget justification for the entire period of performance must be uploaded to the Research & Related Budget after completion of the budget for Period 1.

Partnering PI Option: ***Initiating and Partnering PIs must have a separate budget and justification specific to their distinct portions of the effort that the applicant organization will submit as separate Grants.gov or eBRAP application packages. The Initiating PI should not include budget information for the Partnering PI even if they are located within the same organization. The anticipated combined direct costs budgeted for the entire period of performance for the Initiating and Partnering PIs' applications will not exceed $1.5M. Refer to Section II.D.5, Funding Restrictions, for detailed information.***

Project/Performance Site Location(s) Form: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.6, and for intramural

submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.5, for detailed information.

- **Extramural Applications Only**

  Research & Related Subaward Budget Attachment(s) Form (if applicable): Refer to the General Application Instructions, Section III.A.7, for detailed information.

  - **Extramural Subaward:** Complete the Research & Related Subaward Budget Form through Grants.gov. (Refer to the General Application Instructions, Section III.A.7, for detailed information.) Verify subaward budget(s) and budget justification forms are present in eBRAP during the application verification period. If these components are missing, upload them to eBRAP before the end of the application verification period.

  - **Intramural DoD Collaborator(s):** Complete the DoD Military Budget Form and upload to Grants.gov attachment form as Attachment 10. (Refer to the General Application Instructions, Section IV.A.4, for detailed information.) Each Intramural DoD Collaborator should include costs per year on the Grants.gov Research & Related Budget form under subaward costs.

  DoD Military Budget Form: A military facility collaborating in the performance of the project (but not participating as a Partnering PI) should be treated as a subaward for budget purposes. **Note:** Applicants should complete the **DoD Military Budget Form (Attachment 10)** to show all direct and indirect costs. The costs per year should be included on the Grants.gov Research & Related Budget Form under subaward costs. Refer to the General Application Instructions, Section III.A.8, for detailed information.

Application Components for the Partnering PI, if applying under the Partnering PI Option

The Partnering PI must follow the link in the email from eBRAP and, if not registered in eBRAP, complete the registration process prior to the application submission deadline in order to associate his/her full application package with that of the Initiating PI.

For the Partnering PI, the Initiating PI must identify if the Partnering PI will be named on an extramural or intramural application (in accordance with the guidelines in Section II.C.1.a, Organization) and the appropriate mode of submission (Grants.gov for extramural and eBRAP for intramural). The Partnering PI must verify his/her contact information and mode of submission within eBRAP to ensure proper submission of his/her application.

The application submission process for the Partnering PI uses an abbreviated full application package that includes:

- **Extramural and Intramural Applications**

  Attachments:

  - **Attachment 5: Statement of Work (three-page limit): Upload as "SOW.pdf".** Refer to the General Application Instructions, Section III.A.2, for detailed information on

completing the SOW. Each PI must submit an identical copy of a jointly created SOW. The contributions of the Initiating PI and the Partnering PI should be noted for each task.

- **Attachment 9: Representations (Extramural Submissions only): Upload as "RequiredReps.pdf".** All extramural applicants must complete and submit the Required Representations template available on eBRAP (https://ebrap.org/eBRAP/public/Program.htm). For more information, see the General Application Instructions, Appendix 5, Section B, Representations.

- **Attachment 10: DoD Military Budget Form: Upload as "MFBudget.pdf".** Refer to the General Application Instructions, Section IV.A.4, for detailed information. The costs per year should be included on the Grants.gov Research & Related Budget form under subaward costs.

Research & Related Personal Data: For extramural submissions (via Grants.gov) refer to the General Application Instructions, Section III.A.3, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.2, for detailed information.

Research & Related Senior/Key Person Profile (Expanded): For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.4, and for intramural submissions (via eBRAP), refer to the General Application Instructions, Section IV.A.3, for detailed information.

- PI Biographical Sketch (five-page limit): Upload as "Biosketch_LastName.pdf". The suggested biographical sketch format is available on the "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) in eBRAP. The NIH Biographical Sketch may also be used. All biographical sketches should be submitted in the PDF format that is not editable.

- PI Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf".

- Key Personnel Biographical Sketches (five-page limit each): Upload as "Biosketch_LastName.pdf".

- Key Personnel Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf".

Research & Related Budget: For extramural submissions, refer to the General Application Instructions, Section III.A.5, and for intramural submissions, refer to the General Application Instructions, Section IV.A.4, for detailed information.

Budget Justification (no page limit): Upload as "BudgetJustification.pdf".

Initiating and Partnering PIs must each submit a budget and justification specific to his/her own portion of the efforts as part of his/her separate Grants.gov application packages. The Research & Related Budget for the Partnering PI should not include

budget information for the Initiating PI, even if they are located within the same organization. Refer to Section II.D.5, Funding Restrictions, for detailed information.

Project/Performance Site Location(s) Form: For extramural submissions (via Grants.gov), refer to the General Application Instructions, Section III.A.6, and for intramural submissions (via eBRAP), refer to General Application Instructions, Section IV.A.5, for detailed information.

- **Extramural Applications Only**

  Research & Related Subaward Budget Attachment(s) Form:

  - **Extramural Subaward:** Complete the Research & Related Subaward Budget Form through Grants.gov. (Refer to the General Application Instructions, Section III.A.7, for detailed information.)

  - **Intramural DoD Collaborator(s):** Complete the DoD Military Budget Form and upload to Grants.gov attachment form as Attachment 10. (Refer to the General Application Instructions, Section III.A.8, for detailed information.)

II.D.3. Dun and Bradstreet Data Universal Numbering System (DUNS) Number and System for Award Management (SAM)

Applicant organizations and all sub-recipient organizations must have a DUNS number to submit applications to Grants.gov. The applicant organization must also be registered in the Entity Management functional area of the SAM with an "Active" status to submit applications through the Grants.gov portal. Verify the status of the applicant organization's Entity registration in SAM well in advance of the application submission deadline. Allow several weeks to complete the entire SAM registration process. If an applicant has not fully complied with the requirements at the time the Federal awarding agency is ready to make a Federal award, the Federal awarding agency may determine that the applicant is not qualified to receive a Federal award and use that determination as a basis for making a Federal award to another applicant. Refer to the General Application Instructions, Section III, for further information regarding Grants.gov requirements.

II.D.4. Submission Dates and Times

All submission dates and times are indicated in Section I, Overview of the Funding Opportunity. Pre-application and application submissions are required. The pre-application and application submission process should be started early to avoid missing deadlines. There are no grace periods. Failure to meet either of these deadlines will result in submission rejection.

Applicant Verification of Full Application Submission in eBRAP

Following retrieval and processing of the full application, eBRAP will notify the organizational representatives and PI by email to log into eBRAP to review, modify, and verify the full application submission. eBRAP will validate retrieved files against the specific Program Announcement requirements, and discrepancies will be noted in both the email and in the "Full Application Files" tab in eBRAP. eBRAP does not confirm the accuracy of file content. It is the

applicant's responsibility to review all application components and ensure proper ordering as specified in the Program Announcement. ***If either the Project Narrative or the budget fails eBRAP validation or needs to be modified, an updated full application package must be submitted prior to the application submission deadline.*** The Project Narrative and Budget Form cannot be changed after the application submission deadline.

Extramural Submission: The full application package submitted to Grants.gov may be viewed and modified in eBRAP until the end of the application verification period. During the application verification period, the full application package, ***with the exception of the Project Narrative and Budget Form,*** may be modified.

Intramural DoD Submission: After eBRAP has processed the full application, the organizational Resource Manager/Comptroller/Task Area Manager or equivalent Business Official and PI will receive email notification of the status and will be able to view and modify application components in eBRAP. During the application verification period, the full application package, ***with the exception of the Project Narrative and Budget Form***, may be modified. The Resource Manager/Comptroller/Task Area Manager or equivalent Business Official should log into eBRAP to review and to approve the application package prior to the application verification deadline.

For All Submissions: Verify that subaward budget(s) with budget justification are present in eBRAP during the application verification period. If these components are missing, upload them to eBRAP before the end of the application verification period.

II.D.5. Funding Restrictions

The maximum period of performance is **3** years.

Application submissions with a single PI:

The anticipated direct costs budgeted for the entire period of performance will not exceed **$1.2M**. If indirect cost rates have been negotiated, indirect costs are to be budgeted in accordance with the organization's negotiated rate. No budget will be approved by the Government exceeding **$1.2M** direct costs or using an indirect cost rate exceeding the organization's negotiated rate.

All direct and indirect costs of any subaward or contract must be included in the total direct costs of the primary award.

The applicant may request the entire maximum funding amount for a project that may have a period of performance less than the maximum **3** years.

Application submissions including the Partnering PI Option: The anticipated combined direct costs budgeted for the entire period of performance for the Initiating and Partnering PIs' applications will not exceed **$1.5M**. If indirect cost rates have been negotiated, indirect costs are to be budgeted in accordance with the organization's negotiated rate. Collaborating organizations should budget associated indirect costs in accordance with each organization's

negotiated rate. The combined budgeted direct costs approved by the Government will not exceed **$1.5M** or use an indirect cost rate exceeding each organization's negotiated rate.

A separate award will be made to each PI's organization.

The Initiating and Partnering PIs are expected to be partners in the research, and direct cost funding should be divided accordingly, unless otherwise warranted and clearly justified. Each partner may request no more than 75% (or no less than 25%) of the proposed direct costs.

For this award mechanism, direct costs may be requested for (not all-inclusive):

- Salary
- Research supplies
- Clinical research costs (clinical trials are not allowed)
- Support for multidisciplinary collaborations, including travel
- Travel costs for the PI(s) to disseminate project results at one DoD-supported meeting to be specified by the program office during award negotiations (e.g., the Military Health System Research Symposium)
- Travel costs for one investigator to travel to one scientific/technical meeting per year in addition to the required meeting described above. The intent of travel costs to scientific/technical meeting(s) is to present project information or disseminate project results.

Awards made to extramural organizations will consist solely of assistance agreements (grants and/or cooperative agreements). For extramural awards with an intragovernmental component, direct transfer of funds from an extramural award recipient to a DoD or other Federal agency is not allowed except under very limited circumstances. Funding to intramural DoD and other Federal agencies will be managed through a direct funds transfer. Intramural applicants are responsible for coordinating through their agency's procedures the use of contractual or assistance funding awards or other appropriate agreements to support extramural collaborators. Application packages from associated extramural partners will be funded through assistance agreements.

Refer to the General Application Instructions, Section III.A.5, for budget regulations and instructions for the Research & Related Budget. ***For Federal agencies or organizations collaborating with Federal agencies, budget restrictions apply as are noted in the General Application Instructions, Section III.A.5.***

Funds to be obligated on any award resulting from this funding opportunity will be available for use for a limited time period based on the fiscal year of the funds. The time is considered when establishing the award's period of performance. It is anticipated that awards made from this FY19 funding opportunity will be funded with FY19, which will expire for use on September 30, 2025.

II.D.6. Other Submission Requirements

Refer to the General Application Instructions, Appendix 4, for detailed formatting guidelines.

II.E. Application Review Information

II.E.1. Criteria

II.E.1.a. Peer Review

To determine technical merit, all applications will be evaluated according to the following **scored criteria**, which are listed in decreasing order of importance:

- **Research Strategy and Feasibility**
    - How well the scientific rationale supports the project and its feasibility, as demonstrated by a critical review and analysis of the literature, supporting data, and logical reasoning.
    - How well the hypothesis, specific aims, and objective(s) are developed.
    - How well the experimental design, methods, data collection procedures, and analyses are developed and support completion of the aims.
    - To what degree the statistical plan and power analysis are appropriate for the proposed project.
    - If animal studies are included, how well they are designed to achieve reproducible and rigorous results, including the choice of model and the endpoints/outcomes to be measured.
    - If applicable, whether data will be appropriately reported and documented to support a regulatory filing with the FDA.
    - If applicable, how well the application provides evidence of availability of and access to the necessary study populations and/or resources.
    - How well potential problems are identified and alternative approaches are addressed.
    - Whether the research can be completed within the proposed period of performance.
- **Impact**
    - To what extent the project impacts a central critical problem or question in at least one FY19 PRMRP Topic Area.
    - If applicable, how well the proposed research project addresses one or more of the FY19 PRMRP Areas of Encouragement.

- How the proposed research project, if successful, will make important scientific advances in the relevant field of research.
- To what degree the proposed project could, if successful, make a significant impact on the lives of the relevant patient population(s) in the short term and/or long term.

- **Personnel**
  - How the background and expertise of the PIs and other key personnel demonstrate their ability to perform the proposed work.
  - How the levels of effort by the PIs and other key personnel are appropriate to ensure the successful conduct of the project.
  - How each PI's record(s) of accomplishment demonstrates their ability to accomplish the proposed work.

For applications submitted under the Partnering PI Option:

- **Partnership**
  - How well the research project is supported by the nature of the collaboration.
  - To what extent the proposed partnership will advance research beyond what would be possible through individual efforts.
  - How well the skills and perspectives of the Initiating and Partnering PIs complement each other.
  - How well the application reflects the requirement that the partners have equal intellectual input into the design of the project.

In addition, the following **unscored** criteria will also contribute to the overall evaluation of all IIRA applications:

- **Environment**
  - How the scientific environment is appropriate for the proposed research.
  - How the research requirements are supported by the availability of and accessibility to facilities and resources (including collaborative arrangements).
  - How the quality and extent of organizational support are appropriate for the proposed research.
  - If applicable, to what degree the intellectual and material property plan is appropriate.

- **Budget**
    - Whether the direct maximum costs are equal to or less than the allowable direct maximum costs as published in the Program Announcement.
- **Application Presentation**
    - To what extent the writing, clarity, and presentation of the application components influence the review.

II.E.1.b. Programmatic Review

To make funding recommendations and select the application(s) that, individually or collectively, will best achieve the program objectives, the following criteria are used by programmatic reviewers:

- Ratings and evaluations of the peer reviewers
- Relevance to the mission of the DHP and FY19 PRMRP, as evidenced by the following:
    - Adherence to the intent of the award mechanism
    - Program portfolio composition
    - Relevance to military health
    - Relative impact

II.E.2. Application Review and Selection Process

All applications are evaluated by scientists, clinicians, and consumers in a two-tier review process. The first tier is **peer review**, the evaluation of applications against established criteria to determine technical merit, where each application is assessed for its own merit, independent of other applications. The second tier is **programmatic review**, a comparison-based process in which applications with high scientific and technical merit are further evaluated for programmatic relevance. Final recommendations for funding are made to the Commanding General, USAMRMC, on behalf of the DHA and the OASD(HA). ***The highest-scoring applications from the first tier of review are not automatically recommended for funding. Funding recommendations depend on various factors as described in Section II.E.1.b, Programmatic Review.*** Additional information about the two-tier process used by the CDMRP can be found at https://cdmrp.army.mil/about/2tierRevProcess. A PI Information Paper describing the funding recommendations and review process for the award mechanisms for the PRMRP will be provided to the PI and posted on the CDMRP website.

All CDMRP review processes are conducted confidentially to maintain the integrity of the merit-based selection process. Panel members sign a statement declaring that application and evaluation information will not be disclosed outside the panel. Violations of confidentiality can result in the dissolving of a panel(s) and other corrective actions. In addition, personnel at the

applicant or collaborating organizations are prohibited from contacting persons involved in the review and approval process to gain protected evaluation information or to influence the evaluation process. Violations of these prohibitions will result in the administrative withdrawal of the organization's application. Violations by panel members or applicants that compromise the confidentiality of the review and approval process may also result in suspension or debarment from Federal awards. Furthermore, the unauthorized disclosure of confidential information of one party to another third party is a crime in accordance with 18 USC 1905.

II.E.3. Integrity and Performance Information

Prior to making an assistance agreement award where the Federal share is expected to exceed the simplified acquisition threshold, as defined in 2 CFR 200.88, over the period of performance, the Federal awarding agency is required to review and consider any information about the applicant that is available in the Federal Awardee Performance and Integrity Information System (FAPIIS).

An applicant organization may review FAPIIS, accessible through SAM, and submit comments to FAPIIS on any information about the organization that a Federal awarding agency previously entered and is currently available in FAPIIS.

The Federal awarding agency will consider any comments by the applicant, in addition to other information in the designated integrity and performance system, in making a judgment about the applicant's integrity, business ethics, and record of performance under Federal awards when determining a recipient's qualification prior to award, according to the qualification standards of the Department of Defense Grant and Agreement Regulations (DoDGARs), Section 22.415.

II.E.4. Anticipated Announcement and Federal Award Dates

All application review dates and times are indicated in Section I, Overview of the Funding Opportunity.

Each PI and organization will receive email notification of posting of the funding recommendation in eBRAP. Each PI will receive a peer review summary statement on the strengths and weaknesses of the application.

II.F. Federal Award Administration Information

II.F.1. Federal Award Notices

Awards supported with FY19 funds are anticipated to be made no later than September 30, 2020. Refer to the General Application Instructions, Appendix 2, for additional award administration information.

After email notification of application review results through eBRAP, and if selected for funding, a representative from USAMRAA will contact the Business Official authorized to negotiate on behalf of the PI's organization.

Only an appointed USAMRAA Grants Officer may obligate the Government to the expenditure of funds. No commitment on the part of the Government should be inferred from discussions with any other individual. **The award document signed by the Grants Officer is the official authorizing document.**

Federal Government Organizations: Funding made to Federal Government organizations (to include intramural DoD organizations) will be executed through the Military Interdepartmental Purchase Request (MIPR) or Funding Authorization Document (FAD) process. Transfer of funds is contingent upon appropriate safety and administrative approvals. Intramural applicants and collaborators are reminded to coordinate receipt and commitment of funds through their respective Resource Manager/Task Area Manager/Comptroller or equivalent Business Official.

After email notification of application review results through eBRAP, and if selected for funding, a representative from the CDMRP will contact the Business Official authorized to negotiate on behalf of the PI's organization.

II.F.1.a. PI Changes and Award Transfers

Changes in PI and an organizational transfer of an award supporting the Initiating PI or Partnering PI is discouraged and will be evaluated on a case-by-case basis and only allowed at the discretion of the USAMRAA Grants Officer. An organizational transfer of an award will not be allowed in the last year of the (original) period of performance or any extension thereof.

Refer to the General Application Instructions, Appendix 2, Section B, for general information on organization or PI changes.

II.F.2. Administrative and National Policy Requirements

Applicable requirements in the DoDGARs found in 32 CFR, Chapter I, Subchapter C, and 2 CFR, Chapter XI, apply to grants and cooperative agreements resulting from this Program Announcement.

Refer to the General Application Instructions, Appendix 2, for general information regarding administrative requirements.

Refer to the General Application Instructions, Appendix 5, for general information regarding national policy requirements.

Refer to full text of the latest DoD R&D General Terms and Conditions, the USAMRAA General Research Terms and Conditions with Institutions of Higher Education, Hospitals, and Non-Profit Organizations: Addendum to the DoD R&D General Terms and Conditions and the USAMRAA General Research Terms and Conditions with For-Profit Organizations for further information.

II.F.3. Reporting

Refer to the General Application Instructions, Appendix 2, Section A, for general information on reporting requirements. ***If there are technical reporting requirement delinquencies for any***

existing USAMRAA-sponsored awards at the applicant organization, no new awards will be issued to the applicant organization until all delinquent reports have been submitted.

Annual progress reports as well as a final progress report will be required.

Quarterly technical progress reports may be required.

In addition to written progress reports, annual Award Charts will be required. For the Investigator-Initiated Research Award mechanism, use the format example "Award Chart" available on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm).

Award Expiration Transition Plan: An Award Expiration Transition Plan must be submitted with the final progress report. Use the one-page template "Award Expiration Transition Plan" available on the on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm) under the "Progress Report Formats" section. The Award Expiration Transition Plan must outline if and how the research supported by this award will progress and must include source(s) of funding, either known or pending.

Awards resulting from this Program Announcement will incorporate additional reporting requirements related to recipient integrity and performance matters. Recipient organizations that have Federal contract, grant, and cooperative agreement awards with a cumulative total value greater than $10,000,000 are required to provide information to FAPIIS about certain civil, criminal, and administrative proceedings that reached final disposition within the most recent 5-year period and that were connected with performance of a Federal award. Recipients are required to disclose, semiannually, information about criminal, civil, and administrative proceedings as specified in the applicable Representations (see General Application Instructions, Appendix 5, Section B).

II.G. Federal Awarding Agency Contacts

II.G.1. CDMRP Help Desk

Questions related to Program Announcement content or submission requirements as well as questions related to the pre-application or intramural application submission through eBRAP should be directed to the CDMRP Help Desk, which is available Monday through Friday from 8:00 a.m. to 5:00 p.m. ET. Response times may vary depending upon the volume of inquiries.

Phone: 301-682-5507

Email: help@eBRAP.org

II.G.2. Grants.gov Contact Center

Questions related to extramural application submission through Grants.gov portal should be directed to the Grants.gov Contact Center, which is available 24 hours a day, 7 days a week

(closed on U.S. Federal holidays). Note that the CDMRP Help Desk is unable to provide technical assistance with Grants.gov submission.

Phone: 800-518-4726; International 1-606-545-5035

Email: support@grants.gov

Sign up on Grants.gov for "send me change notification emails" by following the link on the "Synopsis" page for the Program Announcement or by responding to the prompt provided by Grants.gov when first downloading the Grants.gov application package. If the Grants.gov application package is updated or changed, the original version of the application package may not be accepted by Grants.gov.

II.H. Other Information

II.H.1. Program Announcement and General Application Instructions Versions

Questions related to this Program Announcement should refer to the Program name, the Program Announcement name, and the Program Announcement version code 20181120a. The Program Announcement numeric version code will match the General Applications Instructions version code 20181120.

II.H.2. Administrative Actions

After receipt of pre-applications or applications, the following administrative actions may occur:

II.H.2.a. Rejection

The following will result in administrative rejection of the pre-application:

- Preproposal Narrative is missing.

The following will result in administrative rejection of the application:

- Submission of an application for which a letter of invitation was not received.
- Project Narrative exceeds page limit.
- Project Narrative is missing.
- Budget is missing.

II.H.2.b. Modification

- Pages exceeding the specific limits will be removed prior to review for all documents other than the Project Narrative.
- Documents not requested will be removed.

II.H.2.c. Withdrawal

The following may result in administrative withdrawal of the pre-application or application:

- An FY19 PRMRP Programmatic Panel member is named as being involved in the research proposed or is found to have assisted in the pre-application or application processes including, but not limited to, concept design, application development, budget preparation, and the development of any supporting documentation. ***A list of the FY19 PRMRP Programmatic Panel members can be found at https://cdmrp.army.mil/prmrp/panels/panels19***.
- The application fails to conform to this Program Announcement description.
- Inclusion of URLs, with the exception of links in References Cited and Publication and/or Patent Abstract sections.
- Page size is larger than 8.5 inches x 11.0 inches (approximately 21.59 cm x 27.94 cm).
- To preserve the integrity of its peer and programmatic review processes, the CDMRP discourages inclusion of any employee of its review contractors having any role in the preparation, research or other duties for submitted applications. For FY19, the identities of the peer review contractor and the programmatic review contractor may be found at the CDMRP website (https://cdmrp.army.mil/about/2tierRevProcess). Applications that include names of personnel from either of these companies may be administratively withdrawn.
- Personnel from applicant or collaborating organizations are found to have contacted persons involved in the review or approval process to gain protected evaluation information or to influence the evaluation process.
- Applications from extramural organizations, including non-DoD Federal agencies, received through eBRAP may be withdrawn.
- Applications submitted by an intramural DoD organization may be withdrawn if the intramural organization cannot coordinate the use of contractual, assistance, or other appropriate agreements to provide funds to extramural collaborators.
- The proposed research project does not address at least one of the Congressionally directed FY19 PRMRP Topic Areas.
- A clinical trial is proposed.
- The PI does not meet the eligibility criteria.
- Submission of the same research project to different Funding Opportunities within the FY19 PRMRP. Refer to Section II.D, Application and Submission Information, for exceptions.
- **Partnering PI Option:** All associated (Initiating and Partnering PI) applications are not submitted by the deadline.

II.H.2.d. Withhold

Applications that appear to involve research misconduct will be administratively withheld from further consideration pending organizational investigation. The organization will be required to provide the findings of the investigation to the USAMRAA Grants Officer for a determination of the final disposition of the application.

II.H.3. Application Submission Checklist

Application Components	Action	Initiating PI Completed	Partnering PI Completed
SF424 Research & Related Application for Federal Assistance **(Extramural submissions only)**	Complete form as instructed		
Summary (Tab 1) and Application Contacts (Tab 2) **(Intramural submissions only)**	Complete tabs as instructed		
Attachments	Project Narrative: Upload as Attachment 1 with file name "ProjectNarrative.pdf"		
	Supporting Documentation: Upload as Attachment 2 with file name "Support.pdf"		
	Technical Abstract: Upload as Attachment 3 with file name "TechAbs.pdf"		
	Lay Abstract: Upload as Attachment 4 with file name "LayAbs.pdf"		
	Statement of Work: Upload as Attachment 5 with file name "SOW.pdf"		
	Impact Statement: Upload as Attachment 6 with file name "Impact.pdf"		
	Relevance to Military Health Statement: Upload as Attachment 7 with file name "MilRel.pdf"		
	Partnership Statement: Upload as Attachment 8 with file name "Partnership.pdf" if applicable		
	Representations (extramural submissions only): Upload as Attachment 9 with file name "RequiredReps.pdf" if applicable		
	DoD Military Budget Form(s): Upload as Attachment 10 with file name "MFBudget.pdf" if applicable		
Research & Related Personal Data	Complete form as instructed		

Application Components	Action	Initiating PI Completed	Partnering PI Completed
Research & Related Senior/Key Person Profile (Expanded)	Attach PI Biographical Sketch (Biosketch_LastName.pdf) to the appropriate field		
	Attach PI Previous/Current/Pending Support (Support_LastName.pdf) to the appropriate field		
	Attach Biographical Sketch (Biosketch_LastName.pdf) for each senior/key person to the appropriate field		
	Attach Previous/Current/Pending (Support_LastName.pdf) for each senior/key person to the appropriate field		
Research & Related Budget **(Extramural submissions only)**	Complete as instructed. Attach Budget Justification (BudgetJustification.pdf) to the appropriate field		
Budget **(Intramural submissions only)**	Complete the DoD Military Budget Form and Justification		
Project/Performance Site Location(s) Form	Complete form as instructed		
Research & Related Subaward Budget Attachment(s) Form	Complete form as instructed		

APPENDIX 1: ACRONYM LIST

ACOS/R&D	Associate Chief of Staff for Research and Development
ACURO	Animal Care and Use Review Office
B	Billion
CDMRP	Congressionally Directed Medical Research Programs
CFR	Code of Federal Regulations
COI	Conflict of Interest
DHA	Defense Health Agency
DHP	Defense Health Program
DoD	Department of Defense
DoDGARs	Department of Defense Grant and Agreement Regulations
DUNS	Data Universal Numbering System
eBRAP	Electronic Biomedical Research Application Portal
EC	Ethics Committee
ET	Eastern Time
FAD	Funding Authorization Document
FAPIIS	Federal Awardee Performance and Integrity Information System
FDA	Food and Drug Administration
FY	Fiscal Year
HRPO	Human Research Protection Office
IACUC	Institutional Animal Care and Use Committee
IDE	Investigational Device Exemption
IND	Investigational New Drug
IRB	Institutional Review Board
M	Million
MIPR	Military Interdepartmental Purchase Request
NPC	Non-Profit Corporation
OASD(HA)	Office of the Assistant Secretary of Defense for Health Affairs
ORCID	Open Researcher and Contributor ID, Inc.
ORP	Office of Research Protections
PI	Principal Investigator
PRMRP	Peer Review Medical Research Program
RDT&E	Research, Development, Test, and Evaluation
SAM	System for Award Management
SOW	Statement of Work
STEM	Science, Technology, Engineering, and/or Mathematics
USAMRAA	U.S. Army Medical Research Acquisition Activity
USAMRMC	U.S. Army Medical Research and Materiel Command
USC	United States Code
VA	Department of Veterans

APPENDIX 2: AREAS OF ENCOURAGEMENT

Applications addressing any of the FY19 PRMRP Topic Areas are of interest to the program. ***Any aspect of research relevant to one or more FY19 PRMRP Topic Areas may be considered for funding.*** Areas of encouragement related to each FY19 PRMRP Topic Area have been identified by the DoD, VA, and other relevant stakeholders and are listed below under each Topic Area. Applicants are urged to read and consider these areas of encouragement before preparing their applications. ***The information provided is not exhaustive, and applicants are not restricted to submitting applications that address an area of encouragement in this list.***

Acute Lung Injury

- Research on the etiology and prevention of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) caused by host responses to trauma, transfusion, burns, infection, hemorrhagic shock, inhalation, and/or oxygen exposure.

- Novel and/or innovative detection technologies or therapeutics to reduce the incidence and/or severity of ALI/ARDS and/or other lung injury secondary to trauma, transfusion, infection, burns, hemorrhagic shock, inhalation, and/or oxygen exposure.

- Strategies to stabilize and support the safe transport of patients with ALI/ARDS in order to optimize therapeutic interventions, particularly in operational scenarios requiring prolonged field care and/or longer transport times.

- Development of metrics to associate the long-term health outcomes of ALI/ARDS with physiological and physical performance.

Antimicrobial Resistance

- Development of novel and/or innovative interventions to prevent the spread of or treat infections from multi-drug-resistant organisms, focused on hardware-associated infections and biofilms.

- Development and/or testing of new diagnostics to distinguish between viral and bacterial infectious diseases that will inform treatment and limit the spread of antibiotic resistance.

- Development and evaluation of strategies to support healthy gut microbiome (such as prebiotics, probiotics, colostrum, vaccines, and small molecules) to slow emergence of antibiotic-resistant organisms that cause infectious diarrhea in training or deployment settings.

- Development of rapid diagnostics platforms to identify and characterize new pathogen-specific, drug resistance markers that can be used in austere settings and during prolonged field care (e.g., “omic” and systems biology approaches).

- Development of gene-editing tools (e.g., designer nucleases) that optimize treatment and raise the threshold for resistance to anti-infective agents.

Arthritis (other than Rheumatoid Arthritis or Post-Traumatic Osteoarthritis)

- Research quantifying the impacts of obesity, weight loss, physical fitness (all components, e.g., cardiovascular, strength, flexibility, balance), and dietary factors on the development of or prevention/risk reduction of arthritis.
- Studies to examine the use of regenerative medicine techniques and therapies (including cell-based therapies) to prevent or treat osteoarthritis, including dose-response information and the frequency and timing of application.
- Basic and translational research to identify treatments to mitigate and/or reverse osteoarthritis, particularly in the knee, hip, ankle, and shoulder.
- Identification and/or validation of biomarkers for early psoriatic arthritis.
- Research to establish activity recommendations for maximal joint life following joint repair, particularly in young patient populations.

Burn Pit Exposure

- Research on the etiology and treatment of adverse health events related to military deployment to Iraq and Afghanistan that are associated with exposure to airborne hazards and open pit burning of solid waste and other materials.
- Toxicological studies to characterize emissions from open air burns, burn boxes, and incinerators, and to ascertain the toxicity and mechanisms of action of such chemicals and airborne environmental dust and mixtures, as well as interactions among pollutants and particulate materials.
- Identification and validation of biomarkers of both exposure to and health effects of burn pit combustion products, burning biomass and refuse, and geogenic dusts.
- Development and validation of instruments for assessing (including in real time) levels of exposure to airborne hazards for use in research and for occupational and environmental exposure monitoring.

Cardiomyopathy

- Development of novel therapeutic approaches for primary and secondary cardiomyopathies.
- Strategies to identify risk factors associated with the development of cardiomyopathy (i.e., genetic, lifestyle, exposure) in the civilian and/or military populations.
- Research to improve the understanding of the pathophysiology of cardiomyopathies.
- Improvement of noninvasive diagnostic techniques for primary and secondary cardiomyopathies.

- Research on the multiple etiologies of cardiomyopathy (e.g., hypertension, ischemia, hemochromatosis, sleep apnea, radiation therapy, medications, smallpox vaccine, infections).

Cerebellar Ataxia

- Research to identify therapeutic targets and novel therapeutic modalities including gene silencing/gene editing.
- Research to improve the understanding of the causes of cerebellar ataxia.
- Research to improve the understanding of the association between lifestyle, environment, nutrition, and cerebellar ataxia.
- Research to better understand the role of physical rehabilitation/exercise in affecting postural disorders, balance, and coordination in cerebellar ataxia patients.

Chronic Migraine and Post-Traumatic Headache

- Precision medicine research to investigate, develop, and validate biomarkers to diagnose and monitor traumatic brain injury patients with chronic migraine or post-traumatic headache and to characterize individual responses to treatment.
- Epidemiological/natural history studies to characterize specific types of post-traumatic headache and chronic migraine, the pathobiology of these headaches (such as the role of acute cortical spreading depression after injury as a risk factor for chronic headaches of a migrainous type), and the risk factors that might predispose people to certain types of post-traumatic headache and chronic migraine.
- Research on the optimal approaches to effective management of acute and chronic pain and co-occurring psychological health disorders for chronic migraine and post-traumatic headache with a focus on assessing and eliminating adverse outcomes and decreasing polypharmacy.
- Evaluation of the efficacy of existing or emerging pharmaceutical interventions (non-opioid), as well as evaluation of mechanical stimulation and/or other non-pharmaceutical interventions, for the treatment of chronic migraine and post-traumatic headache.
- Evaluation of the differences in etiology, diagnosis, treatment plan, and prevention of migraine headaches between men and women.

Congenital Heart Disease

- Development of approaches, including regenerative medicine, that provide structural support, restore native activity, allow for tissue growth, and prevent the need for reoperation.
- Research to improve understanding of the causes of congenital heart defects, including genomic, proteomic, and metabolomic profiling.

- Research to design and implement disease-in-a-dish and/or microfluidic models with an established phenotype to increase the efficacy of finding novel and/or innovative drug targets, screen existing drugs, perform cardiotoxicity testing, and/or uncover pathogenesis.

- Research both on the risk of neurologic injury and on enhanced neuroprotection before, during, and after surgery for congenital heart disease.

- Population-based and outcomes-based research to assess the health outcomes of individuals with congenital heart disease across their life spans.

Constrictive Bronchiolitis

- Research to understand the role of occupational and environmental exposures, including military relevant volatile compounds, toxic industrial chemicals/materials in dense urban environments, and airborne particulates, such as mineral and soil dusts, in the etiology of constrictive bronchiolitis.

- Research to determine the prevalence and severity of constrictive bronchiolitis and related respiratory diseases in previously deployed military Service members and/or Veterans.

- Development and testing of minimally invasive or non-invasive approaches for diagnosing constrictive bronchiolitis.

- Research to develop novel and/or innovative therapeutics to modify the progression of constrictive bronchiolitis.

- Development and/or validation of animal models for understanding mechanisms and etiology of constrictive bronchiolitis.

Diabetes

- Research to better understand the heterogeneity of diabetes including the identification of novel biomarkers.

- Identification and/or evaluation of interventions to reduce the development of diabetes among individuals meeting the clinical criteria for pre-diabetes.

- Research on interventions to prevent or treat diabetes complications, including diabetic retinopathy, nephropathy, neuropathy, cardiomyopathy, and impaired wound healing.

- Research on the transplantation of allogenic or autologous pancreatic islet cells for long-term natural insulin production, including current good laboratory/clinical/manufacturing practices (as needed) for cell line development.

- Research to design and implement disease-in-a-dish and/or microfluidic models to model pancreatic islets to uncover pathogenesis and improve the efficiency of drug discovery.

Dystonia

- Research to improve identification of delayed onset dystonia following traumatic brain injury.
- Research on interventions to prevent, slow the progression of, or treat dystonia.
- Studies into the natural history, genetics, and/or neurobiology of dystonia.
- Research to identify the relationship between specific molecular/genetic changes and circuitry/network alterations in dystonia.
- Identification and development of novel research tools (such as cellular models, phenotypic models, etc.) to aid dystonia research.

Eating Disorders

- Investigations into the prevalence, diagnosis, and treatment patterns of eating disorders in Service members and their families, including potential relationships with military-unique behaviors or conditions.
- Assessment of patterns of comorbidity between eating disorders and other mental health conditions, including an examination of whether eating disorders are more likely to precede or follow the development of other mental health conditions.
- Studies to identify the most effective treatments (and order of treatment) for patients with an eating disorder and a comorbid disorder.
- Research to advance the understanding of the biological, genetic, lifestyle, and environmental factors that influence eating disorders.
- Studies to elucidate and/or mitigate risk factors for the onset or recurrence of eating disorders, including social influences.

Emerging Infectious Diseases

- Surveillance and predictive modeling tools that leverage artificial intelligence approaches to predict outbreaks and epidemics and support strategies for mitigating the threat of emerging infectious diseases (e.g., dengue, malaria, Hanta).
- Research and development of in silico and/or in vitro tools to assess product manufacturability/developability, efficacy, and toxicity, such as cells-on-a-chip and 3D tissue modeling, to support development of medical countermeasures.
- Development of a wearable sensor that provides real-time diagnostics of naturally occurring infectious diseases to predict illness before onset of symptoms.
- Rapid prediction of protective antigens/epitopes and testable correlates of protection on emerging or novel pathogens.

- Development of highly sensitive diagnostic system for use at the point of need that provides early diagnosis of infection prior to the onset of classical symptoms.

- Research, development, and validation of animal models for the study of infectious diseases that clearly show the pathophysiological mechanism of the disease and provide translational data to advance drug products to human clinical trial.

- Development of risk assessment strategies for vector-borne diseases and novel interventions for vector control, including but not limited to novel insecticides, larvicide applications, and barrier methods.

Epidermolysis Bullosa

- Research, including clinical trials, focused on therapeutics (topical or systemic) or dressings that enhance wound healing in inherited epidermolysis bullosa.

- Development of novel therapeutics to reduce epidermolysis bullosa symptoms, improve quality of life, or lead to a cure.

- Research to provide further insight into those cellular pathways that promote the development of squamous cell carcinomas in recessive dystrophic and junctional epidermolysis bullosa.

- Research, including randomized controlled clinical trials, focused on systemic drugs that prevent, delay the onset, or modify the aggressiveness of squamous cell carcinoma in patients with recessive dystrophic and junctional epidermolysis bullosa.

Focal Segmental Glomerulosclerosis

- Research to improve understanding of the causes of primary and/or secondary focal segmental glomerulosclerosis (especially genetic mutations).

- Development of non-invasive methods to diagnose focal segmental glomerulosclerosis and its variants.

- Development of a curative therapy or treatments to delay or halt the progression of focal segmental glomerulosclerosis and/or prevent post-transplantation recurrence.

- Research to determine the efficacy of medications used off-label (outside the FDA-approved indication) to treat focal segmental glomerulosclerosis.

Frontotemporal Degeneration

- Basic research to establish in vivo and in vitro models for disease pathology, behavioral/cognitive symptoms, and motor dysfunction.

- Research to understand the neurological basis of deficits in social cognition and emotional regulation.

- Research to improve diagnostics of and/or prognostics for frontotemporal degeneration.
- Research to identify risk factors (e.g., gene networks, environmental factors, and family history of dementia).
- Development of evidence-based non-pharmacological and/or pharmacological treatments for frontotemporal degeneration and associated symptoms.

Guillain-Barré Syndrome

- Development of a rapid diagnostic test for Guillain-Barré syndrome.
- Research on the immune system cell types and molecular mechanisms responsible for the pathology of Guillain-Barré syndrome.
- Research to elucidate the characteristics of various exposures (e.g., viruses, bacteria, vaccinations, surgery, trauma) associated with Guillain-Barré syndrome and their effects on the immune system.
- Research to prevent or reduce the effects of residual weakness, relapse of muscle weakness, and other neurological and psychological symptoms of Guillain-Barré syndrome to improve patients' quality of life and increase their independence.
- Development of new treatments and refinement of existing treatments for Guillain-Barré syndrome.

Hemorrhage Control

- Development of new and innovative capabilities to stop non-compressible intracavitary hemorrhage and improved technologies to stop junctional and pelvic bleeding in pre-hospital environments.
- Development of innovative damage control resuscitation and damage control surgical capabilities.
- Research on strategies for early (e.g., pre-hospital) detection (especially internal bleeding) and treatment for hemorrhage, coagulopathy of trauma, and hemodynamic decompensation/ hypovolemic shock.
- Research on novel or engineered blood products that offer physiological, logistical, or cost advantages over current products. Proposals for HBOCs should address nitric oxide scavenging.
- Research on adjunctive pharmacological solutions for hemorrhage, shock, coagulopathy, transfusion, and/or the stabilization of polytrauma, with attention to contraindications and/or impact on TBI.

- Research to evaluate the effects of current combat blood product transfusion guidelines on immunological status and clinical outcomes.

Hepatitis B

- Identification and reduction of hepatitis B in blood products for transfusion.
- Research on strategies to reduce vertical (mother-to-child) transmission of hepatitis B.
- Development of strategies for reliable, non-invasive, early detection of hepatitis-related liver disease and hepatocellular carcinoma.
- Research on strategies to promote reversal of liver fibrosis and/or assess the associated clinical and pathological outcomes.
- Clinical studies to evaluate combination or curative therapies for treatment of hepatitis B infection.

Hereditary Angioedema

- Research toward the development of a cure for hereditary angioedema.
- Development and/or validation of novel and/or innovative therapeutic strategies for the treatment and/or prevention of hereditary angioedema attacks.
- Research to improve early diagnosis of hereditary angioedema.
- Evaluation of existing, innovative, or novel therapeutics in pediatric hereditary angioedema patients.

Hydrocephalus

- Research on the etiology, prevention, diagnosis, and treatment of post-traumatic hydrocephalus.
- Discovery or validation of novel and/or innovative therapies and therapeutic targets for the treatment of hydrocephalus and its sequelae, including therapies directed at myelin regeneration and repair.
- Development or validation of biomarkers and imaging techniques, particularly multimodal approaches, to aid in diagnosis, prognosis, and monitoring of therapeutic efficacy.
- Research on the prevention of shunt failure or the development of novel shunt technologies.
- Development or validation of improved hydrocephalus model systems.

Immunomonitoring of Intestinal Transplants

- Studies to elucidate the role of the mucosal immune system, humoral, innate, and adaptive cellular immunity, other host-derived factors, or gut microbiota-derived factors in maintaining intestinal transplant viability and improving outcomes.
- Development and evaluation of evidence-based intestinal transplant strategies that focus on dampening the immune response against intestinal transplants or circumvent the induction of immunity against the transplant.
- Development and evaluation of implant-associated materials (e.g., scaffolds) with anti-inflammatory properties that protect the intestinal transplant from immune attack.
- Development and evaluation of strategies for inducing and maintaining populations of anti-inflammatory regulatory immune cell populations at the transplant site.
- Studies to improve immunomonitoring of recipient immune responses after intestinal transplantation, with a focus on prospective leukocyte profiling, to aid in diagnosis and treatment of immunological and immunosuppression-related complications.
- Development and/or validation of precise, real-time implanted monitoring devices to improve individualized patient outcomes after intestinal transplantation.

Inflammatory Bowel Diseases

- Studies directed toward understanding how acute enteric infections may trigger chronic inflammatory bowel diseases.
- Studies that leverage genomic, microbiomic, immunological, and systems biology approaches to prevent inflammatory bowel disease (especially inflammatory bowel diseases associated with acute enteric infection).
- Studies to understand the interaction between acute/chronic stress and infection and their influence on inflammatory bowel diseases.
- Research to better characterize the association between the use of drugs, such as isotretinoin and long-term doxycycline, and the development of inflammatory bowel diseases.
- Research on the role of diet in the development and progression of inflammatory bowel diseases.
- Research on treatment strategies for patients with inflammatory bowel diseases to include, but not limited to, rebalancing the microbiome, pre- and probiotics use or colostrum products, including those who are refractory to standard care.

Interstitial Cystitis

- Studies that define the risk, prevalence, and operational impact of interstitial cystitis among active duty personnel.
- Identification and validation of biomarkers for making a definitive diagnosis of interstitial cystitis.
- Evaluation and assessment of novel and/or innovative treatment options for interstitial cystitis patients, including intravesical therapy.
- Research on the etiology and pathogenesis of interstitial cystitis to inform targeted therapy development.

Lung Injury

- Studies to identify the prevalence and associated morbidity and mortality of blast overpressure lung injury.
- Development of improved methods for assessing lung injury due to exposure to chemical or physical (e.g., radiation) hazards and materials in occupational, operational, and training environments to improve surveillance, diagnosis, and prognosis.
- Development of improved methods for monitoring pulmonary exposure to chemical or physical agents that might cause lung injury.
- Identification of pre-existing conditions that predispose an individual's susceptibility to lung injury resulting from environmental exposures (i.e., genetic predisposition).
- Development of novel preventive techniques, detection technologies, and therapeutics to reduce the incidence and/or severity of lung injury.

Metals Toxicology

- Identification and validation of biomarkers to evaluate military Service members' acute exposure to toxic metals in an operational environment and predict potential consequent health risks and associated health outcomes.
- Retrospective studies to evaluate risks and exposure to military-relevant toxic metals among workers at industrial facilities.
- Research on the toxicity of metal combinations and the interaction between different metal components.
- Research on the toxicity of metal-based engineered nanomaterials, including those used in military applications.
- Development of microsurgical techniques to remove embedded toxic metals.

Mitochondrial Disease

- Identification and testing of non-invasive techniques and biomarkers to monitor mitochondrial function, aid in clinical diagnosis, and/or evaluate therapeutic efficacy.
- Development of improved tools and animal models to study primary mitochondrial diseases and evaluate therapeutics.
- Research to better understand the pathology of primary mitochondrial diseases.
- Development of tools and methodologies to assess mitochondrial heteroplasmy on a cellular, tissue, and organ level.
- Research on novel and/or innovative treatments to alleviate symptoms or slow down the progression of mitochondrial diseases.

Musculoskeletal Disorders

- Research on computational models that provide personalized, actionable information regarding the physical resilience of the musculoskeletal system for the prevention and treatment of musculoskeletal disorders such as load carriage capability, effectiveness of physical training and treatments, and effective use of exoskeleton technology.
- Development and/or validation of metrics (clinical-based measures/tools and field-based measures/tools) to inform readiness and/or return-to-duty/work decision making following the management, treatment, and rehabilitation of musculoskeletal disorders.
- Research to increase understanding and to improve diagnosis, prevention, and/or treatment of chronic overuse musculoskeletal disorders.
- Research on back pain treatment and/or management strategies to prevent surgery and recurrence of symptoms, identify factors that predict optimal treatment response for different patients, and encourage self-management.

Myotonic Dystrophy

- Research on the role of epigenetic factors in the onset, progression, and/or severity of myotonic dystrophy in relevant animal models or patients.
- Research into the mechanisms of expanded CTG or CCTG repeat instability in somatic or germ line cells in myotonic dystrophy.
- Identification of biomarkers that can be detected through minimally invasive means to signal early changes in the progression of myotonic dystrophy.
- Development and/or testing of novel and/or innovative treatments, including those utilizing gene editing or silencing.

- Clinical research into the natural history of myotonic dystrophy, to understand disease progression and develop/validate clinical trial endpoint measures across the multiple organ systems involved in the disease.

Nanomaterials for Bone Regeneration

- Research on nanomaterials that stimulate vascularization and new bone growth.
- Research on nanomaterials-based methods to facilitate recruitment of endogenous cell populations for enhanced bone regeneration and osseointegration.
- Development of nanomaterial-based technologies addressing segmental/large bone defects in craniomaxillofacial and/or load-bearing regions.
- Development of nanomaterials for controlled release/extended release of growth factors for bone regeneration.
- Development of nanomaterial-based technologies that repair the soft tissue envelope to enhance bone regeneration.

Nutrition Optimization

- Development and/or validation of nutrition-based strategies that mitigate the consequences of stressors, especially on Service member health, readiness, and performance.
- Development of prolonged nutrition care using oral and/or intravenous approaches including precision nutrition care following injury or illness.
- Research on the impact of the use of dietary supplements (e.g., vitamins, probiotics, protein powders) on the physical and/or cognitive performance, including the readiness of military Service members.
- Development and/or validation of improved nutrition strategies for physical and/or cognitive performance enhancement and sustainment in operational environments and efforts to optimize nutrition in resource-limited settings.
- Research to develop strategies to apply metabolomics to optimize individual nutrition and the development of tools or devices to monitor nutritional intake at an individual level.

Pancreatitis

- Development and testing of novel and/or innovative therapeutics for acute and/or chronic pancreatitis.
- Research on the basic biology and physiology of the pancreas to better understand the etiology and pathology of pancreatitis.
- Research to improve understanding and management of complications of pancreatitis.

Pathogen-Inactivated Blood Products

- Research on lyophilization of pathogen-reduced/-inactivated blood products and derivatives (platelets, plasma, red cells, cryoprecipitate, coagulation factors, etc.).

- Development and advancement of technologies to improve the safety of blood products to include pathogen reduction/inactivation in whole blood for military/civilian blood donor centers and blood banks that meet the requirements for FDA licensure in support of domestic and global contingency/combat operations.

- Expansion and validation of the library of blood-borne pathogens that are reduced/inactivated to include emerging pathogens, genetically modified pathogens, and pathogens designed for biological warfare.

- Advancement in pathogen reduction technology to further improve the log-kill reduction for blood-borne pathogens (e.g., Hepatitis B, Korean Hemorrhagic Fever virus, Bunyan virus, HIV, Rift Valley Fever, Malaria, Babesia, Ebola, West Nile virus, Dengue, Chikungunya, Zika virus).

- Research studies, including clinical trials, to further characterize the effects of pathogen reduction technologies in blood products (e.g., whole blood, platelets, plasma, cryoprecipitate).

- Development and validation of next-generation technologies and/or devices to reduce the production time for pathogen reduction/inactivation in whole blood.

Polycystic Kidney Disease

- Development of improved treatment strategies for polycystic kidney disease, including approaches to identify and monitor patients at higher risk for progressing to end-stage renal disease.

- Research on the underlying pathobiology and molecular mechanisms of polycystic kidney disease, including studies of genetic factors, cyst formation and growth, the role of cilia, and factors that modify disease progression and/or severity.

- Research on the lifestyle factors or comorbidities that may modify the progression of polycystic kidney disease.

Post-Traumatic Osteoarthritis

- Development or validation of innovative regenerative rehabilitation approaches for preventing or mitigating post-traumatic osteoarthritis.

- Studies to evaluate and develop best practices for multidisciplinary team approaches and treatment algorithms for post-traumatic osteoarthritis.

- Intra-articular treatments that offer sustained (two or more months) relief of symptoms and/or disease-modifying effects.

- Research on therapies that target multiple phases of the cellular response pathways that are implicated in the development of post-traumatic osteoarthritis, including cell death, inflammation, matrix changes, and changes in catabolic and anabolic responses.

- Research on biomarkers that can serve as surrogate endpoints for post-traumatic osteoarthritis diagnosis and/or can optimize subject selection in clinical trials.

Pressure Ulcers

- Novel strategies for the treatment of pressure ulcers including mitigation of progression to advanced stages.

- Strategies to prevent or reduce the formation of pressure ulcers during prolonged immobilization of casualties in a pre-hospital environment (e.g., spinal cord injuries) or long-range transport/aeromedical evacuation.

- Research on novel techniques for synthetic production, delivery, and adhesion methodologies leading to permanent closing of pressure ulcers. This might encompass synthetic fibers, novel tissue culture methodologies, growth factors, dermal printing, artificial skin, skin graft substitutes, regenerative medicine, etc.

- Novel strategies for the prevention or early detection of pressure ulcers.

Pulmonary Fibrosis

- Identification of biomarkers of pulmonary injury or early predictors of interstitial lung disease.

- Development and/or validation of improved tools and animal models (excluding mice) to study pulmonary fibrosis and evaluate therapeutics.

- Research into the pathobiology and molecular mechanisms underlying the development and progression of pulmonary fibrosis.

- Retrospective studies to determine the risk and incidence of pulmonary fibrosis among military Service members and/or Veterans.

- Development and/or testing of novel and/or innovative treatments, to include precision medicine approaches, to delay or modify the progression of pulmonary fibrosis.

Resilience Training

- Research to deliver evidence-based interventions and improved comprehensive strategies for building individual, family, and community resilience to physical, psychological, environmental, and social stressors over the military life cycle, including transitions.

- Research that measures the effects of resilience training approaches on a broad array of outcomes and constructs of interest (physical health, performance, well-being, mental health, relationships, etc.) by evaluating the efficacy of resilience training and/or leadership approaches in response to stressors and challenges.

- Development and validation of physical or psychological measures of individual variation and response to stressors and meaningful resilience measures, including non-self-report objective measures.

- Research aimed at understanding the relationship between Service member resilience and successful/unsuccessful recovery from injury.

- Research in psychometric training or pharmacological treatment(s) to manage stress within individuals.

Respiratory Health (excludes lung cancer and mesothelioma)

- Research on the causes, treatment, and prevention of obstructive pulmonary diseases (e.g., chronic obstructive pulmonary disease and bronchiectasis), including identification and validation of biomarkers and disease phenotypes, as well as employing personalized medicine in clinical research and disease management.

- Research on the cause, treatment, and prevention of respiratory symptoms and ailments possibly associated with deployed and redeployed military personnel.

- Research to evaluate the impact of military service, primarily deployment, on the prevalence and severity of respiratory disease.

- Identification and/or validation of biochemical, physiological, or combined biomarkers for evaluating risk or extent of pulmonary injury from either acute or long-term toxic occupational or environmental exposures.

- Research investigating exposure rates, detection, and treatment of diseases related to inhalation of mold and fungi, such as coccidioidomycosis from both indoor and outdoor sources.

Rett Syndrome

- Identification and/or validation of novel and/or innovative biological targets for the treatment of Rett syndrome.

- Development and testing of interventions to improve the neurological symptoms of Rett syndrome.

- Research to understand the relationship between genetic mutations, physical traits, and symptoms in individuals with Rett syndrome.

- Research to understand Rett syndrome's commonalities with, and differences from, other autism spectrum disorders.

- Research on the pathobiology of MeCP2 and associated genes and proteins.

Rheumatoid Arthritis

- Research to better understand the relationship between genetic risk, environmental exposures, and predicted triggers, such as infection or smoking, in developing rheumatoid arthritis.

- Studies that identify or validate biomarkers or personalized medicine strategies that allow for individualized medication choice based on the patient's underlying biology or disease state.

- Research on the long-term use of immunosuppressive and other therapies in patients with rheumatoid arthritis.

- Research to better characterize and understand the preclinical disease stage of rheumatoid arthritis for early diagnosis and treatment.

- Research on management of comorbidities, including biopsychosocial outcomes, for patients with rheumatoid arthritis.

- Research to establish activity recommendations following joint replacement for maximal joint life.

Scleroderma

- Research on the molecular mechanisms and pathogenesis of scleroderma, including the identification of novel and/or innovative therapeutic targets.

- Development and/or validation of novel and/or innovative therapies for scleroderma.

- Identification and/or validation of biomarkers and other approaches for early diagnoses, monitoring disease progression, and/or assessment of treatment response.

- Epidemiologic studies investigating the impact of localized scleroderma (morphea) on duty performance, use of personal protective equipment, and deployability.

- Research on early identification and prevention of scleroderma-associated complications, such as lung, liver, or kidney disease.

Sleep Disorders

- Research on how the disruption of normal sleep and circadian rhythms adversely affects the physical and psychological health, safety, performance, and productivity of military personnel and civilian populations, including sex and gender differences.

- Identification and/or validation of non-Continuous Positive Air Pressure (CPAP)-based treatment regimens that enhance compliance in military personnel and civilian populations.

- Research on the prevention and/or mitigation of sleep disorders that are associated with long aeromedical evacuation flights for both clinical team members and patients.

- Development and/or testing of non-pharmacological treatments for sleep disorders associated with long-term exposure to enclosed environments (e.g., aircraft, submarines, tanks).

- Research on the precision diagnosis and/or treatment of sleep disorders, especially following traumatic brain injury or related to post-traumatic stress disorder.

Spinal Muscular Atrophy

- Research into molecular and proteomic phenotyping the spinal muscular atrophy disease state.

- Research to determine mitochondrial involvement and astrocytic and other non-neuronal contributions to motor neuron vulnerability.

- Exploration of the form and function of SMN-depleted neuromuscular junctions at ultrastructural (e.g., dysregulation of endocytosis), transcriptomic, and proteomic levels, particularly the mildest SMN reduction that leads to consistent quantifiable motor neuron loss.

- Research to find non-SMN-altering spinal muscular atrophy modifying genes that may lead to identification of novel and/or innovative therapeutic targets or treatments.

- Research to further understand SMN gene regulation and post-transcriptional mechanisms leading to synergistic SMN-repleting approaches, as well as to determine whether boosting SMN induction maximizes efficacy.

Tinnitus

- Development and validation of objective tools/methods to diagnose and characterize tinnitus (e.g., imaging techniques to identify functional and structural changes in the brain, biomarkers of resiliency, and susceptibility to tinnitus).

- Research to understand the mechanisms of tinnitus, its relationship to noise-induced hearing loss, and progression to chronic tinnitus, with the focus on developing interventions.

- Research to understand and mitigate the negative impact of tinnitus on operational readiness of the military.

- Identification of novel and/or innovative therapies and/or devices for interventions to prevent, manage, and treat tinnitus, including behavioral approaches, new uses for existing drugs, nutritional and pharmaceutical strategies, and acoustic, electrical, and other stimulation technologies.

Tissue Regeneration

- Development of novel therapies to repair neurosensory damage, maintain the distal end organ interface, or regenerate the neuromuscular junction for reinnervation of end organs during peripheral nerve regeneration.
- Development of novel therapies for regeneration of tendons and musculotendinous junctions.
- Development of novel therapies for regeneration of functional skeletal muscle, particularly (1) stem cell-based approaches and (2) treatments for volumetric muscle loss.
- Research on novel approaches and therapies to understand mechanisms of immune rejection and obviate the need for chronic toxic immunosuppression in reconstructive transplantation and vascularized composite allotransplantation.
- Research into innovative methods for developing biocompatible scaffolds and stem cell therapies for manufacturing and production of tissues.
- Research on novel interventions to regenerate brain tissue and recover neurological function, especially following traumatic brain injury.

Tuberculosis

- Research to understand, diagnose, or treat multi-drug-resistant tuberculosis or extensively drug-resistant tuberculosis.
- Development of novel strategies or therapeutics to treat tuberculosis.
- Development of a diagnostic assay that can be used at the point of care to rapidly and accurately diagnose tuberculosis to include multi-drug-resistant tuberculosis or extensively drug-resistant tuberculosis.
- Development of novel and/or innovative tuberculosis vaccines or optimization of current tuberculosis vaccines.

Vascular Malformations

- Studies into the natural history, genetics, and pathogenesis of vascular malformations.
- Research to develop or improve methods to diagnose and manage vascular malformations.
- Research to discover or develop novel and/or innovative therapeutic targets and treatments to regress or prevent vascular malformations.
- Development of non-invasive or minimally invasive technologies or approaches for the control of internal bleeding, including cerebral arteriovenous malformations, associated with vascular malformations.

- Development of in vivo or in vitro models of vascular malformations for the purpose of identifying novel and/or innovative drug targets, screening existing drugs, and/or elucidating the pathogenesis of the disease.

Women's Heart Disease

- Identification of sex-specific approaches to either develop novel diagnostics and treatments or increase the effectiveness of current practice to improve clinical care of women.

- Research on factors to predict and prevent the long-term impacts of the endocrine system, gestational diabetes, gestational hypertension, menopause, or preeclampsia on the cardiovascular health of women.

- Research on trauma-induced cardiac arrest secondary to hemorrhage and polytrauma in the female population.

- Research focused on elucidating the potential relationship between post-traumatic stress disorder and women's heart disease.

- Studies to determine the risk and incidence of heart disease among current and/or former female Service members.

APPENDIX 3: DOD AND VA WEBSITES

PIs are encouraged to integrate and/or align their research projects with DoD and/or VA research laboratories and programs. Collaboration with DoD or VA investigators is also encouraged. Below is a list of websites that may be useful in identifying additional information about DoD and VA areas of research interest, ongoing research or potential opportunities for collaboration within the FY19 PRMRP Topic Areas.

Air Force Office of Scientific Research
https://www.wpafb.af.mil/afrl/afosr/

Air Force Research Laboratory
https://www.wpafb.af.mil/afrl

Armed Forces Radiobiology Research Institute
https://www.usuhs.edu/afrri/

Clinical and Rehabilitative Medicine Research Program
https://crmrp.amedd.army.mil

Combat Casualty Care Research Program
https://ccc.amedd.army.mil

Congressionally Directed Medical Research Programs
https://cdmrp.army.mil

Defense Advanced Research Projects Agency
https://www.darpa.mil/

Defense Technical Information Center
https://www.dtic.mil

Defense Threat Reduction Agency
http://www.dtra.mil/

Military Health System Research Symposium
https://mhsrs.amedd.army.mil/SitePages/Home.aspx

Military Infectious Diseases Research Program
https://midrp.amedd.army.mil

Military Operational Medicine Research Program
https://momrp.amedd.army.mil

Naval Health Research Center
https://www.med.navy.mil/sites/nhrc

Navy and Marine Corps Public Health Center
https://www.med.navy.mil/sites/nmcphc/Pages/Home.aspx

Office of Naval Research
https://www.med.navy.mil/

Office of the Under Secretary of Defense for Acquisition, Technology and Logistics
https://www.acq.osd.mil/

Telemedicine and Advanced Technology Research Center
http://www.tatrc.org/

Uniformed Services University of the Health Sciences
https://www.usuhs.edu/research

U.S. Army Institute of Surgical Research
https://www.usaisr.amedd.army.mil/

U.S. Army Medical Materiel Development Activity
https://www.usammda.army.mil/

U.S. Army Medical Research and Materiel Command
https://mrmc.amedd.army.mil

U.S. Army Medical Research Institute of Infectious Diseases
https://www.usamriid.army.mil/

U.S. Army Research Institute of Environmental Medicine
https://www.usariem.army.mil/

U.S. Army Research Laboratory
https://www.arl.army.mil

U.S. Department of Defense Blast Injury Research Program
https://blastinjuryresearch.amedd.army.mil/

U.S. Department of Veterans Affairs, Office of Research and Development
https://www.research.va.gov

U.S. Naval Research Laboratory
https://www.nrl.navy.mil

Walter Reed Army Institute of Research
https://www.wrair.army.mil

Please wait...

If this message is not eventually replaced by the proper contents of the document, your PDF viewer may not be able to display this type of document.

You can upgrade to the latest version of Adobe Reader for Windows®, Mac, or Linux® by visiting http://www.adobe.com/go/reader_download.

For more assistance with Adobe Reader visit http://www.adobe.com/go/acrreader.

Windows is either a registered trademark or a trademark of Microsoft Corporation in the United States and/or other countries. Mac is a trademark of Apple Inc., registered in the United States and other countries. Linux is the registered trademark of Linus Torvalds in the U.S. and other countries.

Please wait...

If this message is not eventually replaced by the proper contents of the document, your PDF viewer may not be able to display this type of document.

You can upgrade to the latest version of Adobe Reader for Windows®, Mac, or Linux® by visiting http://www.adobe.com/go/reader_download.

For more assistance with Adobe Reader visit http://www.adobe.com/go/acrreader.

Windows is either a registered trademark or a trademark of Microsoft Corporation in the United States and/or other countries. Mac is a trademark of Apple Inc., registered in the United States and other countries. Linux is the registered trademark of Linus Torvalds in the U.S. and other countries.

Copied from https://research.unc.edu/creativity-hubs/rfp/

Stage 2: Full proposals due April 12, 2019

Please use 1-inch margins and use a font size no smaller than 11pt. to ensure readability.

Proposals should be submitted as one document in PDF format to creativityhubs@unc.edu by 5:00 pm of April 12, 2019.

Full proposals will consist of the following:

Proposal Cover Page

Please use the provided template:

- Include the title of the project;
- A list of 5 key words;
- Principal investigator contact information;
- List of other key personnel and their college/school and/or Center/Institute affiliation, and proposed project effort (both paid and cost-shared);
- Total amount of funding requested;
- Answers to compliance questions; and
- A 200-word (maximum) summary of the proposed research program that is appropriate for a broad faculty readership.

Project description (6 pages):

The project description should include sufficient detail such that reviewers can evaluate the appropriateness and feasibility of the proposed plans. The project description must articulate a clear set of goals that are aspirational yet attainable. Preliminary data are not required but teams must demonstrate that they can successfully advance the project.

The project description should include the following sections:

- Research plan: This section should address how the proposed research program is consistent with the Key Review Criteria. It should include a discussion of the major challenges in the field, a description of innovativeness of the proposed research, how it would solve these challenges, and the impact it would have on the challenge, as well as its benefits to society, culture, community, the environment or the economy. This section should also include a description of convergence: how the project takes advantage of expertise from multiple and diverse disciplines, how the different parts would form a greater whole and contribute to the project's success.
- Sustainability Plan: Detailed plan for sustainability of the proposed research program via the attraction of extramural support. Description of specific sources of extramural funding that will be targeted for future proposals. Plans for extramural funding should be as specific as possible, including name of the private or public sponsor, agency or source names, agency programs that would be the source of funding, timelines for proposal submissions and potential amounts of funding.
- Work and Management Plan: Description of the leadership experience of the PI, how each participating key personnel contributes to the team, how the inter-disciplinary team will function

as a unit and form a greater whole that promote new ways of thinking to push disciplinary boundaries. This section should also include an outline of the plan of work, with milestones to be achieved in the first year of funding, and metrics to gauge success throughout the course of the two years of funding.

Budget

Please use the provided budget template form. A single two-year budget with justification. Funds may be used for any legitimate research-related expense, and may include minimal faculty effort. Funds cannot be used for administrative support, support of workshops/symposia, or F&A costs. Additionally, funds should not be used to develop small grants program. Use of funds for equipment purchase is discouraged, but if new equipment is required to meet the goals and milestones of the project, a detailed justification will need to be included. Since the goal of this program is to grow UNC's research enterprise, if funds are to be requested for subawards, they should be kept at a minimum and clearly justified. Please note that the requested funds for Year 1 should specifically address how the milestones outlined in the Research Plan for Year 1 will be achieved.

Please provide a Budget Justification limited to no more than 2 pages.

Biosketches

Biosketches of the PI(s) and all key personnel that include relevant publications and research funding to date. Please use one of the following formats:

- NIH Biosketch
- NSF Biosketch and a list of currently funded projects from your latest NSF Current and Pending Support document

Additionally, biosketches should provide a statement that describes how the included faculty's contribution and expertise are both required and sufficient to accomplish the proposal's stated goals.

Letters of Support

Applications including any cost-share/match should include a letter of support from the entity providing these funds. Additionally, letters of support from non-funded collaborators may be included.

Review

Full proposals will be reviewed and ranked by a panel that includes university research associate/vice deans, center/institute directors, and faculty with relevant expertise. The Associate Vice Chancellor for Research/Office of Research Development will provide guidance and oversight of the review process.

Rankings for full proposals will be based on the following criteria:

- Innovation and originality of the proposed research program as a solution to the stated challenge in the field.
- Clear articulation of the importance of the challenge and the impact that the ideas for the solution can provide to benefit society, culture, community, the environment or the economy, including the State of North Carolina.
- Impact of the proposed research program on the advancement of interdisciplinary research.

- Qualifications and track record of the PI and project faculty, synergy among team members' expertise to enable research and promote new ways of thinking that push disciplinary boundaries, and how the team will work as a unit.
- Likelihood that the proposed research program will lead to large-scale funding support by government agencies, corporations, private foundations, and/or venture capital and likelihood for long-term sustainability of the research program via extramural funding.
- Likelihood that the proposed research program will be successful.
- How well the proposed research program advances UNC's preeminence in the OVCR's strategic research areas, if relevant.
- Clear description and feasibility of the milestones and metrics.
- A coherent management and work plan that clearly describes feasibility of the milestones and metrics.
- Reasonableness of the budget.

Prior to the finalization of rankings, the proposal teams will be required to make an oral presentation to the review panel and to guest reviewers who are experts in the field. Ranked proposals will be considered by the Vice Chancellor for Research and other UNC Executive Officers, and final selection will be made. PIs of the proposal selected for funding will be notified by the end of June, and funds will be made available July 1, 2019.

Decision Regarding Second Year of Funding

An advisory committee consisting of the representatives from the Office of the Vice Chancellor for Research, the Office or Research Development, Office of Technology Commercialization, and faculty with relevant expertise for each Creativity Hub will advise/track progress against proposed metrics. The Office of Research Development will schedule meetings every 3 months to assess progress, ensure funds are being spent as planned, and help the team overcome any roadblocks that may have arisen. Eleven months after funds are made available, a two-page report describing accomplishments to date, as well as a complete financial accounting of fund expenditures, will be due to the Office of Research Development. Decision regarding the release of funds for the second year of funding will be made by the Vice Chancellor for Research within two weeks. Criteria to be employed to review accomplishments to date and assess research program progress include:

- Are the accomplishments to date consistent with the timeline and metrics presented in the full proposal? If not, do reasonable extenuating circumstances explain lack of progress?
- Are project expenditures to date consistent with progress of the research program and the budget accompanying the full proposal?
- Have there been any changes to the timeline for research or plans for the solicitation of extramural funding?

Assistance from the Office of Research Development for Creativity Hub-Assisted Large-Scale Proposals

Creativity Hubs awardees are guaranteed proposal development assistance from the Office for any large-scale follow-on funding opportunities that spring from Creativity Hub funding. Please contact the Office of Research Development well in advance of the due date for any follow-on funding proposals to ensure that your proposal can be prioritized.

Presentations to Campus and Lay Audiences

Awardees should expect to be called upon to develop and deliver a presentation on their projects suitable for campus leadership or lay audiences.

Final Report

On or before two months after the end of the final year of Creativity Hub funding, a two-page final report will be due to the Office of Research Development that details accomplishments to date. This report should describe research accomplishments as well as tangible results, such as publications, presentations at symposia, patents, and grant submissions/awards that were produced/obtained as a consequence of funding provided by the Creativity Hubs program. Plans for the submission of proposals for extramural funding should be included, and the Office of Research Development should be notified of all subsequent awards.

Questions

For all questions related to this program, please write to creativityhubs@unc.edu. Please also see our FAQ website.

I. OVERVIEW OF THE FUNDING OPPORTUNITY

United States Army Medical Research and Materiel Command

DEPARTMENT OF DEFENSE

BROAD AGENCY ANNOUNCEMENT
for Extramural Medical Research

Funding Opportunity Number: W81XWH18SBAA1

Announcement Type: Initial

Catalog of Federal Domestic Assistance Number: 12.420
Military Medical Research and Development

KEY DATES

Release/Posted Date: October 1, 2017

Closing Date: September 30, 2022, 11:59 p.m. Eastern Time

This Funding Opportunity Announcement is a Broad Agency Announcement (BAA). It is continuously open for a 5-year period, from October 1, 2017 through September 30, 2022, 11:59 p.m. Eastern Time.

This Broad Agency Announcement must be read in conjunction with the General Submission Instructions, which are available for downloading from Grants.gov. The General Submission Instructions are located under the "package tab" and can be downloaded by selecting the "Download Instructions" icon when previewing the submission package.

Table of Contents

I. OVERVIEW OF THE FUNDING OPPORTUNITY 1

I.A. New for Fiscal Year 2018 4

II. DETAILED INFORMATION ABOUT THE FUNDING OPPORTUNITY 5

II.A. Program Descriptions 6

II.A.1. Military Infectious Diseases Research Program 6

II.A.2. Combat Casualty Care Research Program 6

II.A.3. Military Operational Medicine Research Program 7

II.A.4. Clinical and Rehabilitative Medicine Research Program 8

II.A.5. Medical Biological Defense Research Program 9

II.A.6. Medical Chemical Defense Research Program 10

II.A.7. Medical Simulation and Information Sciences Research Program 11

II.A.8. Radiation Health Effects Research Program 12

II.A.9. Other Information 12

II.B. Award Information 14

II.B.1. Funds Available and Anticipated Number of Awards 14

II.B.2. Award Amounts and Periods of Performance 14

II.B.3. Mechanisms of Support 15

II.C. Eligibility Information 15

II.C.1. Eligible Applicants 15

II.C.2. Cost Sharing 16

II.C.3. Other 16

II.D. Proposal/Application Submission Information 17

II.D.1. Where to Obtain the Proposal/Application Submission Package 17

II.D.2. Content and Form of the Proposal/Application Submission for Research Awards Not Including a Clinical Trial 17

II.D.3. Dun and Bradstreet Data Universal Numbering System (DUNS) Number and System for Award Management (SAM) 34

II.D.4. Submission Dates and Times 34

II.D.5. Intergovernmental Review 35

II.D.6. Funding Restrictions 35

II.D.7. Other Submission Requirements 35

II.E. Proposal/Application Review and Selection Information 35

II.E.1. Criteria for Research Proposals/Applications Not Including a Clinical Trial 35

II.E.2. Criteria for Research Proposals/Applications Including a Clinical Trial 37

II.E.3. Criteria for Conference or Symposium Support Proposals 41

II.E.4. Proposal/Application Selection Process 42

II.E.5. Integrity and Performance Information 43

II.E.6. Notification of Proposal/Application Review Results 43
II.F. Award Administration Information 43
II.F.1. Award Notices 43
II.F.2. Administrative and National Policy Requirements 44
II.F.3. Reporting Requirements 44
II.G. Agency Contacts 44
II.G.1. CDMRP Help Desk 44
II.G.2. Grants.gov Contact Center 45
II.H. Other Information 45
II.H.1. Contractor/Recipient Qualification 45
II.H.2. Proprietary Information 45
II.H.3. Administrative Actions 46
II.H.4. Common Submission Problems 47
II.I. Research Proposal/Application Not Including a Clinical Trial Submission Checklist 49
II.J. Research Proposal/Application Including a Clinical Trial Submission Checklist 50
II.K. Conference and Symposium Support Proposal Checklist 52
APPENDIX I: RESEARCH AREAS OF INTEREST 53
I. Military Infectious Diseases Research Program 53
II. Combat Casualty Care Research Program 54
III. Military Operational Medicine Research Program 55
IV. Clinical and Rehabilitative Medicine Research Program 57
V. Medical Biological Defense Research Program 59
VI. Medical Chemical Defense Research Program 61
VII. Medical Simulation and Information Sciences Research Program 63
VIII. Radiation Health Effects Research Program 70
APPENDIX II: CLINICAL TRIALS 71
APPENDIX III: DOD AND VA WEBSITES 87
APPENDIX IV: PROCEDURES FOR ACQUISITION, PROCESSING, STORAGE, AND SHIPMENT OF BIO-FLUIDS 90
APPENDIX V: NATIONAL INSTITUTE OF MENTAL HEALTH DATA ARCHIVE — INFORMED CONSENT 91
APPENDIX VI: ACRONYMS AND ABBREVIATIONS 92

I.A. New for Fiscal Year 2018

The Fiscal Year 2018 – Fiscal Year 2022 (FY18-FY22) U.S. Army Medical Research and Materiel Command's (USAMRMC) Broad Agency Announcement (BAA) for Extramural Medical Research contains several changes from previous USAMRMC BAAs. Read each section carefully. Note the following:

- The open period of the BAA has been extended to 5 years and will be amended annually with any updates.
- The format of the BAA has been revised to conform to Chapter II of Title 2, Code of Federal Regulations (CFR), Section 200.203 "Notices of funding opportunities."
- The "Research Areas of Interest" can be found in Appendix I.
- For assistance agreements:
    - The total period of performance may be proposed for up to 4 years in length; additional periods may be considered.
    - Any assistance agreement (grant or cooperative agreement) awarded under this BAA will be governed by the award terms and conditions that conform to the Department of Defense's (DoD) implementation of Office of Management and Budget (OMB) guidance in 2 CFR part 200, "Uniform Administrative Requirements, Cost Principles, and Audit Requirements for Federal Awards." DoD implementation is located in Chapter XI of Title 2, CFR.
- For contract awards:
    - The total period of performance may be proposed for up to 5 years in length.
    - The total period of performance for conference or symposium support contracts may be proposed for up to 2 years in length.

II. DETAILED INFORMATION ABOUT THE FUNDING OPPORTUNITY

The USAMRMC mission is to provide solutions to medical problems of importance to the American Service member at home and abroad, as well as to the general public at large. The scope of this effort and the priorities attached to specific projects are influenced by changes in military and civilian medical science and technology, operational requirements, military threat assessments, and national defense strategies. Extramural research and development programs play a vital role in the fulfillment of the objectives established by the USAMRMC. General information on USAMRMC can be obtained at http://mrmc.amedd.army.mil/index.cfm.

This BAA is intended to solicit extramural research and development ideas using the authority provided by United States Code, Title 10, Section 2358. The BAA is issued under the provisions of the Competition in Contracting Act (CICA) of 1984 (Public Law 98-369), as implemented in Federal Acquisition Regulation (FAR) 6.102(d)(2) and 35.016 and in DoD Grant and Agreement Regulations (DoDGAR) 22.315. In accordance with FAR 35.016, projects funded under this BAA must be for basic and applied research to support scientific study and experimentation directed toward advancing the state of the art or increasing knowledge or understanding rather than focusing on development of a specific system or hardware solution. Research and development funded through this BAA are intended and expected to benefit and inform both military and civilian medical practice and knowledge.

The selection process is highly competitive, and the quantity of meaningful submissions (both pre-proposals/pre-applications and full proposals/applications) received typically exceeds the number of awards that available funding can support.

This BAA provides a general description of USAMRMC's research and development programs, including Research Areas of Interest, evaluation and selection criteria, pre-proposal/ pre-application and full proposal/application preparation instructions, and general administrative information. Specific submission information and additional administrative requirements can be found in the document titled "General Submission Instructions" available in Grants.gov along with this BAA.

The execution management agent for this BAA will be the Congressionally Directed Medical Research Programs (CDMRP). The CDMRP manages the electronic Biomedical Research Application Portal (eBRAP) system and retrieval and processing of full proposal/application submissions from Grants.gov. Refer to Section II.G, Agency Contacts, for additional information.

The USAMRMC's supporting acquisition office, the U.S. Army Medical Research Acquisition Activity (USAMRAA), will be the awarding and administering office for proposals/applications selected for funding, unless approval is obtained from the USAMRAA Principal Assistant Responsible for Contracting to allow another Federal acquisition office to execute and administer an award.

II.A. Program Descriptions

II.A.1. Military Infectious Diseases Research Program

The Military Infectious Diseases Research Program (MIDRP) focuses on vaccines, drugs, vector detection assays, and novel therapeutics to treat multidrug-resistant organisms in combat wound infections, as well as vector control measures for insect vectors that transmit naturally occurring endemic diseases with demonstrated or potential capability to decrease military operational effectiveness. Malaria, dengue, and bacterial diarrhea are the main areas of interest for the MIDRP. The MIDRP also has programs in multidrug-resistant bacteria and fungi, Rickettsial diseases, emerging infectious diseases (e.g., chikungunya virus, Zika virus) not found on the Defense Threat Reduction Agency (DTRA) biothreat list. The MIDRP does not support proposals/applications for funding research on any biological or chemical warfare threats or cancer.

Research efforts that focus on novel technologies and/or Investigational New Drug (IND)-enabling preclinical and clinical studies to facilitate the development of preventive and treatment therapies for the above-mentioned research areas are of interest to the MIDRP.

The MIDRP is also interested in proposals/applications incorporating a systems biology approach. Systems biology is the study of systems of biological components, which may be molecules, cells, organisms, or entire species. For more information, refer to Section II.D.2.b.i, Attachment 7, Data- and Research Resource-Sharing Plan.

Research Areas of Interest to the MIDRP are found in Appendix I.I. Applicants are urged to read and consider these before preparing their proposals/applications.

II.A.2. Combat Casualty Care Research Program

The Combat Casualty Care Research Program (CCCRP) provides integrated capabilities for current and future operations to reduce the mortality and morbidity associated with major combat-related trauma across the spectrum of combat casualty care including point of injury and pre- or out-of-hospital care, the spectrum of en-route care, and facilities-based treatment. A primary emphasis of the CCCRP is to identify and develop medical techniques, knowledge products, and materiel[1] (medical devices, drugs, and biologics) for early intervention in life-threatening battle injuries and prolonged field care[2] (PFC). Because battlefield conditions impose severe constraints on available manpower, equipment, and medical supplies available for casualty care, the CCCRP places a premium on medical interventions that can be used within the battle area or as close to it as possible, before or during medical evacuation. Preferred medical techniques and materiel that can be used by combat medics must be easily transportable (i.e.,

[1] Materiel is defined as equipment and supplies of a military force.

[2] Prolonged field care is defined as field medical care, applied beyond "doctrinal planning timelines" by a North Atlantic Treaty Organization (NATO) Special Operations Combat Medic (NSOCM) or higher, in order to decrease patient mortality and morbidity. PFC utilizes limited resources and is sustained until the patient arrives at an appropriate level of care. Rasmussen TE, Baer DG, Cap AP, et al. 2015. Ahead of the Curve. *J Trauma Acute Care Surg* 79: S61-64.

small, lightweight, and durable in extreme environments and handling); devices must be easy to use and require low maintenance, with self-contained power sources as necessary. The CCCRP is interested in existing materiel for which concept and/or patient care efficacy have already been demonstrated, but that require improvement to meet military requirements. The CCCRP is also interested in proposals/applications incorporating a systems biology approach. For more information, refer to Section II.D.2.b.i, Attachment 7, Data- and Research Resource-Sharing Plan.

Research efforts are needed in principles and technologies to enhance self- and buddy-aid, also referred to as tactical care; techniques, methods, or materiel to improve basic and advanced life support for all injured persons; monitoring, sustainment, and management of all injured casualties during episodes of delayed care or PFC; and enhanced capability for triage of large numbers of casualties and staged treatment in the field. The principal causes of death among Service members who die within the first hour of wounding are hemorrhage and traumatic brain injury (TBI).

The CCCRP supports additional aspects of casualty care. These include drugs, devices, and/or novel surgical techniques to decontaminate, debride, protect, monitor, repair, and/or stabilize hard and soft tissue wounds to mitigate secondary tissue damage; orthopaedic and maxillofacial trauma repair strategies; and the prevention and/or mitigation of wound infection and disease in austere environments. The CCCRP is also interested in the development of sensors; diagnostic and prognostic algorithms; data gathering or capture modalities; processors to improve our capability for remote triage, monitoring, and management of casualties; and products to maintain casualties during prolonged evacuation.

The CCCRP also supports the conduct of military-relevant clinical research aimed at translating knowledge or materiel from basic and preclinical trauma research into clinical practice. This includes, but is not limited to, single- and multi-center clinical trials performed in the civilian setting to clarify the safety, efficacy, and optimal use of products stemming from the previously mentioned research areas.

The CCCRP supports the conduct of military-relevant, large data research projects including the use of large databases of common elements from trauma research projects (preclinical, translational, and clinical). Such studies should directly contribute to or effectively enable the data-driven conduct of combat casualty care. Examples include, but are not limited to, post-hoc analysis of data from completed trauma research projects, meta-analyses of a number of otherwise separate but completed studies, and the ability to harmonize data from planned or ongoing but otherwise separate research studies.

The Research Areas of Interest to the CCCRP are found in Appendix I.II. Applicants are urged to read and consider these before preparing their proposals/applications.

II.A.3. Military Operational Medicine Research Program

The mission of the Military Operational Medicine Research Program (MOMRP) is to develop effective countermeasures against military-relevant stressors and to prevent physical and psychological injuries during training and operations in order to maximize the health, readiness,

and performance of Service members and their families, in support of the Army Human Performance Optimization and Enhancement, Human Dimension, Multi-Domain Battle, and the DoD Total Force Fitness concepts.

The MOMRP provides the planning, programming and budgeting of biomedical research to deliver products and solutions to Service members and their families that address readiness, health, and performance throughout the deployment cycle and Service member lifecycle. The MOMRP is centered on cutting-edge scientific research and bringing "Science to the Service member" on the battlefield and at home in a relevant, timely manner.

The MOMRP supports research focused on solving critical problems facing the military today and in the future. Service- and platform-specific issues are addressed through close coordination with all Services to prevent unnecessary duplication of effort.

The MOMRP is divided into four Research Areas of Interest (with examples of types of research efforts shown in parentheses): (1) *Environmental Health and Protection* (performance optimization and biomarker validation during heat/cold/altitude exposures); (2) *Injury Prevention and Reduction* (countermeasures against aviation stressors; blast, blunt trauma, and accelerative injury prevention strategies; neurosensory injury protection; injury return-to-duty standards and strategies; and physiological mechanisms of musculoskeletal injury); (3) *Physiological Health and Performance* (performance and recovery nutrition, weight balance optimization, cognitive health and performance sustainment in the face of operational challenges, restorative sleep, and establishment of a physiological basis for resilience to operational and environmental stressors); and (4) *Psychological Health and Resilience* (post-traumatic stress disorder [PTSD], suicide prevention, resilience [including military families], substance abuse prevention, and violence prevention within the military).

More information on these Research Areas of Interest can be found in Appendix I.III.
Applicants are urged to read and consider these before preparing their proposals/applications.

The MOMRP is particularly interested in proposals/applications and products that incorporate integrated biomedical approaches (e.g., systems biology). Proposals/Applications are encouraged to leverage existing resources and infrastructure to support lifecycle logistics and sustainability. For more information, refer to Section II.D.2.b.i, Attachment 7, Data- and Research Resource-Sharing Plan.

II.A.4. Clinical and Rehabilitative Medicine Research Program

The Clinical and Rehabilitative Medicine Research Program (CRMRP) focuses on the innovations required to reset our wounded Service members, both in terms of duty performance and quality of life. Innovations developed from CRMRP-supported research efforts are expected to improve restorative treatments and rehabilitative care to maximize function for return to duty (RTD) or civilian life. Medical technologies (drugs, biologics, and devices) and treatment/rehabilitation strategies (methods, guidelines, standards, and information) that will significantly improve the medical care our wounded Service members receive within the DoD healthcare system are of particular interest. Implementation of these technologies and strategies should improve the rate of RTD of Service members, the time to RTD, clinical outcome

measures, and quality of life, as well as reduce the hospital stay lengths, clinical workload (patient encounters, treatments, etc.), and initial and long-term costs associated with restorative and rehabilitative or acute care.

Development and validation of in-vitro and in-vivo assessment models that represent military-relevant conditions in wounded Service members, as well as those that incorporate a systems biology approach, are of interest to the CRMRP when they can be used to identify and describe, in a predictable manner, the safety and efficacy of novel technologies in patients. For more information, refer to Section II.D.2.b.i, Attachment 7, Data- and Research Resource-Sharing Plan.

The CRMRP focuses its efforts on the following research areas: neuromusculoskeletal injury (including limb trauma and amputation), sensory systems impairment (including hearing, balance, tinnitus, and vision), acute and chronic pain, and regenerative medicine. While research topics of highest priority and interest are listed in Appendix I.IV for each of these areas, proposals/applications for topics that align within an overall research area will also be considered, except as specifically noted. TBI research proposals/applications will only be considered if the focus is related to one or more of the following: hearing, balance, tinnitus, vision, or pain related to TBI. Novel manufacturing technologies necessary to translate innovative therapies or devices into clinical development are a focus.

All projects should adhere to a core set of reporting standards for rigorous study design. The CRMRP strongly encourages award recipients to follow the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines (http://www.elsevier.com/__data/promis_misc/622936arrive_guidelines.pdf). While these standards are written for animal studies, the basic principles of randomization, blinding, sample size estimation, and data handling derive from well-established best practices in clinical studies and should be applied to those projects as well.

The Research Areas of Interest to the CRMRP are found in Appendix I.IV. Applicants are urged to read and consider these before preparing their proposals/applications.

II.A.5. Medical Biological Defense Research Program

The DTRA Joint Science and Technology Office for Chemical and Biological Defense (JSTO-CBD) manages research directed toward medical biological defense. The DTRA JSTO-CBD has limited funding for proposals/applications submitted through the USAMRMC BAA. DTRA also seeks proposals/applications for its requirements through the Federal Business Opportunities (FedBizOpps) and the DoD Small Business Innovation Research (SBIR) Program solicitations. For information regarding DTRA business opportunities, visit the website at http://www.dtra.mil/Contracts/BusinessOpportunities.aspx.

The Medical Biological Defense Research Program (MBDRP) provides medical countermeasures for biological warfare agents. These countermeasures include specialized medical materiel or procedures designed to enhance protection. The priorities of the program are (a) prophylaxis or pretreatment to prevent any casualty; (b) identification and diagnosis of biological agents; and (c) treatment or supportive care regimens. The MBDRP is interested in

applications and products incorporating a systems biology approach. For more information, refer to Section II.D.2.b.i, Attachment 7, Data- and Research Resource-Sharing Plan.

Examples of some of the infectious agents of interest are those causing anthrax, plague, and glanders; the Ebola, Marburg, Venezuelan, Western and Eastern equine encephalitis viruses; and poxvirus models of variola virus. Examples of toxins of interest include those from plants (Ricin) and bacteria (Staphylococcal enterotoxins, botulinum).

The Research Areas of Interest to the MBDRP are found in Appendix I.V. Applicants are urged to read and consider these before preparing their proposals/applications.

II.A.6. Medical Chemical Defense Research Program

The DTRA JSTO-CBD manages research directed toward medical chemical defense; the program has limited funding for proposals/applications submitted through the USAMRMC BAA. DTRA also seeks proposals/applications for its requirements through FedBizOpps and DoD SBIR program solicitations. For information on DTRA business opportunities, visit its website at http://www.dtra.mil/Contracts/BusinessOpportunities.aspx.

The Medical Chemical Defense Research Program (MCDRP) seeks to preserve combat effectiveness through timely provision of medical countermeasures in response to Joint Service Chemical Warfare Defense Requirements. The fundamental orientation of the program is to protect U.S. Forces from the effects of chemical warfare agents by developing protective, pretreatment, and prophylactic products, providing products usable by the individual Service member for immediate treatment of chemical warfare agent exposures, developing antidotes/therapeutics to chemical warfare agents, defining care procedures for chemical warfare agent casualties, and advancing management of these casualties. The medical countermeasures are intended to preserve and sustain the Service members' combat effectiveness in the face of combined threats from chemical and conventional munitions on the integrated battlefield. The MCDRP is interested in applications and products incorporating a systems biology approach. For more information, refer to Section II.D.2.b.i, Attachment 7, Data- and Research Resource-Sharing Plan.

The broad goals of this program are described below:

a. Maintain the technological capability to meet present requirements and counter future chemical warfare agent threats: The program will maintain the scientific base and technological capability to develop timely medical countermeasures for both current and future chemical warfare agent threats. Research funded by this program will be used to identify concepts and candidate medical countermeasures for use by the individual Service member or by medical personnel. Basic and applied research are both supported and may address topics as diverse as determining sites/mechanisms of action and effects of exposure to chemical warfare agents with emphasis on exploitation of neuroscience technology and respiratory, ocular, and dermal pathophysiology; identifying sites and biochemical mechanisms of action of medical countermeasures; exploiting molecular biological and biotechnological approaches for development of new approaches for medical countermeasures to chemical warfare agents; and

exploiting molecular modeling and quantitative structure-activity relationships in support of drug discovery and design.

b. Provide medical countermeasures for the individual Service member to maintain combat effectiveness and prevent or reduce injury from chemical warfare agents: This goal encompasses research supporting development of new concepts for prophylaxes, pretreatments, antidotes, and therapeutic countermeasures; development of skin protectants and decontaminants; identification of factors that influence safety and efficacy of candidate medical countermeasures; and development and maintenance of preformulation, formulation, and radiolabeling capabilities.

c. Provide medical management of chemical casualties to enhance survival and expedite the RTD of chemical warfare agent casualties through definitive therapies and life support technologies: This goal includes developing concepts and therapeutic regimens and procedures for the management of chemical warfare agent casualties; developing diagnostic and prognostic indicators for chemical warfare agent casualties; and developing life-support equipment for definitive care of chemical warfare agent casualties.

Recent changes in the security situation facing the U.S. have not materially reduced the threat that chemical weapons present to American Forces in the field. Many countries and terrorist groups have the capability of producing and delivering chemical warfare agents, thus posing a substantial and serious threat to the Armed Forces of the U.S.

Classical chemical agent threat categories include vesicant or blister agents (e.g., sulfur mustard), blood agents (e.g., cyanide), respiratory agents (e.g., phosgene), and nerve agents (e.g., GA or Tabun, GB or Sarin, GD or Soman, and VX).

The Research Areas of Interest to the MCDRP are found in Appendix I.VI. Applicants are urged to read and consider these before preparing their proposals/applications.

II.A.7. Medical Simulation and Information Sciences Research Program

The mission of the Medical Simulation and Information Sciences Research Program (MSISRP) is to explore the implications of models and technology for medical education and for the provision, management, and support of health services in the military. The MSISRP plans, coordinates, and oversees a responsive world-class, tri-Service science and technology program focused on three areas of research. The first area is focused on improving military medical training through medical modeling, simulation, educational gaming, assessment systems, interoperable training platforms, and objective training metrics. The second area is focused on developing, researching, and/or improving technologies and informatics that support Theater and Operational Medicine, such as the capture, movement, storage, usability, use, and sharing of health-related data for better clinical care, strategic planning, process development, and software applications. The third area is focused on the Multi-Domain Battle, an operational environment involving greater dispersion and near isolation over great distances, which is likely to cause severe restrictions on mobility for medical missions and shortfalls in both human and materiel human resources due to area denial challenges. Combat units will need to be more self-sufficient and less dependent on logistical support. Combatant commanders with increased sick or

wounded Soldiers will face degradation of medical resources and encumbered combat effectiveness without new combat casualty management and Force multiplication strategies.

MSISRP is organized into three portfolios, one for each of the three focus areas.

The sub-focus areas and Research Areas of Interest to the MSISRP are found in Appendix I.VII. Applicants are urged to read and consider these before preparing their proposals/applications.

II.A.8. Radiation Health Effects Research Program

The Radiation Health Effects Research Program (RHERP) focuses on developing medical countermeasures for acute ionizing radiation injury. The program has interest in the following research focus areas: post-exposure mitigation of radiation injury; protection and prevention of injury from ionizing radiation exposure (prophylaxis); mechanism of radiation injury; and development of novel biodosimetry tools. Research Areas of Interest to the RHERP are found in Appendix I.VIII. The RHERP is interested in applications and products incorporating a systems biology approach. For more information, refer to Section II.D.2.b.i, Attachment 7, Data- and Research Resource-Sharing Plan.

II.A.9. Other Information

II.A.9.i. Clinical Trial Support

Investigator(s) proposing a clinical trial should refer to Appendix II for a detailed description of the requirements for such an application. The pre-application/pre-proposal submission process should be performed as described in Section II.D.2.a.i. Full applications submission is described in Appendix II.B. Refer to the Clinical Trial Submission Checklist in preparing the full application/proposal.

A clinical trial is defined as a prospective accrual of human subjects where one or more intervention(s) (e.g., device, drug, biologic, surgical procedure, rehabilitative modality, behavioral intervention, or other) is tested on a human subject for a measurable outcome with respect to safety, effectiveness, and/or efficacy. This outcome represents a direct effect on the human subject of that intervention or interaction. The term "human subjects" is used in this BAA to refer to individuals who will be recruited for or who will participate in the proposed clinical trial. For more information, a Human Subject Resource Document is provided at https://ebrap.org/eBRAP/public/Program.htm.

If the proposed clinical trial involves the use of a drug that has not been approved by the U.S. Food and Drug Administration (FDA) for the proposed investigational use, then an IND application to the FDA that meets all requirements under the Code of Federal Regulations, Title 21, Part 312 (21 CFR 312) may be required. *If invited to submit a full application, the investigator(s) must submit an IND application to the FDA on or before the date of application submission. If the investigational product is a device, evidence that an Investigational Device Exemption (IDE) application that meets all requirements under 21 CFR 812 has been submitted to the FDA by the application submission deadline, or that the device is exempt or qualifies for an abbreviated IDE, is required.* The Government reserves the right to withdraw funding if an IND or IDE is necessary but has not been submitted to the FDA prior to the grant submission

deadline, or if documented status of the IND or IDE has not been obtained within 6 months of the award date.

II.A.9.ii. Conference or Symposium Support

The USAMRMC may, on a very limited basis, provide financial support for the management and execution of conferences or symposia that are critical to USAMRMC's mission. Funding for conference or symposium support may require approval outside of USAMRMC and will only be considered if the event significantly furthers the mission of the DoD and has a quantifiable benefit or return on investment. If a conference or symposium proposal is selected for funding, the award will be issued as a contract.

II.A.9.iii. Use of Military and Department of Veterans Affairs (VA) Populations or Resources

PIs are encouraged to integrate and/or align their research projects with DoD and/or VA research laboratories and programs. Collaboration with the DoD or VA is also encouraged. A list of websites that may be useful in identifying additional information about ongoing DoD and VA areas of research interest or potential opportunities for collaboration can be found in Appendix III.

If the proposed research involves access to active duty military and/or VA population(s) and/or resource(s), the PI is responsible for establishing access. If possible, access to target active duty military and/or VA patient population(s) should be confirmed at the time of proposal/application submission. A letter of support, signed by the lowest ranking person with approval authority, should be included for studies involving Service members, Veterans, military and/or VA-controlled study materials, and military and/or VA databases. Use Attachment 2, Supporting Documentation, to provide this documentation.

II.a.9.iv. Data Sharing

The USAMRMC intends that information, data, and research resources generated under awards funded by this BAA be made available to the research community and to the public at large. The Government reserves the right to identify repositories for submission of data for archive. For additional guidance, refer to the General Submission Instructions, Appendix 2, Section K.

A number of research areas utilize Common Data Elements (CDEs) to facilitate the sharing of data to promote collaboration, accelerate research, and advance knowledge in specific topic areas. In accordance with the White House Office of Science and Technology Policy memorandum "Increasing Access to the Results of Federally Funded Research" and Executive Order (EO) 13625 and the National Research Action Plan (NRAP) responding to the EO "Improving Access to Mental Health Services for Veterans, Service Members, and Families (August 31, 2012)," federally funded research is required to be conducted in a manner that promotes public access to scientific data. In cases of psychological health and traumatic brain injury, use of CDEs is required under the NRAP. The USAMRMC strongly encourages applicants to incorporate CDE measures appropriate to each field of study, such as:

- PhenX Core and Specialty collections, which are available in the Mental Health Research, Substance Abuse and Addiction, and Research Domains Collections of the PhenX Toolkit, https://www.phenxtoolkit.org/index.php, in all studies involving human subjects, as applicable.

- TBI CDEs related to Federal Interagency Traumatic Brain Injury Research (FITBIR) informatics system data sharing requirements, which can be found at http://fitbir.nih.gov.

- Spinal cord injury research CDEs developed through the collaboration of the International Spinal Cord Society, the American Spinal Injury Association, and the National Institute of Neurological Disorders and Stroke CDE team, as referenced at http://www.commondataelements.ninds.nih.gov/SCI.aspx.

  If the project includes the collection of bio-fluids, such as blood, saliva, urine, etc., the PI may be required to include a set of collection variables and patient phenotypic data in order to standardize the quality of bio-fluid studies. The suggested procedures for acquisition, processing, storage, and shipment of bio-fluids can be found in Appendix IV.

II.B. Award Information

II.B.1. Funds Available and Anticipated Number of Awards

The funding amount for this BAA is unspecified, and the number of awards is indeterminate and contingent upon funding availability. Selection of research projects is a highly competitive process and is based on the evaluation of the proposal/application's technical merit, programmatic considerations, and the availability of funds. The quantity of meaningful submissions received normally exceeds the number of awards that the available funding can support. Any funding that is received by the USAMRMC and is appropriate for a research area described within this BAA may be utilized to fund proposals/applications.

II.B.2. Award Amounts and Periods of Performance

There are no specified funding limitations identified for a proposal/application submitted under this BAA. A budget should be commensurate with the nature and complexity of the proposed research. Researchers should submit budgets that include the entire period of performance of the research project. Budgets should include all direct and indirect costs, based on supportable, verifiable estimates. The budget for the full proposal/application should not differ significantly from the Pre-Application Budget Summary Form provided in the pre-proposal/pre-application submission.

Period of performance may differ depending upon the type of funding mechanism awarded under this BAA. For an assistance agreement, the total period of performance may be proposed for up to 4 years in length; additional periods may be considered. For research support contract awards, the total period of performance may be proposed for up to 5 years in length. For conference or symposium support contract awards, the total period of performance may be proposed for up to 2 years in length. Because the nature and scope of each proposed research project will vary, it is anticipated that the size and duration of each award will vary. Start dates will vary, depending

on when proposals/applications were submitted and reviewed and the negotiation process. However, no proposal/application submitted under this BAA will be considered for funding after 24 months from the date of submission.

Principal Investigators (PIs) seeking additional or continuation funding must submit new pre-proposals/pre-applications and be invited to submit full proposals/applications.

Refer to the General Submission Instructions, Section III.A.4, for additional information regarding the research and related budget.

II.B.3. Mechanisms of Support

The USAMRMC executes its extramural research program primarily through the awarding of contracts and assistance agreements (grants and cooperative agreements). The type of instrument used to reflect the business relationship between the organization and the Government is at the discretion of the Government, in accordance with the Federal Grant and Cooperative Agreement Act of 1977, as amended, 31 USC[3] 6301-6308, which provides the legal criteria to select a procurement contract or an assistance agreement. Refer to the General Submission Instructions, Appendix 2, for additional information.

II.C. Eligibility Information

II.C.1. Eligible Applicants

II.C.1.a. Organizations

Awards are made to organizations only. Organizations eligible to apply include national, international, for-profit, non-profit, public, and private organizations. Refer to the General Submission Instructions, Appendix 3, for general eligibility information.

NOTE: In accordance with FAR 35.017, Federally Funded Research and Development Centers (FFRDCs) are not eligible to directly receive awards under this BAA. However, teaming arrangements between FFRDCs and eligible organizations are allowed so long as they are permitted under the sponsoring agreement between the Federal Government and the specific FFRDC.

The USAMRMC is committed to supporting small businesses. Small business, Veteran-owned small business, Service-disabled Veteran-owned small business, HUBZone small business, small disadvantaged business, and woman-owned small business concerns must be given the maximum practical opportunity to participate through subawards on research proposals/ applications submitted through the BAA.

Government Agencies Within the United States: Local, state, and Federal Government agencies are eligible to the extent that proposals/applications do not overlap with their fully

[3] United States Code

funded internal programs. Such agencies are required to explain how their proposals/ applications do not overlap with their internal programs.

II.C.1.b. Investigator(s)

Eligible investigators include all individuals, regardless of ethnicity, nationality, or citizenship status, who are employed by, or affiliated with, an eligible organization.

Investigators are cautioned that awards are made to organizations only, not individuals.

II.C.2. Cost Sharing

Cost sharing or matching is not required under this BAA.

II.C.3. Other

Organizations must be able to access .gov and .mil websites in order to fulfill the financial and technical deliverable requirements of the award and submit invoices for payment.

Use of the System for Award Management (SAM) and the Federal Awardee Performance and Integrity Information System (FAPIIS): To protect the public interest, the Federal Government ensures the integrity of Federal programs by striving to conduct business only with responsible organizations. The USAMRMC uses the "Exclusions" within the Performance Information functional area of the SAM and data from FAPIIS, a component within SAM, to verify that an organization is eligible to receive Federal awards. More information about SAM and FAPIIS is available at https://www.sam.gov/. Refer to the General Submission Instructions, Appendix 3, for additional information.

Conflicts of Interest: All awards must be free of conflicts of interest (COIs) that could bias the research results. Prior to award of an assistance agreement or contract, applicants will be required to disclose all potential or actual COIs along with a plan to manage them. An award may not be made if it is determined by the Grants Officer or Contracting Officer that COIs cannot be adequately managed. Refer to the General Submission Instructions, Appendix 1, for additional information.

Review of Risk: The following areas may be reviewed in evaluating the risk posed by an applicant: Financial stability; quality of management systems and operational controls; history of performance; reports and findings from audits; ability to effectively implement statutory, regulatory, or other requirements imposed on non-Federal entities; degree of institutional support; integrity; adequacy of facilities; and conformance with safety and environmental statutes and regulations.

Subcontracting Plan: If the resultant award is a contract that exceeds $700,000 and the offeror is other than a small business, the contractor will be required to submit a subcontracting plan for small business and small disadvantaged business concerns, in accordance with FAR 19.704 and DFARS 219.704. A mutually agreeable plan will be incorporated as part of the resultant contract.

II.D. Proposal/Application Submission Information

II.D.1. Where to Obtain the Proposal/Application Submission Package

To obtain the complete Grants.gov proposal/application package (hereinafter, submission package), including all required forms, perform a Grants.gov (http://www.grants.gov/) basic search using the Funding Opportunity Number **W81XWH18SBAA1**.

Submission is a two-step process requiring both (1) pre-proposal/pre-application submission through eBRAP (https://eBRAP.org/) and (2) full proposal/application submission through Grants.gov or eBRAP, depending on the type of application being submitted.

II.D.2. Content and Form of the Proposal/Application Submission for Research Awards Not Including a Clinical Trial

Investigator(s) proposing a clinical trial should refer to Appendix II for a detailed description of the requirements for such an application.

Submission of a pre-proposal/pre-application is required and must be submitted through eBRAP (https://eBRAP.org/). If the USAMRMC is interested in receiving a full proposal/application, the PI will be sent an invitation via eBRAP to submit. Investigator(s) proposing a clinical trial should refer to Appendix II for a detailed description of the requirements for such an application. eBRAP is a multifunctional web-based system that allows PIs to submit their pre-proposals/ pre-applications electronically through a secure connection, to view and edit the content of their pre-proposals/pre-applications and full proposals/applications, to receive communications from the CDMRP, and to submit documentation during award negotiations and period of performance.

A key feature of eBRAP is the ability of an organization's representatives and PIs to view and modify the full proposal/application submissions associated with them. eBRAP will validate full proposal/application files against the BAA requirements and discrepancies will be noted in an email to the PI and in the Full Application Files tab in eBRAP. It is the applicant's responsibility to review all proposal/application components for accuracy as well as ensure proper sequence as specified in this BAA.

The proposal/application title, eBRAP log number, and all information for the PI, Business Official(s), performing organization, and contracting organization must be consistent throughout the entire pre-proposal/pre-application and proposal/application submission process. Inconsistencies may delay proposal/application processing and limit or negate the ability to view, modify, and verify the proposal/application in eBRAP. If any changes need to be made, the applicant should contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507 prior to the proposal/application deadline.

Because the invitation to submit a proposal/application is based on the contents of the pre-proposal/pre-application, a PI should not change the title or research objectives after the pre-proposal/pre-application is submitted. A PI and organization identified in the pre-proposal/pre-application should be the same as those intended for the full proposal/ application submission. If any changes are necessary after submission of the pre-proposal/

pre-application, the PI must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507. A change in PI or organization after submission of the pre-proposal/pre-application will be allowed only at the discretion of the USAMRAA Contracting or Grants Officer.

The organization, Business Official, and PI must register in eBRAP before submitting a pre-proposal/pre-application. Upon completion of an organization's registration in eBRAP and approval by the CDMRP Help Desk, the organization name will be displayed in eBRAP to assist the organization's Business Officials and PIs as they register. The organization, Business Officials, and PIs must all be registered and affiliated in eBRAP. (See *eBRAP User Guide* at https://ebrap.org/eBRAP/public/UserGuide.pdf.)

Pre-proposals/Pre-applications may be submitted at any time prior to the BAA closing date. Pre-proposals/Pre-applications should describe specific ideas or projects that pertain to any of the areas described under "Program Description" in this BAA. A pre-proposal/pre-application must include a brief description of the scientific methods and design to address the problem as described below. Brochures or other descriptions of general organizational or individual capabilities will not be accepted as a pre-proposal/pre-application. ***DO NOT include any proprietary information in the pre-proposal/pre-application.***

II.D.2.a.i. Step 1: Pre-Proposal/Pre-Application Submission Content

During the pre-proposal/pre-application process, each submission is assigned a unique log number by eBRAP. This unique eBRAP log number will be needed during the full proposal/application submission process.

All pre-proposal/pre-application components must be submitted through eBRAP (https://eBRAP.org/). Because the invitation to submit a proposal/application is based on the contents of the pre-proposal/pre-application, investigators should not change the title or research objectives after the pre-proposal/pre-application is submitted.

PIs and organizations identified in the pre-proposal/pre-application should be the same as those intended for the subsequent proposal/application submission. If any changes are necessary after submission of the pre-proposal/pre-application, the PI must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

The pre-proposal/pre-application consists of the following components, which are organized in eBRAP by separate tabs for each proposal/application type. Select "Research," "Research Including Clinical Trial," or "Conference/Symposium" when initiating the application. Refer to the General Submission Instructions, Section II, for additional information on pre-proposal/ pre-application submission.

- **Tab 1 – Application Information**

  Enter the information as described in eBRAP before continuing the pre-proposal/ pre-application.

- **Tab 2 – Application Contact**

  Enter contact information for the PI. Enter the organization's Business Official responsible for sponsored program administration (the "person to be contacted on matters involving this application" in Block 5 of the Grants.gov SF424 Form). The Business Official must either be selected from the eBRAP list or invited for the pre-proposal/pre-application to be submitted.

  Select the performing organization (site at which the PI will perform the proposed work) and the contracting organization (organization submitting on behalf of the PI, which corresponds to Block 5 on the Grants.gov SF424 (R&R) Form), and click on "*Add Organizations to this Pre-application.*" The organization(s) must be either selected from the eBRAP drop-down list or invited in order for the pre-proposal/pre-application to be submitted.

  It is recommended that PIs identify an Alternate Submitter in the event that assistance with pre-proposal/pre-application submission is needed.

- **Tab 3 – Collaborators and Key Personnel**

  Enter the name, organization, and role of all collaborators and key personnel associated with the pre-proposal/pre-application.

- **Tab 4 – Conflicts of Interest**

  List all individuals other than collaborators and key personnel who may have a conflict of interest (COI) in the review of the pre-proposal/pre-application (including those with whom the PI has a personal or professional relationship).

  Federal agency personnel involved in the review process and/or with making funding recommendations are prohibited from being involved in the research proposed or assisting in any pre-proposal/pre-application, including, but not limited to, concept design, proposal/ application development, budget preparation, and the development of any supporting documentation. ***If formal collaboration with Military Facility personnel is planned (i.e., included in the proposal/application in performance of the research), this prohibition is not applicable. Military Facility is defined as Military Health System (MHS) facility, research laboratory, medical treatment facility, dental treatment facility, or a DoD activity embedded with a civilian medical center. However, these Military Facility personnel cannot be involved in the review process and/or with making funding recommendations.***

  Refer to the General Submission Instructions, Appendix 3, for additional information. For questions related to COI, contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

- **Tab 5 – Required Files**

  NOTE: No figures, charts, graphs, or other additional material will be accepted during the pre-proposal/pre-application process.

Provide responses in the appropriate data fields for the following in eBRAP. **EVERY DATA FIELD MUST CONTAIN COMPLETE INFORMATION.** eBRAP will truncate characters exceeding the limit specified for each data field. Enter "none" if there is no information to be included.

- Problem To Be Studied (4,000 character limit, including spaces).
- Theoretical Rationale, Scientific Methods, and Design (4,000 character limit, including spaces).
- Significance and/or Uniqueness of the Proposed Effort (4,000 character limit, including spaces).
- Military Relevance and Impact (4,000 character limit, including spaces).
- Brief Description of Research Involving Animals, Human Anatomical Substances and/or Human Subjects (4,000 character limit, including spaces).
- Plans and Strategy for Translation, Implementation, and/or Commercialization (4,000 character limit, including spaces).

Upload document(s) as individual PDF file(s). eBRAP will not allow a document to be uploaded in the Required Files tab if the number of pages exceeds the limits specified below.

- Budget Summary: Upload as "BudgetSummary.pdf." Complete the two-page Budget Summary Form (available for download in eBRAP) as instructed.
- PI and Key Personnel Biographical Sketches (five-page limit per individual): Use boldfaced type or highlight titles of publications relevant to the proposed project. All biographical sketches should be uploaded as a single combined file.

Refer to the General Submission Instructions, Section II.C, for detailed information.

- **Tab 6 – Submit Pre-Application**

  This tab must be completed for the pre-proposal/pre-application to be accepted and processed.

Pre-Proposal/Pre-Application Screening

USAMRMC scientists or outside experts will screen pre-proposals/pre-applications for technical merit and programmatic considerations. Based on the screening of the pre-proposal/ pre-application, a PI may be invited to submit a full proposal/application.

Notification of Pre-Proposal/Pre-Application Screening Results

Following the pre-proposal/pre-application screening, PIs will be notified as to whether or not they are invited to submit full proposals/applications; however, they will not receive feedback

(e.g., a critique of strengths and weaknesses) on their pre-proposals/pre-applications. Within 120 days of submission, PIs should receive email notification via eBRAP regarding disposition of their pre-proposals/pre-applications.

II.D.2.a.ii. Step 2: Full Proposal/Application Submission Content

Proposals/Applications will not be accepted unless the PI has received notification of invitation.

All contributors and administrators to the proposal/application must use matching compatible versions of Adobe software when editing and preparing proposal/application components. The use of different software versions will result in corruption of the submitted file. Refer to the General Submission Instructions, Section III, for details on compatible Adobe software.

The CDMRP cannot make allowances/exceptions to its policies for submission problems encountered by the applicant organization using system-to-system interfaces with Grants.gov.

Each proposal/application submission must include the completed full submission package for this BAA. The full submission package is submitted by the Authorized Organizational Representative through Grants.gov (***http://www.grants.gov/***)

Proprietary information should only be included if necessary for evaluation of the proposal/ application. Conspicuously and legibly mark any proprietary information that is included in the proposal/application.

The organization's Business Official or Authorized Organizational Representative (or Resource Manager/Comptroller) should approve/verify the full proposal/application submission prior to the proposal/application verification deadline.

II.D.2.a.iii. Full Proposal/Application Submission Components

The Grants.gov submission package includes the following components (refer to the General Submission Instructions, Section III, for additional information on proposal/application submission). Note that components for a proposal/application including a clinical trial are provided in Appendix II.

- **SF424 (R&R) Application for Federal Assistance Form:** Refer to the General Submission Instructions, Section III.A.1, for detailed information.

  Attachments

  Each attachment to the full proposal/application components must be uploaded as an individual file in the format specified and in accordance with the formatting guidelines listed in the General Submission Instructions, Appendix 4.

  For all attachments, ensure that the file names are consistent with the guidance. Attachments will be rejected if the file names are longer than 50 characters or if they have incorrect file names, i.e., containing characters other than the following: A-Z, a-z, 0-9, underscore, hyphen, space, and period. In addition, there are file size limits that may apply in some

circumstances. Individual attachments may not exceed 20 MB and the file size for the entire full submission package may not exceed 200 MB.

- **Attachment 1: Project Narrative (20-page limit):** Upload as "ProjectNarrative.pdf." There is no form for this information. The attachments must be PDF files in accordance with the formatting guidelines specified for full proposal/application preparation.

  A detailed description of the research to be undertaken should be submitted. This should include the areas provided below and address their relationship to the state of knowledge in the field and to comparable work in progress elsewhere. Evaluation of the proposed research will be influenced by the adequacy of this information.

  Literature references and curriculum vitae will be shown in separate addenda entries. The following general outline should be followed:

  - Background: Provide a brief statement of ideas and theoretical reasoning behind the proposed study. Describe previous experience most pertinent to this proposal/ application. Cite relevant literature references. Include discussion of any findings (if available) from relevant pilot or preliminary work or any related work underway. For development of devices and technologies, provide an intellectual property plan as part of the supporting documentation.

  - Hypothesis: State the hypothesis to be tested and the expected results. For development of devices and technologies, discuss the technical feasibility of the proposed project including background of the problem, previous and current solutions, similar projects previously undertaken, and related development activities.

  - Technical Objectives: State concisely the question to be answered by each research objective.

  - Project Milestones: Identify timelines for critical events that must be accomplished in order for the project to be successful in terms of cost, schedule, and performance. For development of devices and technologies, discuss the timelines and provide a commercialization strategy/plan for the technology being developed.

  - Military Significance: State precisely the estimates as to the immediate and/or long-range usefulness of this study to the Armed Forces, as distinguished from general advancement of knowledge in medicine.

  - Public Purpose: If appropriate, provide a concise, detailed description of how this research project will benefit the general public.

  - Methods: Give details about the experimental design and methodology. If the methodology is new or unusual, describe it in sufficient detail for evaluation. For synthetic chemistry applications, include a clear statement of the rationale for the proposed syntheses. Outline and document the routes to the syntheses. For development of devices and technologies, discuss the engineering/technical design to achieve the project goals demonstrating the feasibility of the proposed product

development. Discuss the perceived engineering/design strengths and flaws and recommendations for overcoming/preventing them. For studies involving human subjects, describe the recruitment plan and access to populations. The proposal/ application should describe a plan for data access and sharing. (Access to subjects and data is the sole responsibility of the investigator.) As relevant, describe plans for addressing issues unique to working with military populations. For studies involving human and animal research, provide a statistical and data analysis plan. Describe the statistical model and data analysis plan with respect to the study objectives as appropriate to the type of study. For clinical studies and applied research involving human subjects, specify the approximate number of human subjects that will be enrolled. If multiple study sites are involved, state the approximate number to be enrolled at each site. Include a complete power analysis to demonstrate that the sample size is appropriate to meet the objectives of the study. When possible, investigators should write protocols for research with human subjects and/or human anatomical substances that are specific to the DoD-supported effort outlined in the submitted application. Submission to HRPO of protocols covering more than the scope of work in the DoD-funded proposal will require HRPO review of the entire protocol as DoD-supported research and may include extensive modifications to meet DoD human subjects protection requirements.

- Additional Information: If human subjects, animals, or cadavers are involved in the research, proposals/applications may be submitted prior to obtaining human, animal, or cadaver protocol institutional approvals. However, protocols with required institutional approvals must be submitted no later than 60 days after award to demonstrate continued progress and ensure continuation of payment. The Contracting or Grants Officer may make exceptions in situations where human and/or animal use is not expected to begin until after the first year of the research project. In such cases, a timeframe for submission of the appropriate protocols and institutional approvals will be established prior to award.

PIs and collaborating organizations may not use, employ, or subcontract for the use of any human participants, including the use of human anatomical substances, human data, and/or human cadavers, or laboratory animals until applicable regulatory documents are reviewed and approved by the USAMRMC Office of Research Protections (ORP) to ensure that DoD regulations have been met.

- For studies with prospective accrual of human subjects, indicate quarterly enrollment targets.

- For use of human anatomical substances, identify the commercial or organizational source(s) of the material. For cell lines, identify cell line(s) to be used. If human anatomical substances (including cell lines) will be used, specify whether or not identifiable information is accessible to the research team by any means.

- If applicable, indicate time required for submission and/or approval of documents (e.g., IND and IDE) to the FDA or appropriate Government regulatory agency.

- For studies involving human subjects, allow at least 2 to 3 months for regulatory review and approval by the USAMRMC Human Research Protection Office (HRPO); this does not include the additional time required for local Institutional Review Board (IRB) review and approval, as stated above.

- For animal studies, allow at least 2 to 3 months for regulatory review and approval by the USAMRMC Animal Care and Use Review Office; this does not include the additional time required for local Institutional Animal Care and Use Committee review and approval, as stated above.

- Refer to the General Submission Instructions, Appendix 1, for additional regulatory information.

- **Attachment 2: Supporting Documentation:** The attachment should be in PDF in accordance with the formatting guidelines specified for full proposal/application preparation. Start each document on a new page. Combine and upload as a single file named "Support.pdf." If documents are scanned to pdf, the lowest resolution (100 to 150 dpi) should be used. ***There are no page limits for any of these components unless otherwise noted.***

  - Bibliography and References Cited: List the references in the order they appear in the proposal/application narrative. Use a reference format that gives the title of the citation. Do not send or attach copies of articles in print. There is no form for this information.

  - List of Abbreviations, Acronyms, and Symbols: Provide a list of abbreviations, acronyms, and symbols.

  - Facilities and Other Resources: Describe the facilities available for performance of the proposed research project and any additional resources proposed for acquisition at no cost to the award. Indicate if a Government-owned facility is proposed for use. Reference should be made to the original or present award under which the facilities or resources are now accountable. There is no form for this information.

  - Equipment: Include a description of existing equipment to be used for the proposed research project.

  - Publications and/or Patent Abstracts (five-document limit): Include relevant publication URLs and/or patent abstracts. If publications are not publicly available, then a copy/copies of the published manuscript(s) must be attached.

  - Letters of Organizational Support: Provide a letter (or letters, if applicable), signed by the Department Chair or appropriate organization official, confirming the laboratory space, equipment, and other resources available for the project. A letter from each organization involved in the project should be provided.

  - Collaboration: Provide letter(s) supporting stated collaborative efforts necessary for the project's success, even if the collaboration is offered at no cost.

- Letter of Support for Use of Military and VA Populations or Resources (if applicable; one-page limit per letter is recommended): If the proposed research plan involves access to active duty military and/or VA patient populations or resources, include a letter(s) of support, signed by the lowest-ranking person with approval authority, confirming such access. If access cannot be confirmed at the time of proposal/ application submission, the Government reserves the right to withhold or revoke funding until the PI has demonstrated support for and access to the relevant population(s) and/or resources.

- *If the project involves collaboration with a Military Facility, special requirements apply.* A DoD researcher, to include collaborating DoD PIs, must obtain a letter from his/her commanding officer or Military Facility director authorizing his/her participation in the research project. This letter must be included with the proposal/application.

- Joint Sponsorship: Describe present or prospective joint sponsorship of any portion of the program outlined in the proposal/application. In the absence of agreements among sponsors for joint support, the proposal/application should be structured so that the research can be carried out without the resources of any other sponsor. If, however, it is desirable to request partial support from another agency, the proposed plan should be stated and the reasons documented. If the plan cannot be formulated at the time the proposal/application is submitted, information should be sent later as an addendum to the proposal/application. Prior approval from both agencies must be secured for research to be undertaken under joint sponsorship. Provide letters of support related to recruitment, subject access, and data access plans.

- Intellectual Property (if applicable):

  - Background and Proprietary Information: All software and data first produced under the award are subject to a Federal purpose license. A term of the award requires the recipient to grant the Government all necessary and appropriate licenses, which could include licenses to background and proprietary information that have been developed at private expense.

    Therefore, it is important to disclose/list any intellectual property (software, data, patents, etc.) that will be used in performance of the project or provide a statement that none will be used. If applicable, all proprietary information to be provided to the Government should be stated and identified; the applicant should indicate whether a waiver of the Federal purpose license will be required.

  - Intellectual and Material Property Plan: Provide a plan for resolving intellectual and material property issues among participating organizations.

- **Attachment 3: Technical Abstract (one-page limit):** Upload as "TechAbs.pdf." The abstract is vitally important to both the scientific peer and programmatic review processes. In accordance with Section 8123 of the Consolidated and Further Continuing Appropriations Act, 2015 (Public Law 113-235), the PI is required to submit a technical

abstract that fully describes the proposed work. The abstract must contain the title of the project and the name of the PI. Do not include figures or tables in the abstract. Use only characters available on a standard QWERTY keyboard. Spell out all Greek or other non-English letters. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information.*** .

The structured technical abstract should be clear and concise and, at a minimum, provide the following information:

- Background: Provide a brief statement of the ideas and theoretical reasoning behind the proposed work.
- Objective/Hypothesis: State the objective/hypothesis to be tested. Provide evidence or rationale that supports the objective/hypothesis.
- Specific Aims: State concisely the specific aims of the study.
- Study Design: Briefly describe the study design.
- Relevance: Provide a brief statement explaining the potential relevance of the proposed work to the specific topic area being addressed and its impact on health outcomes.

- **Attachment 4: Lay Abstract (one-page limit):** Upload as "LayAbs.pdf." The lay abstract is used by all reviewers. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information.*** Use only characters available on a standard QWERTY keyboard. Spell out all Greek letters, other non-English letters, and symbols. Graphics are not allowed.

  Lay abstracts should be written using the outline below. Do not duplicate the technical abstract.

  - Clearly describe the objectives and theoretical reasoning behind the proposed work in a manner readily understood by readers without a background in science or medicine.
  - Clearly describe the problem or question to be addressed and the ultimate applicability and impact of the research.
    - What types of patients will it help, and how will it help them? Include the current available statistics to the related injury/condition.
    - What are the potential clinical applications, benefits, and risks?
    - What is the projected timeline it may take to achieve the expected patient-related outcome?
    - Describe how the proposed project will benefit Service members, Veterans, and/or their family members.

- **Attachment 5: Statement of Work (SOW) (two-page limit):** Upload as "SOW.pdf." The SOW outlines and establishes the performance expectations and milestones for which the USAMRMC may provide funding. The SOW will be incorporated into the award document and, as such, is subject to release under the Freedom of Information Act. The SOW should identify all collaborating research sites involved in the performance of the research. Suggested SOW formats and examples specific to different types of research projects are available on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm).

  A series of relatively short statements should be included that comprise the approach to each of the major goals or objectives of the proposed research. The statements should outline the specific tasks, systems, key assessments/techniques, and materials that are reasonable estimates for testing the proposed hypotheses of the study. A timeline should be included that shows the work statements to be accomplished in each year of the award. Any animal use and/or human subjects recruitment should be included. Allow at least 2 to 3 months for the USAMRMC ORP regulatory review and approval processes for studies involving human subjects and 2 to 3 months for studies involving animal subjects.

- **Attachment 6: Impact/Outcomes Statement (one-page limit):** Upload as "Impact.pdf." Explain the potential impact of the research in the field, the significance of this impact, and when it can be anticipated. Explain how the results of this research are expected to impact the intended beneficiaries. Expand upon the dual (military and public) purpose for the research, as appropriate.

- **Attachment 7: Data- and Research Resource-Sharing Plan (one-page limit):** ***All data must be shared while ensuring appropriate protection of information.*** Upload as "Sharing.pdf." Describe how unique and/or final research data will be shared with the research community, along with any resulting research resources. This includes cases where pre-existing data or research resources will be utilized and/or modified during the course of the proposed project. If there are limitations associated with a pre-existing agreement for the original data or research resources that preclude subsequent sharing, the applicant should explain this in the data- and/or research resource-sharing plan. For projects involving clinical trials, PIs may be required to register their clinical trials on Clinicaltrials.gov (https://clinicaltrials.gov/). For projects involving TBI, PIs may be required to report data to the FITBIR informatics system (http://fitbir.nih.gov/). If the project includes systems biology-related research, the PI may be required to make the systems biology data, generated via an award, available to the research community by depositing research data into the SysBioCube system (https://sysbiocube-abcc.ncifcrf.gov). For studies that will enroll subjects with psychological health disorders, awardees may be requested to submit data to the National Institute of Mental Health Data Archive (NDA) https://data-archive.nimh.nih.gov. The NDA is a data repository run by the National Institute of Mental Health (NIMH) that allows researchers studying mental illness to collect and share de-identified information with each other. Such studies may require the inclusion of specific language in the informed consent form which references the NDA (see Appendix V).

  Refer to the General Submission Instructions, Appendix 2.K, for additional information.

- **Attachment 8: Transition Plan and Regulatory Strategy:** Upload as "Transition.pdf." Provide information on the methods and strategies proposed to move the anticipated research outcomes (e.g., knowledge, models, devices, technologies, interventions) from this project to the next level (e.g., additional research, clinical trials, delivery to the military or civilian market, incorporation into clinical practice) after successful completion of the award. Applicants are encouraged to work with their organization's Technology Transfer Office to develop the transition plan. The transition plan should include the components listed below, as applicable.

  - Details of the funding strategy that will be used to bring the outcomes to the next level (e.g., specific potential industry partners, specific funding opportunities to be pursued).

  - Description of collaborations and other resources that will be used to provide continuity of development.

  - Brief schedule/timeline and milestones for bringing the outcome(s) to the next level.

  - For knowledge outcomes, a description of what will be further developed and how it will be disseminated, and incorporated into clinical care.

  - Identification of the anticipated regulatory strategy (e.g., additional non-clinical or clinical studies anticipated/required, FDA or regulatory authority meetings desired, industry partnerships) for movement of the research into later phases of development and to support a potential marketing application (e.g., New Drug Application, Biologics License Application).

  - Outline of ownership rights and/or access to the intellectual property necessary for the development and/or commercialization of products or technologies supported with this award and the Government's ability to access such products or technologies in the future.

  - Evidence for involvement of appropriate intellectual property, licensing, and/or business professionals.

  - A risk analysis for cost, schedule, manufacturability, and sustainability.

  - A commercialization strategy including intellectual property, market size, financial analysis, strengths and weaknesses, barriers to market, competitors, and management team. Discuss the significance of this development effort, when it can be anticipated, and the potential commercial use for the technology being developed.

- **Attachment 9: Collaborating DoD Military Facility Budget Form(s), *if applicable*:** Upload as "MFBudget.pdf." If a Military Facility will be a collaborator in performance of the project, complete the Collaborating DoD Military Facility Budget Form (available for download on eBRAP "Funding Opportunities and Forms" web page), including a budget justification for each year. If more than one Military Facility is proposed, submit

a separate budget form for each site. Refer to the General Submission Instructions, Section II.A.4, for detailed information.

Research & Related Senior/Key Person Profile (Expanded): Refer to the General Submission Instructions, Section III.A.3, for detailed information.

- PI Biographical Sketch (five-page limit): Upload as "Biosketch_LastName.pdf."
- PI Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."
- Key Personnel Biographical Sketches (five-page limit each): Upload as "Biosketch_LastName.pdf."
- Key Personnel Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."

Research & Related Budget: Refer to the General Submission Instructions, Section III.A.4, for detailed information.

Budget Justification (no page limit): Upload as "BudgetJustification.pdf." The budget justification for the entire period of performance must be uploaded to the Research & Related Budget after completion of the budget for Period 1.

Project/Performance Site Location(s) Form: Refer to the General Submission Instructions, Section III.A.5, for detailed information.

R & R Subaward Budget Attachment(s) Form (if applicable): Refer to the General Submission Instructions, Section III.A.6, for detailed information.

NOTE: Proposals/Applications from **Federal agencies** must include in their budget justifications a **Federal Financial Plan**. Proposals/Applications from organizations that include **collaborations with DoD Military Facilities** must comply with special requirements. Refer to the General Submission Instructions, Section III.A.4, Research & Related Budget, for detailed information.

II.D.2.b. Content and Form of the Proposal/Submission Information for Conference or Symposium Support

II.D.2.b.i. Where to Obtain the Proposal/Application Submission Package

To obtain the complete Grants.gov proposal package (hereinafter, submission package), including all required forms, perform a Grants.gov (http://www.grants.gov/) basic search using the Funding Opportunity Number **W81XWH18SBAA1**.

Submission is a two-step process requiring both pre-proposal submission and full proposal submission through eBRAP (https://eBRAP.org/).

II.D.2.b.ii. Content and Form of the Proposal Submission

Refer to instructions for research proposal submission for details on the use of eBRAP.

II.D.2.b.iii. Step 1: Pre-Proposal Submission Content

During the pre-proposal process, each submission is assigned a unique log number by eBRAP. This unique eBRAP log number will be needed during the full proposal submission process.

All pre-proposal components must be submitted through eBRAP (https://eBRAP.org/). Because the invitation to submit a proposal is based on the contents of the pre-proposal, investigators should not change the title or research objectives after the pre-proposal is submitted.

PIs and organizations identified in the pre-proposal should be the same as those intended for the subsequent proposal submission. If any changes are necessary after submission of the pre-proposal, the PI must contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

The pre-proposal consists of the following components, which are organized in eBRAP by separate tabs. Refer to the General Submission Instructions, Section II, for additional information on pre-proposal submission.

- **Tab 1 – Application Information**
    - Enter the information as described in eBRAP before continuing the pre-proposal.
- **Tab 2 – Application Contact**
    - Enter contact information for the PI. Enter the organization's Business Official responsible for sponsored program administration. The Business Official must either be selected from the eBRAP list or invited for the pre-proposal to be submitted.
    - Select the performing organization (site at which the PI will perform the proposed work) and contracting organization (organization submitting on behalf of the PI), and click on "Add Organizations to this Pre-application." The organization(s) must either be selected from the eBRAP drop-down list or invited for the pre-proposal to be submitted.
    - It is recommended that PIs identify an Alternate Submitter in the event that assistance with pre-proposal submission is needed.

NOTE: The eBRAP system does not require approval of the pre-proposal by the PI's organization.

- **Tab 3 – Collaborators and Key Personnel**
    - To enable the USAMRMC to avoid COI during the screening and review processes, list the name, organization, and role of all participants in the proposed conference or symposium support, including collaborators, consultants, and subrecipients/subawardees.

- **Tab 4 – Conflicts of Interest**

    - List all individuals other than collaborators and key personnel who may have a COI in the review of the pre-proposal (including those with whom the PI has a personal or professional relationship).

Federal agency personnel involved in the review process and/or with making funding recommendations are prohibited from being involved in the conference or symposium support proposed or assisting in any pre-proposal, including, but not limited to, proposal development, budget preparation, and the development of any supporting documentation. ***If formal collaboration with Military Facility personnel is planned (i.e., included in the proposal), this prohibition is not applicable. However, these Military Facility personnel cannot be involved in the review process and/or with making funding recommendations.***

Refer to the General Submission Instructions, Appendix 3, for additional information. For questions related to COI, contact the CDMRP Help Desk at help@eBRAP.org or 301-682-5507.

- **Tab 5 – Required Files**

    NOTE: Figures, charts, graphs, or other additional material will not be accepted during the pre-proposal process.

    Provide responses in the appropriate data fields for the following in eBRAP. DO NOT LEAVE ANY FIELDS BLANK. eBRAP will truncate characters exceeding the limit specified for each data field. Enter "none" if there is no information to be included.

    - Conference or Symposium Objectives: (4,000 character limit, including spaces).

    - Impact and Outcomes of the Proposed Conference or Symposium: (2,000 character limit, including spaces).

    - Proposed Budget to Include Direct and Indirect Costs: (1,000 character limit, including spaces).

    - PI and Key Personnel Biographical Sketches (five-page limit per individual): Use boldfaced type or highlight titles of publications or specific expertise relevant to the proposed conference or symposium support. All biographical sketches should be uploaded in a single combined file.

    - Refer to the General Submission Instructions, Section II, for detailed information.

- **Tab 6 – Submit Pre-Application:** This tab must be completed for the pre-proposal to be accepted and processed.

Pre-Proposal Screening

USAMRMC scientists or outside experts will screen pre-proposals for technical merit and programmatic considerations. Based on the screening of the pre-proposal, a PI may be invited to submit a full proposal.

Notification of Pre-Proposal Screening Results

Following the pre-proposal screening, PIs will be notified as to whether or not they are invited to submit full proposals; however, they will not receive feedback (e.g., a critique of strengths and weaknesses) on their pre-proposals. Within 120 days of submission, PIs should receive email notification via eBRAP regarding disposition of their pre-proposals.

II.D.2.b.iv. Step 2: Full Proposal Submission Components for Conference or Symposium Support

eBRAP submission package components: For the FY18-FY22 BAA Conference or Symposium Support contract award, the eBRAP submission package includes the following components, which are organized in eBRAP by separate tabs. To access these tabs, go to "My Applications" and click on "Start Full Application" for the log number under which the pre-proposal was submitted.

- **Tab 1 – Summary:** Provides a summary of the proposal information.
- **Tab 2 – Application Contacts:** This tab will be populated by eBRAP. Add Authorized Organizational Representative.
- **Tab 3 – Full Application Files:** Under each Application Component in eBRAP, upload each as an individual PDF file. Refer to the General Submission Instructions, Appendix 4, for detailed formatting guidelines.

  Proposal Component: Attachments: Each attachment must be uploaded as an individual PDF file unless otherwise stated.

  - **Attachment 1: Conference or Symposium Objectives: (five-page limit):** Upload as "Objectives.pdf." The page limit applies to text and non-text elements (e.g., figures, tables, graphs, photographs, diagrams, chemical structures, drawings, etc.) used to describe the conference or symposium. Inclusion of URLs that provide additional information to expand the Conference or Symposium Objectives and could confer an unfair competitive advantage is prohibited and may result in administrative withdrawal of the proposal.

    A detailed description of the conference or symposium to be undertaken should be submitted. This should include the areas listed below and address the relevance to the USAMRMC mission. Evaluation of the proposed conference or symposium will be influenced by the adequacy of this information.

- Literature-relevant publications and curriculum vitae will be shown in separate addenda entries. The following general outline should be followed:

  - Background: Provide a brief statement of ideas and theoretical reasoning behind the proposed conference or symposium. Describe previous experience most pertinent to this proposal such as participation in professional societies and attendance at other relevant meetings. Cite relevant literature references.

  - Technical Objectives: State concisely the question(s) to be answered during the conference or symposium, and how the outcomes of the conference or symposium will benefit the USAMRMC mission.

  - Project Milestones: Identify timelines for critical events that must be accomplished in order for the conference or symposium to be successful in terms of cost, schedule, performance, and outcomes.

- **Attachment 2: Agenda (two-page limit):** Upload as "Agenda.pdf." The proposed agenda should include potential topics and speakers and a schedule format that will meet the conference or symposium objectives.

- **Attachment 3: List of Relevant Publications (two-page limit):** Upload as "References.pdf." Relevant publications should provide evidence for why the conference or symposium, with the proposed agenda and speakers, should be supported.

- **Attachment 4: Statement of Work (two-page limit):** Upload as "SOW.pdf." The SOW outlines and establishes the performance expectations and milestones for which the USAMRMC may provide funding. The SOW will be incorporated into the award document and, as such, is subject to release under the Freedom of Information Act. A series of relatively short statements should be included that comprise the approach to each of the major goals or objectives of the proposed effort. The statements should outline the specific tasks, including but not limited to: planning committee meeting dates, speaker selection, meeting publication plan, invitation and Save-the-Date release dates, compilation of abstract submission guidelines, etc.

- **Attachment 5: Impact/Outcomes Statement (two-page limit):** Upload as "Impact/Outcomes.pdf." Describe the potential impact of the conference or symposium content in the field and the significance of this impact. Describe the potential impact of the conference or symposium for individual attendees at various levels. Provide the milestones and methods for publication and/or peer review of the conference or symposium results and recommendations.

- **Attachment 6: Key Personnel Qualifications (two-page limit):** Upload as "Personnel.pdf." Describe the qualifications, capabilities, and experience of the proposed key personnel to demonstrate that they will be able to achieve the proposed objectives.

- **Attachment 7: Key Personnel Biographical Sketches (five-page limit per biographical sketch):** Upload all biographical sketches as a single document as "Biosketch.pdf."

- **Attachment 8: Budget (no page limit):** Upload as "Budget.pdf." Prepare the budget using the Conference/Symposium Budget form and include a detailed budget justification.

- **Tab 4 – Application and Budget Data:** Review and edit Proposed Project Start Date, Proposed End Date, and Budget Data pre-populated from the Conference/Symposium Budget form.

- **Tab 5 – Submit/Request Approval Full Proposal**: Once all components have been uploaded and prior to the full proposal submission deadline, enter your password in the space provided next to "Enter Your Password Here" and press the "Submit Full Application" button. eBRAP will validate files against the BAA requirements and discrepancies will be noted. If no discrepancies are noted, press the "Confirm Submission" button to complete the proposal submission. eBRAP will notify your Business Official by email to log into eBRAP to review and to approve the proposal prior to the Approval deadline.

  The PI should receive disposition regarding the proposal via an email from eBRAP within 180 days of submission.

II.D.3. Dun and Bradstreet Data Universal Numbering System (DUNS) Number and System for Award Management (SAM)

Applicant organizations and all subrecipient organizations must have a DUNS number to submit proposals/applications to Grants.gov. The applicant organization must also be registered in the Entity Management functional area of the SAM with an "Active" status to submit proposals / applications through the Grants.gov portal. Verify the status of the applicant organization's Entity registration in SAM well in advance of the proposal/applications submission deadline. Allow 3 to 4 weeks to complete the entire SAM registration process.

Applicants must maintain an "Active" SAM status to be qualified to receive federal awards. If an applicant is not fully compliant with the requirements at the time the Federal awarding agency is ready to make a Federal award, the Federal awarding agency may determine that the applicant is not qualified to receive a Federal award and use that determination as a basis for making a Federal award to another applicant.

Refer to the General Submission Instructions, Section III, for further information regarding Grants.gov requirements.

II.D.4. Submission Dates and Times

This is a continuously open announcement through September 30, 2022; therefore, reviews occur throughout the year. Pre-proposals/Pre-applications may be submitted at any time throughout the 5-year period noted above. An invited full proposal/application should be submitted within 90 days of the PI's receipt of an invitation to submit. No pre-proposal/pre-application or full proposal/application may be submitted under this BAA after September 30, 2022, 11:59 p.m. Eastern Time. If an invited proposal/application is not submitted by September 30, 2022, 11:59 p.m. Eastern Time, the applicant must wait for the next available opportunity for submission, i.e., the release of the FY23 BAA (to be posted to Grants.gov October 1, 2022). No

proposal/application received under this BAA will be considered for funding after 24 months from the date of submission.

II.D.5. Intergovernmental Review

This BAA is not subject to EO 12372, "Intergovernmental Review of Federal Programs." The EO provides for state and local government coordination and review of proposed Federal financial assistance and direct Federal development. The EO allows each state to designate an entity to perform this function. This coordination and review is not required under this BAA.

II.D.6. Funding Restrictions

There are no specified funding limitations identified for a proposal/application submitted under this BAA. Refer to the General Submission Instructions, Section III.A.4, "Research & Related Budget," for discussion of allowable costs, including pre-award costs and collaborations with Military Facilities.

II.D.7. Other Submission Requirements

Refer to the General Submission Instructions, Appendix 4, for detailed formatting guidelines.

II.E. Proposal/Application Review and Selection Information

II.E.1. Criteria for Research Proposals/Applications Not Including a Clinical Trial

II.E.1.a. Peer and Programmatic Review

1. **Peer Review:** To determine technical merit, all proposals/applications will be evaluated according to the following scored criteria, which are listed in descending order of importance:

    - **Research Objectives:** The degree to which the stated objectives are clear, valid, and logical. For development of devices and technologies, the degree to which the performance objectives are plausible; the proposed effort demonstrates familiarity with the historical background of the problem and previous/current solutions; and the awareness of similar projects previously undertaken and related activities. The extent to which the proposed research project demonstrates an innovative approach and relates to the Research Areas of Interest identified in Section II.A and Appendix I.

    - **Scientific Design Excellence:** The degree to which proposed plans, methods, techniques, and procedures are feasible, clear, valid, adequately referenced, and state-of-the-art; the merit of the statistical features of the study; the extent to which literature searches were used to document the strengths of the proposed project. For development of devices and technologies, the feasibility of the proposed product/technology development plan; how well the engineering/technical design is likely to achieve the goals indicated; adequacy of the engineering/design solutions; and how well the perceived engineering/design strengths and flaws are addressed. To what degree the

intellectual and material property plan is appropriate, and demonstrates cooperating institutions' willingness and ability to resolve intellectual and material property issues.

- **Impact/Outcomes:** The potential impact of the research in the field, the significance of this impact, and when it can be anticipated. For development of devices and technologies, the potential translation, implementation, and/or commercial use for the product/technology being developed, including whether the funding strategy described to bring the product(s) to the next level of development (e.g., clinical trial, transition to industry, delivery to the market, progression toward incorporation into standard practice) is appropriate and proposed milestones are appropriate and feasible.

- **PI and Key Personnel Qualifications:** The qualifications, capabilities, and experience of the proposed PI and other key personnel to demonstrate that the proposed staff has the knowledge, technical expertise, and management skills to achieve the proposed objectives as well as the time available for the percentage of efforts indicated for the project.

- **Facilities and Resources:** The proposed facilities and equipment, population resources, or unique combinations of these, to demonstrate that the organization has the necessary facilities and resources required for accomplishing the proposed objectives.

- **Budget:** The degree to which the budget reflects the actual needs of the proposed work and is thoroughly detailed and fully justified so that the Government can evaluate and determine the costs to be allocable, allowable and reasonable, and commensurate with the complexity and nature of the research proposed.

2. **Programmatic Review:** To make funding recommendations, the following criteria will be used by programmatic reviewers:

   - Scientific peer review results

   - Adherence to the intent of the award mechanism

   - Program portfolio balance and priorities

   - Relative military benefit

   - Relative innovation, impact, and translatability

   NOTE: Military-relevant research must be responsive to the healthcare needs of the Armed Forces, family members of the Armed Forces, and the U.S. Veteran population. Proposals/ Applications must address a military-relevant health problem responsive to one of the Research Areas of Interest identified in Section II.A and Appendix I.

II.E.2. Criteria for Research Proposals/Applications Including a Clinical Trial

II.E.2.a. Peer and Programmatic Review

1. **Peer Review:** To determine technical merit, all proposals/ applications will be evaluated according to the following scored criteria, which are listed in descending order of importance:

   - **Clinical Impact**
     - How relevant the anticipated outcomes of the proposed clinical trial are to the targeted population.
     - How well the sample population represents the targeted patient population that might benefit from the proposed intervention.
     - How the potential outcomes of the proposed clinical trial will provide/improve short-term benefits for individuals.
     - How significantly the long-term benefits for implementation of the intervention may impact patient care and/or quality of life.
   - **Research Strategy**
     - How well the scientific rationale for testing the intervention is supported by the preliminary data, critical review and analysis of the literature, and/or laboratory/ preclinical evidence.
     - How well the study aims, hypotheses or objectives, experimental design, methods, data collection procedures, and analyses are designed to answer clearly the clinical objective.
     - How well the inclusion and randomization criteria meet the needs of the proposed clinical trial.
     - How well the exclusion criteria are justified.
     - How well plans to collect specimens and conduct laboratory evaluations are addressed, if applicable.
     - To what degree the data collection instruments (e.g., surveys, questionnaires), if applicable, are appropriate to the proposed study.
   - **Statistical Plan**
     - To what degree the statistical model and data analysis plan are suitable for the planned study.

- How the statistical plan, including sample size projections and power analysis, is adequate for the study and all proposed correlative studies.

- Whether the statistical plan compensates for the use of a subpopulation of a recruited sample population to ensure appropriate power can be achieved within the subpopulation study.

- **Intervention**

  - Whether there is evidence of support, indicating availability of the intervention from its source, for the duration of the proposed clinical trial (if applicable).

  - To what degree the intervention addresses the clinical need(s) described.

  - How the intervention compares with currently available interventions and/or standards of care.

  - To what degree t preclinical and/or clinical evidence to support the safety of the intervention is provided.

  - Whether a member of the study team holds the IND/IDE for the indication proposed or whether the timeline proposed for obtaining the IND/IDE is appropriate (if applicable).

  - For investigator-sponsored INDs, whether there is evidence of appropriate institutional support, including capabilities to ensure monitoring as required by the FDA.

  - Whether plans to comply with Good Manufacturing Practices (GMP), Good Laboratory Practices (GLP), and Good Clinical Practices (GCP) guidelines are appropriate.

  - Whether measures are described to ensure the consistency of dosing of active ingredients for nutritional supplements (if applicable).

- **Recruitment, Accrual, and Feasibility**

  - How well the availability of human subjects for the clinical trial and the prospect of their participation is addressed.

  - Whether access to the proposed human subjects population is demonstrated.

  - The degree to which the recruitment, informed consent, screening, and retention processes for human subjects will meet the needs of the proposed clinical trial.

  - How well the application identifies possible delays (e.g., slow accrual, attrition) and presents adequate contingency plans to resolve them.

- To what extent the proposed clinical trial might affect the daily lives of the individual human subjects participating in the study (e.g., Will human subjects still be able to take their regular medications while participating in the clinical trial? Are human subjects required to stay overnight in a hospital?).

- **Ethical Considerations**

  - How the level of risk to human subjects is minimized and how the safety monitoring and reporting plan is appropriate for the level of risk.

  - Whether a research monitor with expertise consistent with the nature of the potential risk(s) is identified, if applicable.

  - How well the evidence shows that the procedures are consistent with sound research design and, when appropriate, that these procedures are already in use for diagnostic or treatment purposes.

  - To what degree privacy issues are appropriately considered.

  - To what degree the process for seeking informed consent is appropriate and whether safeguards are in place for vulnerable populations.

- **Personnel and Communication**

  - Whether the composition of the study team (e.g., study coordinator, statistician) is appropriate.

  - To what degree the study team's background and expertise are appropriate to accomplish the proposed work (e.g., statistical expertise, expertise in the disease, and clinical studies).

  - How the levels of effort of the study team members are appropriate for successful conduct of the proposed trial.

  - How well the logistical aspects of the proposed clinical trial (e.g., communication plan, data transfer and management, standardization of procedures) meet the needs of the proposed clinical trial.

- **Transition Plan and Regulatory Strategy**

  - Whether the identified next level of development and/or commercialization is realistic.

  - Whether the funding strategy described to bring the intervention to the next level of development (e.g., specific industry partners, specific funding opportunities to be applied for) is reasonable and realistic.

- How the regulatory strategy and development plan to support a product label change, if applicable, is appropriate and well described.

- Whether the proposed collaborations and other resources for providing continuity of development, including proposed development or modification of clinical practice guidelines (CPG) and recommendations, provider training materials, patient brochures, and other clinical support tools, scientific journal publications, models, simulations, and applications are established and/or achievable.

- Whether the schedule and milestones for bringing the intervention to the next level of development (next-phase clinical trials, transition to industry, delivery to the market, incorporation into standard practice, and/or approval by the FDA) are achievable.

- Whether the potential risk analysis for cost, schedule, manufacturability, and sustainability is realistic and reasonable.

- How well the application identifies intellectual property ownership, describes any appropriate intellectual and material property plan among participating organizations (if applicable), and addresses any impact of intellectual property issues on product development and subsequent Government access to products supported by this Program Announcement/Funding Opportunity.

- Whether the applicant has demonstrated that they have access to all intellectual property rights necessary for development and commercialization and evidence that the Government has the ability to access such products or technologies.

- **Budget**

  - Whether the budget is appropriate for the proposed research.

- **Environment**

  - To what degree the scientific environment, clinical setting, and accessibility of institutional resources support the clinical trial at each participating center or institution (including collaborative arrangements).

  - Whether there is evidence for appropriate institutional commitment from each participating institution.

  - If applicable, to what degree the intellectual and material property plan is appropriate.

2. **Programmatic Review:** To make funding recommendations, the following criteria will be used by programmatic reviewers:

   - Scientific peer review results

   - Adherence to the intent of the award mechanism

- Program portfolio balance and priorities
- Relative military benefit
- Relative innovation, impact, and translatability

NOTE: Military-relevant research must be responsive to the healthcare needs of the Armed Forces, family members of the Armed Forces, and the U.S. Veteran population. Proposals/ Applications must address a military-relevant health problem responsive to one of the Research Areas of Interest identified in Section II.A and Appendix I.

II.E.3. Criteria for Conference or Symposium Support Proposals

II.E.3.a. Peer and Programmatic Review

1. **Peer Review:** To determine technical merit, proposals will be evaluated according to the following scored criteria, which are listed in descending order of importance:

   - **Conference or Symposium Objectives and Plan:** The degree to which the stated objectives are clear, valid, and logical. The degree to which the performance objectives are plausible; the proposed effort demonstrates familiarity with the historical background of the problem and previous/current solutions (such as professional society meetings). The degree of quality and completeness of the overall management and execution plan. The extent to which the conference or symposium relates to the Research Areas of Interest identified in Section II.A and Appendix I. The extent to which the conference or symposium circumstances do not create the appearance of Government impropriety.

   - **Scientific Excellence:** The degree to which proposed conference or symposium procedures are state-of-the-art; the degree to which the conference or symposium includes expert peer review in rating and selection of presentation through methods such as abstract submission and established processes for invitation of appropriate participants.

   - **Impact/Outcomes:** The potential impact of the conference or symposium content in the field and for individual attendees, the significance of this impact, and when it can be anticipated, as well as methods for publication and peer review of conference or symposium results and recommendations.

   - **Key Personnel Qualifications:** The qualifications, capabilities, and experience of the proposed key personnel to demonstrate that the proposed staff has the knowledge, technical expertise, and management skills to achieve the proposed objectives.

   - **Budget:** The degree to which the budget reflects the actual needs of the proposed work and is thoroughly detailed and fully justified so that the Government can evaluate and determine the costs to be allocable, allowable and reasonable, and commensurate with the complexity and nature of the meeting proposed. The extent to which the costs reflect common sense and good judgment as to appropriate and necessary conference or symposium expenses and do not include expenses that are prohibited (including, but not limited to, entertainment-related expenses including hiring musicians or other

entertainers, procurement of extraneous promotional items, decorations or other goods and services for participants that are unrelated to the purpose of the conference or symposium, procuring tickets to recreational activities outside of the conference setting, or using DoD funds to produce non-substantive audio/visual materials).

2. **Programmatic Review:** The following criteria will be used by programmatic reviewers:

- Programmatic relevance
- Scientific merit and relative impact
- Relevance to the DoD mission

II.E.4. Proposal/Application Selection Process

All invited proposals/applications are evaluated by USAMRMC scientists, other Federal agency representatives, outside scientists with diverse expertise, clinicians, consumers, or combinations thereof, using a two-tier review process. The first tier is peer review of proposals/applications against established criteria for determining technical merit. The second tier is programmatic review based on established criteria for determining relevance to the mission of the USAMRMC and its programs.

All USAMRMC review processes are conducted confidentially to maintain the integrity of the merit-based selection process. Panel members sign agreements to (1) protect the confidentiality of the information in the proposal/application and (2) not disclose evaluation information outside the panel. Violations of confidentiality can result in the dissolving of a panel(s) and other corrective actions. In addition, personnel at the applicant or collaborating organizations are prohibited from contacting persons involved in the review process to gain protected evaluation information or to influence the evaluation process. Violations of these prohibitions will result in the administrative withdrawal of the organization's proposal/application. Violations by panel members or applicants that compromise the confidentiality of the review process may also result in suspension or debarment from Federal awards. Furthermore, the unauthorized disclosure of confidential information of one party to another third party by military personnel or employee of the Federal Government is a crime in accordance with 18 USC 1905.

After the two-tier evaluation, proposals/applications recommended for funding may be prioritized. A prioritized listing of alternates (deferred decisions) may also be prepared, when warranted. Subsequent awards depend upon the availability of funds and fulfillment of requirements and priorities determined to exist at the time of award. In some cases, funding priorities may change as certain scientific tasks are addressed and new mission assignments arise.

If selected for funding, the award may also be dependent on the organization providing adequate additional regulatory documentation, such as human subjects/anatomical substances/use of cadavers protocols and approvals, animal subjects protocols and approvals, and environmental information. The award may also be dependent on additional supporting administrative and budgetary information.

II.E.5. Integrity and Performance Information

Prior to making an award where the Federal share is expected to exceed the simplified acquisition threshold (currently $150,000) over the period of performance, the Federal awarding agency is required to review and consider any information about the applicant that is available in the Federal Awardee Performance and Integrity Information System (FAPIIS).

An applicant, at its option, may review FAPIIS, accessible through SAM, and submit comments to FAPIIS on any information about itself that a Federal awarding agency previously entered and is currently available in FAPIIS.

The Federal awarding agency will consider any comments by the applicant, in addition to other information in the designated integrity and performance system, in making a judgment about the applicant's integrity, business ethics, and record of performance under Federal awards when determining an organization's qualification prior to award, according to the qualification standards of the DoDGAR or the FAR.

II.E.6. Notification of Proposal/Application Review Results

Each PI and organization will receive email notification via eBRAP of the proposal/application status. Notifications should be sent within 180 days of submission. Each PI will receive a peer review summary statement on the strengths and weaknesses of the proposal/application.

A recommended for funding notification is NOT an authorization to begin performance or a guarantee of an award. Awards are contingent upon availability of funding, adequacy of supporting documentation submitted, fulfillment of all requirements, and upon completion of successful negotiations. Authorization to begin performance will be received via an award document (contract, grant, or cooperative agreement, as applicable) signed by the USAMRAA Contracting or Grants Officer. Awards may be issued at any time throughout the year.

II.F. Award Administration Information

II.F.1. Award Notices

The PI should receive disposition regarding the full proposal/application via an email from eBRAP within 180 days of submission. **A recommended for funding notification is NOT an authorization to begin performance nor a guarantee of an award.**

The awarding agency will be the USAMRAA. The USAMRAA Contracting and Grants Officers are the only individuals authorized to obligate funds and bind the Federal Government.

Authorization to begin performance will be received via an award document (contract, grant, or cooperative agreement, as applicable) signed by the USAMRAA Contracting or Grants Officer. No commitment on the part of the Government should be inferred from discussions with any other individual.

Awards will be made at any time throughout the year and are contingent upon availability of funding, adequacy of supporting documentation submitted, fulfillment of requirements, and

completion of successful negotiations. No proposal/application submitted under this BAA will be considered for funding after 24 months from the date of submission to Grants.gov.

Refer to the General Submission Instructions, Appendix 2, Section D, Award Notices, for additional information. Applicable requirements in the DoDGAR are found in 32 CFR, Chapter I, subchapter C, and 2 CFR, Chapter XI, apply to grants and cooperative agreements resulting from this BAA. Refer to the full text of the USAMRAA General Research Terms and Conditions for Institutions of Higher Education, Hospitals, and Non-Profit Organizations and the USAMRAA General Research Terms and Conditions for For-Profit Organizations available at http://www.usamraa.army.mil/Pages/Resources.aspx for further information.

II.F.1.a. PI Changes and Award Transfers

Refer to the General Submission Instructions, Appendix 4, Sections L and M, for general information on changes to PIs and organizational transfers.

II.F.2. Administrative and National Policy Requirements

Applicable requirements in the DoDGAR apply to grants and cooperative agreements resulting from this BAA. See 2 CFR, Chapter XI, and 32 CFR, Chapter I.

Refer to the General Submission Instructions, Appendix 2, for general information regarding administrative requirements.

Refer to the General Submission Instructions, Appendix 5, for general information regarding national policy requirements.

II.F.3. Reporting Requirements

Refer to the General Submission Instructions, Appendix 2, Section A, for general information on reporting requirements.

II.G. Agency Contacts

II.G.1. CDMRP Help Desk

Questions related to BAA content or submission requirements as well as questions related to the submission of the pre-proposal/pre-application through eBRAP should be directed to the CDMRP Help Desk, which is available Monday through Friday from 8:00 a.m. to 5:00 p.m. Eastern Time. Response times may vary depending upon the volume of inquiries.

Phone: 301-682-5507

Email: help@eBRAP.org

II.G.2. Grants.gov Contact Center

Questions related to full proposal/application submission through the Grants.gov portal should be directed to the Grants.gov Contact Center, which is available 24 hours a day, 7 days a week (closed on U.S. Federal holidays). Note that the CDMRP Help Desk is unable to provide technical assistance with Grants.gov submission.

Phone: 800-518-4726 (International: 1-606-545-5035)

Email: support@grants.gov

Sign up on Grants.gov for "send me change notification emails" by following the link on the Synopsis page for the BAA or by responding to the prompt provided by Grants.gov when first downloading the submission package. If the submission package is updated or changed, the original version of the submission package may not be accepted by Grants.gov.

II.H. Other Information

II.H.1. Contractor/Recipient Qualification

Refer to the General Submission Instructions, Appendix 3, for general information on required qualifications.

In addition to other information provided herein, by submitting a proposal/application and accepting an award, the organization is: (1) certifying that the investigators' credentials have been examined; (2) verifying that the investigators are qualified to conduct the proposed study and to use humans or animals as research subjects, if proposed. Investigators include all individuals, regardless of ethnicity, nationality, or citizenship status, who are employed by, or affiliated with, an eligible organization.

Investigators are cautioned that awards are made to organizations, not individuals. A PI must submit a proposal/application through an organization in order to receive support.

Should the PI of a funded project leave the award organization, both the PI and organization must contact the USAMRAA as soon as possible to discuss options for continued support of the research project. Every effort should be made to notify the USAMRAA prior to the PI leaving the organization.

II.H.2. Proprietary Information

Do not include any proprietary information in the pre-proposal/pre-application. Proprietary information should ***only be included*** in the full proposal/application ***if necessary for evaluation purposes.*** Abstracts of all funded proposals/applications will be posted publicly. ***Therefore, do not include proprietary information in the abstract.***

Conspicuously and legibly, mark any proprietary information that is included in the full proposal/application. Identify any proprietary information to be provided to the Government and indicate whether the applicant will request a waiver of Government purpose rights.

II.H.3. Administrative Actions

After agency receipt of proposals/applications from Grants.gov, the following administrative actions may occur:

II.H.3.a. Rejection

The following will result in administrative rejection of the proposal/application:

- Project Narrative exceeds the page limit.
- Project Narrative is missing.
- Budget form contains only zeros.
- Full proposal/application submission in the absence of an invitation.
- For proposals/applications including a clinical trial, the following may result in administrative rejection:
  - Human Subject Recruitment and Safety Procedures (Attachment 6) is missing.
  - Intervention (Attachment 7) is missing.
  - Data Management (Attachment 8) is missing

II.H.3.b. Modification

- Pages exceeding the specific limits may be removed prior to review for all documents other than the Project Narrative.
- Documents not requested may be removed.
- Following proposal/application submission to Grants.gov, the PI will receive an email request from eBRAP to review, modify, and verify the proposal/application submitted to Grants.gov. During this verification period, the PI may upload missing documents (excluding those listed above, Section II.H.3.a, Rejection), replace files, and re-categorize files. These modifications must be completed by the end of the proposal/application verification period; otherwise, the proposal/application will be reviewed as submitted. If either the Project Narrative exceeds the page limit or the Budget form contains only zeros, an updated Grants.gov submission package must be submitted via Grants.gov as a "Changed/Corrected Application" with the previous Grants.gov Tracking ID.

II.H.3.c. Withdrawal

The following may result in administrative withdrawal of the pre-proposal/pre-application or proposal/application:

- Federal agency personnel involved in the review process and/or with making funding recommendations are named as being involved in the research proposed or found to have assisted in the pre-proposal/pre-application or proposal/application processes, including, but not limited to, concept design, proposal/application development, budget preparation, and the development of any supporting documentation. ***If formal collaboration with Military Facility personnel is planned (i.e., included in the proposal/application in performance of the research), this prohibition is not applicable. However, these Military Facility personnel are prohibited from being involved in the review process and/or with making funding recommendations.***

- Inclusion of any employee of USAMRMC review contractors in pre-proposal/ pre-applications or full proposals/applications for funding without adequate plans to manage COIs. Refer to General Submission Instructions, Appendix 3.D, for detailed information.

- Personnel from applicant or collaborating organizations are found to have contacted persons involved in the review process to gain protected evaluation information or to influence the evaluation process.

- The full proposal/application does not propose the same research project as described in the pre-proposal/pre-application.

- The full proposal/application budget differs significantly from the budget included in the pre-proposal/pre-application.

- Proposed research of work that was or is currently funded may result in withdrawal.

- For studies requiring an IND or IDE, documentation of IND/IDE submission and/or approval is not provided

- Applications may be administratively withdrawn from further consideration if the applicant cannot demonstrate access to the relevant study population and resources.

II.H.3.d. Withhold

Proposals/Applications that appear to involve research misconduct will be administratively withheld from further consideration pending organizational investigation. The organization will be required to provide the findings of the investigation to the USAMRAA Contracting or Grants Officer for a determination of the final disposition of the proposal/application.

II.H.4. Common Submission Problems

- Failure to enter an email address for change notifications under the BAA in Grants.gov for notifications on any modification made to the initial posting.

- Attachments are uploaded into the incorrect form on Grants.gov forms. (See the appropriate Proposal/Application Submission Checklist below.)

- Failure to contact the Grants.gov Help Desk when needed.

- Failure to send attachments.
- Inability to locate attachment forms. (Select "Search Grants" at http://www.grants.gov and enter W81XWH18SBAA1 in the "Opportunity Number" block; or in the "CFDA" block, enter 12.420, and perform a "Search." Under the "Opportunity Number" column, look for number W81XWH18SBAA1 in the list.)
- Use of "illegal" characters in attachment titles.
- Attachments exceed size limits.
- Upload attempts of unacceptable attachments: bitmap, TIFF, etc.
- Duplicate upload of documents.

II.I. Research Proposal/Application Not Including a Clinical Trial Submission Checklist

Grants.gov Application Components	Upload Order	Action	Completed
SF424 (R&R) Application for Federal Assistance		Complete form as instructed.	
Attachments Form	1	Project Narrative: Upload as Attachment 1 with file name "ProjectNarrative.pdf."	
	2	Supporting Documentation: Upload as Attachment 2 with file name "Support.pdf."	
	3	Technical Abstract: Upload as Attachment 3 with file name "TechAbs.pdf."	
	4	Lay Abstract: Upload as Attachment 4 with file name "LayAbs.pdf."	
	5	Statement of Work: Upload as Attachment 5 with file name "SOW.pdf."	
	6	Impact/Outcomes Statement: Upload as Attachment 6 with file name "Impact.pdf."	
	7	Data- and Research Resource-Sharing Plan: Upload as Attachment 7 with file name "Sharing.pdf."	
	8	Transition Plan: Upload as Attachment 8 with file name "Transition.pdf."	
	9	Collaborating DoD Military Facility Budget Form(s): Upload as Attachment 9 with file name "MFBudget.pdf," if applicable.	
Research & Related Senior/Key Person Profile (Expanded)		Attach PI Biographical Sketch (Biosketch_LastName.pdf) to the appropriate field.	
		Attach PI Previous/Current/Pending Support (Support_LastName.pdf) to the appropriate field.	
		Attach Biographical Sketch (Biosketch_LastName.pdf) for each senior/key person to the appropriate field.	
		Attach Previous/Current/Pending (Support_LastName.pdf) for each senior/key person to the appropriate field.	
Research & Related Budget		Complete as instructed. Attach Budget Justification (BudgetJustification.pdf) to the appropriate field.	
Project/Performance Site Location(s) Form		Complete form as instructed.	
R & R Subaward Budget Attachment(s) Form		Complete form as instructed.	

II.J. Research Proposal/Application Including a Clinical Trial Submission Checklist

Application Components	Action	Completed
SF424 (R&R) Application for Federal Assistance	Complete form as instructed.	
Attachments	Project Narrative: Upload as Attachment 1 with file name "ProjectNarrative.pdf."	
	Supporting Documentation: Upload as Attachment 2 with file name "Support.pdf."	
	Technical Abstract: Upload as Attachment 3 with file name "TechAbs.pdf."	
	Lay Abstract: Upload as Attachment 4 with file name "LayAbs.pdf."	
	Statement of Work: Upload as Attachment 5 with file name "SOW.pdf."	
	Human Subject Recruitment and Safety Procedures: Upload as Attachment 6 with file name "HumSubProc.pdf."	
	Intervention: Upload as Attachment 7 with file name "Intervention.pdf."	
	Data Management: Upload as Attachment 8 with file name "Data_Manage.pdf."	
	Study Personnel and Organization: Upload as Attachment 9 with file name "Personnel.pdf."	
	Surveys, Questionnaires, and Other Data Collection Instruments: Upload as Attachment 10 with file name "Surveys.pdf."	
	Impact Statement: Upload as Attachment 11 with file name "Impact.pdf."	
	Transition Plan and Regulatory Strategy: Upload as Attachment 12 with file name "Transition.pdf."	
	IND/IDE Documentation: Upload as Attachment 13 with file name "IND-IDE.pdf."	
	Military Relevance Statement: Upload as Attachment 14 with file name "MilRel.pdf."	
	DoD Military Budget Form(s): Upload as Attachment 15 with file name "MFBudget.pdf," as applicable.	

Application Components	Action	Completed
Research & Related Senior/Key Person Profile (Expanded)	Attach PI Biographical Sketch (Biosketch_LastName.pdf) to the appropriate field.	
	Attach PI Previous/Current/Pending Support (Support_LastName.pdf) to the appropriate field.	
	Attach Biographical Sketch (Biosketch_LastName.pdf) for each senior/key person to the appropriate field.	
	Attach Previous/Current/Pending (Support_LastName.pdf) for each senior/key person to the appropriate field.	
Research & Related Budget	Complete as instructed. Attach Budget Justification (BudgetJustification.pdf) to the appropriate field.	
Project/Performance Site Location(s) Form	Complete form as instructed.	
R&R Subaward Budget Attachment(s) Form, if applicable	Complete form as instructed.	

II.K. Conference and Symposium Support Proposal Checklist

eBRAP Application Components	Upload Order	Action	Completed
Attachments Form	1	Conference or Symposium Objectives: Upload as Attachment 1 with file name "Objectives.pdf."	
	2	Agenda: Upload as Attachment 2 with file name "Agenda.pdf."	
	3	List of Relevant Publications: Upload as Attachment 3 with file name "References.pdf."	
	4	Statement of Work: Upload as Attachment 4 with file name "SOW.pdf."	
	5	Impact/Outcomes Statement: Upload as Attachment 5 with file name "Impact/Outcomes.pdf."	
	6	Key Personnel Qualifications: Upload as Attachment 6 with file name "Personnel.pdf."	
	7	Key Personnel Biographical Sketches: Upload as Attachment 7 with file name "Biosketch.pdf."	
	8	Budget: Upload as Attachment 8 with file name "Budget.pdf."	

APPENDIX I: RESEARCH AREAS OF INTEREST

I. Military Infectious Diseases Research Program

A. Research and Development Toward Preventive Measures for Infectious Diseases

- **Vaccines:** The development of vaccines to protect the Warfighter is a priority for the MIDRP. Vaccines can be administered prior to deployment, thereby protecting the Warfighter by preventing disease, obviating the need for prophylactic medication, and reducing the medical logistic burden. The MIDRP supports studies to characterize infectious disease agents and their mechanisms of pathogenesis, utilizing various approaches to identify and evaluate vaccine candidates in animal models and humans, as well as studies of protective immune responses to these agents.

- **Anti-Parasitic Drugs:** The MIDRP supports research efforts for discovery, design, and development of drugs to prevent malarial infections. This includes drug synthesis, characterization of modes of action, screening for antimalarial activity, in vitro and in vivo research using animal models, and drug resistance mechanisms. Investigations into parasite metabolism, structural biology, genomics, proteomics, and metabolomics focused on identification of potential novel targets for drug intervention are also relevant to the MIDRP.

- **Vector Control Products:** The MIDRP supports investigations focusing on arthropod vectors and arthropod vector-borne diseases (primary malaria, dengue, and scrub typhus). Current studies target vector-pathogen-human interactions, vector control measures (including personal protective equipment), and risk assessment (including identification and classification of vectors, improved surveillance techniques, and field-deployable assays for detecting pathogens in their vectors).

- **Combat Wound Infections:** The MIDRP supports research to inform and/or facilitate product development to detect, prevent, treat, and manage combat wound infections. Investigations in this area include characterization of the mechanisms of biofilm formation in wounds, the mitigation of biofilm formation in wounds, novel therapies for wound infection treatment, and methods to understand the dynamics of microbial communities in infected and healing wounds. In addition, novel chemical classes and/or biologics to prevent or treat combat wound infection and/or biofilm formation are of interest.

B. Research and Development of Therapeutic Measures for Infectious Diseases

Therapeutic drug development (including studies to screen, synthesize, and develop therapeutic drugs for malaria and other military-relevant infectious agents) is secondary to prophylactic drug development within the MIDRP (see above). However, efforts to advance novel drug delivery systems (i.e., sustained-release reduce toxicity or targeted drug delivery) would be considered. In addition, MIDRP supports investigations into the development of novel medical countermeasures and innovative treatment approaches (e.g., chelators, phage, antimicrobial peptides, quorum-sensing inhibitors, and host immune augmentation) against multidrug-resistant organisms in combat wound infections and pathogenic biofilm formation. Given modest pharmaceutical industry interest in developing and marketing vaccines for orphan diseases, the

MIDRP is also interested in applications for the development of products leading to FDA-licensed treatment options and broadly active therapeutics against multiple endemic disease threats.

II. Combat Casualty Care Research Program

A. Research and development of technologies to stop blood loss, resuscitate the casualty, and limit the immediate, short-, and long-term deleterious consequences of severe hemorrhage: Research focused on the pre-hospital setting including point of injury, the initial "Golden Hour" after injury, and scenarios in which a casualty cannot be transported through traditional levels of care (i.e., prolonged field care [PFC]) are of high interest. Included in this area of interest are diagnostics and therapeutics to predict, diagnose, prevent, and treat coagulopathy of trauma and non-invasive or minimally invasive sensors to detect and warn of impending vascular collapse and/or significant tissue damage due to perfusion deficits. Examples of specific products include local and systemic hemostatic agents or devices (exovascular or endovascular) for control of vascular disruption and subsequent compressible and non-compressible hemorrhage, treatments to sustain or enhance oxygen delivery and perfusion of vital tissues and organs, and equipment and procedures for effective fluid resuscitation and enhanced resuscitation fluids. Also of interest are the improved preservation, storage, transportability, and processing of red blood cells, platelets and plasma, and other blood or blood-like substitutes.

B. Research and development of technologies to diagnose and to limit the immediate, short-, and long-term impairments that follow TBI and spinal cord injury: Research specializing in "polytrauma" accounting for the impact of hemorrhagic shock and failure to oxygenate and/or ventilate as brain injury progresses is of interest to provide insight leading to improvements in clinical practice guidelines. Included in this area of interest are non-invasive or minimally invasive sensors or assays to rapidly diagnose the severity of brain and neurological injury within the battle area (or as close to it as possible) and drugs, biologics, or other agents to mitigate the progression of neurotrauma/secondary brain injury such as post-injury neural and immune cell overstimulation, inflammation, cell loss, and/or neurologic dysfunction.

C. Research and development of technologies to diagnose and reduce acute secondary organ damage: Secondary damage to organs frequently occurs after severe trauma and resuscitation. The CCCRP is interested in materiel and/or devices that can reduce acute secondary organ damage such as ischemia/reperfusion injury, cell death, general organ failure, and secondary brain/spinal cord damage. Technologies to sustain or support single- and multiple-organ injury and failure are also of interest to the CCCRP. These objectives include methods to reduce cellular demand for oxygen and metabolic substrates and therapeutics to modulate the immune response to traumatic injury as well as single- and multiple-organ support or replacement technologies (extracorporeal). In addition, the utilities of these modalities during (and the effects of longer distance) en-route care on the critically injured casualty are also of interest.

D. Research and development into the impacts of transport: An important element of combat casualty care is the transport of patients from the initial point of injury and

throughout the continuum of care. Accordingly, the CCCRP is interested in improving and maintaining optimal clinical outcomes for en-route care including PFC. Identifying feasible ways to mitigate the stresses of flight and/or transport (such as hypobaria, hypoxia, vibration, and g-forces) in an austere/constrained environment and the impact on clinical outcomes (such as healing rates, pain, infection rates, etc.) are particularly important. Additionally, establishing either timeframes or ways to measure the appropriate time to transport patients with critical injuries (including neurotrauma, burns, lung injuries, and musculoskeletal injuries) are a critical element to improving outcomes.

III. Military Operational Medicine Research Program

A. Injury Prevention and Reduction: This area of research addresses the requirement to provide the biomedical basis for countermeasures that prevent and mitigate Service member injuries and performance decrements occurring in training and operational environments, decrease attrition and medical cost, minimize personal impact to the Service member, and promote readiness. The primary need of the Injury Prevention and Reduction portfolio is the development of validated injury criteria utilizing animal models and postmortem human subjects against blunt and ballistic threats that will inform the development of helmets and body armor. Coordination with DoD labs such as the U.S. Army Aeromedical Research Laboratory, U.S. Army Research Laboratory (ARL) Survivability, Lethality, and Analysis Directorate and/or ARL Weapons and Materials Research Directorate is encouraged. Other research areas of interest include: developing and validating return-to-duty (RTD) clinical assessments and physical performance standards following neurosensory and musculoskeletal injury; understanding the physiological impact of exposure to repetitive low-level blast in order to validate military safety standards and health hazard assessment prediction tools to prevent brain, sensory, and lung injury; understanding the bioeffects from exposure to, and developing injury thresholds against, directed energy threats (e.g., laser, microwave, and radiofrequency waves); and developing interventions to mitigate neurosensory and musculoskeletal injury risk and optimize performance in complex military systems, and develop and validate assessment tools and strategies to promote Service member physical performance optimization (individual and group). In addition, this research includes the development of policy, training, planning/management tools, knowledge and materiel solutions, status monitoring systems, interventions, and reset solutions to sustain Service member health and operational effectiveness.

B. Psychological Health and Resilience: The Psychological Health and Resilience research program area is interested in research aimed at maximizing resilience and psychological health and decreasing PTSD, depression and anxiety disorders, suicide, and risk behaviors (e.g., substance abuse, anger/aggression, sexual harassment and assault, and interpersonal violence within the military). Additional psychological health areas of interest include military-related grief, guilt, or loss issues; moral injury; interdisciplinary and comprehensive prevention and life-skills training strategies to reduce negative psychological health trajectories; and reduction of stigma and other barriers to psychological healthcare-seeking. MOMRP has interest in understanding and addressing psychosocial/psychological health challenges unique to military families, women Service members, Reserve and Guard, and lesbian, gay, bisexual, and transgender Service members. This research area focuses on the development and validation of effective training and prevention interventions, screening and

assessment strategies, and treatment and rehabilitation interventions that address the psychological health topic areas. In addition, this research area focus may include development and validation of effective evidence-based training and prevention interventions for concussion/mild TBI. Research areas of particular interest include foundational studies to generate and validate theories and elucidate underlying mechanisms of psychological disorders and treatment response; studies addressing comorbidities (including, but not limited to, PTSD, concussion, alcohol and other drug abuse, sleep disturbance, mood disorders, suicidality, and psychosocial factors); studies focused on enhancing translation, implementation, and uptake of evidence-based strategies and treatments; research focused on establishing validated objective RTD standards following psychological injury; and research focused on systems approaches to psychological health. Proposals/Applications are encouraged that incorporate and evaluate leveraging of technology (e.g., telemedicine, remote monitoring, biosensors, advance immunologic testing, and health information technologies), and leverage existing resources and infrastructure to support lifecycle logistics and sustainability. Also of interest are rigorous studies on integrative medicine and complementary and alternative medicine (CAM) approaches spanning mind/body, movement, natural products, non-Western medicine approaches and spiritual practices, along with validation studies of CAM therapies. Research topics of particular interest include those directed at evaluating efficacy of cognitive training approaches to promote resilience and prevent/mitigate acute negative responses to psychological trauma and promote readiness; and the development of a systematically applied set of therapeutic services designed to reduce cognitive dysfunction and restore functions that can be restored and/or improve quality of life.

C. **Physiological Health and Performance:** This area of research develops biomedical countermeasures to sustain Service member health and operational effectiveness. It informs military policy, training, clinical practice guidelines, and the development of materiel solutions to establish, sustain, optimize, and monitor Service member health, physiological factors of resilience, and cognitive and physical performance throughout the military lifecycle, including training, deployment, reset, and injury recovery cycles. This research area aims to prevent or mitigate the negative effects of operational and training stressors on the readiness, performance, and fitness of Service members, as well as safely enhance performance with evidence-based pharmacological and non-pharmacological personalized strategies based on a systems medicine approach. Studies may include, but are not limited to, those that investigate the use of dietary supplements and nutritional and behavioral interventions to mitigate threats to readiness, operational health, and performance. Research also aims to develop healthy sleep and fatigue management strategies, strategies that exploit individual differences in sleep loss resilience, and strategies that promote individualized resilience to various operational stressors and injuries. Physiological health and performance research also encompasses work focusing on overall brain and cognitive fitness. Basic, applied, and advanced research studies utilizing technologies and strategies to monitor and promote Service member and family readiness and health to support the Army Surgeon General's Performance Triad and U.S. Army Training and Doctrine Command Human Dimension Initiative are of interest.

D. **Environmental Health and Protection:** This area of research includes assessment and sustainment of health, Force readiness, and the operational effectiveness of Service members

exposed to harsh operational environments including altitude, cold, heat, undersea, and exposure to environmental toxicant health hazards or combination of environmental stressors. Studies may include, but are not limited to, methods for effective monitoring of environmental exposures in individuals and populations and assessment of health risks following exposures to environmental stressors. In addition, this research includes development of policy, training, planning/management tools, decision aids, knowledge and materiel solutions, physiological status monitoring systems, interventions, and reset solutions to sustain Service member readiness, and health and operational effectiveness to environmental stressors encountered during training or operations. Additional research identifies biomarkers of exposure, dosimetry, and risk management to environmental toxicant health hazards, neurological and physical assessment tools for optimizing performance of the Service member exposed to environmental toxicant hazards, and development of portable devices for rapid identification of biomarkers of complex exposures and health effects in support of military operational requirements. Additional research focuses on developing wearable solutions that are small size, low weight, cube, and have minimal power requirements for real-time physiological status monitoring in harsh operational environments.

IV. Clinical and Rehabilitative Medicine Research Program

A. Neuromusculoskeletal (NMS) Injury Rehabilitation: The NMS Injury Rehabilitation program area seeks research efforts directed toward optimal treatment, rehabilitation, and reintegration following Service-related NMS injury including: Service-related acute and repetitive overuse injury management, limb loss rehabilitation and prosthetic management, and limb trauma rehabilitation and orthotic management. Areas of encouragement include: validation of therapeutic exercise dosage (frequency, intensity, timing, and/or type) and rehabilitation strategies to improve Warfighter function, performance, and quality of life; understanding confounders of optimal rehabilitation; research to develop and validate standardized metrics of function, performance, and quality of life across and beyond the continuum of rehabilitative care; validation of existing short- and long-term reintegration strategies; research to predict and mitigate secondary health deficits following NMS injury; and efforts to develop and validate rehabilitation technologies (e.g., rehabilitation tools, interoperability of devices, prosthetic sockets, orthotic devices, advanced prosthetic control, sensory/proprioception, etc.) for Warfighters with NMS injuries to restore function and quality of life.

B. Vision Restoration and Rehabilitation: The Sensory Systems program area is seeking research efforts aimed at understanding and treating traumatic and Service-related injuries (e.g., blast, burn, penetrating, chemical, etc.) to ocular structures and the visual system (e.g., optic neuropathy, corneal scarring, retinal injury, lids and adnexal injuries, and ocular polytrauma, etc.). Additional areas of interest include studies to improve or advance visual system diagnostic/assessment capabilities, and restoration/rehabilitation strategies (including, but not limited to, rehabilitation for multisensory dysfunctions, low vision and blindness, and oculomotor vision disorders) following traumatic injury.

C. Hearing Loss/Dysfunction, Balance Disorders, and Tinnitus: The Sensory Systems program area is seeking research efforts to support the development of strategies and technologies (including, but not limited to, medical devices, pharmaceuticals, rehabilitation

strategies, and regenerative medicine-based approaches) to restore and/or rehabilitate patients with hearing loss/dysfunction, balance disorders, and/or tinnitus due to trauma (including TBI). This includes research focused on the etiology of injury including studies to support an understanding of the molecular, cellular, and physiological mechanisms underlying hearing loss/dysfunction, balance disorders, and tinnitus. Additional areas of interest include research supporting the development, advancement, and/or validation of objective diagnostics, and treatment/rehabilitative strategies for hearing loss/dysfunction, balance disorders, multisensory dysfunction, and tinnitus after traumatic or Service-related injuries.

D. Pain Management: The primary interest of the Pain Management program area is management of acute and chronic pain associated with traumatic or combat-related injuries. The CRMRP's specific needs include development of alternative interventions to current opioid analgesics for pain management by the medic/corpsman on the battlefield/remote locations; development of intervention strategies for acute pain management in deployed locations, including battlefield and resource-limited environments; development of strategies for management of acute pain under the care of a clinician in non-deployed settings; development of intervention strategies for chronic pain management in deployed locations, including battlefield and resource-limited environments; development of intervention strategies for management of chronic pain under the care of a clinician in non-deployed settings; identification of pain generators and etiology of pain; development of strategies for identifying and addressing biopsychosocial aspects of pain; and development of substance misuse and abuse assessments and treatments in pain management.

E. Regenerative Medicine and Composite Tissue Engineering: Regenerative medicine involves the use of innovative technologies such as scaffolds and tissue engineering, growth factors, and cell-based treatments to restore Service members who have suffered combat-related injuries. Research topics of particular interest include those directed toward the use of regenerative medicine-based technologies to repair functional neural deficits (to include all peripheral nerves, visual system, and auditory system but excluding other deficits associated with central nervous system or spinal cord), repair/replace neuromuscular tissue units of the extremities or face including composite facial features (eyelids, lips, and nares), regenerate bone defects (weight-bearing and alveolar), regenerate skin, address vascular repair/revascularization, regenerate cartilage/musculoskeletal connective tissues for the prevention of post-traumatic arthritis, muscle protection/regeneration, vascularized tissue allotransplantation, immunomodulation, and tolerization related to vascularized tissue allotransplantation and wound management and tissue preservation such as promotion of scarless wound healing (not to include infection control).

F. TBI Rehabilitation: The sensorimotor clinical rehabilitation line of effort within the TBI program seeks to develop, evaluate, and/or validate rehabilitation intervention strategies (e.g., multitask/dual-task) to address TBI-related sequelae. Interventions should remediate TBI-related deficits including, but not limited to, dizziness, cognitive dysfunction (e.g., attention, memory, or impaired executive function), or sensorimotor deficits (e.g., gaze or gait instability). Intervention strategies should aim to increase patient tolerance for rehabilitation and result in clinically meaningful and measurable improvement in targeted impairments, functional deficits, and barriers to participation. Competitive proposals/applications will address theorized physiologic mechanisms underlying treatment

strategy. Clinical research is needed to investigate the optimal delivery of rehabilitation interventions and prescription (i.e., frequency, intensity, timing, and type) and to investigate comparative effectiveness of standard of care and novel intervention strategies. Studies that characterize or investigate proposed neural mechanisms of recovery with rehabilitation will be favorably considered. Cognitive rehabilitation studies that utilize a practice-based evidence design to identify clinical best practices and optimal outcomes among active duty Service members and like-aged Veterans with persistent post-traumatic cognitive sequelae are encouraged.

G. Ecological Assessment: The Ecological Assessment line of effort within the TBI program aims to develop, evaluate, and/or validate RTD outcome measures following rehabilitation in patients with mild TBI. Outcome measures may integrate novel or commercially available assessment technologies to enhance sensitivity and/or specificity. Outcome measures should be both ecologically valid (i.e., focused on military-specific tasks) and clinically feasible, with an aim to inform RTD/participation decisions in either an operational or garrison-based environment. Outcome measures should quantify and characterize patient impairments, functional limitations, and barriers to participation and have a strong plan for validation in the Warfighter population.

V. Medical Biological Defense Research Program

A. Viral, Toxin, and Bacterial Studies

- Identification and characterization of organisms and toxins. Molecular antigenic analysis; development of diagnostic assays; studies on structure and function that are related to mechanisms of action, binding, internalization, and interaction with the immune system and neutralizing antibodies; investigation of pathogenesis and immunology that will inform and enable decisions regarding the optimal approach to disease prevention and control. Specific long-term goals include development of physiological support methodologies, diagnostic tests, rational prevention and control strategies, and improvement of existing products.

- Vaccine development, with emphasis on protection from aerosolized agents, molecular approaches for development of vaccines, measurement of relevant cellular and humoral protective immune responses, and expression or production of protective antigens using recombinant technology. Development of vaccines for specific toxins and disease agents involving the generation, selection, and characterization of attenuated strains or inactivated purified antigen preparations, to include polyvalent vaccines that are more broadly effective. Safer means of passive immunization such as production of human monoclonal or modified antibodies that are de-speciated are also of interest. Identification of surrogate markers of protection for the agents identified above and development of assays to assess such protection are needed.

- Development of improved methods for delivery of vaccines, including adjuvants, nucleic acid vaccines, methods for oral or nasal immunization with inactivated, live, and subunit antigens; sustained release formulations; and development of methods for delivery of antigens for specific induction of mucosal immunity and development of methods to enhance appropriate immune responses to include co-delivery of cytokines.

- Preparation of research quantities of highly purified and characterized toxins as well as studies on basic chemistry, mechanisms of action, metabolism, and excretion.

B. Drug Development: Development, synthesis, and testing of compounds that possess antiviral, antibacterial, immunomodulatory, or antitoxin activities, with emphasis on compounds that provide broad, non-specific protection against viruses, bacteria, and toxins as described above. Studies of their pharmacokinetics and other measurements relevant to more effective drug use are also of interest, as is the development of lead compound(s) that are potent, active-site inhibitors that may include combinatorial-derived organic molecules and/or rationally designed transition-state substrate analogs. Testing for potency is required. Approaches that will be considered include, but are not limited to, computational chemistry, combinatorial organic synthesis, high-throughput in vitro screening, and x-ray analysis of ligand-toxin co-crystals.

Research areas of interest include:

- Discovery of novel or unique biochemical elements or compounds with antiviral, antibacterial, or antitoxin activity against biological organisms.

- Development of testing models for evaluation of compounds effective against toxins of several classes, including pre- and post-synaptic toxins, membrane-damaging toxins, and toxins that inhibit protein synthesis and others.

- Mechanism of action studies of immunomodulators, including characterization of effector cells (lymphocytes, macrophages), effector mechanisms, ancillary effects on other cells of the immune system, and production and characterization of cytokines released as a consequence of immunomodulation.

- Development of novel treatments to reverse paralysis in nerve terminals exposed to botulinum neurotoxin (BoNT) serotypes A, B, E, or F, with emphasis on the following objectives: (a) development of clinically feasible treatments to block the catalytic activity of the BoNT light chain; (b) treatments to accelerate recovery from paralysis-induced atrophy by targeting skeletal muscle regenerative pathways; and (c) identification and development of novel chemical scaffolds for small molecule inhibitors of the catalytic activity of the light chain of BoNT serotype A. While there may be limited funds available for development of promising approaches, this effort is predominantly focused on the accelerated development of clinically viable treatments with existing efficacy data.

C. Identification and Diagnosis: The investigation and evaluation of sensitive and specific methods of identifying and diagnosing both antigens and antibodies of viruses, bacteria, and rickettsia in biological materials. Development of sensitive and specific immunologic, chemical, or biological assays for the rapid (within minutes) and reliable (1) diagnoses of acute diseases due to agents of potential biological threat and (2) identification of toxins or their metabolites in biological samples. Assay may include antigen, antibody, or metabolite detection or the use of nucleic acid probes or synthetic antigens. In addition, there is interest in the development of rapid identification and diagnostic methods for the assay of toxins, metabolites, and analogs in clinical specimens.

D. Biosurveillance (BSV): The process of gathering, integrating, analyzing, and communicating a range of information that relates to health threats for people, animals, and plants to help inform decisions and provide for increased global health security. The Joint Biosurveillance Common Framework (JBCF) will be the first materiel solution and provides a single enterprise environment that supports collaboration, data sharing, and coordination between multiple BSV stakeholders. The JBCF and future BSV applications, tools, and devices will provide a conduit between the medical, physical, and operational communities. This topic includes:

- Algorithms for rapid identification of baseline deviation; novel/unknown pathogens, naturally occurring versus intentional release.
- Models to predict the likelihood of an outbreak, forecast the associated epidemic curves and impacts of interventions, and update forecast based on field (and simulated) data.
- Applications to engage citizens via social media, crowd sourcing, gaming, etc.

In addition, two specific topics currently of interest are:

- Next-generation analytic capabilities for BSV: The objective is to develop next-generation methodologies to enhance analytic capabilities in the detect-identify-respond timeline for a bioevent. Research should be exploratory, with a low-technology readiness level, and should address long-term challenges in threat surveillance. Efforts should significantly contribute to the current body of knowledge and lead to new concepts for technology application that may have impact on future BSV analytic capabilities.
- Biosurveillance Ecosystem (BSVE) Analytics 2.0: The objective is to ensure state-of-the-art technologies are made rapidly accessible through the BSVE. This topic seeks to develop analytic applications to synthesize and interrogate multiple sources of data to provide high confidence in the prediction, early warning, and forecasting (inclusive of mitigation strategies) of disease events. Metrics shall be devised such that successful utilization of these analytic tools will result in a measurable impact on the bioevent timeline. Efforts in this area should result in flexible, extensible, and sustainable analytics and models that are designed to plug into the BSVE as a-la-carte services rather than as standalone capabilities.

VI. Medical Chemical Defense Research Program

- Characterizing the mechanisms of vesicant agent pathophysiology to identify medical countermeasures against vesicant agents.
- Developing innovative models of the pathophysiology of vesicant agent injury.
- Identifying and/or evaluating innovative candidate medical countermeasures against vesicant agents.
- Identifying, exploring, and developing innovative clinical diagnostic, prognostic, and management approaches to vesicant agent casualties.

- Characterizing the ocular lesions associated with vesicant agent exposures; developing treatments to ameliorate these injuries.
- Characterizing the mechanisms of nerve agent-induced seizures and resulting pathophysiology to identify medical countermeasures against nerve agent-induced seizures.
- Identifying, synthesizing, and/or evaluating innovative candidate medical countermeasures against nerve agent-induced seizures.
- Developing innovative models of the pathophysiology of nerve agent-induced seizures.
- Developing catalytic and/or stoichiometric chemical warfare agent scavengers from biological molecules (e.g., antibodies and enzymes) that provide protection against nerve agent incapacitation and lethality for extended periods following their administration.
- Developing innovative models for evaluation of chemical warfare agent scavengers.
- Identifying, expressing, synthesizing, and/or evaluating biotechnologically derived or pharmaceutically based scavengers as candidate medical countermeasures against chemical warfare agents.
- Developing and evaluating custom-synthesized pharmaceuticals based on a detailed understanding of the pathophysiology and mechanisms of action of the chemical warfare agent structure and the function of the intended target molecule.
- Developing catalytic and/or stoichiometric additives for use in skin protectants, or decontaminants, to protect against chemical warfare agents, especially vesicant and nerve agents.
- Developing innovative models for evaluation of catalytic and/or stoichiometric additives in skin protectants or decontaminants.
- Developing candidate formulations for skin protectants or decontaminants containing catalytic and/or stoichiometric additives and evaluating these formulations against chemical warfare agents.
- Characterizing the pathophysiology and natural progression of chemical warfare agent-induced damage to human tissues.
- Developing and validating innovative techniques for rapid and accurate analysis of human tissues and body fluids for detection of chemical warfare agent exposures.
- Characterizing the effects of long-term or chronic exposures to chemical warfare agents and/or medical countermeasures to these agents.
- Identifying, exploring, and developing innovative clinical diagnostic, prognostic, and management approaches to nerve agent casualties.

- Developing and validating field-usable procedures for diagnosis, prognosis, and treatment of chemical warfare agent casualties under both field and laboratory conditions.

VII. Medical Simulation and Information Sciences Research Program

A. The Medical Simulation (MedSim) Portfolio

The Medical Simulation (MedSim) Portfolio is focused on three overarching initiatives: Combat Casualty Simulation Initiative, Medical Readiness Initiative, and Tools for Medical Education. These three science and technology initiatives feed into broader Defense Health Agency programs with programmatic goals in Joint Evacuation and Transport Simulation (JETS) and Point of Injury Training Simulation (POINTS). The JETS program seeks to standardize patient movement training within the Military Health System (MHS) continuum of care while sustaining clinical standards of patient management. The POINTS program seeks to enable Point of Demand, mass-customizable training.

- **Combat Casualty Simulation Initiative (CCSI):** This initiative focuses on simulations that advance combat casualty care. Research in this area will examine the efficacy of modern simulation system technology versus current training models with emphasis on multi-trauma and mass casualty scenarios. The CCSI supports research to inform simulation development and acquisition in ways to develop appropriate fidelity material properties and characteristics that best mimic tissue and respond appropriately to users' actions; develop training assets for high state of combat medical readiness; provide resiliency training prior to deployment to better elicit higher performance under pressure; and to create and evaluate efficient and effective ways to deliver team (collective) training. Goals include:

    - Optimizing critical lifesaving skills and procedures through training and educational simulation systems

    - Developing adaptable, flexible, and interoperable training assets to reflect the continuous changes and modifications in combat-related injuries for high state of combat casualty medical readiness

    - Increasing psychological resilience into pre-deployment training and increasing emphasis on mastery of skills and procedures through simulation system training tools

    - Creating and integrating more physiologically based algorithms and models into simulation systems (mannequins and/or virtual/augmented/immersive reality) to appropriately and accurately represent tissue behaviors and characteristics

    - Increasing emphasis on PFC training and researching and integrating simulation systems to substantially improve communicating and connecting with each other to transfer and accept information/data from one system to another to support a system of system training and education interactive environment

    - Developing system of systems interoperable architecture to allow all Services at all roles within the continuum of care to train collectively, either with a modular training system

or a collection of training systems to better understand clinical outcomes from a holistic patient perspective instead of as individual skills or procedures

- **Medical Readiness Initiative:** This initiative focuses on medical provider training systems and assessment of competence for sustained military and public medical readiness. Research efforts are aligned with maximizing healthcare professionals' training and investigating how existing medical cognitive and psychomotor skills might degrade. The initiative seeks to research improved intelligent automated assessment systems that will assist in directing and catering the type of training courses an individual needs as well as systems that connect medical training to real-world patient outcomes. This initiative invites research and development toward near-time, pre-intervention rehearsal. Goals include:

    - Identifying, researching, and developing predictive models that may accelerate cognitive, psychomotor, and healthcare behavioral skills (tasks) to a level of proficiency and develop reliable and predictable tools to accelerate development of clinical skills or to minimize skill decay (or degradation)

    - Identifying, researching, and developing simulation system tools that will improve (or allow) ethical, patient-focused, and more predictable pre-surgical/intervention models and pre-surgical/intervention training systems to optimize clinical outcomes

    - Identifying and researching potential predictors (data, markers, classifiers, etc.) for how training or use of any type of simulation system transitions to the real-world and patient outcomes

    - Improving assessment systems of users' cognition, psychomotor skills, and affective behavior before, during, and after (retention) training

    - Leading the effort to develop a sustainable medical education lifecycle

- **Tools for Medical Education:** Research and develop next-generation inter-professional, open-source platforms, toolkits, and models to deliver future training systems in combat casualty care and medical readiness to improve the overall health of the Force. Focus is on promoting deliberate practice, enhancing mastery learning, enabling instructors, and preventing skill decay. Outcomes will result in resource sharing, collaborative research, and wide dissemination of knowledge and products to the medical modeling, simulation, training, and education community at large. Goals include:

    - Ensuring that advanced medical simulation tools and system capabilities are ubiquitous and support a diverse set of skills as well as allowing an individual or team to learn more advanced skills on a single device

    - Creating medical models and repositories that can be openly shared for medical simulation system developers and for the medical simulation community at large

    - Researching effective, efficient, elegant, accurate, appropriate, and robust medical models (anatomical, physiological, and/or behavioral) for developing next-generation mannequin prototypes and virtual/augmented/immersive reality simulation systems

- Democratizing of knowledge and products through training platforms and tools that deliver healthcare content and advocate open-source/open-architectures to allow limited resources to be shared

B. The Health Information Technology and Informatics (HITI) Portfolio

- **Theater/Operational Medicine:** This is the main priority research domain for technologies and data management that improve and document clinical care and support services to the Armed Forces combat and deployment Warfighters. Focusing on providing high-quality healthcare services by improving information accessibility, data management, data movement, remote healthcare delivery, and decision support for Joint Casualty Management, Joint Patient Movement, Joint Performance Enhancement, Joint Medical Logistics and Infrastructure Support, and Joint Theater Medical Command and Control and research for the Armed Forces to promote, improve, conserve, or restore the mental or physical well-being of personnel.

  The HITI Theater/Operational Medicine focus area provides technology solutions, software, decision support tools, algorithms, data management, knowledge, and HITI services to enhance the efficiency of healthcare operations in combat and operational environments. The objective of HITI Theater/Operational Medicine research is to ensure delivery of high-quality healthcare services through technologies for improved information accessibility and information management and use of emerging/advanced technologies by clinicians caring for injured Service members. The Government plans to use research outcomes to assess critical technology elements and technology maturity, system integration risk, future use feasibility, and, where necessary, technology maturation and demonstration to fulfill critical capability gaps in theater/operational medicine healthcare delivery and support. Particular focus is placed on advanced information management and use of emerging and next generation technologies. The goal is to research technology and data tools/strategies to support Theater/Operation Medicine within the following categories:

  - **Joint Casualty Management (JCM)** directly supports medical care in theater/operational environments and sustains a healthy and fit Force. JCM delivers five of the seven Joint Health Services Support capabilities outlined in the new Taxonomy Continuum of Health Care Capabilities developed in Joint Publication 4-02, Health Service Support. The capabilities delivered are expected to be technology and informatics solutions for first responders, forward resuscitative care, theater hospitalization, definitive care, and en route care.

  - **Joint Medical Logistics Infrastructure Support (JMLIS**) must establish the principles and practices that will move medical logistics into focused logistics. To integrate into the focused logistics system and to provide the highest quality of care, the medical logistics system must be able to accomplish two primary tasks. The first, accomplished in conjunction with Service Force management and Force design organizations, is to ensure that the medical supplies, materiel, and equipment with which U.S. medical forces deploy include the latest technologies and advances in the medical field. The second task is to ensure that medical supplies, materiel, and equipment are delivered to the right person, at the right place, and at the right time. JMLIS must incorporate new technologies and

informatics solutions to provide real-time medical and operational capability and situational awareness of medical logistics operations within the joint area of operations. JMLIS technology and informatics will rapidly integrate advances in research, technology, and doctrine from science, medicine, engineering, information technology, and other areas into fielded capabilities.

- **Joint Patient Movement** supplies and develops solutions for interoperable device data to improve ground and air evacuation capabilities, standardization for device data to improve ability to evacuate the casualty to a treatment site and autonomous device interoperability to support reception/staging en route care operations, and prolonged care in place.

- **Joint Performance Enhancement** supplies and develops innovative data capture, storage, analytics, management, and movement solutions for real-time, non-invasive monitoring of vigilance, subject performance, and enhancement of cognitive abilities for optimal decision making in Warfighter readiness.

- **Joint Theater Medical Command and Control** will research and validate a Joint Medical Command and Control solution that will synchronize and integrate health and medical force-related information from disparate databases and systems into one efficient, effective command and control capability. The resulting medical decision-based application will utilize intelligence, algorithms, decision support, or other novel approaches to organize and synthesize planning information queried from existing databases. It is anticipated that the data will eventually be migrated to a cloud solution to enable real-time response to anticipated, emerging, or contingency (crisis) situations. The research will enable direction, management, and/or coordination of assigned medical forces, assets, and resources in the accomplishment of assigned missions.

- **Military Healthcare Services:** Research into how healthcare providers and patients can better use health services and population health-related data, information, and technologies to improve health. Efforts to directly impact the way healthcare is provided to the patient, improve medical providers' ability to treat patients, promote health through readiness-centric patient engagement, patient safety-driven medical device information, interoperability, and connected healthcare services. Focus areas are as follows:

  - **Readiness-Centric Patient Engagement Applied Solutions:** Improve readiness through patient-initiated healthcare activities. Provide a user view of information that is comprehensive of healthcare and patient-generated data that can apply analytic and trending algorithms to help providers and patients make better decisions. Provide large volumes of data from agnostic input and devices, from any environment, in real time, to enable usable, actionable information. Develop and apply methods for analysis, interpretation, prediction, and modeling of health system and patient-generated data. The objective is to use mathematical and/or intelligent learning/machine learning tools to extract practical information, usable/actionable clinical knowledge, and/or predict disease or adverse events from health system and patient-generated data.

- **Patient Safety-Driven Medical Device Information and Interoperability:** Improve systems or applications that will enable medical devices to interact with each other for the purpose of improving patient care, and to integrate health system or patient-generated medical device information seamlessly with an Electronic Health Record system for the purpose of providing effective clinical decision support to assist healthcare professionals and patients in making better clinical and/or lifestyle decisions.

- **Connected Healthcare Services:** Promote "Healthcare Anywhere" through mobile and telehealth solutions that are usable, and facilitate point-of-injury care and documentation of care in theater/operational medicine environments for Roles 1, 2, and 3.

  – Role 1

    This includes the provision of primary care, emergency treatment (resuscitation and stabilization), and preparation for transfer, usually under the guidance of a medical officer. This capability is normally integral to a major land-based unit and also reflects the provision of medical support inherent to an afloat platform.

  – Role 2

    This includes the reception and sorting of patients as well as the ability to provide elements of damage control resuscitation and the treatment of casualties. This is bolstered by a wider range of medical and nursing interventions and enhanced laboratory and imaging facilities. In addition, this level of care will prepare patients for further transfer with a limited holding capacity to prepare casualties for onward evacuation or for RTD.

  – Role 3

    This incorporates reception from Role 2 Military Treatment Facilities as well as direct receipt from local incidents. Major specialist facilities are available at this level of care with intensive care, holding, and nursing capabilities. Final sorting of casualties for transfer to Role 4 or RTD will occur here.

- **Health Information Technology Infrastructure and Data Management:** Research to enhance health enterprise infrastructure by implementing superior information technology and communications infrastructure.

  - **Health Data Management:** Improvements to data availability, management, storage, and operational use of Enterprise Health Data. Proposed objectives will ensure the unique identification of each patient, as well as aggregated data strategies for population health and big data.

  - **Health Informatics or Information Technology (HIT) Infrastructure:** Research into system interfaces that will ensure that products or systems work efficiently with other products or systems, present or future, without any unintended restrictions. Improve the ability of medical devices to securely and reliably exchange health system or patient-generated data/information with other devices and with medical documentation and

management systems. Research to examine technology integration and clinical/business process integration to reduce implementation barriers with regard to remote health monitoring.

- **Medical Resourcing:** Research to improve financial and personnel management for better delivery of healthcare services. Distribution of healthcare resources around the globe through solutions for personnel management and personnel support functions, or interoperable Joint Force research outcomes with concentration on:

    - **Medical Personnel Resource Planning and Allocation:** Deliver technologies and solutions for personnel management functions. Research to harness potential novel HITI approaches to efficiently and effectively match incoming patients with a provider and efficiencies gained through HITI mobile technologies for budgetary planning and execution.

    - **Education and Training:** Explore technologies to streamline the access to, and management of, educational systems across the MHS. Conduct research to explore the use of HIT in the provision of training. Develop best approaches to leveraging HIT and discovering efficient training delivery across the enterprise. Research to harness potential efficiencies gained through e-textbook interoperability.

    - **Financial Planning and Budget Execution:** Research on efficiencies gained through HITI technologies and data management for budgetary planning and execution.

C. Medical Capabilities to Support Dispersed Operations Portfolio

The Multi-Domain Battle, an operational environment involving greater dispersion and near-isolation over great distances, is likely to cause severe restrictions on mobility for medical missions and shortfalls in both human and materiel human resources due to area denial challenges. Combat units will need to be more self-sufficient and less dependent on logistical support. Combatant commanders with an increased number of sick or wounded Soldiers will face degradation of medical resources and encumbered combat effectiveness without new combat casualty management and Force multiplication strategies.

- **Medical Robotics Research** focuses on man-machine teaming in the delivery of medical care and patient handling to include automated patient monitoring and assessment functions for patient hold capabilities, aid in patient extraction from contested areas, and autonomous closed-loop interventions. As robotic capabilities continue to evolve, autonomous and tele-operated semi-autonomous robotic patient support systems could serve as Force multipliers for medical operations for closed-loop patient monitoring and triage as for robotics intervention. In addition to what may be considered contemporary robotics, i.e., an integration of knowledge-based software with electrical and mechanical engineering technologies, or so-called "hard robotics," one approach to employing robotics for medical applications is the emerging field of so-called "soft robotics." Soft robotics involves the use of soft and deformable structures in the robotics systems that deal with uncertain and dynamic task environments, e.g., grasping and manipulation of unknown objects, locomotion in rough terrains, and physical contacts with living cells and human bodies. Soft robotics

faces a number of fundamental scientific challenges: the studies of unconventional materials are still in their exploration phase; it has not been fully clarified what materials are available and useful for robotics applications; tools and methods for fabrication and assembly are not established; the Government does not have broadly agreed upon methods of modeling and simulation of soft continuum bodies; it is not fully understood how to achieve sensing, actuation and control in soft bodied robots; and the Government is still exploring ways to test, evaluate, and communicate the soft robotics technologies.

- **Medical Autonomous and Unmanned Systems** focuses on delivering enhanced autonomous and unmanned medical capabilities such as the delivery of forward resuscitation treatment and emergency resupply of Class VIII products like whole blood as well as closed-loop en route care modules that could enable general purpose unmanned vehicles to perform casualty evacuation (CASEVAC) missions with some level of on-board medical capability and bi-directional interface with remote medical personnel. Focus areas include automating critical care procedures such as physical (pressure-controlled, palpable, tactile) diagnostics and automated therapeutics in the field and to integrate those capabilities into ground and air unmanned systems platforms to enable unmanned CASEVAC missions. Areas of basic research include:

    - Intelligent agent approaches to integration of intelligent algorithms from multiple critical care systems during PFC or extended evacuation. This effort focuses on new approaches for autonomous clustering of individual closed-loop medical systems and intelligent agent algorithms that interact with each other to enable future development of systems that provide care for multiple injuries (polytrauma) while maximizing patient outcomes through physiologic optimization and deconflicting multiple medical procedures.

    - Strategies for interfacing medical intelligent systems, closed-loop diagnostic, and critical care systems into autonomous unmanned ground and air platforms that allow for unmanned and expedited care of combat casualties and extended evacuations in the dispersed field environment.

    - Research in the area of artificial intelligence and perception systems for accurate detection, monitoring, and modeling of human physiology to enable future applied research in autonomous casualty extraction and en route care systems. Autonomous systems for these applications require high-fidelity mapping and identification of the human body, organs, and structures in near real-time for safe physical contact with casualties and interaction with Soldiers.

- **Virtual Health/Telehealth and Decision Support Tools for the Combat Medic** focuses on enabling greater field medical capabilities for the combat medic in the far-forward future battlefield in PFC and austere environments. The task is to research, design, and develop advanced telehealth capabilities and tools as well as automated decision support systems that operate in a pre-hospital context capable of providing greater support for the combat medic in the diagnosis, triage, and treatment stages, as well as reachback, if available, either synchronously (real time) or asynchronously (store/forward) to specialty care providers and other expert medical consultants from remote locations (both in-garrison and in-theater).

VIII. Radiation Health Effects Research Program

A. Medical Countermeasures (MCMs) for Acute Radiation Exposure: Radiation MCMs include radioprotectants (pre-exposure) and mitigators and therapeutics (post-exposure). Both categories of MCMs are focused on preventing or treating the effects of acute radiation exposure, including the resultant development of acute radiation syndrome (ARS). The focus area examines ARS resulting from exposure to ionizing radiation from radioactive sources or a nuclear detonation, including low linear energy transfer (LET) sources (gamma and x-rays) and high LET sources (neutrons). Research objectives include, but are not limited to, identifying mechanisms of action, obtaining efficacy and safety data in animal models for MCMs for ARS, and demonstrating improved survivability following high doses of radiation with treatment either before exposure or within 24 hours after exposure. Research in biodosimetry in support of MCM development may also be considered.

APPENDIX II: CLINICAL TRIALS

A. Important Aspects of Clinical Trials

A clinical trial is defined as a prospective accrual of human subjects in which one or more intervention(s) (e.g., device, drug, biologic, surgical procedure, rehabilitative modality, behavioral intervention, or other) is tested on a human subject for a measurable outcome with respect to safety, effectiveness, and/or efficacy. This outcome represents a direct effect on the human subject of that intervention or interaction. The term "human subjects" is used in this Broad Agency Announcement to refer to individuals who will be recruited for or who will participate in the proposed clinical trial. For more information, a Human Subject Resource Document is provided at https://ebrap.org/eBRAP/public/Program.htm.

The following are important aspects of submission for the Clinical Trial Award:

- The proposed clinical trial is expected to begin no later than 12 months after the award date.
- The proposed intervention to be tested should offer significant potential impact for the targeted population.
- Inclusion of preliminary data relevant to the proposed clinical trial is required.
- The proposed clinical trial must be based on sound scientific rationale that is established through logical reasoning and critical review and analysis of the literature.
- The application should describe the planned indication for the product label, if appropriate, and include an outline of the development plan required to support that indication.
- The application should demonstrate availability of, and access to, a suitable patient population that will support a meaningful outcome for the study. The PI should discuss how accrual goals will be achieved and how standards of care may impact the study population.
- The application should demonstrate documented availability of and access to the drug/ compound, device, and/or other materials needed, as appropriate. The quality of the product should be commensurate with U.S. Food and Drug Administration (FDA) manufacturing standards applicable to the type and phase of product being developed (i.e., Quality System Regulation, Good Manufacturing Practices).
- The proposed clinical trial design should include clearly defined and appropriate endpoints, and follow Good Clinical Practice (GCP) guidelines.
- The application should include a clearly articulated statistical analysis plan, appropriate statistical expertise on the research team, and a power analysis reflecting sample size projections that will clearly answer the objectives of the study.
- The application should include a clearly articulated data management plan and use of an appropriate database to safeguard and maintain the integrity of the data.

- The application should include a clearly articulated safety management plan outlining how safety pharmacovigilance will be conducted, as applicable.

- The application should include a clearly articulated clinical monitoring plan outlining how the study will be monitored for GCP compliance.

- The application should include a study coordinator(s), who will guide the clinical protocol through the local IRB of record and other Federal agency regulatory approval processes, coordinate activities from all sites participating in the trial, and coordinate participant accrual.

- The application should include a Transition Plan (including potential funding and resources) showing how the product will progress to the next clinical trial phase and/or delivery to the market after the successful completion of the award.

- The application should clearly demonstrate strong institutional support.

Funded studies are required to file the study in the National Institutes of Health (NIH) clinical trials registry, www.clinicaltrials.gov. Refer to the General Application Instructions, Appendix 6, Section C, for further details.

Multi-Institutional Clinical Trials: If the proposed clinical trial is multi-institutional, plans for the multi-institutional structure governing the research protocol(s) should be outlined in the Attachment 9: Study Personnel and Organization. The lead organization responsible for developing the master protocol and master consent form should be identified and should be the single point of contact for regulatory submissions and requirements. A single Institutional Review Board (IRB) or Ethics Committee (EC) pathway is strongly recommended whenever possible. The master protocol and consent form must be reviewed by the Human Research Protection Office prior to distribution to the additional sites for IRB/EC review. Communication and data transfer among the collaborating institutions, as well as how specimens and/or imaging products obtained during the study will be handled, should be included in the appropriate sections of the application. A separate intellectual and material property plan agreed upon by all participating institutions is also required for multi-institutional clinical trials.

If the IRB determines that a trial presents greater than minimal risk to human subjects, the DoD requires an independent research monitor with expertise consistent with the nature of risk(s) identified within the research protocol.

B. Full Proposal/Application Submission Components

The Grants.gov submission package includes the following components (refer to the General Submission Instructions, Section III, for additional information on proposal/application submission):

SF424 (R&R) Application for Federal Assistance Form: Refer to the General Submission Instructions, Section III.A.1, for detailed information.

Attachments:

Each attachment to the full proposal/application components must be uploaded as an individual file in the format specified and in accordance with the formatting guidelines listed in the General Submission Instructions, Appendix 4.

For all attachments, ensure that the file names are consistent with the guidance. Attachments will be rejected if the file names are longer than 50 characters or incorrect file names that contain characters other than the following: A-Z, a-z, 0-9, underscore, hyphen, space, and period. In addition, there are file size limits that may apply in some circumstances. Individual attachments may not exceed 20 MB and the file size for the entire full submission package may not exceed 200 MB.

The Project Narrative is NOT the formal clinical trial protocol. Instead, all essential elements of the proposed clinical trial necessary for scientific review must be included as directed in Attachment 1 (the Project Narrative) and Attachments 6-8 described below. Failure to submit these attachments as part of the application package will result in rejection of the entire application.

- **Attachment 1: Project Narrative (20-page limit):** Upload as "ProjectNarrative.pdf." The page limit of the Project Narrative applies to text and non-text elements (e.g., figures, tables, graphs, photographs, diagrams, chemical structures, drawings) used to describe the project. Inclusion of URLs that provide additional information to expand the Project Narrative and could confer an unfair competitive advantage is prohibited and may result in administrative withdrawal of the application.

  Describe the proposed project in detail using the outline below.

  - **Background:** Describe in detail the rationale for the study. Provide a literature review and describe the preliminary studies and/or preclinical data that led to the development of the proposed clinical trial. Provide a summary of other relevant ongoing, planned, or completed clinical trials and describe how the proposed study differs. Include a discussion of any current clinical use of the intervention under investigation and/or details of its study in clinical trials for other indications (as applicable). The Background section should clearly support the choice of study variables and should explain the basis for the study questions and/or study hypotheses. This section should establish the relevance of the study and explain the applicability of the proposed findings.

    If the proposed clinical trial was initiated using other funding prior to this application, explain the history and background of the clinical trial and declare the source of prior funding. Specifically, identify the portions of the study that will be supported with funds from this award.

  - **Objectives/Specific Aims/Hypotheses:** Provide a description of the purpose and objectives of the study with detailed specific aims and/or study questions/hypotheses. The aims should agree with the primary aims and associated tasks described in the Statement of Work.

- **Study Design:** Describe the type of study to be performed (e.g., prospective, randomized, controlled) and outline the proposed methodology in sufficient detail to show a clear course of action. Describe potential challenges and alternative strategies where appropriate.

    - Identify the intervention to be tested and describe the projected outcomes.

    - Define the study variables, outline why they were chosen, and describe how they will be measured. Include a description of appropriate controls and the endpoints to be tested.

    - Describe the study population and inclusion and exclusion criteria that will be used.

    - Describe the methods that will be used to recruit a sample of human subjects from the accessible population (e.g., convenience, simple random, stratified random).

    - Describe the human subject-to-group assignment process (e.g., randomization, block randomization, stratified randomization, age-matched controls, alternating group, or other procedures), if applicable. Explain the specific actions to accomplish the group assignment (e.g., computer assignment, use of table of random numbers).

    - If using psychometric measures, describe their reliability and validity.

- **Statistical Plan and Data Analysis:** Describe the statistical model and data analysis plan with respect to the study objectives. Specify the approximate number of human subjects to be enrolled. If multiple study sites are involved, state the approximate number to be enrolled at each site. Include a complete power analysis to demonstrate that the sample size is appropriate to meet the objectives of the study. If a subpopulation of a recruited sample population will be used for analysis, complete a statistical analysis to ensure appropriate power can be achieved within the subpopulation study.

- **Attachment 2: Supporting Documentation:** Combine and upload as a single file named "Support.pdf." Start each document on a new page. If documents are scanned to pdf, the lowest resolution (100 to 150 dpi) should be used. The Supporting Documentation attachment should not include additional information such as figures, tables, graphs, photographs, diagrams, chemical structures, or drawings. These items should be included in the Project Narrative. Any additional material viewed as an extension of the Project Narrative will be removed or may result in administrative withdrawal of the application. ***There are no page limits for any of these components unless otherwise noted.***

    - References Cited: List the references cited (including URLs, if available) in the Project Narrative using a standard reference format that includes the full citation (i.e., author[s], year published, title of reference, source of reference, volume, chapter, page numbers, and publisher, as appropriate).

- List of Abbreviations, Acronyms, and Symbols: Provide a list of abbreviations, acronyms, and symbols.

- Facilities, Existing Equipment, and Other Resources: Describe the facilities and equipment available for performance of the proposed project and any additional facilities or equipment proposed for acquisition at no cost to the award. Indicate whether or not Government-furnished facilities or equipment are proposed for use. If so, reference should be made to the original or present Government award under which the facilities or equipment items are now accountable. There is no form for this information.

- Publications and/or Patents: Include a list of relevant publication URLs and/or patent abstracts. If publications are not publicly available, then copies of up to five published manuscripts may be included in Attachment 2. Extra items will not be reviewed.

- Letters of Organizational Support: Provide a letter (or letters, if applicable), signed by the Department Chair or appropriate organization official, confirming the laboratory space, equipment, and other resources available for the project. Letters of support not requested in the Program Announcement, such as those from members of Congress, do not impact application review or funding decisions.

- Letters of Collaboration (if applicable): Provide a signed letter from each collaborating individual or organization that will demonstrate that the PI has the support or resources necessary for the proposed work. If an investigator at a Military Facility is named as a collaborator on a proposal/application, the proposal/application must include a letter from the collaborator's Commander or Commanding Officer at the intramural organization that authorizes the collaborator's involvement.

- Letters of Commitment (if applicable): If the proposed study involves use of a commercially produced investigational drug, device, or biologic, provide a letter of commitment from the commercial entity indicating availability of the product for the duration of the study, support for the proposed phase of research, and support for the indication to be tested.

- Letter of Support for Use of Military and VA Populations or Resources (if applicable; one-page limit per letter is recommended): If the proposed research plan involves access to active duty military and/or VA patient populations or resources, include a letter(s) of support, signed by the lowest-ranking person with approval authority, confirming such access. If access cannot be confirmed at the time of proposal/ application submission, the Government reserves the right to withhold or revoke funding until the PI has demonstrated support for and access to the relevant population(s) and/or resources.

- *If the project involves collaboration with a Military Facility, special requirements apply.* A DoD researcher, to include collaborating DoD PIs, must obtain a letter from his/her commanding officer or Military Facility director authorizing his/her

participation in the research project. This letter must be included with the proposal/application.

- Joint Sponsorship: Describe present or prospective joint sponsorship of any portion of the program outlined in the proposal/application. In the absence of agreements among sponsors for joint support, the proposal/application should be structured so that the research can be carried out without the resources of any other sponsor. If, however, it is desirable to request partial support from another agency, the proposed plan should be stated and the reasons documented. If the plan cannot be formulated at the time the proposal/application is submitted, information should be sent later as an addendum to the proposal/application. Prior approval from both agencies must be secured for research to be undertaken under joint sponsorship. Provide letters of support related to recruitment, subject access, and data access plans

- Intellectual Property: Information can be found in Code of Federal Regulations, Title 2, Part 200.315 (2 CFR 200.315), "Intangible Property."

    - Intellectual and Material Property Plan (if applicable): Provide a plan for resolving intellectual and material property issues among participating organizations.

- Data and Research Resources Sharing Plan: Describe how data and resources generated during the performance of the project will be shared with the research community. Refer to the General Application Instructions, Appendix 2, Section K, for more information about the CDMRP expectations for making data and research resources publicly available.

- **Attachment 3: Technical Abstract (one-page limit):** Upload as "TechAbs.pdf." The abstract is vitally important to both the scientific peer and programmatic review processes. In accordance with Section 8123 of the Consolidated and Further Continuing Appropriations Act, 2015 (Public Law 113-235), the PI is required to submit a technical abstract that fully describes the proposed work. The abstract must contain the title of the project and the name of the PI. Do not include figures or tables in the abstract. Use only characters available on a standard QWERTY keyboard. Spell out all Greek or other non-English letters. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information***.

  The structured technical abstract should be clear and concise and, at a minimum, provide the following information:

  - Background: Provide a brief statement of the ideas and theoretical reasoning behind the proposed work.

  - Objective/Hypothesis: State the objective/hypothesis to be tested. Provide evidence or rationale that supports the objective/hypothesis.

  - Specific Aims: State concisely the specific aims of the study.

- Study Design: Briefly describe the study design.

- Relevance: Provide a brief statement explaining the potential relevance of the proposed work to the specific topic area being addressed and its impact on health outcomes.

- Clinical Impact: Briefly describe how the proposed project will have an impact on research and patient care.

- **Attachment 4: Lay Abstract (one-page limit):** Upload as "LayAbs.pdf." The lay abstract is used by all reviewers. Abstracts of all funded research projects will be posted publicly. ***Do not include proprietary or confidential information***. Use only characters available on a standard QWERTY keyboard. Spell out all Greek letters, other non-English letters, and symbols. Graphics are not allowed.

  Lay abstracts should be written using the outline below. Do not duplicate the technical abstract.

  - Clearly describe the objectives and rationale for the proposed study in a manner readily understood by readers without a background in science or medicine.

  - Describe the ultimate applicability and impact of the research.

    - What types of patients will it help, and how will it help them? Include the current available statistics to the related injury/condition.

    - What are the potential clinical applications, benefits, and risks?

    - What is the projected timeline it may take to achieve the expected patient-related outcome?

    - Describe how the proposed project will benefit Service members, Veterans, and/or their family members.

- **Attachment 5: Statement of Work (SOW) (three-page limit):** Upload as "SOW.pdf." The SOW outlines and establishes the performance expectations and milestones for which the USAMRMC may provide funding. The SOW will be incorporated into the award document and, as such, is subject to release under the Freedom of Information Act. The SOW should identify all collaborating research sites involved in the performance of the research. Suggested SOW formats and examples specific to different types of research projects are available on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm).

  A series of relatively short statements should be included that comprise the approach to each of the major goals or objectives of the proposed research. The statements should outline the specific tasks, systems, key assessments/techniques, and materials that are reasonable estimates for testing the proposed hypotheses of the study. A timeline should be included that shows the work statements to be accomplished in each year of the award.

Any animal use and/or human subjects recruitment should be included. Allow at least 2 to 3 months for the USAMRMC ORP regulatory review and approval processes for studies involving human subjects and 2 to 3 months for studies involving animal subjects.

If applicable, indicate timelines required for regulatory approvals relevant to human subjects research (e.g., Investigational New Drug [IND] and Investigational Device Exemption [IDE] applications) by the FDA or other Government agency.

- **Attachment 6: Human Subject Recruitment and Safety Procedures (no page limit): Upload as "HumSubProc.pdf."** The Human Subject Recruitment and Safety Procedures attachment should include the components listed below.

  - **Study Population:** Describe the target population (to whom the study findings will be generalized) and the nature, approximate number, and pertinent demographic characteristics of the accessible population at the study site(s) (population from whom the sample will be recruited/drawn). Demonstrate that the research team has access to the proposed study population. Furthermore, discuss past efforts in recruiting human subjects from the target population for previous clinical trials (if applicable). Address any potential barriers to accrual and plans for addressing unanticipated delays. Include justification of any age, race, ethnicity, or sex limitations provided.

  - **Inclusion/Exclusion Criteria:** List the inclusion and exclusion criteria for the proposed clinical trial. Inclusion/exclusion criteria should take into consideration the specific risk profile of the studies to be conducted and the standard of care for that patient population. Provide detailed justification for exclusions.

    Inclusion of Women and Minorities in Study: Consistent with the Belmont Report, "Ethical Principles and Guidelines for the Protection of Human Subjects," and Congressional legislation, special attention is given to inclusion of women and/or minorities in studies funded or supported by the USAMRMC. This policy is intended to promote equity both in assuming the burdens and in receiving the benefits of human subjects research. Include an appropriate justification if women and/or minorities will be excluded from the clinical trial.

  - **Description of the Recruitment Process:** Explain methods for identification of potential human subjects (e.g., medical record review, obtaining sampling lists, healthcare provider identification).

    - Describe the recruitment process in detail. Address who will identify potential human subjects, who will recruit them, and what methods will be used to recruit them.

    - If human subjects will be compensated for participation in the study, include a detailed description of and justification for the compensation plan.

    - Describe the recruitment and advertisement materials. The recruitment materials should not be coercive or offer undue inducements and should accurately reflect the study.

- **Description of the Informed Consent Process:** Specifically describe the plan for obtaining informed consent from human subjects.

  - ***For the proposed clinical trial, provide a draft, in English, of the Informed Consent Form.***

  - Identify who is responsible for explaining the study, answering questions, and obtaining informed consent. Include a plan for ensuring that human subjects' questions will be addressed during the consent process and throughout the trial.

  - Include information regarding the timing and location of the consent process.

  - Address issues relevant to the mental capacity of the potential human subject (e.g., altered capacity due to administration of any mind-altering substances such as tranquilizers, conscious sedation or anesthesia, brain injury, stress/life situations, or human subject age), if applicable.

  - Address how privacy and time for decision making will be provided and whether or not the potential human subject will be allowed to discuss the study with anyone before making a decision.

  - Consider the need for obtaining ongoing consent or for re-assessing capacity over the course of a long-term study and describe any relevant procedures to assure continued consent.

  - Describe the plan for the consent of the individual's Legally Authorized Representative (LAR) to be obtained prior to the human subject's participation in the study. State law defines who may act as the LAR. The local IRB of record should be consulted for guidance regarding who can serve as LAR for research at the study site. Note: The PI must describe a clear intent to benefit for human subjects who cannot give their own consent to participate in the proposed clinical trial to be in compliance with Title 10 United States Code Section 980 (10 USC 980) (http://www.gpo.gov/fdsys/pkg/USCODE-2011-title10/pdf/USCODE-2011-title10-subtitleA-partII-chap49-sec980.pdf). If applicable, please refer to the General Application Instructions, Appendix 6, for more information.

  - ***Assent:*** If minors or other populations that cannot provide informed consent are included in the proposed clinical trial, a plan to obtain assent (agreement) from those with capacity to provide it, or a justification for a waiver of assent, should be provided. PIs should consult with their local IRB to identify the conditions necessary for obtaining assent.

- **Screening Procedures:** List and describe any evaluations (e.g., laboratory procedures, history, or physical examination) that are required to determine eligibility/suitability for study participation and the diagnostic criteria for entry. Please note that some screening procedures may require a separate consent or a two-stage consent process. Informed consent must be obtained prior to initiation of any procedures for the purpose of determining eligibility.

- **Risks/Benefits Assessment:**

  - **Foreseeable risks:** Clearly identify all study risks, including potential safety concerns and adverse events. Study risks include any risks that the human subject is subjected to as a result of participation in the clinical trial. Consider psychological, legal, social, and economic risks as well as physical risks. If the risks are unknown, this should be stated. If applicable, any potential risk to the study personnel should be identified.

  - **Risk management and emergency response:**

    - Describe how safety surveillance and reporting to the IRB and FDA (if applicable) will be managed and conducted.

    - Describe all safety measures to minimize and/or eliminate risks to human subjects and study personnel or to manage unpreventable risks. Include safeguards and planned responses such as dose reduction or stopping criteria based on toxicity grading scales or other predetermined alert values.

    - Discuss the overall plan for provision of emergency care or treatment for an adverse event for study-related injuries, to include who will be responsible for the cost of such care.

    - Address any special precautions to be taken by the human subjects before, during, and after the study (e.g., medication washout periods, dietary restrictions, hydration, fasting, pregnancy prevention).

    - Describe any special care (e.g., wound dressing assistance, transportation due to side effects of study intervention impairing ability to drive) or equipment (e.g., thermometers, telemedicine equipment) needed for human subjects enrolled in the study.

    - If the IRB determines that a trial presents greater than minimal risk to human subjects, the DoD requires an independent research monitor with expertise consistent with the nature of risk(s) identified within the research protocol. If applicable, please refer to the General Application Instructions, Appendix 6, for more information on study reporting authorities and responsibilities of the research monitor.

  - **Potential benefits:** Describe known and potential benefits of the study to the human subject, a specific community, or society.

- **Attachment 7: Intervention (no page limit): Upload as "Intervention.pdf."** The Intervention attachment should include the components listed below.

  - **Description of the Intervention:** Identify the intervention to be tested and describe the particular outcomes. As applicable, the description of the intervention should include the following components: complete name and composition, storage and

handling information, source, dose, schedule, administration route, washout period, duration of the intervention, and concomitant medications allowed. Description of devices should include general concept of design, detailed operational instructions, any potential risks to users, and intended benefits. Other types of interventions should be fully described. Indicate who holds the intellectual property rights to the intervention, if applicable, and how the PI has obtained access to those rights for the conduct of the clinical trial.

Summarize key preclinical pharmacological findings, dosage studies, and other clinical studies (if applicable) that examine the safety of the intervention.

- **Study Procedures:** Describe the interaction with the human subject to include the study intervention that he/she will experience. Provide sufficient detail in chronological order for a person uninvolved in the study to understand what the human subject will experience. Provide a schedule (e.g., flowchart or diagram) of study evaluations and follow-up procedures. Discuss how compliance with Good Laboratory Practices (GLP), Good Manufacturing Practices (GMP), and other regulatory considerations will be established, monitored, and maintained, as applicable.

- **Clinical Monitoring Plan:** Describe how the study will be conducted by and monitored for ICH E6 (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) GCP compliance, by an independent clinical trial monitor (or clinical research associate). The monitoring plan should describe the types of monitoring visits to be conducted, the intervals (based on level of risk), how corrective actions will be reported to the Sponsor and PI, and how they will be corrected and prevented by the clinical trial site/PI.

- **Attachment 8: Data Management (no page limit): Upload as "Data_Manage.pdf."** The Data Management attachment should include the components listed below.

  - **Data Management:** Describe all methods used for data collection to include the following:

    - **Identifiers:** Describe the unique identifiers or specific code system to be used to identify human subjects, if applicable.

    - **Confidentiality:**

      - Explain measures taken to protect the privacy of human subjects and maintain confidentiality of study data. Strategies to protect the privacy and confidentiality of study records, particularly those containing identifying information, should be addressed.

      - Address who will have access to study records, data, and specimens, including an acknowledgment that representatives of the DoD are eligible to review study records.

- ❖ Address requirements for reporting sensitive information to state or local authorities.

- **Data capture, verification, and disposition:** Describe how data will be captured and verified. Describe where data (both electronic and hard copy) will be stored, who will keep the data, how the data will be stored, the process for locking the database at study completion, and the length of time data will be stored. Describe the proposed database, how it will be developed and validated, and its capability to safeguard and maintain the integrity of the data. For FDA-regulated studies, compliance with 21 CFR 11 is required.

- **Sharing study results:** In cases where the human subject could possibly benefit medically or otherwise from the information, explain whether or not the results of screening and/or study participation will be shared with human subjects or their primary care provider, to include results from any screening or diagnostic tests performed as part of the study.

– **Laboratory Evaluations:**

- **Specimens to be collected, schedule, and amount:** All specimens that will be collected for study purposes must be clearly stated. The collection schedule and amount of material collected must also be clearly described.

- **Evaluations to be made:** Describe all evaluations that will be made for study purposes. Explain how the results of laboratory evaluations will be used to meet the objectives of the study (or to monitor safety of human subjects).

- **Storage:** Describe specimen storage, to include location of storage, how long specimens will be stored, any special conditions required, labeling, and specimen disposition. Outline the plan to store specimens for future use to include considerations for informed consent and providing human subjects with an opportunity to decline participation in the study.

- **Labs performing evaluations and special precautions:** Identify the laboratory performing each evaluation, as well as any special precautions that should be taken in handling the samples. Special precautions that should be taken by the human subject before, during, or after the laboratory procedure should be clearly defined. If transport of samples is required, describe provisions for ensuring proper storage during transport.

○ **Attachment 9: Study Personnel and Organization (no page limit): Start each document on a new page. Combine into one document and upload as "Personnel.pdf."** The Study Personnel and Organization attachment should include the components listed below.

– **Organizational Chart:** Provide an organizational chart that identifies key members of the study team and provides an outline of the governing structure for multi-institutional studies. Identify collaborating organizations, centers, and/or departments

and name each person's position on the project. Identify the data and clinical coordinating center(s) and note any involvement from Contract Research Organizations, as appropriate. If applicable, identify the FDA regulatory sponsor and any external consultants or other experts who will assist with FDA applications. While there is no specified format for this information, a table(s) or diagram is recommended.

- **Study Personnel Description:** Briefly describe the roles of the individuals listed in the organizational chart on the project. Describe relevant experience and qualifications that demonstrate appropriate expertise for the given role. An external research monitor (if applicable) and study coordinator(s) should be included.

- **Study Management Plan:** Provide a plan for ensuring the standardization of procedures among staff and across sites (if applicable). If the proposed clinical trial is multi-institutional, clearly describe the multi-institutional structure governing the research protocol(s) across all participating institutions. Provide a regulatory submission plan for the master protocol and master consent form by the lead organization; include a single IRB/EC pathway whenever possible. If applicable, describe how communication and data transfer between the collaborating institutions will occur, as well as how data, specimens, and/or imaging products obtained during the study will be handled and shared.

- **Attachment 10: Surveys, Questionnaires, and Other Data Collection Instruments, if applicable (no page limit): Upload as "Surveys.pdf."** The Surveys, Questionnaires, and Other Data Collection Instruments attachment should include a copy of the most recent version of surveys, questionnaires, data collection forms, rating scales, interview guides, or other instruments. For each instrument, describe how the information collected is related to the objectives of the study. Describe how and when the instrument(s) will be administered. Describe how the instrument(s) will be adapted to the subject population, if applicable.

- **Attachment 11: Impact Statement (two-page limit): Upload as "Impact.pdf."**

  - Identify the volunteer population(s) that will participate in the proposed intervention, describe how they represent the target population that would benefit from the intervention, and describe the potential impact of the proposed clinical trial on the outcomes of individuals with the targeted disease or condition.

  - Describe the short-term impact: Detail the anticipated outcomes that will be directly attributed to the results of the proposed clinical trial.

  - Describe the long-term impact: Explain the long-range vision for implementation of the intervention in the clinic or field, and describe the anticipated long-term benefits for the targeted population.

  - Describe any relevant controversies or treatment issues that will be addressed by the proposed clinical trial.

- Describe any potential issues that might limit the impact of the proposed clinical trial.

- Describe how the intervention represents an improvement over currently available interventions and/or standards of care.

- **Attachment 12: Transition Plan and Regulatory Strategy (three-page limit): Upload as "Transition.pdf."** Describe/discuss the methods and strategies proposed to move the intervention to the next phase of development (clinical trials, commercialization, and/or delivery to the civilian or military market) after successful completion of the award. Applicants are encouraged to work with their organization's Technology Transfer Office (or equivalent) to develop the transition plan. PIs are encouraged to explore developing relationships with industry and/or other funding agencies to facilitate moving the product into the next phase of development. The post-award transition plan should include the components listed below.

  - The planned indication for the product label, if appropriate, and an outline of the development plan required to support that indication. Describe in detail the FDA regulatory strategy, to include considerations for compliance with GMP, GLP, and GCP (if appropriate).

  - Details of the funding strategy to transition to the next level of development and/or commercialization (e.g., partners, internal/external funding opportunities to be applied for). Include a description of collaborations and other resources that will be used to provide continuity of development.

  - For Knowledge Products, a description of collaborations and other resources that will be used to provide continuity of development including proposed development or modification of clinical practice guidelines (CPGs) and recommendations, provider training materials, patient brochures, and other clinical support tools, scientific journal publications, models, simulations, and applications. A "Knowledge Product" is a non-materiel product that addresses an identified need, topic area, or capability gap, is based on current evidence and research, aims to transition into medical practice, training, tools, or to support materiel solutions (systems to develop, acquire, provide, and sustain medical solutions and capabilities), and educates or impacts behavior throughout the continuum of care, including primary prevention of negative outcomes.

  - A brief schedule and milestones for transitioning the intervention to the next phase of development (next-phase clinical trials, commercialization, delivery to the military or civilian market, incorporation into clinical practice, and/or approval by the FDA).

  - Ownership rights/access to the intellectual property necessary for the development and/or commercialization of products or technologies supported with this award and the Government's ability to access such products or technologies in the future.

  - A risk analysis for cost, schedule, manufacturability, and sustainability.

- **Attachment 13: IND/IDE Documentation:** If submitting multiple documents, start each document on a new page. **Combine and upload as a single file named "IND-IDE.pdf."**

  - Complete the IND/IDE Documentation Form, which is available for download on the Full Announcement page for the Broad Agency Announcement on Grants.gov.

  - State whether the trial requires regulation by the FDA. If FDA regulation is required, describe the planned indication for the proposed product and whether an IND/IDE is necessary. Identify the IND/IDE sponsor. If an IND or IDE is required, it must be submitted to the FDA ***prior to submission of the full proposal/application.***

  - If an IND or IDE has already been obtained for the investigational drug or device pertaining to the indication to be studied, provide evidence in the form of formal communication (e.g., letterhead correspondence) from the FDA.

  - If an IND or IDE application has been submitted, provide an explanation of the status of the IND or IDE application (e.g., past the critical 30-day period, pending response to questions raised by the Agency, on clinical hold). Provide a summary of previous meetings with the FDA on development of this product, if appropriate. A copy of the Agency meeting minutes should be included if available. Provide copies of communications from the FDA relevant to the most recent status of the IND or IDE application.

  - If an IND or IDE is not required for the proposed study, or if it qualifies for an abbreviated IDE, provide evidence in the form of formal communication (e.g., letterhead correspondence) from the FDA or the IRB of record to that effect. Devices qualifying for an abbreviated IDE must comply with the abbreviated IDE requirements but do not require the submission of an IDE application to the FDA.

- **Attachment 14: Military Relevance Statement (one-page limit): Upload as "MilRel.pdf."**

  - Describe how the proposed study is responsive to the healthcare needs of military – Service members, Veterans, and/or beneficiaries. Provide information about the incidence and/or prevalence of the disease or condition in the general population as well as in military Service members, Veterans, and/or beneficiaries.

  - If active duty military, military families, and/or Veteran population(s) will be used in –the proposed research project, describe the population(s) and the appropriateness of the population(s) for the proposed study. If a non-military population will be used for the proposed research project, explain how the population simulates the targeted population (i.e., military Service members, Veterans, and/or beneficiaries).

  - If applicable, show how the proposed research project aligns with DoD and/or VA – areas of research interests. Provide a description of how the knowledge or technology gained from the research could be implemented in a dual-use capacity to benefit the civilian population and address a military need, as appropriate.

- **Attachment 15: DoD Military Budget Form(s), if applicable: Upload as "MFBudget.pdf."** If a military facility (military health system facility, research laboratory, treatment facility, dental treatment facility, or a DoD activity embedded with a civilian medical center) will be a collaborator in performance of the project, complete the DoD Military Budget Form, available for download on the eBRAP "Funding Opportunities & Forms" web page (https://ebrap.org/eBRAP/public/Program.htm), including a budget justification, for each military facility as instructed. The costs per year should be included on the Grants.gov Research and Related Budget form under subaward costs. Refer to the General Application Instructions, Section III.A.7., for detailed information.

Research & Related Senior/Key Person Profile (Expanded): Refer to the General Submission Instructions, Section III.A.3, for detailed information.

- PI Biographical Sketch (five-page limit): Upload as "Biosketch_LastName.pdf."
- PI Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."
- Key Personnel Biographical Sketches (five-page limit each): Upload as "Biosketch_LastName.pdf."
- Key Personnel Previous/Current/Pending Support (no page limit): Upload as "Support_LastName.pdf."

Research & Related Budget: Refer to the General Submission Instructions, Section III.A.4, for detailed information.

Budget Justification (no page limit): Upload as "BudgetJustification.pdf." The budget justification for the entire period of performance must be uploaded to the Research & Related Budget after completion of the budget for Period 1.

Project/Performance Site Location(s) Form: Refer to the General Submission Instructions, Section III.A.5, for detailed information.

R & R Subaward Budget Attachment(s) Form (if applicable): Refer to the General Submission Instructions, Section III.A.6, for detailed information.

NOTE: Proposals/Applications from **Federal agencies** must include in their budget justifications a **Federal Financial Plan**. Proposals/Applications from organizations that include **collaborations with DoD Military Facilities** must comply with special requirements. Refer to the General Submission Instructions, Section III.A.4, Research & Related Budget, for detailed information.

APPENDIX III: DOD AND VA WEBSITES

PIs are encouraged to integrate and/or align their research projects with DoD and/or VA research laboratories and programs. Collaboration with DoD or VA investigators is also encouraged. Below is a list of websites that may be useful in identifying additional information about DoD and VA areas of research interest, ongoing research or potential opportunities for collaboration.

Air Force Office of Scientific Research
http://www.wpafb.af.mil/afrl/afosr/

Air Force Research Laboratory
http://www.wpafb.af.mil/afrl

Armed Forces Radiobiology Research Institute
http://www.usuhs.edu/afrri/

Clinical and Rehabilitative Medicine Research Program
https://crmrp.amedd.army.mil

Combat Casualty Care Research Program
https://ccc.amedd.army.mil

Congressionally Directed Medical Research Programs
http://cdmrp.army.mil

Defense Advanced Research Projects Agency
http://www.darpa.mil/

Defense Health Agency
https://health.mil/dha

Defense Technical Information Center
http://www.dtic.mil

Defense Threat Reduction Agency
http://www.dtra.mil/

Military Health System Research Symposium
https://mhsrs.amedd.army.mil/SitePages/Home.aspx

Military Infectious Diseases Research Program
https://midrp.amedd.army.mil

Military Operational Medicine Research Program
https://momrp.amedd.army.mil

Naval Health Research Center
http://www.med.navy.mil/sites/nhrc

Navy Bureau of Medicine and Surgery
http://www.med.navy.mil/

Naval Medical Research Center
www.med.navy.mil/sites/nmrc

Navy and Marine Corps Public Health Center
http://www.nmcphc.med.navy.mil/

Office of Naval Research
http://www.onr.navy.mil/

Office of the Under Secretary of Defense for Acquisition, Technology and Logistics
http://www.acq.osd.mil/

Telemedicine and Advanced Technology Research Center
http://www.tatrc.org/

Uniformed Services University of the Health Sciences
http://www.usuhs.edu/research

U.S. Army Aeromedical Research Laboratory
www.usaarl.army.mil

U.S. Army Center for Environmental Health Research
http://usacehr.amedd.army.mil

U.S. Army Institute of Surgical Research
http://www.usaisr.amedd.army.mil/

U.S. Army Research Institute of Environmental Medicine
http://www.usariem.army.mil/

U.S. Army Medical Research Institute of Infectious Diseases
http://www.usamriid.army.mil/

U.S. Army Medical Research and Materiel Command
http://mrmc.amedd.army.mil

U.S. Army Research Institute
https://sslweb.hqda.pentagon.mil/ari/

U.S. Army Research Laboratory
http://www.arl.army.mil

U.S. Army Research, Development and Engineering Command
www.army.mil/rdecom

U.S. Army Resiliency Directorate
http://www.army.mil/readyandresilient/

U.S. Department of Defense Blast Injury Research Program
https://blastinjuryresearch.amedd.army.mil/

U.S. Department of Veterans Affairs, Office of Research and Development
http://www.research.va.gov

U.S. Naval Research Laboratory
http://www.nrl.navy.mil

Walter Reed Army Institute of Research
http://www.wrair.army.mil/

APPENDIX IV: PROCEDURES FOR ACQUISITION, PROCESSING, STORAGE, AND SHIPMENT OF BIO-FLUIDS

The following pre-analytical variables should be recorded when collecting bio-fluids:

- Time of sample collection
- Time of sample freezing and the interval between collection and freezing
- Temperature of freeze
- Needle size and type (21 G preferred)
- Tube collection order
- Tube labels
- Centrifugation parameters
- Handling, shipping, and storage temperature
- Tube handling
- Small aliquot size
- Sample storage quality control

The following subject characteristics should be recorded when collecting bio-fluids:

- Fasting and diet
- Therapy
- Time of day
- Physical activity
- Acute state (rested?)
- Body position

APPENDIX V: NATIONAL INSTITUTE OF MENTAL HEALTH DATA ARCHIVE — INFORMED CONSENT

Psychological Health, Human Subjects Studies
Informed Consent and Data Repository

Data from this study may be submitted to the National Institute of Mental Health Data Archive (NDA). NDA is a data repository run by the National Institute of Mental Health (NIMH) that allows researchers studying mental illness to collect and share deidentified information with each other. A data repository is a large database where information from many studies is stored and managed. Deidentified information means that all personal information about research participants such as name, address, and phone number is removed and replaced with a code number. With an easier way to share, researchers hope to learn new and important things about mental illnesses more quickly than before.

During and after the study, the researchers will send deidentified information about your health and behavior and in some cases, your genetic information, to NDA. Other researchers nationwide can then file an application with the NIMH to obtain access to your deidentified study data for research purposes. Experts at the NIMH who know how to protect health and science information will look at every request carefully to minimize risks to your privacy.

You may not benefit directly from allowing your information to be shared with NDA. The information provided to NDA may help researchers around the world treat future children and adults with mental illnesses so that they have better outcomes. NIMH will also report to Congress and on its web site about the different studies that researchers are conducting using NDA data. However, you will not be contacted directly about the data you contributed to NDA.

You may decide now or later that you do not want to share your information using NDA. If so, contact the researchers who conducted this study, and they will tell NDA, which can stop sharing the research information. However, NDA cannot take back information that was shared before you changed your mind. If you would like more information about NDA, this is available on-line at https://data-archive.nimh.nih.gov.

APPENDIX VI: ACRONYMS AND ABBREVIATIONS

ARS	Acute Radiation Syndrome
BAA	Broad Agency Announcement
BoNT	Botulinum Neurotoxin
BSV	Biosurveillance
BSVE	Biosurveillance Ecosystem
CAM	Complementary and Alternative Medicine
CASEVAC	Casualty Evacuation
CCCRP	Combat Casualty Care Research Program
CCSI	Combat Casualty Simulation Initiative
CDE	Common Data Element
CDMRP	Congressionally Directed Medical Research Programs
CFR	Code of Federal Regulations
CPG	Clinical Practice Guidelines
COI	Conflict of Interest
CRMRP	Clinical and Rehabilitative Medicine Research Program
DoD	Department of Defense
DoDGAR	DoDGAR Grant and Agreement Regulations
DTRA	Defense Threat Reduction Agency
DUNS	Data Universal Numbering System
eBRAP	Electronic Biomedical Research Application Portal
EO	Executive Order
FAPIIS	Federal Awardee Performance and Integrity Information System
FAR	Federal Acquisition Regulation
FDA	U.S. Food and Drug Administration
FedBizOpps	Federal Business Opportunities
FFRDC	Federally Funded Research and Development Center
FITBIR	Federal Interagency Traumatic Brain Injury Research
FY	Fiscal Year
GA	Tabun
GB	Sarin
GD	Soman
GCP	Good Clinical Practices
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
HIT	Health Informatics or Information Technology
HITI	Health Information Technology and Informatics

JBCF	Joint Biosurveillance Common Framework
JCM	Joint Casualty Management
JETS	Joint Evacuation and Transport Simulation
JMLIS	Joint Medical Logistics and Infrastructure Support
JSTO-CBD	Joint Science and Technology Office for Chemical and Biological Defense
LET	Linear Energy Transfer
MBDRP	Medical Biological Defense Research Program
MCDRP	Medical Chemical Defense Research Program
MCM	Medical Countermeasure
MIDRP	Military Infectious Diseases Research Program
MOMRP	Military Operational Medicine Research Program
MSISRP	Medical Simulation and Information Sciences Research Program
NATO	North Atlantic Treaty Organization
NDA	National Institute of Mental Health Data Archive
NIH	National Institutes of Health
NIMH	National Institute of Mental Health
NMS	Neuromusculoskeletal
NRAP	National Research Action Plan
NSOCM	NATO Special Operations Combat Medic
OMB	Office of Management and Budget
PFC	Prolonged Field Care
PI	Principal Investigator
POINTS	Point of Injury Training Simulation
PTSD	Post-Traumatic Stress Disorder
RHERP	Radiation Health Effects Research Program
RTD	Return to Duty
SAM	System for Award Management
SBIR	Small Business Innovation Research
SOW	Statement of Work
TBI	Traumatic Brain Injury
USAMRAA	U.S. Army Medical Research Acquisition Activity
USAMRMC	U.S. Army Medical Research and Materiel Command
USC	United States Code
VA	Department of Veterans Affairs

Acta Neuropathol (2004) 108: 453–460
DOI 10.1007/s00401-004-0916-1

REGULAR PAPER

Herbert Weissenböck · Tamás Bakonyi
Sonja Chvala · Norbert Nowotny

Experimental Usutu virus infection of suckling mice causes neuronal and glial cell apoptosis and demyelination

Received: 13 May 2004 / Revised: 22 July 2004 / Accepted: 26 July 2004 / Published online: 14 September 2004
Springer-Verlag 2004

Abstract Usutu virus (USUV), a mosquito-borne flavivirus of the Japanese encephalitis virus group has been responsible for avian mortality in Austria since 2001. In the present study, the neuropathogenicity and neuroinvasiveness of USUV for 1-week-old suckling mice was investigated. After intraperitoneal inoculation, clinical signs like depression, disorientation, paraplegia, paralysis and coma were observed between 6 and 11 days post infection. Histologically, there was widespread neuronal apoptosis especially in the brain stem. Inflammatory infiltrates were scarce. Apoptosis was also present in white matter of cerebellum, medulla and spinal cord, and was frequently accompanied by primary demyelination. While apoptosis of neurons was clearly associated with presence of viral signals, the cause of apoptosis of white matter cells was more ambiguous. However, focal immunostaining was found in the white matter, especially in the spinal cord. As with all flaviviruses, USUV proved to be neuropathogenic for mice. In contrast to other flaviviruses, neuroinvasion occurred only in animals that were not older than 1 week at the time of inoculation. While neuronal apoptosis is a general aspect of flavivirus pathogenicity, demyelination seems to be a unique feature of USUV infection.

Keywords Usutu virus · Mouse · Experimental infection · Apoptosis · Demyelination

H. Weissenböck (✉) · S. Chvala
Institute of Pathology and Forensic Veterinary Medicine,
Department of Pathobiology, University of Veterinary Medicine,
Veterinärplatz 1, 1210 Vienna, Austria
E-mail: herbert.weissenboeck@vu-wien.ac.at
Tel.: +43-1-250772418
Fax: +43-1-250772490

T. Bakonyi · N. Nowotny
Clinical Virology Group,
Clinical Department of Diagnostics,
University of Veterinary Medicine,
Veterinärplatz 1, 1210 Vienna, Austria

T. Bakonyi
Department of Microbiology and Infectious Diseases,
Faculty of Veterinary Science, Szent István University,
1143 Budapest, Hungary

N. Nowotny
Department of Medical Microbiology,
Faculty of Medicine and Health Sciences,
United Arab Emirates University,
Al Ain, United Arab Emirates

Introduction

Usutu virus (USUV), a mosquito-borne flavivirus of the Japanese encephalitis virus group, has been responsible for avian mortality in Austria since 2001 [28]. The virus is highly pathogenic for Eurasian blackbirds (Turdus merula), which succumbed in high numbers, but also affects other bird species [29]. Most members of this virus group are pathogenic for human beings, inducing febrile illnesses, meningitis or encephalitis. The pathogenicity of USUV for humans seems to be low with so far only one published case of fever and rash in Central Africa [1]. Except for birds in Austria, clinical disease due to USUV infection has not been reported from any animal species and any other country. Practically all members of the Flavivirus and Alphavirus genera are neuropathogenic for mice, and experimental infections with some of these viruses serve as models for the study of apoptosis, inflammation or demyelination induced by neuronotropic viruses [4, 9, 14, 18, 27]. The neuropathogenicity of USUV for mice has never been demonstrated. In this report we describe the effects of peripheral infection with USUV in suckling mice, and show that neuronal apoptosis and multifocal demyelination are key features in this experimental disease.

Materials and methods

Experimental animals

For all experiments 1-week-old specified pathogen-free (SPF) NMRI mice were used. The animals were

purchased from Charles River Hungary Ltd. (Budapest) and were kept in the experimental animal house of the Department of Microbiology, Faculty of Veterinary Science, Budapest. The principles of laboratory animal care (NIH publication No. 86–23, revised 1985) were followed and the animals were kept and handled according to the standard requirements and regulations of the Experimental Animal Welfare Codex of the Faculty of Veterinary Science, Budapest (project permit number: 70/2003).

Virus

USUV strain 939/01, designated Vienna 2001-blackbird, was isolated in Vero cell culture, which had been inoculated with a brain homogenate of a blackbird. This blackbird was found dead in Perchtoldsdorf near Vienna on September 4, 2001, and USUV-specific nucleic acid and antigen had been identified in several organs of the bird by reverse transcription (RT)-PCR, sequencing, in situ hybridization (ISH) and immunohistochemistry (IHC). The virus isolate was passaged twice in Vero cells and then stored frozen at ff 80 C.

Experimental schedule, euthanasia, necropsy, histology

Forty-two (experiment 1) and 10 (experiment 2) mice were infected intraperitoneally (i.p.) into the abdominal cavity with 1,000 tissue culture infectious doses 50% ($TCID_{50}$) of virus in 100 l l minimal essential medium (MEM). The amount of virus was checked by back-titration of the working dilution on Vero cell cultures. Control animals were inoculated i.p. with 100 l l MEM without virus. Preliminary experiments (not shown) had revealed that this inoculation procedure resulted in reliable induction of encephalitis in 1-week-old animals, but not in animals aged 2 weeks and more. Additional pilot studies (not shown) demonstrated that intracerebral (i.c.) inoculation of adult and suckling mice resulted in mortality and widespread neuronal death, which was accompanied by non-suppurative encephalitis in adult animals. To study both neuroinvasion and neurovirulence of USUV, we decided to use the i.p. route for the main experiments.

For the study of sequential development of lesions and viral distribution, 42 mice were used. It was planned to process 2 infected animals and one control animal daily, starting at day 1 p.i. until the planned end of the experiment at day 14 (experiment 1).

An additional experiment (experiment 2) with 10 animals treated as above was performed to study spinal cord changes with the plan to euthanize the animals after development of clinical symptoms. In both experiments the mice were checked twice daily for clinical signs.

According to the experimental schedule, the mice were killed by overdosing of CO_2. Animals showing severe clinical signs were also killed, regardless of the schedule. The mice from experiment 1 were necropsied, and brain, lung, heart, liver, spleen, kidneys and small intestine were collected; some tissues were fixed in 4% buffered formaldehyde and others—for virological examinations—frozen at ff 80 C. From the mice of experiment 2 only brain and spinal cord were fixed. The fixed organs were embedded in paraffin wax and a series of 4-l m sections were cut. The brains of experiment 1 animals were cut into paramedian sagittal sections. The brains of experiment 2 animals were cut into transversal sections at three levels and the entire vertebral column was cut at four transversal levels corresponding to C3, C5, L1 and L3 segments. The sections were stained with hematoxylin and eosin for general histomorphology, cresyl echt violet for neuronal morphology, and Luxol-fast blue (Klu¨ver-Barrera) for myelin. In addition, they were subjected to IHC, ISH and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) as described below.

Immunohistochemistry

The avidin biotin complex (ABC) technique and a rabbit USUV antiserum (produced at the Department of Microbiology, Faculty of Veterinary Science, Budapest) were used to localize USUV antigen in tissue samples. Additional primary antibodies for detection of T cells (anti-CD3; Dako, Glostrup, Denmark), macrophages (anti-lysozyme; Dako), astrocytes (anti-glial fibrillary acidic protein, GFAP; Dako) and neurofilament (anti-neurofilament 200; Sigma, St. Louis, MO) were applied. Paraffin wax sections (4 l m) were mounted on positively charged glass slides (Superfrost plus; Menzel-Gläser, Braunschweig, Germany). After dewaxing and rehydration, antigen retrieval was performed by protease digestion (protease type XIV, Sigma; lysozyme, GFAP, neurofilament) or heating the slides in citrate buffer (pH 6.0) in a microwave oven (USUV, CD3). Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min. The sections were then incubated with a 1:10 dilution of normal goat serum (Vector Laboratories, Burlingame, CA) for 60 min, followed by an overnight incubation with the primary antibodies at 4 C in the following dilutions: anti-USUV 1:2,500; anti-GFAP 1:1,000; and anti-CD3, anti-lysozyme, and anti-neurofilament all at 1:200. After washing in phosphate-buffered saline (PBS), the tissues were incubated with a 1:400 dilution of biotinylated anti-rabbit IgG (Vector Laboratories) for 30 min, followed by the Vectastain Elite ABC Kit (Vector Laboratories) for 60 min. The reaction was visualized with a DAB Substrate Kit for peroxidase (Vector Laboratories). After counterstaining with hemalum and dehydrating, the slides were mounted under coverslips with Neomount (VWR, Vienna, Austria).

In situ hybridization

ISH was used to show the distribution of USUV-specific nucleotide sequences After dewaxing and rehydration,

the tissueswere incubated in TRIS-buffered saline with 2.5 l g/ml proteinase K (Roche, Basel, Switzerland) at 37 C for 30 min. After rinsing, the slides were covered with hybridization mixture, 100 l l of which contained 50 l l formamide, 20 l l 20· standard saline citrate buffer (SSC), 2 l l Denhardt's, 10 l l dextran sulfate, 5 l l boiled herring sperm DNA, 11.5 l l distilled water and 1.5 l l USUV-specific digoxigenin-labeled oligonucleotide probe (MWG Biotech, Ebersberg, Germany) with a final concentration of approximately 0.35 ng/l l. The probe sequence was: 5'-TCGCATAACTTTCACCACCTT-GTGTTTGTAGGTCAGCTC-3'. The slides were hybridized for 16 h in a humid chamber at 40 C. After hybridization, the slideswere washedin 2· SSC, 1· SSC and 0.1· SSC (10 min each) at room temperature. For detection of digoxigenin-labeled hybrids, the slides were incubated for 60 min with anti-digoxigenin-AP Fab fragments (dilution 1:100; Roche). The slides were rinsed twice and the signal was visualized using BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (4-nitro blue tetrazolium chloride) (both Roche). After 4 h, the color development was stopped with TE buffer (pH 8.0); the slides were counterstained with hemalum, dehydrated, and mounted under coverslipswith Aquatex (VWR).

TUNEL

Detection of nuclear DNA fragmentation indicative of apoptosis was achieved using DeadEnd Colorimetric TUNEL System (Promega, Madison, WI). The assay was performed according to the manufacturer's instructions.

RT-PCR

Those samples, which were negative by histology, IHC and ISH (infected mice from days 1 to 4 and controls) as well as selectedIHC- and ISH-positive sampleswere investigated by RT-PCR to detect USUV-specific nucleic acid. The organs of the animals were homogenized in ceramic mortars using sterile sand, and the homogenates were suspended in 1,500 l l diethyl pyrocarbonate (DEPC)-treated distilled water. The suspension was centrifuged at 8,000 g for 10 min and RNA was isolated from 140 l l of the supernatant using QiaAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT and amplification were performed with a continuous RT-PCR method using the Qiagen One-Step RT-PCR Kit (Qiagen). Each 25-l l reaction mixture contained 5 l l of 5· buffer (final $MgCl_2$ concentration 2.5 mM), 0.4 mM of each deoxynucleoside triphosphate (dNTP), 10 U RNasin RNase inhibitor (Promega), 20 pmol of the USUV-specific forward (5'-AGGACCATTGGTTAGGAAGA-3') and reverse (5'-GGCTTGACAACACAATCATC-3') primers, 1 l l of enzyme mix (containing Omniscript and Sensiscript Reverse Transcriptases and HotStarTaq DNA polymerase) and 2.5 l l of template RNA. RT was carried out at 50 C for 30 min, followed by a denaturation step at 95 C for 15 min. Thereafter the cDNA was amplified in 40 cycles (heat denaturation at 94 C for 40 s, primer annealing at 57 C for 50 s, and DNA extension at 72 C for 1 min), and the reaction was completed by a final extension for 7 min at 72 C. The sampleswere kept at 4 C until electrophoresiswas carried out. The reactions were performed in a Perkin Elmer GeneAmp PCR System 2400 thermocycler. Following RT-PCR, 10 l l of the amplicons were electrophoresed in a 1.2% TRIS acetate-EDTA-agarose gel at 5 V/cm for 80 min. The gel was stained with ethidium bromide; bands were visualized by UV transillumination at 312 nm using a TFX 35 M UV transilluminator (Life Technologies, Paisley, UK) and photographed with a Kodak DS Electrophoresis Documentation and Analysis System using the Kodak Digital Science 1D software program. Product sizes were determined with reference to a 100-bp DNA ladder (Promega).

Results

Clinical signs

Experiment 1

The animals investigated from days 1 to 5 of the experiment had no clinical signs. At day 6, seven mice showed depression or disorientation. In three mice identical clinical signs started at day 7, in one at day 8, in three at day 9 and in two eachat days 10 and 11. The signs were progressiveand led to paraplegia, paralysis, and coma. Death occurred within at least 2 days after onset, if the animal had not beenkilled before. All mice that were still in the experiment after day 5 developed clinical disease. Diseased animals did not gain weight and by the end of the experimenthad only about half the weight of controls.

The experiment could not be performed as initially designed, becausebetween days 7 and 8 of the experiment eight animals died. There were two more spontaneousdeathsat days 9 and 11. Thus, there wereno more infected animals left after day 11.

There were no clinical signs in control animals.

Experiment 2

Clinical signs were noticed between days 6 and 9 p.i. They were identical to the signs described above. After the development of clinical signs, four animals were killed on day 7 p.i. and three animals eachon days 8 and 9 p.i.

Neuropathology and TUNEL assay

Experiment 1

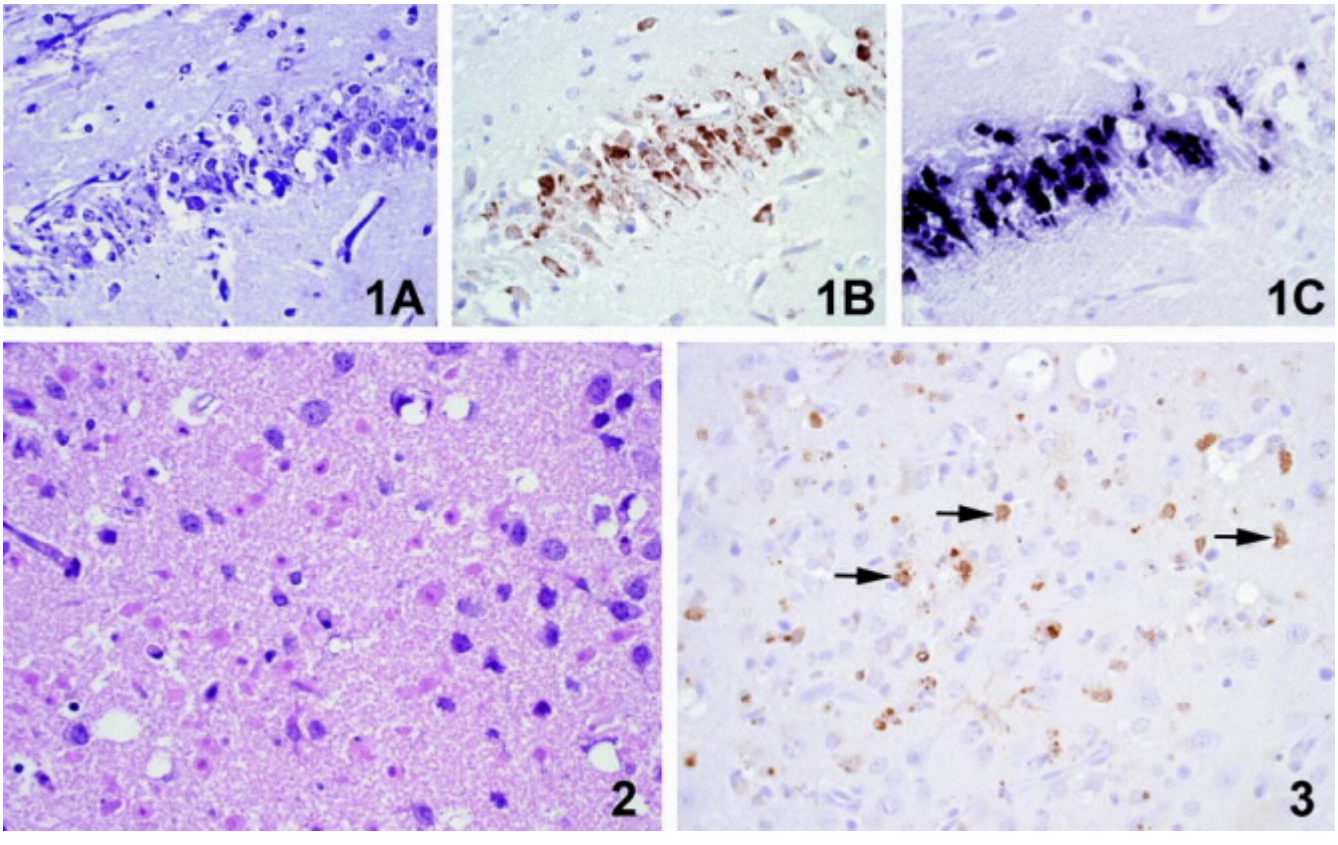
All control animals and the infected animals killed between days 1 and 4 p.i. showed no pathological lesions, not more than five TUNEL-positive cells per · 100 field, and no viral signals by IHC and ISH.

Infected animals which were killed or died from day 5 p.i. onwards showed multifocal or diffuse neuronal death widely distributed in the brain. Neuronal death was identified as nuclear shrinkage and condensation, frequently followed by breaking into chromatin fragments (Fig. 1A). In larger neurons the cytoplasm was frequently distinctly hypereosinophilic, suggesting lytic necrosis (Fig. 2). Using TUNEL, however, in areas with neuronal death, the majority of neurons had positive signals und TUNEL staining showed a clear correlation to morphological changes. The fragmented DNA was usually confined to the nuclei; sometimes, however, diffuse, brownish-staining patterns of the cytoplasm were also observed, suggesting leakage of DNA fragments into the cytoplasm or extracellular space (Fig. 3). From days 5 to 6 p.i., lesions and TUNEL signals were mild to moderate and multifocal. They were predominantly found in nuclei of the medulla oblongata (such as the medial parabrachial nucleus, spinal trigeminal nucleus, rostral periolivary region, spinal vestibular nucleus, lateral vestibular nucleus), in the cerebellum, affecting Purkinje cells, cells of the internal granule layer and white matter cells, in the mesencephalon, especially in external cortex of the inferior colliculus, deep mesencephalic nucleus, and deep gray layer of the superior colliculus, in thalamic nuclei, and, to a limited extent, in the CA3 region of the hippocampus. From days 7 to 9 p.i., the lesions were generally severe and more widespread but had still a multifocal distribution pattern. In addition to the regions already mentioned, they were also present in the dentate gyrus and the CA1 and CA2 region of the hippocampus. In the medulla, the lesions were more widespread, including also the motor trigeminal nucleus, the parvicellular reticular nucleus, the nucleus of the solitary tract, and the spinal trigeminal nucleus. In other brain regions the pararubral nucleus of the mesenceph-

Fig. 1 A Abundant neuronal death in the CA1 region of the hippocampus, discernible by shrunken pale perikarya and different forms of nuclear degradation in a USUV-infected mouse, day 8 p.i.; Cresyl echt violet staining. B, C Adjacent sections to Fig. 1A. Neuronal damage correlates with viral replication in the affected cells; viral protein is demonstrated by IHC with USUV-specific antibodies (B), and viral nucleic acid by ISH with a USUV-specific oligonucleotide probe (C) in the cytoplasm of the affected cells; (USUV Usutu virus, p.i. post infection, IHC immunohistochemistry, ISH in situ hybridization). A–C · 270

Fig. 2 Extensive neuronal death indicated by hypereosinophilic perikarya, karyopyknosis and karyolysis in the thalamus of a USUV-infected mouse, day 9 p.i.; hematoxylin and eosin staining. · 260

Fig. 3 Numerous TUNEL-positive cells in the thalamus of a USUV-infected mouse. Note that signal is not always strictly nuclear, but frequently seems to be present in the entire cells (arrows); day 7 p.i.; TUNEL staining. · 260



alon, the nucleus of the horizontal limb of the diagonal band, the caudate putamen, the claustrum, the accumbens nucleus, the anterior olfactory nucleus, and foci in the motor, sensory and visual cortex were affected. At days 10 and 11 p.i., lesions and TUNEL signals were significantly reduced and only focally found in the cerebellum, thalamus, motor cortex, dentate gyrus, caudate putamen and medulla. The distribution of neuronal damage was not uniform in all animals investigated per time point. The regions mentioned above and shown in Fig. 4 represent a compilation of the affected areas of all animals available per time point.

Subtle and inconsistent demyelinating lesions were observed by day 7 p.i. By day 8 p.i., the demyelination was robust and consistently found through the last time points examined. The demyelination was present in the cerebellar white matter (Fig. 5), where it was predominantly multifocal, and in some white matter tracts of the medulla oblongata. Immunostaining for neurofilament in demyelinated areas revealed no significant axonal damage or axonal loss. This finding is indicative of primary demyelination. Numerous TUNEL-positive cells were frequently found in demyelinated areas (Fig. 6).

There were only minor inflammatory changes at all time points examined, as evidenced by immunostaining for T cells and macrophages; these changes consisted of infrequent and mild perivascular infiltrates of mononuclear cells. There was no obvious glial response.

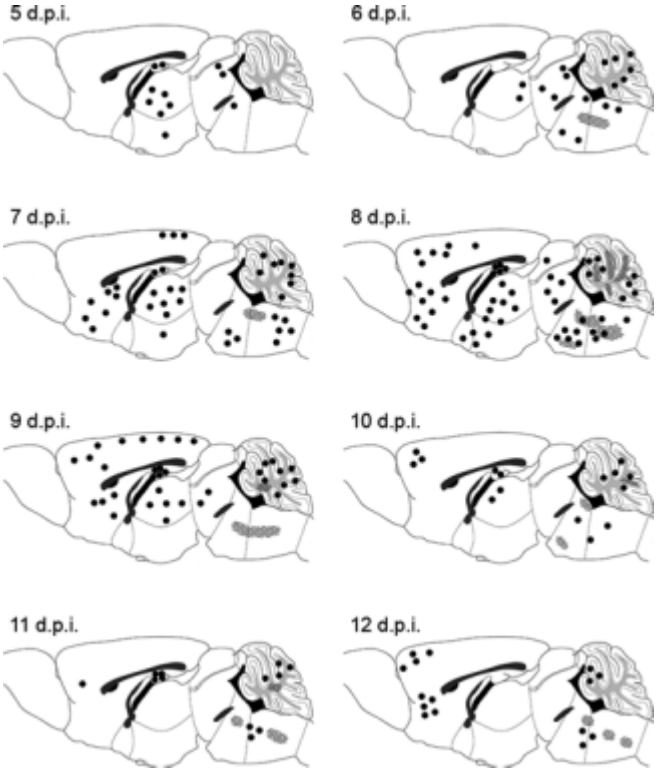


Fig. 4 Distribution of cerebral lesions during the time course of the experiment from days 5 to 12 p.i.: number and location of dots represent intensity of neuronal apoptosis and associated viral signals and their distribution; hatched areas represent locations of demyelination

Experiment 2

The brain lesions closely matched the changes of experiment 1. The spinal cords showed moderate neuronal death, especially in the ventral horns, and considerable demyelination especially of the dorsal (Fig. 7) and dorsolateral funiculi. Nuclear fragmentation characteristic of apoptosis (Fig. 8A–C) was present in gray matter. GFAP immunostaining showed that fragmented nuclei were not attributable to astrocytes (Fig. 9). Moderate numbers of TUNEL-positive nuclei were present in demyelinated white matter (Fig. 10). In demyelinated areas, axons were preserved (Fig. 11). Immunostaining did not reveal significant numbers of T cells or macrophages.

Distribution of viral signals

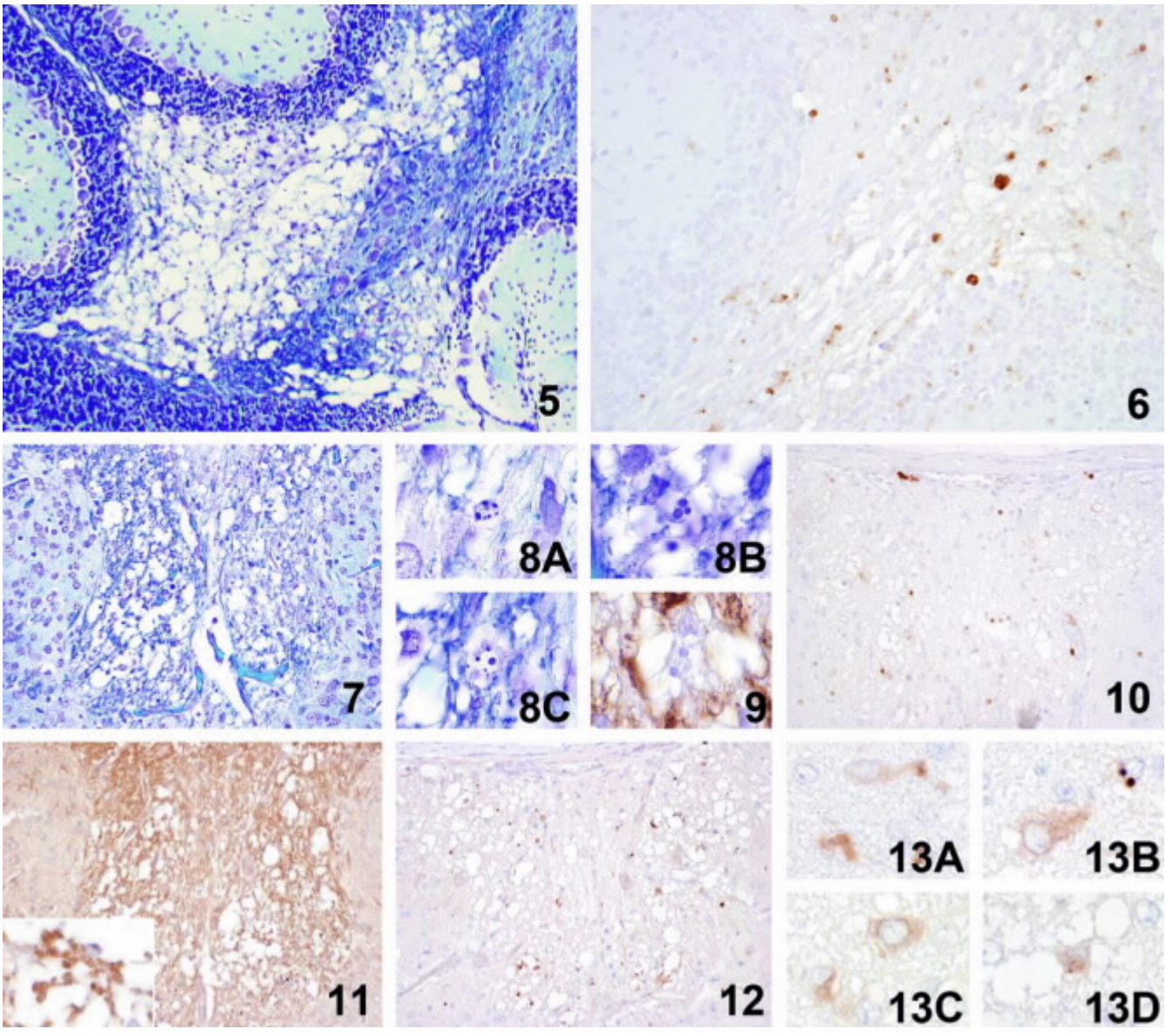
Experiment 1

IHC and ISH did not detect viral replication in brain neurons before day 5 p.i. From this time point until the end of the experiment viral signals were present in the perikarya of neurons, occasionally extending into neuronal processes, of many brain regions (Fig. 1B, C). The positive cells were usually arranged in clusters, occasionally they were found singly. The locations of infected neurons generally correlated well with areas of neuronal apoptosis. However, the number of cells with viral signals was by far exceeded by the number of apoptotic nuclei in the respective regions. Exceptions were the Purkinje cells, which frequently showed viral infection, but never displayed TUNEL signals. The white matter, in unchanged as well as demyelinated regions, showed inconsistent and generally weak viral signals. They were generally present as diffuse or granular deposits close to nuclei of white matter cells.

By RT-PCR, USUV-specific nucleic acid was detected in the organ homogenates of the infected mice of all time points examined, even in those processed from days 1 to 4 p.i. The control animals were all negative by RT-PCR until the end of the experiment.

Experiment 2

In the spinal cord, signals were found in the cytoplasm of gray matter neurons and their processes, especially of the ventral horn. In addition, there was multifocal weak to moderate staining in the white matter, both of demyelinated (Figs. 12, 13D) and unchanged areas (Fig. 13A–C). The signal was predominantly dotted or granular and not easily attributable to cells; sometimes, the staining was clearly intracellular with spared nuclei within white matter cells, probably oligodendrocytes (Fig. 13A–D).



Discussion

In suckling mice, USUV led to central nervous system (CNS) infection after peripheral inoculation; in this model the virus proved neuroinvasive and neurovirulent. Viral replication and associated cell damage was obvious in neurons. Whether other gray matter cells are affected remains to be shown. Damage to white matter cells, presumably oligodendrocytes, was also present, although viral signals were generally scarce. Mice of 2 weeks of age were resistant to peripheral USUV infection: neither peripheral viral replication nor neuroinvasion were observed. The age dependence of neuroinvasiveness is well known from numerous experiments with other flavi- and alphaviruses. While peripheral infection of suckling mice is generally lethal, older animals are frequently resistant to neuroinvasion. The age until which neuroinvasion by peripheral inoculations can be achieved ranges from 12 days (Semliki forest virus, SFV) [7], over 6 weeks (West Nile virus) [6], and 4 months (Saint Louis encephalitis virus) [2, 23] to even adult age (Banzi virus) [3, 16]. However, different strains of the same virus can exhibit dramatic differences in neuroinvasiveness. Highly invasive strains of SFV kill mice of all ages after i.p. infection, while strains of low neuroinvasiveness kill only mice aged 12 days and younger [7].

In the present USUV model, apoptosis (as evidenced by the TUNEL method) seems to be the major mode of neuronal death. This clearly correlates with other flavivirus and alphavirus infections, where neuronal apoptosis plays a pivotal role in pathogenesis [12, 15, 21, 30]. The TUNEL method has been claimed to be superior to other in situ labeling techniques for the detection of

b

Fig. 5 Severe vacuolation of the cerebellar white matter due to demyelination in a USUV-infected mouse, day 11 p.i.; Luxol-fast blue-cresyl echt violet staining. · 65

Fig. 6 Demyelination is paralleled by apoptosis of white matter cells, presumably oligodendroglia; USUV-infected mouse, day 8 p.i.; TUNEL staining. · 260

Fig. 7 Spinal cord; demyelination within the dorsal funiculi, indicated by irregular vacuolation of white matter associated with condensation and shrinkage of cell nuclei; USUV-infected mouse, day 7 p.i.; Luxol-fast blue-cresyl echt violet staining. · 90

Fig. 8 A–C Nuclear fragmentation indicative of apoptosis in demyelinated areas of the spinal cord; USUV-infected mouse, day 7 p.i.; Luxol-fast blue-cresyl echt violet staining. · 385

Fig. 9 Cell with fragmented nucleus is negative for glial fibrillary acidic protein immunostaining; indicating that apoptotic cells in demyelinated areas are not astrocytes; USUV-infected mouse, day 7 p.i. · 385

Fig. 10 Several TUNEL-positive nuclei are demonstrated within the demyelinated white matter; USUV-infected mouse, day 7 p.i. · 90

Fig. 11 Neurofilament immunoreactivity is preserved within demyelinated areas, suggesting primary demyelination; USUV-infected mouse, day 7 p.i. Inset shows intact axons. · 90; inset · 385

Fig. 12 IHC shows several USUV-positive signals within the demyelinated white matter; USUV-infected mouse, day 7 p.i. · 90

Fig. 13 A–D Morphology of virus-infected cells within the white matter of spinal cord, in unchanged (A–C) and demyelinated (D) areas. USUV-infected mouse, day 7 p.i. IHC, · 385

DNA fragmentation specific for apoptosis [10, 24]. However, TUNEL staining may also occur in very late stages of necrosis, although at reduced intensity [10, 24]. Thus, it appears possible that some cells displaying morphological features of lytic necroses may also have been stained by TUNEL. Purkinje cells, however, although frequently positive for viral signals, were never labeled by TUNEL. This phenomenon was also observed by Jackson and Rossiter [15] in experimental Venezuelan equine encephalitis and was explained by specific anti-apoptotic mechanisms in place in these particular neurons.

Peripheral USUV infection of mice older than 1 week did not result in neuroinvasion, whereas younger mice developed widespread neuronal death and succumbed to infection. The higher susceptibility to virus-induced neuronal apoptosis in very young animals has also been observed with other arboviruses, e.g., Sindbis virus and SFV [7, 13]. The neurons of newborn mice may simply be more prone to activation of an apoptotic cascade as a general response to stress, because programmed cell death is a crucial part of normal nervous system development, and thus the intracellular machinery is already in place. Neurons of more mature mice have down-regulated these cell death pathways [17].

On the other hand, the age of mice also affected neuroinvasion; due to the not fully developed blood brain barrier (BBB), neuroinvasion may take place more frequently in newborn or suckling mice [5, 20, 22]. The neuroinvasiveness of USUV tested here seems to be low compared to other viruses, as it does not cross the BBB when inoculated i.p. at the age of 2 weeks.

The occurrence of neuronal apoptosis in a flavivirus model of suckling mice is not surprising; however, infection of white matter cells with resulting apoptosis and demyelination is intriguing. So far, no flavivirus model has been reported in which demyelination has been described. The authors of the MODV hamster model do not describe demyelination, although in figures showing spinal cord of animals with acute disease, lesions suggestive of demyelination are present [19]. The only well-characterized arboviral model of demyelination is the murine SFV model [7, 8, 9]. Here demyelination became apparent at 14 days p.i. and seemed to be an immune-mediated event as a result of $CD8^+$ T cell-mediated destruction of virus-infected oligodendrocytes.

In USUV-infected mice, however, demyelination occurred comparatively early, was not associated with considerable inflammatory infiltrates, and was preceded by viral infection and apoptosis of adjacent cells. Phenotyping of these cells has yet to be done, but they did not display cell markers of astrocytes, macrophages or T cells, which most likely identified them as oligodendroglial cells. However, viral products were very scarce in white matter compared to the focal abundance of TUNEL-positive nuclei. Hence, we conclude that viral replication may only trigger myelin breakdown, which is finally maintained by other, as yet unidentified, factors. Thus also an indirect, but not primarily immunopathological mechanism of oligodendrocyte damage, as shown in other models, e.g., morbillivirus, may be occur [11, 31]. This particular aspect of the USUV mouse model deserves to be studied in more detail to gain more insight into the mechanism by which demyelination occurs during acute flaviviral encephalomyelitis.

There has been considerable debate concerning the route of entry into the CNS of neurovirulent flavi- and alphaviruses. While St. Louis encephalitis virus in animals infected i.p. has been shown to enter the brain via the olfactory neuroepithelium with the early viral replication taking place in the rostral brain regions [23], SFV seems to enter the brain across cerebral endothelial cells, leading to initial perivascular foci of infection. In USUV-infected mice, the disseminated distribution of early lesions in thalamic, midbrain, and hindbrain areas does not favor the olfactory entry route; hematogenous spread is more likely.

Animals from the later time points of the experiment had limited viral load. It is not entirely clear whether this reflects attempts at viral clearance or whether the animals survived longer because viral replication and resulting lesions occurred at a lower rate in these individuals.

Skeletal muscle has been identified as the only site of peripheral viral replication, a site where also SFV multiplies [7]. Interestingly, all investigated internal organs remained negative, although some replication at these sites has been found in this type of models [7, 25, 26]. However, virus titrations demonstrated that peripheral replication was less efficient and was probably not found because it was below the sensitivity level of the assays used.

Acknowledgements We thank Karin Fragner, Ingrid Friedl and Gudrun Kurz for excellent technical assistance and Klaus Bittermann for the professional digital artwork.

References

1. Adam F, Digoutte J-P Virus d'Afrique (base de donnes). Centre collaborateur OMS de référence et de recherche pour les arbovirus et les virus de fièvres hémorrhagiques (CRORA). URL:http://www.pasteur.fr/recherche/banques/CRORA
2. Andersen AA, Hanson RP (1974) Influence of sex and age on natural resistance to St. Louis encephalitis virus infection in mice. Infect Immun 9:1123–1125
3. Bhatt PN, Jacoby RO (1976) Genetic resistance to lethal flavirus encephalitis.II. Effect of immunosuppression. J Infect Dis 134:166–173
4. Chiba N, Iwasaki T, Mizutani T, Kariwa H, Kurata T, Takashima I (1999) Pathogenicity of tick-borne encephalitis virus isolated in Hokkaido, Japan in mouse model. Vaccine 17:779–787
5. Dubuisson J, Lustig S, Ruggli N, Akov Y, Rice CM (1997) Genetic determinants of Sindbis virus neuroinvasiveness. J Virol 71:2636–2646
6. Eldadah AH, Nathanson N, Sarsitis R (1967) Pathogenesis of West Nile virus encephalitis in mice and rats. 1. Influence of age and species on mortality and infection. Am J Epidemiol 86:765–775
7. Fazakerley JK (2002) Pathogenesis of Semliki Forest virus encephalitis. J Neurovirol 8 Suppl 2:66–74
8. Fazakerley JK, Walker R (2003) Virus demyelination. J Neurovirol 9:148–164
9. Fazakerley JK, Khalili-Shirazi A, Webb HE (1988) Semliki Forest virus (A7[74]) infection of adult mice induces an immune-mediated demyelinating encephalomyelitis. Ann N Y Acad Sci 540:672–673
10. Gold R, Schmied M, Giegerich G, Breitschopf H, Hartung HP, Toyka KV, Lassmann H (1994) Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques. Lab Invest 71:219–225
11. Graber HU, Mu¨ller CF, Vandevelde M, Zurbriggen A (1995) Restricted infection with canine distemper virus leads to down-regulation of myelin gene transcription in cultured oligodendrocytes. Acta Neuropathol 90:312–318
12. Griffin DE, Hardwick JM (1998) Apoptosis in alphavirus encephalitis. Semin Virol 8:481–489
13. Griffin DE, Levine B, Tyor WR, Tucker PC, Hardwick JM (1994) Age-dependent susceptibility to fatal encephalitis: alphavirus infection of neurons. Arch Virol Suppl 9:31–39
14. Griffin DE, Ubol S, Despres P, Kimura T, Byrnes A (2001) Role of antibodies in controlling alphavirus infection of neurons. Curr Top Microbiol Immunol 260:191–200
15. Jackson AC, Rossiter JP (1997) Apoptotic cell death is an important cause of neuronal injury in experimental Venezuelan equine encephalitis virus infection of mice. Acta Neuropathol 93:349–353
16. Jacoby RO, Bhatt PN (1976) Genetic resistance to lethal flavivirus encephalitis.I. Infection of congenic mice with Banzi virus. J Infect Dis 134:158–165
17. Kerr DA, Larsen T, Cook SH, Fannjiang YR, Choi E, Griffin DE, Hardwick JM, Irani DN (2002) BCL-2 and BAX protect adult mice from lethal Sindbis virus infection but do not protect spinal cord motor neurons or prevent paralysis. J Virol 76:10393–10400
18. Lewis J, Wesselingh SL, Griffin DE, Hardwick JM (1996) Alphavirus-induced apoptosis in mouse brains correlates with neurovirulence. J Virol 70:1828–1835
19. Leyssen P, Croes R, Rau P, Heiland S, Verbeken E, Sciot R, Paeshuyse J, Charlier N, De Clercq E, Meyding-Lamade U, Neyts J (2003) Acute encephalitis, a poliomyelitis-like syndrome and neurological sequelae in a hamster model for flavivirus infections. Brain Pathol 13:279–290
20. Mann MA, Knipe DM, Fischbach GD, Fields BN (2002) Type 3 reovirus neuroinvasion after intramuscular inoculation: direct invasion of nerve terminals and age-dependent pathogenesis. Virology 303:222–231
21. Matthews V, Robertson T, Kendrick T, Abdo M, Papadimitriou J, McMinn P (2000) Morphological features of Murray Valley encephalitis virus infection in the central nervous system of Swiss mice. Int J Exp Pathol 81:31–40
22. Medeot SI, Contigiani MS, Sabattini MS, Diaz G (1992) Experimental neuroinvasiveness of wild and laboratory Junin virus strains. Res Virol 143:259–267
23. Monath TP, Cropp CB, Harrison AK (1983) Mode of entry of a neurotropic arbovirus into the central nervous system. Reinvestigation of an old controversy. Lab Invest 48:399–410
24. Mundle SD, Gao XZ, Khan S, Gregory SA, Preisler HD, Raza A (1995) Two in situ labeling techniques reveal different patterns of DNA fragmentation during spontaneous apoptosis in vivo and induced apoptosis in vitro. Anticancer Res 15:1895–1904
25. Odelola HA, Oduye OO (1977) West Nile virus infection of adult mice by oral route. Arch Virol 54:251–253
26. Wang Y, Lobigs M, Lee E, Mullbacher A (2003) $CD8^+$ T cells mediate recovery and immunopathology in West Nile virus encephalitis. J Virol 77:13323–13334
27. Weiner LP, Cole GA, Nathanson N (1970) Experimental encephalitis following peripheral inoculation of West Nile virus in mice of different ages. J Hyg 68:435–446
28. Weissenbo¨ck H, Kolodziejek J, Url A, Lussy H, Rebel-Bauder B, Nowotny N (2002) Emergence of Usutu virus, an African mosquito-borne flavivirus of the Japaneseencephalitis virus group, central Europe. Emerg Infect Dis 8:652–656
29. Weissenbo¨ck H, Kolodziejek J, Fragner K, Kuhn R, Pfeffer M, Nowotny N (2003) Usutu virus activity in Austria, 2001–2002. Microbes Infect 5:1132–1136
30. Xiao SY, Guzman H, Zhang H, Travassos da Rosa AP, Tesh RB (2001) West Nile virus infection in the golden hamster (Mesocricetus auratus): a model for West Nile encephalitis. Emerg Infect Dis 7:714–721
31. Zurbriggen A, Schmid I, Graber HU, Vandevelde M (1998) Oligodendroglial pathology in canine distemper. Acta Neuropathol 95:71–77

Antiviral Research 160 (2018) 137–142

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Favipiravir inhibits in vitro Usutu virus replication and delays disease progression in an infection model in mice

Nidya A. Segura Guerrero[a,b,1], Sapna Sharma[a,1], Johan Neyts[a,*], Suzanne J.F. Kaptein[a]

[a] KU Leuven, Department of Microbiology and Immunology, Rega Institute for Medical Research, Laboratory of Virology and Chemotherapy Leuven, Belgium
[b] Universidad Pedagógica y Tecnológica de Colombia, Tunja, Colombia

ARTICLE INFO

Keywords:
Emerging flavivirus
Usutu virus
Mouse model
AG129 mice
Antivirals

ABSTRACT

Usutu virus (USUV) is an emerging flavivirus that causes Usutu disease mainly in birds, but infection of mammals such as rodents, bats and horses has also been demonstrated. In addition, human cases (both in immunocompromised and -competent individuals) were also reported. Large outbreaks with other flaviviruses, such as West Nile virus and Zika virus, indicate that one should be vigilant for yet other outbreaks. To allow the identification of inhibitors of USUV replication, we established in vitro antiviral assays, which were validated using a small selection of known flavivirus inhibitors, including the broad-spectrum viral RNA polymerase inhibitor favipiravir (T-705). Next, an USUV infection model in AG129 (IFN-α/β and IFN-γ receptor knockout) mice was established. AG129 mice proved highly susceptible to USUV; an inoculum as low as 10^2 PFU (1.3×10^5 $TCID_{50}$) resulted in the development of symptoms as early as 3 days post infection with viral RNA being detectable in various tissues. Treatment of mice with favipiravir (150 mg/kg/dose, BID, oral gavage) significantly reduced viral load in blood and tissues and significantly delayed virus-induced disease. This USUV mouse model is thus amenable for assessing the potential in vivo efficacy of (novel) USUV/flavivirus inhibitors.

1. Main text

Usutu virus (USUV) is a mosquito-borne flavivirus originating from Africa, where it was first detected in the late 1950's. This virus is a relatively unknown member of the Japanese encephalitis serocomplex and is thus closely related to human pathogens such as Japanese encephalitis virus (JEV), yellow fever virus (YFV), Saint Louis encephalitis virus (SLEV), West Nile virus (WNV) and Murray Valley encephalitis virus (MVEV) (Moureau et al., 2015). The Culex mosquitoes are the primary vectors although other mosquito species such as Aedes albopictus have also been proposed as potential vectors (Eiden et al., 2018; Calzolari et al., 2010; Vázquez et al., 2011). USUV most likely emerged for the first time in Europe in 1996 causing massive deaths among blackbirds (Turdus merula) (Weissenböck et al., 2013). Currently, the virus is ubiquitous in Europe as its presence has been demonstrated in various animal species (e.g. birds, rodents, bats and horses) in more than ten European countries (Becker et al., 2012; Busquets et al., 2008; Cadar et al., 2014; Calzolari et al., 2012; Chvala et al., 2007; Garigliany et al., 2017; Hubálek et al., 2008; Lecollinet et al., 2016; Rijks et al., 2016; Steinmetz et al., 2011). Potentially even more concerning is the presence of USUV in humans. Seroprevalence studies showed that antibodies against USUV could be detected in the blood of 0.02% and 0.23%–1.1% of otherwise healthy blood donors in respectively Germany and Italy (Allering et al., 2012; Gaibani et al., 2012; Pierro et al., 2013). However, a higher seroprevalence was detected in serum samples from both healthy and sick individuals in Italy (6.6% seroprevalence) and healthy individuals in Serbia (7.5% seroprevalence), respectively (Cvjetković et al., 2016; Gaibani and Rossini, 2017; Grottola et al., 2017). USUV infections in humans remain largely asymptomatic. However, as of 2009, the number of USUV infections that are associated with disease, ranging from mild (rash, fever, headache) to severe disease (encephalitis, meningoencephalitis, meningitis), is slowly increasing in Europe. Although the first cases were reported on the African continent, more recent cases have been reported in Italy, Croatia, Germany, and France (Allering et al., 2012; Cavrini et al., 2009, 2011; Grottola et al., 2017; Nikolay et al., 2011; Pecorari et al., 2009; Simonin et al., 2018; Vilibic-Cavlek et al., 2014). The widespread distribution in different hosts in Europe provides evidence that the virus can jump between species and adapt to a new host. Hence, USUV should be considered a potential health concern. Given the recent -and

* Corresponding author. KU Leuven, Department of Microbiology and Immunology, Rega Institute for Medical Research, Laboratory of Virology and Chemotherapy, Herestraat 49 - box 1043, 3000, Leuven, Belgium.
E-mail address: johan.neyts@kuleuven.be (J. Neyts).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.antiviral.2018.10.026
Received 2 August 2018; Received in revised form 26 October 2018; Accepted 28 October 2018
Available online 29 October 2018
0166-3542/ © 2018 Published by Elsevier B.V.

mostly unexpected-outbreaks of related flaviviruses such as WNV, YFV, dengue virus and Zika virus (Musso et al., 2018; Weaver, 2018), it will thus be important to have broad-spectrum antivirals at hand that are also active against USUV.

To allow the identification of molecules that can inhibit in vitro USUV replication, antiviral assays were established. First, various cell types were tested for their permissiveness to USUV replication: Vero E6 (African Green monkey kidney) cells, BHK (baby hamster kidney) cells and C6/36 mosquito (from Ae. albopictus larvae) cells. Vero cells were highly permissive to USUV (strain V18; kindly provided by Dr. Stephan Günther, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany; Becker et al., 2012), resulting in a full cytopathic effect (CPE) at day 7 post infection (pi) (data not shown). Hence, prior to performing subsequent experiments, USUV was propagated to a maximum of two passages in Vero E6 cells after which infectious titers were determined by performing plaque assays on BHK cells, essentially as described previously (Zmurko et al., 2016). The in vitro kinetics of USUV replication is comparable with that of other flaviviruses (Mastrangelo et al., 2012; Zmurko et al., 2016), with USUV RNA becoming first detectable by RT-qPCR at 6–8 h pi (Supplemental Fig. 1, grey bars). Next, a CPE-based assay followed by colorimetric read out (i.e. MTS/PMS; Promega) as well as a virus yield reduction assay followed by RT-qPCR was established using Vero E6 cells. Both assays were then validated using a selection of molecules with known antiviral activity against other flaviviruses (reviewed in Boldescu et al., 2017). All compounds were able to inhibit CPE formation as well as USUV RNA replication, with favipiravir as well as the 2′-C-methyl nucleoside analogs 2′-C-methyladenosine (2′CMA) and 7-deaza-2-C-methyladenosine (7DMA) being the best, albeit not very potent, inhibitors, as based on the selectivity index (i.e. ratio CC_{50}/EC_{50}) (Table 1). More in detail, favipiravir was most effective in inhibiting USUV replication when added to the infected cells during the first 6 h after infection (Supplemental Fig. 1, black bars). The antiviral activity was lost when the compound was added after the onset of viral RNA synthesis, which corroborates with its proposed mechanism of action against other RNA viruses, i.e. acting as a mimetic nucleobase by interfering with the viral RNA synthesis (Delang et al., 2014; Furuta et al., 2005). The in vitro assays that were established are thus capable of identifying inhibitors of USUV replication.

To evaluate the in vivo efficacy of molecules against USUV, we aimed to establish a small animal model. Since USUV is an avian (flavi-) virus, we anticipated that the virus would replicate well in embryonated chicken eggs. USUV (10^4, 10^5 or 10^6 PFU) was injected in the allantoic cavity of chicken eggs ten days after fertilization, after which the allantoic fluid was harvested at various days (d2, d4 and d6) pi to determine infectious virus content. Surprisingly, only low USUV titers (i.e. 10^3 PFU/ml) were detected in only 14% of the chicken eggs, which may represent remnants of the input virus. In addition, all embryonated chicks developed normally (data not shown). Embryonated chicken eggs are thus not suitable to serve as a replication model for USUV.

Table 1
Antiviral activity of a selection of compounds against USUV and their anti-metabolic effect.

Compound	EC_{50} (μM)		CC_{50} (μM)
	CPE	RT-qPCR	
2′CMA	7.2 ± 0.1	12 ± 7.7	69 ± 1.3
2′CMC	13 ± 1.0	24 ± 9.1	71 ± 14
2′CMG	26 ± 0.8	43 ± 7.5	> 168
7DMA	45 ± 7.1	130 ± 2.1	> 357
Favipiravir	90 ± 26	151 ± 28	> 637
Ribavirin	139 ± 30	ND	> 410

Antiviral activities were determined in CPE reduction assays (CPE) followed by MTS/PMS read out or in virus yield reduction assay followed by real time quantitative PCR (RT-qPCR). Anti-metabolic activities were determined in toxicity assays followed by MTS read out. Data represent mean values ± standard deviations from at least three independent experiments with three replicates for each experiment (n = 9). 2′-C-methyladenosine, 2′CMA; 2′-C-methylcytidine, 2′CMC; 2′-C-methylguanosine, 2′CMG; 7-deaza-2-C-methyladenosine, 7DMA. ND, not determined.

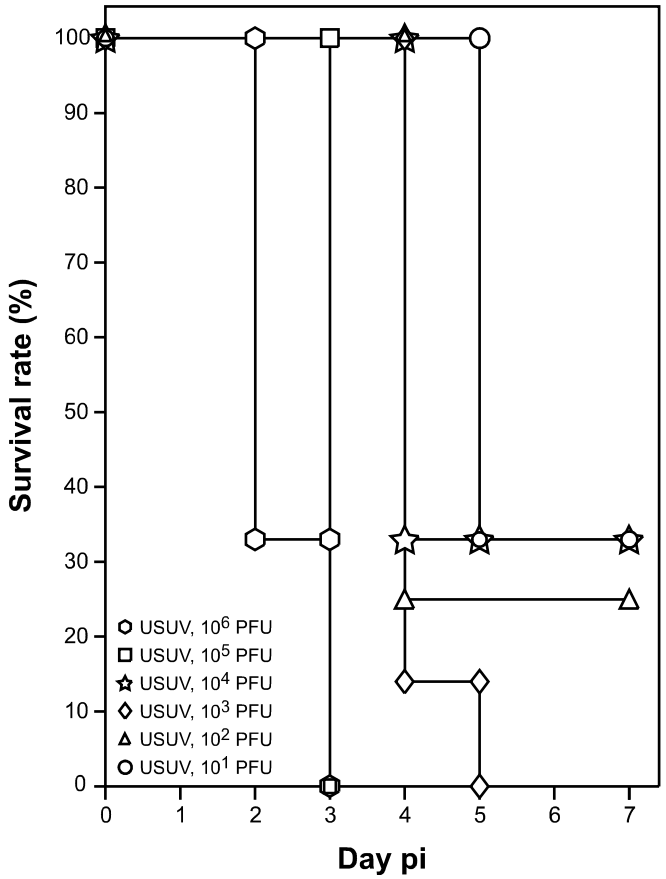


Fig. 1. Kaplan-Meier survival curve for mice infected with different inoculums of USUV. Male (8–14 weeks of age) 129/Sv mice deficient in both IFN-alpha/beta (IFN-α/β) and IFN-gamma (IFN-γ) receptors (AG129) were inoculated intraperitoneally (ip) with USUV V18 using different inoculums (ranging from 10^1 PFU - 10^6 PFU, corresponding to 1.3×10^4 $TCID_{50}$ - 1.3×10^9 $TCID_{50}$). Mice were observed daily for body weight loss and the development of virus-induced disease and were euthanized when reaching humane endpoints. In case of a body weight loss of ≥ 20% and/or severe illness (neurotropic symptoms such as movement impairment, lower limb paralysis), mice were euthanized with pentobarbital (Dolethal). All animal experiments were performed with approval of and under the guidelines of the Ethical Committee of KU Leuven [P099-2013].

We next studied whether AG129 mice can be infected with USUV. AG129 mice are deficient in IFN-α/β and IFN-γ receptors, which makes them highly susceptible to other (flavi-)viruses (Couderc et al., 2008; Rocha-Pereira et al., 2013; Schul et al., 2007; Zmurko et al., 2016), Moreover, it was previously reported that an USUV infection is partly regulated by the interferon response (Blázquez et al., 2015; Cacciotti et al., 2015; Martín-Acebes et al., 2016; Scagnolari et al., 2013), which is impaired in AG129 mice. Indeed, intraperitoneal (ip) infection of AG129 mice (8–14 weeks old) with USUV resulted in a rapid development of disease requiring euthanasia in the majority of the mice within a few days after infection (Fig. 1). When inoculated with 10^6 PFU (corresponding to 1.3×10^9 $TCID_{50}$), mice developed the first signs of disease 2 days pi and all mice had to be euthanized by day 3 pi. At the lowest inoculum used (10^1 PFU/1.3×10^4 $TCID_{50}$), first signs of disease appeared a few days later (day 4 pi) and most of the mice had to be euthanized by day 7 pi (33% survival). Disease signs include general

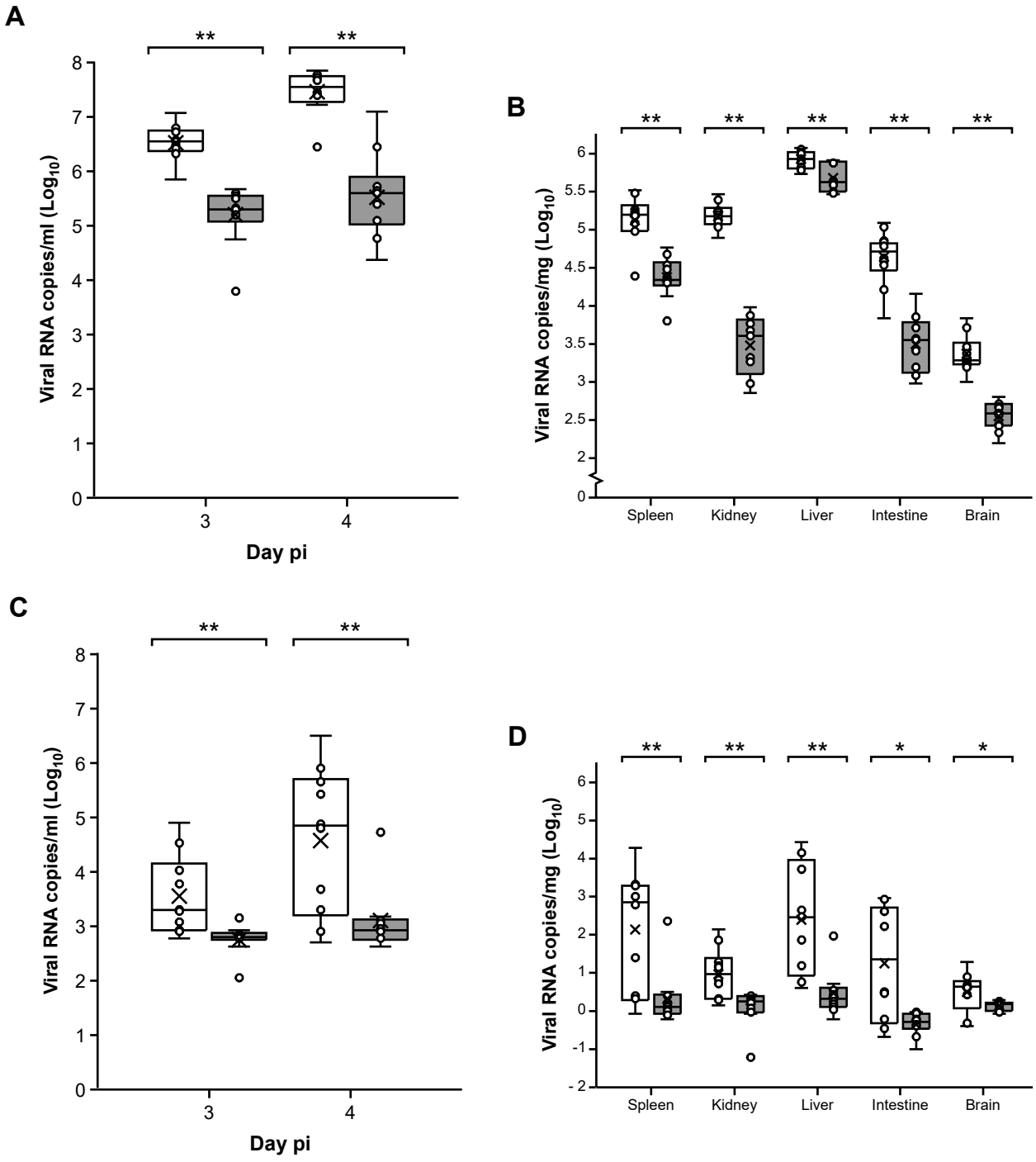


Fig. 2. Significant reduction in viral RNA load in serum and tissues of USUV-infected mice treated with favipiravir compared to those treated with vehicle. Male (8–14 weeks old) AG129 mice (n = 10/group) were inoculated ip with either 10^2 PFU/1.3 × 10^5 $TCID_{50}$ (A and B) or 10^0 PFU/1.3 × 10^3 $TCID_{50}$ (C and D) of USUV V18. Mice were treated twice-daily by oral gavage with favipiravir (T-705, 150 mg/kg/dose; grey bars) or vehicle (0.4% sodium carboxymethylcellulose, CMC-Na; white bars) for 5 consecutive days, starting one day prior to infection. Viral RNA load in serum (A and C) was determined on d3 and d4 pi, whereas viral RNA load in tissues (B and D) was determined at d4 pi, a time point at which all mice were sacrificed. Viral RNA was extracted from tissues using the RNeasyMini Kit (Qiagen, The Netherlands) followed by viral RNA quantification by means of RT-qPCR.Statistical analysis was performed using the Shapiro–Wilk test for normality followed by the one-tailed Mann-Whitney U test. **, $p < 0.01$; *, $p < 0.05$ (Statistical tools, available from http://sdittami.altervista.org/shapirotest/ShapiroTest.html and http://in-silico.online , accessed May 2018).

symptoms (such as rapid body weight loss, hunched posture, and lethargy), conjunctivitis as well as neurotropic symptoms (such as movement impairment, lower limb paralysis). Next, viral RNA levels were monitored in the blood at d3-d5 pi to determine the day of peak viremia. In mice infected with either 10^2 PFU (1.3 × 10^5 $TCID_{50}$) or 10^3 PFU (1.3 × 10^6 $TCID_{50}$), comparable high levels of viral RNA were detected in the blood of infected mice at day 3 pi: 6.6 log_{10} and 6.5 log_{10}, respectively (Supplemental Fig. 2). Viral RNA levels increased further in the following days, reaching a peak level of 8.3log_{10} at d4 pi and 8.8log_{10} at d5 pi for mice infected with 10^2 PFU (1.3 × 10^5 $TCID_{50}$) and 10^3 PFU (1.3 × 10^6 $TCID_{50}$), respectively. At d5, pi the majority of the mice infected with the higher inoculum were euthanized, whereas

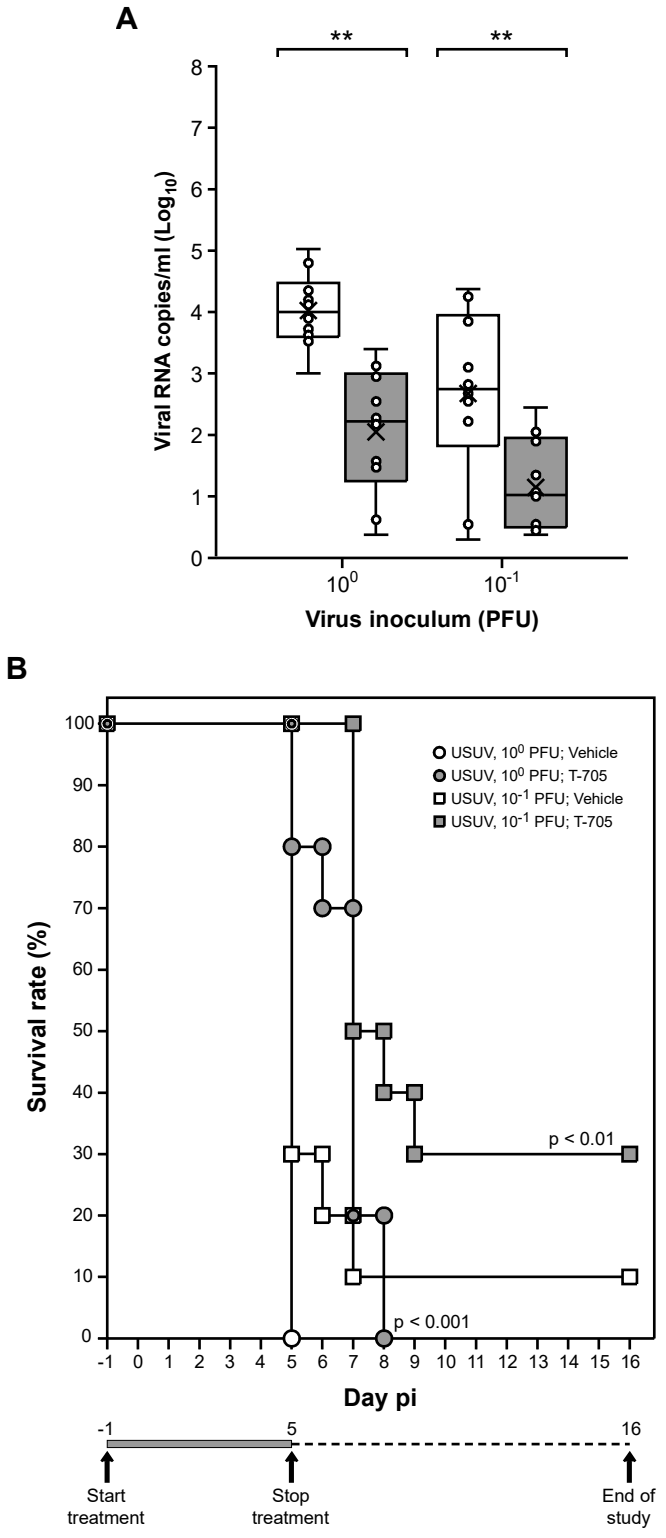


Fig. 3. Significant reduction in viremia (A) translates in significant delay in USUV-induced disease(B) in mice treated with favipiravir. Male (8–14 weeks old) AG129 mice (n = 10/group) were inoculated ip with either 10^0 PFU/ 1.3×10^3 $TCID_{50}$ (depicted by circles in B) or 10^{-1} PFU/1.3×10^2 $TCID_{50}$ (depicted by squaresin B) of USUV V18. Mice were treated twice-daily by oral gavage with favipiravir (T-705, 150 mg/kg/dose; grey bars, squaresor circles) or vehicle (0.4% CMC-Na;white bars, squaresor circles) for 6 consecutive days, starting one day prior to infection. Mice were observed daily for body weight loss and the development of virus-induced disease.In caseof a body weight loss of ≥ 20% and/or severe illness, mice were euthanized with pentobarbital (Dolethal). Viral RNA load in serum was determined at the day of peak viremia (d4 pi) by RT-qPCR.Statistical analysis was performed using the Shapiro–Wilk test for normality followed by the one-tailed Mann-Whitney U test (viremia) or the Log rank test (survival). **, $p < 0.01$ (Statistical tools, available from http://sdittami.altervista.org/shapirotest/ShapiroTest.html and http://in-silico.online , accessedMay 2018).

this was the case for only one mouse in the lower inoculum group. Based on these findings, it was decided to quantify viremia in blood at d3 and d4 pi and in tissues at d4 pi in all further experiments using an inoculum of 10^2 PFU (1.3×10^5 $TCID_{50}$) or lower.

The USUV mouse model was then validated for suitability in antiviral studies. To this end, favipiravir was used, which was earlier shown to be active in mouse infection models with other (related) viruses (Delang et al., 2014; Furuta et al., 2002; Gowen et al., 2007; Julander et al., 2009; Morrey et al., 2008; Rocha-Pereiraet al., 2012). Treatment of mice infected with 10^2 PFU (1.3×10^5 $TCID_{50}$) USUV for 5 consecutive days with favipiravir (150 mg/kg/dose, BID, oral gavage) resulted in a statistically significant reduction of viral RNA levels in the blood as compared to vehicle-treated mice at both d3 and d4 pi: $1.3\log_{10}$ and 1.9 $\log_{10}$, respectively (Fig. 2A). In addition, viral RNA levels were also statistically significantly reduced in tissues (spleen, kidney, liver, intestine and brain) as compared to vehicle-treated mice (Fig. 2B). The reduction in viremia appeared to be independent on the inoculum used, as significant reductions in viral RNA load were also observed in favipiravir-treated mice infected with a 100 fold lower virus inoculum (i.e. 10^0 PFU/1.3×10^3 $TCID_{50}$) (Fig. 2C and D).

Finally, it was assessedwhether the reduction in viremia that is brought about by favipiravir translates into a significant delay of virus-induced disease progression. Mice were infected with 10^0 PFU (1.3×10^3 $TCID_{50}$; as in Fig. 2C and D) or a lower inoculum (i.e., 10^{-1} PFU/1.3×10^2 $TCID_{50}$). Treatment with favipiravir significantly reduced viremia by $1.8\log_{10}$ and 1.7 $\log_{10}$ in mice infected with 10^0 PFU respectively 10^{-1} PFU (Fig. 3A), as was shown before. In addition, treatment with the compound significantly delayed virus-induced diseasein both groups (Fig. 3B). In mice infected with 10^0 PFU, the mean day of euthanasia (MDE) was 5.0 and 6.7 for mice treated with vehicle or favipiravir, respectively, whereas the MDE was 6.4 (vehicle) and 10 (favipiravir) in mice infected with a 10-fold lower inoculum. Favipiravir could not halt the progression of the diseaseand ultimately all 10 mice (inoculated with 10^0 PFU/1.3×10^3 $TCID_{50}$), respectively 7 out 10 mice (inoculated with 10^{-1} PFU/1.3×10^2 $TCID_{50}$), had to be euthanized.

In conclusion, the USUV infection model in AG129 mice represents a suitable model for use in antiviral studies. USUV causesa particularly fulminant infection in AG129 mice, which may also explain the rather modest protection that is achieved by the reference compound favipiravir.

Declarations of interest

None.

Acknowledgements

We thank Carolien De Keyzer, Ruben Pholien, Charlotte Vanderheydt and Joanna Zmurko for excellent technical assistance.

This researchwas partially funded by the European Union's Horizon 2020 Researchand Innovation Programme under Grant Agreement no. 734584 (ZikaPLAN) and 734548 (ZIKAlliance).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

doi.org/10.1016/j.antiviral.2018.10.026 .

References

Allering, L., Jöst, H., Emmerich, P., Günther, S., Lattwein, E., Schmidt, M., Seifried, E., Sambri, V., Hourfar, K., Schmidt-Chanasit, J., 2012. Detection of Usutu virus infection in a healthy blood donor from South-West Germany, 2012. Euro Surveill. 17, pii=20341. https://doi.org/10.2807/ese.17.50.20341-en .

Becker, N., Jöst, H., Ziegler, U., Eiden, M., Höper, D., Emmerich, P., Fichet-Calvet, E., Ehichioya, D.U., Czajka, C., Gabriel, M., Hoffmann, B., Beer, M., Tenner-Racz, K., Racz, P., Günther, S., Wink, M., Bosch, S., Konrad, A., Pfeffer, M., Groschup, M.H., Schmidt-Chanasit, J., 2012. Epizootic emergence of Usutu virus in wild and captive birds in Germany. PloS One 7. https://doi.org/10.1371/journal.pone.0032604 .

Blázquez, A.B., Escribano-Romero, E., Martín-Acebes, M.A., Petrovic, T., Saiz, J.C., 2015. Limited susceptibility of mice to Usutu virus (USUV) infection and induction of flavivirus cross-protective immunity. Virology 482, 67–71. https://doi.org/10.1016/j.virol.2015.03.020 .

Boldescu, V., Behnam, M.A.M., Vasilakis, N., Klein, C.D., 2017. Broad-spectrum agents for flaviviral infections: dengue, Zika and beyond. Nat. Rev. Drug Discov. 16, 565–586. https://doi.org/10.1038/nrd.2017.33 .

Busquets, N., Alba, A., Allepuz, A., Aranda, C., Nuñez, J., 2008. Usutu virus sequences in Culex pipiens (Diptera: Culicidae), Spain. Emerg. Infect. Dis. 14, 861–863. https://doi.org/10.3201/eid1405.071577 .

Cacciotti, G., Caputo, B., Selvaggi, C., la Sala, A., Vitiello, L., Diallo, D., Ceianu, C., Antonelli, G., Nowotny, N., Scagnolari, C., 2015. Variation in interferon sensitivity and induction between Usutu and West Nile (lineages 1 and 2) viruses. Virology 485, 189–198. https://doi.org/10.1016/j.virol.2015.07.015 .

Cadar, D., Becker, N., de Mendonca Campos, R., Borstler, J., Jost, H., Schmidt-Chanasit, J., 2014. Usutu virus in bats, Germany, 2013. Emerg. Infect. Dis. 20, 1771–1773. https://doi.org/10.3201/eid2010.140909 .

Calzolari, M., Bonilauri, P., Bellini, R., Albieri, A., Defilippo, F., Maioli, G., Galletti, G., Gelati, A., Barbieri, I., Tamba, M., Lelli, D., Carra, E., Cordioli, P., Angelini, P., Dottori, M., 2010. Evidence of simultaneous circulation of West Nile and Usutu viruses in mosquitoes sampled in Emilia-Romagna region (Italy) in 2009. PloS One 5, 1–10. https://doi.org/10.1371/journal.pone.0014324 .

Calzolari, M., Gaibani, P., Bellini, R., Defilippo, F., Pierro, A., Albieri, A., Maioli, G., Luppi, A., Rossini, G., Balzani, A., Tamba, M., Galletti, G., Gelati, A., Carrieri, M., Poglayen, G., Cavrini, F., Natalini, S., Dottori, M., Sambri, V., Angelini, P., Bonilauri, P., 2012. Mosquito, bird and human surveillance of West Nile and Usutu viruses in Emilia-Romagna region (Italy) in 2010. PloS One 7. https://doi.org/10.1371/journal.pone.0038058.

Cavrini, F., Gaibani, P., Longo, G., Pierro, A.M., Rossini, G., Bonilauri, P., Gerundi, P., Benedetto, F. Di, Passetto, A., Girardis, M., Dottori, M., Landini, M.P., Sambri, V., 2009. Usutu virus infection in a patient who underwent orthotropic liver transplantation, Italy, August-September 2009. Euro Surveill. 14 pii=19448. https://doi.org/10.2807/ese.14.50.19448-en.

Cavrini, F., Pepa, M.E. Della, Gaibani, P., Pierro, A.M., Rossini, G., Landini, M.P., Sambri, V., 2011. A rapid and specific real-time RT-PCR assay to identify Usutu virus in human plasma, serum, and cerebrospinal fluid. J. Clin. Virol. 50, 221–223. https://doi.org/10.1016/j.jcv.2010.11.008 .

Chvala, S., Bakonyi, T., Bukovsky, C., Meister, T., Brugger, K., Rubel, F., Nowotny, N., Weissenböck, H., 2007. Monitoring of Usutu virus activity and spread by using dead bird surveillance in Austria, 2003-2005. Vet. Microbiol. 122, 237–245. https://doi.org/10.1016/j.vetmic.2007.01.029 .

Couderc, T., Chrétien, F., Schilte, C., Disson, O., Brigitte, M., Guivel-Benhassine, F., Touret, Y., Barau, G., Cayet, N., Schuffenecker, I., Desprès, P., Arenzana-Seisdedos, F., Michault, A., Albert, M.L., Lecuit, M., 2008. A mouse model for Chikungunya: young age and inefficient type-I interferon signaling are risk factors for severe disease. PLoS Pathog. 4, e29. https://doi.org/10.1371/journal.ppat.0040029 .

Cvjetković, I.H., Petrović, T., Petrić, D., Cvjetković, D., Kovačević, G., Radovanov, J., 2016. Seroprevalence of mosquito-borne and tick-born microorganisms in human population of south Backa district. Arhiv. veterinarske Med. 9, 23–30. http://niv.ns.ac.rs/wp-content/uploads/2016/11/3.pdf .

Delang, L., SeguraGuerrero, N., Tas, A., Quérat, G., Pastorino, B., Froeyen, M., Dallmeier, K., Jochmans, D., Herdewijn, P., Bello, F., Snijder, E.J., de Lamballerie, X., Martina, B., Neyts, J., van Hemert, M.J., Leyssen, P., 2014. Mutations in the chikungunya virus non-structural proteins cause resistance to favipiravir (T-705), a broad-spectrum antiviral. J. Antimicrob. Chemother. 69, 2770–2784. https://doi.org/10.1093/jac/dku209.

Eiden, M., Gil, P., Ziegler, U., Rakotoarivony, I., Marie, A., Frances, B., L'Ambert, G., Simonin, Y., Foulongne, V., Groschup, M.H., Gutierrez, S., Eloit, M., 2018. Emergence of two Usutu virus lineages in Culex pipiens mosquitoes in the Camargue, France, 2015. Infect. Genet. Evol. 61, 151–154. https://doi.org/10.1016/j.meegid.2018.03.020.

Furuta, Y., Takahashi, K., Fukuda, Y., Kuno, M., Kamiyama, T., Kozaki, K., Nomura, N., Egawa, H., Minami, S., Watanabe, Y., Shiraki, K., Narita, H., 2002. In vitro and in vivo activities of anti-influenza virus compound T-705 in vitro and in vivo activities of anti-influenza virus compound T-705. Antimicrob. Agents Chemother. 46, 977–981. https://doi.org/10.1128/AAC.46.4.977 .

Furuta, Y., Takahashi, K., Kuno-maekawa, M., Sangawa, H., Uehara, S., Kozaki, K., Nomura, N., Egawa, H., Shiraki, K., 2005. Mechanism of action of T-705 against influenza virus. Antimicrob. Agents Chemother. 49, 981–986. https://doi.org/10.1128/AAC.49.3.981.

Gaibani, P., Pierro, A., Alicino, R., Rossini, G., Cavrini, F., Landini, M.P., Sambri, V., 2012. Detection of Usutu-virus-specific IgG in blood donors from northern Italy. Vector Borne Zoonotic Dis. 12, 431–433. https://doi.org/10.1089/vbz.2011.0813 .

Gaibani, P., Rossini, G., 2017. An overview of Usutu virus. Microb. Infect. 19, 382–387. https://doi.org/10.1016/j.micinf.2017.05.003 .

Garigliany, M., Linden, A., Gilliau, G., Levy, E., Sarlet, M., Franssen, M., Benzarti, E., Derouaux, A., Francis, F., Desmecht, D., 2017. Usutu virus, Belgium, 2016. Infect. Genet. Evol. 48, 116–119. https://doi.org/10.1016/j.meegid.2016.12.023 .

Gowen, B.B., Wong, M.H., Jung, K.H., Sanders, A.B., Mendenhall, M., Bailey, K.W., Furuta, Y., Sidwell, R.W., 2007. In vitro and in vivo activities of T-705 against arenavirus and bunyavirus infections. Antimicrob. Agents Chemother. 51, 3168–3176. https://doi.org/10.1128/AAC.00356-07 .

Grottola, A., Marcacci, M., Tagliazucchi, S., Gennari, W., Di Gennaro, A., Orsini, M., Monaco, F., Marchegiano, P., Marini, V., Meacci, M., Rumpianesi, F., Lorusso, A., Pecorari, M., Savini, G., 2017. Usutu virus infections in humans: a retrospective analysis in the municipality of Modena, Italy. Clin. Microbiol. Infect. 23, 33–37. https://doi.org/10.1016/j.cmi.2016.09.019 .

Hubálek, Z., Halouzka, J., Juricová, Z., Sikutová, S., Rudolf, I., Honza, M., Janková, J., Chytil, J., Marec, F., Sitko, J., 2008. Serologic survey of birds for West Nile flavivirus in southern Moravia (Czech Republic). Vector Borne Zoonotic Dis. 8, 659–666. https://doi.org/10.1089/vbz.2007.0283 .

Julander, J.G., Shafer, K., Smee, D.F., Morrey, J.D., Furuta, Y., 2009. Activity of T-705 in a hamster model of yellow fever virus infection in comparison with that of a chemically related compound, T-1106. Antimicrob. Agents Chemother. 53, 202–209. https://doi.org/10.1128/AAC.01074-08 .

Lecollinet, S., Blanchard, Y., Manson, C., Lowenski, S., Laloy, E., Quenault, H., Touzain, F., Lucas, P., Eraud, C., Bahuon, C., Zientara, S., Beck, C., Decors, A., 2016. Dual emergence of Usutu virus in common blackbirds, Eastern France, 2015. Emerg. Infect. Dis. 22, 2225. https://doi.org/10.3201/eid2212.161272 .

Martín-Acebes, M.A., Blázquez, A.B., Cañas-Arranz, R., Vázquez-Calvo, Á., Merino-Ramos, T., Escribano-Romero, E., Sobrino, F., Saiz, J.C., 2016. A recombinant DNA vaccine protects mice deficient in the alpha/beta interferon receptor against lethal challenge with Usutu virus. Vaccine 19 (34), 2066–2073. https://doi.org/10.1016/j.vaccine.2016.03.015.

Mastrangelo, E., Pezzullo, M., De Burghgraeve, T., Kaptein, S., Pastorino, B., Dallmeier, K., de Lamballerie, X., Neyts, J., Hanson, A.M., Frick, D.N., Bolognesi, M., Milani, M., 2012. Ivermectin is a potent inhibitor of flavivirus replication specifically targeting NS3 helicase activity: new prospects for an old drug. J. Antimicrob. Chemother. 67, 1884–1894. https://doi.org/10.1093/jac/dks147 .

Morrey, J.D., Taro, B.S., Siddharthan, V., Wang, H., Smee, D.F., Christensen, A.J., Furuta, Y., 2008. Efficacy of orally administered T-705 pyrazine analog on lethal West Nile virus infection in rodents. Antivir. Res. 80, 377–379. https://doi.org/10.1016/j.antiviral.2008.07.009 .

Moureau, G., Cook, S., Lemey, P., Nougairede, A., Forrester, N.L., Khasnatinov, M., Charrel, R.N., Firth, A.E., Gould, E.A., de Lamballerie, X., 2015. New insights into flavivirus evolution, taxonomy and biogeographic history, extended by analysis of canonical and alternative coding sequences. PloS One 10, e0117849. https://doi.org/10.1371/journal.pone.0117849 .

Musso, D., Rodriguez-Morales, A.J., Levi, J.E., Cao-Lormeau, V.M., Gubler, D.J., 2018. Unexpected outbreaks of arbovirus infections: lessons learned from the Pacific and tropical America. Lancet Infect. Dis. https://doi.org/10.1016/S1473-3099(18)30269-X. Jun 19. pii: S1473-3099(18)30269-X.

Nikolay, B., Diallo, M., Boye, C.S.B., Sall, A.A., 2011. Usutu virus in Africa. Vector Borne Zoonotic Dis. 11, 1417–1423. https://doi.org/10.1089/vbz.2011.0631 .

Pecorari, M., Longo, G., Gennari, W., Grottola, A., Sabbatini, A.M.T., Tagliazucchi, S., Savini, G., Monaco, F., Simone, M., Lelli, R., Rumpianesi, F., 2009. First human case of Usutu virus neuroinvasive infection, Italy, August-September 2009. Euro Surveill. 50 pii=19446. https://doi.org/10.2807/ese.14.50.19446-en .

Pierro, A., Gaibani, P., Spadafora, C., Ruggeri, D., Randi, V., Parenti, S., Finarelli, A.C., Rossini, G., Landini, M.P., Sambri, V., 2013. Detection of specific antibodies against West Nile and Usutu viruses in healthy blood donors in northern Italy, 2010–2011. Clin. Microbiol. Infect. 19, E451–E453. https://doi.org/10.1111/1469-0691.12241 .

Rijks, J., Slaterus, R., Stroo, A., IJzer, J., Gröne, A., Koopmans, M., van der Jeugd, H., Reusken, C., 2016. Widespread Usutu virus outbreak in birds in The Netherlands, 2016. Euro Surveill. 21 pii=30391. https://doi.org/10.2807/1560-7917.ES.2016.21.45.30391.

Rocha-Pereira, J., Jochmans, D., Dallmeier, K., Leyssen, P., Nascimento, M.S.J., Neyts, J., 2012. Favipiravir (T-705) inhibits in vitro norovirus replication. Biochem. Biophys. Res. Commun. 424, 777–780. https://doi.org/10.1016/j.bbrc.2012.07.034 .

Rocha-Pereira, J., Jochmans, D., Debing, Y., Verbeken, E., Nascimento, M.S., Neyts, J., 2013. The viral polymerase inhibitor 2′-C-methylcytidine inhibits Norwalk virus replication and protects against norovirus-induced diarrhea and mortality in a mouse model. J. Virol. 87, 11798–11805. https://doi.org/10.1128/JVI.02064-13 .

Scagnolari, C., Caputo, B., Trombetti, S., Cacciotti, G., Soldà, A., Spano, L., Villari, P., della Torre, A., Nowotny, N., Antonelli, G., 2013. Usutu virus growth in human cell lines: induction of and sensitivity to type I and III interferons. J. Gen. Virol. 94, 789–795. https://doi.org/10.1099/vir.0.046433-0 .

Schul, W., Liu, W., Xu, H.Y., Flamand, M., Vasudevan, S.G., 2007. A dengue fever viremia model in mice shows reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drugs. J. Infect. Dis. 195, 665–674. https://doi.org/10.1086/511310 .

Simonin, Y., Sillam, O., Carles, M.J., Gutierrez, S., Gil, P., Constant, O., Martin, M.F., Girard, G., Van de Perre, P., Salinas, S., Leparc-Goffart, I., Foulongne, V., 2018. Human Usutu virus infection with atypical neurologic presentation, Montpellier, France, 2016. Emerg. Infect. Dis. 24, 875–878. https://doi.org/10.3201/eid2405.171122.

Steinmetz, H.W., Bakonyi, T., Weissenböck, H., Hatt, J.M., Eulenberger, U., Robert, N., Hoop, R., Nowotny, N., 2011. Emergence and establishment of Usutu virus infection

in wild and captive avian speciesin and around Zurich, Switzerland-Genomic and pathologic comparison to other central European outbreaks. Vet. Microbiol. 148, 207–212. https://doi.org/10.1016/j.vetmic.2010.09.018 .

Vázquez, A., Ruiz, S., Herrero, L., Moreno, J., Molero, F., Magallanes, A., Sánchez-Seco, M.P., Figuerola, J., Tenorio, A., 2011. West Nile and Usutu viruses in mosquitoes in Spain, 2008-2009. Am. J. Trop. Med. Hyg. 85, 178–181. https://doi.org/10.4269/ajtmh.2011.11-0042.

Vilibic-Cavlek, T., Kaic, B., Barbic, L., Pem-Novosel,I., Slavic-Vrzic, V., Lesnikar, V., Kurecic-Filipovic, S., Babic-Erceg,A., Listes, E., Stevanovic, V., Gjenero-Margan, I., Savini, G., 2014. First evidence of simultaneous occurrence of West Nile virus and Usutu virus neuroinvasive diseasein humans in Croatia during the 2013 outbreak. Infection 42, 689–695. https://doi.org/10.1007/s15010-014-0625-1 .

Weaver, S.C.,2018. Prediction and prevention of urban arbovirus epidemics: a challenge for the global virology community. Antivir. Res.156, 80–84. https://doi.org/10.1016/j.antiviral.2018.06.009 .

Weissenböck,H., Bakonyi, T., Rossi,G., Mani, P., Nowotny, N., 2013. Usutu virus, Italy, 1996. Emerg. Infect. Dis. 19, 274–277. https://doi.org/10.3201/eid1902.121191 .

Zmurko, J., Marques, R., Schols,D., Verbeken, E., Kaptein, S.J.F., Neyts, J., 2016. The viral polymerase inhibitor 7-deaza-2-C-methyladenosine is a potent inhibitor of in vitro Zika virus replication and delays diseaseprogressionin a robust mouseinfection model. PLoSNeglected Trop. Dis. 10, 1–15. https://doi.org/10.1371/journal.pntd.0004695.

viruses

MDPI

Review

Usutu Virus: An Arbovirus on the Rise

Ferdinand Roesch[1], Alvaro Fajardo[2], Gonzalo Moratorio [1,2,3] and Marco Vignuzzi [1,*]

1 Institut Pasteur, Viral Populations and Pathogenesis Unit, CNRS UMR 3569, 75015 Paris, France
2 Laboratorio de Virología Molecular, Centro de Investigaciones Nucleares, Facultad de Ciencias, Universidad de la República, Montevideo 11400, Uruguay
3 Laboratorio de Inmunovirología, Institut Pasteur de Montevideo, Montevideo 11400, Uruguay
* Correspondence: marco.vignuzzi@pasteur.fr

Received: 11 June 2019; Accepted: 9 July 2019; Published: 12 July 2019

Abstract: The Usutu virus (USUV) is a flavivirus that is drawing increasing attention because of its potential for emergence. First isolated in Africa, it was introduced into Europe where it caused significant outbreaks in birds, such as in Austria in 2001. Since then, its geographical distribution has rapidly expanded, with increased circulation, especially in the last few years. Similar to West Nile virus (WNV), the USUV enzootic transmission cycle involves Culex mosquitoes as vectors, and birds as amplifying reservoir hosts, with humans and other mammals likely being dead-end hosts. A similarity in the ecology of these two viruses, which co-circulate in several European countries, highlights USUV's potential to become an important human pathogen. While USUV has had a severe impact on the blackbird population, the number of human cases remains low, with most infections being asymptomatic. However, some rare cases of neurological disease have been described, both in healthy and immuno-compromised patients. Here, we will discuss the transmission dynamics and the current state of USUV circulation in Europe.

Keywords: USUV; Usutu; WNV; flavivirus; emergence

1. Introduction

The dramatic outbreaks and global spread of the chikungunya virus (CHIKV) and the Zika virus (ZIKV) illustrate the danger that emerging arboviruses represent. Given the plethora of mosquito-transmitted viruses, which have been identified to date, and the known ability of RNA viruses to mutate rapidly to adapt to new environments, it is likely that other arboviruses will emerge in the next decades. The Usutu virus (USUV), an arbovirus from the Flaviviridae family, genus Flavivirus, has recently garnered a great deal of attention from the scientific community, particularly since its emergence in Europe. Similar to other flaviviruses, its (+)-strand RNA genome of 11,064 nucleotides encodes a single polyprotein of 3,434 amino acids that is subsequently cleaved into structural (C, prM and E) and non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins. The natural life cycle of USUV involves Culex mosquitoes as its main vector, and multiple bird species as a natural viral reservoir. To date, USUV has been mostly associated with disease in birds, with spectacular episodes of mass mortality occurring in Central Europe; but it can also infect humans, and in some rare cases, it can be associated with neurological complications.

In this review, we will summarize the current knowledge on USUV, with particular emphasis on the increased circulation in Europe in recent years, the dynamics of transmission, and the human clinical cases described to date.

2. Introduction of USUV in Europe and Genetic Diversity

USUV belongs to the Japanese encephalitis virus (JEV) antigenic complex, together with West Nile virus (WNV) and Murray Valley encephalitis virus (MVEV). It was isolated for the first time in

1959 from a Culex neavei mosquito caught near the Usutu river in Swaziland [1]. Since then, the virus has continuously circulated within Africa, having been detected in Senegal, Uganda, Central African Republic, Nigeria, Burkina Faso, and Côte d'Ivoire (reviewed in [2]). USUV was observed in Europe for the first time in Vienna in 2001 [3], where it was responsible for massive outbreaks in blackbirds (Turdus merula) and gray owls (Strix nebulosa). A retrospective study analyzing dead birds conducted in the Tuscany region of Italy showed that USUV has been circulating in Europe since 1996 [4].

The genetic variability of USUV has been explored through phylogenetic studies performed over full-length sequences, as well as the envelope and NS5 genes [5–9]. These analyses cluster USUV sequences into distinct lineages which are designated on the basis of their geographic origin of isolation: Africa 1, 2 and 3 and Europe 1, 2, 3 and 4 (Figure 1). A recent phylogenetic study focused on partial NS5 gene sequences (265 bp) revealed a novel lineage (Europe 5) composed of strains isolated from birds in Germany in 2016 [8]. The prototype African strain SAAR-1776 (represented with a star in Figure 1) is distantly related to the European lineages. In addition to these genetic differences, SAAR-1776 differs from other USUV strains in that it was generated by intracerebral inoculation of newborn mice [1].

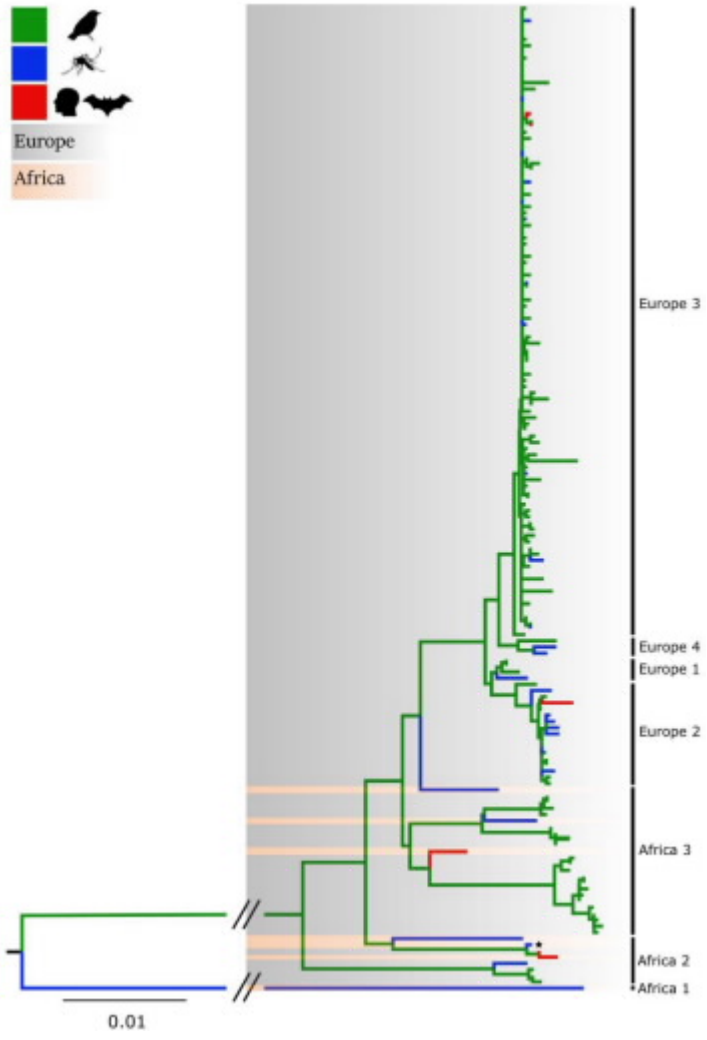


Figure 1. Maximum-likelihood phylogenetic tree analysis of the NS5 gene of USUV strains (n_1 = 158) circulating worldwide. Strains isolated from birds, mosquitoes and mammals are indicated in green, blue and red, respectively. African and European variants are shaded in pink and gray, respectively. The prototype SAAR-1776 strain is indicated by a star. The bar at the bottom of the tree denotes evolutionary distance, as number of base substitutions per site. The interrupted branches (indicated by oblique lines) were shortened by 50% for better graphic representation. The list of USUV strains used to generate the tree are provided in Supplemental Materials: Table S1.

Figure 1. Maximum-likelihood phylogenetic tree analysis of the NS5 gene of USUV strains (n_1 = 158) circulating worldwide. Strains isolated from birds, mosquitoes and mammals are indicated in green, blue and red, respectively. African and European variants are shaded in pink and gray, respectively. The prototype SAAR-1776 strain is indicated by a star. The bar at the bottom of the tree denotes evolutionary distance, as number of base substitutions per site. The interrupted branches (indicated by oblique lines) were shortened by 50% for better graphic representation. The list of USUV strains used to generate the tree are provided in Supplemental Materials: Table S1.

3. Transmission Dynamics

3.1. WNV and USUV Co-Circulation and Co-Infection Dynamics

The transmission dynamics of arboviruses are generally influenced by biological and environmental factors, such as the identity and population density of vector and reservoir species,

USUV is thought to have been introduced in Europe multiple times through bird migration, starting in the 1950s [7]. The Europe 1 lineage is thought to derive from a virus in Senegal that reached Spain, and is likely the origin of the first epizoosis in Central Europe. According to the same study, the Europe 2 and 3 lineages probably originated in Austria in 1993, and in Italy in 2007, respectively (Figure 2). Since these introductions, the virus has spread rapidly around the Mediterranean basin and Central Europe, reaching Tunisia [10], Morocco [11], Israel [12], Greece [13], France [14], Spain [15], Poland [16], Hungary [17], Czech Republic [18], Serbia [5], the United Kingdom [19], Croatia [20], the Netherlands [21], Switzerland [22], Italy [23], where it gave rise to the Europe 4 lineage, and Germany [24], where 3 different lineages have been recently shown to co-circulate [25]. Recent evidence suggests that introduction events into Europe from Africa are still happening. Indeed, viruses from African lineages were recently detected in Germany [26] and in the Camargue area of France [14].

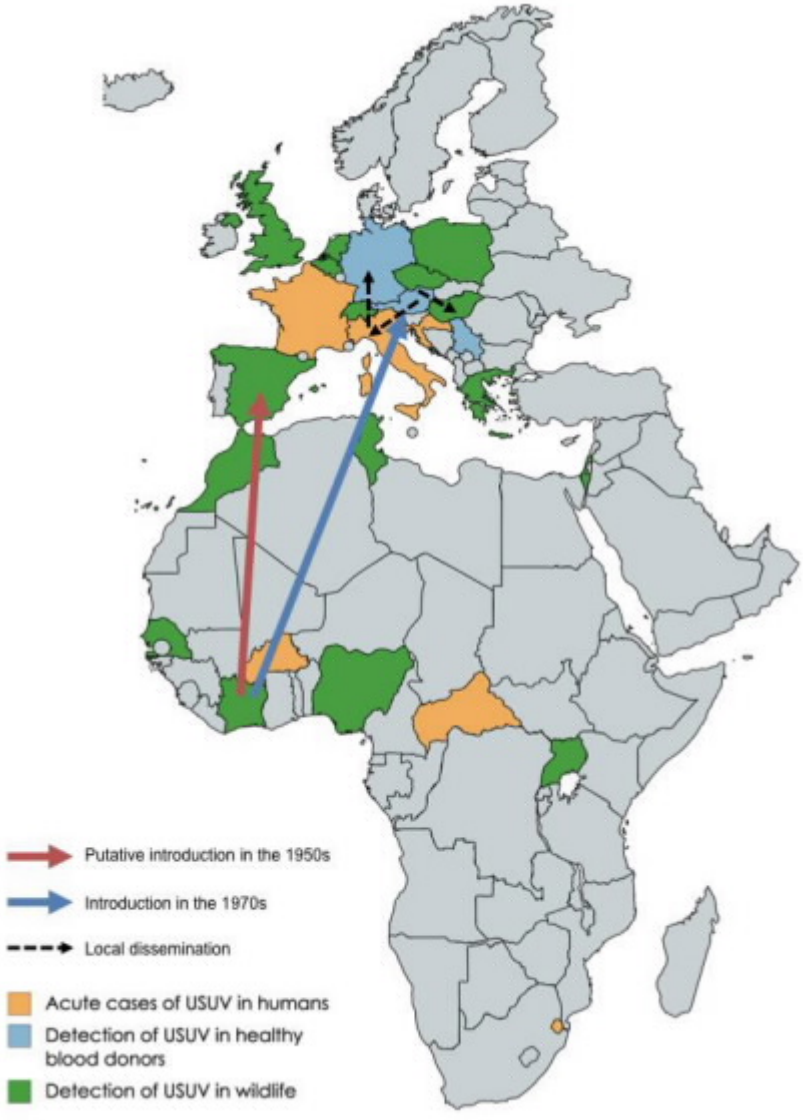


Figure 2. Geographical distribution of USUV. Countries where USUV was detected in wildlife (birds, mosquitoes, horses, etc.) are indicated in green; countries where USUV was detected in healthy blood donors are represented in blue; and countries where USUV caused symptomatic, acute infections are shown in orange. The arrows represent the presumed bird migration event that led to introduction of USUV in Europe, according to Engel et al. [7].

In one human case, viral RNA was detected simultaneously for both viruses [31,32], confirming that co-infection does occur. How significant USUV and WNV co-circulation and co-infections are at

The viral or immunological determinants responsible for USUV adaptation to birds and humans remain unclear. Similar to other arboviruses alternating between the insect vector and mammalian host species, evidence of strong purifying selection was detected across the entire viral genome [7]. In the case of USUV, European lineages seem to be subjected to a stronger negative selection than African lineages [7]. Nevertheless, one position in the viral polymerase gene NS5 (amino acid 898) evolved under significant positive selection, possibly reflecting an ongoing host/pathogen evolutionary conflict. Interestingly, another substitution in the NS5 gene has been observed in the Bologna/09 viral strain [27] derived from an USUV-positive patient recovering from orthotropic liver transplant [28]. As more viral sequences are made available, the viral determinants influencing USUV pathogenesis may be identified, as well as whether or not other factors such as evasion from the IFN response participate in this process.

3. Transmission Dynamics

3.1. WNV and USUV Co-Circulation and Co-Infection Dynamics

The transmission dynamics of arboviruses are generally influenced by biological and environmental factors, such as the identity and population density of vector and reservoir species, the extrinsic incubation period, humidity, temperature, host immunity, etc. In this regard, USUV shares a few common features with WNV: both viruses are mainly transmitted by Culex mosquitoes, with migratory birds acting as the major amplifying host (Table 1). Therefore, it is not surprising that in Europe, where WNV has also recently re-emerged, the two viruses co-circulate in 10 European countries and in 34 species of birds [29], as well as in horses [10]. Furthermore, 9 cases of birds [19,30] and 3 cases of humans [31,32] with positive serology for both viruses have been reported to date. This may be the result of local co-circulation of WNV and USUV, although cross-reactivity of antibodies complicates interpretation of results. Indeed, the envelope protein of USUV shares some structural features with the WNV envelope [33,34], and antibody cross-reactivity has been observed [35]. Thus, serological tests, such as ELISAs, cannot always distinguish USUV from WNV, which has led to the hypothesis that USUV circulation may be underestimated [36]. Other assays, such as protein microarray based on NS1 [37] or quantitative multiplex real-time RT-PCR [38], have been developed in recent years.

In one human case, viral RNA was detected simultaneously for both viruses [31,32], confirming that co-infection does occur. How significant USUV and WNV co-circulation and co-infections are at the population level, and whether a previous flavivirus infection influences subsequent WNV or USUV infections, remain to be determined. An in vivo study suggested that the immunization of mice with recombinant WNV particles induced low levels of antibodies cross-reacting with USUV [39]. Similarly, a previous infection with USUV was shown to reduce the susceptibility of adult mice to WNV [40]. Future work should determine whether such processes of cross-protection, or in the opposite sense, whether antibody enhancement of infection, may occur and be relevant for USUV transmission in birds and humans.

3.2. Vector Species for USUV

While USUV has been detected in a wide range of mosquitoes, it seems to be most frequently associated with *Culex pipiens* [41,42], which is thought to be the main vector for USUV in Europe. Indeed, competence studies have shown that *Culex pipiens* supports high levels of USUV infection [43–46]. Notably, competence of *Culex pipiens* for USUV and WNV is comparable at 18 $^{+}$C and 23 $^{+}$C, but not at 28 $^{+}$C, where mosquitoes had higher susceptibility for USUV [43]. This may indicate some interesting temperature-related di (erences in the ability of the two viruses to overcome anatomical barriers and bottlenecks. As noted for other arboviruses, there may be some variation in the ability of USUV to infect di (erent *Culex pipiens* populations. Indeed, the prototype strain SAAR-1776 could only infect one of the two *Culex pipiens* colonies from the United Kingdom [44], suggesting that host factors or

genetics may restrict infection in these mosquitoes. Whether this is specific to the SAAR-1776 strain, or whether this happens with European USUV strains, remains to be established.

Other species of Culex have been described to be competent for USUV: for instance, both Culex neavei and Culex quinquefasciatus show high infection and dissemination rates [45,47]. USUV has been detected in pools of Aedes albopictus [41,48,49], an invasive mosquito species involved as an alternative vector for the transmission of many arboviruses, such as CHIKV. This prompted further investigation into Aedes albopictus competence for USUV. The results indicated that Aedes albopictus exhibits a lower competence for USUV [45], although the virus can replicate in the body and escape the midgut barrier [50]. In more rare cases, USUV was also detected in other mosquito species such as Aedes japonicus, Aedes vexans, Anopheles maculipennis, Anopheles plumbeus, Coquillettidia richiardii, Culiseta annulata, and some Ochlerotatus species [41,42,46]. USUV has never been detected in ticks, even in areas such as Italy where USUV circulation is known to be significant [51,52]. Since a single mutation was required for CHIKV adaptation to Aedes albopictus [53], sustained surveillance efforts may be required to rapidly detect potential adaptation of USUV to new vector species, which may allow viral dissemination into new geographic regions.

3.3. Reservoir of USUV

The main USUV natural hosts are birds, with infection being reported to date in 93 different species belonging to 35 families [54]. USUV is particularly pathogenic in a few species such as blackbirds (Turdus merula), gray owls (Strix nebulosa), and house sparrows (Passer domesticus) [17,55]. In these birds, systemic USUV replication is likely the cause of the severe pathogenesis, as the virus was detected in many organs such as the liver, heart, brain, and spleen [56]. Necrotic lesions were noted in these organs [57]. It is currently not known whether USUV can infect American robins (Turdus migratorius), a bird species that was critical for WNV dissemination across the US [58]. However, USUV potently infects its European counterpart Turdus merula, and many other Passeriformes birds known to be susceptible to WNV, such as the common starling (Sturnus vulgaris) or the house sparrow [29].

USUV induced mass mortality in blackbirds and grey owls in Austria [57], Germany [56], France [59], and the Netherlands [21], with dramatic consequences on bird populations. For instance, in Germany, the blackbird population declined by 15% within 5 years of USUV's arrival [8]. In Austria, however, bird mortality sharply dropped after 2004, while the number of infected birds with low viral titers increased [17]. This may have resulted from the acquisition of herd immunity: in owls and birds of prey, the proportion of seropositive birds reached levels above 50% [60]. Interestingly, only a relatively low number of bird species have been subjected to large die-off episodes, and in some avian species such as chickens or geese, experimental inoculation of USUV only caused mild pathogenicity [61,62]. The basis for these species-specific differences in pathogenicity is currently unknown. The co-infection of birds with USUV and other pathogens may increase disease severity: for instance, co-infection of birds with USUV and Plasmodium spp. can lead to a more severe outcome than when they carry only one of these pathogens [21,63]. Future investigation will help determine which virological and immunological factors influence USUV pathogenesis and co-morbidities.

3.4. Other Dead-End Hosts

Beyond infection of birds and humans, USUV has been detected in many mammalian species considered to be dead-end hosts. As is the case with WNV, horses are considered to be a sensitive host for USUV: viral circulation has been detected in Tunisia [10], Spain [64], Serbia [65], Poland [16] and Croatia [20]. USUV can also infect dogs [66,67], dear [68] and wild boars [69]. USUV was also detected in bats [70], squirrels [71] and rodents [72], in which the virus did not seem to cause the same severe pathogenicity as it does in birds, raising concerns that these species could act as secondary reservoirs. Whether USUV can reach a viremia in these animals to sustain a new mosquito infection cycle is unclear. The expanding range of wild animals susceptible to USUV highlights its potential as an important emerging pathogen.

4. Human Clinical Cases and Disease

The first human cases of USUV infection were described in Central African Republic and Burkina Faso in the 1980s and in 2004, respectively, with mild symptoms such as fever and rash [2]. To date, a total of 28 acute USUV infections have been reported in humans [73], including some serious complications such as meningoencephalitis [28,74] and facial paralysis [75]. Seroprevalence studies suggested that USUV infections in humans may have been largely underestimated, and many of them may be asymptomatic. Indeed, antibodies against USUV were detected in the blood of 0.01% to 1% of healthy blood donors in Germany and Italy, respectively [49,76,77]. Higher seroprevalence was detected in serum samples collected from healthy individuals in Serbia (7.5%) [78] and from forestry workers in Italy (18%) [79]. A large retrospective study, conducted on over 900 patients, of which about a third were suspected for encephalitis or meningoencephalitis, found a prevalence of 6.5% [80]. In contrast, two studies failed to detect USUV in the cerebrospinal fluid of patients with encephalitis in Italy and Switzerland [81,82]. These studies indicate that USUV already circulates to significant levels in human populations, although it is rarely associated with neurological complications.

While most USUV infections are mild or asymptomatic, neurotropism represents a growing concern for human health, especially since two immuno-compromised patients tested positive for USUV. One of them was recovering from a liver transplant [28], and the other was diagnosed with a B cell lymphoma [74]: both showed signs of neuropathogenesis that were thought to be caused by USUV. Both survived the infection. USUV RNA was also detected by RT-PCR in cerebrospinal fluid of patients su(ering from meningoencephalitis, suggesting that the virus may establish an infection in the brain [83]. Several other lines of evidence suggest that USUV can be neurotropic. First, this virus was detected in the brains of dead bats and birds in the wild [57,70]. Furthermore, experimental infection of 1-week-old suckling mice leads to paraplegia and paralysis, and is associated with apoptosis and demyelination of neuronal and glial cells [84]. Moreover, the USUV virus isolated from a patient diagnosed with facial paralysis could infect primary astrocytes [75]. An in vitro study confirmed these findings and demonstrated that USUV can establish productive infection and induce apoptosis in a wide range of neural cells such as neurons, astrocytes, microglial cells and neuronal stem cells [85]. USUV was even shown to be associated with levels of infection and apoptosis in neuronal cells that were higher than ZIKV, suggesting that USUV may potentially cause significant neurological defects [85].

5. Cellular Responses to USUV Infection

USUV infection triggers the induction of pro-inflammatory and antiviral responses, including cytokine secretion. In primary human nasal epithelial cells, USUV induces a modest expression of IL6, IL8 and IP10, albeit to lower levels than JEV [86]. In dendritic cells (DCs), USUV induces high levels of TNF-([87], and of both IFN-(and IFN-) [87,88], leading to the expression of Interferon Stimulated Genes (ISGs). Interestingly, cells infected by USUV induce IFN to higher levels than cells infected by WNV (10- to 100-fold, depending on the multiplicity of infection and time point considered) [88]. USUV is also very sensitive to the antiviral e(ect of IFN: in A549 cells, USUV replication is restricted 10 times more potently than WNV by a wide variety of IFN-I and IFN-III subtypes [88]. Consistent with these findings, while adult Swiss mice are highly susceptible to WNV infection, they are not to USUV [40]. In contrast, USUV infection induced high mortality rates in suckling mice, which have not yet developed a functional IFN response [40,84], or in AG129 mice, knocked-out for the IFN-(and IFN- pathways [89]. Taken together, these results suggest that USUV is potently controlled by the IFN response, which may account (at least in part) for its low viral pathogenicity in humans.

Conversely, USUV may also hijack cellular responses to its advantage: for instance, it triggers the autophagy pathway in infected cells, stimulating viral replication [90]. The interplay between autophagy and flaviviruses, which has been described before [91], could be particularly interesting, since it may provide cellular drug targets. Indeed, the autophagy inhibitors 3-methyladenine and wortmanin significantly reduced USUV replication in Vero cells (from 3-fold to 5-fold) [90]. Host lipid

biosynthesis pathways are also required for the production of infectious viral particles: inhibition of the acetyl-CoA carboxylase (ACC) enzyme by two different drugs very potently (up to 3 logs) inhibited WNV and USUV [92]. Finally, favipiravir, a broad-spectrum viral RNA polymerase inhibitor, has shown some efficacy in restricting USUV replication in the AG129 model [89].

6. USUV Circulation in the Future: Surveillance and Mathematical Modeling

The circulation of USUV in bird and human populations is already solidly established in Europe, as evidenced by the massive decline in blackbird populations [8] and the significant levels of seroconversion in human blood donors [76–80]. However, the extent of USUV circulation in 2018 is unprecedented [93]. In France, USUV was detected from dead birds in 46 administrative districts, versus only 4 in 2017 (Cécile Beck, personal communication). In Austria, 18 blood donors (16 of them asymptomatic) tested positive for USUV, while only 6 did in 2017 [32]. According to the European Centre for Disease Prevention and Control (EDCC), WNV circulation also reached historically high levels in Europe in 2018, with 2083 human cases, representing a 7-fold increase compared to 2017. Some countries, such as Bulgaria (15-fold increase), France (13.5-fold increase) and Italy (11-fold increase) were particularly impacted by the uptick in WNV circulation. A likely hypothesis for this is that some environmental and ecological factors influencing both USUV and WNV account for the exceptional levels of viral circulation in 2018. However, the possibility that viral adaptation may have contributed to this increased circulation of USUV cannot be ruled out. Therefore, surveillance of USUV circulation, as well as monitoring USUV evolution, will be crucial to try to prevent future outbreaks. This goal remains difficult to achieve, notably because of the lack of large-scale cohort studies and the absence of commercially available diagnostic reagents for USUV. In this context, mathematical modeling can help estimate the potential levels of USUV circulation. Habitat models have recently been used to predict the number of USUV cases in under-sampled areas [94]. Two models have been developed to study USUV transmission dynamics: a mechanistic epidemiological model, aimed at estimating USUV transmission in Austria [95,96]; and a correlation-based environmental model, based on data collected in Germany [8]. Both models were used in a recent study to generate risk maps for USUV [97]. Large areas of Central and Western Europe including northern Italy, Austria, southern France, and a large area spanning the North East of France, Belgium, and the North West of Germany, were predicted as suitable for USUV replication. As temperatures are likely to increase in Europe because of the effects of climate change, these risk maps are even likely underestimating future USUV transmission rates.

Given the similarities in the ecology of WNV and USUV (Table 1), and the relatively recent emergence of USUV in Europe, it is possible that USUV has the potential to reach levels of viral circulation similar to those of WNV. For now, USUV has seemingly not reached the United States, but a study established that North American colonies of Culex pipiens and Culex quinquefasciatus are competent vectors for USUV [45]. As stated earlier, USUV also infects multiple bird species very closely related to the ones that were involved in dissemination of WNV in the United States. Therefore, USUV emergence outside of Europe should be considered a real possibility, prompting research and surveillance efforts.

On the other hand, it is possible that WNV and USUV are competing with each other, and that the co-existence of both viruses at high levels in the same geographical areas and ecological niches is impossible. Such competition between two related flaviviruses has been observed before, for instance in the case of WNV and Saint Louis encephalitis virus (SLEV). Indeed, prior to WNV emergence in the US, SLEV was endemic in southern California, but circulation drastically dropped after WNV invasion in 2003 [104]. This led to the hypothesis that SLEV, while still capable of sporadically re-emerging [105], has been outcompeted by WNV, which infects similar bird and vector species with greater success.

Table 1. Comparison of WNV and USUV main characteristics. Some important features of West Nile Virus (WNV) and Usutu Virus (USUV) are listed here, with the corresponding bibliographical references.

	WNV	USUV
Geographical Distribution	Africa, Europe, Middle East, North America and West Asia	Africa, Europe
Main Vector	Culex spp.	Culex spp.
Putative Secondary Vector	A. vexans [98], A. japonicus [99], A. albopictus [100]	A. albopictus [45,50]
Amplifying Host	Migratory birds	Migratory birds
Main Route of Transmission	Mosquito bite	Mosquito bite
Alternate Route of Transmission	Rare cases of contamination through organ transplant and transfusion [101] and of mother-to-child transmission [102]	Not described
Sensitivity to IFN	Strong [88]	Very strong [88]
Antagonism of IFN Response	Through NS4B [103]	Not described

7. Concluding Remarks

While USUV has caused significant outbreaks in birds since 2001, its limited circulation and overall low pathogenicity in humans explain why researchers and public health authorities are not yet too concerned about this pathogen. In the context of unprecedented viral circulation in 2018, and since USUV has now clearly been shown to be associated with neurological disorders, increased surveillance and research efforts are in order. In particular, larger cohort studies of populations of interest, such as wildlife workers and patients suffering from encephalitis, will help more accurately estimate USUV prevalence. Identifying molecular determinants associated with virulence, host tropism, and adaptation to new vectors may help anticipate key events leading to the possible emergence of USUV. Finally, developing antiviral approaches, or other preemptive or control measures in humans or at the reservoir-vector interface, will help control USUV circulation and reduce the economic and sanitary burden it may pose in the future.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/11/7/640/s1, Table S1: List of USUV strains used to build the phylogenetic tree.

Funding: This work was funded by the DARPA PREEMPT program managed by Jim Gimlett, Bradley Ringeisen and Amy Jenkins and administered though DARPA Cooperative Agreement #HR001118S0017-PREEMPT-FP001 (the content of the information does not necessarily reflect the position or the policy of the U.S. government, and no official endorsement should be inferred).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Williams, M.C.; Simpson, D.I.; Haddow, A.J.; Knight, E.M. THE ISOLATION OF WEST NILE VIRUS FROM MAN AND OF USUTU VIRUS FROM THE BIRD-BITING MOSQUITO MANSONIA AURITES (THEOBALD) IN THE ENTEBBE AREA OF UGANDA. Ann. Trop. Med. Parasitol. **1964**, 58, 367–374. [CrossRef] [PubMed]
2. Nikolay, B.; Diallo, M.; Boye, C.S.; Sall, A.A. Usutu virus in Africa. Vector Borne Zoonotic Dis. **2011**, 11, 1417–1423.
3. Weissenbock, H.; Kolodziejek, J.; Url, A.; Lussy, H.; Rebel-Bauder, B.; Nowotny, N. Emergence of Usutu virus, an African mosquito-borne flavivirus of the Japanese encephalitis virus group, central Europe. Emerg. Infect. Dis. **2002**, 8, 652–656. [CrossRef] [PubMed]
4. Weissenbock, H.; Bakonyi, T.; Rossi, G.; Mani, P.; Nowotny, N. Usutu virus, Italy, 1996. Emerg. Infect. Dis. **2013**, 19, 274–277. [CrossRef] [PubMed]

5. Kemenesi, G.; Buzas, D.; Zana, B.; Kurucz, K.; Krtinic, B.; Kepner, A.; Foldes, F.; Jakab, F. First genetic characterization of Usutu virus from Culex pipiens mosquitoes Serbia, 2014. Transbound Emerg. Dis. **2018**, 63, 58–61. [CrossRef] [PubMed]
6. Bakonyi, T.; Jungbauer, C.; Aberle, S.W.; Kolodziejek, J.; Dimmel, K.; Stiasny, K.; Allerberger, F.; Nowotny, N. Usutu virus infections among blood donors, Austria, July and August 2017-Raising awareness for diagnostic challenges. Euro Surveill. **2017**, 22, 17-00644. [CrossRef] [PubMed]
7. Engel, D.; Jost, H.; Wink, M.; Borstler, J.; Bosch, S.; Garigliany, M.M.; Jost, A.; Czajka, C.; Luhken, R.; Ziegler, U.; et al. Reconstruction of the Evolutionary History and Dispersal of Usutu Virus, a Neglected Emerging Arbovirus in Europe and Africa. MBio **2016**, 7, e01938-15. [CrossRef]
8. Cadar, D.; Luhken, R.; van der Jeugd, H.; Garigliany, M.; Ziegler, U.; Keller, M.; Lahoreau, J.; Lachmann, L.; Becker, N.; Kik, M.; et al. Widespread activity of multiple lineages of Usutu virus, western Europe, 2016. Euro Surveill. **2017**, 22, 30452. [CrossRef]
9. Calzolari, M.; Chiapponi, C.; Bonilauri, P.; Lelli, D.; Baioni, L.; Barbieri, I.; Lavazza, A.; Pongolini, S.; Dottori, M.; Moreno, A. Co-circulation of two Usutu virus strains in Northern Italy between 2009 and 2014. Infect. Genet. Evol. **2017**, 51, 255–262. [CrossRef]
10. Ben Hassine, T.; De Massis, F.; Calistri, P.; Savini, G.; BelHaj Mohamed, B.; Ranen, A.; Di Gennaro, A.; Sghaier, S.; Hammami, S. First detection of co-circulation of West Nile and Usutu viruses in equids in the south-west of Tunisia. Transbound Emerg. Dis. **2014**, 61, 385–389. [CrossRef]
11. Durand, B.; Haskouri, H.; Lowenski, S.; Vachiery, N.; Beck, C.; Lecollinet, S. Seroprevalence of West Nile and Usutu viruses in military working horses and dogs, Morocco, 2012: Dog as an alternative WNV sentinel species? Epidemiol. Infect. **2016**, 144, 1857–1864. [CrossRef] [PubMed]
12. Mannasse, B.; Mendelson, E.; Orshan, L.; Mor, O.; Shalom, U.; Yeger, T.; Lustig, Y. Usutu Virus RNA in Mosquitoes, Israel, 2014–2015 Emerg. Infect. Dis. **2017**, 23, 1699–1702. [CrossRef] [PubMed]
13. Chaintoutis, S.C.; Dovas, C.I.; Papanastassopoulou, M.; Gewehr, S.; Danis, K.; Beck, C.; Lecollinet, S.; Antalis, V.; Kalaitzopoulou, S.; Panagiotopoulos, T.; et al. Evaluation of a West Nile virus surveillance and early warning system in Greece, based on domestic pigeons. Immunol. Microbiol. Infect. Dis. **2014**, 37, 131–141. [CrossRef] [PubMed]
14. Eiden, M.; Gil, P.; Ziegler, U.; Rakotoarivony, I.; Marie, A.; Frances, B.; L'Ambert, G.; Simonin, Y.; Foulongne, V.; Groschup, M.H.; et al. Emergence of two Usutu virus lineages in Culex pipiens mosquitoes in the Camargue, France, 2015. Infect. Genet. Evol. **2018**, 61, 151–154. [CrossRef] [PubMed]
15. Bakonyi, T.; Busquets, N.; Nowotny, N. Comparison of complete genome sequences of Usutu virus strains detected in Spain, Central Europe, and Africa. Vector Borne Zoonotic Dis. **2014**, 14, 324–329. [CrossRef]
16. Bazanow, B.; Jansen van Vuren, P. A Survey on West Nile and Usutu Viruses in Horses and Birds in Poland. Viruses **2018**, 10, 87. [CrossRef]
17. Bakonyi, T.; Erdelyi, K.; Ursu, K.; Ferenczi, E.; Csorgo, T.; Lussy, H.; Chvala, S.; Bukovsky, C.; Meister, T.; Weissenbock, H.; et al. Emergence of Usutu virus in Hungary. J. Clin. Microbiol. **2007**, 45, 3870–3874. [CrossRef]
18. Rudolf, I.; Bakonyi, T.; Sebesta, O.; Mendel, J.; Pesko, J.; Betasova, L.; Blazejova, H.; Venclikova, K.; Strakova, P.; Nowotny, N.; et al. Co-circulation of Usutu virus and West Nile virus in a reed bed ecosystem. Parasit Vectors **2015**, 8, 520. [CrossRef]
19. Buckley, A.; Dawson, A.; Moss, S.R.; Hinsley, S.A.; Bellamy, P.E.; Gould, E.A. Serological evidence of West Nile virus, Usutu virus and Sindbis virus infection of birds in the UK. J. Gen. Virol. **2003**, 84, 2807–2817. [CrossRef]
20. Barbic, L.; Vilibic-Cavlek, T.; Listes, E.; Stevanovic, V.; Gjenero-Margan, I.; Ljubin-Sternak, S.; Pem-Novosel, I.; Listes, I.; Mlinaric-Galinovic, G.; Di Gennaro, A.; et al. Demonstration of Usutu virus antibodies in horses, Croatia. Vector Borne Zoonotic Dis. **2013**, 13, 772–774. [CrossRef]
21. Rijks, J.M.; Kik, M.L.; Slaterus, R.; Foppen, R.; Stroo, A.; Jzer, J.I.; Stahl, J.; Grone, A.; Koopmans, M.; van der Jeugd, H.P.; et al. Widespread Usutu virus outbreak in birds in the Netherlands, 2016. Euro Surveill. **2016**, 21, 30391. [CrossRef] [PubMed]
22. Steinmetz, H.W.; Bakonyi, T.; Weissenbock, H.; Hatt, J.M.; Eulenberger, U.; Robert, N.; Hoop, R.; Nowotny, N. Emergence and establishment of Usutu virus infection in wild and captive avian species in and around Zurich, Switzerland–genomic and pathologic comparison to other central European outbreaks. Vet. Microbiol. **2011**, 148, 207–212. [CrossRef] [PubMed]

23. Manarolla, G.; Bakonyi, T.; Gallazzi, D.; Crosta, L.; Weissenbock, H.; Dorrestein, G.M.; Nowotny, N. Usutu virus in wild birds in northern Italy. Vet. Microbiol. **2010**, 141, 159–163. [CrossRef] [PubMed]
24. Jost, H.; Bialonski, A.; Maus, D.; Sambri, V.; Eiden, M.; Groschup, M.H.; Gunther, S.; Becker, N.; Schmidt-Chanasit, J. Isolation of usutu virus in Germany. Am. J. Trop. Med. Hyg. **2011**, 85, 551–553. [CrossRef] [PubMed]
25. Sieg, M.; Schmidt, V.; Ziegler, U.; Keller, M.; Hoper, D.; Heenemann, K.; Ruckner, A.; Nieper, H.; Muluneh, A.; Groschup, M.H.; et al. Outbreak and Cocirculation of Three Di(erent Usutu Virus Strains in Eastern Germany. PLoSNegl. Trop. Dis. **2017**, 17, 662–664. [CrossRef] [PubMed]
26. Ziegler, U.; Fast, C.; Eiden, M.; Bock, S.; Schulze, C.; Hoeper, D.; Ochs, A.; Schlieben, P.; Keller, M.; Zielke, D.E.; et al. Evidence for an independent third Usutu virus introduction into Germany. BMC Vet. Res. **2016**, 192, 60–66. [CrossRef] [PubMed]
27. Gaibani, P.; Cavrini, F.; Gould, E.A.; Rossini, G.; Pierro, A.; Landini, M.P.; Sambri, V. Comparative genomic and phylogenetic analysis of the first Usutu virus isolate from a human patient presenting with neurological symptoms. PLoSONE **2013**, 8, e64761. [CrossRef] [PubMed]
28. Cavrini, F.; Gaibani, P.; Longo, G.; Pierro, A.M.; Rossini, G.; Bonilauri, P.; Gerunda, G.E.; Di Benedetto, F.; Pasetto, A.; Girardis, M.; et al. Usutu virus infection in a patient who underwent orthotropic liver transplantation, Italy, August-September 2009. Euro Surveill. **2009**, 14. [CrossRef]
29. Nikolay, B. A review of West Nile and Usutu virus co-circulation in Europe: How much do transmission cycles overlap? Trans. R. Soc. Trop. Med. Hyg. **2015**, 109, 609–618. [CrossRef]
30. Tamba, M.; Bonilauri, P.; Bellini, R.; Calzolari, M.; Albieri, A.; Sambri, V.; Dottori, M.; Angelini, P. Detection of Usutu virus within a West Nile virus surveillance program in Northern Italy. Vector Borne Zoonotic Dis. **2011**, 11, 551–557. [CrossRef]
31. Vilibic-Cavlek, T.; Kaic, B.; Barbic, L.; Pem-Novosel, I.; Slavic-Vrzic, V.; Lesnikar, V.; Kurecic-Filipovic, S.; Babic-Erceg, A.; Listes, E.; Stevanovic, V.; et al. First evidence of simultaneous occurrence of West Nile virus and Usutu virus neuroinvasive disease in humans in Croatia during the 2013 outbreak. Infection **2014**, 42, 689–695. [CrossRef] [PubMed]
32. Aberle, S.W.; Kolodziejek, J.; Jungbauer, C.; Stiasny, K.; Aberle, J.H.; Zoufaly, A.; Hourfar, M.K.; Weidner, L.; Nowotny, N. Increase in human West Nile and Usutu virus infections, Austria, 2018. Euro Surveill. **2018**, 23. [CrossRef] [PubMed]
33. Nikolay, B.; Fall, G.; Boye, C.S.; Sall, A.A.; Skern, T. Validation of a structural comparison of the antigenic characteristics of Usutu virus and West Nile virus envelope proteins. Virus Res. **2014**, 189, 87–91. [CrossRef] [PubMed]
34. Chen, Z.; Ye, F.; Lin, S.; Yang, F.; Cheng, Y.; Cao, Y.; Chen, Z.; Lu, G. Crystal structure of Usutu virus envelope protein in the pre-fusion state. Virol. J. **2018**, 15, 183. [CrossRef] [PubMed]
35. Beck, C.; Jimenez-Clavero, M.A.; Leblond, A.; Durand, B.; Nowotny, N.; Leparc-Go(art, I.; Zientara, S.; Jourdain, E.; Lecollinet, S. Flaviviruses in Europe: Complex circulation patterns and their consequences for the diagnosis and control of West Nile disease. Int. J. Environ. Res. Public Health **2013**, 10, 6049–6083. [CrossRef] [PubMed]
36. Llorente, F.; Garcia-Irazabal, A.; Perez-Ramirez, E.; Cano-Gomez, C.; Sarasa, M.; Vazquez, A.; Jimenez-Clavero, M.A. Influence of flavivirus co-circulation in serological diagnostics and surveillance: A model of study using West Nile, Usutu and Bagaza viruses. Transbound Emerg. Dis. **2019**. [CrossRef] [PubMed]
37. Cleton, N.B.; van Maanen, K.; Bergervoet, S.A.; Bon, N.; Beck, C.; Godeke, G.J.; Lecollinet, S.; Bowen, R.; Lelli, D.; Nowotny, N.; et al. A Serological Protein Microarray for Detection of Multiple Cross-Reactive Flavivirus Infections in Horses for Veterinary and Public Health Surveillance. Transbound Emerg. Dis. **2017**, 64, 1801–1812. [CrossRef]
38. Del Amo, J.; Sotelo, E.; Fernandez-Pinero, J.; Gallardo, C.; Llorente, F.; Aguero, M.; Jimenez-Clavero, M.A. A novel quantitative multiplex real-time RT-PCR for the simultaneous detection and di(erentiation of West Nile virus lineages 1 and 2, and of Usutu virus. J. Virol. Methods **2013**, 189, 321–327. [CrossRef]
39. Merino-Ramos, T.; Blazquez, A.B.; Escribano-Romero, E.; Canas-Arranz, R.; Sobrino, F.; Saiz, J.C.; Martin-Acebes, M.A. Protection of a single dose west nile virus recombinant subviral particle vaccine against lineage 1 or 2 strains and analysis of the cross-reactivity with Usutu virus. PLoSONE **2014**, 9, e108056. [CrossRef]

40. Blazquez, A.B.; Escribano-Romero, E.; Martin-Acebes, M.A.; Petrovic, T.; Saiz, J.C. Limited susceptibility of mice to Usutu virus (USUV) infection and induction of flavivirus cross-protective immunity. Virology **2015**, 482, 67–71. [CrossRef]
41. Mancini, G.; Montarsi, F.; Calzolari, M.; Capelli, G.; Dottori, M.; Ravagnan, S.; Lelli, D.; Chiari, M.; Santilli, A.; Quaglia, M.; et al. Mosquito species involved in the circulation of West Nile and Usutu viruses in Italy. Vet. Ital. **2017**, 53, 97–110. [PubMed]
42. Lebl, K.; Zittra, C.; Silbermayr, K.; Obwaller, A.; Berer, D.; Brugger, K.; Walter, M.; Pinior, B.; Fuehrer, H.P.; Rubel, F. Mosquitoes (Diptera: Culicidae) and their relevance as disease vectors in the city of Vienna, Austria. Parasitol. Res. **2015**, 114, 707–713. [CrossRef] [PubMed]
43. Fros, J.J.; Miesen, P.; Vogels, C.B.; Gaibani, P.; Sambri, V.; Martina, B.E.; Koenraadt, C.J.; van Rij, R.P.; Vlak, J.M.; Takken, W.; et al. Comparative Usutu and West Nile virus transmission potential by local Culex pipiens mosquitoes in north-western Europe. One Health **2015**, 1, 31–36. [CrossRef] [PubMed]
44. Hernandez-Triana, L.M.; de Marco, M.F.; Mansfield, K.L.; Thorne, L.; Lumley, S.; Marston, D.; Fooks, A.A.; Johnson, N. Assessment of vector competence of UK mosquitoes for Usutu virus of African origin. PLoS Negl. Trop. Dis. **2018**, 11, 381. [CrossRef] [PubMed]
45. Cook, C.L.; Huang, Y.S.; Lyons, A.C.; Alto, B.W.; Unlu, I.; Higgs, S.; Vanlandingham, D.L. North American Culex pipiens and Culex quinquefasciatus are competent vectors for Usutu virus. PLoS Negl. Trop. Dis. **2018**, 12, e0006732. [CrossRef]
46. Camp, J.V.; Kolodziejek, J.; Nowotny, N. Targeted surveillance reveals native and invasive mosquito species infected with Usutu virus. Parasit Vectors **2019**, 12, 46. [CrossRef] [PubMed]
47. Nikolay, B.; Diallo, M.; Faye, O.; Boye, C.S.; Sall, A.A. Vector competence of Culex neavei (Diptera: Culicidae) for Usutu virus. Am. J. Trop. Med. Hyg. **2012**, 86, 993–996. [CrossRef]
48. Calzolari, M.; Bonilauri, P.; Bellini, R.; Albieri, A.; Defilippo, F.; Maioli, G.; Galletti, G.; Gelati, A.; Barbieri, I.; Tamba, M.; et al. Evidence of simultaneous circulation of West Nile and Usutu viruses in mosquitoes sampled in Emilia-Romagna region (Italy) in 2009. PLoS ONE **2010**, 5, e14324. [CrossRef]
49. Calzolari, M.; Gaibani, P.; Bellini, R.; Defilippo, F.; Pierro, A.; Albieri, A.; Maioli, G.; Luppi, A.; Rossini, G.; Balzani, A.; et al. Mosquito, bird and human surveillance of West Nile and Usutu viruses in Emilia-Romagna Region (Italy) in 2010. PLoS ONE **2012**, 7, e38058. [CrossRef]
50. Puggioli, A.; Bonilauri, P.; Calzolari, M.; Lelli, D.; Carrieri, M.; Urbanelli, S.; Pudar, D.; Bellini, R. Does Aedes albopictus (Diptera: Culicidae) play any role in Usutu virus transmission in Northern Italy? Experimental oral infection and field evidences. Acta. Trop. **2017**, 172, 192–196. [CrossRef]
51. Llopis, I.V.; Tomassone, L.; Grego, E.; Silvano, F.; Rossi, L. Investigation into Usutu and West Nile viruses in ticks from wild birds in Northwestern Italy, 2012-2014. New Microbiol. **2017**, 40, 56–57. [PubMed]
52. Mancini, F.; Toma, L.; Ciervo, A.; Di Luca, M.; Faggioni, G.; Lista, F.; Rezza, G. Virus investigation in ticks from migratory birds in Italy. New Microbiol. **2013**, 36, 433–434. [PubMed]
53. Schu(enecker, I.; Iteman, I.; Michault, A.; Murri, S.; Frangeul, L.; Vaney, M.C.; Lavenir, R.; Pardigon, N.; Reynes, J.M.; Pettinelli, F.; et al. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. PLoS Med. **2006**, 3, e263. [CrossRef] [PubMed]
54. Benzarti, E.; Linden, A.; Desmecht, D.; Garigliany, M. Mosquito-borne epornitic flaviviruses: An update and review. J. Gen. Virol. **2019**, 100, 119–132. [CrossRef] [PubMed]
55. Weissenbock, H.; Kolodziejek, J.; Fragner, K.; Kuhn, R.; Pfe(er, M.; Nowotny, N. Usutu virus activity in Austria, 2001–2002. Microbes. Infect. **2003**, 5, 1132–1136. [CrossRef]
56. Becker, N.; Jost, H.; Ziegler, U.; Eiden, M.; Hoper, D.; Emmerich, P.; Fichet-Calvet, E.; Ehichioya, D.U.; Czajka, C.; Gabriel, M.; et al. Epizootic emergence of Usutu virus in wild and captive birds in Germany. PLoS ONE **2012**, 7, e32604. [CrossRef]
57. Chvala, S.; Kolodziejek, J.; Nowotny, N.; Weissenbock, H. Pathology and viral distribution in fatal Usutu virus infections of birds from the 2001 and 2002 outbreaks in Austria. J. Comp. Pathol. **2004**, 131, 176–185. [CrossRef]
58. Bergsman, L.D.; Hyman, J.M.; Manore, C.A. A mathematical model for the spread of west nile virus in migratory and resident birds. Math. Biosci. Eng. **2016**, 13, 401–424. [CrossRef]
59. Lecollinet, S.; Blanchard, Y.; Manson, C.; Lowenski, S.; Laloy, E.; Quenault, H.; Touzain, F.; Lucas, P.; Eraud, C.; Bahuon, C.; et al. Dual Emergence of Usutu Virus in Common Blackbirds, Eastern France, 2015. Emerg. Infect. Dis. **2016**, 22, 2225. [CrossRef]

60. Meister, T.; Lussy, H.; Bakonyi, T.; Sikutova, S.; Rudolf, I.; Vogl, W.; Winkler, H.; Frey, H.; Hubalek, Z.; Nowotny, N.; et al. Serological evidence of continuing high Usutu virus (Flaviviridae) activity and establishment of herd immunity in wild birds in Austria. Vet. Microbiol. **2008**, 127, 237–248.[CrossRef]
61. Chvala, S.; Bakonyi, T.; Hackl, R.; Hess, M.; Nowotny, N.; Weissenbock, H. Limited pathogenicity of Usutu virus for the domestic chicken (Gallus domesticus). Avian Pathol. **2005**, 34, 392–395.[CrossRef] [PubMed]
62. Chvala, S.; Bakonyi, T.; Hackl, R.; Hess, M.; Nowotny, N.; Weissenbock, H. Limited pathogenicity of usutu virus for the domestic goose (Anser anser f. domestica) following experimental inoculation. J. Vet. Med. B Infect. Dis. Vet. Public Health **2006**, 53, 171–175.[CrossRef] [PubMed]
63. Rou(aer, L.O.; Steensels, M.; Verlinden, M.; Vervaeke, M.; Boonyarittichaikij, R.; Martel, A.; Lambrecht, B. Usutu Virus Epizootic and Plasmodium Coinfection in Eurasian Blackbirds (Turdus merula) in Flanders, Belgium. J. Wildl. Dis. **2018**, 54, 859–862.[CrossRef] [PubMed]
64. Vanhomwegen, J.; Beck, C.; Despres, P.; Figuerola, A.; Garcia, R.; Lecollinet, S.; Lopez-Roig, M.; Manuguerra, J.C.; Serra-Cobo, J. Circulation of Zoonotic Arboviruses in Equine Populations of Mallorca Island (Spain). VectorBorneZoonoticDis. **2017**, 17, 340–346.[CrossRef] [PubMed]
65. Lupulovic, D.; Martin-Acebes, M.A.; Lazic, S.; Alonso-Padilla, J.; Blazquez, A.B.; Escribano-Romero, E.; Petrovic, T.; Saiz, J.C. First serological evidence of West Nile virus activity in horses in Serbia. Vector Borne ZoonoticDis. **2011**, 11, 1303–1305[CrossRef] [PubMed]
66. Montagnaro, S.; Piantedosi, D.; Ciarcia, R.; Loponte, R.; Veneziano, V.; Fusco, G.; Amoroso, M.G.; Ferrara, G.; Damiano, S.; Iovane, G.; et al. Serological Evidence of Mosquito-Borne Flaviviruses Circulation in Hunting Dogs in Campania Region, Italy. VectorBorneZoonoticDis. **2019**, 19, 142–147.[CrossRef] [PubMed]
67. Busani, L.; Capelli, G.; Cecchinato, M.; Lorenzetto, M.; Savini, G.; Terregino, C.; Vio, P.; Bonfanti, L.; Pozza, M.D.; Marangon, S. West Nile virus circulation in Veneto region in 2008-2009 Epidemiol.Infect. **2011**, 139, 818–825.[CrossRef] [PubMed]
68. Garcia-Bocanegra, I.; Paniagua, J.; Gutierrez-Guzman, A.V.; Lecollinet, S.; Boadella, M.; Arenas-Montes, A.; Cano-Terriza, D.; Lowenski, S.; Gortazar, C.; Hofle, U. Spatio-temporal trends and risk factors a(ecting West Nile virus and related flavivirus exposure in Spanish wild ruminants. BMC Vet. Res. **2016**, 12, 249. [CrossRef]
69. Escribano-Romero, E.; Lupulovic, D.; Merino-Ramos, T.; Blazquez, A.B.; Lazic, G.; Lazic, S.; Saiz, J.C.; Petrovic, T. West Nile virus serosurveillance in pigs, wild boars, and roe deer in Serbia. Vet. Microbiol. **2015**, 176, 365–369.[CrossRef]
70. Cadar, D.; Becker, N.; Campos Rde, M.; Borstler, J.; Jost, H.; Schmidt-Chanasit, J. Usutu virus in bats, Germany, 2013. Emerg. Infect. Dis. **2014**, 20, 1771–1773[CrossRef]
71. Romeo, C.; Lecollinet, S. Are tree squirrels involved in the circulation of flaviviruses in Italy? Transbound Emerg. Dis. **2018**, 65, 1372–1376[CrossRef] [PubMed]
72. Diagne, M.M.; Ndione, M.H.D.; Di Paola, N.; Fall, G.; Bedekelabou, A.P. Usutu Virus Isolated from Rodents in Senegal. Viruses **2019**, 11, 181. [CrossRef] [PubMed]
73. Cle, M.; Salinas, S.; Lecollinet, S.; Beck, C.; Gutierrez, S.; Baldet, T.; Vande Perre, P.; Foulongne, V.; Simonin, Y. Usutu virus: The phantom menace. Med. Sci. (Paris) **2018**, 34, 709–716.
74. Pecorari, M.; Longo, G.; Gennari, W.; Grottola, A.; Sabbatini, A.; Tagliazucchi, S.; Savini, G.; Monaco, F.; Simone, M.; Lelli, R.; et al. First human case of Usutu virus neuroinvasive infection, Italy, August-September 2009. Euro Surveill. **2009**, 14. [CrossRef]
75. Simonin, Y.; Sillam, O.; Carles, M.J.; Gutierrez, S.; Gil, P.; Constant, O.; Martin, M.F.; Girard, G.; Van de Perre, P.; Salinas, S.; et al. Human Usutu Virus Infection with Atypical Neurologic Presentation, Montpellier, France, 2016. Emerg. Infect. Dis. **2018**, 24, 875–878.[CrossRef] [PubMed]
76. Allering, L.; Jost, H.; Emmerich, P.; Gunther, S.; Lattwein, E.; Schmidt, M.; Seifried, E.; Sambri, V.; Hourfar, K.; Schmidt-Chanasit, J. Detection of Usutu virus infection in a healthy blood donor from south-west Germany, 2012. Euro Surveill. **2012**, 17. [CrossRef]
77. Pierro, A.; Gaibani, P.; Spadafora, C.; Ruggeri, D.; Randi, V.; Parenti, S.; Finarelli, A.C.; Rossini, G.; Landini, M.P.; Sambri, V. Detection of specific antibodies against West Nile and Usutu viruses in healthy blood donors in northern Italy, 2010–2011. Clin. Microbiol. Infect. **2013**, 19, E451–E453[CrossRef]
78. Cvjetković, I.H.; Petrović, T.; Petrić, D.; Cvjetković, D.; Kovačević, G.; Radovanov, J.; Galović, A.J.; Patić, A.; Nikolić, N.; Mikić, S.S. Seroprevalence of mosquito-born and tick-born microorganisms in human population of South Backa District. Arhiv. Veterinarske Med. **2016**, 9, 23–30.

79. Percivalle, E.; Sassera, D.; Rovida, F.; Isernia, P.; Fabbi, M.; Baldanti, F.; Marone, P. Usutu Virus Antibodies in Blood Donors and Healthy Forestry Workers in the Lombardy Region, Northern Italy. Vector Borne ZoonoticDis. **2017**, 17, 658–661. [CrossRef]
80. Grottola, A.; Marcacci, M.; Tagliazucchi, S.; Gennari, W.; Di, A.G.; Orsini, M.; Monaco, F.; Marchegiano, P.; Marini, V.; Meacci, M.; et al. Usutu virus infections in humans: A retrospective analysis in the municipality of Modena, Italy. Clin. Microbiol. Infect. **2017**, 23, 33–37. [CrossRef]
81. Maggi, F.; Mazzetti, P.; Focosi, D.; Macera, L.; Scagnolari, C.; Manzin, A.; Antonelli, G.; Nelli, L.C. Lack of usutu virus RNA in cerebrospinal fluid of patients with encephalitis of unknown etiology, Tuscany, Italy. J. Med. Virol. **2015**, 87, 913–916. [CrossRef] [PubMed]
82. Cordey, S.; Vieille, G.; Turin, L.; Kaiser, L. Usutu virus in cerebrospinal fluid: A 2-year survey in a Tertiary Care Hospital, Geneva, Switzerland. J. Med. Virol. **2018**, 90, 609–611. [CrossRef] [PubMed]
83. Cavrini, F.; Della Pepa, M.E.; Gaibani, P.; Pierro, A.M.; Rossini, G.; Landini, M.P.; Sambri, V. A rapid and specific real-time RT-PCR assay to identify Usutu virus in human plasma, serum, and cerebrospinal fluid. J. Clin. Virol. **2011**, 50, 221–223. [CrossRef] [PubMed]
84. Weissenbock, H.; Bakonyi, T.; Chvala, S.; Nowotny, N. Experimental Usutu virus infection of suckling mice causes neuronal and glial cell apoptosis and demyelination. Acta. Neuropathol **2004**, 108, 453–460. [CrossRef] [PubMed]
85. Salinas, S.; Constant, O.; Desmetz, C. Deleterious e(ect of Usutu virus on human neural cells. PLoS Negl. Trop. Dis. **2017**, 11, e0005913. [CrossRef] [PubMed]
86. Vielle, N.J.; Garcia-Nicolas, O.; Oliveira Esteves, B.I.; Brugger, M.; Summerfield, A.; Alves, M.P. The Human Upper Respiratory Tract Epithelium Is Susceptible to Flaviviruses. Front. Microbiol. **2019**, 10, 811. [CrossRef] [PubMed]
87. Sembene, P.M.; Faye, O.; Zanotto, P.M.A.; Sall, A.A.; Garcia-Nicolas, O.; Lewandowska, M.; Ricklin, M.E.; Summerfield, A. Monocyte-Derived Dendritic Cells as Model to Evaluate Species Tropism of Mosquito-Borne Flaviviruses. Viruses **2019**, 9, 5.
88. Cacciotti, G.; Caputo, B.; Selvaggi, C.; la Sala, A.; Vitiello, L.; Diallo, D.; Ceianu, C.; Antonelli, G.; Nowotny, N.; Scagnolari, C. Variation in interferon sensitivity and induction between Usutu and West Nile (lineages 1 and 2) viruses. Virology **2015**, 485, 189–198. [CrossRef]
89. Segura Guerrero, N.A.; Sharma, S.; Neyts, J.; Kaptein, S.J.F. Favipiravir inhibits in vitro Usutu virus replication and delays disease progression in an infection model in mice. Antiviral Res. **2018**, 160, 137–142. [CrossRef]
90. Blazquez, A.B.; Escribano-Romero, E.; Merino-Ramos, T.; Saiz, J.C.; Martin-Acebes, M.A. Infection with Usutu virus induces an autophagic response in mammalian cells. PLoS Negl. Trop. Dis. **2013**, 7, e2509. [CrossRef]
91. Echavarria-Consuegra, L.; Smit, J.M.; Reggiori, F. Role of autophagy during the replication and pathogenesis of common mosquito-borne flavi- and alphaviruses. Open Biol. **2019**, 9, 190009. [CrossRef] [PubMed]
92. Merino-Ramos, T.; Vazquez-Calvo, A.; Casas, J.; Sobrino, F.; Saiz, J.C.; Martin-Acebes, M.A. Modification of the Host Cell Lipid Metabolism Induced by Hypolipidemic Drugs Targeting the Acetyl Coenzyme A Carboxylase Impairs West Nile Virus Replication. Antimicrob. Agents. Chemother. **2016**, 60, 307–315. [CrossRef] [PubMed]
93. Papa, A. Emerging arboviruses of medical importance in the Mediterranean region. J. Clin. Virol. **2019**, 115, 5–10. [CrossRef]
94. Walter, M.; Brugger, K.; Rubel, F. Usutu virus induced mass mortalities of songbirds in Central Europe: Are habitat models suitable to predict dead birds in unsampled regions? Prev. Vet. Med. **2018**, 159, 162–170. [CrossRef] [PubMed]
95. Rubel, F.; Brugger, K.; Hantel, M.; Chvala-Mannsberger, S.; Bakonyi, T.; Weissenbock, H.; Nowotny, N. Explaining Usutu virus dynamics in Austria: Model development and calibration. Prev. Vet. Med. **2008**, 85, 166–186. [CrossRef] [PubMed]
96. Brugger, K.; Rubel, F. Simulation of climate-change scenarios to explain Usutu-virus dynamics in Austria. Prev. Vet. Med. **2009**, 88, 24–31. [CrossRef]
97. Cheng, Y.; Tjaden, N.B.; Jaeschke, A.; Luhken, R.; Ziegler, U.; Thomas, S.M.; Beierkuhnlein, C. Evaluating the risk for Usutu virus circulation in Europe: Comparison of environmental niche models and epidemiological models. Int. J. Health Geogr. **2018**, 17, 35. [CrossRef]

98. Tiawsirisup, S.; Kinley, J.R.; Tucker, B.J.; Evans, R.B.; Rowley, W.A.; Platt, K.B. Vector competence of Aedes vexans (Diptera: Culicidae) for West Nile virus and potential as an enzootic vector. J. Med. Entomol. **2008**, 45, 452–457. [CrossRef]
99. Veronesi, E.; Paslaru, A.; Silaghi, C.; Tobler, K.; Glavinic, U.; Torgerson, P.; Mathis, A. Experimental evaluation of infection, dissemination, and transmission rates for two West Nile virus strains in European Aedes japonicus under a fluctuating temperature regime. Parasitol. Res. **2018**, 117, 1925–1932. [CrossRef]
100. Fortuna, C.; Remoli, M.E.; Severini, F.; Di Luca, M.; Toma, L.; Fois, F.; Bucci, P.; Boccolini, D.; Romi, R.; Ciufolini, M.G. Evaluation of vector competence for West Nile virus in Italian Stegomyia albopicta (= Aedes albopictus) mosquitoes. Med. Vet. Entomol. **2015**, 29, 430–433. [CrossRef]
101. Kusne, S.; Smilack, J. Transmission of West Nile virus by organ transplantation. Liver Transpl. **2005**, 11, 239–241. [CrossRef] [PubMed]
102. Hayes, E.B.; O'Leary, D.R. West Nile virus infection: A pediatric perspective. Pediatrics **2004**, 113, 1375–1381. [CrossRef] [PubMed]
103. Zmurko, J.; Neyts, J.; Dallmeier, K. Flaviviral NS4b, chameleon and jack-in-the-box roles in viral replication and pathogenesis, and a molecular target for antiviral intervention. Rev. Med. Virol. **2015**, 25, 205–223. [CrossRef] [PubMed]
104. Reisen, W.K.; Lothrop, H.D.; Wheeler, S.S.; Kennsington, M.; Gutierrez, A.; Fang, Y.; Garcia, S.; Lothrop, B. Persistent West Nile virus transmission and the apparent displacement St. Louis encephalitis virus in southeastern California, 2003–2006 J. Med. Entomol. **2008**, 45, 494–508.
105. Diaz, A.; Co(ey, L.L.; Burkett-Cadena, N.; Day, J.F. Reemergence of St. Louis Encephalitis Virus in the Americas. Emerg. Infect. Dis. **2018**, 24, 2150. [CrossRef] [PubMed]

' 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

VACCINES AND ANTIVIRAL AGENTS

β-D-N⁴-Hydroxycytidine Is a Potent Anti-alphavirus Compound That Induces a High Level of Mutations in the Viral Genome

Nadya Urakova[a], Valeriya Kuznetsova[a], David K. Crossman[b], Arpine Sokratian[a], David B. Guthrie[c], Alexander A. Kolykhalov[c], Mark A. Lockwood[c], Michael G. Natchus[c], Michael R. Crowley[b], George R. Painter[c,d], Elena I. Frolova[a], Ilya Frolov[a]

[a]Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama, USA
[b]Heflin Center for Genomic Science, University of Alabama at Birmingham, Birmingham, Alabama, USA
[c]Emory Institute for Drug Development, Emory University, Atlanta, Georgia, USA
[d]Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia, USA

ABSTRACT Venezuelan equine encephalitis virus (VEEV) is a representative member of the New World alphaviruses. It is transmitted by mosquito vectors and causes highly debilitating disease in humans, equids, and other vertebrate hosts. Despite a continuous public health threat, very few compounds with anti-VEEV activity in cell culture and in mouse models have been identified to date, and rapid development of virus resistance to some of them has been recorded. In this study, we investigated the possibility of using a modified nucleoside analog, β-D-N⁴-hydroxycytidine (NHC), as an anti-VEEV agent and defined the mechanism of its anti-VEEV activity. The results demonstrate that NHC is a very potent antiviral agent. It affects both the release of genome RNA-containing VEE virions and their infectivity. Both of these antiviral activities are determined by the NHC-induced accumulation of mutations in virus-specific RNAs. The antiviral effect is most prominent when NHC is applied early in the infectious process, during the amplification of negative- and positive-strand RNAs in infected cells. Most importantly, only a low-level resistance of VEEV to NHC can be developed, and it requires acquisition and cooperative function of more than one mutation in nsP4. These adaptive mutations are closely located in the same segment of nsP4. Our data suggest that NHC is more potent than ribavirin as an anti-VEEV agent and likely can be used to treat other alphavirus infections.

IMPORTANCE Venezuelan equine encephalitis virus (VEEV) can cause widespread epidemics among humans and domestic animals. VEEV infections result in severe meningoencephalitis and long-term sequelae. No approved therapeutics exist for treatment of VEEV infections. Our study demonstrates that β-D-N⁴-hydroxycytidine (NHC) is a very potent anti-VEEV compound, with the 50% effective concentration being below 1 μM. The mechanism of NHC antiviral activity is based on induction of high mutation rates in the viral genome. Accordingly, NHC treatment affects both the rates of particle release and the particle infectivity. Most importantly, in contrast to most of the anti-alphavirus drugs that are under development, resistance of VEEV to NHC develops very inefficiently. Even low levels of resistance require acquisition of multiple mutations in the gene of the VEEV-specific RNA-dependent RNA polymerase nsP4.

KEYWORDS alphaviruses, antivirals, N-hydroxycytidine, RNA-dependent RNA polymerase, Venezuelan equine encephalitis virus, drug-resistant mutant, lethal mutagenesis

Alphaviruses represent a group of mosquito-borne viruses that are widely distributed on all continents. Most of them are maintained in nature by continuous transmission between mosquito vectors and vertebrates that serve as amplifying hosts.

Received 10 November 2017 **Accepted** 10 November 2017

Accepted manuscript posted online 22 November 2017

Citation Urakova N, Kuznetsova V, Crossman DK, Sokratian A, Guthrie DB, Kolykhalov AA, Lockwood MA, Natchus MG, Crowley MR, Painter GR, Frolova EI, Frolov I. 2018. β-D-N⁴-Hydroxycytidine is a potent anti-alphavirus compound that induces a high level of mutations in the viral genome. J Virol 92:e01965-17. https://doi.org/10.1128/JVI.01965-17.

Editor Susana López, Instituto de Biotecnologia/UNAM

Copyright © 2018 American Society for Microbiology. All Rights Reserved.

Address correspondence to Elena I. Frolova, efrolova@uab.edu or Ilya Frolov, ivfrolov@uab.edu.

N.U. and V.K. contributed equally to this article.

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

Based on their geographical distribution, the currently known members of the Alphavirus genus are divided into the New World (NW) and the Old World (OW) alphaviruses (1). Although the OW alphaviruses cause less severe diseases characterized by rash, fever, and arthritis, most of the NW alphaviruses exhibit an encephalitogenic phenotype (1). The NW representative members include Venezuelan (VEEV), eastern (EEEV), and western (WEEV) equine encephalitis viruses (2–6). In humans, these viruses cause debilitating diseases, and the overall mortality rates are ! 1% (7), 30 to 80% (8), and 1 to 5% (9) for VEEV, EEEV and WEEV, respectively.

Alphaviruses replicate to very high titers in many commonly used cell lines of vertebrate and mosquito origins. They can be produced on a large scale, stored in lyophilized form for a long period of time, and efficiently distributed by aerosol. Moreover, mortality rates are amplified with aerosol infection (10). Therefore, EEEV and some VEEV strains can potentially be used by bioterrorists, and thus they are on the U.S. Select Agents and Toxins list. Nevertheless, despite continuous circulation in Central, South, and North America and the public health threat, no safe and efficient vaccines or therapeutics exist against VEEV or other NW alphavirus infections.

VEEV is a relatively small enveloped virus. Its genome consists of a single-stranded RNA (G RNA) of positive polarity. Upon release into the cytoplasm from the nucleocapsids, G RNA serves directly as a template for the translation of nonstructural proteins that mediate replication of viral G RNA and transcription of the subgenomic RNA (SG RNA). The latter RNA is translated into viral structural proteins that ultimately form infectious, G RNA-containing virions. The nonstructural proteins are initially synthesized as precursor polyproteins P123 and P1234 and are later processed by nsP2-associated protease activity into individual nsP1, nsP2, nsP3, and nsP4. This stepwise processing of the polyproteins regulates synthesis of the negative-strand RNA genome intermediates, G RNAs and SG RNAs (11–13).

To date, many functions of individual nsPs in RNA and virus replication have been identified. nsP2, which exhibits protease and helicase activities, and nsP4, which contains the predicted RNA-dependent RNA polymerase (RdRp) domain (1), attracted most of the attention as possible targets for the development of antiviral drugs. However, few screens have yielded positive results thus far. In one study, the identified CID15997213 compound demonstrated a strong inhibitory effect on VEEV replication in vitro and in a small animal model via its targeting of the nsP2 protein and ultimately viral RNA replication (14). Some other quinazolinone-derived molecules also exhibited antiviral activity against VEEV (15, 16). For example, compound ML336 inhibited several VEEV strains in the nanomolar range in vitro and also protected mice from a lethal VEEV infection. Although the exact mechanism of action for this compound has not been fully elucidated, the mutations that determined drug-resistant phenotypes of VEEV occurred in nsP2 and nsP4 proteins (15).

Compounds that indirectly interfere with VEEV replication by modifying intracellular processes in the host were also investigated as potential therapeutic options to modulate the course of alphavirus infections (17–20). These included (i) an inhibitor of glycogen synthase kinase-3! (GSK-3!) that governs proinflammatory responses in the host cell (17); (ii) acriflavine, an inhibitor of Ago2 (18); (iii) D-(") enantiomer of carbodine, which inhibited cellular CTP synthetase and was capable of decreasing the levels of intracellular CTP (19); (iv) VX-497, a reversible uncompetitive inhibitor of inosine-5=monophosphate dehydrogenase, that demonstrated a moderately negative effect on VEEV replication in vitro (20); and (v) selective inhibitors of nuclear export, which enhanced accumulation of the capsid protein in cell nuclei and thus decreased the titers of released VEEV (21). Ribavirin, a broad-spectrum antiviral drug, exhibited a very limited effect against VEEV even if applied at concentrations higher than 500 " M (20).

Although several compounds show anti-VEEV activity in cell culture and in mouse models, it is unlikely that a single drug can be sufficiently potent and/or applied without rapid selection of resistant mutants. Therefore, in this study, we investigated the possibility of using a modified nucleoside analog, ! -D-N^4-hydroxycytidine (NHC) as

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

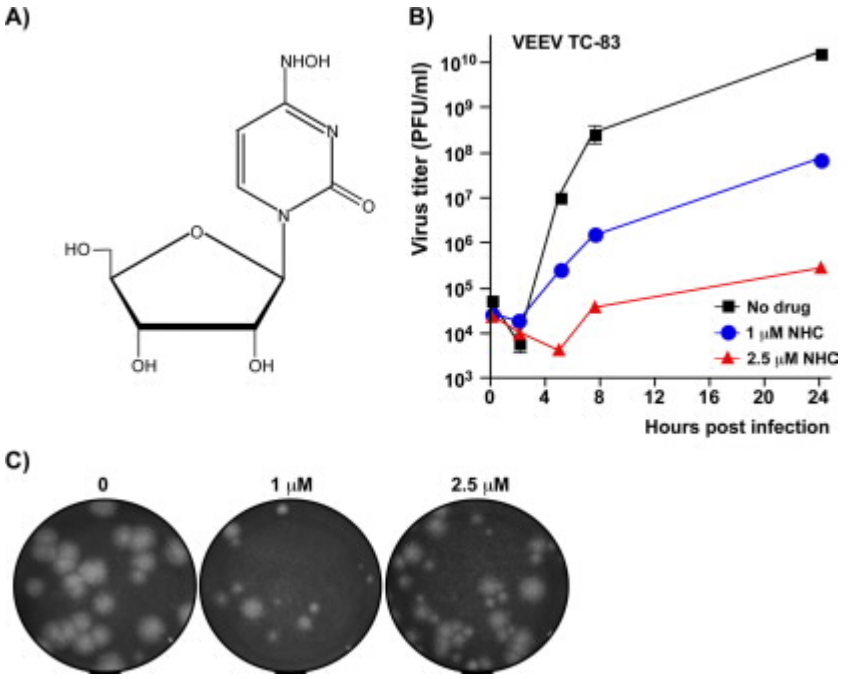


FIG 1 NHC has a strong negative effect on VEEV TC-83 replication. (A) Chemical structure of NHC. (B) Vero cells were infected with VEEV TC-83 at an MOI of 0.5 PFU/cell and were either treated with NHC at the indicated concentrations starting from 0 h postinfection (p.i.) throughout the 24 h of incubation period or they remained mock treated. At the indicated times p.i., media were harvested, and viral titers were determined by a plaque assay on Vero cells. Starting from 5 h p.i., virus titers in the samples harvested from the drug-treated cells were significantly different from those collected from the mock-treated cells (P # 0.0001). (C) Morphology of the plaques formed on Vero cells by VEEV TC-83 variants from the samples harvested at 24 h p.i. from mock- and NHC-treated cells. Due to profound differences in infectious titers, different dilutions are presented.

an anti-VEEV agent. Previous studies demonstrated that NHC significantly inhibits replication of hepatitis C virus (HCV) replicon and bovine viral diarrhea virus in Huh7 and MDBK cells, respectively (22). NHC was also capable of inhibiting human norovirus replicon in HG23 cells (23), human coronavirus (24), and chikungunya virus (25). It was reasonable to expect that the antiviral effect of this nucleoside analog is not limited to these few viruses, and might also be effective against other viral species with RNA genomes, such as VEEV. We tested the effect of NHC on VEEV replication, defined optimal in vitro conditions for its application, and the mechanism of its antiviral activity. Our data demonstrate several important features of NHC. (i) NHC is a very potent anti-VEEV agent, with an EC_{50} below 1 " M. (ii) Most VEE virions released from the NHC-treated cells contain mutated viral genomes, which are incapable of replication. (iii) The antiviral effect of NHC is more prominent if it is applied during the first 4 h postinfection, when amplification of negative-strand RNA intermediates and G RNA takes place. (iv) The resistance to NHC develops very inefficiently and is determined by a cooperative function of multiple mutations in nsP4. Even after 20 passages of VEEV under increasing concentrations of NHC, the pool of VEEV mutants remained highly sensitive to NHC treatment.

RESULTS

NHC has a strong negative effect on infectious titers of the released VEEV TC-83. In initial experiments, we assessed the antiviral potential of NHC (Fig. 1A) by testing whether it was capable of inhibiting VEEV replication. Indeed, NHC demonstrated a strong negative effect on the replication of VEEV TC-83 in Vero cells. In the applied experimental conditions (see the legend to Fig. 1B), NHC at 1 and 2.5 " M concentrations reduced titers of released virus progeny by 2 and 4 orders of magnitude, respectively. Moreover, in the plaque assay, samples harvested from NHC-treated cells,

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

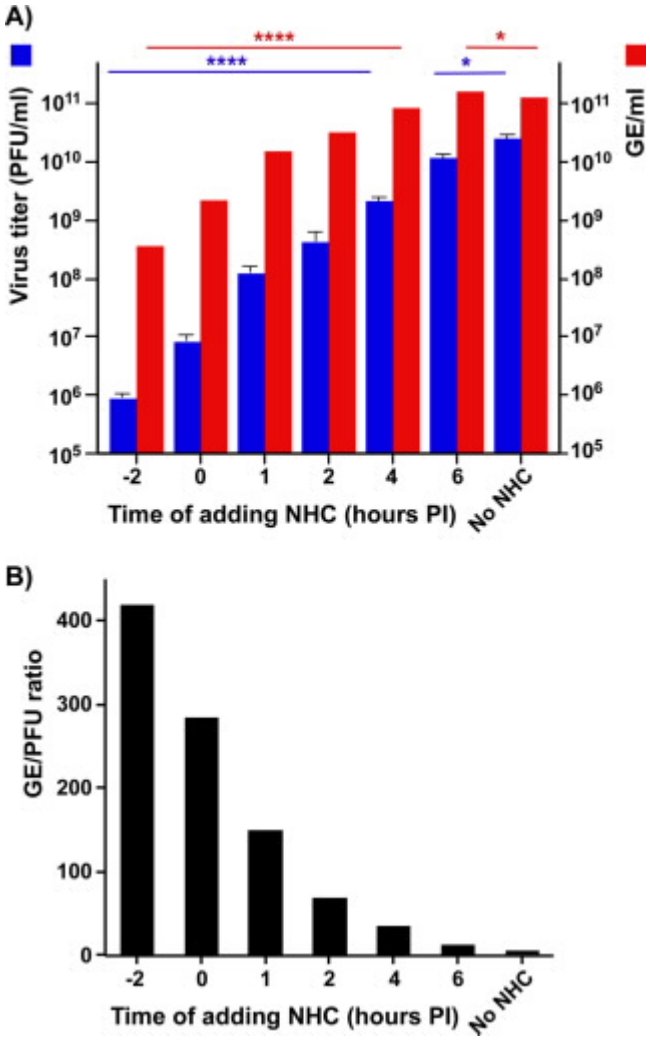


FIG 2 The antiviral effect of NHC depends on its application time. (A) Vero cells were infected with VEEV TC-83 at an MOI of 0.5 PFU/cell. At the time points indicated, cells were treated with 2 " M NHC and then harvested at 24 h p.i. Viral titers were determined using a plaque assay on Vero cells. The data are presented as mean values and standard deviations. In the same samples, the number of genome copies of VEEV TC-83 (GE/ml) was measured by qPCR. (B) GE/PFU ratio in the samples presented in panel A. This experiment was repeated twice with consistent results.

but not from the mock-treated cells, contained a large fraction of small-plaque-forming VEEV variants (Fig. 1C). The heterogeneity of plaques suggested that the antiviral effect of NHC is likely, at least partially, determined by its RNA-mutagenic activity.

Antiviral effect of NHC depends on the timing of its application. In subsequent experiments, Vero cells were infected with VEEV at a multiplicity of infection (MOI) of 0.5 PFU/cell. NHC was added to cells at 2 " M either 2 h prior to VEEV infection, at the moment of infection, or at different times postinfection (p.i.). Infectious titers of the released virus were determined at 24 h p.i. using a plaque assay (PFU/ml), and the concentrations of genome-containing viral particles in the same samples were assessed by reverse transcription-quantitative PCR (RT-qPCR) as genome equivalents (GE) per ml.

NHC was most potent when applied 2 h before infection, causing a reduction in infectious virus titers of more than 4 orders of magnitude. If it was added at later times p.i., the negative effect on released viral progeny titers gradually decreased (Fig. 2A). Addition of the compound at 6 h p.i. had a very small, # 5-fold, negative effect on final infectious titers. NHC treatment prior to infection or at early times p.i. also had a negative effect on the release of genome-containing viral particles. However, the

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

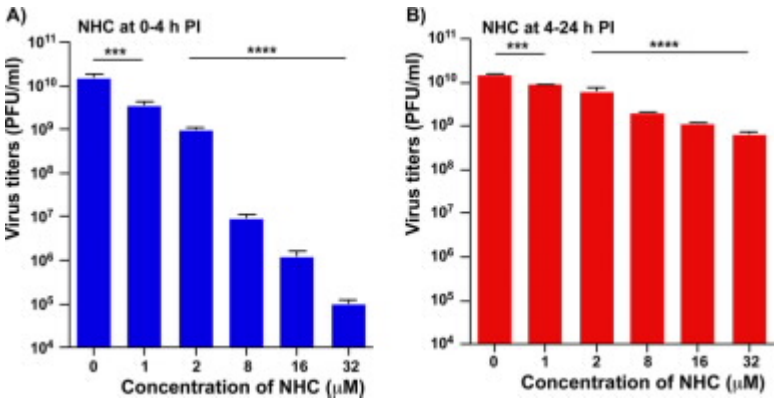


FIG 3 The first 4 h p.i. are a critical time for the antiviral effect of NHC. Vero cells in six-well Costar plates were infected at room temperature with VEEV TC-83 at an MOI of 20 PFU/cell. (A) Cells were incubated with NHC at the concentrations indicated from 0 to 4 h p.i. At 4 h p.i., the cells were washed, and media were replaced with NHC-free medium. (B) Infected cells were incubated in NHC-free medium until 4 h p.i., and then media were replaced by those supplemented with NHC at indicated concentrations. All of the samples indicated in panels A and B were harvested at 24 h p.i., and viral titers were determined by a plaque assay on Vero cells. The experiments were performed in triplicates. The reductions in viral titers in all NHC treated samples were statistically significant, as assessed by the one-way ANOVA.

measured changes in concentrations of GE in the same samples were less prominent than the decreases in the infectious titers (Fig. 2A). Accordingly, samples from the cells treated with NHC starting from 2 h prior to infection, 0 h p.i., 1 h p.i., and 2 h p.i. exhibited higher GE/PFU ratios than those obtained for mock-treated cells or in samples harvested from cells treated with NHC from 6 h p.i. (Fig. 2B). In the media from cells incubated with the compound starting from 2 h prior to infection, the GE/PFU ratio was $ 100-fold higher than in media harvested from mock-treated cells. This was an indication that early treatment led to release of mostly noninfectious virions. Thus, these experiments demonstrated that NHC treatment has strong negative effects on both the efficiency of genome-containing virion release and their infectivity. However, the antiviral effect of NHC is strongly dependent on the timing of its application in relation to viral infection.

To further evaluate the time-dependent effect of NHC treatment, the compound was added at different concentrations (1 to 32 " M) to the media either (i) for the first 4 h p.i. only, followed by incubation in drug-free media (Fig. 3A), or (ii) incubation in the presence of NHC started at 4 h p.i. (Fig. 3B) and continued until 24 h p.i.. All of the samples were harvested at 24 h p.i. to assess infectious titers. NHC treatment within the first 4 h caused profound reduction in infectious titers at 24 h p.i., and there was a strong dose dependence. In contrast, treatment started after 4 h p.i. caused a relatively small negative effect on the infectious titers, irrespective of NHC concentration and despite its presence in the media for a longer period of time (20 h) compared to the samples incubated with this compound for only the first 4 h p.i.

Taken together, these data strongly suggested that NHC is most effective against VEEV in vitro when applied early in the viral replication cycle. As a result, measuring the 50% effective concentration (EC_{50}) and other antiviral characteristics of NHC is very dependent on the time of its application and the MOI used. Therefore, for detailed evaluation of the antiviral effect of NHC on its concentration, subconfluent Vero cells were synchronously infected at an MOI of 20 PFU/cell, and drug was applied at different concentrations at 0 h p.i. The media were harvested at 24 h p.i., and the virus titers were determined by plaque assay on Vero cells. The results are presented in Fig. 4A. Based on these data, the EC_{50}, EC_{90}, and EC_{99} were 0.426, 1.036, and 2.5 " M, respectively.

In parallel experiments, we also assessed the cytotoxicity of NHC (Fig. 4B). Vero cells were incubated in the media supplemented with NHC at 0 to 200 " M for 24 h. Cell viability was assessed using Cell Titer-GLO 2.0 assay. In this range of concentrations,

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

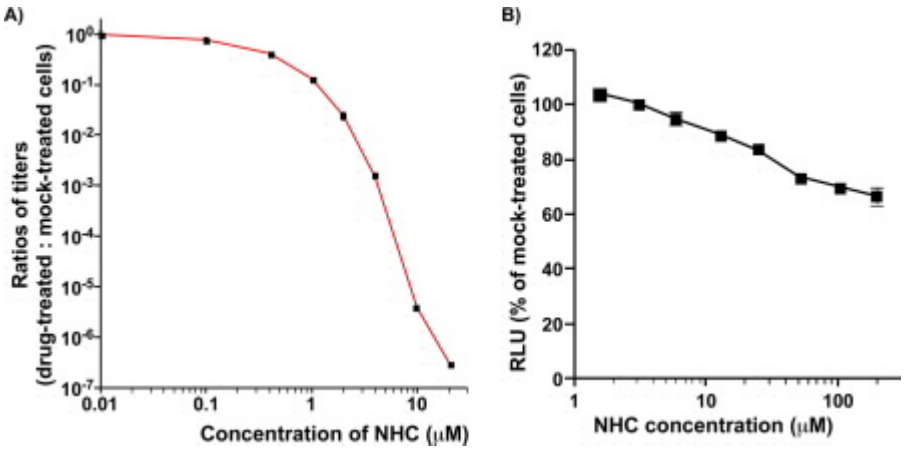


FIG 4 NHC is a potent anti-VEEV compound with low cytotoxicity. (A) Vero cells were infected with VEEV TC-83 at an MOI of 20 PFU/cell. The indicated concentrations of NHC were applied at 0 h p.i., and media were harvested at 24 h p.i. Infectious titers were determined by a plaque assay on Vero cells and normalized to those in the mock-treated samples. The experiment was performed three times. The standard deviation are too low to be visible on the plot. (B) Vero cells were incubated for 24 h with indicated concentrations of NHC in the media. Cell viability was assessed using the Cell Titer-GLO 2.0 assay. Data were normalized to those generated on the mock-treated cells. RLU, relative light units.

NHC demonstrated a cytotoxicity that was too low to determine a 50% cytotoxic concentration (CC_{50} $ 200 " M).

NHC induces high mutation rates in VEEV G RNA. Next, we tested whether the presence of NHC increases the mutation rates of viral G RNA. Vero cells were infected with VEEV TC-83 at an MOI of 2 PFU/cell and incubated for 24 h either in NHC-free media or in media supplemented with 2 " M NHC at the time of infection. G RNAs were purified from harvested stocks, and a 941-nucleotide (nt) fragment of viral genome in both samples was amplified by RT-PCR. The PCR products were cloned into a plasmid, and for each sample, the plasmid insertions of 50 randomly selected clones were sequenced. We identified 42 mutations (8.9 mutations per 10,000 nt) in sequences derived from the virus population generated on NHC-treated cells, and only 4 mutations were detected in the sequences generated from virus harvested on mock-treated cells (0.85 mutations per 10,000 nt). Thus, within a single passage, NHC treatment at 2 " M concentration led to at least a 10-fold increase in accumulation of mutations. However, this ratio is probably even higher, since both pools likely contained equal numbers of mutations introduced by RT and PCRs. The majority of mutations acquired in the presence of NHC were transition mutations. U-to-C or C-to-U transitions were the most prevalent and were likely generated during positive-strand RNA synthesis. The number of A-to-G and G-to-A transitions was ! 4-fold lower, and these likely resulted from NHC incorporation into the negative-strand RNA.

VEEV resistance to NHC develops very inefficiently. Characterization of the drug's antiviral effects requires assessing the possibility for development of resistant viral mutants. Moreover, identification of the adaptive mutations provides critical information about the mechanism of antiviral function. In this study, we passaged the cDNA-derived VEEV TC-83 20 times in the presence of incrementally increasing concentrations of NHC (see Materials and Methods for details). Drug resistance developed very inefficiently and became clearly detectable only after 15 passages, as evidenced by more efficient cytopathic effect (CPE) development in the presence of NHC. This was suggestive that the drug-resistant phenotype likely required multiple adaptive mutations, and a high resistance is probably impossible to generate. After 20 passages, we selected a viral population, which was capable of replication and CPE production in the presence of 3.2 " M NHC. Starting from passage 1, harvested virus stocks produced heterogeneous plaques, and no homogeneous viral population was found at any passage, including passage 20. This was indicative of continuous viral evolution, even after acquiring partial resistance to NHC.

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

After passage 20, two plaques were randomly selected from the agarose overlay supplemented with 2 " M NHC. After extraction from the agarose, plaque-purified viruses were amplified once in the presence of 1 " M NHC to avoid appearance of true or second-site revertants with an NHC-sensitive phenotype. Total RNA was isolated from Vero cells, which were used for this virus amplification. These cell-derived RNAs were used to sequence the genome fragment encoding nsP1-, nsP2-, nsP3-, and nsP4-coding genes (nt 540 to 7700 of viral G RNA). This genome fragment encodes all viral nonstructural proteins, which form a replication complex and thus are directly involved in RNA synthesis. Mutations in these genes could directly determine viral resistance to NHC.

Both plaque isolates contained large numbers of mutations. The nsP-coding sequence of plaque isolate 1 (PP1) contained 34 synonymous and nonsynonymous mutations (47.5 mutations per 10,000 nt), and the plaque isolate 2 (PP2), which was used in the following experiments, had 41 mutations in the same fragment (57.3 mutations per 10,000 nt). The mutations resulting in amino acid substitutions in the nsPs are presented in Fig. 5A. Similarly, in the PP2 genome, the RNA sequence encoding viral structural proteins contained 21 mutations (55 mutations per 10,000 nt) with 4 of them causing amino acid changes (H96R and F389L in E2; P8S and V388M in E1). These mutations in structural genes had no effect on virus infectivity, and the PP2 isolate demonstrated the same GE/PFU ratio as did the original VEEV TC-83 (see Fig. 7B). Therefore, these mutations were not further investigated. In agreement with the results described above, obtained after treating the infected cells with NHC for 24 h, all but one mutations found in viruses passaged 20 times in the presence of NHC were transition mutations, with prevalent U-to-C or C-to-U transitions (Fig. 5B). Importantly, a large number of synonymous and nonsynonymous mutations in the nsP-coding sequence were the same in both randomly selected plaque isolates. This was a strong indication that development of any resistance to NHC is likely to be a rare event and requires a cooperative/synergistic effect of more than one adaptive mutation. Such a mutant likely appeared in one of the intermediate, earlier passages; however, it continued to accumulate other mutations, which distinguished nucleotide sequences of plaque isolates selected after final passage 20.

As a control, VEEV TC-83 was passaged in parallel 20 times in the absence of NHC. After this serial passage, neither changes in the plaque sizes nor changes in virus titers were detected (data not shown). Sequencing of the nsP-coding fragment of a plaque purified after passage 20 virus identified only two mutations (2.8 mutations per 10,000 nt). Both mutations found in the virus passaged without drug were transversions. Thus, the passaging in the presence of NHC resulted in accumulation ! 20-fold more mutations than in the absence of the compound. These results correlate well with the above data, showing a strong difference in the mutation rates during a single passage of VEEV TC-83 in the presence or absence of NHC.

To complete evaluation of the drug resistance development process, we tested the efficiency of reverting of the NHC-resistant isolate back to an NHC-sensitive phenotype. The PP2 isolate was passaged 20 times in Vero cells in NHC-free media. After the last passage, the resulting virus population was homogeneous in terms of plaque size, and these plaques were indistinguishable from those formed by VEEV TC-83 (see Fig. 6C). A plaque-purified variant (PREV1) from this population was used to sequence the nsP-coding genes. Surprisingly, it retained all of the PP2-specific mutations in nonstructural genes, but accumulated 12 additional mutations, from which 6 were nonsynonymous (Fig. 5A).

In the absence of NHC, PREV1 growth rates were very similar to those of the original VEEV TC-83 (Fig. 6A) and were strongly affected by the presence of NHC in the media (Fig. 6B). In side-by-side experiments, without NHC, the PP2 mutant exhibited delayed replication and achieved lower titers (Fig. 6A) compared to both wild-type (wt) VEEV and PREV1 but reproducibly yielded higher titers in NHC-containing media (Fig. 6B). Similarly, in a plaque assay when NHC was present in the agarose overlay, the PP2 mutant was capable of forming plaques in the presence of NHC at 1.2 and 2.5 " M

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

A)

Mutant Plaque1 (PP1)	Mutant Plaque2 (PP2)	PREV1	nsP
V263A	V263A	V263A	nsP1
V280A	V280A	V280A	
A514T	A514T	A514T	
Y15H	Y15H	Y15H	nsP2
		Q80R	
	K202E	K202E	
	V293A	V293A	
	V340M	V340M	
		D492N	
N499D	N499D	N499D	
T770A			
		I92V	nsP3
V237I			
		I359T	
T360I	T360I	T360I	
	I372T	I372T	
	S412P	S412P	
F419L			
L424S			
	S480P	S480P	
V502A	V502A	V502A	
	V119I	V119I	nsP4
	A126V	A126V	
P187S	P187S	P187S	
A189V	A189V	A189V	
I190T	I190T	I190T	
		A201V	
M347I			
C465R	C365R	C365R	
		V496A	

B)

PP1

	U	C	G	A
U		18		
C	7			
G				4
A			5	

PP2

	U	C	G	A
U		23		
C	7			
G				4
A			7	

PREV1

	U	C	G	A
U		4		
C	1			
G				3
A			4	

VEEV p20, pl. purified

	U	C	G	A
U				1
C				1
G				
A				

FIG 5 VEEV TC-83 accumulates a large number of mutations when exposed to NHC. (A) VEEV TC-83 was serially passaged in the presence of NHC (see Materials and Methods for details), and the nsP-coding fragments in the genomes of two randomly selected plaque isolates (PP1 and PP2) were sequenced. PP2 isolate was then passaged 20 times in NHC-free media, and the same nsP-coding fragment in the genome of plaque-purified variant PREV1 was sequenced. Only the nonsynonymous mutations are presented. The mutations identified in both PP1 and PP2 are highlighted in gray. Additional mutations identified in PREV1 are highlighted in red. (B) Matrixes of nucleotide substitutions identified in plaque purified variants PP1, PP2, PREV1, and plaque-purified VEEV TC-83 after 20 passages in the absence of NHC.

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

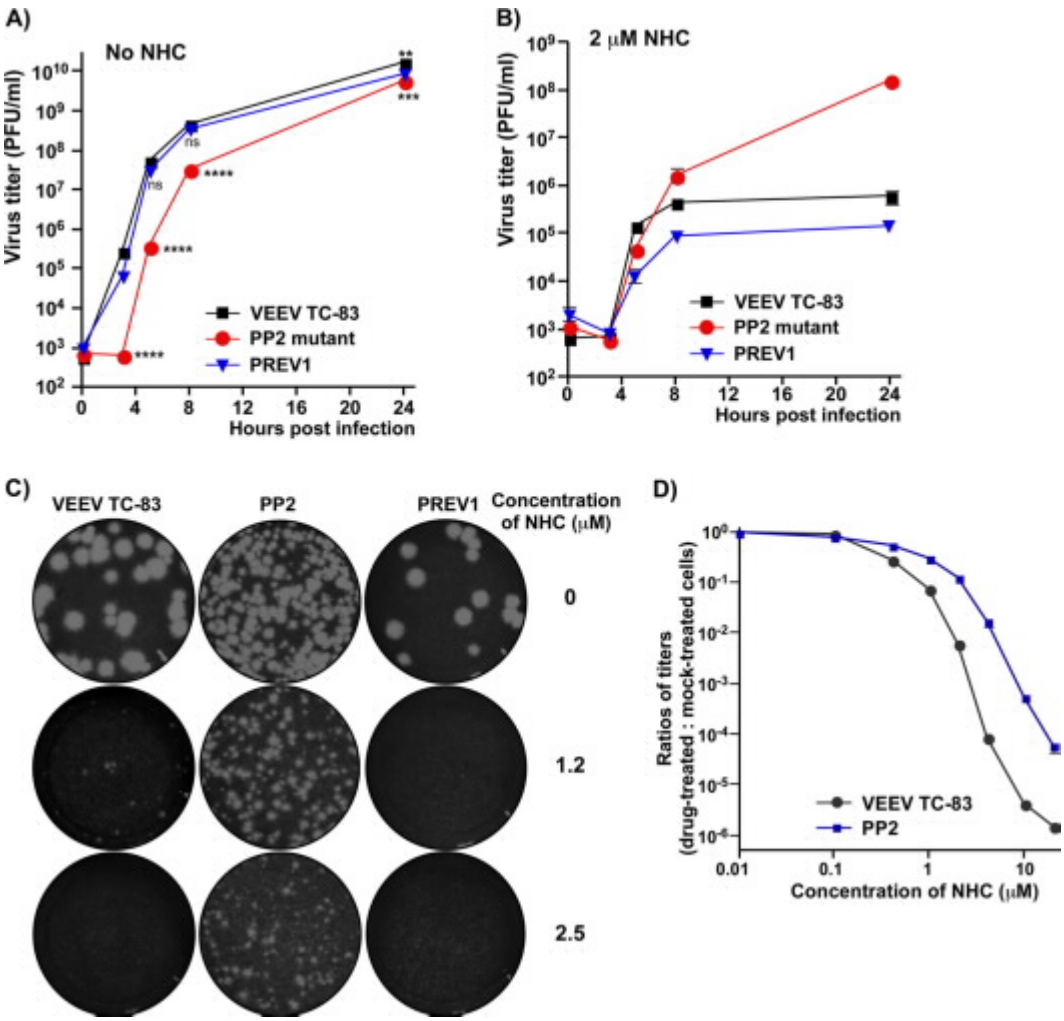


FIG 6 Drug-resistant VEEV isolate PP2 replicates more efficiently than parental VEEV TC-83 and pseudorevertant PREV1 in the presence, but not in the absence of NHC. Subconfluent Vero cells were infected with parental VEEV TC-83, selected PP2 mutant or PREV1 viruses at an MOI of 1 PFU/cell. Starting from 0 h p.i., cells were incubated either in the absence (A) or in the presence (B) of 2 " M NHC. At the indicated time points, media were harvested, and viral titers were determined using a plaque assay on Vero cells. The experiment was repeated three times. Most of the standard deviations are too small to be visible on the plots. On panel B, starting from 5 h p.i., titers of PP2 and PREV1 are significantly different from those of VEEV TC-83 and from each other (P # 0.0001). (C) In a standard plaque assay, cell monolayers infected with the same dilution samples were overlaid with 0.5% agarose-containing media (see Materials and Methods for details) without or with the indicated concentrations of NHC. Plaques were visualized by crystal violet staining at 2 days p.i. (D) Vero cells were infected with VEEV TC-83 and PP2 mutant at an MOI of 20 PFU/cell. The indicated concentrations of NHC were applied at 0 h p.i., and media were harvested at 24 h p.i. Infectious titers were determined by a plaque assay on Vero cells and normalized to those in the mock-treated samples. The standard deviations are too low to be visible on the plot.

concentrations. In contrast, wt VEEV TC-83 could not develop plaques in the presence of 2.5 " M NHC, and PREV1 did not produce plaques under the drug at both concentrations (Fig. 6C).

Taken together, our results demonstrated that (i) in the presence of NHC, the selected PP2 mutant replicated more efficiently than parental VEEV TC-83 and PREV1; (ii) the PP2 drug resistance was unstable and in the absence of NHC, this mutant reverted to an NHC-sensitive phenotype; (iii) the evolution to higher replication rates of the pseudorevertant PREV1 and its sensitivity to drug were the results of accumulation of additional mutations in the PP2 genome, but not the reversal of those developed under the prior NHC treatment.

NHC inhibits VEEV replication via a dual mechanism. To further evaluate the mechanism of NHC-specific inhibition of VEEV replication, we performed a detailed

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

comparative analysis of VEEV TC-83, the PP2 mutant, and the PREV1 pseudorevertant for their ability to produce both infectious virus and G RNA-containing viral particles. Vero cells were infected with these viruses at the same MOI and incubated either with or without 2 " M NHC added at the time of infection. At the indicated time points, media were harvested and used for assessment of both infectious titers (PFU/ml) and concentrations of released viral particles (GE/ml). The results of these experiments (Fig. 7A) demonstrate that the presence of NHC in the media caused a dramatic ($ 1,000-fold) decrease of VEEV TC-83 and PREV1 infectious titers. However, depending on the time p.i., we detected only a 5- to 50-fold decrease in PP2 infectious titers in media harvested from the drug-treated cells compared to mock-treated cells. (ii) In agreement with the results presented in Fig. 2, NHC at 2 " M had a relatively small, but readily detectable negative effect on the release of genome-containing viral particles (GE/ml) of both the original VEEV TC-83 and PREV1. For both viruses, at any time p.i., the G RNA concentrations (GE/ml) in the media of drug-treated cells were 10- to 20-fold lower than in the mock-treated controls. In contrast, the NHC-resistant PP2 mutant produced essentially the same level of particles (GE) in the presence or absence of NHC (Fig. 7A).

Thus, NHC treatment had a dual effect on parental VEEV TC-83 and PREV1 replication. This compound caused a modest decrease in virion release and a strong decrease in virion infectivity. The latter effect likely resulted from the introduction of lethal mutations into G RNA. On the other hand, in the presence of 2 " M NHC, PP2 mutant was able to release viral particles to higher infectious titers compared to VEEV TC-83 and PREV1. However, the negative effect of the compound on infectious titers of PP2, but not on particle release, was also detectable. This resulted in an increase in the PP2 GE/PFU ratio (Fig. 7B) but not to the levels detected in VEEV TC-83 and PREV1 samples. Thus, a large fraction of released PP2 virions also contained noninfectious G RNA, and this was an additional indication that the resistance of PP2 to NHC was only partial.

NHC induces rapid evolution of the VEEV population. Sequencing the plaque-purified variants provided important information about virus evolution under NHC pressure. To complement these data, we applied next-generation sequencing (NGS) to additionally characterize the effect of NHC treatment and/or virus passaging on accumulation of mutations in VEEV TC-83 pools. We sequenced genomes in virus samples harvested at passages 5, 10, 15, and 20, performed either in the absence or presence of NHC. The read sequences were aligned against the VEEV TC-83 genome and analyzed as described in Materials and Methods. The results presented in Fig. 8A, were in agreement with sequencing of the plaque-purified variants. Even after 20 passages in the absence of drug, the VEEV TC-83 population demonstrated relatively low diversity. Only 7 mutations with frequency above 5% were found after 20 passages. None of the mutations became prevalent (with frequencies above 50%). In contrast, passaging VEEV TC-83 in the presence of NHC induced high levels of mutations and led to high heterogeneity of the viral population. After passage 5, four mutations with frequencies above 5% were already present. After passage 20, more than 200 mutations with frequencies above 5% were detected in the virus pool, and 30 of them became prevalent ($ 50%). These data correlated with finding of one synonymous or nonsynonymous mutation per every 150 to 200 nt in the genomes of plaque-purified variants (Fig. 5A), which were isolated from the same passage 20 viral pool.

Using NGS, we detected the accumulation of mutations in essentially all viral genome sequences. However, it was reasonable to expect the mutations responsible for resistance to NHC to be localized in nsP4 and to become dominant by passage 20. In the NHC passage 20 stock, NGS detected four nonsynonymous nsP4-specific mutations, which were present in the viral pool with frequencies higher than 50%. Most importantly, the same mutations were identified in the plaque-purified variants, PP1 and PP2 (Fig. 5A). This was an additional indication that they may be responsible for development of resistance to NHC. Three out of four mutations, namely, P187S, A189V, and I190T, were closely located on the index finger in a three-dimensional (3D) structural model of nsP4 (see Fig. 10).

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

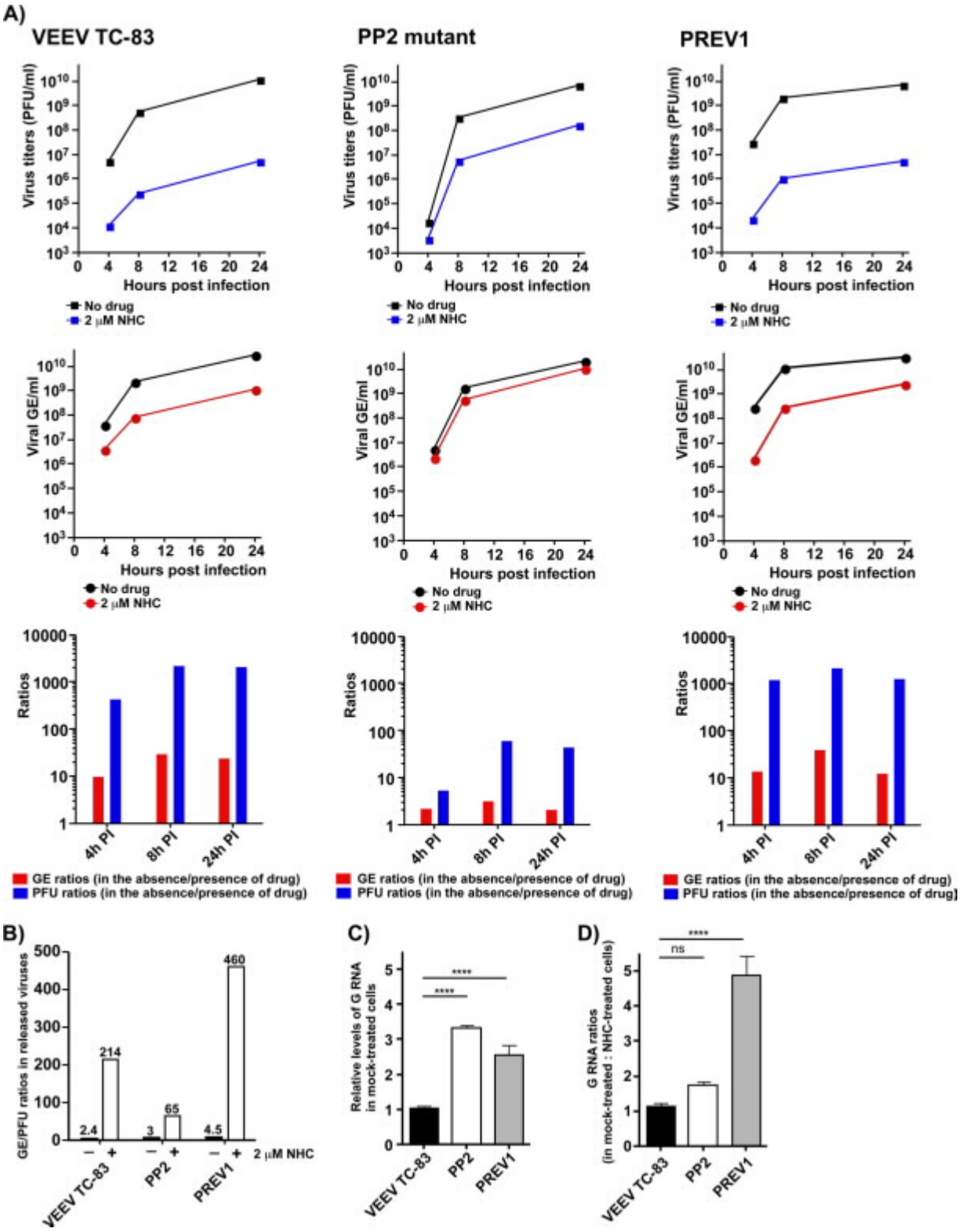


FIG 7 NHC has stronger negative effects on the release and infectivity of VEEV TC-83 and PREV1 particles than those of the PP2 mutant. A total of 5 % 10^5 Vero cells in six-well plates were infected with VEEV TC-83, PP2, or PREV1 at an MOI of 1 PFU/cell and were incubated with or without 2 " M NHC. At the indicated time points, media were collected, and the infectious titers (PFU/ml) and concentrations of genome-containing viral particles (GE/ml) were determined by a plaque assay on Vero cells and RT-qPCR, respectively. Experiments were performed in triplicates. The standard deviations are too small to be visible on the plots. (B) GE/PFU ratios in virus samples harvested at 24 h p.i. of mock-treated and NHC-treated cells in the experiment presented in panel A. (C) Levels of viral G RNAs in the mock-treated cells at 24 h p.i. The cells were harvested after collecting the medium samples described in panel A at 24 h p.i. Data were normalized to the G RNA levels in VEEV TC-83-infected cells. (D) Ratio of G RNAs in mock-treated versus NHC-treated cells at 24 p.i. with the indicated viruses. Cells were collected in the experiment presented in panel A.

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

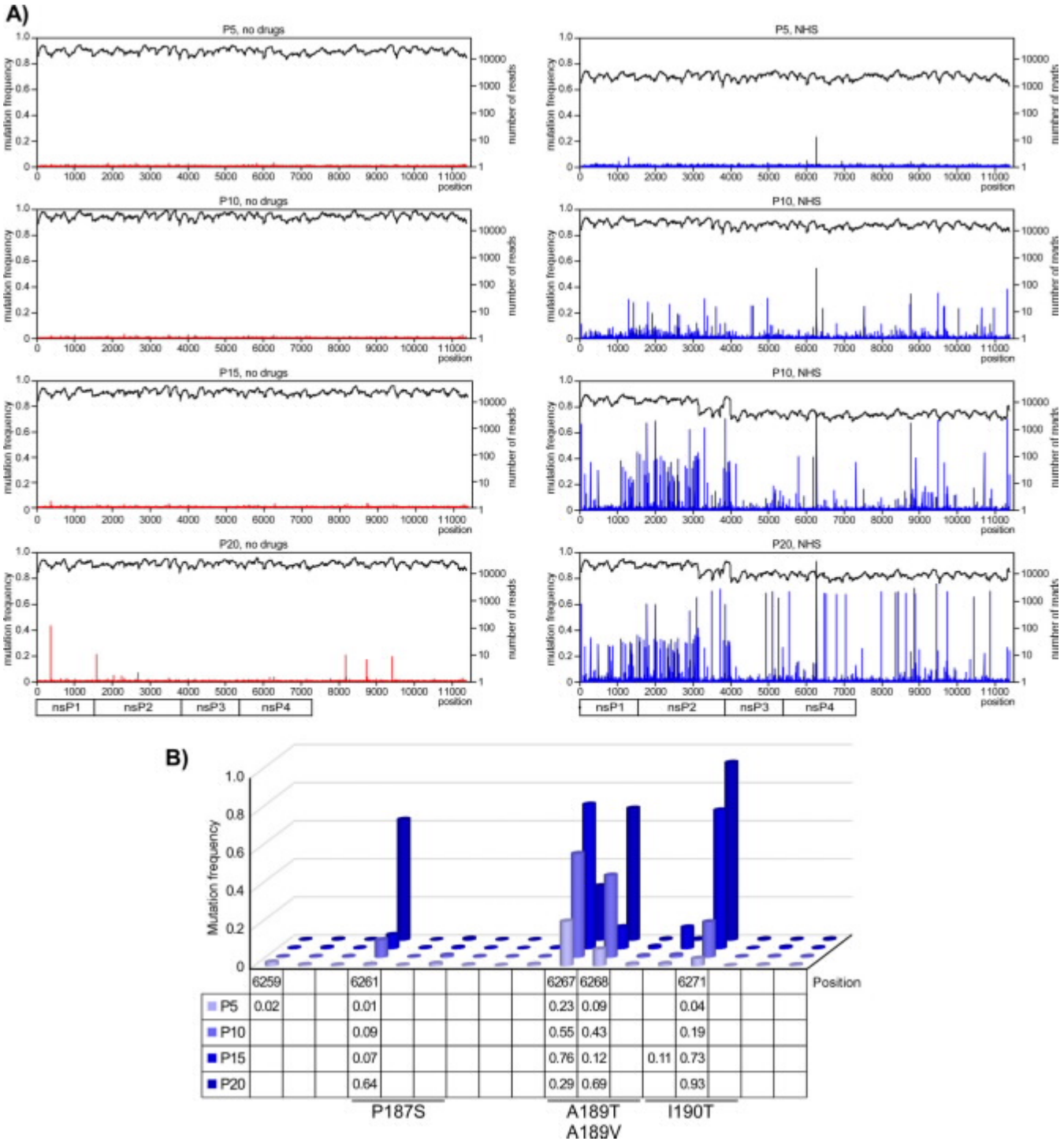


FIG 8 VEEV TC-83 passaging in the presence of increasing concentrations of NHC leads to rapid accumulation of mutations in viral pool. (A) Frequencies of mutations in viral pools at passage 5, 10, 15, and 20 in the absence or presence of NHC, determined by NGS (see Materials and Methods for details). (B) Dynamics of the increase in the frequencies of mutations at the indicated positions of VEEV nsP4.

Further analysis of the NGS data revealed an interesting dynamic in nsP4 evolution (Fig. 8B). The presence of VEEV genomes encoding a nonconservative amino acid substitution I190T steadily increased with virus passaging. After passage 20, the mutation was present in more than 90% of G RNAs. The prevalence of variants with another substitution (A189T) was 23% at passage 5 and steadily increased to 76% by passage 15. However, by passage 20, the A189T mutation was no longer prevalent and

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

was replaced in the virus pool by a combination of two other amino acid substitutions, P187S and A189V. The dynamics of mutation accumulation in the index finger suggested the possibility of its function in regulating nucleotide incorporation by VEEV nsP4.

NHC-resistant VEEV variant continued to rapidly evolve in the absence of NHC. NGS was also applied for analysis of mutation accumulation in the NHC-resistant PP2 plaque isolate during its serial passage in NHC-free media. As in the above-described experiments, we sequenced population of G RNAs isolated from samples of PP2-originated viruses after passages 5, 10, 1, and 20 in drug-free media. Interestingly, in the absence of NHC, the replication complex of PP2 continued to introduce additional mutations, which were equally distributed throughout the viral genome (Fig. 9A). After passage 5, the frequencies of the detected mutations were already higher than those in similarly passaged VEEV TC-83 (Fig. 8A, left panel). Mutations continued to accumulate during the following passages, whereas the viral pools retained all of the PP2-specific mutations. These data correlated with the findings of numerous new mutations in the plaque-purified PREV1 isolate from the passage 20 stock (Fig. 5A).

At passage 20 in drug-free media, only two new prevalent mutations that led to amino acid substitutions in nsP4 (A201V and V308I) were identified (Fig. 9B). Interestingly, in the predicted 3D structure of VEEV nsP4, they were located very close to each other (Fig. 10) and to the mutations found in the NHC-resistant virus population and in the plaque isolates PP1 and PP2. Based on the frequencies of A201V and V308I mutations in the passage 20 viral pool and on the different dynamics of their appearance (Fig. 9B), it is reasonable to expect that they function independently and are present in different viral genomes. Moreover, substitution A201V, but not V308I, was identified in the PREV1 variant (Fig. 5A), which had reverted to an NHC-sensitive phenotype. Therefore, the effects of A201V on loss of resistance to NHC were analyzed further.

The identified nsP4-specific mutations are the determinants of resistance and sensitivity of selected VEEV variants to NHC. To design VEEV variants with mutated nsP4, we used the following information. The I190T mutation in VEEV nsP4 was the most prevalent in the virus pool after 20 passages in the presence of NHC (Fig. 8B). Based on the predicted 3D structure of VEEV nsP4, the second identified mutation, P187S, changed the same amino acid as that previously implicated in ribavirin resistance development by foot-and-mouth disease virus (26). Combination of I190T, P187S, and A189V mutations was identified in both the pool of NHC-resistant mutants (Fig. 8B) and the genomes of plaque-purified variants (Fig. 5). Thus, based on these data, we designed single (VEEV/P187S and VEEV/I190T) and triple (VEEV/3x) mutants (Fig. 11A) and evaluated the effects of the introduced mutations on the ability of VEEV TC-83 to replicate in the presence of NHC.

In the absence of drug, all of the reengineered mutants were viable, replicated at essentially the same rates as VEEV TC-83 (Fig. 11C), and formed large plaques in Vero cells (Fig. 11B). However, in the presence of 1.5 " M NHC in the agarose overlay (Fig. 11B), (i) the parental VEEV TC-83 formed almost undetectable, pinpoint plaques; (ii) the VEEV/P187S mutant, surprisingly, was found to be more sensitive to compound than parental VEEV TC-83 and did not form plaques at all; (iii) the VEEV/I190T mutant produced readily detectable plaques, which remained relatively small; (iv) the combination of all three mutations in nsP4 made the VEEV/3x variant capable of forming midsize plaques in the presence of NHC, which was suggestive of its higher resistance to the drug. To confirm this result, Vero cells were infected with the indicated mutants at an MOI of 2 PFU/cell, and NHC at the indicated concentrations was added to the media at the time of infection. Figure 11D presents the ratios of infectious viral titers produced in the presence or absence of NHC at 8 h p.i. As in the above-described experiments, the VEEV/I190T mutant was more resistant to NHC than the parental VEEV, VEEV/P187S was more sensitive to this compound, and the VEEV/3x mutant was the most resistant to the NHC treatment. The NGS data also suggested that during passaging in the presence of NHC (Fig. 8B), the virus population became

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

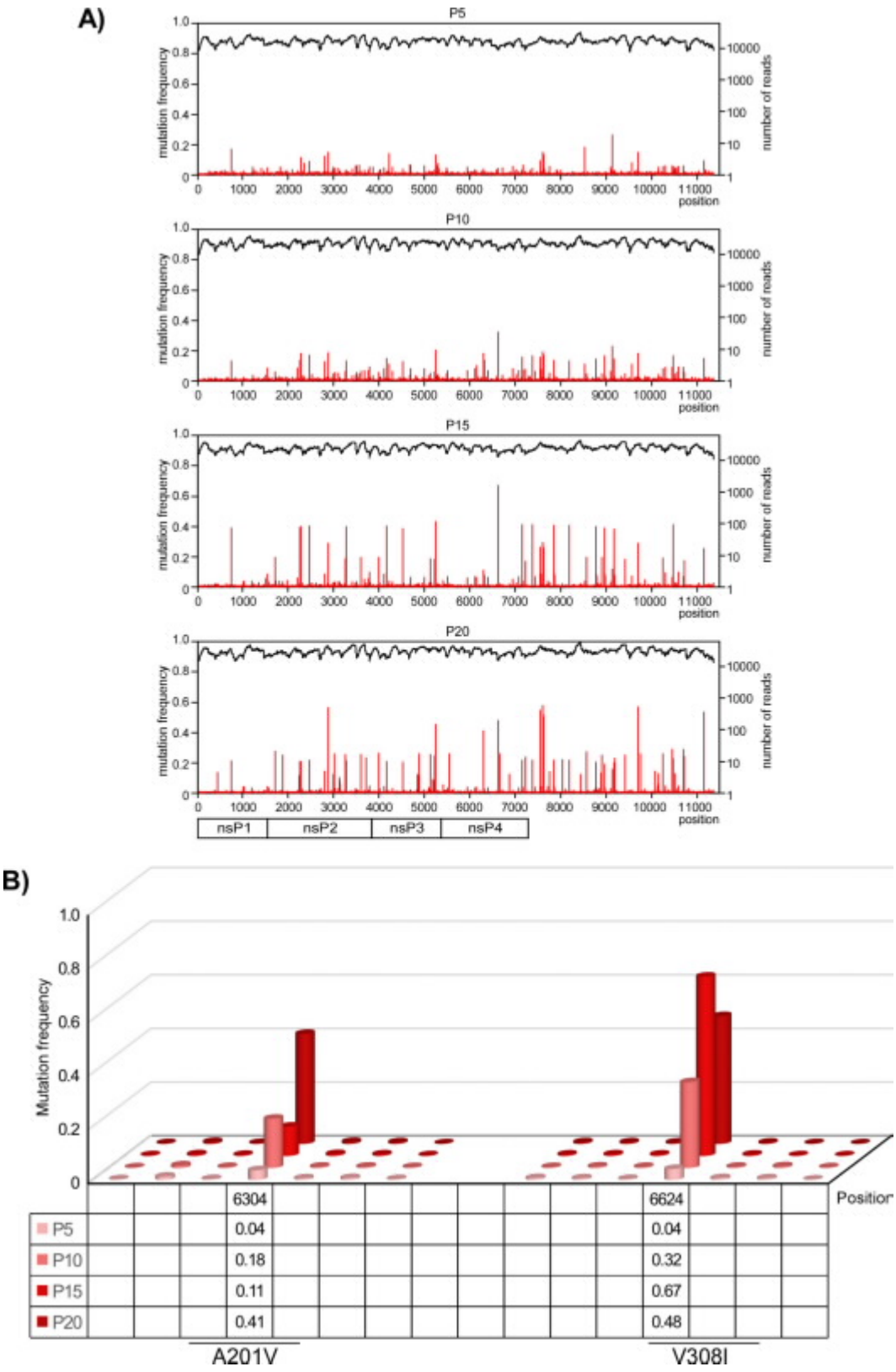


FIG 9 Accumulation of mutations in the genome of plaque-purified, NHC-resistant VEEV isolate PP2 (see Fig. 5) during its serial passaging in NHC-free media. (A) Frequencies of mutations in viral populations at passage 5, 10, 15, and 20, determined by NGS. (B) Dynamics of increase in mutation frequencies at the indicated positions of nsP4.

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

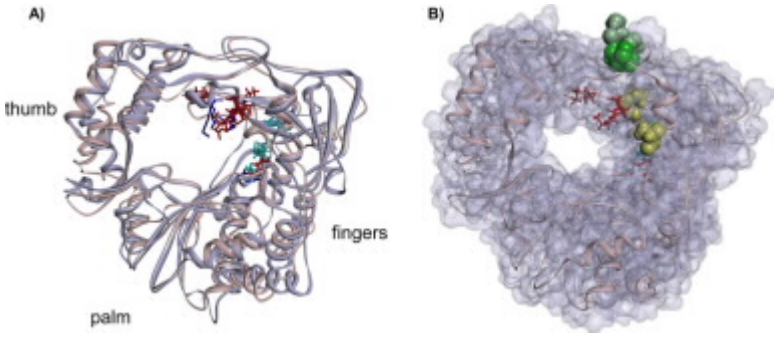


FIG 10 The mutations that lead to NHC-resistant and NHC-sensitive phenotypes of VEEV TC-83 are closely located in the 3D structure of the catalytic domain of VEEV nsP4. (A) Structural alignment of the predicted 3D structure of VEEV nsP4 RdRp domain with FMDV 3D polymerase (2E9T). The VEEV RdRp structure is presented as a solid, light red ribbon. The structure of FMDV 3D protein is presented as a solid, light blue ribbon. The amino acids involved in coordination of NTP phosphates are presented as red sticks (VEEV) or blue sticks (FMDV). The amino acids that bind RNA template are presented as turquoise sticks for both polymerases. (B) Positions of mutations identified in nsP4 of NHC-resistant and NHC-sensitive mutants of VEEV TC-83 on the predicted structure of the catalytic domain. The amino acids that were substituted in the NHC-resistant viruses are shown in medium green (I190) and light green (A189 and P187). The amino acids that were substituted in the pool of PP2 virus passaged 20 times in NHC-free media (A201V and V308I, see Fig. 9 for details) are shown in yellow.

initially enriched with the variants containing A189T mutation in nsP4. During further passaging, the latter mutation was mostly replaced in virus pool by A189V. Therefore, in additional experiments, we compared the effects of both mutations on development of virus resistance to drug. As expected, the ultimate A189V mutation induced significantly higher resistance to NHC than the early A189T mutation in nsP4 (Fig. 11E).

Analysis of the NGS data and sequencing of the PREV1 isolate suggested the possibility that the A201V mutation in nsP4 plays a critical role in reversion of PP2 to an NHC-sensitive phenotype. Indeed, cloning of A201V into the genome of the VEEV/3x mutant (Fig. 12A) had a profound negative effect on viral resistance to NHC. The VEEV/3x/A201V variant replicated in Vero cells to the same titers and at the same rates as the parental VEEV TC-83 and VEEV/3x mutant but was reproducibly less resistant to NHC than both of these viruses (Fig. 12B and C). Thus, the identified A201V mutation in nsP4 was sufficient for reverting the drug-resistant phenotype of VEEV/3x to NHC sensitive.

DISCUSSION

Currently, the most widely used nucleoside analog with the antiviral activity is ribavirin (27, 28). It demonstrates a broad-spectrum inhibitory effect against a number of RNA viruses and low cytotoxicity and functions as an RNA mutagen, since it is incorporated into virus-specific RNAs by viral RdRp (22, 28). This leads to the accumulation of random mutations in viral genomes, which either affect virus replication rates at the next round of infection or make them nonviable (29–31). One of the potential concerns about application of ribavirin is that its antiviral activity only becomes evident when this drug is applied at relatively high concentrations of 100 to 1,000 " M (32) and when it is applied hours before the beginning of infection (33). To detectably inhibit CHIKV replication in vitro, this drug was used at 100 " M (34) and, thus far, no antiviral effect of ribavirin has been unambiguously demonstrated for VEEV (20). Another complication of using ribavirin is that at least some viruses develop drug-resistant mutants (35). This resistance is most often determined by single point mutations in RdRp-coding sequences, and they were intensely investigated in several positive-strand RNA viruses (34, 36).

In the present study, we characterized the antiviral activity of another nucleoside analog, NHC (Fig. 1A). This compound exhibited a very strong inhibitory effect on VEEV infection in cell culture. The antiviral function was readily detectable when NHC was

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

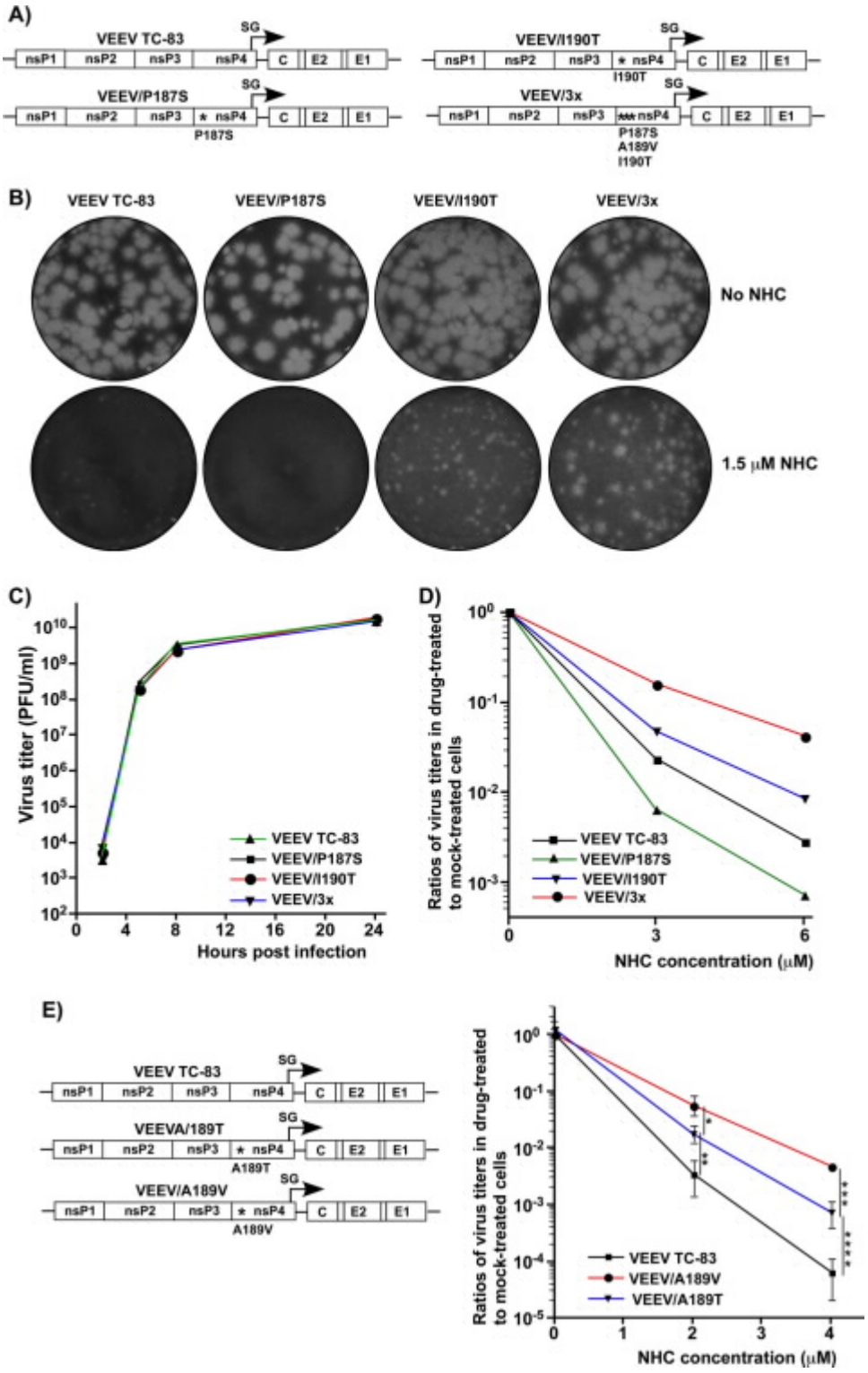


FIG 11 nsP4-specific mutations determine the resistance of VEEV replication to NHC. (A) Schematic presentations of VEEV genomes containing the indicated nsP4-specific mutations. (B) In a standard plaque assay performed on the indicated viruses, Vero cells were overlaid with 0.5% agarose-containing medium either with or without 1.5 " M NHC. After 2 days, plaques were visualized by staining with crystal violet. (C) A total of 4 % 10^5 Vero cells were infected with the indicated viruses at an MOI of 2 PFU/cell. At the indicated times p.i., media were harvested, and viral titers were determined by a plaque assay on Vero cells. (D) A total of 5 % 10^5 Vero cells were infected with the indicated viruses at an MOI of 2 PFU/cell. Cells were incubated with NHC starting from 0 h p.i. At 8 h p.i., media were harvested, and viral titers were determined by a plaque assay on Vero cells. The titers were normalized to those in the samples harvested from mock-treated cells. This experiment was repeated three times using different

(Continued on next page)

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

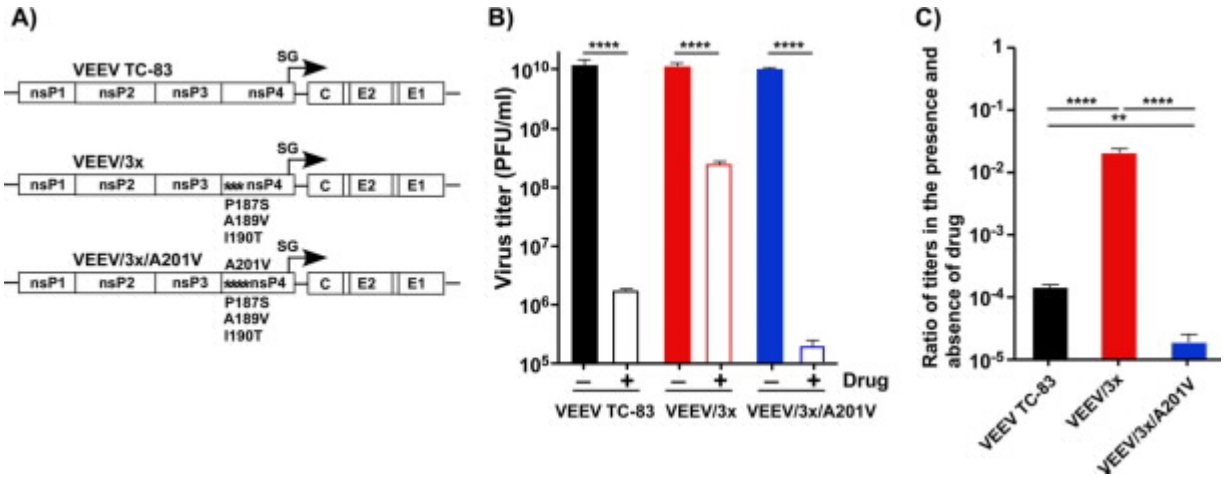


FIG 12 A201V mutation in VEEV nsP4 reverts the NHC-resistant phenotype of the VEEV/3x mutant to drug sensitive. (A) Schematic presentation of VEEV genomes containing the indicated nsP4 mutations. (B) Vero cells were infected with indicated mutant viruses at an MOI of 1 PFU/cell. Cells were incubated in the presence of 2 " M NHC starting from 0 h p.i. At 8 h p.i., media were harvested, and viral titers were determined by a plaque assay on Vero cells. (C) Titers in samples from NHC-treated cells were normalized to those in samples from mock-treated cells. This experiment was repeated three times using different concentrations of drug with consistent results. The data from one of the representative experiments are presented in this figure.

applied at 1 to 2 " M prior to, at the time of, or within 4 h after VEEV infection (Fig. 2). Previous studies showed that in cultured cells, NHC is rapidly converted into mono-, di-, and triphosphate forms, along with other metabolites, such as CTP and UTP (22, 25, 37). Because the levels of the NHC-triphosphate form (NHC-TP) were highest among the intracellular metabolites, it was proposed as the metabolite primarily responsible for the antiviral effect of NHC (22). In vitro experiments using purified HCV NS5B polymerase demonstrated that NHC-TP can serve as a substrate with a low efficiency of incorporation (22). Because NHC-TP can be incorporated into RNA to replace either CTP or UTP (38), one would expect the appearance of C-to-U and U-to-C transition mutations in synthesized viral G RNAs if NHC-TP is introduced into G RNA during positive-strand synthesis and A-to-G and G-to-A mutations if NHC-MP is incorporated during synthesis of the negative strand of the viral genome.

Our data support a notion that the primary antiviral effect of NHC is based on its potent RNA-mutagenic activity, which can force VEEV replication into an “error catastrophe” or “lethal defection” (39). Accordingly, viral pools released from Vero cells treated with NHC starting at early times p.i. demonstrated GE/PFU ratios that were almost 2 orders of magnitude higher than those from mock-treated cells. This was an indication that most of the released virions contained noninfectious viral genomes that were incapable of producing a spreading infection. The released viral pools also produced heterogeneous plaques, with a high proportion of these plaques being small and pinpoint in size. A $ 10-fold increase in the VEEV genome mutation rate was detected in the presence of 2 " M NHC compared to drug-free media. In agreement with these data, both NGS of viral pools and Sanger sequencing of the genomes in randomly selected plaque-purified variants also demonstrated that NHC treatment during VEEV replication resulted in the generation of large numbers of synonymous and nonsynonymous mutations in all of the nonstructural and structural viral genes.

On the other hand, in the presence of NHC, infected cells produced 10- to 20-fold

FIG 11 Legend (Continued)
concentrations of drug with consistent results. The data from one of the representative experiments are presented in this figure. (E) A total of 5 % 10^5 Vero cells were infected with the indicated viruses at an MOI of 2 PFU/cell. Cells were incubated with NHC starting from 0 h p.i. At 20 h p.i., media were harvested, and viral titers were determined by a plaque assay on Vero cells. Titers were normalized to those in the samples harvested from mock-treated cells.

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

fewer G RNA-containing viral particles (GE). We detected only a small decrease in G RNA accumulation in the cells treated with NHC compared to the mock-treated cells (Fig. 7D). Therefore, there is some possibility that similar to favipiravir (40), NHC can function as a direct, weak inhibitor of RdRp. However, another, more plausible explanation for less efficient release of G RNA-containing virions is that during intracellular viral replication, NHC-TP is responsible for accumulation of mutations in nonstructural and structural proteins rather than for direct inhibition of RNA synthesis. Similarly, prior in vitro studies using HCV RdRp did not find evidence that the presence of NHC could lead to direct inhibition of the polymerization reaction. It has also been proposed that the incorporation of NHC-TP may change the thermodynamics of RNA secondary structures and thereby affect the initiation of replication and/or protein translation (22). Although this is a considerable possibility for VEEV RdRp, such an assumption needs experimental support.

The anti-VEEV effect of NHC is dramatically more prominent when it is applied a few hours before or within the first 4 h p.i. There are two possible explanations for such temporal dependence of the inhibitory effect. First, the immature replication complex, which is composed of P123 and nsP4, is only present within the first 4 h p.i., at the amplification stage of viral RNA. In this complex, RdRp might utilize NHC-TP more efficiently than in a mature RC that contains completely processed, individual nsPs. The second and more likely possibility is that during the early stage of replication, when G RNA undergoes multiple rounds of amplification, incorporation of NHC-MP into both positive- or negative-strand RNAs generates large numbers of mutations in the viral progeny. Moreover, early in the infection, the synthesized mutated G RNAs are also translated into defective nsPs, and this in turn affects VEEV replication rates and the efficiency of particle release.

Another major characteristic of NHC is that the development of resistance to this compound by VEEV is a highly inefficient process. The difficulty in selection and the poor resistance of selected mutants suggested that (i) the acquisition of a single mutation in nsP4 has a relatively small positive effect on virus resistance to NHC and (ii) the accumulation of a few mutations appears to be required for advanced resistance to the drug. In two independent selections, one of which is presented in this study, the low-level resistance to NHC only became evident after 15 passages.

Further experiments clearly demonstrated that this low-level resistance is determined by a combination of nsP4-specific mutations. The NGS of G RNAs in viral pools at different passages, sequencing of the genomes of plaque-purified viruses and subsequent reverse genetics experiments demonstrated that resistance to NHC resulted from sequential selection of several mutations in VEEV nsP4. Acquisition of I190T substitution was likely the most critical step. It was detected as early as passage 5 (4% frequency) and became present in almost 100% of the viral genomes by passage 20 (in the presence of NHC). Moreover, the same mutation was prevalent in viral genomes after the second independent selection of NHC-resistant variants (data not shown). Although I190T substitution alone caused detectable resistance of VEEV to NHC (Fig. 11), the ultimately developed triple mutant contained a combination of P187S, A189V, and I190T substitutions in nsP4. Their presence made virus replication clearly more resistant to the drug (Fig. 11). Based on a predicted structural model of VEEV nsP4 RdRp (Fig. 10), all three mutated amino acids were located in the RdRp index finger domain near the template entrance channel. Other research teams, using a variety of picornaviruses, have previously identified several RdRp-specific mutations that generate resistance to different nucleoside analogs (32, 40–42). However, only the P44S substitution in the RdRp of foot-and-mouth disease virus (FMDV) appeared in the same position as P187S in the structural model of VEEV-specific RdRp (26). The indicated FMDV-specific mutation has been proposed to amplify the resistance of this virus to ribavirin. The authors of that study suggested that the substitution of P44S increased the frequency of ribavirin incorporation instead of guanosine by affecting the shape of the template channel and the template transition rates through the active site. This in turn resulted in a

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

detectable reduction of the FMDV mutant replication rates. The NHC-resistant PP2 variant selected in our study also demonstrated lower replication rates than both the original VEEV TC-83 and the drug-sensitive pseudorevertant PREV1. This suggests that less efficient RNA replication may be an important contributor to VEEV resistance to the compound. This assumption needs more detailed investigation because, in the case of PP2, the lower rate of viral replication was likely determined by a synergistic effect of numerous mutations accumulated in viral nsPs. Importantly, upon the withdrawal of NHC, the selected PP2 mutant further evolved to a more efficiently replicating phenotype. It was also acquiring mutations dramatically more efficiently than the original VEEV TC-83 in the absence of drug. At this stage, we cannot definitively distinguish whether the higher evolution rates of PP2 in the absence of NHC were the direct result of accumulation of previous mutations in nsP4 or its higher replication rates. However, the more efficiently replicating phenotype of PREV1 correlated with a complete loss of NHC resistance, and it became even more sensitive to this compound than the original VEEV TC-83.

Based on the results of NGS, after 20 passages of the NHC-resistant PP2 isolate in NHC-free media, the resulting viral pool retained all of the original PP2-specific mutations and also acquired A201V and V308I substitutions in nsP4. Based on their frequencies and the presence of only the A201V substitution in PREV1-specific nsP4, we hypothesized that A201V and V308I substitutions were likely encoded by different genomes rather than present together. Spatially, they are located close to each other and to the mutations leading to NHC resistance (Fig. 10), suggesting their possible function in both increasing the viral replication rates and lowering the resistance levels to the drug. Indeed, the addition of a single A201V-encoding mutation into the genome of NHC-resistant triple mutant (VEEV/3x) resulted in reversion to a drug-sensitive phenotype. Interestingly, in a predicted model of the nsP4 RdRp, A201 in the F motif is located next to K200, which, based on a structural alignment with FMDV 3D polymerase, may interact with the RNA template (43). Therefore, this mutation may compensate for the defect caused by a triple substitution in the index finger domain. Thus, the amino acid substitutions that lead to NHC resistance and its reversion possibly affect the interaction of template RNA with VEEV-specific RdRp.

In summary, this study demonstrated several key features of NHC. (i) NHC is a very potent anti-VEEV compound. (ii) NHC induces high mutation rates in the genome of replicating VEEV. (iii) NHC application has negative effects on both the release of viral particles and their infectivity. (iv) The antiviral effect of NHC is most prominent when it is applied at early times postinfection, during the rapid amplification of negative and positive-strand RNAs and assembly of replication complexes. (v) VEEV resistance to NHC develops very inefficiently. Even a low-level resistance requires acquisition of more than one mutation in VEEV nsP4. (vi) The development of such mutations correlates with lower replication rates of the virus. (vii) In the absence of NHC, the resistant mutants of VEEV revert to an NHC-sensitive phenotype and higher replication rates by acquiring additional mutations in nsP4. Taken together, our data suggest that NHC is a very promising and potent anti-VEEV agent that can likely also be used against other alphavirus infections.

MATERIALS AND METHODS

NHC was synthesized at Emory Institute for Drug Development. Its synthesis and additional characterization will be presented elsewhere.

Cell cultures. Vero cells were obtained from the American Type Culture Collection (Manassas, VA). BHK-21 cells were kindly provided by Paul Olivo (Washington University, St. Louis, MO). These cell lines were maintained at 37°C in alpha minimum essential medium supplemented with 10% fetal bovine serum and vitamins.

Plasmid constructs. The original plasmids containing the infectious cDNAs of VEEV TC-83 were described elsewhere (44). Mutations were introduced into nsP4 using standard PCR-based techniques. Schematic representations of all of the modified genomes are presented in the corresponding figures. The sequences of the plasmids and details regarding the cloning procedures can be provided upon request.

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

In vitro RNA transcription and transfection. Plasmids with the VEEV TC-83 genome and its derivatives were purified by ultracentrifugation in CsCl gradients. They were then linearized using a MluI restriction site located downstream of the poly(A) sequence. RNAs were synthesized by SP6 RNA polymerase in the presence of a cap analog, as described elsewhere (45). Electroporation of BHK-21 cells by in vitro-synthesized RNA was performed under previously described conditions (45, 46). In all of the experiments, viral titers were determined by a plaque assay on Vero cells as described elsewhere (47).

Selection of VEEV TC-83 mutants. A total of 2.5 % 10^6 Vero cells in 100-mm dishes were infected at an MOI of 1 PFU/cell with either original, electroporation-derived VEEV TC-83 or stocks harvested at the prior passage. The starting concentration of NHC was 1 " M, and it was gradually increased by passage 20 to 3.2 " M. Viral stocks were harvested at each passage after developing signs of a CPE, either at 24 or 48 h p.i. After the final passage, the plaques were randomly selected from the agarose cover, supplemented with 2 " M NHC. Viruses from two agarose plugs (PP1 and PP2) were extracted and used to infect Vero cells in the presence of 1 " M NHC to avoid the possible selection of true and pseudorevertants. At 24 h p.i., cells were harvested for RNA isolation and sequencing of viral genomes, and media were used as stocks for further analysis of viral isolates. One of the plaque-purified variants, PP2, was additionally passaged 20 times in Vero cells in the absence of NHC. During this serial passage, the volume of inoculum harvested from the previous passage was being gradually decreased from 100 to 0.01 " l. After the last passage, the viral population was homogeneous in terms of plaque size. One of the plaques (PREV1) was selected, and the extracted virus was used to infect Vero cells. Harvested medium was used for further experiments, and RNA from the infected cells was used for sequencing of viral nsP genes. Passaging of the original VEEV TC-83 in the absence of NHC was performed in parallel under the same conditions. After passage 20, one of plaques was also isolated, and the nsP-coding sequence was sequenced. For all isolates, DNA fragments were synthesized using RT-PCR and sequenced in the UAB Heflin Center Genomics Core Facility.

RT-qPCR. The viral RNA was isolated using ZR viral RNA kit (Zymo Research) from 100-" l portions of medium samples harvested at different time points p.i. with the indicated viruses grown in the absence or presence of NHC. As an internal control for normalization of the data, 10 " l of media containing the same amount of Sindbis virus (SINV) was added to each sample before RNA purification. VEEV- and SINV-specific cDNAs were synthesized using a QuantiTect reverse transcription kit (Qiagen) and further used for quantitative PCR (qPCR) analysis using VEEV and SINV nsP2-specific primers. The qPCRs were performed in triplicates using SsoFast EvaGreen Supermix (Bio-Rad) and a CFX96 real-time PCR detection system (Bio-Rad). The specificities of the products were tested by measuring their melting temperatures, and the data were normalized to the mean threshold cycle of the SINV control in each sample. Averages from three reactions and standard deviations were calculated and plotted on the graphs. Total cellular RNAs were isolated from infected Vero cells using a ZR viral RNA kit. cDNAs synthesis and qPCR analyses were performed using the above-described protocols, and the data were normalized to the concentrations of 18S rRNA present in the same samples.

NGS. Viral G RNAs were isolated from 0.6-ml portions of viral stocks indicated in the corresponding figures using ZR viral RNA or Direct-Zol RNA Mini Prep kits (Zymo Research). Purified viral RNAs were used to prepare NGS libraries using an Agilent SureSelect strand-specific mRNA library kit according to the manufacturer's instructions (Agilent, Santa Clara, CA), except that poly(A) selection was omitted. Briefly, 40-ng portions of viral RNAs were randomly fragmented with cations and heat, followed by the first-strand cDNA synthesis using random primers with the inclusion of actinomycin D (2.4 ng/" l final concentration). Second-strand cDNA was synthesized using standard techniques for stranded libraries. Adaptors were ligated for indexing to allow for multiplexing during sequencing. The size selection and purification of the libraries was performed using the Agilent 2100 bioanalyzer. The average amplicon length was 400 bp. Prior to cluster generation, the cDNA libraries were quantitated using qPCR in a Roche LightCycler 480 with a Kapa Biosystems kit for Illumina library quantitation (Kapa Biosystems, Woburn, MA). The 300-bp paired-end sequencing was performed on a MiSeq Illumina machine in the UAB Heflin Center Genomics Core Facility.

Illumina BaseSpace was used for base-calling and read demultiplexing. Trim Galore (v0.4.1) was used to remove adaptor contaminants and reads shorter than 15 bp and to trim low-quality bases (Q-score # 30). The high-quality, trimmed, paired-end fastq reads were them merged into one single-end read fastq file. The single-end trimmed fastq reads were then aligned using BWA-MEM (v0.7.15-r1140) to the VEEV reference genome in order to create the aligned SAM file. Naive Variant Caller (Galaxy, v0.0.2) was used to generate a variant file from the sorted BAM file on all positions of the VEEV genome. To create the count lists containing the number of bases seen at each position, Variant Annotator (Galaxy, v1.2) was used on the Naive Variant Caller's vcf file. The list was further processed in Excel to account for all possible mutations at each position and to generate figures. Table 1 presents the total and aligned read numbers.

Analysis of viral replication. A total of 5 % 10^5 Vero cells were seeded into six-well Costar plates and infected at MOIs indicated in the figure legends. NHC was added to the cells at the indicated times, media were harvested, and viral titers in the samples were determined by a plaque assay on Vero cells as described elsewhere (47).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 7 software. The two-way analysis of variance (ANOVA) with the Tukey correction was used for statistical analysis of virus replication rates. The one-way ANOVA with the Dunnett's correction was used for statistical analysis of infectious virus titers and concentrations of GE. The data are presented as means & the standard

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

TABLE 1 Number of total and mapped reads

Sample	Virus	Treatment	Passage	Total no. of raw reads	No. (%) mapped reads to the genome
W0	VEEV TC-83	None	0	1,694,499	1,473,387 (86.95)
W5	VEEV TC-83	None	5	1,932,410	1,320,488 (68.33)
W10	VEEV TC-83	None	10	3,055,210	2,575,881 (84.31)
W15	VEEV TC-83	None	15	2,436,142	1,720,526 (70.63)
W20	VEEV TC-83	None	20	2,239,203	1,697,248 (75.80)
d5	VEEV TC-83	NHC	5	1,583,429	168,026 (10.61)
d10	VEEV TC-83	NHC	10	2,748,574	1,291,652 (46.99)
d15	VEEV TC-83	NHC	15	1,312,641	456,518 (34.78)
d20	VEEV TC-83	NHC	20	2,102,043	949,366 (45.16)
r5	VEEV TC-83, PP2	None	5	1,588,152	1,171,668 (73.78)
r10	VEEV TC-83, PP2	None	10	1,893,413	1,699,941 (89.78)
r15	VEEV TC-83, PP2	None	15	2,059,806	1,691,743 (82.13)
r20	VEEV TC-83, PP2	None	20	2,438,228	2,170,0771 (89.03)

deviations. The differences with P $ 0.05 were considered nonsignificant (ns). P values of # 0.05, # 0.01, 0.001, and 0.0001 are indicated by asterisks (*, **, ***, and ****, respectively).

Accession number(s). The RNA-seq data have been deposited in the NCBI Gene Expression Omnibus under accession number GSE106752.

ACKNOWLEDGMENTS

We thank Maryna Akhrymuk for technical assistance.

This study was supported by Defense Threat Reduction Agency contract HDTRA1-15-C-0075 to G.R.P. and Public Health Service grants AI118867 to E.I.F. and AI095449 to I.F.

REFERENCES

1. Strauss JH, Strauss EG. 1994. The alphaviruses: gene expression, replication, evolution. Microbiol Rev 58:491–562.
2. Zacks MA, Paessler S. 2010. Encephalitic alphaviruses. Vet Microbiol 140:281–286. https://doi.org/10.1016/j.vetmic.2009.08.023.
3. Weaver SC, Ferro C, Barrera R, Boshell J, Navarro JC. 2004. Venezuelan equine encephalitis. Annu Rev Entomol 49:141–174. https://doi.org/10.1146/annurev.ento.49.061802.123422.
4. Gibbs EP. 1976. Equine viral encephalitis. Equine Vet J 8:66–71. https://doi.org/10.1111/j.2042-3306.1976.tb03293.x.
5. Gibney KB, Robinson S, Mutebi JP, Hoenig DE, Bernier BJ, Webber L, Lubelczyk C, Nett RJ, Fischer M. 2011. Eastern equine encephalitis: an emerging arboviral disease threat, Maine, 2009. Vector Borne Zoonotic Dis 11:637–639. https://doi.org/10.1089/vbz.2010.0189.
6. Go YY, Balasuriya UB, Lee CK. 2014. Zoonotic encephalitides caused by arboviruses: transmission and epidemiology of alphaviruses and flaviviruses. Clin Exp Vaccine Res 3:58–77. https://doi.org/10.7774/cevr.2014.3.1.58.
7. Weaver SC. 2001. Venezuelan equine encephalitis, p 539–548. In Service MW (ed), The encyclopedia of arthropod-transmitted infections. CAB International, Wallingford, UK.
8. Weaver SC. 2001. Eastern equine encephalitis, p 151–159. In Service MW (ed), The encyclopedia of arthropod-transmitted infections. CAB International, Wallingford, UK.
9. Reisen WK. 2001. Western equine encephalitis, p 558–563. In Service MW (ed), The encyclopedia of arthropod-transmitted infections. CAB International, Wallingford, UK.
10. Reed DS, Larsen T, Sullivan LJ, Lind CM, Lackemeyer MG, Pratt WD, Parker MD. 2005. Aerosol exposure to western equine encephalitis virus causes fever and encephalitis in cynomolgus macaques. J Infect Dis 192:1173–1182. https://doi.org/10.1086/444397.
11. Lemm JA, Rice CM. 1993. Roles of nonstructural polyproteins and cleavage products in regulating Sindbis virus RNA replication and transcription. J Virol 67:1916–1926.
12. Shirako Y, Strauss JH. 1994. Regulation of Sindbis virus RNA replication: uncleaved P123 and nsP4 function in minus strand RNA synthesis whereas cleaved products from P123 are required for efficient plus strand RNA synthesis. J Virol 185:1874–1885.
13. Gorchakov R, Frolova E, Sawicki S, Atasheva S, Sawicki D, Frolov I. 2008. A new role for ns polyprotein cleavage in Sindbis virus replication. J Virol 82:6218–6231. https://doi.org/10.1128/JVI.02624-07.
14. Chung DH, Jonsson CB, Tower NA, Chu YK, Sahin E, Golden JE, Noah JW, Schroeder CE, Sotsky JB, Sosa MI, Cramer DE, McKellip SN, Rasmussen L, White EL, Schmaljohn CS, Julander JG, Smith JM, Filone CM, Connor JH, Sakurai Y, Davey RA. 2014. Discovery of a novel compound with anti-Venezuelan equine encephalitis virus activity that targets the nonstructural protein 2. PLoS Pathog 10:e1004213. https://doi.org/10.1371/journal.ppat.1004213.
15. Schroeder CE, Yao T, Sotsky J, Smith RA, Roy S, Chu YK, Guo H, Tower NA, Noah JW, McKellip S, Sosa M, Rasmussen L, Smith LH, White EL, Aube J, Jonsson CB, Chung D, Golden JE. 2014. Development of (E)-2-((1,4-dimethylpiperazin-2-ylidene)amino)-5-nitro- N-phenylbenzamide, ML336: novel 2-amidinophenylbenzamides as potent inhibitors of Venezuelan equine encephalitis virus. J Med Chem 57:8608–8621. https://doi.org/10.1021/jm501203v.
16. Selvam P, Vijayalakshimi P, Smee DF, Gowen BB, Julander JG, Day CW, Barnard DL. 2007. Novel 3-sulphonamido-quinazolin-4(3H)-one derivatives: microwave-assisted synthesis and evaluation of antiviral activities against respiratory and biodefense viruses. Antivir Chem Chemother 18:301–305. https://doi.org/10.1177/095632020701800506.
17. Kehn-Hall K, Narayanan A, Lundberg L, Sampey G, Pinkham C, Guendel I, Van Duyne R, Senina S, Schultz KL, Stavale E, Aman MJ, Bailey C, Kashanchi F. 2012. Modulation of GSK-3! activity in Venezuelan equine encephalitis virus infection. PLoS One 7:e34761. https://doi.org/10.1371/journal.pone.0034761.
18. Madsen C, Hooper I, Lundberg L, Shafagati N, Johnson A, Senina S, de la Fuente C, Hoover LI, Fredricksen BL, Dinman J, Jacobs JL, Kehn-Hall K. 2014. Small molecule inhibitors of Ago2 decrease Venezuelan equine encephalitis virus replication. Antiviral Res 112:26–37. https://doi.org/10.1016/j.antiviral.2014.10.002.
19. Julander JG, Bowen RA, Rao JR, Day C, Shafer K, Smee DF, Morrey JD, Chu CK. 2008. Treatment of Venezuelan equine encephalitis virus infection with (")-carbodine. Antiviral Res 80:309–315. https://doi.org/10.1016/j.antiviral.2008.07.002.
20. Markland W, McQuaid TJ, Jain J, Kwong AD. 2000. Broad-spectrum antiviral activity of the IMP dehydrogenase inhibitor VX-497: a comparison with ribavirin and demonstration of antiviral additivity with alpha

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

interferon. Antimicrob Agents Chemother 44:859–866. https://doi.org/10.1128/AAC.44.4.859-866.2000.
21. Lundberg L, Pinkham C, de la Fuente C, Brahms A, Shafagati N, Wagstaff KM, Jans DA, Tamir S, Kehn-Hall K. 2016. Selective inhibitor of nuclear export (SINE) compounds alter New World alphavirus capsid localization and reduce viral replication in mammalian cells. PLoS Negl Trop Dis 10:e0005122. https://doi.org/10.1371/journal.pntd.0005122.
22. Stuyver LJ, Whitaker T, McBrayer TR, Hernandez-Santiago BI, Lostia S, Tharnish PM, Ramesh M, Chu CK, Jordan R, Shi J, Rachakonda S, Watanabe KA, Otto MJ, Schinazi RF. 2003. Ribonucleoside analogue that blocks replication of bovine viral diarrhea and hepatitis C viruses in culture. Antimicrob Agents Chemother 47:244–254. https://doi.org/10.1128/AAC.47.1.244-254.2003.
23. Costantini VP, Whitaker T, Barclay L, Lee D, McBrayer TR, Schinazi RF, Vinje J. 2012. Antiviral activity of nucleoside analogues against norovirus. Antivir Ther 17:981–991. https://doi.org/10.3851/IMP2229.
24. Pyrc K, Bosch BJ, Berkhout B, Jebbink MF, Dijkman R, Rottier P, van der Hoek L. 2006. Inhibition of human coronavirus NL63 infection at early stages of the replication cycle. Antimicrob Agents Chemother 50:2000–2008. https://doi.org/10.1128/AAC.01598-05.
25. Ehteshami M, Tao S, Zandi K, Hsiao HM, Jiang Y, Hammond E, Amblard F, Russell OO, Merits A, Schinazi RF. 2017. Characterization of β-D-N4-hydroxycytidine as a novel inhibitor of chikungunya virus. Antimicrob Agents Chemother 61:e02395-16. https://doi.org/10.1128/AAC.02395-16.
26. Agudo R, Ferrer-Orta C, Arias A, de la Higuera I, Perales C, Perez-Luque R, Verdaguer N, Domingo E. 2010. A multi-step process of viral adaptation to a mutagenic nucleoside analogue by modulation of transition types leads to extinction-escape. PLoS Pathog 6:e1001072. https://doi.org/10.1371/journal.ppat.1001072.
27. Beaucourt S, Vignuzzi M. 2014. Ribavirin: a drug active against many viruses with multiple effects on virus replication and propagation: molecular basis of ribavirin resistance. Curr Opin Virol 8:10–15. https://doi.org/10.1016/j.coviro.2014.04.011.
28. Freistadt MS, Meades GD, Cameron CE. 2004. Lethal mutagens: broad-spectrum antivirals with limited potential for development of resistance? Drug Resist Updat 7:19–24. https://doi.org/10.1016/j.drup.2003.12.003.
29. Crotty S, Maag D, Arnold JJ, Zhong W, Lau JY, Hong Z, Andino R, Cameron CE. 2000. The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. Nat Med 6:1375–1379. https://doi.org/10.1038/82191.
30. Crotty S, Cameron C, Andino R. 2002. Ribavirin's antiviral mechanism of action: lethal mutagenesis? J Mol Med 80:86–95. https://doi.org/10.1007/s00109-001-0308-0.
31. Crotty S, Gohara D, Gilligan DK, Karelsky S, Cameron CE, Andino R. 2003. Manganese-dependent polioviruses caused by mutations within the viral polymerase. J Virol 77:5378–5388. https://doi.org/10.1128/JVI.77.9.5378-5388.2003.
32. Pfeiffer JK, Kirkegaard K. 2003. A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleotide analogs via increased fidelity. Proc Natl Acad Sci U S A 100:7289–7294. https://doi.org/10.1073/pnas.1232294100.
33. Crotty S, Cameron CE, Andino R. 2001. RNA virus error catastrophe: direct molecular test by using ribavirin. Proc Natl Acad Sci U S A 98:6895–6900. https://doi.org/10.1073/pnas.111085598.
34. Coffey LL, Beeharry Y, Borderia AV, Blanc H, Vignuzzi M. 2011. Arbovirus high fidelity variant loses fitness in mosquitoes and mice. Proc Natl Acad Sci U S A 108:16038–16043. https://doi.org/10.1073/pnas.1111650108.
35. Vignuzzi M, Stone JK, Andino R. 2005. Ribavirin and lethal mutagenesis of poliovirus: molecular mechanisms, resistance, and biological implications. Virus Res 107:173–181. https://doi.org/10.1016/j.virusres.2004.11.007.
36. Ferrer-Orta C, Sierra M, Agudo R, de la Higuera I, Arias A, Perez-Luque R, Escarmis C, Domingo E, Verdaguer N. 2010. Structure of foot-and-mouth disease virus mutant polymerases with reduced sensitivity to ribavirin. J Virol 84:6188–6199. https://doi.org/10.1128/JVI.02420-09.
37. Hernandez-Santiago BI, Beltran T, Stuyver L, Chu CK, Schinazi RF. 2004. Metabolism of the anti-hepatitis C virus nucleoside β-D-N4-hydroxycytidine in different liver cells. Antimicrob Agents Chemother 48:4636–4642. https://doi.org/10.1128/AAC.48.12.4636-4642.2004
38. Les A, Adamowicz L, Rode W. 1993. Structure and conformation of N^4-hydroxycytosine and N^4-hydroxy-5-fluorocytosine: a theoretical ab initio study. Biochim Biophys Acta 1173:39–48. https://doi.org/10.1016/0167-4781(93)90240-E.
39. Tejero H, Montero F, Nuno JC. 2016. Theories of lethal mutagenesis: from error catastrophe to lethal defection. Curr Top Microbiol Immunol 392:161–179. https://doi.org/10.1007/82_2015_463.
40. Delang L, Segura Guerrero N, Tas A, Querat G, Pastorino B, Froeyen M, Dallmeier K, Jochmans D, Herdewijn P, Bello F, Snijder EJ, de Lamballerie X, Martina B, Neyts J, van Hemert MJ, Leyssen P. 2014. Mutations in the chikungunya virus nonstructural proteins cause resistance to favipiravir (T-705), a broad-spectrum antiviral. J Antimicrob Chemother 69:2770–2784. https://doi.org/10.1093/jac/dku209.
41. Kempf BJ, Peersen OB, Barton DJ. 2016. Poliovirus polymerase Leu420 facilitates RNA recombination and ribavirin resistance. J Virol 90:8410–8421. https://doi.org/10.1128/JVI.00078-16.
42. Sierra M, Airaksinen A, Gonzalez-Lopez C, Agudo R, Arias A, Domingo E. 2007. Foot-and-mouth disease virus mutant with decreased sensitivity to ribavirin: implications for error catastrophe. J Virol 81:2012–2024. https://doi.org/10.1128/JVI.01606-06.
43. Ferrer-Orta C, Arias A, Perez-Luque R, Escarmis C, Domingo E, Verdaguer N. 2007. Sequential structures provide insights into the fidelity of RNA replication. Proc Natl Acad Sci U S A 104:9463–9468. https://doi.org/10.1073/pnas.0700518104.
44. Michel G, Petrakova O, Atasheva S, Frolov I. 2007. Adaptation of Venezuelan equine encephalitis virus lacking 51-nt conserved sequence element to replication in mammalian and mosquito cells. Virology 362:475–487. https://doi.org/10.1016/j.virol.2007.01.009.
45. Gorchakov R, Hardy R, Rice CM, Frolov I. 2004. Selection of functional 5= cis-acting elements promoting efficient Sindbis virus genome replication. J Virol 78:61–75. https://doi.org/10.1128/JVI.78.1.61-75.2004.
46. Liljeström P, Lusa S, Huylebroeck D, Garoff H. 1991. In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. J Virol 65:4107–4113.
47. Volkova E, Frolova E, Darwin JR, Forrester NL, Weaver SC, Frolov I. 2008. IRES-dependent replication of Venezuelan equine encephalitis virus makes it highly attenuated and incapable of replicating in mosquito cells. Virology 377:160–169. https://doi.org/10.1016/j.virol.2008.04.020.

Downloaded from http://jvi.asm.org/ on March 25, 2019 by guest

Application #:

Principal Investigator(s):

OVERALL IMPACT

Reviewers will provide an overall impact score to reflect their assessment of the likelihood for the project to exert a sustained, powerful influence on the research field(s) involved, in consideration of the following five scored review criteria, and additional review criteria. An application does not need to be strong in all categories to be judged likely to have major scientific impact.

Overall Impact *Write a paragraph summarizing the factors that informed your Overall Impact score.*

SCORED REVIEW CRITERIA

Reviewers will consider each of the five review criteria below in the determination of scientific and technical merit, and give a separate score for each.

1. Significance
Strengths • **Weaknesses** •

2. Investigator(s)
Strengths • **Weaknesses** •

3. Innovation
Strengths • **Weaknesses** •

4. Approach
Strengths • **Weaknesses** •

5. Potential for Fit/Collaboration with SIG-U19 Program
Strengths • **Weaknesses** •

Please wait...

If this message is not eventually replaced by the proper contents of the document, your PDF viewer may not be able to display this type of document.

You can upgrade to the latest version of Adobe Reader for Windows®, Mac, or Linux® by visiting http://www.adobe.com/go/reader_download.

For more assistance with Adobe Reader visit http://www.adobe.com/go/acrreader.

Windows is either a registered trademark or a trademark of Microsoft Corporation in the United States and/or other countries. Mac is a trademark of Apple Inc., registered in the United States and other countries. Linux is the registered trademark of Linus Torvalds in the U.S. and other countries.

Name

NIH_BudgetJustification.pdf
NIH_BudgetJustification_final.pdf

Comment	Size	Modified
RR_Budget_1_4_P4.optionalFile0	146,383	4/10/2019 11:08 AM
RR_Budget_1_4_P4.optionalFile0	276,729	4/10/2019 5:10 PM

Pandemic Prevention Platform (P3)

Volume II C: Technical Plan

C.7 METHODS FOR VALIDATION AND COMPLETION FOR TA3

TA3: (C.8) System to evolve antibodies

RFP Page 9-15, 24,

Page count allocations: See CCM spreadsheet for details:

Authors: Ken Tucker, Nigel Horscroft / Philip Santangelo / Mike Sailor / Malcolm Finlayson

Use or disclosure of data contained on this sheet is subject to the restriction on the title page of this proposal.

Pandemic Prevention Platform (P3)

Volume II A: Executive Summary Writer's Template

RFP Page 23

Max 2 Pages

	Requirements
II.A	Executive Summary
II.A.1	What the Proposed Work is Attempting to Accomplish / Do
II.A.2	How It is Done Today and the Limitations
II.A.3	Innovations in Our Approach
II.A.4	Key Technical Challenges and Plan to Overcome Them
II.A.5	Who / What Will Be Affected and What the Impact Will Be
II.A.6	

	How Much Will It Cost and How Long Will It Take

Use or disclosure of data contained on this sheet is subject to the restriction on the title page of this proposal.

VOLUME II A: EXECUTIVE SUMMARY (PAGE COUNT 2) RFP PAGE 23

A.1 WHAT THE PROPOSED WORK IS ATTEMPTING TO ACCOMPLISH/DO

A.2 HOW IT IS DONE TODAY AND THE LIMITATIONS

A.3 INNOVATIONS IN OUR APPROACH

A.4 KEY TECHNICAL CHALLENGES AND PLAN TO OVERCOME THEM

A.5 WHO/WHAT WILL BE AFFECTED AND WHAT THE IMPACT WILL BE

A.6 HOW MUCH WILL IT COST AND HOW LONG WILL IT TAKE

Use or disclosure of data contained on this sheet is subject to the restriction on the title page of this proposal.

CHEMICAL REVIEWS

Review

pubs.acs.org/CR

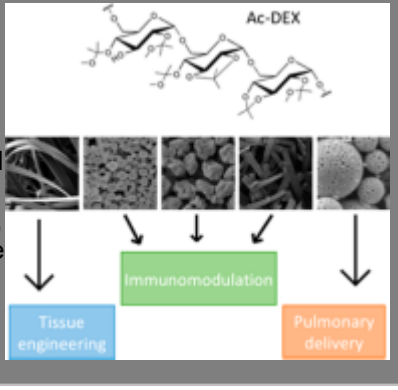
Acetalated Dextran: A Tunable and Acid-Labile Biopolymer with Facile Synthesis and a Range of Applications

Eric M. Bachelder*, Erica N. Pino, and Kristy M. Ainslie

Division of Pharmacoengineering and Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599, United States

* Supporting Information

Acetalated dextran (Ac-DEX) is a tunable acid-labile biopolymer with facile synthesis, aptly designed for the formulation of microparticles for vaccines and immunemodulation. Tunability of degradation is achieved based on the kinetics of reaction and the molecular weight of the parent dextran polymer. This tunability translated to differential rates of activation of CD8+ T cells in an in vitro ovalbumin model and illustrated that acid-labile polymer can activate CD8+ T cells at an increased rate compared to acid-insensitive polymers. In addition, Ac-DEX has been used to encapsulate small molecules, deliver nucleotides, transport inorganic molecules, formulate immune modulating therapies and vaccines, and trigger pH responsive constructs for therapy. Here we highlight the properties and results of Ac-DEX nano-/microparticles as well as the use of the polymer in other constructs and chemistries.



CONTENTS

1. Introduction 1915
2. Adjuvant and Vaccine Delivery 1917
2.1. Adjuvant Encapsulation 1917
2.2. Vaccine Formulations 1917
3. Delivery to Macrophages 1920
4. Controlled Release 1920
5. Devices 1922
6. Copolymer and Other Acetalated Polymers 1922
7. Conclusion 1923
Associated Content 1923
Supporting Information 1923
Author Information 1923
Corresponding Author 1923
ORCID 1923
Notes 1923
Biographies 1923
Acknowledgments 1924
References 1924

1. INTRODUCTION

Dextran is an FDA approved polysaccharide that has been used for decades as a plasma expander. The acetalation of dextran was first reported by Bachelder et al. wherein 2-methoxypropene was used to modify dextran, resulting in a biopolymer with dextran, methanol, and acetone as degradation products (Figure 1).[1] The acetalation of the dextran pendant hydroxyl group results in the polymer being hydrophobic, which allows the encapsulation of drugs through emulsion chemistry, similar to what is done with polyester polymers. This acetalized polymer (Ac-DEX) facilitated the encapsulation of hydrophobic and other drugs that would typically be difficult to deliver. Additionally, acetalated dextran (Ac-DEX) is an acid-sensitive polymer that degrades more rapidly at lower pH, such as in the endosome of phagocytic cells, tumors, or areas of inflammation, allowing for site-specific degradation of the polymer and subsequent release of the encapsulated therapeutic.

In the reaction of dextran with 2-methoxypropene, the kinetically favored acyclic acetals will initially form, followed by the thermodynamically stable cyclic acetals. Acyclic acetals degrade rapidly during hydrolysis and result in the generation of methanol and acetone. In contrast, cyclic acetals degrade more slowly through hydrolysis and result in the generation of acetone only. This differential acetal formation was shown to be dependent on the reaction time of the polymer and to result in varying degradation rates based on the ratio of cyclic to acyclic acetals. With the differential tunability of Ac-DEX came a marked difference in T cell activation using β-galactosidase reporter cells and the model antigen protein ovalbumin. Most notably, faster degrading materials at lower pHs resulted in the most significant major histocompatibility complex class I (MHC I) and MHC II presentation. When comparing fast and slow degrading Ac-DEX to other acid-sensitive (polyacrylamide), degradable (poly(lactic-*co*-glycolic acid) (PLGA)), and nondegradable (iron oxide) carriers,[2] fast degrading Ac-DEX displayed enhanced MHC I presentation and the best MHC II presentation over other carriers. CD8+ T cells are cytotoxic T cells that can kill infected cells indirectly by inducing apoptosis in the target cell, or can kill cells directly through the secretion of perforin. Therefore, CD8+ T cell

Received: August 9, 2016
Published: December 29, 2016

© 2016 American Chemical Society

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

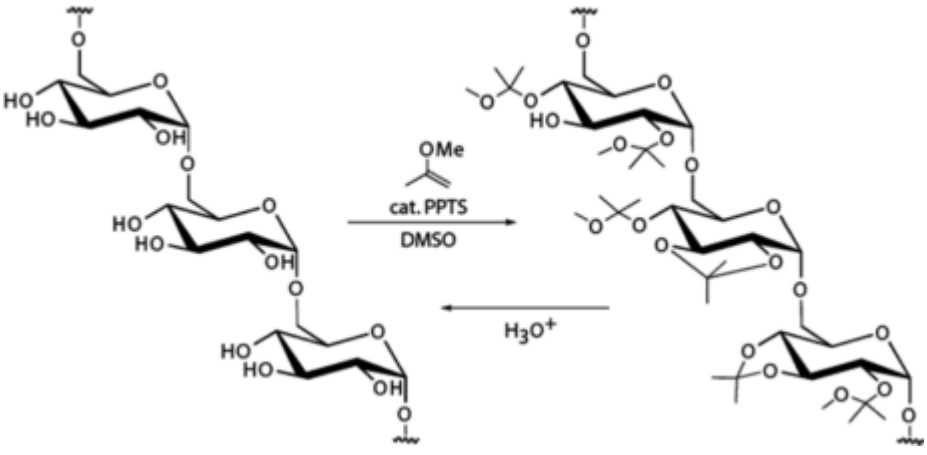


Figure 1. Reaction scheme for the formation of Ac-DEX with 2-methoxypropene. Reprinted with permission from ref 1. Copyright 2008 American Chemical Society.

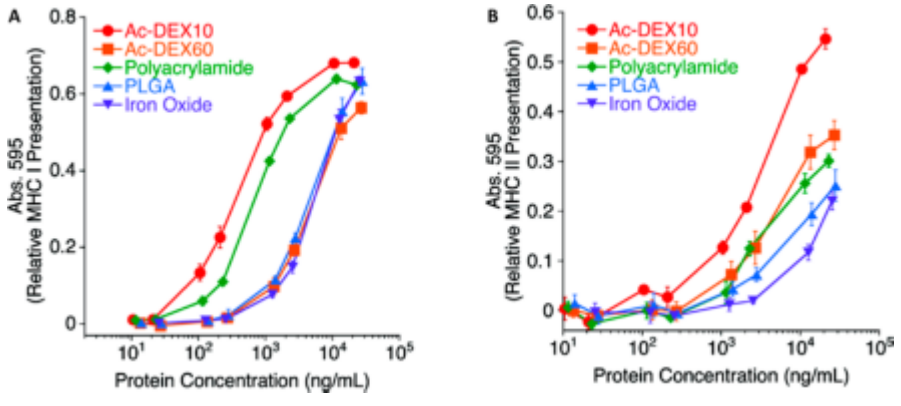


Figure 2. MHC I and MHC II class presentation of OVA with biopolymers of different degradation rates. Ac-DEX10 and Ac-DEX60 correspond to degradation half-lives of 1.7 and 16 h, respectively. Reprinted with permission from ref 2. Copyright 2009 National Academy of Sciences.

activation, via MHC I, is desirable to generate protective vaccine mediated immune responses against intracellular pathogens such as viruses, as well as some bacteria, parasites, and fungi. Moreover, MHC I presentation is required for the development of a therapeutic cancer vaccine. This tunability and enhanced MHC I class presentation illustrates Ac-DEX is a well-designed polymer for vaccine applications (Figure 2). Moreover, Ac-DEX particles displayed significantly greater CD4+ (MHC II) presentation in comparison to other biomaterials, exhibiting that Ac-DEX particles cross-presented to both CD4+ and CD8+ T cells, arming both the cellular and humoral responses that would be needed for many vaccines.[2]

Apart from enhanced activation of cellular immune responses, Ac-DEX has several other benefits when compared to other commonly used biopolymers. A primary benefit is its facile synthesis. Ac-DEX synthesis is a single vessel reaction with subsequent purification through precipitation.[3] Moreover, the degradation products of Ac-DEX are pH-neutral, unlike commonly used polyesters (e.g., poly beta amino esters (PBAEs), poly(lactic-co-glycolic) acid (PLGA), polycaprolactone (PCL)) and several polyanhydrides. This allows for minimal degradation of the payload due to low pH, without the addition of excipients.[4−6]

Other acid-sensitive polymers have been developed for drug delivery applications, including PBAEs, poly(ortho esters) (POEs), and polyketals. PBAEs, while associated with increased cellular internalization and transfection efficiencies over PLGA particles, have illustrated increased cytotoxicity, likely due to their highly positive zeta potentials from their cationic amines in the backbone.[7] In contrast to PBAEs, POEs are very biocompatible but have relatively slow degradation at pH 5.[8] Polyketals, another acid-sensitive polymer class, offer great polymer degradation control, but their difficult synthesis is less appealing than that of Ac-DEX.[9] In comparison to these polymers, Ac-DEX's inherent acid-sensitivity allows for tunable degradation profiles based on reaction time, thus allowing precise control over payload release. Since both sustained and burst release can be selectively achieved, Ac-DEX is an ideal carrier for a wide range of therapeutics. Ac-DEX is a unique biopolymer in that where other commonly used polymers degrade into acidic byproducts, all byproducts of Ac-DEX degradation are pH-neutral. This property gives Ac-DEX the following two advantages: first, a neutral pH should not adversely affect the encapsulated payloads; second, the environment surrounding Ac-DEX should not be harmed upon degradation as it could by the degradation of polymers with acidic byproducts, such as PLGA.

Ac-DEX stands alone as an easily made, tunable, and acid-sensitive polymer, which makes it understandable why it has been used in over 30 publications since 2008 and has been mentioned in five reviews.[10−14] Here we report its application to deliver a variety of compounds, including small molecules, proteins, peptides, nucleotides, and inorganic molecules. Moreover, the most common formulation of Ac-DEX, nano-/

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

microparticles, has been studied extensively in vitro and in vivo and has been delivered through parenteral and needle-free routes. In addition to the particulate constructs, Ac-DEX has been incorporated into a variety of copolymers and advanced designed delivery devices which rely on the polymer's acid sensitivity and tunable degradation rates (Supporting Information Table 1). The many publications that use Ac-DEX, as well as its broad range of applications, make it an emerging biomaterial for drug delivery.

2. ADJUVANT AND VACCINE DELIVERY

The original proposed application of Ac-DEX was the formation of nano-/microparticles as a subunit vaccine. Since subunit vaccines often rely on adjuvant activity for innate immune activation,[15] it goes hand-in-hand that adjuvant encapsulation in Ac-DEX would also be studied. If Ac-DEX microparticles (MPs) are of a certain size, they can passively target antigen presenting cells (APCs). Unlike most cell types in vivo, phagocytes (e.g., dendritic cells (DCs), macrophages) can internalize particles larger than 100 nm, thereby allowing uptake only by phagocytic cells if particles are greater than 100 nm.[16,17] Furthermore, passive targeting of professional APCs (DCs) is possible with further sizing. Studies have shown that MPs in the range of 1−2 μm are four times more likely to be phagocytosed by CD11c+/CD11b+ cells (DCs, professional APCs) than CD11c-/CD11b+ cells (e.g., macrophages). Also, in this same size range, when observing cells extracted from the local draining lymph nodes, approximately 50 times more CD11c+CD11b+ or CD11c+CD11b+ cells (DCs) phagocytosed the MPs, compared to CD11c-CD11b+ cells (macrophages). These results indicate that large particles (1−2 μm) are predominately transported by DCs from the injection site to the lymph nodes, whereas smaller particles are cleared by resident macrophages.[18] Moreover, once the MPs are taken up, the acid-sensitivity of particles leads to an enhanced release of cargo through more rapid degradation of the polymer, compared to degradation at neutral pH. This combination of acid-sensitivity and passive targeting makes Ac-DEX MPs apt carriers for adjuvants and vaccines.

2.1. Adjuvant Encapsulation

Primarily, toll-like receptor (TLR) agonists are the type of adjuvants that have been reported to be encapsulated in Ac-DEX particles. Of the TLRs studied, TLR 3 and TLR 7/8 are the most common, since these are intracellular Pathogen-Associated Molecular Patterns (PAMPs) found within the cell's phagolysosome, which is where Ac-DEX particles would reside after phagocytosis. Imiquimod, the active ingredient in Aldera cream and a TLR 7/8 agonist was first encapsulated into Ac-DEX particles by Bachelder et al.[19] A dose sparing of the hydrophobic adjuvant was observed in vitro in both macrophages and bone-marrow derived dendritic cells (BMDCs). A statistically significant difference in both mRNA and soluble cytokine responses was observed when BMDCs and macrophages were cultured with Ac-DEX MPs encapsulating imiquimod in comparison to empty Ac-DEX MPs and soluble imiquimod, at the same concentrations (Figure 3).[19] Resiquimod, a more hydrophilic imiquimod derivative, was later encapsulated into Ac-DEX MPs, and also displayed a dose sparing response in cytokine production in macrophages.[20] Moreover, by creating a library of polymers with unique cyclic acetal coverage and molecular weight, it has been illustrated that the loading of resiquimod as well as its release and bioactivity in macrophages can be controlled and illustrate dose sparing.[21]

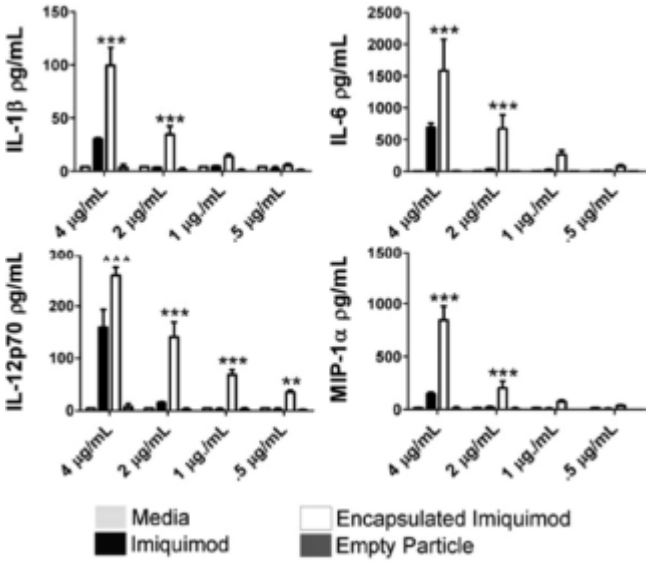


Figure 3. Pro-inflammatory cytokine response of a bone-marrow derived dendritic cell after 24 h of culturing with varying concentrations of media, soluble imiquimod, Ac-DEX particles encapsulating imiquimod, or empty Ac-DEX particles. Reprinted with permission from ref 19. Copyright 2010 American Chemical Society.

In addition to the encapsulation of small molecule adjuvants, nucleotide adjuvants have been formulated into Ac-DEX MPs. Two adjuvant-based nucleotides, in particular, have been used: Poly I:C, which is an agonist for TLR 3, and CpG, which activates TLR 9. In contrast to TLR 8 expression, which is uniform across species, TLR 9 activity differs between mice and humans, where in it is significantly reduced in humans because it is limited to only plasmacytoid DCs and B cells.[22] Despite this, CpG is heavily studied and therefore a practical adjuvant for vaccine formulation. In the comparison of poly I:C encapsulation with Ac-DEX versus PLGA, there was a significant increase in adjuvant encapsulation efficacy (EE) at intermediate (71K) dextran molecular weights. Also, the more quickly degrading poly I:C loaded Ac-DEX MPs resulted in increased cellular activation compared to more slowly degrading Ac-DEX and PLGA. One or both degradation rates of Ac-DEX resulted in a dose sparing compared to soluble drug.[23] Increased EE in comparison to PLGA and dose sparing compared to soluble drug was also observed with the encapsulation of CpG in Ac-DEX MPs. Moreover, the viability of Ac-DEX MPs was shown to be comparable to that of PLGA MPs, indicating that increased cytotoxicity is not the cause of the increased adjuvant activity observed with Ac-DEX MPs. The encapsulation of adjuvant in Ac-DEX particles paves the way for formulation of subunit vaccines using the biopolymer.

2.2. Vaccine Formulations

The model antigen ovalbumin (OVA) was encapsulated into Ac-DEX to evaluate the effect of Ac-DEX encapsulation[1] and tunability[2] on MHC I and MHC II presentation using model cell lines (B3Z for MHC I presentation and KZO for MHC II presentation). Both cell lines will express β-galactosidase downstream of CD3 signaling when the immunodominant peptide of OVA is presented on APCs. The enhanced MHC I presentation (indicator of cellular activity) observed by

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

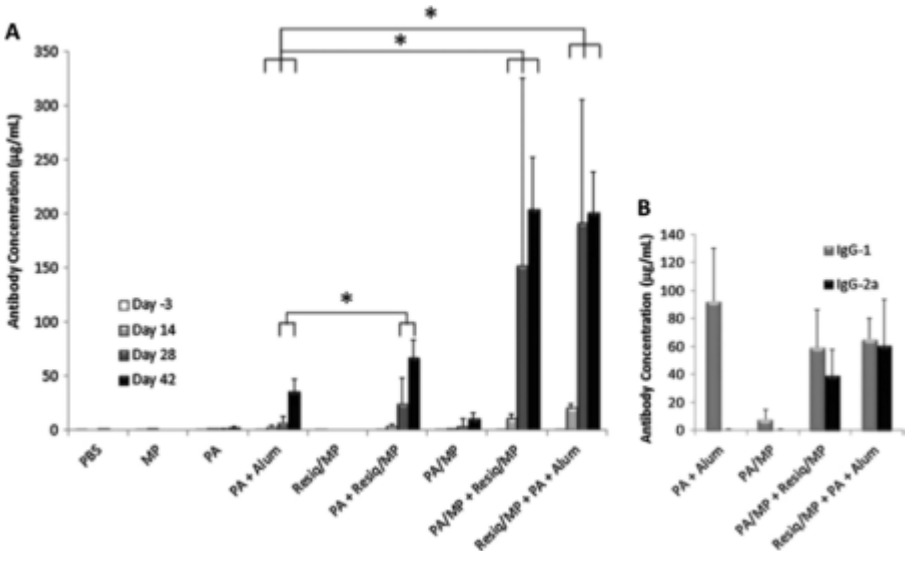


Figure 4. (A) Total IgG antibody and (B) antibody isotype generated when AJ mice were vaccinated at day 0 and 7 with Ac-DEX microparticles (MP) containing recombinant protective antigen (PA) and/or resiquimod (Resiq). A backslash (/) indicates encapsulation, whereas a plus sign (+) indicates addition of component. Statistically significant differences between groups are indicated by *. Reprinted with permission from ref 25. Copyright 2013 Springer.

Bachelder et al. in the initial reporting of Ac-DEX is due in part because particle cross-presentation of protein antigen has resulted in both MHC II and MHC I expression of antigen.[24] Broaders et al. then illustrated that the acid-sensitivity of Ac-DEX generates a greater cellular response at more rapid degradation rates and in comparison to non-acid-sensitive formulations. It is this foundation in enhanced cellular responses compared to free protein as well as other biomaterials that leads to the formation of vaccines with Ac-DEX MPs.[2]

Using separate particles, a complete subunit vaccine comprised of Ac-DEX was formulated for protection against anthrax. Separate emulsion based particles were used for adjuvant and antigen because the adjuvant had poor EE using homogenization compared to sonication, but homogenization was less damaging to the protein antigen. For the study, ~400 nm homogenized particles encapsulated recombinant protective antigen (rPA) and ~200 nm sonicated particles loaded with resiquimod were used. Ac-DEX MPs were shown to be an efficacious vaccine carrier for both antigen and adjuvant over unencapsulated delivery. The study also indicated that high levels of total IgG antibody, significantly greater than Alum and rPA, were generated in the particle vaccine group; however, these antibodies were non-neutralizing, likely due to the denaturation of the protein during the emulsion fabrication process. Despite the poor neutralizing response, a Type 1 helper T-cell (Th1) response was observed in both the antigen recall study as well as by the generation of IgG2 antibodies (Figure 4). This illustrated that the protection observed with the lethal challenge could be the result of a cellular immunity,[25] which corresponds with the results of both Bachelder et al.[1] and Broaders et al.[2]

The work with the anthrax vaccine paved the way for the evaluation of a Burkholderia pseudomallei vaccine for the prevention of melioidosis. Using cell lysate as a crude antigen and resiquimod, again in separate particles, vaccination with Ac-DEX particles was shown to significantly delay time to death, after lethal challenge, in both a rapid and more traditional vaccine schedule. The particle formulation generated humoral and cellular immune responses greater than those of alum and lysate. Additionally, approximately 10% of mice were sterile (blood, liver, spleen) after challenge when treated with encapsulated resiquimod Ac-DEX particles, with and without antigen. This indicates a potential post exposure treatment for this bioterrorism agent.[26]

With the use of emulsion for both the anthrax and B. pseudomallei vaccine work, there were several inherent manufacturing limitations that needed to be addressed prior to additional development of the Ac-DEX vaccine platform. These limitations included: (1) batch processing, (2) high energy methods (e.g., sonication, homogenization) which could denature the protein antigen, (3) extended solvent and protein antigen contact times during the solvent evaporation process which can also cause denaturation of the protein; (4) limited control over the amount of drug and efficacy at which it is encapsulated, and (5) broad polydispersity of particles. The effects of protein denaturation can result in poor B cell activation, which was illustrated by the lack of neutralizing antibodies observed with the emulsion anthrax work.[25] To address these limitations, an Ac-DEX MP anthrax vaccine was formulated using scalable electrospray.[27] In contrast to emulsion, electrospray can be scaled by multiplexing the electrospray heads. Electrospray can have limited interaction with solvents, offers a broader range of control of EE of the payload, produces fairly monodisperse MPs, and uses slow flow rates, which translates to reduced shear stress (Figure 5). When electrospray was used to encapsulate PA, decreased protein denaturation was observed compared to emulsion, which resulted in increased toxin neutralization of generated antibodies (Figure 5). Also due to the flexibility of particle fabrication via electrospray, we were able to encapsulate both protein and adjuvant into a single MP. In comparison to separately encapsulated adjuvant and antigen, the coencapsulated group provided decreased survival in response to a lethal challenge (Figure 5), although both groups outperformed the FDA approved anthrax vaccine formulation, BioThrax. This work illustrates the feasibility of manufacturing an Ac-DEX MP

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

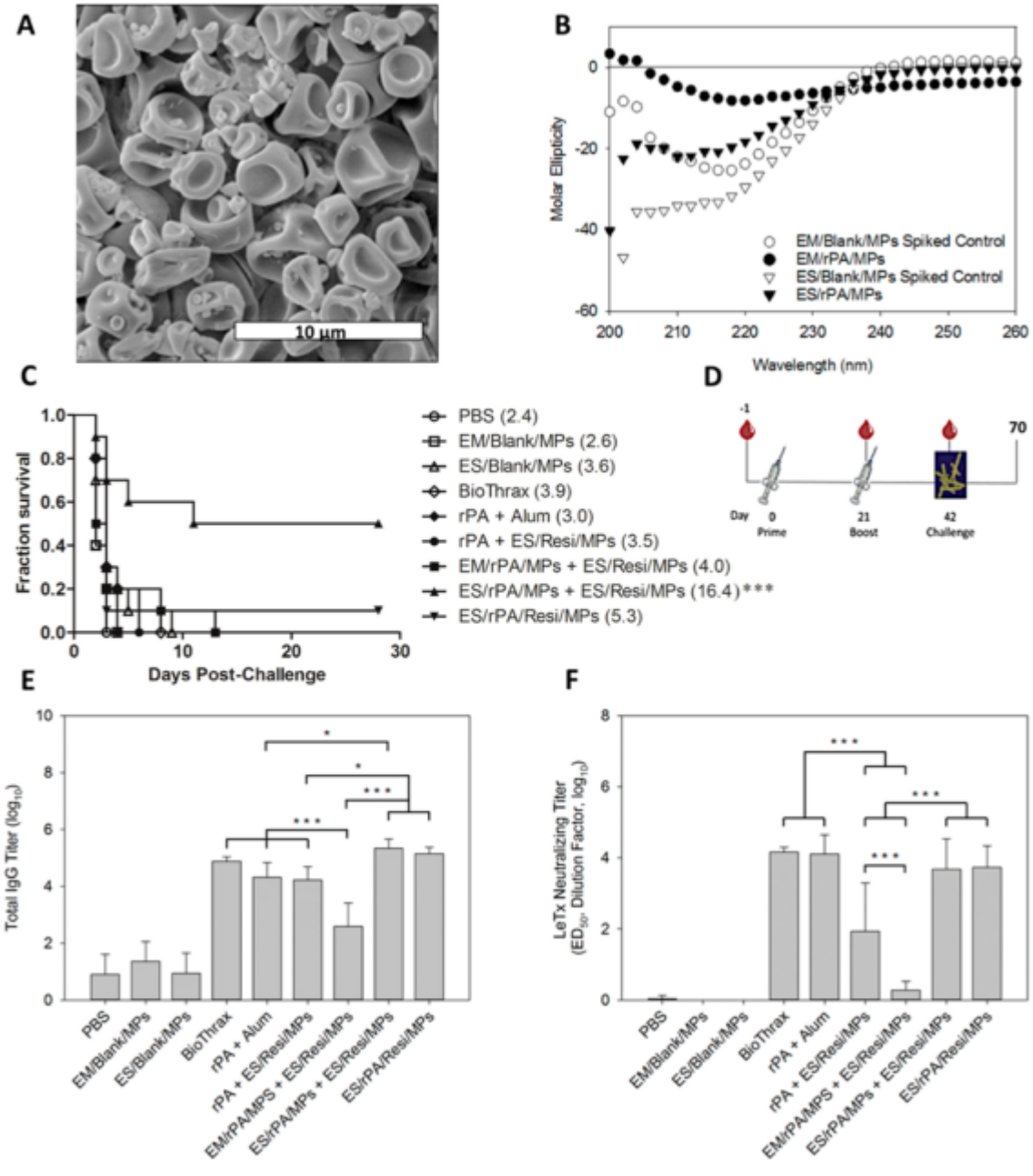


Figure 5. (A) Scanning electron micrograph of electrosprayed particles encapsulating recombinant protective antigen (rPA). (B) Circular dichroism spectra for rPA microparticles (MPs) fabricated by emulsion (EM/rPA/MPs) and electrospray (ES/rPA/MPs). (C) Survival curves of mice (n = 10) intratracheally challenged with 12x LD_{50} of B. anthracis Ames spores. ES = electrospray, EM = emulsion, Resi = Resiquimod (R848); /MPs indicates encapsulation in Ac-DEX. (D) Vaccination schedule of BALB/c mice. (E) Total IgG anti-PA antibodies in mouse sera collected on Day 42. Data are displayed as the mean logarithmic transformation (base 10) of the titers (ng/mL) + 95% confidence interval (n = 10). (F) Lethal toxin (LeTx) neutralizing titers for Day 42 mouse sera. Data are reported as the mean of the log-transformed (base 10) reciprocals of the sera dilution that inhibits LeTx cytotoxicity by 50% (ED_{50}) + 95% confidence interval (n = 5−10). Reprinted with permission from ref 27. Copyright 2016 Wiley.

vaccine platform, and the platform's ability to protect better than the current standard vaccine.

Ac-DEX particles have not only been used to stimulate an effector immune response, but they have also been used to attenuate immune responses in an antigen-specific manner. By using a tolerizing agent (e.g., dexamethasone) with an antigen (Myelin Oligodendrocyte Glycoprotein (MOG)), the costimulatory signals required for generation of an effector response are absent in the education of the T cell by the DC. This can result in antigen-specific attenuation of the immune response via mechanisms such as the formation of T-regulatory cells, anergy, or T cell deletion. Using Ac-DEX particles to encapsulate MOG and dexamethasone into one particle, via emulsion, it was shown using a Multiple Sclerosis (MS) model (experimental autoimmune encephalomyelitis (EAE)) that mice could be taken, on average, from full hind and partial front paralysis to only exhibiting a limp tail. The application of Ac-DEX for these autoimmune therapies is reviewed by Chen et al.[10] and, later, by Northrup et al.[14] The application of Ac-DEX particles has gone beyond the model antigen to illustrate protective responses in

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

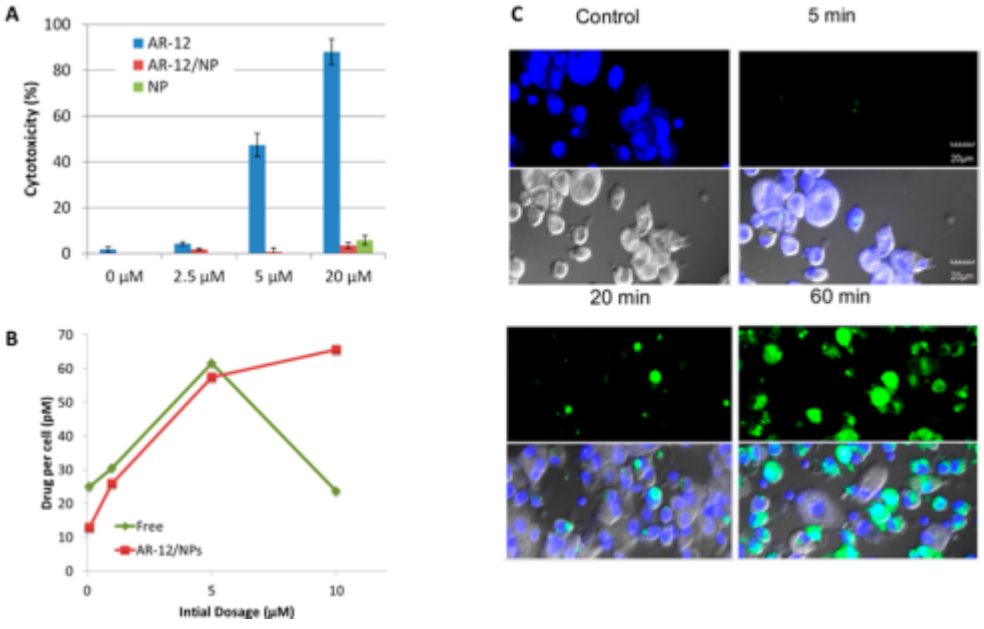


Figure 6. (A) Cytotoxicity, as measured with an LDH, of varying doses of empty Ac-DEX NPs, soluble AR-12, or AR-12 loaded Ac-DEX nanoparticles (NPs) when cultured with human monocyte derived macrophages. (B) Intracellular drug concentration of AR-12 in macrophages as measured with HPLC. (C) Uptake of Ac-DEX NPs when cultured with macrophages. Reprinted with permission from ref 33. Copyright 2014 Elsevier.

disease models, further illustrating the strong application of this biopolymer in a vaccine formulation.

3. DELIVERY TO MACROPHAGES

Like DCs, macrophages can be targeted by particles that are too large to be taken up by nonphagocytic cells. Moreover, since smaller particles are taken up and cleared by macrophages, one can also have a concentrated uptake in macrophages by sizing the particles between 100 nm and 1,000 nm.[18] Since macrophages serve as host cells to a variety of pathogens, including viral, bacterial, parasitic, and fungal, they can act as interdiction points for therapy. By disrupting the mechanisms the pathogen usurps to reside intracellularly, pathogen growth and survival can be decreased. These host-directed therapies (HDT) are thought to mitigate the emergence of drug resistance, since they are not targeting the pathogen directly. Ac-DEX has been used to formulate one HDT, AR-12 (also known as OSU-03012). AR-12 has illustrated broad spectrum activity against multiple bacteria, viruses, and the parasite Leishmania donovani,[12,28−33] and its activity has been reviewed by Collier et al.[31] Compared to unencapsulated drug, AR-12 in Ac-DEX particles had significantly reduced cytotoxicity and higher intracellular drug loading. Additionally, the particles were taken up freely by human monocyte derived macrophages (Figure 6).[33] In vivo, AR-12 in Ac-DEX particles displayed enhanced clearance of Francisella tularensis infection compared to the unencapsulated compound. When AR-12 in Ac-DEX particles were co-delivered with a suboptimum dose of antibiotic intranasally, there was a significant increase in mouse survival compared to free drug and free suboptimum antibiotic. In the host-directed treatment of intracellular parasite, L. donovani used intravenously delivered AR-12 in Ac-DEX particles and displayed a significant reduction in parasite burden over free AR-12, both alone and when co-delivered with amphotericin B. Overall, this work displays that Ac-DEX particles can more aptly deliver HDTs that target macrophages to reduce drug toxicity and increase drug efficacy.

4. CONTROLLED RELEASE

Probably the broadest application of Ac-DEX is its use as a controlled release drug delivery vehicle. Similar to the payloads seen with vaccine formulations, small molecules, proteins, peptides, and nucleotides have all been encapsulated or adsorbed to Ac-DEX to achieve a desired effect. In addition, inorganic based silver carbine has been encapsulated into Ac-DEX to serve as an antibacterial agent.[24] Many of these formulations are the traditional emulsion based nano-/microparticles that are used for both vaccine and macrophage targeting applications. By varying emulsion parameters with Ac-DEX, it has been shown that the encapsulation of rapamycin is dependent on the molecular weight of the base dextran,[35] which corresponds to what was observed with the encapsulation of poly I:C.[23] Also using traditional emulsion chemistry, it was shown that a model protein enzyme (horseradish peroxidase), encapsulated in Ac-DEX particles, had enhanced stability compared to unencapsulated protein. Moreover, Ac-DEX protected the enzyme and its activity from −20 to 45 °C, while PLGA melted at these higher temperatures. As expected, homogenized particles maintained better activity of the enzyme over sonicated particles (Figure 7).[36]

Using Ac-DEX to encapsulate proteins has advantages over polyesters and polyanhydrides because the degradation products are pH-neutral and can therefore be less damaging to the encapsulated protein. In contrast to Ac-DEX, when PLGA degrades, the core of the PLGA particle becomes extremely acidic.[4] It is then not surprising that Ac-DEX has been used to deliver proteins and peptides for therapeutic treatment. Henniget al. compared the EE of a peptide in Ac-DEX particles versus PLGA. They observed a decreased EE with Ac-DEX over PLGA for positively charged peptides for PLGA terminated with a carboxylic acid. The higher

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

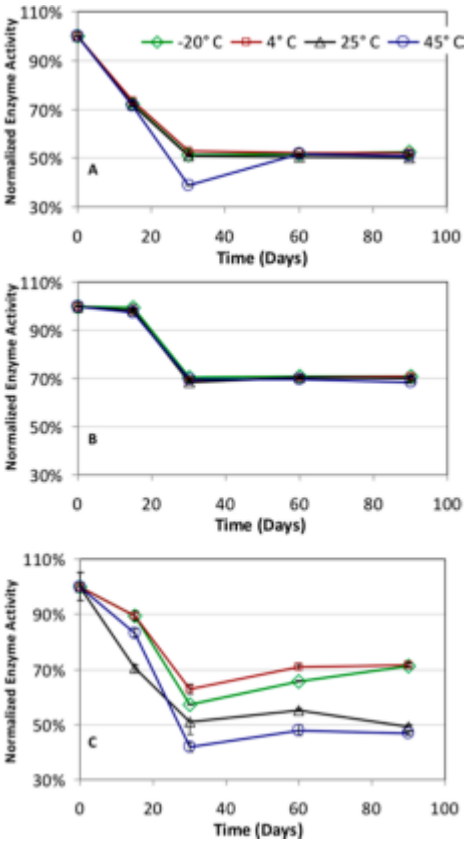


Figure 7. Activity of horseradish peroxidase when (A) encapsulated in sonicated Ac-DEX particles, (B) encapsulated in homogenized Ac-DEX particles, or (C) unencapsulated and stored at different temperatures as a dry powder. Reprinted with permission from ref 36. Copyright 2012 Elsevier.

encapsulation for PLGA is probably due to the interaction between the positive charge of the peptide and the negative charge of the carboxylic acid.[37] For neutral charged peptides and small molecules, Ac-DEX had higher EE. Other studies have encapsulated peptide into Ac-DEX particles[38] but have not compared their encapsulation to PLGA. Despite differences in EE, another advantage of Ac-DEX over polyesters is the tunable delivery. Suarez et al. illustrate this as an advantage in the delivery of basic fibroblast growth factor (bFGF) and myoglobin for treatment after myocardial infarction (MI). The tunability of release of these proteins afforded optimization of the release rates for the best therapeutic effect.[39] The temporal control of Ac-DEX was also illustrated to deliver a hepatocyte growth factor fragment for cardioprotective therapy.[40] As with the MI treatment, an optimum therapeutic effect was determined by varying the degradation rate of the polymer, underlining the importance of this Ac-DEX property.

For the delivery of nucleotides, polycations have been covalently attached to Ac-DEX to facilitate ionic absorption of siRNA,[41] resulting in increased knock-down compared to the free siRNA control. The increased intracellular trafficking over soluble siRNA of the Ac-DEX particles likely contributed to the increased siRNA efficacy.[41] Similarly, poly beta amino esters (PBAEs) were blended with Ac-DEX to deliver plasmid. Plasmid transfection in both HeLa and RAW macrophages was found to be dependent on the weight fraction of PBAE to Ac-DEX.[42]

Other constructs of Ac-DEX have been generated to offer controlled release, including porous microparticles, composite spray dried particles, electrosprayed microparticles, electrospun mats, and electrospun mat fragments called microconfetti (Figure 8). To mimic the large porous PLGA microparticles originally developed by Edwards et al.[43] Ac-DEX was made into porous microparticles, which display a significantly reduced density to volume ratio, compared to traditional emulsion particles. When modeled in a cascade impactor, the porous Ac-DEX particles had aerosol properties for deep lung penetration. The porous particles displayed a controlled release of the chemotherapeutic camptothecin as well as degradation-dependent release.[44] To advance lung delivery using Ac-DEX, nanoparticles made of the biopolymer were loaded with either tobramycin or paclitaxel[45,46] and then spray dried in a secondary matrix to form composite microparticles. Ac-DEX adjuvant microparticles, albeit not composite, have also been made through electrospray. Compared to emulsion, electrospray

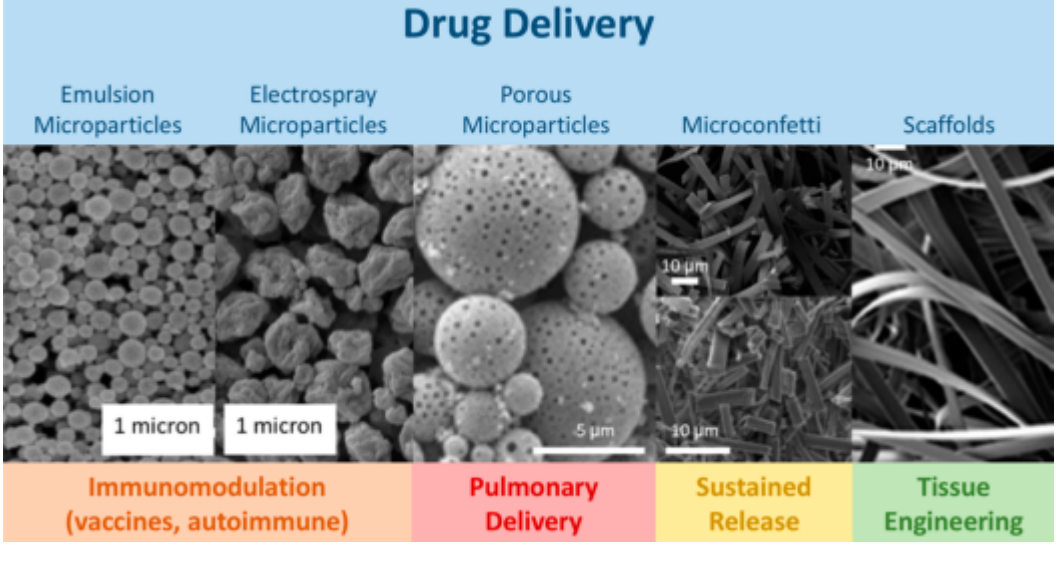


Figure 8. Various constructs of Ac-DEX and their general applications.[22,41−43,60]

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

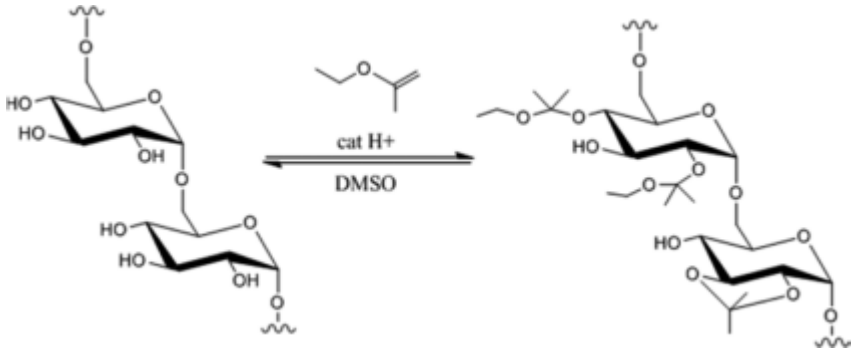


Figure 9. Ace-DEX made from 2-ethoxypropene. Reprinted with permission from ref 63. Copyright 2012 American Chemical Society.

affords a more scalable method that uses air as a continuous phase, in place of an aqueous or organic solution, resulting in increased EEs of compounds. The EE of resiquimod was increased almost 10-fold over emulsion with electrospray to form microparticles that could be delivered intravenously to treat L. donovani infection.[47] A method similar to electrospray, electrospinning, has also been used to generate controlled release nanofibrous scaffolds for Ac-DEX. By electrospinning Ac-DEX with different degradation rates, controlled release of resiquimod was observed, as well as a differential effect of resiquimod on macrophages.[47] The differential effect of Ac-DEX degradation rates aligns with the cardioprotective effects with the MI treatment with bFGF and myoglobin and also the release of saquinavir from electrospun fibers ground into injectable fragments called microconfetti.[48] Collier et al. illustrated that fragments of nanofibrous Ac-DEX scaffolds that are milled can afford nearly week-long release of the protease inhibitor saquinavir when injected subcutaneously. Microconfetti could not be fabricated from PLGA or PCL, even when milled under liquid nitrogen. The high glass transition temperature of Ac-DEX (~160−190 °C)[36] allowed electrospun nanofibrous scaffolds, loaded up to 50 w/w % with saquinavir, to be milled into small injectable pieces.[48]

Ac-DEX has also been used in the formation of Janus particles with PLGA.[49] By using electrohydrodynamic (EHD; electrospray) cojetting, Rahman et al. formed microparticles where one-half of the particle consists of PLGA, and the other half consists of a PLGA/Ac-DEX blend. They loaded the chemotherapeutic irinotecan into the part of the particle that consisted of Ac-DEX, and they were capable of releasing in a pH-sensitive manner.[50] Expanding on their previous work, Ross et al. then proceeded to use their Janus particles for the delivery of drugs to the cochlea in an animal model of cochlear implants. By using piribedil loaded microparticles, they were able to show extended release in vivo.[51] These controlled release studies illustrate that the range of degradation and processability afforded with Ac-DEX is much greater than that which is observed with PLGA, leading to better controlled release systems, as included in reviews by Miladi et al.[13] and Chifiriuc et al.[11]

5. DEVICES

The acid-sensitive acceleration in degradation as well as Ac-DEX's broadly tunable degradation make it an appealing candidate for incorporation into highly engineered devices. Ac-DEX has been used to more rapidly release insulin in response to changes in glucose,[52] as valves in mesoporous silicon,[53,54] and as vehicles to trap water for laser-assisted release of payload.[55] Gu et al. coated Ac-DEX microparticles with chitosan or alginate to give alternating charges. By creating a low acid environment with gluconic acid, one product of glucose and glucose oxidase, insulin encapsulated in the Ac-DEX particles is released at an accelerated rate in the presence of glucose. This device was shown to attenuate blood sugar in diabetic mice.[52] This is similar to the work that was done in the 1990s using poly ortho-esters as the pH-sensitive material.[8] Additionally, Wang et al. has incorporated anti-PD1 antibodies into Ac-DEX for microneedle applications.[56] By incorporating anti-PD1 into a polymeric microneedle, they show enhanced antibody efficacy. Also taking advantage of Ac-DEX's acid-sensitivity, two laboratories have reported the incorporation of the biopolymer into mesoporous silicon to work as valves to control the release of drug that has been absorbed into the inorganic material. Either by using different degradation rates of polymer or by exposing the composite particles to an acid-environment, drug can be released in a more controlled manner, compared to drug absorbed on mesoporous silicon alone.[53] Furthermore, Ac-DEX was used to functionalize the silicon, by attaching cell penetrating peptide to the biopolymer incorporated in the mesoporous silicon.[54] Beyond mesoporous silicon, Viger et al. trapped water in Ac-DEX microparticles fabricated via electrospray. They then heated the water into vapor using a near-IR laser, which results in destabilization of the Ac-DEX matrix and more triggered drug release.[55] These devices display the broad applicability of Ac-DEX.

6. COPOLYMER AND OTHER ACETALATED POLYMERS

To further enhance Ac-DEX, functional groups have been covalently attached to the polymer and copolymers have been formed to create new micelles and other drug delivery vehicles. Initially Ac-DEX was modified with oxime chemistry to covalently link cell penetrating peptide to the particle surface. This chemistry was used to attach the peptide with a fluorophore that then displayed strong covalent attachment to the peptide.[57] Also for enhanced targeting of Ac-DEX particles, mannose was ligated to the biopolymer to actively target the mannose receptor, which is highly expressed in immune cells. Mannose targeting resulted in increased MHC I presentation of OVA over conventional Ac-DEX particles.[58] To cationically charge the Ac-DEX particles, spermine[59] and diethylenetriamine (DET) grated poly(L-aspartic acid)[60] have been covalently bound to Ac-DEX. The spermine functionalized Ac-DEX resulted in better knock-down than sham siRNA, without a marked difference in cell viability.[59] For the DET grated poly(L-aspartic acid) functionalized to Ac-DEX particles,

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

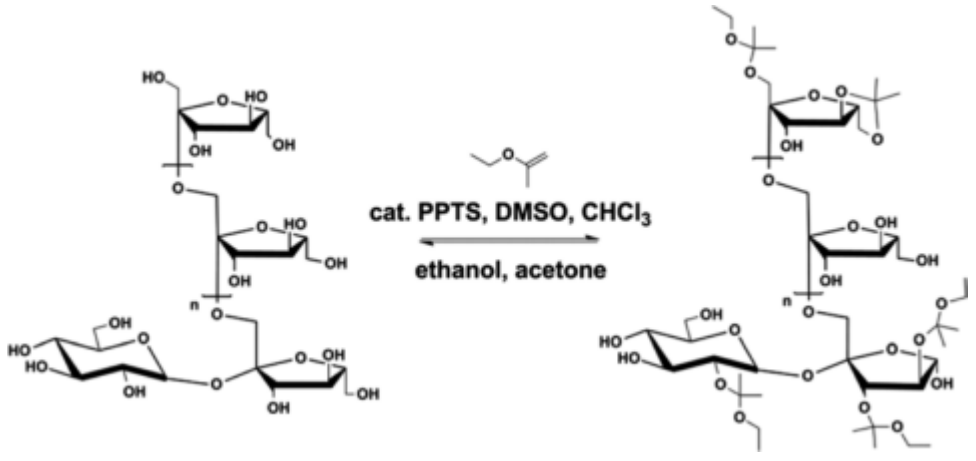


Figure 10. Ace-Inulin made from 2-ethoxypropene and inulin from dahlia. Reprinted with permission from ref 64. Copyright 2016 Royal Society of Chemistry.

both doxorubicin and plasmid were delivered to cancer cells with polymeric particles formed through emulsion and micelles. These carriers resulted in significant uptake of doxorubicin in cancer cells.[60] In addition to the peptide-DET-Ac-DEX micelles formed, poly(ethylene glycol) (PEG) has been covalently attached in two ways to Ac-DEX to form copolymer micelles with pH-sensitive triggers. With the addition of PEG to Ac-DEX with varying degradation rates, micelles were formed to deliver doxorubicin. The Ac-DEX-PEG micelles released drug in a pH-sensitive manner and illustrated increased cell death compared to PEG-b-polylactide (PLA) pH-insensitive micelles at some concentrations when cultured with HeLa cells.[61] PEG was also attached to Ac-DEX by adenine termination of the biopolymer and thymine termination of PEG to form a triblock polymer. Using Ac-DEX at different degradation rates resulted in differential pH-sensitive release of doxorubicin. All triblock micelles loaded with doxorubicin resulted in a significant decrease in cell viability when cultured with HeLa cells.[62] In each of these cases, the acid-sensitivity and/or degradation tunability of Ac-DEX was used to help facilitate better drug delivery.

In addition to copolymers, two acetalated polymers have been produced that display similar properties as the original Ac-DEX. Ace-DEX, which was used in several of the previously mentioned results,[21,31,33,52] is formed by the reaction of dextran and 2-ethoxypropene (leading to the use of the e in Ace over Ac), resulting in ethanol, in place of methanol, as a degradation product. Ace-DEX displays similar degradation tunability as Ac-DEX as well as similar cytocompatibility (Figure 9).[63] Using similar chemistry, Ace-Inulin was formed (Figure 10). Whereas dextran is immunologically inert, inulin has naturally occurring immune stimulating properties, allowing Ace-Inulin to serve as its own adjuvant in vaccine formulations. Blending Ace-Inulin with Ace-DEX results in better encapsulation of protein, but decreased immune stimulatory activity, as measured through tumor necrosis factor-alpha (TNF-α) production by RAW macrophages. Overall, an Ace-Inulin blended OVA vaccine did elicit strong total IgG antibodies on order with OVA and alum.[64] Similarly, silylated dextran and inulin have shown tunable properties and generation of IgG.[65] These second generation Ac-DEX polymers have the same beneficial properties of Ac-DEX with some added features, including more benign degradation products and immune stimulating capabilities.

7. CONCLUSION

In conclusion, we have reviewed the emerging biopolymer acetalated dextran (Ac-DEX). Ac-DEX has several features which make it a unique biodegradable polymer, including facile synthesis, acid-sensitivity, and tunable degradation rates. For these reasons it has emerged as a top candidate for applications in vaccines, targeted host-directed therapies to macrophages, controlled release of drug, chemotherapeutic delivery, engineered drug delivery devices, and as copolymers in drug and vaccine carriers. Ac-DEX has displayed delivery of small molecules, proteins, peptides, nucleotides, and inorganic compounds, illustrating the broad applicability of this material. Moreover, it has been used to modify inorganic mesoporous silicon, spun into nanofibrous mats, made into microconfetti, and covalently bound to other polymers to form micelles. Current uses of this biopolymer are merely the tip of the iceberg, the applications of Ac-DEX will certainly expand in the coming years.

ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrev.6b00532

Uses of Ac-DEX currently in the literature (PDF)

AUTHOR INFORMATION

Corresponding Author

* Eric M. Bachelder, Ph.D., 4210 Marsico Hall, 125 Mason Farm Road, Chapel Hill, NC 27599. E-mail, ebacheld@email.unc.edu; phone, 919-962-4857.

ORCID

Eric M. Bachelder 0000-0002-8572-888X

Notes

The authors declare no competing financial Interest.

Biographies

Eric M. Bachelder gained extensive immunological experience while completing his graduate studies in Dr. Polly Matzinger's lab at the National Institutes of Health (NIH), National Institute of Allergy and Infectious Diseases (NIAID). During his graduate studies, he studied

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

the interaction of T-cells and dendritic cells. After completing his Ph.D. in Chemical Engineering, he joined Prof. Jean Fréchet's lab at the University of California, Berkeley's chemistry department for a postdoc. While there, Dr. Bachelder invented various biodegradable polymers for vaccine and drug delivery applications, including Acetalated Dextran (Ac-DEX). Dr. Bachelder continued his work on Ac-DEX as an Assistant Professor at the Ohio State University and currently at the University of North Carolina at Chapel Hill. The focus of Dr. Bachelder's research program is to develop novel drug delivery moieties for the formulation of vaccines, infectious disease treatments, and autoimmune therapies.

Erica N. Pino received her B.S. in Biology from the Massachusetts Institute of Technology in 2013. Her undergraduate thesis work in the laboratories of Dr. Robert Desimone and Dr. Edward Boyden centered on characterizing heat and light propagation through rodent cortex to establish safe guidelines for optogenetic studies in nonhuman primates. Following graduation, she continued research in the Desimone lab, focusing on optogenetic inactivation of frontal eye field neurons during memory-guided saccade tasks. She is now a graduate student in the Ainslie lab, working toward her Ph.D. in the Division of Pharmacoengineering and Molecular Pharmaceutics at the University of North Carolina at Chapel Hill.

Kristy M. Ainslie is an Associate Professor with appointments in the Division of Pharmacoengineering and Molecular Pharmaceutics, within the Eshelman School of Pharmacy, and the Department of Biomedical Engineering at the University of North Carolina at Chapel Hill. Her research focuses on the use of novel micro- and nanoscaled carriers for the modulation of immune responses to create vaccines for and therapies against infectious diseases. Previously, she was an Assistant Professor in the Division of Pharmaceutics and Pharmaceutical Chemistry at The Ohio State University. As a postdoctoral scholar at the University of California, San Francisco, she researched the microfabrication of oral drug delivery vehicles and the immune response of nanobiomaterials. Previously, she studied biosensors at the Naval Research Laboratory in Washington, DC, and completed a Ph.D. in Chemical Engineering at The Pennsylvania State University in the application of nanobiomaterials for antifouling implant surfaces.

ACKNOWLEDGMENTS

This work was supported by internal funds from the Eshelman School of Pharmacy at UNC-Chapel Hill.

REFERENCES

(1) Bachelder, E. M.; Beaudette, T. T.; Broaders, K. E.; Dashe, J.; Frechet, J. M. Acetal-Derivatized Dextran: An Acid-Responsive Biodegradable Material for Therapeutic Applications. J. Am. Chem. Soc. 2008, 130, 10494–10495.
(2) Broaders, K. E.; Cohen, J. A.; Beaudette, T. T.; Bachelder, E. M.; Frechet, J. M. J. Acetalated Dextran Is a Chemically and Biologically Tunable Material for Particulate Immunotherapy. Proc. Natl. Acad. Sci. U. S. A. 2009, 106, 5497–5502.
(3) Helaly, F. M.; Hashem, M. S. Preparation and Characterization of Poly(B-Amino Ester) Capsules for Slow Release of Bioactive Material. J. Encapsulation Adsorpt. Sci. 2013, 2013, 65.
(4) Ding, A. G.; Schwendeman, S. P. Acidic Microclimate pH Distribution in Plga Microspheres Monitored by Confocal Laser Scanning Microscopy. Pharm. Res. 2008, 25, 2041–2052.
(5) Li, L.; Schwendeman, S. P. Mapping Neutral Microclimate pH in Plga Microspheres. J. Controlled Release 2005, 101, 163–173.
(6) Schwendeman, S. P.; Tobio, M.; Joworowicz, M.; Alonso, M. J.; Langer, R. New Strategies for the Microencapsulation of Tetanus Vaccine. J. Microencapsulation 1998, 15, 299–318.
(7) Fields, R. J.; Cheng, C. J.; Quijano, E.; Weller, C.; Kristofik, N.; Duong, N.; Hoimes, C.; Egan, M. E.; Saltzman, W. M. Surface Modified Poly(B Amino Ester)-Containing Nanoparticles for Plasmid DNA Delivery. J. Controlled Release 2012, 164, 41–48.
(8) Heller, J.; Barr, J. Poly(Ortho Esters) from Concept to Reality. Biomacromolecules 2004, 5, 1625–1632.
(9) Paramonov, S. E.; Bachelder, E. M.; Beaudette, T. T.; Standley, S. M.; Lee, C. C.; Dashe, J.; Fréchet, J. M. Fully Acid-Degradable Biocompatible Polyacetal Microparticles for Drug Delivery. Bioconjugate Chem. 2008, 19, 911–919.
(10) Chen, N.; Peine, K.; Bachelder, E.; Ainslie, K. Micro- and Nano-Particulate Strategies for Antigen Specific Immune Tolerance to Treat Autoimmune Diseases. Pharm. Nanotechnol. 2015, 3, 85–100.
(11) Chifiriuc, M. C.; Grumezescu, A. M.; Grumezescu, V.; Bezirtzoglou, E.; Lazar, V.; Bolocan, A. Biomedical Applications of Natural Polymers for Drug Delivery. Curr. Org. Chem. 2014, 18, 152–164.
(12) Collier, M. A.; Gallovic, M. D.; Peine, K. J.; Duong, A. D.; Bachelder, E. M.; Gunn, J. S.; Schlesinger, L. S.; Ainslie, K. M. Delivery of Host Cell-Directed Therapeutics for Intracellular Pathogen Clearance. Expert Rev. Anti-Infect. Ther. 2013, 11, 1225–1235.
(13) Miladi, K.; Ibraheem, D.; Iqbal, M.; Sfar, S.; Fessi, H.; Elaissari, A. Particles from Preformed Polymers as Carriers for Drug Delivery. EXCLI J. 2014, 13, 28.
(14) Northrup, L.; Christopher, M. A.; Sullivan, B. P.; Berlind, C. Combining Antigen and Immunomodulators: Emerging Trends in Antigen-Specific Immunotherapy for Autoimmunity. Adv. Drug Delivery Rev. 2016, 98, 86–98.
(15) Janeway, C.; Travers, P.; Walport, M.; Shlomchik, M. Immunobiology, 5th ed.; Garland Science: New York, 2001.
(16) Foged, C.; Brodin, B.; Frokjaer, S.; Sundblad, A. Particle Size and Surface Charge Affect Particle Uptake by Human Dendritic Cells in an in Vitro Model. Int. J. Pharm. 2005, 298, 315–322.
(17) Hirota, K.; Hasegawa, T.; Hinata, H.; Ito, F.; Inagawa, H.; Kochi, C.; Soma, G.; Makino, K.; Terada, H. Optimum Conditions for Efficient Phagocytosis of Rifampicin-Loaded Plga Microspheres by Alveolar Macrophages. J. Controlled Release 2007, 119, 69–76.
(18) Manolova, V.; Flace, A.; Bauer, M.; Schwarz, K.; Saudan, P.; Bachmann, M. F. Nanoparticles Target Distinct Dendritic Cell Populations According to Their Size. Eur. J. Immunol. 2008, 38, 1404–1413.
(19) Bachelder, E. M.; Beaudette, T. T.; Broaders, K. E.; Frechet, J. M. J.; Albrecht, M. T.; Mateczun, A. J.; Ainslie, K. M.; Pesce, J. T.; Keane-Myers, A. M. In Vitro Analysis of Acetalated Dextran Microparticles as a Potent Delivery Platform for Vaccine Adjuvants. Mol. Pharmaceutics 2010, 7, 826–835.
(20) Duong, A. D.; Collier, M. A.; Bachelder, E. M.; Wyslouzil, B. E.; Ainslie, K. M. One Step Encapsulation of Small Molecule Drugs in Liposomes Via Electrospray-Remote Loading. Mol. Pharmaceutics 2015, 13, 92.
(21) Chen, N.; Collier, M. A.; Gallovic, M. D.; Collins, G. C.; Sanchez, C. C.; Fernandes, E. Q.; Bachelder, E. M.; Ainslie, K. M. Degradation of Acetalated Dextran Can Be Broadly Tuned Based on Cyclic Acetal Coverage and Molecular Weight. Int. J. Pharm. 2016, 512, 147–157.
(22) Hochrein, H.; Wagner, H. Of Men, Mice and Pigs: Looking at Their Plasmacytoid Dendritic Cells [Corrected]. Immunology 2004, 112, 26–27.
(23) Peine, K. J.; Bachelder, E. M.; Vangundy, Z.; Papenfuss, T.; Brackman, D. J.; Gallovic, M. D.; Schully, K.; Pesce, J.; Keane-Myers, A.; Ainslie, K. M. Efficient Delivery of the Toll-Like Receptor Agonists Polyinosinic:Polycytidylic Acid and Cpg to Macrophages by Acetalated Dextran Microparticles. Mol. Pharmaceutics 2013, 10, 2849–2857.
(24) Kovacsovics-Bankowski, M.; Clark, K.; Benacerraf, B.; Rock, K. L. Efficient Major Histocompatibility Complex Class I Presentation of Exogenous Antigen Upon Phagocytosis by Macrophages. Proc. Natl. Acad. Sci. U. S. A. 1993, 90, 4942–4946.
(25) Schully, K. L.; Sharma, S.; Peine, K. J.; Pesce, J.; Elberson, M. A.; Fonseca, M. E.; Prouty, A. M.; Bell, M. G.; Borteh, H.; Gallovic, M.;

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915–1926

et al. Rapid Vaccination Using an Acetalated Dextran Microparticulate Subunit Vaccine Confers Protection against Triplicate Challenge by Bacillus Anthracis. Pharm Res 2013, 30, 1349−1361.
(26) Schully, K. L.; Bell, M. G.; Prouty, A. M.; Gallovic, M. D.; Gautam, S.; Peine, K. J.; Sharma, S.; Bachelder, E. M.; Pesce, J. T.; Elberson, M. A.; et al. Evaluation of a Biodegradable Microparticulate Polymer as a Carrier for Burkholderia Pseudomallei Subunit Vaccines in a Mouse Model of Melioidosis. Int. J. Pharm. 2015, 495, 849−861.
(27) Gallovic, M. D.; Schully, K. L.; Bell, M. G.; Elberson, M. A.; Palmer, J. R.; Darko, C. A.; Bachelder, E. M.; Wyslouzil, B. E.; Keane-Myers, A. M.; Ainslie, K. M. Acetalated Dextran Microparticulate Vaccine Formulated Via Coaxial Electrospray Preserves Toxin Neutralization and Enhances Murine Survival Following Inhalational Bacillus Anthracis Exposure. Adv. Healthcare Mater. 2016, 5, 2617.
(28) Booth, L.; Roberts, J. L.; Cash, D. R.; Tavallai, S.; Jean, S.; Fidanza, A.; Cruz-Luna, T.; Siembiba, P.; Cycon, K. A.; Cornelissen, C. N.; et al. Grp78/Bip/Hspa5/DnaK Is a Universal Therapeutic Target for Human Disease. J. Cell. Physiol. 2015, 230, 1661−1676.
(29) Chiu, H. C.; Soni, S.; Kulp, S. K.; Curry, H.; Wang, D.; Gunn, J. S.; Schlesinger, L. S.; Chen, C. S. Eradication of Intracellular Francisella Tularensis in Thp-1 Human Macrophages with a Novel Autophagy Inducing Agent. J. Biomed. Sci. 2009, 16, 110.
(30) Chiu, H. C.; Kulp, S. K.; Soni, S.; Wang, D.; Gunn, J. S.; Schlesinger, L. S.; Chen, C. S. Eradication of Intracellular Salmonella Enterica Serovar Typhimurium with a Small-Molecule, Host Cell-Directed Agent. Antimicrob. Agents Chemother. 2009, 53, 5236−5244.
(31) Collier, M. A.; Peine, K. J.; Gautam, S.; Oghumu, S.; Varikuti, S.; Borteh, H.; Papenfuss, T. L.; Sataoskar, A. R.; Bachelder, E. M.; Ainslie, K. M. Host-Mediated Leishmania Donovani Treatment Using Ar-12 Encapsulated in Acetalated Dextran Microparticles. Int. J. Pharm. 2016, 499, 186−194.
(32) Hoang, K. V.; Curry, H.; Collier, M. A.; Borteh, H.; Bachelder, E. M.; Schlesinger, L. S.; Gunn, J. S.; Ainslie, K. M. Needle-Free Delivery of Acetalated Dextran-Encapsulated Ar-12 Protects Mice from Francisella Tularensis Lethal Challenge. Antimicrob. Agents Chemother. 2016, 60, 2052−2062.
(33) Hoang, K. V.; Borteh, H. M.; Rajaram, M. V. S.; Peine, K. J.; Curry, H.; Collier, M. A.; Homsy, M. L.; Bachelder, E. M.; Gunn, J. S.; Schlesinger, L. S.; et al. Acetalated Dextran Encapsulated Ar-12 as a Host-Directed Therapy to Control Salmonella Infection. Int. J. Pharm. 2014, 477, 334−343.
(34) Ornelas-Megiatto, C.; Shah, P. N.; Wich, P. R.; Cohen, J. L.; Tagaev, J. A.; Smolen, J. A.; Wright, B. D.; Panzner, M. J.; Youngs, W. J.; Frechet, J. M.; et al. Aerosolized Antimicrobial Agents Based on Degradable Dextran Nanoparticles Loaded with Silver Carbene Complexes. Mol. Pharmaceutics 2012, 9, 3012−3022.
(35) Kauffman, K. J.; Kanthamneni, N.; Meenach, S. A.; Pierson, B. C.; Bachelder, E. M.; Ainslie, K. M. Optimization of Rapamycin-Loaded Acetalated Dextran Microparticles for Immunosuppression. Int. J. Pharm. 2012, 422, 356−363.
(36) Kanthamneni, N.; Sharma, S.; Meenach, S. A.; Billet, B.; Zhao, J. C.; Bachelder, E. M.; Ainslie, K. M. Enhanced Stability of Horseradish Peroxidase Encapsulated in Acetalated Dextran Microparticles Stored Outside Cold Chain Conditions. Int. J. Pharm. 2012, 431, 101−110.
(37) Hennig, D.; Schubert, S.; Dargatz, H.; Kostenis, E.; Fahr, A.; Schubert, U. S.; Heinzel, T.; Imhof, D. Novel Insights into Appropriate Encapsulation Methods for Bioactive Compounds into Polymers: A Study with Peptides and Hdac Inhibitors. Macromol. Biosci. 2014, 14, 69−80.
(38) Peine, K. J.; Guerau-de-Arellano, M.; Lee, P.; Kanthamneni, N.; Severin, M.; Probst, G. D.; Peng, H. Y.; Yang, Y. H.; Vangundy, Z.; Papenfuss, T. L.; et al. Treatment of Experimental Autoimmune Encephalomyelitis by Codelivery of Disease Associated Peptide and Dexamethasone in Acetalated Dextran Microparticles. Mol. Pharmaceutics 2014, 11, 828−835.
(39) Suarez, S.; Grover, G. N.; Braden, R. L.; Christman, K. L.; Almutairi, A. Tunable Protein Release from Acetalated Dextran Microparticles: A Platform for Delivery of Protein Therapeutics to the Heart Post-Mi. Biomacromolecules 2013, 14, 3927−3935.
(40) Suarez, S. L.; Munoz, A.; Mitchell, A. C.; Braden, R. L.; Luo, C. L.; Cochran, J. R.; Almutairi, A.; Christman, K. L. Degradable Acetalated Dextran Microparticles for Tunable Release of an Engineered Hepatocyte Growth Factor Fragment. ACS Biomater. Sci. Eng. 2016, 2, 197−204.
(41) Wich, P. R.; Fréchet, J. M. J. Degradable Dextran Particles for Gene Delivery Applications. Aust. J. Chem. 2012, 65, 15−19.
(42) Cohen, J. A.; Beaudette, T. T.; Cohen, J. L.; Broaders, K. E.; Bachelder, E. M.; Frechet, J. M. Acetal-Modified Dextran Microparticles with Controlled Degradation Kinetics and Surface Functionality for Gene Delivery in Phagocytic and Non-Phagocytic Cells. Adv. Mater. 2010, 22, 3593−3597.
(43) Edwards, D. A.; Hanes, J.; Caponetti, G.; Hrkach, J.; Ben-Jebria, A.; Eskew, M. L.; Mintzes, J.; Deaver, D.; Lotan, N.; Langer, R. Large Porous Particles for Pulmonary Drug Delivery. Science 1997, 276, 1868−1871.
(44) Meenach, S. A.; Kim, Y. J.; Kauffman, K. J.; Kanthamneni, N.; Bachelder, E. M.; Ainslie, K. M. Synthesis, Optimization, and Characterization of Camptothecin-Loaded Acetalated Dextran Porous Microparticles for Pulmonary Delivery. Mol. Pharmaceutics 2012, 9, 290−298.
(45) Wang, Z. M.; Meenach, S. A. Ieee Dry Powders Based on Mucus-Penetrating Nanoparticle Entrapped Microparticles for Pulmonary Delivery of Tobramycin. 2014 40th Annual Northeast Bioengineering Conference (Nebec) 2014.
(46) Torrico-Guzman, E. A.; Meenach, S. Ieee In 2015 41st Annual Northeast Biomedical Engineering Conference, 2015.
(47) Duong, A. D.; Sharma, S.; Peine, K. J.; Gupta, G.; Satoskar, A. R.; Bachelder, E. M.; Wyslouzil, B. E.; Ainsie, K. M. Electrospray Encapsulation of Toll-Like Receptor Agonist Resiquimod in Polymer Microparticles for the Treatment of Visceral Leishmaniasis. Mol. Pharmaceutics 2013, 10, 1045−1055.
(48) Collier, M. A.; Gallovic, M. D.; Bachelder, E. M.; Sykes, C. D.; Kashuba, A.; Ainslie, K. M. Saquinavir Loaded Acetalated Dextran Microconfetti − a Long Acting Protease Inhibitor Injectable. Pharm. Res. 2016, 33, 1998−2009.
(49) Rahmani, S.; Park, T.-H.; Dishman, A. F.; Lahann, J. Multimodal Delivery of Irinotecan from Microparticles with Two Distinct Compartments. J. Controlled Release 2013, 172, 239−245.
(50) Rahmani, S.; Ross, A. M.; Park, T. H.; Durmaz, H.; Dishman, A. F.; Prieskorn, D. M.; Jones, N.; Altschuler, R. A.; Lahann, J. Dual Release Carriers for Cochlea Delivery. Adv. Healthcare Mater. 2016, 5, 94−100.
(51) Ross, A. M.; Rahmani, S.; Prieskorn, D. M.; Dishman, A. F.; Miller, J. M.; Lahann, J.; Altschuler, R. A. Persistence, Distribution, and Impact of Distinctly Segmented Microparticles on Cochlear Health Following in Vivo Infusion. J. Biomed. Mater. Res., Part A 2016, 104, 1510−1522.
(52) Gu, Z.; Aimetti, A. A.; Wang, Q.; Dang, T. T.; Zhang, Y.; Veiseh, O.; Cheng, H.; Langer, R. S.; Anderson, D. G. Injectable Nano-Network for Glucose-Mediated Insulin Delivery. ACS Nano 2013, 7, 4194−4201.
(53) Lin, Z.; Li, J. Z.; He, H. Y.; Kuang, H. H.; Chen, X. S.; Xie, Z. G.; Jing, X. B.; Huang, Y. B. Acetalated-Dextran as Valves of Mesoporous Silica Particles for Ph Responsive Intracellular Drug Delivery. RSC Adv. 2015, 5, 9546−9555.
(54) Liu, D.; Zhang, H.; Makila, E.; Fan, J.; Herranz-Blanco, B.; Wang, C. F.; Rosa, R.; Ribeiro, A. J.; Salonen, J.; Hirvonen, J.; et al. Microfluidic Assisted One-Step Fabrication of Porous Silicon@Acetalated Dextran Nanocomposites for Precisely Controlled Combination Chemotherapy. Biomaterials 2015, 39, 249−259.
(55) Viger, M. L.; Sheng, W. Z.; Dore, K.; Alhasan, A. H.; Carling, C. J.; Lux, J.; Lux, C. D.; Grossman, M.; Malinow, R.; Almutairi, A. Near-Infrared-Induced Heating of Confined Water in Polymeric Particles for Efficient Payload Release. ACS Nano 2014, 8, 4815−4826.
(56) Wang, C.; Ye, Y.; Hochu, G. M.; Sadeghifar, H.; Gu, Z. Enhanced Cancer Immunotherapy by Microneedle Patch-Assisted Delivery of Anti-Pd1 Antibody. Nano Lett. 2016, 16, 2334−2340.

DOI: 10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

(57) Beaudette, T. T.; Cohen, J. A.; Bachelder, E. M.; Broaders, K. E.; Cohen, J. L.; Engleman, E. G.; Frechet, J. M. Chemoselective Ligation in the Functionalization of Polysaccharide-Based Particles. J. Am. Chem. Soc. 2009, 131, 10360−10361.
(58) Cui, L. N.; Cohen, J. A.; Broaders, K. E.; Beaudette, T. T.; Frechet, J. M. J. Mannosylated Dextran Nanoparticles: A Ph-Sensitive System Engineered for Immunomodulation through Mannose Targeting. Bioconjugate Chem. 2011, 22, 949−957.
(59) Cohen, J. L.; Schubert, S.; Wich, P. R.; Cui, L.; Cohen, J. A.; Mynar, J. L.; Frechet, J. M. J. Acid-Degradable Cationic Dextran Particles for the Delivery of Sirna Therapeutics. Bioconjugate Chem. 2011, 22, 1056−1065.
(60) Li, Q.; Liu, W.; Dai, J.; Zhang, C. Synthesis of Polysaccharide-Block-Polypeptide Copolymer for Potential Co-Delivery of Drug and Plasmid DNA. Macromol. Biosci. 2015, 15, 756−764.
(61) Zhang, Z.; Chen, X.; Chen, L.; Yu, S.; Cao, Y.; He, C.; Chen, X. Intracellular Ph-Sensitive Peg-Block-Acetalated-Dextran as Efficient Drug Delivery Platforms. ACS Appl. Mater. Interfaces 2013, 5, 10760−10766.
(62) Kuang, H. H.; Wu, Y. J.; Zhang, Z. Y.; Li, J. Z.; Chen, X. S.; Xie, Z. G.; Jing, X. B.; Huang, Y. B. Double Ph-Responsive Supramolecular Copolymer Micelles Based on the Complementary Multiple Hydrogen Bonds of Nucleobases and Acetalated Dextran for Drug Delivery. Polym. Chem. 2015, 6, 3625−3633.
(63) Kauffman, K. J.; Do, C.; Sharma, S.; Gallovic, M. D.; Bachelder, E. M.; Ainslie, K. M. Synthesis and Characterization of Acetalated Dextran Polymer and Microparticles with Ethanol as a Degradation Product. ACS Appl. Mater. Interfaces 2012, 4, 4149−4155.
(64) Gallovic, M. D.; Montjoy, D. G.; Collier, M. A.; Do, C.; Wyslouzil, B. E.; Bachelder, E. M.; Ainslie, K. M. Chemically Modified Inulin Microparticles Serving Dual Function as a Protein Antigen Delivery Vehicle and Immunostimulatory Adjuvant. Biomater. Sci. 2016, 4, 483−493.
(65) Gallovic, M. D.; Bandyopadhyay, S.; Borteh, H.; Montjoy, D. G.; Collier, M. A.; Peine, K. J.; Wyslouzil, B. E.; Bachelder, E. M.; Ainslie, K. M. Microparticles Formulated from a Family of Novel Silylated Polysaccharides Exhibit Tunable Hydrolytic Degradability. J. Mater. Chem. B 2016, 4, 4302.

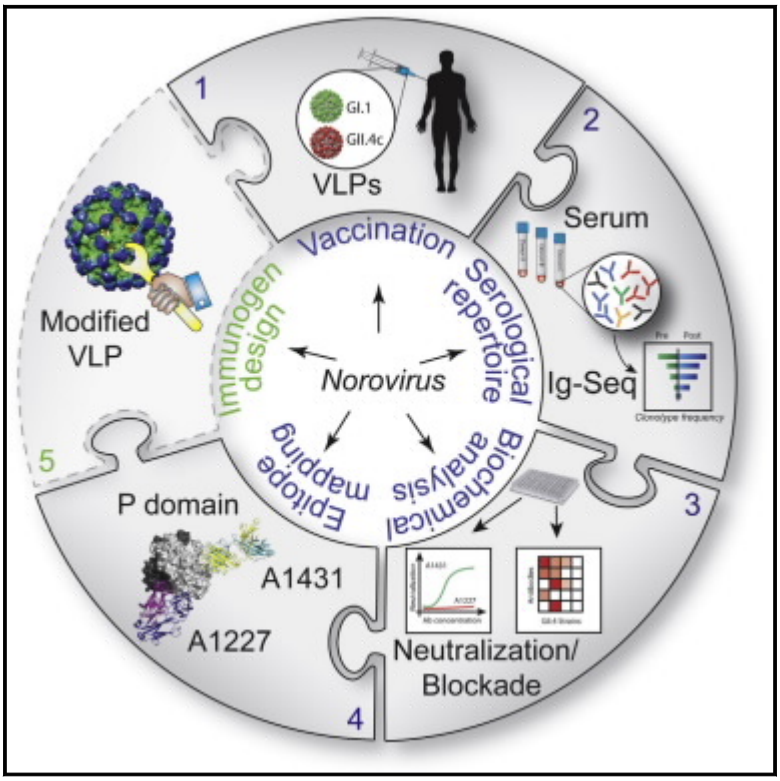
DOI:10.1021/acs.chemrev.6b00532
Chem. Rev. 2017, 117, 1915−1926

Article

Immunity

Sera Antibody Repertoire Analyses Reveal Mechanisms of Broad and Pandemic Strain Neutralizing Responses after Human Norovirus Vaccination

Graphical Abstract



Highlights

- Serum vaccine response is dominated by a small number of abundant antibody clonotypes
- Vaccine-boosted antibodies predominantly target conserved norovirus epitopes
- Identified cross-genogroup and strain-specific epitopes
- Discovered a pandemic-genotype neutralizing antibody recognizing a conserved epitope

Authors

Lisa C. Lindesmith, Jonathan R. McDaniel, Anita Changela, ..., Peter D. Kwong, George Georgiou, Ralph S. Baric

Correspondence

gg@che.utexas.edu (G.G.), rbaric@email.unc.edu (R.S.B.)

In Brief

Human norovirus (HuNoV) is a leading cause of gastroenteritis. Lindesmith et al. identify circulating serum antibodies following experimental HuNoV vaccination in humans and map them to viral epitopes. One antibody recognizes a neutralizing epitope conserved across three decades of pandemic strains and neutralizes virus in vitro, demonstrating that vaccination can elicit pandemic-strain neutralizing antibody responses in some individuals.

Lindesmith et al., 2019, Immunity 50, 1530–1541
June 18, 2019 a 2019 The Author(s). Published by Elsevier Inc.
https://doi.org/10.1016/j.immuni.2019.05.007

CellPress

Sera Antibody Repertoire Analyses Reveal Mechanisms of Broad and Pandemic Strain Neutralizing Responses after Human Norovirus Vaccination

Lisa C. Lindesmith, [1,7] Jonathan R. McDaniel, [2,7] Anita Changela, [3,7] Raffaello Verardi, [3,7] Scott A. Kerr, [4,7,9] Veronica Costantini, [6] Paul D. Brewer-Jensen, [1] Michael L. Mallory, [1] William N. Voss, [2] Daniel R. Boutz, [4] John J. Blazeck, [2] Gregory C. Ippolito, [5] Jan Vinje, [6] Peter D. Kwong, [3] George Georgiou, [2,4,5,8,*] and Ralph S. Baric [1,8,10,*]

[1]Department of Epidemiology, University of North Carolina, Chapel Hill, NC 27599, USA
[2]Department of Chemical Engineering, University of Texas at Austin, Austin, TX 78712, USA
[3]Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA
[4]Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, TX 78712, USA
[5]Department of Molecular Biosciences, University of Texas at Austin, Austin, TX 78712, USA
[6]Division of Viral Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA
[7]These authors contributed equally
[8]These authors contributed equally
[9]Present address: BioFire Inc, Salt Lake City, UT 84108, USA
[10]Lead Contact
*Correspondence: gg@che.utexas.edu (G.G.), rbaric@email.unc.edu (R.S.B.)
https://doi.org/10.1016/j.immuni.2019.05.007

SUMMARY

Rapidly evolving RNA viruses, such as the GII.4 strain of human norovirus (HuNoV), and their vaccines elicit complex serological responses associated with previous exposure. Specific correlates of protection, moreover, remain poorly understood. Here, we report the GII.4-serological antibody repertoire—pre- and post-vaccination—and select several antibody clonotypes for epitope and structural analysis. The humoral response was dominated by GII.4-specific antibodies that blocked ancestral strains or by antibodies that bound to divergent genotypes and did not block viral-entry-ligand interactions. However, one antibody, A1431, showed broad blockade toward tested GII.4 strains and neutralized the pandemic GII.P16-GII.4 Sydney strain. Structural mapping revealed conserved epitopes, which were occluded on the virion or partially exposed, allowing for broad blockade with neutralizing activity. Overall, our results provide high-resolution molecular information on humoral immune responses after HuNoV vaccination and demonstrate that infection-derived and vaccine-elicited antibodies can exhibit broad blockade and neutralization against this prevalent human pathogen.

INTRODUCTION

Human norovirus (HuNoV) is the leading cause of acute gastroenteritis, a disease responsible for an estimated 200,000 deaths per year, mostly in children less than five years old and the elderly (Collaborators, 2017; Patel et al., 2008). The human norovirus genome is a single-stranded, plus-sensed RNA with 3 open reading frames (ORFs), including an ORF2 major capsid protein (viral protein 1, VP1) composed of shell and protruding (P) domains. Expression of VP1 in insect and mammalian systems leads to self-assembly of 90 VP1 dimers into icosahedral virus-like particles (VLPs)that are antigenically indistinguishable from virions (Baric et al., 2002; Green et al., 1993; Jiang et al., 1995; Prasad et al., 1999). Despite more than 30 known human norovirus genotypes, 60% of norovirus outbreaks are caused by GII.4 genotype strains (Burke et al., 2019; Cannon et al., 2017). Efforts to develop an effective vaccine against human norovirus are complicated by the large number of antigenically distinct genotypes and the rapid evolution of GII.4 viruses (Atmar et al., 2018; Bull et al., 2007; Debbink et al., 2014a; Kocher et al., 2018; Lindesmith et al., 2008). Increased understanding of which capsid epitopes should be targeted to protect against different genotypes and of the impact of pre-exposure history on the elicitation of protective immunity after vaccination are important considerations in vaccine design and efficacy (Havenar-Daughton et al., 2018; Lindesmith et al., 2015, 2017; Snijder et al., 2018).

Antigenic drift within GII.4 norovirus strains has prompted serial pandemic outbreaks in 1995, 2002, 2006, 2009, and 2012, each correlating with the emergence of new GII.4 antigenic variants that appeared to have escaped pre-existing immunity (Debbink et al., 2012b; Lindesmith et al., 2008; Siebenga et al., 2007). Human norovirus attaches to the cell surface via binding to histoblood group antigens (HBGAs; typically A, B, O, or Lewis antigens) on mucosal surfaces of the gut (Lindesmith et al., 2003; Tan and Jiang, 2011). Extensive studies have established that antibodies that block norovirus VLP binding to HBGAs strongly correlate with protective immunity in chimpanzees and humans (Atmar et al., 2011; Bok et al., 2011; Malm et al., 2014; Reeck et al., 2010). Immune escape frequently relies on mutations at key residues in the immunodominant A, D, and E epitopes targeted by blockade

 2019 The Author(s). Published by Elsevier Inc.
This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

antibodies, with blockade measured by a surrogate neutralization assay based on the antibody-dependent inhibition of VLP binding to HBGAs (Debbink et al., 2012a; Harrington et al., 2002; Lindesmith et al., 2012a, 2012b). Carbohydrate ligand blockade assays are the primary functional assay used to predict serological immunity and represent the clinical endpoint for evaluating norovirus infection and vaccination outcomes (Atmar et al., 2011; Lindesmith et al., 2015, 2017; Malm et al., 2014; Reeck et al., 2010). Recent development of human intestinal enteroids (HIEs) as an *in vitro* system that successfully supports the replication of HuNoV also allows for direct evaluation of neutralization (Costantini et al., 2018; Ettayebi et al., 2016).

Multivalent VLP immunization broadens the breadth of blockade antibodies produced after immunization of mice (Debbink et al., 2014b; LoBue et al., 2006) and humans (Lindesmith et al., 2015). Currently, the leading human norovirus vaccine candidate is in phase IIb clinical trials (Bernstein et al., 2015; Leroux-Roels et al., 2018). This vaccine comprises a mixture of two VLPs: GI.1, the prototypical genogroup 1 strain, and GII.4c (GII.4 consensus), a VLP based on the consensus sequence of the GII.4 strains Houston (2002), Yerseke (2006a), and Den Haag (2006b) (Bernstein et al., 2015; Parra et al., 2012; Treanor et al., 2014). GII.4.2006a was used as the default sequence where all three of the strains diverged, most notably within the evolving immunodominant epitopes A, D, and E. The vaccine induces rapid (evident at day 7 post vaccination) antibody responses against both genogroup I and genogroup II VLPs (Lindesmith et al., 2015). The rapid blockade antibody response after vaccination suggests that the vaccine may activate a memory B cell or recall response in adults (Lindesmith et al., 2015), though the mechanisms governing this immune outcome have remained unknown.

Here, we report on the serological repertoire to the GII.4c VLP component of the bivalent human norovirus vaccine pre- and post-immunization. We determined the serological repertoire using immunoglobulin sequencing (Ig-Seq), a proteomics-based serum antibody repertoire analysis methodology (Georgiou et al., 2014; Lee et al., 2016; Williams et al., 2017). We focused on the GII.4c component of the vaccine because GII.4 strains have proven more clinically significant and display a higher rate of evolutionary drift, which potentially complicates the elicitation of protective immunity. Our results (1) provide clear evidence of the dominant effect of pre-existing immunity arising from earlier exposure on the humoral response to the vaccine; (2) define three classes of HuNoV circulating antibodies: one class comprising antibodies with very extensive binding breadth recognizing GI strains and GII strains but having no blockade activity, a second class that is specific to GII.4 and has blockade activity towards historical pandemic strains, and finally, a third class represented by one virus-neutralizing antibody with potent blockade activity towards historical GII.4 strains and contemporary strains that emerged well after the strains which were used to design the vaccine GII.4c VLP and human trials; (3) characterize in molecular detail VP1 epitopes that are targeted to enable broad binding or broad blockade with neutralizing activity, and (4) highlight the impact of pre-existing serological repertoire breadth and titers on the response to the vaccine.

RESULTS

The GII.4 HuNoV Serological Repertoire after HuNoV Bivalent Vaccination Is Highly Polarized and Shaped by Previous GII.4 Infection

We analyzed three donors that experienced a significant increase in GII.4 titer after immunization with the bivalent GII.4c + GI.1 VLP vaccine (Figure 1A; Table S1). The serological repertoire was delineated using Ig-Seq, a proteomic methodology in which serum antibodies are purified by affinity chromatography against an immobilized antigen—in this case, immobilized GII.4c VLP—then proteolytically digested into peptides and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Peptide spectral matches were obtained using a custom database of heavy-chain-variable (VH) genes encoded by peripheral B cells from the respective donor. To construct the VH database, peripheral blood mononuclear cells (PBMCs) collected at each time point were split into two aliquots: one aliquot was sequenced using high-throughput single-cell flow-focusing technology (DeKosky et al., 2015; McDaniel et al., 2016) to determine the native VH/VL (heavy-chain-variable/light-chain-variable) paired repertoire, and the second aliquot was processed to determine the VH repertoire. Relative quantitation of serum anti-GII.4c antibodies was determined from the LC peak intensity of high-confidence peptide-spectrum matches (PSMs) derived from peptides of the third complementarity determining region of the heavy chain (CDR-H3). Antibodies belonging to a CDR-H3 clonotype likely recognize the same epitope, given the dominant role of CDR-H3 in antigen recognition. As we established earlier, the Ig-Seq workflow identifies the majority (>70%) of the most abundant antigen-specific antibodies in the serum, and quantitation calibrations using isobaric peptide spike-ins show that peak intensities correlate well with absolute peptide concentrations (Lavinder et al., 2014). The antibody repertoire composition and relative quantities at the clonotypic level are plotted as a histogram with each bar representing an individual clonotype and the horizontal axis showing its relative abundance (Figure 1B). All three donors had a titer to GII.4c at day 0, presumably a consequence of prior exposure to GII.4 infection and consistent with earlier data indicating that the vast majority of asymptomatic adults have antibodies to HuNoV (Carmona-Vicente et al., 2015; Nurminen et al., 2011). As has been the case with the serological repertoire to other antigens reported so far (e.g., influenza hemagglutinin [Lee et al., 2016], tetanus toxoid [Lavinder et al., 2014], or desmoglin in pemphigus patients [Chen et al., 2017]), the repertoire to GII.4c was found to be oligoclonal and highly polarized, in that <3 antibodies dominated the response, contributing more than 50% of the antigen-specific titer (Figure 1B). In particular, at day 0, the dominant CDR-H3 clonotype in each repertoire accounted for between 58% and 86% of the total CDR-H3 peptide intensity, suggesting that the long-term serological memory to GII.4 is dominated by a single antibody. Other examples of highly skewed serological repertoire polarization with one clonotype accounting for >60% of the response have been reported previously (Lee et al., 2016).

Antibody clonotypes present in both the pre-vaccination repertoire (day 0) and the post-vaccination repertoire were defined as "pre-existing," whereas clonotypes observed exclusively in the post-vaccination repertoire were defined as "emergent." The term "emergent antibodies" refers only to the

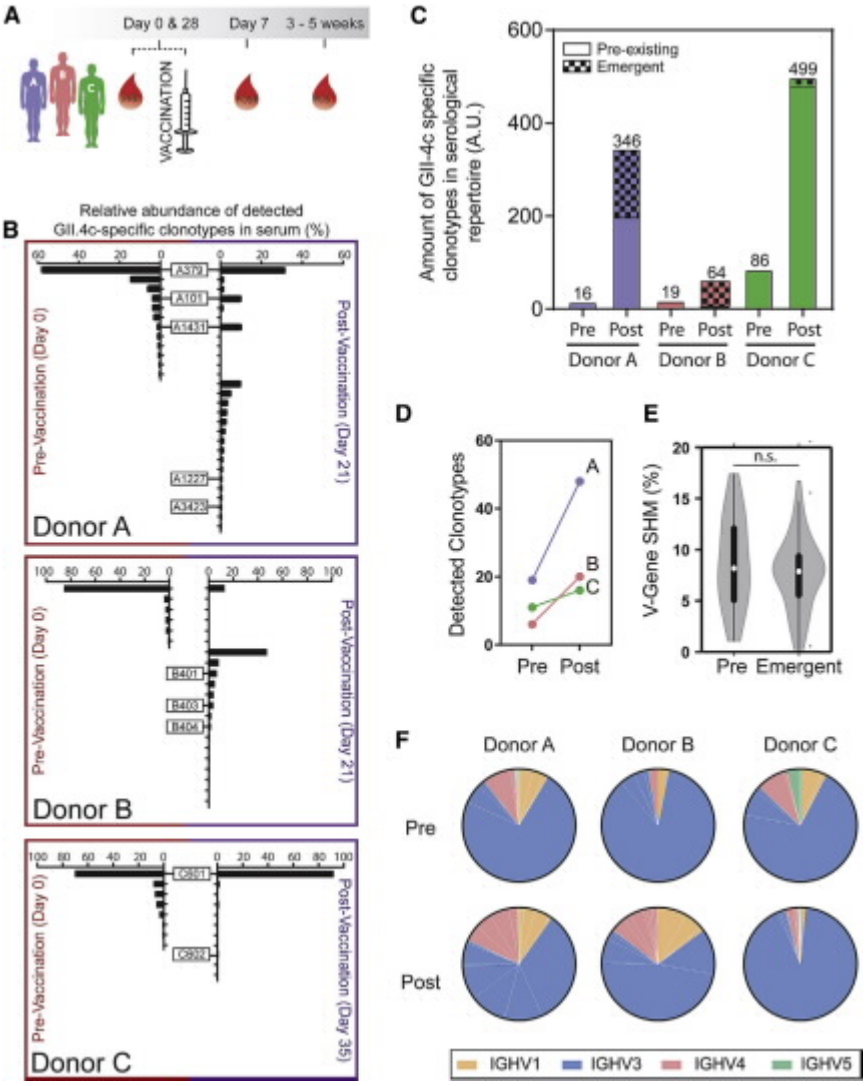


Figure 1. The Anti-NoV GII.4c IgG Serum Antibody Repertoire following Vaccination Is Polarized and Shaped by Previous HuNoV Infection

(A) Donors (n = 3) were immunized with experimental HuNoV vaccine and serum and PBMCs were collected at the times indicated.

(B) Relative abundance of GII.4c-specific IgG clonotypes pre- and post-vaccination. Each bar represents a CDR-H3 antibody clonotype observed at a 0.5% of the GII.4c antigen-specific antibody repertoire.

(C) Amount of anti-GII.4c serum antibodies (i.e., the product of anti-GII.4c IgG ELISA titer 3 relative antibody abundance as determined by LS-MS/MS; see STAR Methods) pre- and post-vaccination; ELISA titer shown over each bar.

(D) Number of detected antibody clonotypes before and after vaccination.

(E) Anti-GII.4c antibody V-gene somatic hypermutation rates for pre-existing and emergent antibodies; n.s. represents not statistically significant (two-tailed t test; $p > 0.3$).

(F) V-gene family usage in the serological repertoire. Related to Table S1.

fact that they become detectable in the serum post vaccination and should not be interpreted in terms of B cell ontogeny (i.e., whether they arise from stimulated naive cells or memory B cells). Although vaccination increased the diversity of the serological repertoire in all donors, both the number and abundance of vaccine-elicited clonotypes were substantially lower in donor C, who had a 4- to 5-fold higher titer to GII.4c before vaccination (day 0) compared to the other two donors (Figures 1B–1D). In this donor, the increase in the GII.4c titer 4 weeks post vaccination was overwhelmingly due to the boosting of the dominant antibody clonotype C601, which accounted for >90% of the serum response and increased in absolute abundance by more than 7-fold relative to the level observed at day 0. The low level of emergent antibodies in donor C, who had the highest pre-vaccination titer, is consistent with the strong inverse correlation between day 0 serum titer and the number of emergent antibody clonotypes in the serological memory after immunization with the seasonal influenza vaccine (Lee et al., 2016).

Pre-existing and emergent antibodies displayed similar somatic hypermutation profiles (Figure 1E). Although emergent antibodies (7.1 ± 0.5% mean rate of somatic hypermutation) were slightly less mutated than the pre-existing antibodies (8.2 ± 1.1%), this difference did not reach statistical significance. The anti-GII.4c serological repertoire was predominantly comprised of antibodies using the immunoglobulin heavy variable 3 (IGHV3)family (80%; Figure 1F) compared to 30% in naive or mature B cells (DeKosky et al., 2016; Mroczek et al., 2014). These findings suggest that the serological IgG response to human norovirus vaccination is largely dominated by the boosting of a limited number of pre-existing antibody clonotypes.

Recombinant HuNoV Serum Antibodies Demonstrate Neutralization against GII.4 and Breadth of Binding across Strains

We expressed and characterized a set of 10 representative serum antibodies for which both VH and VL sequences were detected by paired VH/VL sequencing. These included the top most abundant clonotypes in donors A and C (A379 and C601), two strongly boosted pre-existing clones (A101 and A1431, boosted by 123 and 1263 , respectively, in terms of abundance in the post-vaccination serum relative to day 0), and six emergent antibodies (Figure 1B). Binding and blockade activity were tested using an antigenically diverse panel of norovirus genogroup I and genogroup II VLPs, including a time-ordered panel of pandemic GII.4 VLPs representing historical variant strains, including US95_96 (GII.4 1997), Farmington Hills (GII.4 2002), and Den Haag (GII.4 2006b), as well as prospective strains that emerged following vaccine design (2006) and the

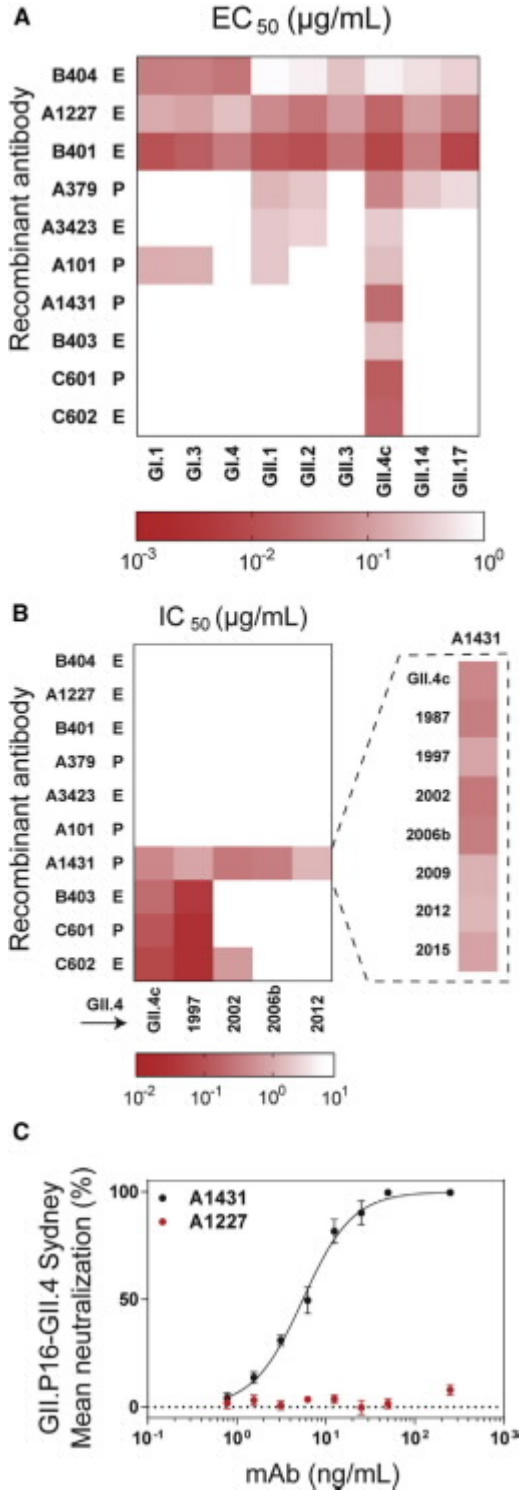


Figure 2. HuNoV Recombinant Serum Antibodies Recognize Conserved Binding, Ligand-Blocking, and GII.4 Neutralizing Epitopes

Sigmoidal dose response curves were fit to the mean percent control binding for determination of (A) half-maximum binding (EC_{50}) or (B) half-maximum blocking of HBGA binding (half-maximum inhibitory concentration, IC_{50}) of pre-existing (P) and emergent (E) antibodies. Titers >2 mg/mL were scored as negative (white box). The HBGA blocking assay was used to characterize A1431 against a panel of pandemic norovirus GII.4 strains. Antibodies were tested in duplicate in a minimum of two independent experiments. (C) Sigmoidal dose response curves were fit to the mean percent viral genomic copies control for determination of IC_{50} in a neutralization assay. The neutralization assay was used to characterize A1431 and A1227 against the GII.P16-GII 4 Sydney strain. Error bars represent mean ± SEM from four independent experiments with 2 technical replicates for each dilution. Related to Figure S1.

clinical trial (2010), such as GII.Pe-GII.4 Sydney (GII.4 2012) and GII.P16-GII.4 Sydney (GII.4 2015) (Lindesmith et al., 2018a). As expected, all antibodies bound to the vaccine strain VLP (Figure 2A), with half-maximal effective concentration (EC_{50}) values between 8 and 717 ng/mL. The potential for neutralization by inhibition of cellular ligand binding was evaluated by determining blockade of VLP-ligand binding (blockade assay) (Figure 2B). The 10 serum antibodies could be categorized into three classes. The first class consisted of 6 antibodies that displayed broad binding but no blocking activity toward multiple GI and GII strains; three of these, namely A1227, B401, and B404, showed binding to all strains tested, including a variety of GI and GII genotypes such as GI.I, GII.2, and GII.17. The VP1 protein among these strains shows only 50% amino acid identity (Zheng et al., 2006). The second class consisted of antibodies B403, C601, and C602, which had strong blockade activity towards the vaccine strain and the earlier pandemic GII.4 1997 strain but not to GII.4 strains that emerged post vaccination. Finally, the third class of serum antibodies had a single member, A1431, which had strong blockade activity to all known historical GII.4 strains from 1987 onwards. A1431 had very extensive breadth and potency not only towards all historical strains available but also towards prospective GII.4 strains that appeared after the vaccine trial, including GII.4 2012 and 2015, neither of which had circulated in the general population at the time of sample collection (Figure 2B). Furthermore, A1431 potently neutralized GII.P16-GII.4 Sydney virus, a GII.4 strain that emerged 5 years post sample collection, in a human intestinal enteroid culture system (Costantini et al., 2018; Ettayebi et al., 2016) (IC_{50} 5.5 ng/mL), supporting the correlation between blockade of ligand-binding potency and antibody-mediated neutralization (Figures 2C and S1) (Alvarado et al., 2018; Ettayebi et al., 2016).

We sought to define biochemically the epitopes recognized by the antibodies with blockade activity to historical strains, namely B403, C601, and C602; as discussed below, the epitope of A1431 that has broad blockade breadth was determined by crystallography. We first evaluated antibody mediated blockade against a panel of VLPs with epitopes A, D, and E exchanged from GII.4 2012 (non-binding VLP) into GII.4 1987 (binding VLP). Exchange of epitope A residues ablated blockade potency of B403 and diminished potency of C601 by 13.5-fold but had little effect on C602 blockade (1.8-fold decrease). Exchange of epitope D or E residues had little impact on blockade of any of the three antibodies, suggesting that the major binding determinants for B403 and C601 likely reside within epitope A (Figures 3A–3C). VLPs displaying an acidic residue at 298, Val at position 356, and His at 357 had lower EC_{50} titers than VLPs without these residues for all three antibodies (Figures 3B and 3C). Exchange of V298E/A356V/E357H into the null backbone VLP 581 (Lindesmith et al., 2018b) (VLP 581.A4A) gained binding of B403 and C601 and improved binding of C602 by 11.6-fold compared to

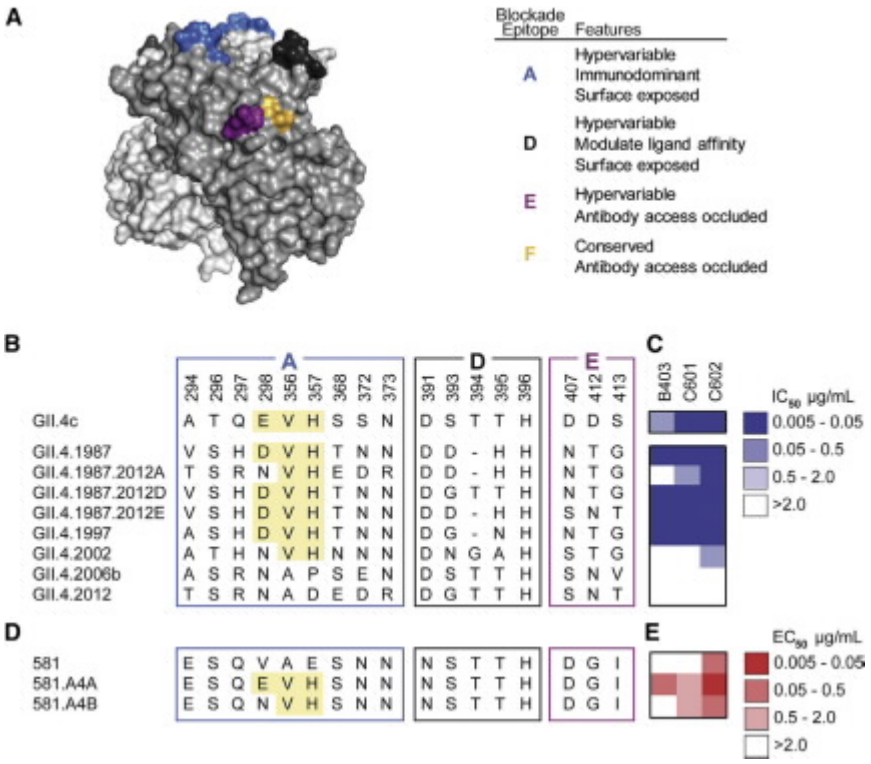


Figure 3. Serum Antibodies that Block Historical GII.4 Strains Recognize Overlapping Epitopes within Epitope A

(A) Homology model based on PDB: 3SLD (Shanker et al., 2011) of the GII.4 2006a P domain dimer of the HuNoV major capsid protein, VP1 with known P domain blockade antibody epitopes A (blue), D (dark gray), E (purple), and F (orange) highlighted.

(B) Amino acid sequences of pandemic strains and select mutants of GII.4 1987.

(C) Blockade potency binned by IC_{50}.

(D) Select mutants of GII.4 2006a.581 VP1. Highlighted areas indicate sequences important for antibody binding.

(E) Antibody binding binned by EC_{50} values. Antibodies were tested in duplicate in a minimum of two independent experiments.

the 581 VLP (Figures 3D and 3E). Substitution of V298N/A356V/E357H (VLP 581.A4B) did not gain binding for B403, yielded similar binding to that to 581.A4A for C601, and decreased C602 binding by 7.6-fold compared to 591.A4A. These data confirm E or D at position 298 as an anchor residue for B403. For antibody C601, binding was heavily influenced by V356/H357, residues that are predicted to contribute to epitope A but have not been previously verified. Further, our analysis also revealed that C602 recognizes a previously undefined blockade epitope. Together, these data categorize antibodies boosted by HuNoV VLP vaccination into three classes: (1) genogroup I and genogroup II broadly binding, non-neutralizing antibodies; (2) ancestral GII.4 binding and ligand-blocking antibodies; and (3) GII.4 broadly binding and neutralizing antibodies. Genetic mapping indicated that antibodies displaying a limited breadth of GII.4 ligand-blockade potency (class 2 above) recognized the immunodominant epitope A antigenic site (Mallory et al., 2019) and accounted for previously observed back-boost antibody responses in HuNoV vaccinated donors (Lindesmith et al., 2015). These data demonstrate that HuNoV vaccination can elicit serum antibodies capable of neutralizing GII.4 pandemic strains.

Structural Studies Define a Highly Conserved Non-Blocking Epitope and a Conserved GII.4 Neutralizing Epitope within the Capsid P Domain

To gain insight into the molecular mechanisms underlying cross-reactive, non-blockade activity, we selected the pan-reactive antibody A1227 for crystallographic analysis. We purified the antigen-binding fragment (Fab) of A1227 in complex with soluble GII.4 P domains from 2002, 2006, 2009, or 2012 pandemic strains and screened for crystallization. Complexes with the GII.4.2002 P domain (P2002) yielded diffracting crystals suitable for structural analysis.

Single crystals of the complex between A1227 and P2002 diffracted to 2.6 Å, and the structure was solved by molecular replacement using the apo P2002 structure (PDB: 4OOV) (Singh et al., 2015) (Table S2). A1227 Fab bound to each of the conserved P1 subdomains of the P2002 dimer (Figure 4A). Each Fab bound at the interface between monomers in the P2002 dimer, with the heavy chain recognizing both protomers and the light chain recognizing only a single protomer (Figure 4B). A1227 recognized a region of P2002 which was mostly conserved across genogroups GI and GII (Figures 4C and S2), thus explaining the broad cross-reactivity of antibody A1227. Overall, each Fab buried 950 $Å^2$ by establishing polar contacts and favorable van der Waals interactions with P2002 (Figures 4D and S2). There were no substantial conformational changes upon A1227 binding to P2002, with a root-mean-square deviation (RMSD) of 0.6 Å between apo-P2002 and A1227-P2002 complex structures.

To map the A1227 epitope within the context of the VLP, the crystal structure of the A1227-P2002 complex was superimposed onto the GI.1 capsid structure (PDB: 1IHM) (Prasad et al., 1999). The GI.1 capsid structure was chosen as a template for modeling due to similarities between GI.1 and GII.4 capsid structures indicated by prior electron microscopy (EM) studies (Chen et al., 2004). Modeling revealed that the conserved region recognized by A1227 was located at an occluded site on the norovirus capsid, at the interface between the P domain and shell domain. Overlay of Fab A1227 onto the GI.1 capsid structure (Figures 4E and 4F) indicated that other P domains on the assembled norovirus capsid would clash with both heavy and light chains of the Fab, with only minor clashes with the shell domains (Figure 4G), suggesting that structural rearrangements of the P domain could either expose the A1227 binding site or allow binding at a neighboring P domain. Its lack of blockade or neutralizing activity likely results from antibody A1227 only recognizing defects or maturation intermediates (Pogan et al., 2018).

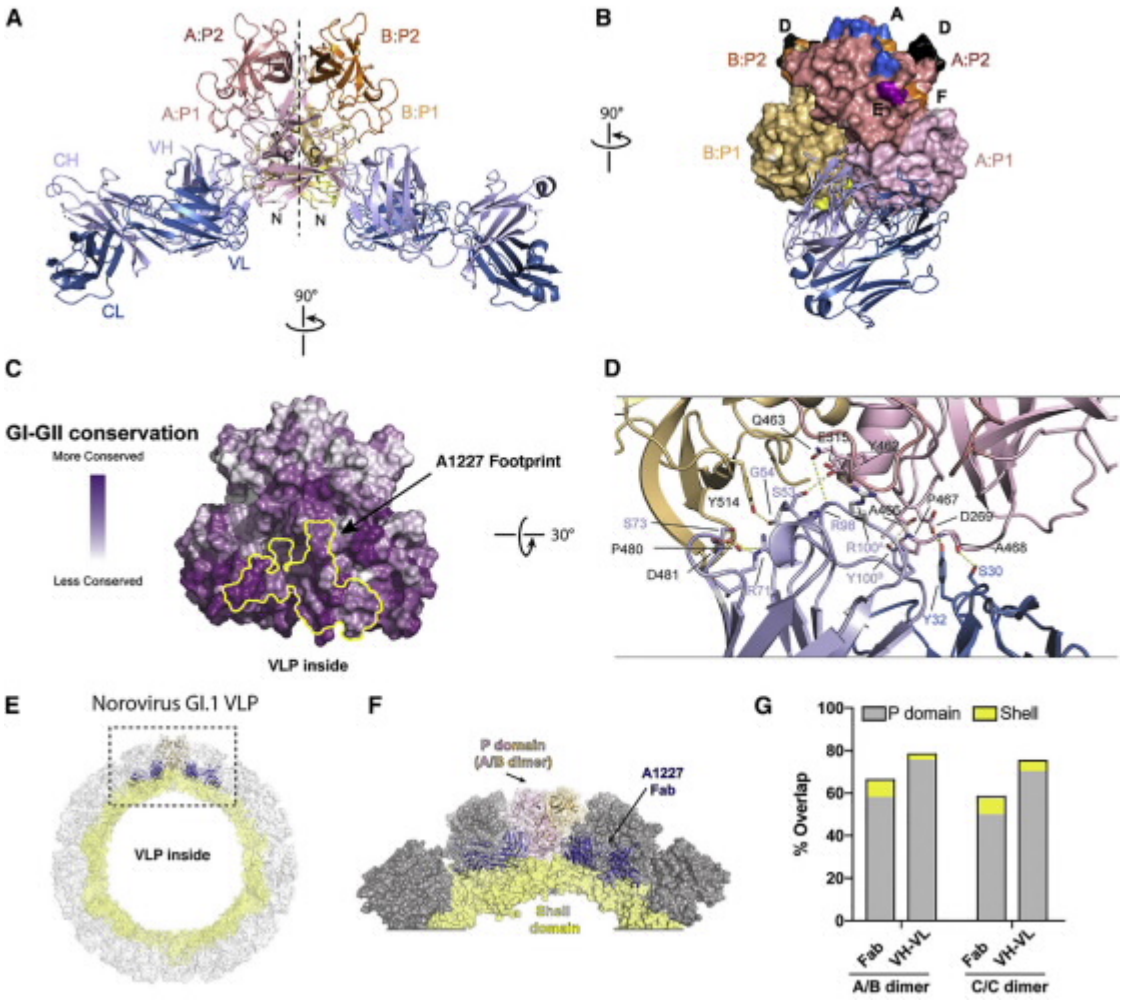


Figure 4. The Broadly Binding Non-Blockade Antibody A1227 Binds to a Highly Conserved Epitope within the P Domain

(A) Crystal structure of A1227 Fab bound to GII.4.2002 P domain dimer shown in cartoon representation, with P domain monomers colored pink and orange and A1227 heavy and light chains in gray and blue, respectively.

(B) The A1227 antibody binds near the P1 dimeric interface (P domain dimer in surface representation and A1227 Fab in cartoon).

(C) A1227 Fab footprint is outlined in yellow on the P domain surface, mapped by GI-GII sequence conservation, with degree of conservation colored from variable (white) to conserved (purple).

(D) Detailed view of A1227 Fab-P domain binding interface, with interacting residues depicted in stick and hydrogen bonds shown in yellow dotted lines.

(E) Modelling of the GII.4.2002 P domain-A1227 Fab crystal structure onto the GI.1 capsid structure (PDB: 1IHM) reveals an antibody binding site that is occluded on the assembled VLP.

(F) Close-up view from (E) showing the occlusion of A1227 Fab within the GI.1 capsid.

(G) Quantification of volume overlap between A1227 Fab (or VH/VL) and GI.1 capsid proteins. Over 60% of the volume of A1227 Fab overlaps with neighboring P domains when modelled onto either A/B or C/C dimers. Only small overlaps are due to clashing with shell domains. Overlaps from P domain and shell domain are colored in gray and yellow, respectively. Volumes were calculated using Voss-Void-Voxelator program (Voss and Gerstein, 2010).

Related to Table S2 and Figure S2.

To provide a structural basis for recognition by the GII.4 broad blockade antibody A1431, we solved the crystal structure of A1431 Fab in complex with the GII.4.2002 P domain at 3.1 Å resolution (Table S2). The asymmetric unit contained two P-domain-A1431 Fab complexes, with each dimer generated by crystallographic 2-fold symmetry. Both complexes were nearly identical, with an RMSD of 0.3 Å. Electron density for both P2002 and A1431 variable domains was well defined, although regions of A1431 constant domains were not well ordered.

Antibody A1431 bound each P domain monomer at a cleft between the P1 and P2 subdomains (Figure 5A). Similar to A1227, A1431 binding did not induce substantial conformational changes in the P domain, with an overall RMSD of 0.4 Å between the complex and apo (PDB: 4OOV) structures. Antibody A1431 recognized an epitope located near previously mapped blockade epitopes D and F and distinct from the site of HBGA binding (Figure 5B). Both A1431 heavy and light chains contributed to the binding interface with a total buried surface area of 985 $Å^2$ on the P domain. The CDR-H3 loop of A1431 was

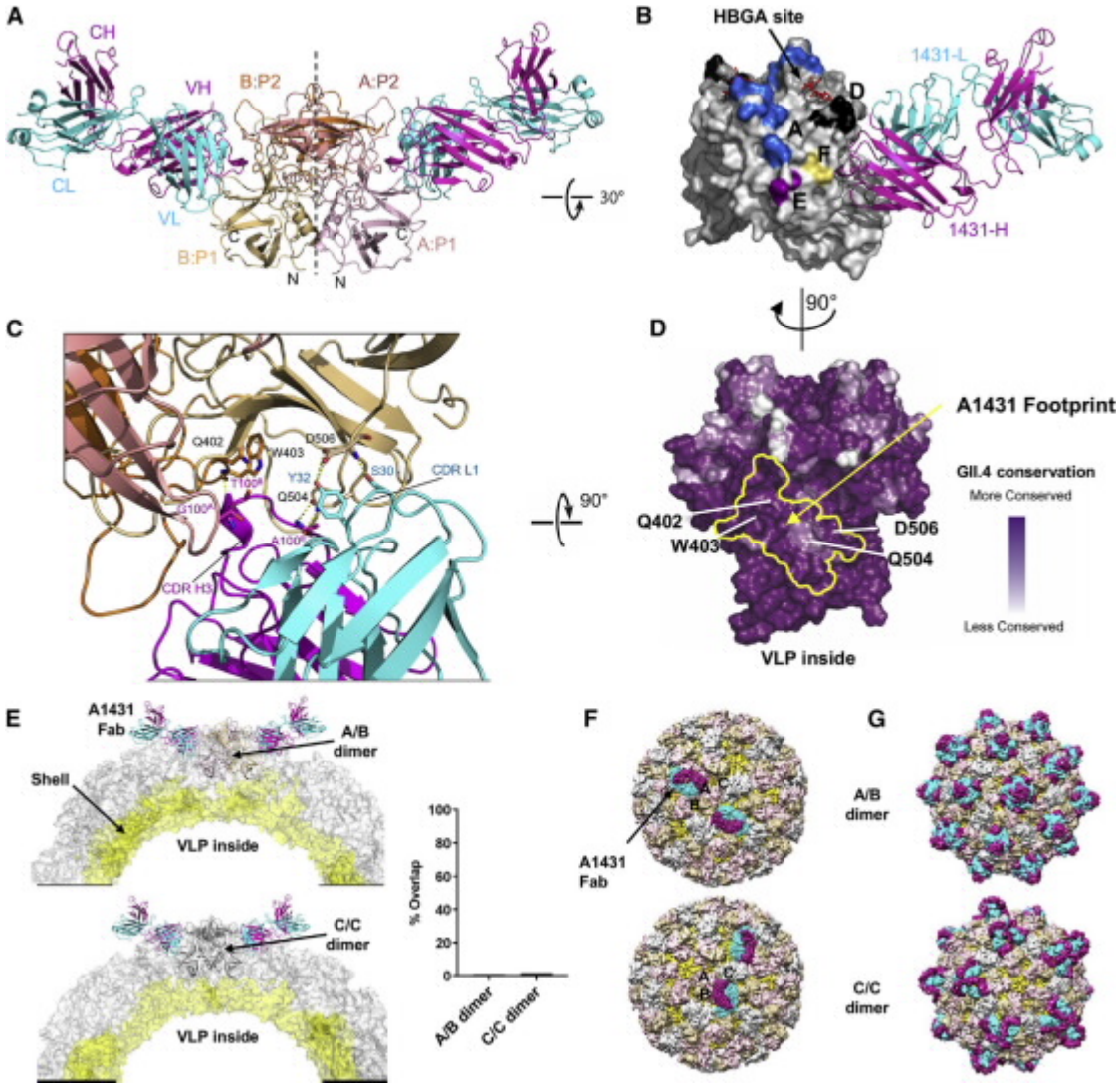


Figure 5. Broad GII.4-Blockade Antibody A1431 Recognizes an Accessible Semi-Conserved Site of Vulnerability

(A) Overall structure of A1431 Fab bound to GII.4.2002 P domain dimer is shown in ribbon representation, with P domain monomers in pink and orange and A1431 heavy and light chains in magenta and cyan, respectively.

(B) Mapped blockade epitopes are highlighted on a surface representation of GII.4.2002 P domain dimer (colored as in Figure 3A) with the A1431 Fab shown in cartoon. The crystal structure of GII.4 (Farmington Hills, 2004) P domain complexed to HBGA type B ligand (PDB: 4X05) was superimposed onto the GII.4.2002 P domain + A1431 complex to show location of HBGA binding site.

(C) Details of A1431 Fab interactions with P domain residues are highlighted with interacting residues depicted in stick and hydrogen bonds shown in yellow dotted lines.

(D) A1431 Fab footprint is outlined in yellow on the P domain dimer surface, mapped by GII.4 sequence conservation, with degree of conservation colored from variable (white) to conserved (purple).

(E) The GII.4.2002 P domain-A1431 Fab crystal structure is modeled onto the GI.1 capsid structure (1IHM; Prasad et al., 1999), with a cross-section showing the P domain fit onto the A/B dimer (top) or the C/C dimer (bottom). Overlap between A1431 Fab and P domain, as modeled onto either GI.1 capsid A/B or C/C dimers, is minimal and shown on right, as in Figure 4G.

(F) Modeling of A1431 Fab to the A/B dimer (top) or C/C dimer (bottom) on the GI.1 capsid structure, with A/B dimers colored pink and tan, C/C dimers colored in gray and shell domain shown in yellow.

(G) GI.1 capsid models, colored as in (F), showing occupancy of A1431 binding sites on all A/B dimers (top) or all C/C dimers (bottom).

Related to Table S2 and Figure S3.

inserted into a cleft between the P1:P2 subdomains and was held in place by hydrogen bonding between main-chain atoms of the CDR-H3 and P domain residues Gln402, Trp403, and Gln504 (Figure 5C). Ser30 and Tyr32 from the CDR-L1 loop provide additional stabilization via hydrogen bonds to Asp506 on the P1 subdomain. The recognized surface, including P domain residues Gln402, Trp403, Gln504, and Asp506, was nearly completely conserved in GII.4 (Figure 5D) but only semi-conserved between GI and GII genogroups, with Asp506 changed to Pro506 in most other GI and GII strains (Figure S3),

providing the structural basis for the broad recognition of A1431 of GII.4 viruses but not of other norovirus genotypes.

To gain insight into the molecular mechanism of blockade activity by antibody A1431, we superimposed the crystal structure of the P2002-A1431 Fab complex onto the GI.1 capsid structure (Figure 5E). On the assembled VLP, the P domain dimer exists in two distinct structural environments, defined by A/B and C/C dimers (Prasad et al., 1999). A1431 Fab binding to the A/B dimer showed no overlap between Fab and either P or S domains, whereas binding to the C/C dimer showed minor overlap (Figures 5E and 5F). Binding of the A1431 Fab to all equivalent sites on the VLP showed recognition at the A/B dimer to cover most of the VLP surface, whereas recognition of the C/C dimer left the 5-fold vertices potentially capable of attaching to HBGAs at the cell surface (Figure 5G). In both cases, binding at all sites would lead to substantial antibody-antibody overlap (Figures 5F and 5G), suggesting that A1431 exerts its blockade and neutralizing activity by binding to only a fraction of potential binding sites on the VLP. Overall, in contrast to the A1227 binding site, modeling suggested the A1431 blockade epitope to be mostly accessible on the capsid surface. Notably, the A1431 epitope did not overlap with identified sites of HBGA recognition, and therefore, the observed blockade functionality likely stems either from antibody A1431 sterically inhibiting the approach of the VLP to the >500 kDa multivalent HBGAs found on MUC2 in PGM and in human gastric and duodenal tissues (Park et al., 2015) or from subtle allosteric effects, as reported for another antibody that docks near to the 1431 binding site (Lindesmith et al., 2014, 2018b), as defined by epitope mapping. High-resolution structures of broadly reactive and/or neutralizing epitopes expand our understanding of the HuNoV antigenic landscape and narrow the search for optimal vaccine candidates. For instance, whereas antibodies targeting hypervariable P2 epitopes (i.e., epitope A) have limited cross-strain ligand-blocking potency and antibodies recognizing highly conserved epitopes near the P1-shell-domain interface are highly cross-reactive but have no blocking potency, the antibodies targeting genotype-conserved epitopes at the P1-P2 interface display broad ligand-binding blocking potency. It should be noted that each of these antibody classes may contribute to vaccine-induced protection, and further studies and *in vivo* models are required to fully understand the interplay between the antibody targets and the physiological response.

DISCUSSION

Vaccine-induced immunity relies on the development and maintenance of long-term protective antibody titers or serological memory. The best studied correlate for protective immunity after HuNoV infection is the development of robust blockade antibody responses (Lindesmith et al., 2015; Malm et al., 2014; Reeck et al., 2010). Immune responses to rapidly evolving viral infections are complex and heavily impacted by pre-exposure histories that shape the antibody response to newly evolved strains (Belongia et al., 2017; Gostic et al., 2016; Lessler et al., 2012; Lindesmith et al., 2017). These studies demonstrate that bivalent HuNoV vaccines can elicit GII.4 neutralizing antibody responses in humans, while also structurally defining the highly conserved GII.4 (1987–2015)epitope recognized by a neutralizing antibody.

Here, we demonstrate that the serological repertoire to HuNoV is oligoclonal and dominated by IGHV3 family antibodies in three pre-immune individuals who received the vaccine. A large fraction (6/10) of the serum antibodies that we biochemically characterized in detail displayed significant binding breadth, with 4/6 recognizing both GI and GII genotype VLPs. Although these high-binding-breadth antibodies did not have blockade activity, they may play a role in protection against HuNoV infection via Fc-mediated mechanisms. Non-neutralizing antibodies that protect against infection in animal models are observed at significant levels in circulation after influenza vaccination (Lee et al., 2016), and recent studies report the isolation of such antibodies for other viruses as well (Bootz et al., 2017; Henry Dunand et al., 2016). Current technologies do not allow direct evaluation of HuNoV Fc-mediated protection.

In donor A, we showed that the most dramatically back-boosted blockade antibody following vaccination is capable of blocking VLP-HBGA binding of all examined GII.4 strains circulating between 1987 and 2015 and is potently neutralizing in the human intestinal enteroid culture system. This antibody was present in appreciable amounts in the serum prior to vaccination, suggesting that it must have been elicited by an earlier HuNoV infection. Although lack of successful propagation of historic GII.4 strains limits our ability to test the breadth of GII.4 neutralization, all tested blockade monoclonal antibodies have also been neutralizing antibodies (Alvarado et al., 2018), indicating that A1431 is also likely cross-GII.4 neutralizing. These findings reveal that HuNoV infection can induce broad blockade and neutralizing antibodies and that these antibodies can be boosted by vaccination (Lindesmith et al., 2012a, 2015).

A1431 is a broad blockade neutralizing human antibody for which the binding site has now been structurally defined. The structures of antibodies A1431 and A1227 in complex with the P domain described here, as well as structures of the GI.1-specific, HBGA-blocking 5I2 IgA (Shanker et al., 2016) and of the broadly GII-reactive 5B18 IgG (Hansman et al., 2012) described previously, support multiple mechanisms of antibody-mediated recognition and potentially protection from norovirus infection (Koromyslova et al., 2018). The most abundant ligand-blocking antibodies recognize hypervariable surface-exposed epitopes that are frequently mutated as a result of antigenic drift (Debbink et al., 2012a; Lindesmith et al., 2013, 2015). Antibody binding of these epitopes blocks the HBGA binding pocket (Shanker et al., 2016). In contrast, A1431 recognizes conserved residues distal to the HBGA binding pocket but accessible on the assembled VLP. Blocking of ligand binding and neutralization likely results not from direct interference at the ligand-binding site but rather from either prevention of attachment to the ligand-displaying surface (i.e., steric effects) or modulation of capsid dynamics (Lindesmith et al., 2014, 2018b). We note that the unprecedented blockade breadth of A1431 may be useful for therapeutic purposes, as currently there are no treatment options for chronic infection or life-threatening HuNoV infection (Ahmed et al., 2012; Cardemil et al., 2017; Petrignani et al., 2018). The co-crystal structure of A1431 with the P domain potentially provides avenues by which this antibody may be further improved for breadth and potency (Diskin et al., 2011; Kwon et al., 2018). Antibodies like A1227 with broad cross-genogroup binding have not yet been shown to neutralize or block VLP binding to

HBGAs. These antibodies likely bind to occluded epitopes available for antibody access during phases of particle assembly and/or disassembly, intermediates, or rearrangements. Although mechanisms of protection driven by this class of antibodies are undefined, short-term boosting of cross-blockade antibodies after vaccination (Lindesmith et al., 2015) and mouse norovirus (MNV) monoclonal antibodies with cross-serotype neutralization (Kolawole et al., 2017) support the potential for HuNoV cross-genotype neutralization.

These data provide structural and mechanistic evidence supporting that the GII.4 component of the Takeda multivalent norovirus VLP vaccine recalls a memory antibody response that persists for at least six months, indicating that, similar to other rapidly evolving RNA viruses, early-life norovirus strain exposure shapes antibody responses to subsequent norovirus strains (Lindesmith et al., 2015, 2017). As with influenza, exposure history and genetic background are expected to impact outcomes differently by age groups, potentially impacting vaccine efficacy (Gostic et al., 2016; Lessler et al., 2012). Future studies are needed to determine additional mechanisms of the antibody boost to prospective GII.4 strains, as well as the antibody response in key vaccine target populations, including the aged, with a life-time of norovirus exposure but waning immune responsiveness, and the very young, with no pre-exposure history and possibly with maternal antibodies. These data are critical for understanding vaccine performance and population-based immunity against future pandemic isolates. Cross-blocking antibodies like A1431 provide one mechanism of breadth, and serological repertoire analyses offer robust strategies to dissect the molecular basis for this and other responses in human populations. Coupled with blockade, neutralization potency in the enteroid culture system (Costantini et al., 2018; Ettayebi et al., 2016), and structural determination of antibody binding sites, recombinant serum antibodies can provide invaluable information for immunogen design and vaccination strategies that phenocopy infection and provide broad, long-term protection to HuNoV pandemics.

STAR+ METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCESTABLE
- CONTACT FOR REAGENTAND RESOURCESHARING
- EXPERIMENTALMODEL AND SUBJECT DETAILS
  - Humans Subjects
  - Cell lines
  - Microbes
- METHOD DETAILS
  - Ten percent stool filtrate
  - Human intestinal enteroid culture (HIE)
  - High-throughput sequencing of BCR transcripts
  - VH Sequencing
  - VH/VL Sequencing
  - Isolation of HuNoV specific antibodies
  - Sample preparation and LC-MS/MS analysis
  - Antibody expression and purification
  - Production of Virus Like Particles
  - Antibody functional assays
  - P domain expression and purification
  - Crystallization of Fab complex with P2002
  - Structure determination and analysis
  - Neutralization Assay
- QUANTIFICATIONAND STATISTICALANALYSIS
- DATA AND SOFTWAREAVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.immuni.2019.05.007.

ACKNOWLEDGMENTS

The authors would like to thank Takeda Vaccines for provision of human samples; the Genome Sequencing and Analysis Facility at the University of Texas at Austin for performing Illumina next-generation sequencing; the Center for Biomedical Research Support Proteomics Facility at the University of Texas at Austin for providing mass spectrometry analysis; and Victoria Madden of Microscopy Services Laboratory, Department of Pathology and Laboratory Medicine, University of North Carolina–Chapel Hill for expert technical support. Support for this work was provided by the Intramural Research Program of" the Vaccine Research Center, NIAID, NIH, and the intramural food safety program at CDC and by grants from the NIH (R56AI106006, G.G. and R.S.B. and U19 AI109761 CETR, R.S.B.), Defense Threat Reduction Agency (DTRA) (contract HDTRA1-12-C-0105, G.G.), and the Wellcome Trust [203268/Z/16/Z] (R.S.B.). Use of sector 22 (Southeast Region Collaborative Access team) at the Advanced Photon Source was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Science, under contract number W-31-109-Eng-38. The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

AUTHOR CONTRIBUTIONS

Conceptualization: R.S.B., G.G., L.C.L., J.R.M., A.C., R.V., S.A.K., and P.D.K. Investigation and Formal Analysis: L.C.L., J.R.M., A.C., R.V., S.A.K., V.C., P.D.J.B., M.L.M., W.N.V., D.R.B., J.J.B., G.C.I., and J.V. Writing, All Drafts: L.C.L., J.R.M., A.C., R.V., P.D.K., G.G., and R.S.B. with input from all authors. Funding Acquisition and Supervision: R.S.B., G.G., and P.D.K.

DECLARATION OF INTERESTS

J.R.M., S.A.K., A.C., R.V., V.C., P.D.B.J., M.L.M., W.N.V., D.R.B., J.J.B., G.C.I., J.V., P.D.K., and G.G. declare no competing interests. L.C.L. and R.S.B. have consulted and collaborated with Takeda on norovirus vaccines. They are also inventors on patents filed on issues relevant to noroviruses. G.G. is an inventor on patents related to the analysis of the serological antibody repertoire.

Received: January 3, 2019
Revised: March 15, 2019
Accepted: May 15, 2019
Published: June 13, 2019

SUPPORTING CITATIONS

The following references appear in the Supplemental Information: Word et al. (1999).

REFERENCES

Adams, P.D., Gopal, K., Grosse-Kunstleve, R.W., Hung, L.W., Ioerger, T.R., McCoy, A.J., Moriarty, N.W., Pai, R.K., Read, R.J., Romo, T.D., et al. (2004).

Recent developments in the PHENIX software for automated crystallographic structure determination. J. Synchrotron Radiat. *11*, 53–55.

Agnihothram, S., Menachery, V.D., Yount, B.L., Jr., Lindesmith, L.C., Scobey, T., Whitmore, A., Schäfer, A., Heise, M.T., and Baric, R.S. (2018). Development of a Broadly Accessible Venezuelan Equine Encephalitis Virus Replicon Particle Vaccine Platform. J. Virol. *92*, e00027–e00018.

Ahmed, S.F., Klena, J.D., Mostafa, M., Dogantemur, J., Middleton, T., Hanson, J., and Sebeny, P.J. (2012). Viral gastroenteritis associated with genogroup II norovirus among U.S. military personnel in Turkey, 2009. PLoS ONE *7*, e35791.

Alvarado, G., Ettayebi, K., Atmar, R.L., Bombardi, R.G., Kose, N., Estes, M.K., and Crowe, J.E., Jr. (2018). Human Monoclonal Antibodies That Neutralize Pandemic GII.4 Noroviruses. Gastroenterology *155*, 1898–1907.

Atmar, R.L., Bernstein, D.I., Harro, C.D., Al-Ibrahim, M.S., Chen, W.H., Ferreira, J., Estes, M.K., Graham, D.Y., Opekun, A.R., Richardson, C., and Mendelman, P.M. (2011). Norovirus vaccine against experimental human Norwalk Virus illness. N. Engl. J. Med. *365*, 2178–2187.

Atmar, R.L., Ramani, S., and Estes, M.K. (2018). Human noroviruses: recent advances in a 50-year history. Curr. Opin. Infect. Dis. *31*, 422–432.

Baric, R.S., Yount, B., Lindesmith, L., Harrington, P.R., Greene, S.R., Tseng, F.C., Davis, N., Johnston, R.E., Klapper, D.G., and Moe, C.L. (2002). Expression and self-assembly of norwalk virus capsid protein from venezuelan equine encephalitis virus replicons. J. Virol. *76*, 3023–3030.

Belongia, E.A., Skowronski, D.M., McLean, H.Q., Chambers, C., Sundaram, M.E., and De Serres, G. (2017). Repeated annual influenza vaccination and vaccine effectiveness: review of evidence. Expert Rev. Vaccines *16*, 1–14.

Bernstein, D.I., Atmar, R.L., Lyon, G.M., Treanor, J.J., Chen, W.H., Jiang, X., Vinjé, J., Gregoricus, N., Frenck, R.W., Jr., Moe, C.L., et al. (2015). Norovirus vaccine against experimental human GII.4 virus illness: a challenge study in healthy adults. J. Infect. Dis. *211*, 870–878.

Bok, K., Parra, G.I., Mitra, T., Abente, E., Shaver, C.K., Boon, D., Engle, R., Yu, C., Kapikian, A.Z., Sosnovtsev, S.V., et al. (2011). Chimpanzees as an animal model for human norovirus infection and vaccine development. Proc. Natl. Acad. Sci. USA *108*, 325–330.

Bootz, A., Karbach, A., Spindler, J., Kropff, B., Reuter, N., Sticht, H., Winkler, T.H., Britt, W.J., and Mach, M. (2017). Protective capacity of neutralizing and non-neutralizing antibodies against glycoprotein B of cytomegalovirus. PLoS Pathog. *13*, e1006601.

Bull, R.A., Tanaka, M.M., and White, P.A. (2007). Norovirus recombination. J. Gen. Virol. *88*, 3347–3359.

Burke, R.M., Shah, M.P., Wikswo, M.E., Barclay, L., Kambhampati, A., Marsh, Z., Cannon, J.L., Parashar, U.D., Vinjé, J., and Hall, A.J. (2019). The Norovirus Epidemiologic Triad: Predictors of Severe Outcomes in US Norovirus Outbreaks, 2009-2016. J. Infect. Dis. *219*, 1364–1372.

Cannon, J.L., Barclay, L., Collins, N.R., Wikswo, M.E., Castro, C.J., Magaña, L.C., Gregoricus, N., Marine, R.L., Chhabra, P., and Vinjé, J. (2017). Genetic and Epidemiologic Trends of Norovirus Outbreaks in the United States from 2013 to 2016 Demonstrated Emergence of Novel GII.4 Recombinant Viruses. J. Clin. Microbiol. *55*, 2208–2221.

Cardemil, C.V., Parashar, U.D., and Hall, A.J. (2017). Norovirus Infection in Older Adults: Epidemiology, Risk Factors, and Opportunities for Prevention and Control. Infect. Dis. Clin. North Am. *31*, 839–870.

Carmona-Vicente, N., Fernández-Jiménez, M., Ribes, J.M., Téllez-Castillo, C.J., Khodayar-Pardo, P., Rodríguez-Díaz, J., and Buesa, J. (2015). Norovirus infections and seroprevalence of genotype GII.4-specific antibodies in a Spanish population. J. Med. Virol. *87*, 675–682.

Chen, R., Neill, J.D., Noel, J.S., Hutson, A.M., Glass, R.I., Estes, M.K., and Prasad, B.V. (2004). Inter- and intragenus structural variations in caliciviruses and their functional implications. J. Virol. *78*, 6469–6479.

Chen, J., Zheng, Q., Hammers, C.M., Ellebrecht, C.T., Mukherjee, E.M., Tang, H.Y., Lin, C., Yuan, H., Pan, M., Langenhan, J., et al. (2017). Proteomic Analysis of Pemphigus Autoantibodies Indicates a Larger, More Diverse, and More Dynamic Repertoire than Determined by B Cell Genetics. Cell Rep. *18*, 237–247.

Collaborators, G.B.D.D.D.; GBD Diarrhoeal Diseases Collaborators (2017). Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015. Lancet Infect. Dis. *17*, 909–948.

Costantini, V., Morantz, E.K., Browne, H., Ettayebi, K., Zeng, X.L., Atmar, R.L., Estes, M.K., and Vinjé, J. (2018). Human Norovirus Replication in Human Intestinal Enteroids as Model to Evaluate Virus Inactivation. Emerg. Infect. Dis. *24*, 1453–1464.

Debbink, K., Donaldson, E.F., Lindesmith, L.C., and Baric, R.S. (2012a). Genetic mapping of a highly variable norovirus GII.4 blockade epitope: potential role in escape from human herd immunity. J. Virol. *86*, 1214–1226.

Debbink, K., Lindesmith, L.C., Donaldson, E.F., and Baric, R.S. (2012b). Norovirus immunity and the great escape. PLoS Pathog. *8*, e1002921.

Debbink, K., Costantini, V., Swanstrom, J., Agnihothram, S., Vinje, J., Baric, R., and Lindesmith, L. (2013). Human norovirus detection and production, quantification, and storage of virus-like particles. Curr Protoc Microbiol *31*, 15K.1.1–15K.1.45.

Debbink, K., Lindesmith, L.C., and Baric, R.S. (2014a). The state of norovirus vaccines. Clin. Infect. Dis. *58*, 1746–1752.

Debbink, K., Lindesmith, L.C., Donaldson, E.F., Swanstrom, J., and Baric, R.S. (2014b). Chimeric GII.4 norovirus virus-like-particle-based vaccines induce broadly blocking immune responses. J. Virol. *88*, 7256–7266.

DeKosky, B.J., Kojima, T., Rodin, A., Charab, W., Ippolito, G.C., Ellington, A.D., and Georgiou, G. (2015). In-depth determination and analysis of the human paired heavy- and light-chain antibody repertoire. Nat. Med. *21*, 86–91.

DeKosky, B.J., Lungu, O.I., Park, D., Johnson, E.L., Charab, W., Chrysostomou, C., Kuroda, D., Ellington, A.D., Ippolito, G.C., Gray, J.J., and Georgiou, G. (2016). Large-scale sequence and structural comparisons of human naive and antigen-experienced antibody repertoires. Proc. Natl. Acad. Sci. USA *113*, E2636–E2645.

Diskin, R., Scheid, J.F., Marcovecchio, P.M., West, A.P., Jr., Klein, F., Gao, H., Gnanapragasam, P.N., Abadir, A., Seaman, M.S., Nussenzweig, M.C., and Bjorkman, P.J. (2011). Increasing the potency and breadth of an HIV antibody by using structure-based rational design. Science *334*, 1289–1293.

Emsley, P., and Cowtan, K. (2004). Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. *60*, 2126–2132.

Ettayebi, K., Crawford, S.E., Murakami, K., Broughman, J.R., Karandikar, U., Tenge, V.R., Neill, F.H., Blutt, S.E., Zeng, X.L., Qu, L., et al. (2016). Replication of human noroviruses in stem cell-derived human enteroids. Science *353*, 1387–1393.

Georgiou, G., Ippolito, G.C., Beausang, J., Busse, C.E., Wardemann, H., and Quake, S.R. (2014). The promise and challenge of high-throughput sequencing of the antibody repertoire. Nat. Biotechnol. *32*, 158–168.

Gostic, K.M., Ambrose, M., Worobey, M., and Lloyd-Smith, J.O. (2016). Potent protection against H5N1 and H7N9 influenza via childhood hemagglutinin imprinting. Science *354*, 722–726.

Green, K.Y., Lew, J.F., Jiang, X., Kapikian, A.Z., and Estes, M.K. (1993). Comparison of the reactivities of baculovirus-expressed recombinant Norwalk virus capsid antigen with those of the native Norwalk virus antigen in serologic assays and some epidemiologic observations. J. Clin. Microbiol. *31*, 2185–2191.

Hansman, G.S., Biertümpfel, C., Georgiev, I., McLellan, J.S., Chen, L., Zhou, T., Katayama, K., and Kwong, P.D. (2011). Crystal structures of GII.10 and GII.12 norovirus protruding domains in complex with histo-blood group antigens reveal details for a potential site of vulnerability. J. Virol. *85*, 6687–6701.

Hansman, G.S., Taylor, D.W., McLellan, J.S., Smith, T.J., Georgiev, I., Tame, J.R., Park, S.Y., Yamazaki, M., Gondaira, F., Miki, M., et al. (2012). Structural basis for broad detection of genogroup II noroviruses by a monoclonal antibody that binds to a site occluded in the viral particle. J. Virol. *86*, 3635–3646.

Harrington, P.R., Lindesmith, L., Yount, B., Moe, C.L., and Baric, R.S. (2002). Binding of Norwalk virus-like particles to ABH histo-blood group antigens is blocked by antisera from infected human volunteers or experimentally vaccinated mice. J. Virol. *76*, 12335–12343.

Havenar-Daughton, C., Sarkar, A., Kulp, D.W., Toy, L., Hu, X., Deresa, I., Kalyuzhniy, O., Kaushik, K., Upadhyay, A.A., Menis, S., et al. (2018). The human naive B cell repertoire contains distinct subclasses for a germline-targeting HIV-1 vaccine immunogen. Sci Transl Med *10*, eaat0381.

Henry Dunand, C.J., Leon, P.E., Huang, M., Choi, A., Chromikova, V., Ho, I.Y., Tan, G.S., Cruz, J., Hirsh, A., Zheng, N.Y., et al. (2016). Both Neutralizing and Non-Neutralizing Human H7N9 Influenza Vaccine-Induced Monoclonal Antibodies Confer Protection. Cell Host Microbe *19*, 800–813.

Ippolito, G.C., Hoi, K.H., Reddy, S.T., Carroll, S.M., Ge, X., Rogosch, T., Zemlin, M., Shultz, L.D., Ellington, A.D., Vandenberg, C.L., and Georgiou, G. (2012). Antibody Repertoires in Humanized NOD-scid-IL2Rgamma(null) Mice and Human B Cells Reveals Human-Like Diversification and Tolerance Checkpoints in the Mouse. PLoS One *7*, e35497.

Jiang, X., Matson, D.O., Ruiz-Palacios, G.M., Hu, J., Treanor, J., and Pickering, L.K. (1995). Expression, self-assembly, and antigenicity of a snow mountain agent-like calicivirus capsid protein. J. Clin. Microbiol. *33*, 1452–1455.

Kocher, J.F., Debbink, K., Lindesmith, L.C., Graham, R.L., Hugues, B., Goodwin, R.R., and Baric, R.S. (2018). Noroviurs Vaccines. In Plotkin's Vaccines, P.A.O, S.A. Plotkin, W.A. Orenstein, and K.M. Edwards, eds. (Philadelphia, PA: Elsevier), pp. 698–703.

Kolawole, A.O., Smith, H.Q., Svoboda, S.A., Lewis, M.S., Sherman, M.B., Lynch, G.C., Pettitt, B.M., Smith, T.J., and Wobus, C.E. (2017). Norovirus Escape from Broadly Neutralizing Antibodies Is Limited to Allostery-Like Mechanisms. MSphere *2*, e00334–e17.

Koromyslova, A.D., Morozov, V.A., Hefele, L., and Hansman, G.S. (2018). Human Norovirus Neutralized by a Monoclonal Antibody Targeting the Histo-Blood Group Antigen Pocket. J. Virol. *93*, e02174–e18.

Krissinel, E., and Henrick, K. (2007). Inference of macromolecular assemblies from crystalline state. J. Mol. Biol. *372*, 774–797.

Kwon, Y.D., Chuang, G.Y., Zhang, B., Bailer, R.T., Doria-Rose, N.A., Gindin, T.S., Lin, B., Louder, M.K., McKee, K., O'Dell, S., et al. (2018). Surface-Matrix Screening Identifies Semi-specific Interactions that Improve Potency of a Near Pan-reactive HIV-1-Neutralizing Antibody. Cell Rep. *22*, 1798–1809.

Lavinder, J.J., Wine, Y., Giesecke, C., Ippolito, G.C., Horton, A.P., Lungu, O.I., Hoi, K.H., DeKosky, B.J., Murrin, E.M., Wirth, M.M., et al. (2014). Identification and characterization of the constituent human serum antibodies elicited by vaccination. Proc. Natl. Acad. Sci. USA *111*, 2259–2264.

Lee, J., Boutz, D.R., Chromikova, V., Joyce, M.G., Vollmers, C., Leung, K., Horton, A.P., DeKosky, B.J., Lee, C.H., Lavinder, J.J., et al. (2016). Molecular-level analysis of the serum antibody repertoire in young adults before and after seasonal influenza vaccination. Nat. Med. *22*, 1456–1464.

Leroux-Roels, G., Cramer, J.P., Mendelman, P.M., Sherwood, J., Clemens, R., Aerssens, A., De Coster, I., Borkowski, A., Baehner, F., and Van Damme, P. (2018). Safety and Immunogenicity of Different Formulations of Norovirus Vaccine Candidate in Healthy Adults: A Randomized, Controlled, Double-Blind Clinical Trial. J. Infect. Dis. *217*, 597–607.

Lessler, J., Riley, S., Read, J.M., Wang, S., Zhu, H., Smith, G.J., Guan, Y., Jiang, C.Q., and Cummings, D.A. (2012). Evidence for antigenic seniority in influenza A (H3N2) antibody responses in southern China. PLoS Pathog. *8*, e1002802.

Lindesmith, L., Moe, C., Marionneau, S., Ruvoen, N., Jiang, X., Lindblad, L., Stewart, P., LePendu, J., and Baric, R. (2003). Human susceptibility and resistance to Norwalk virus infection. Nat. Med. *9*, 548–553.

Lindesmith, L.C., Donaldson, E.F., Lobue, A.D., Cannon, J.L., Zheng, D.P., Vinje, J., and Baric, R.S. (2008). Mechanisms of GII.4 norovirus persistence in human populations. PLoS Med. *5*, e31.

Lindesmith, L.C., Beltramello, M., Donaldson, E.F., Corti, D., Swanstrom, J., Debbink, K., Lanzavecchia, A., and Baric, R.S. (2012a). Immunogenetic mechanisms driving norovirus GII.4 antigenic variation. PLoS Pathog. *8*, e1002705.

Lindesmith, L.C., Debbink, K., Swanstrom, J., Vinjé, J., Costantini, V., Baric, R.S., and Donaldson, E.F. (2012b). Monoclonal antibody-based antigenic mapping of norovirus GII.4-2002. J. Virol. *86*, 873–883.

Lindesmith, L.C., Costantini, V., Swanstrom, J., Debbink, K., Donaldson, E.F., Vinjé, J., and Baric, R.S. (2013). Emergence of a norovirus GII.4 strain correlates with changes in evolving blockade epitopes. J. Virol. *87*, 2803–2813.

Lindesmith, L.C., Donaldson, E.F., Beltramello, M., Pintus, S., Corti, D., Swanstrom, J., Debbink, K., Jones, T.A., Lanzavecchia, A., and Baric, R.S. (2014). Particle conformation regulates antibody access to a conserved GII.4 norovirus blockade epitope. J. Virol. *88*, 8826–8842.

Lindesmith, L.C., Ferris, M.T., Mullan, C.W., Ferreira, J., Debbink, K., Swanstrom, J., Richardson, C., Goodwin, R.R., Baehner, F., Mendelman, P.M., et al. (2015). Broad blockade antibody responses in human volunteers after immunization with a multivalent norovirus VLP candidate vaccine: immunological analyses from a phase I clinical trial. PLoS Med. *12*, e1001807.

Lindesmith, L.C., Mallory, M.L., Jones, T.A., Richardson, C., Goodwin, R.R., Baehner, F., Mendelman, P.M., Bargatze, R.F., and Baric, R.S. (2017). Impact of Pre-exposure History and Host Genetics on Antibody Avidity Following Norovirus Vaccination. J. Infect. Dis. *215*, 984–991.

Lindesmith, L.C., Brewer-Jensen, P.D., Mallory, M.L., Debbink, K., Swann, E.W., Vinjé, J., and Baric, R.S. (2018a). Antigenic Characterization of a Novel Recombinant GII.P16-GII.4 Sydney Norovirus Strain With Minor Sequence Variation Leading to Antibody Escape. J. Infect. Dis. *217*, 1145–1152.

Lindesmith, L.C., Mallory, M.L., Debbink, K., Donaldson, E.F., Brewer-Jensen, P.D., Swann, E.W., Sheahan, T.P., Graham, R.L., Beltramello, M., Corti, D., et al. (2018b). Conformational Occlusion of Blockade Antibody Epitopes, a Novel Mechanism of GII.4 Human Norovirus Immune Evasion. MSphere *3*, e00518–e17.

LoBue, A.D., Lindesmith, L., Yount, B., Harrington, P.R., Thompson, J.M., Johnston, R.E., Moe, C.L., and Baric, R.S. (2006). Multivalent norovirus vaccines induce strong mucosal and systemic blocking antibodies against multiple strains. Vaccine *24*, 5220–5234.

Mallory, M.L., Lindesmith, L.C., Graham, R.L., and Baric, R.S. (2019). GII.4 Human Norovirus: Surveying the Antigenic Landscape. Viruses *11*, 177.

Malm, M., Uusi-Kerttula, H., Vesikari, T., and Blazevic, V. (2014). High serum levels of norovirus genotype-specific blocking antibodies correlate with protection from infection in children. J. Infect. Dis. *210*, 1755–1762.

McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C., and Read, R.J. (2007). Phaser crystallographic software. J. Appl. Cryst. *40*, 658–674.

McDaniel, J.R., DeKosky, B.J., Tanno, H., Ellington, A.D., and Georgiou, G. (2016). Ultra-high-throughput sequencing of the immune receptor repertoire from millions of lymphocytes. Nat. Protoc. *11*, 429–442.

McLellan, J.S., Pancera, M., Carrico, C., Gorman, J., Julien, J.P., Khayat, R., Louder, R., Pejchal, R., Sastry, M., Dai, K., et al. (2011). Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9. Nature *480*, 336–343.

Mroczek, E.S., Ippolito, G.C., Rogosch, T., Hoi, K.H., Hwangpo, T.A., Brand, M.G., Zhuang, Y., Liu, C.R., Schneider, D.A., Zemlin, M., et al. (2014). Differences in the composition of the human antibody repertoire by B cell subsets in the blood. Front. Immunol. *5*, 96.

Nurminen, K., Blazevic, V., Huhti, L., Rasanen, S., Koho, T., Hytönen, V.P., and Vesikari, T. (2011). Prevalence of norovirus GII-4 antibodies in Finnish children. J. Med. Virol. *83*, 525–531.

Otwinowski, Z., and Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. *276*, 307–326.

Park, J.S., Yeom, J.S., Seo, J.H., Lim, J.Y., Park, C.H., Woo, H.O., Youn, H.S., Jun, J.S., Park, J.H., Ko, G.H., et al. (2015). Immunohistochemical Expressions of MUC2, MUC5AC, and MUC6 in Normal, Helicobacter pylori Infected and Metaplastic Gastric Mucosa of Children and Adolescents. Helicobacter *20*, 260–268.

Parra, G.I., Bok, K., Taylor, R., Haynes, J.R., Sosnovtsev, S.V., Richardson, C., and Green, K.Y. (2012). Immunogenicity and specificity of norovirus Consensus GII.4 virus-like particles in monovalent and bivalent vaccine formulations. Vaccine *30*, 3580–3586.

Patel, M.M., Widdowson, M.A., Glass, R.I., Akazawa, K., Vinjé, J., and Parashar, U.D. (2008). Systematic literature review of role of noroviruses in sporadic gastroenteritis. Emerg. Infect. Dis. *14*, 1224–1231.

Pei, J., and Grishin, N.V. (2001). AL2CO: calculation of positional conservation in a protein sequence alignment. Bioinformatics *17*, 700–712.

Petrignani, M., Verhoef, L., de Graaf, M., Richardus, J.H., and Koopmans, M. (2018). Chronic sequelae and severe complications of norovirus infection: A systematic review of literature. J. Clin. Virol. *105*, 1–10.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. (2004). UCSF Chimera–a visualization system for exploratory research and analysis. J. Comput. Chem. *25*, 1605–1612.

Pogan, R., Dülfer, J., and Uetrecht, C. (2018). Norovirus assembly and stability. Curr. Opin. Virol. *31*, 59–65.

Prasad, B.V., Hardy, M.E., Dokland, T., Bella, J., Rossmann, M.G., and Estes, M.K. (1999). X-ray crystallographic structure of the Norwalk virus capsid. Science *286*, 287–290.

Reeck, A., Kavanagh, O., Estes, M.K., Opekun, A.R., Gilger, M.A., Graham, D.Y., and Atmar, R.L. (2010). Serological correlate of protection against norovirus-induced gastroenteritis. J. Infect. Dis. *202*, 1212–1218.

Shanker, S., Choi, J.M., Sankaran, B., Atmar, R.L., Estes, M.K., and Prasad, B.V. (2011). Structural analysis of histo-blood group antigen binding specificity in a norovirus GII.4 epidemic variant: implications for epochal evolution. J. Virol. *85*, 8635–8645.

Shanker, S., Czakó, R., Sapparapu, G., Alvarado, G., Viskovska, M., Sankaran, B., Atmar, R.L., Crowe, J.E., Jr., Estes, M.K., and Prasad, B.V. (2016). Structural basis for norovirus neutralization by an HBGA blocking human IgA antibody. Proc. Natl. Acad. Sci. USA *113*, E5830–E5837.

Siebenga, J.J., Vennema, H., Renckens, B., de Bruin, E., van der Veer, B., Siezen, R.J., and Koopmans, M. (2007). Epochal evolution of GGII.4 norovirus capsid proteins from 1995 to 2006. J. Virol. *81*, 9932–9941.

Singh, B.K., Leuthold, M.M., and Hansman, G.S. (2015). Human noroviruses' fondness for histo-blood group antigens. J. Virol. *89*, 2024–2040.

Snijder, J., Ortego, M.S., Weidle, C., Stuart, A.B., Gray, M.D., McElrath, M.J., Pancera, M., Veesler, D., and McGuire, A.T. (2018). An Antibody Targeting the Fusion Machinery Neutralizes Dual-Tropic Infection and Defines a Site of Vulnerability on Epstein-Barr Virus. Immunity *48*, 799–811.e9.

Tan, M., and Jiang, X. (2011). Norovirus-host interaction: multi-selections by human histo-blood group antigens. Trends Microbiol. *19*, 382–388.

Treanor, J.J., Atmar, R.L., Frey, S.E., Gormley, R., Chen, W.H., Ferreira, J., Goodwin, R., Borkowski, A., Clemens, R., and Mendelman, P.M. (2014). A novel intramuscular bivalent norovirus virus-like particle vaccine candidate–reactogenicity, safety, and immunogenicity in a phase 1 trial in healthy adults. J. Infect. Dis. *210*, 1763–1771.

Voss, N.R., and Gerstein, M. (2010). 3V: cavity, channel and cleft volume calculator and extractor. Nucleic Acids Res. *38*, W555–W562.

Williams, L.D., Ofek, G., Schatzle, S., McDaniel, J.R., Lu, X., Nicely, N.I., Wu, L., Lougheed, C.S., Bradley, T., Louder, M.K., et al. (2017). Potent and broad HIV-neutralizing antibodies in memory B cells and plasma. Sci Immunol *2*, eaal2200.

Word, J.M., Lovell, S.C., LaBean, T.H., Taylor, H.C., Zalis, M.E., Presley, B.K., Richardson, J.S., and Richardson, D.C. (1999). Visualizing and quantifying molecular goodness-of-fit: small-probe contact dots with explicit hydrogen atoms. J. Mol. Biol. *285*, 1711–1733.

Zheng, D.P., Ando, T., Fankhauser, R.L., Beard, R.S., Glass, R.I., and Monroe, S.S. (2006). Norovirus classification and proposed strain nomenclature. Virology *346*, 312–323.

STAR+ METHODS

KEY RESOURCES TABLE

REAGENTor RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-human IgG Fc peroxidase antibody	Invitrogen	Cat# A18817
Donkey anti-rabbit IgG-HRP	GE Healthcare	Cat# NA934; RRID: AB_772206
Bacterial and Virus Strains		
E.coli One Shot TOP10	Invitrogen	Cat# C404010
E.coli BL21 (DE3)	Agilent Technologies	Cat# 200131
Biological Samples		
Rabbit anti-norovirus capsid protein polyclonal serum	CoCalico Biologicals Custom Synthesis	N/A
GII.P16-GII.4 Sydney	Positive stool sample	N/A
Chemicals, Peptides, and Recombinant Proteins		
GI.1 Virus like particles	R. Baric, UNC-CH	GenBank: AFJ38516.1
GI.3 Virus like particles	R. Baric, UNC-CH	GenBank: AFK75851.1
GI.4 Virus like particles	R. Baric, UNC-CH	GenBank: AFK75852.1
GII.4 1987 Virus like particles	R. Baric, UNC-CH	GenBank: AAK50355.1
GII.4 1997 Virus like particles	R. Baric, UNC-CH	GenBank: AFJ04707.1
GII.4 2002 Virus like particles	R. Baric, UNC-CH	GenBank: AFJ04708.1
GII.4 2006b Virus like particles	R. Baric, UNC-CH	GenBank: AFJ04709.1
GII.4 2009 Virus like particles	R. Baric, UNC-CH	GenBank: ADD10375.1
GII.4 2012 Virus like particles	R. Baric, UNC-CH	GenBank: AFV08795.1
GII.4 2015 Virus like particles	R. Baric, UNC-CH	GenBank: APA31970.1
GII.1 Virus like particles	R. Baric, UNC-CH	GenBank: AFS33555.1
GII.2 Virus like particles	R. Baric, UNC-CH	GenBank: AAN08112.1
GII.3 Virus like particles	R. Baric, UNC-CH	GenBank: AFK75854.1
GII.14 Virus like particles	R. Baric, UNC-CH	GenBank: AAN05735.1
GII.17 Virus like particles	R. Baric, UNC-CH	GenBank: AKB94547.1
GII.4c Virus like particles	This study	GenBank: MK614455
GII.4 2006a.581 Virus like particles	R. Baric, UNC-CH	GenBank: MK614084
GII.4 2006a.581.A4A Virus like particles	This study	GenBank: MK614085
GII.4 2006a.581.A4B Virus like particles	This study	GenBank: MK614086
GII.4 1987.2012A Virus like particles	This study	GenBank: MK614081
GII.4 1987.2012D Virus like particles	This study	GenBank: MK614082
GII.4 1987.2012E Virus like particles	This study	GenBank: MK614083
Pig gastric mucin	Sigma-Aldrich	Cat# M1778
Matrigel	Corning	Cat# 356231
IntestiCult Organoid Growth Medium (Human)	STEMCELL Technologies	Cat# 06010
Y-27632	Sigma-Aldrich	Cat# Y0503-1MG
Advanced Dulbecco Modified Eagle Medium (DMEM)/F12	Invitrogen	Cat# 12634-028
GlutaMAX	Invitrogen	Cat# 35050-061
Penicillin/Streptomycin	Invitrogen	Cat# 15140-122
1M HEPES	Invitrogen	Cat# 15630-080
B27 supplement	Invitrogen	Cat# 17504-044
N2 supplement	Invitrogen	Cat# 17502-048
mouse epidermal growth factor	Invitrogen	Cat# PMG8043

(*Continued on next page*)

Continued

REAGENTor RESOURCE	SOURCE	IDENTIFIER
n-Acetyl-L-cysteine	Sigma-Aldrich	Cat# A9165-5G
[Leu15]-Gastrin	Sigma-Aldrich	Cat# G9145
Noggin media	Ettayebi et al 2016	N/A
A83-01	Tocris	Cat# 2939
DPBS, no calcium, no magnesium	Invitrogen	Cat# 14190-250
Sodium glycochenodeoxycholate	Sigma-Aldrich	Cat# G0759-100MG
Collagen from human placenta (collagen type IV)	Sigma-Aldrich	Cat# C5533-5MG
C2 Ceramide	Santa Cruz Biotechnology	Cat# sc-201375A
5 M Ethylenediaminetetraacetic (EDTA)	Invitrogen	Cat# 15575-020
0.05% Trypsin/0.5mM EDTA	Gibco	Cat# 25300054
Fetal bovine serum certified	Life Technologies	Cat# 16000-044
cOmplete, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	Cat# 5056489001
Lysozyme from chicken egg white	Sigma-Aldrich	Cat# L6876
cOmplete His-Tag Purification Resin	Sigma-Aldrich	Cat# 5893801001
HRV-3C Protease	Sigma-Aldrich	Cat# SAE0045
Turbo293 Transfection Reagent	Speed Biosystems	Cat# PXX1002
AbBooster, Antibody Expression Enhancer	Abi Scientific	Cat# PB2668
Expi293 Expression Medium	Thermo Fisher Scientific	Cat# A1435101
GE MabSelect Protein A Resin	Sigma-Aldrich	Cat# GE17-5199-01
Pierce IgG Elution Buffer	Thermo Fisher Scientific	Cat# 21004
Polyethylene Glycol 4000	Rigaku	Cat# 1008059
Tris-HCl buffer	Rigaku	Cat# HR2-237
0.1 M L-Proline	Hampton Research	Cat# HR2-428-26
Polyethylene Glycol 8000	Rigaku	Cat# 1008062
CHES/Sodium Hydroxide	Rigaku	Cat#1008158
Critical Commercial Assays		
mMESSAGE MACHINE T7 Transcription kit	Invitrogen	Cat# AM1344
Deoxyribonuclease I	Sigma Aldrich	Cat# D4513-1VL
TRIzol	Thermo Fisher Scientific	Cat# 15596-026
Rneasy Kit	Qiagen	Cat# 74004
SuperScript II	Invitrogen	Cat# 11904-018
Human Memory B Cell Isolation Kit	Miltenyi	Cat# 130-094-350
Oligo d(T)25magnetic beads	NEB	Cat# S1419S
One step fast qRT-PCR solution	VWR	Cat# 95039-940
N-hydroxysuccinimide (NHS)-agarose beads	Thermo Fisher Scientific	Cat# 26197
Melon gel purification	Thermo Fisher Scientific	Cat# 45212
Tris(2-carboxyethyl)phosphine	Thermo Fisher Scientific	Cat# 77720
Iodoacetamide	Sigma Aldrich	Cat# I1149-5G
Trifluoroethanol	Thermo Fisher Scientific	Cat# AC13975-1000
Trypsin Gold	Thermo Fisher Scientific	Cat# PR-V5280
5x MagMAX - 96 Viral RNA Isolation Kit	Applied Biosystems	Cat# AMB1836-5
AgPath-ID One-Step RT-PCR	Applied Biosystems	Cat# 4387391
Deposited Data		
Crystal structure of GII.4.2002 P domain in complex with A1227 Fab	This paper	PDB: 6N81
Crystal structure of GII.4.2002 P domain in complex with A1431 Fab	This paper	PDB: 6N8D
GII.4c Virus like particles	This study	GenBank: MK614455

(*Continued on next page*)

Continued

REAGENTor RESOURCE	SOURCE	IDENTIFIER
GII.4 2006a.581 Virus like particles	R. Baric, UNC-CH	GenBank: MK614084
GII.4 2006a.581.A4A Virus like particles	This study	GenBank: MK614085
GII.4 2006a.581.A4B Virus like particles	This study	GenBank: MK614086
GII.4 1987. 2012A Virus like particles	This study	GenBank: MK614081
GII.4 1987. 2012D Virus like particles	This study	GenBank: MK614082
GII.4 1987. 2012E Virus like particles	This study	GenBank: MK614083
Experimental Models: Cell Lines		
BHK cell line	ATCC	Cat# CCL-10
Human intestinal enteroids (J3 cell line)	Ettayebi et al., 2016	N/A
Human: Expi293F cells	Thermo Fisher Scientific	Cat# A14527
Oligonucleotides		
Cog 1F (5′-CGYTGGATGCGITTYCATGA-3′)	Cannon et al., 2017	N/A
Cog 1R (5′-CTTAGACGCCATCATCATTYAC-3′)	Cannon et al., 2017	N/A
Probe Ring 1E (5′-FAM-TGGACAGGRGAYCGC-MGBNFQ-3′)	Cannon et al., 2017	N/A
MS2.F (5′-TGGCACTACCCCTCTCCGTATTCACG-3′)	Cannon et al., 2017	N/A
MS2.R (5′-GTACGGGCGACCCCACGATGAC-3′)	Cannon et al., 2017	N/A
Probe MS2.P (5′-HEX-CACATCGATAGATCAAGG TGCCTACAAGC-BHQ1-3′)	Cannon et al., 2017	N/A
Cog 2F (5′-CARGARBCNATGTTYAGRTGGATGAG-3′)	Cannon et al., 2017	N/A
Cog 2R (5′-TCGACGCCATCTTCATTCACA-3′)	Cannon et al., 2017	N/A
Probe Ring 2 (5′-Cy5-TGGGAGGGCGATCGCAATCT-BHQ2-3′)	Cannon et al., 2017	N/A
Recombinant DNA		
pVR21	R. Baric, UNC	N/A
pUC57 with norovirus capsid inserted	BioBasic Custom Synthesis	N/A
pVRC8400 Vector	NIH/VRC	N/A
pMal-C5X plasmid	New England Biolabs	Cat# N8108S
Software and Algorithms		
Prism	https://www.graphpad.com/scientific-software/prism/	RRID: SCR_002798
Sequest, Percolator on Proteome Discoverer 1.4	Thermo Fisher Scientific	https://www.thermofisher.com/order/catalog/product/OPTON-30795
HKL2000	HKL Research	http://www.hkl-xray.com/
Phenix	Adams et al., 2004	https://sbgrid.org/software/
Coot	Emsley and Cowtan, 2004	https://sbgrid.org/software/
Pymol	Schrödinger	https://pymol.org/2
Chimera	Pettersen et al., 2004	https://www.cgl.ucsf.edu/chimera/
AL2CO	Pei and Grishin, 2001	http://prodata.swmed.edu/al2co/al2co.php
PDBePISA	Krissinel and Henrick, 2007	http://www.ebi.ac.uk/pdbe/pisa/
Other		
KingFisher Flex Purification System	ThermoFisher Scientific	Cat# 5400610
GII.4 Sydney RNA transcripts	CDC	N/A
7500 Real-Time PCR System	Applied Biosystems	Cat# 4351104

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ralph Baric (rbaric@email.unc.edu). All human samples remain property of Takeda Vaccines.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Humans Subjects

Serum and PBMC samples

This study used de-identified human samples under University of North Carolina institutional review board exemption approval #11-0883. Serum and PBMC samples were collected from two female ages 25 and 46 and one male age 30 participants in a study conducted by Takeda Vaccines Inc. (Deerfield, IL) (Treanor et al., 2014). Participants in Group A1 received an intramuscular injection of 5 mg of both GI.1 and GII.4c VLP adjuvanted with 3-O-deacylated monophosphoryl lipid A (MPL, GlaxoSmithKline) and alhydrogel on day 0 (dose 1) followed by an identical booster vaccination on day 28 (dose 2). This study was done under IRB approval and subjects provided informed consent. The trial was registered at ClinicalTrials.gov (NCT01168401).

Cell lines

BHK -21 cells (ATCC CCL-10, derived from unsexed hamsters) were grown at 37! C in 5% CO_2 in Minimum Essential Medium with Earles salts and L-glutamine (Gibco) supplemented with 7% fetal Clone II (Hyclone), non-essential amino acids (Gibco), sodium pyruvate (Gibco) and Antibiotic-Antimycotic (Gibco).

Microbes

E coli Top 10 with pVR21 plasmid containing norovirus capsid genes were propagated at 30! C in the presence of 0.1mg/ml carbenicillin in Luria-Bertani broth. For full IgG expression and characterization, VH and VL plasmids were transfected into 30 mL cultures of Expi293F cells (Invitrogen) at a 1:2 ratio and incubated at 37! C and 8% CO_2 for 7 days.

For protein crystallization, P-domains were transfected into *E.coli* BL21 (DE3)(Agilent Technologies) and plated onto Luria-Bertani plates with 50mg/mL Ampicillin (LB-Amp). VH and VL chains were cotransfected into Expi293F cells (ThermoFisher)using a 3:1 ratio of Turbo293 transfection reagent (Speed Biosystem) to plasmid. Cells were incubated at 37! C, 5% CO_2, 130 rpm shaking, 70% humidity for 18 h and subsequently boosted with 80mL of AbBooster (Abi Scientific).

METHOD DETAILS

Ten percent stool filtrate

This investigation was determined by CDC to be public health non-research, and therefore was not subject to institutional review board review. Ten percent stool suspension was prepared by adding 0.5 g of GII.P16-GII.4 Sydney positive whole stool to 4.5 mL of PBS. The stool suspension was vortexed for 30 seconds, kept at room temperature for 5 min and vortexed again. Sample was sonicated and solids were removed by centrifugation for 10 min at 10,000 x g. The supernatant was serially filtered through 5 mm, 1 mm, 0.45 mm, and 0.22 mm filters. The resulting 10% stool filtrate was aliquoted and stored at 70! C.

Human intestinal enteroid culture (HIE)

HIE cells were kindly provided by Dr. Mary K. Estes, Baylor College of Medicine, Houston, Texas. Adult (female) secretor positive jejunal HIE cultures were grown as undifferentiated 3D cultures as described previously with minor modifications (Costantini et al., 2018; Ettayebi et al., 2016). Briefly, HIEs were recovered from liquid nitrogen (LN_2), suspended in 20 ml of Matrigel (Corning), plated in a single well of a 24 well plate, and grown as 3D cultures in 500 mL IntestiCult Organoid Growth Medium (Human) (STEMCELL Technologies) supplemented with 10 mM Y-27632 (Sigma-Aldrich). Media was refreshed every other day. Highly dense 3D cultures were passaged once per week. To passage, HIE were taken up in ice-cold complete medium without growth factors (CMGF-), broken up by repeating pipetting cycles, suspended in Matrigel (Corning), and plated in 24 well plates. CMGF- compromises Advanced Dulbecco Modified Eagle Medium (DMEM)/F12 supplemented with 1% GlutaMAX, 1% Penicillin/Streptomycin, 1% 1M HEPES. If single cell suspension was required, HIE were taken up in ice-cold 0.5mM EDTA and broken up by repeating pipetting cycles. After centrifugation (200 x g for 5 min at 4! C), single cell suspension was obtained upon treatment of the HIE with 0.05% Trypsin/0.5mM EDTA (Invitrogen) for 3–4 min at 37! C. Trypsin was then inactivated by adding CMGF- /10% FBS to the cell suspension. Cell suspensions were prepared by pipetting with a P1000 pipet and passing the cells through a 40 mm cell strainer. The cells were pelleted for 5 min at 400 x *g*, suspended in 100 ml of in IntestiCult Organoid Growth Medium (Human)(STEMCELL Technologies) supplemented with 10 mM Y-27632 (Sigma-Aldrich) and plated as undifferentiated monolayers in collagen IV (Sigma-Aldrich) pre-coated 96 well plates. After 24 h, culture medium was replaced with differentiation medium to induce cell differentiation. Differentiation media compromises CMGF- ,1:100 B27 supplement, 1:50 N2 supplement, 1:1000 mouse epidermal growth factor (all Invitrogen), 1 mM n-Acetyl-L-cysteine, 10 nM [Leu15]-Gastrin, 10 mM Y-27632 (all Sigma-Aldrich), Noggin 20%, (conditioned home-made medium), 500 nM A83-01 (Tocris). Cells were differentiated for 4 days. Media was refreshed every other day.

High-throughput sequencing of BCR transcripts

Frozen PBMCs were thawed at 37! C, resuspended in RPMI-1640 (Fisher) supplemented with 10% Fetal Bovine Serum (FBS) and 20 U/mL deoxyribonuclease I (Roche), concentrated by centrifugation (300 x g for 15 min at 20! C), and allowed to recover in 4 mL media at 37! C for 30 min. The cells were then split: half were dedicated to BCR heavy chain transcript sequencing, and half were used to identify the native VH/VL repertoire.

VH Sequencing

Cells were diluted with 10 mL of cold buffer (phosphate buffered saline supplemented with 0.5% BSA and 2 mM EDTA)and pelleted via centrifugation (300 x g for 15 min at 4! C). The supernatant was decanted, and the cells were resuspended in 1mL of TRIzol reagent (Thermo Fisher). RNA was extracted using RNeasy (Qiagen), and first strand cDNA was synthesized from 500 ng total RNA using SuperScript II (Invitrogen). IgM and IgG heavy chain repertoires were amplified using a multiplex primer set (Table S3) under the following conditions: 2 min at 94! C; 4 cycles of 94! C for 30 s, 50! C for 30 s, 72! C for 30 s; 4 cycles of 94! C for 30 s, 55! C for 30 s, 72! C for 30 s; 22 cycles of 94! C for 30 s, 63! C for 30 s, 72! C for 30 s; 72! C for 7 min; hold at 4! C (Ippolito et al., 2012) and sequenced by 2x300 paired-end Illumina MiSeq.

VH/VL Sequencing

Total B cells were isolated from PBMCs using the Human Memory B Cell Isolation Kit (Miltenyi Biotec) with an LD column according to the manufacturer's instructions. B cells were then coemulsified with oligo d(*T*)25 magnetic beads (New England Biolabs) and lysis buffer (100 mM Tris pH 7.5, 500 mM LiCl, 10 mM EDTA, 1% lithium dodecyl sulfate, and 5 mM dithiothreitol) using a custom-designed flow focusing device as described (McDaniel et al., 2016). The magnetic beads were washed, resuspended in One step fast qRT-PCR solution (VWR)supplemented with an overlap extension VH and VL primer set as described (McDaniel et al., 2016), emulsified, and subjected to overlap-extension RT-PCR under the following conditions: 30 min at 55 ! C followed by 2 min at 94 ! C; 4 cycles of 94 ! C for 30 s, 50 ! C for 30 s, 72 ! C for 2 min; 4 cycles of 94 ! C for 30 s, 55 ! C for 30 s, 72 ! C for 2 min; 32 cycles of 94 ! C for 30 s, 60 ! C for 30 s, 72 ! C for 2 min; 72 ! C for 7 min; hold at 4 ! C. VH/VL amplicons were then extracted from the emulsions, amplified using nested PCR, and sequenced by 2x300 Illumina MiSeq.

Isolation of HuNoV specific antibodies

HuNoV VLP (GII.4c, 1 mg) was immobilized on 250 mg N-hydroxysuccinimide (NHS)-agarose beads (Thermo Fisher) by overnight rotation at 4! C. The conjugated resin was washed with PBS, and unreacted NHS groups were blocked with 1M ethanolamine, pH 8.3, for 20 min at RT. The beads were washed with PBS, packed into a 5 mL column, washed with 10 column volumes (cv) of 4M urea in PBS, and finally washed with 20 cv PBS. Total IgG was isolated from each serum sample using melon gel purification (Thermo Fisher) according to the manufacturer's instructions. IgG was then passed over the affinity column in gravity flow mode to capture GII.4c VLP-specific antibodies. Flow through was collected and reapplied to the column five times. The column was washed with PBS with 2 cv followed by 1 cv of water before elution in 12-1 mL fractions of 5% formic acid. 10 mL of each elution fraction was neutralized with 100 ml of 25x PBS in 2% milk and analyzed via ELISA against GII.4c VLP (antibodies bound to immobilized GII.4c were detected using goat anti-human IgG Fc peroxidase antibody (Invitrogen)). Fractions containing antigen-specific antibodies were pooled, concentrated under vacuum, and resuspended into 50 mL of 100 mM Tris-pH 8.

Sample preparation and LC-MS/MS analysis

For each sample, the flow through and antigen enriched elution were denatured in 1:1 (v/v) trifluoroethanol, reduced with 5 mL tris(2-carboxyethyl)phosphine (TCEP,110 mM) in the dark at 55! C for 45 min, and alkylated with 32 mM iodoacetamide by incubating in the dark for 30 min. Samples were diluted 10x into 50 mM Tris (pH 8.0) and digested with trypsin (1:10 trypsin/protein) for 5 h at 37! C. Formic acid (0.1%) was used to quench the reaction. The solution was then concentrated, desalted using a C18 Hypersep SpinTip (Thermo) according to the manufacturer's instructions, and submitted for LC-MS/MS.

Peptides were separated by reversed-phase chromatography (Dionex UltiMate 3000 RSLCnano System with Dionex Acclaim PepMap RSLC C18 column, Thermo Scientific) and analyzed online by nano–electrospray ionization–MS/MS on Orbitrap Velos Pro (Thermo Scientific). MS1 scans were collected in the orbitrap at a resolution of 60,000 Å, and the ions with >+1 charge were fragmented by collision-induced dissociation with up to 20 MS2 spectra collected per MS1.

SEQUEST (Proteome Discoverer 1.4, Thermo Scientific) with previously described settings (Williams et al., 2017) was used to search the spectra against a patient-specific protein database constructed from the full-length VH and VL sequences, Ensembl human protein-coding sequences, and common contaminants (maxquant.org). PSMs were filtered with Percolator (Proteome Discoverer 1.4) at a false discovery rate of <1% and filtered for average mass deviations <1.5 parts per million. Peptides mapping to the CDR-H3 region of unique antibody lineages were grouped, and the relative abundances of the corresponding peptide matches were determined by the sum of the extracted ion chromatograms (XIC)of the respective precursor ions. Relative abundances were multiplied by the patient specific anti-GII.4c titer to calculate the amount of GII.4c specific clonotypes in serum.

Antibody expression and purification

Selected antibody sequences were purchased as gBlocks gene fragments (Integrated DNA Technologies) and cloned into a customized pcDNA3.4 vector (Invitrogen) containing human IgG1 Fc regions. VH and VL plasmids were transfected into 30 mL cultures of Expi293F cells (Invitrogen) at a 1:2 ratio and incubated at 37! C and 8% CO_2 for 7 days. The supernatant containing secreted antibodies was collected following centrifugation (1000 g for 10 min at 4! C), neutralized, and filtered. Antibodies were isolated using Protein G Plus agarose (Pierce Thermo Fisher Scientific) affinity chromatography, washed with 20 column volumes of PBS, eluted with 100 mM glycine-HCl pH 2.7, and neutralized with 1 M Tris-HCl pH 8.0. The antibodies were then concentrated and buffer exchanged into PBS using 10,000 MWCO Vivaspin centrifugal spin columns (Sartorius).

Production of Virus Like Particles

For production of the VLP for this study, HuNoV ORF2 genes were synthesized by Bio Basic Inc (Markham, ON) with ApaI and AcsI restriction sites for direct insertion into the VEE replicon vector pVR21. The sequence-verified pDNA was propagated in Ecoli Top 10 (Invitrogen). pDNA was purified with QIAprep Miniprep kit (Qiagen). Purified pDNA was linearized with NotI, agarose gel purified with QIAquick Gel Extraction kit (Qiagen) and mRNA generated using mMESSAGE mMachine T7 transcription kit (Invtirogen). Message RNA was electroplated into BHK cells (ATCC CCL-10) for production of VLP (Agnihothram et al., 2018; Debbink et al., 2013). After 27 h, cells were washed and lysed in 1% Triton X-100 in PBS plus cOmplete EDTA-free protease inhibitor cocktail (Roche) and cell lysates layered above a cushion of 40% sucrose in PBS. VLP were purified into the cushion by ultracentrifugation at 120,000 x g for 75 min and stored at -80! C in 40% sucrose/PBS. Protein concentration was determined by BCA assay (Pierce). VLPs were verified for particle integrity by ligand and antibody binding and visualization by electron microscopy of 40nm particles.

Antibody functional assays

EIA and blockade antibody assays were performed at described (Lindesmith et al., 2018a) at 0.25 mg/ml VLP. EIA plates were coated with VLP in PBS for 4 h and blocked over night at 4! C in 5% dry milk in PBS-0.05% Tween-20 before the addition of decreasing two-fold serial dilutions of mAb at 37! C. Bound mAb was detected by anti-human IgG-HRP (GE Healthcare) and color developed with 1-Step Ultra TMB ELISA HRP substrate solution (Thermo-Fisher). Similarly, VLPs were pretreated with decreasing concentrations of mAb for 1 h and added to pig gastric mucin type III (Sigma Aldrich, St. Louis, MO) coated plates for 1 h. Bound VLP was detected by anti-VLP rabbit hyperimmune sera followed by anti-rabbit IgG-HRP (GE Healthcare) and color developed as above. Percent control binding is defined as the binding in the presence of antibody pretreatment compared to the binding in the absence multiplied by 100.

P domain expression and purification

The gene for the P-domain of HuNoV GII.4.2002 (accession number: AFJ04708) corresponding to amino acids 225-530 was synthesized by GenScript and codon optimized for *E. coli* expression. The P-domain gene was cloned in pMal-C5X plasmid (NEB) with a HRV-3C cleavage site between the MBP-6xHis and the P-domain (Nucleotide sequence: CATCACCACCACCAT CACGGTTCTGGTCTGGAAGTTC-TGTTCCAGGGGCCCTCC).The plasmid was transformed into *E.coli* BL21 (DE3) (Agilent Technologies) and plated onto Luria-Bertani plates with 50mg/mL Ampicillin (LB-Amp). A single colony was used to inoculate 100mL of LB-Amp. After incubation for 18 h at 37! C and 210 rpm shaking, 10 mL of culture was used to inoculate 1L of LB-Amp at 37! C and 210 rpm shaking. When OD_{600} reached 0.7, flasks were cooled for 30 min in ice/water bath. Flasks were subsequently incubated at 18! C and 210 rpm shaking for 30 min before induction with 0.5mM IPTG (Isopropyl b-D-1-thiogalactopyranoside). After 18 h, cells were harvested by centrifugation at 10,000 x g for 20 min at 20! C. Pellets from 1L cell culture were resuspended in 40mL of PBS with addition of 150mM of NaCl and flash frozen in liquid nitrogen. Cell pellets were stored at -80! C until further use.

Pellets from 1L of cell culture were thawed on ice and supplemented with one tablet of protease inhibitors (Roche cOmplete EDTA-free cocktail) and 1mg/mL of Lysozyme (Sigma-Aldrich). Cell slurry was kept on ice and sonicated with a Branson Sonifier SFX150 equipped with micro-tip (5 seconds ON, 30 seconds OFF for a total process time of 2 min). Lysed cells were centrifuged at 38,000 x g for 45 min at 4! C. Clarified lysate was batch bound to 4mL of Ni-resin (Roche cOmplete His-Tag Purification Resin) for 1 h at 4! C with end-over-end rotation. Resin slurry was loaded on a plastic column and allowed to flow by gravity. Resin was sequentially washed with 40mL of PBS and 40mL of PBS with 20mM Imidazole (pH 8.0). Bound protein was eluted with PBS with 200mM Imidazole (pH 8.0) and elution collected in 5mL fractions. Protein concentration was determined by absorption at 280nm using a calculated extinction coefficient of 102010 M^{-1} cm^{-1}. Fractions with highest concentration were pooled and 20U of HRV-3C protease (Millipore-Sigma) were added. Cleavage reaction was subsequently dialyzed in 10 kDa MWCO cassette (Thermofisher Slide-A-Lyzer) overnight against 4L of buffer containing 25mM Tris-HCl (pH 7.5), 150mM NaCl and 5mM b-mercaptoethanol. Complete cleavage of MBP-6His from P-domain was confirmed by SDS-PAGE gel shift after Coomassie staining. Cleavage reaction was bound to 2mL of Ni-resin and incubated with end-over-end rotation for 1 h. Resin was loaded onto plastic column and flow-through containing cleaved P-domain was collected. Resin was washed with 5mL of dialysis buffer and both flow-throughs were pooled and concentrated to around 10mg/mL in 10kDa MWCO Amicon Ultra (Millipore-Sigma). Concentrated sample was loaded onto Superdex-200 Increase 10/300 GL (GE-healthcare) equilibrated with 25mM Tris-HCl (pH 7.5), 150mM NaCl and the peak corresponding to P-domain (elution volume 15.5mL) was collected. Purification was assessed by SDS-PAGE and purified P-domain was concentrated to 5mg/mL in 10kDa MWCO Amicon Ultra and stored at 4! C until further use.

Antibodies A1227 and A1431 were produced as previously described (McLellan et al., 2011). Briefly, heavy chain plasmids, containing the HRV3C cleavage site in the hinge region, and light chain plasmids were cotransfected in Expi293F cells (ThermoFisher) using a 3:1 ratio of Turbo293 transfection reagent (Speed Biosystem) to plasmid. Cells were incubated at 37! C, 5% CO_2, 130 rpm shaking, 70% humidity for 18 h and subsequently boosted with 80mL of AbBooster (Abi Scientific). Temperature was lowered to 33! C and expression was continued for additional 6 days. Cells were pelleted at 10000 x g, 20! C for 45 min and pellet discarded. Supernatant containing secreted antibody was supplemented with two tablets of protease inhibitors (Roche cOmplete EDTA-free). All subsequent steps were performed at 4! C. Cell supernantant was loaded by gravity onto 5ml of Protein A slurry (GE MabSelect) pre-equilibrated with PBS. Resin was washed with 50 mL of PBS and eluted in 1mL fractions with Pierce IgG Elution buffer. Elution was immediately neutralized with 1M Tris-Cl, pH 8.0 (final concentration 0.1M). Fractions with highest concentration (determined by A280 absorption) were pooled and 40U of HRV-3C protease (Millipore-Sigma) were added. Cleavage reaction was allowed to

proceed for 18 h at 4! C with end-over-end rotation. Cleavage reaction was assessed by SDS-PAGE before proceeding to next step. One mL of Ni-resin (Roche cOmplete His-Tag Purification Resin) loaded onto plastic column and overlaid with two mL of protein A resin. Ni-resin was used to capture His-tag, while protein A resin was used to capture free Fc. Both resins were equilibrated with PBS. Cleavage reaction was loaded by gravity onto resins and flow-through was collected. Resins were washed with 10mL of PBS and the second flow-through pooled with the first. Protein concentration was assessed by absorption at 280nm and removal of Fc and His-tag was verified after SDS-page and Coomassie staining. Purified Fab were concentrated to 10mg/mL in 10kDa MWCO Amicon Ultra (Millipore-Sigma) and purified on a Superdex-200 Increase 10/300 GL (GE-healthcare). Fractions containing pure Fab were pooled, concentrated to 10mg/mL in 10kDa MWCO Amicon Ultra (Millipore-Sigma) and stored at 4! C until further use.

Crystallization of Fab complex with P2002

Purified P2002 was mixed with 1.5-fold molar excess of purified Fab A1227 or Fab A1431 and incubated at 20! C for 1 h with end-over-end rotation. The mixture was injected onto Supredex 200 Increase 10/300 GL equilibrated with 25mM Tris pH 7.5, 150mM NaCl. Both complexes had a retention volume of around 12.3mL, consistent with two Fabs binding to one P-domain dimer. The fractions containing the complex were pooled and concentrated to 5mg/mL in MWCO 10kDa and immediately used for crystallization trials. The P2002-A1227 Fab complex was mixed with an equal volume of precipitant solution (10% polyethylene glycol 4000, 0.1M Tris pH 8.5, 50mM proline) and crystallized by sitting-drop vapor diffusion over the precipitant solution at 20! C. Rod-shaped crystals formed after 2 days. Crystals were harvested using Dual-Thickness MicroLoops (MiTeGen), cryoprotected using 30% ethylene glycol and immediately flash frozen in liquid nitrogen. The same procedure was used to crystallize the P2002-A1431 Fab complex, except the precipitant solution contained 10% polyethylene glycol 8000 and 0.1M CHES pH 9.5. Rod-shaped crystals formed after 2 days.

Structure determination and analysis

All diffraction data were collected at 100 K and 1.000 Å wavelength at the SER-CAT beamline ID-22 (Advanced Photon Source, Argonne National Laboratory) and processed with the HKL2000 suite (Otwinowski and Minor, 1997). A complete dataset was collected for the GII.4.2002 P domain-A1227 Fab complex to 2.6 Å using 0.25! oscillations and translating the crystal to different spots throughout data collection. A complete dataset was collected for the GII.4.2002 P domain-A1431 Fab complex to 3.1 Å from a single crystal using 0.5! oscillations. Each structure was solved by molecular replacement with the program PHASER (McCoy et al., 2007) using PDB ID 4OOV for the apo structure of GII.4.2002 P domain and PDB ID 3EYQ for the Fab as search models. Iterative cycles of model building and refinement were performed using COOT (Emsley and Cowtan, 2004) and PHENIX (Adams et al., 2004). Data collection and refinement statistics are shown in Table S2.

GI, GII and GII.4 sequence conservation scores were calculated using the AL2CO server (Pei and Grishin, 2001) and mapped onto the GII.4 P domain surface as described previously (Hansman et al., 2011). Buried surface area calculations and interface analyses were carried out with PISA (Krissinel and Henrick, 2007). Structure figures were prepared using the programs The PyMOL Molecular Graphics System, Version 2.0.5 Schrödinger, LLC and Chimera (Pettersen et al., 2004).

Neutralization Assay

Norovirus antibody neutralization

Serial dilutions of each mAb were prepared in complete medium without growth factors (CMGF) and pre-incubated with GII.P16-GII.4 Sydney stool filtrate (3.1 x 10^5 viral genomic copies). After 1 h incubation at 37! C and 5% CO_2, samples were diluted 1:2 in CMGF supplemented with 1000 mM sodium glycochenodeoxycholate (GCDCA; Sigma-Aldrich) plus 100 mM ceramide (Santa Cruz Biotechnology). Duplicated, 100% confluent 4-day old differentiated monolayers, were inoculated. After 1 h incubation at 37! C and 5% CO_2, monolayers were washed twice with CMGF and 100 ml of differentiation medium containing 500 mM GCDCA plus 50 mM ceramide was added to each well. For each set of infections, one plate was immediately frozen at -70! C and a duplicate plate was incubated at 37! C, 5% CO_2 for 24 h and frozen at -70! C.

For each monoclonal antibody, 4 independent experiments with 2 technical replicates for each mAb dilution on each experiment were performed. Neutralization was expressed as a measure of the reduction in viral genomic copies (as percentage) when compared to a control (no mAb) within each assay using real-time RT-qPCR.

Norovirus detection and quantification

Viral RNA was extracted from cultures (cells and media) at 1 h post-infection (hpi) and 24 hpi using the KingFisher instrument and MagMAX - 96 Viral RNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. Norovirus RNA was detected by GI/GII TaqMan real-time RT-PCR (Cannon et al., 2017). Standard curves were generated using 10-fold serial dilutions of GII.4 Sydney RNA transcripts.

QUANTIFICATION AND STATISTICAL ANALYSIS

Antibody EC_{50} and IC_{50} were determined from log-transformed non-linear, dose-response sigmoidal curve fit data using GraphPad 7.02 (Lindesmith et al., 2015; Lindesmith et al., 2017). Antibody binding by EIA less than 0.2 OD at 2 mg/ml or blockade assays IC_{50} >2mg/ml was scored as negative for binding. Samples were tested in duplicate in a minimum of two independent experiments and the results reported in Figures 2 and 3.

DATA AND SOFTWARE AVAILABILITY

The datasets generated during the current study are available from the Lead Contact upon reasonable request. The accession numbers for the amino acids sequence of HuNoV strain capsids reported in this paper are GenBank: AAB50466.2 (GI.1), AFK75851.1 (GI.3), AFK75852.1 (GI.4), AFJ04707.1 (GII.4 1997), AFJ04708.1 (GII.4 2002), AFJ04709.1 (GII.4 2006b), AFV08795.1 (GII.4 2012), APA31970.1 (GII.4 2015), AFS33555.1 (GII.1), AAN08112.1 (GII.2), AFK75854.1 (GII.3), AAN05735.1 (GII.14), AKB94547.1 (GII.17), MK614455 (GII.4c), MK614084 (GII.4 2006a.581 virus-like particles), MK614085 (GII.4 2006a.581.A4A virus-like particles), MK614086 (GII.4 2006a.581.A4B virus-like particles), MK614081 (GII.4 1987. 2012A virus-like particles), MK614082 (GII.4 1987. 2012D virus-like particles), MK614083 (GII.4 1987. 2012E virus-like particles). The accession numbers for the atomic coordinates and structure factors for the reported crystal structures are Protein Data Bank (PDB): 6N81 and 6N8D.

The purpose of Modification 2 to HR001117S0019, the Pandemic Prevention Platform (P3) Broad Agency Announcement, is to change the requirement for the quantity of virus that must be grown as part of the effort. Specifically, the requirement to grow 10^{13} infectious viral units is no longer applicable. The new requirement states that performers must demonstrate the ability to grow pathogen quantities that are sufficient to complete medical countermeasure development, including "bait" for antibody or countermeasure discovery as well as *in vitro* and *in vivo* assays to determine countermeasure efficacy.

The corresponding changes can be found on pages 12 and 13 of the BAA and are bolded below:

Platform Integration and Testing

To ensure end-to-end functional platform integration, the performer will be required to transition the technological advances from the three TAs into capability demonstrations. A central component of this program will be the demonstration of an end-to-end platform functionality. The first capability demonstration will occur in month 12 of the program. Performer groups will be allotted 90 days to:

- **Grow pathogen quantities sufficient to complete medical countermeasure discovery, testing, and evaluation**
- Discover pathogen-specific antibodies
- Successfully transition both antibody/therapy and virus to the antibody evolution group
- Evolve antibody/therapy to 100-fold increased potency
- Deliver genetic sequence of highly potent antibody/therapy to the manufacturing group (data)
- Manufacture research grade material for animal studies
- Demonstrate both product delivery and protection from viral challenge in an animal model

At month 24, performers will conduct a capability demonstration with a pathogen of performer choice. At 36 months, performers will conduct a third 60-day capability demonstration with a DARPA selected pathogen. It is required that during one of the capability demonstrations, GMP manufacturing of clinical grade material will be completed for use in a human clinical trial. The proposal must include a discussion of the number of doses to be manufactured during the demonstration, and the time for manufacturing of these doses must fall within the 60-day timeline.

Using the lessons learned in the previous capability demonstrations, performers will conducted two final capability demonstrations with pathogens that are unidentified to the groups to simulate a "real–world" response to an unknown pathogen. The final deliverable will be the ability to produce at a minimum 1000 doses. The platform should be capable to scale-up manufacturing to 20,000 doses of gene-encoded antibody product for Phase I/II human clinical trials in <60 days, with a delivery platform able to achieve protective levels inside the body.

Finally, the proposal must include a discussion of where the capability platform will be located. It is anticipated that the platform will either be centrally located in one facility run by one of the performer teams, or that aspects of the platform will be physically located at different facilities which are integrated through a system of agreements allowing for the facile transfer of material between locations. In the latter case, the proposal must discuss the coordination and management of the decentralized platform.

Proposers must provide the following in the proposal:

- The proposed manufacturing plan, demonstrating the ability to GMP manufacture products in addition to being able to scale-up manufacturing and distribute 20,000 doses
- The proposed transition plan describing the product/data pipeline from receipt of pathogen and antibody input to final product
- The regulatory plan for phase I clinical safety trials

Table 1. Program metrics and milestones

Technical Metrics
TA 1: On-demand platform to grow virus **1. Grow pathogen quantities sufficient to complete medical countermeasure discovery, testing, and evaluation** 2. Pathogen genetic drift less than 0.1% after 5 successive culturing (growth) iterations (mutations per base pair per culturing cycle) 3. Progeny virus bio-identical to original isolate in lipid, protein, and carbohydrate content 4. Apply platform to five viruses representing at least one RNA virus, one DNA virus, and both (+)-sense and (-)-sense single stranded viral genomes
TA 2: System to evolve antibodies. 1. Ready for use in less than 72 hours 2. Demonstrate the ability to couple antibody discovery capability with the antibody evolution capability either through new discovery technologies or physical coupling with existing technologies 3. Support rapid potency maturation improving antibody/therapy greater than 100-fold in under 8 days 4. Apply technology to mature five different antibodies/therapies targeting at a minimum three (3) different pathogens representing at least one RNA virus, one DNA virus, and both (+)-sense and (-)-sense single stranded viral genomes *While it is assumed that the development of antibody therapies may be necessary to achieve a protective countermeasure in <60 days, all innovative approaches that meet the time and quality metrics stated above will be considered.

Technical Metrics
TA 3: Deliver medical countermeasure(s) 1. Achieve greater than 10 μg/mL serum concentration of target medical countermeasure with peak production in <3 days post-injection in large animals 2. Minimally or low-invasive simple delivery method given at 1 delivery site 3. Demonstrate reproducible production (less than 10% variance in serum concentration) in large animals 4. Demonstrate 100% protection against performer defined pathogen challenge in animal models by day 3 post countermeasure administration 5. Demonstrate longevity of protection lasts for greater than 30 days *While it is assumed that the development of antibody therapies may be necessary to achieve a protective countermeasure in <60 days, all innovative approaches that meet the time and quality metrics stated above will be considered.
Platform Integration: All of the following metrics must be achieved within 60 days and must include full integration with the metrics for TA1-3: 1. Demonstrate the ability to identify a pathogen specific antibody/therapy for rapid affinity maturation in TA2 within 14 days 2. Demonstrate the ability to manufacture research grade genetic constructs for use in animal studies 3. Demonstrate the ability to manufacture clinical grade GMP material for human safety trials – this metric must be met for only one of the phase II demonstrations. The ability to scale-up manufacturing and distribution to 20,000 doses must also be addressed as part of this metric 4. Demonstrate safety and efficacy in animal models and in human clinical trials