Response to GOF Pause Letter – 2R01AI 089728–06, Li (PI)/Baric (co-I)

On March 31, 2016, NIAID informed us that our grant application 2R01AI089728–06 may include GOF research that is subject to the recently-announced U S Government funding pause issued on October 17, 2014 Program has identified subaim 2 1 that may involve research covered under the pause Therefore, we have been requested to address (1) whether the mutations introduced to bat SLCoVs as well as passaging of bat SLCoVs in cell culture may result in SLCoVs that have enhanced pathogenicity and/or transmissibility in mammals, (2) information on the strains of SARS-CoV that we have engineered with "super binding affinity" for human and civet ACE2 receptors, (3) if subaim 2 1 does include GOF work, what changes (either in research design or in budget) can be made to maintain subaim 2 1

Summary of subaim 2.1:

Rationale for subaim 2 1 Recently three bat SLCoVs have been identified that can infect human cells and use human ACE2 as their receptor (PMID 24172901) The overall goal of this subaim is to identify the receptor and viral determinants of the cross-species transmission of these three bat SLCoVs

Experiment #1 We will construct, express, and purify the RBDs from these three bat SLCoVs and ACE2 from different species (including human and bat) We will then measure the binding affinity between each of the RBDs and each of the ACE2 molecules The research tools we will use include ELISA, surface plasmon resonance, AlphaScreen, and pseudovirus entry

Experiment #2 Based on the sequence comparisons of ACE2 from different species (Fig 10A), we can identify a number of residue differences among them that potentially regulate cross-species determinants for bat SLCoVs We will introduce mutations into bat ACE2 corresponding to the residue differences between bat ACE2 and ACE2 from other species, and measure the binding between the mutant bat ACE2 and bat SLCoV RBDs In addition, we will introduce the reverse mutations into ACE2 from non-bat species, and measure the binding between bat SLCoV RBDs and mutant ACE2 from other species

A

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ACE2   21          31          41
Human  IEEQAKTFLD  KFNHEAEDLF  YQSSLASWNY
Civet  TEELAKTFLE  TFNYEAQELS  YQSSVASWNY
Bat    TEDEAKMFLD  KFNTKAEDLS  HQSSLASWDY
Mouse  TEENAKTFLN  NFNQEAEDLS  YQSSLASWNY
         *  ** **   ** :*: *   *** ***·*

ACE2   71          81          321         351
Human  AFLKEQSTLA  QMYPLQEIQN  PNMTQGFWEN  LGKGDFRILM
Civet  AYYEEQSKLA  QTYPLAEIQD  PNMTQGFWEN  LGKGDFRIKM
Bat    AFYEEQSKLA  KNYSLEQIQN  PNMTEGFWNN  LGKGDFRIKM
Mouse  AFYEEQSKTA  QSFSLQEIQT  PHMTQGFWAN  LGHGDFRIKM
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B

SARS-CoV	436	442	472	479	480	487
Human (Tor02)	Y	Y	L	N	D	T
Civet (Sz02)	Y	Y	L	K	D	S
Human/Civet (Gd03)	Y	Y	P	N	G	S
Mouse (PMID 22072787)	H	Y	L	N	D	T
Bat (rs3366)	Y	S	F	N	D	N
Bat (shc014)	Y	W	P	R	P	A
Bat (wiv1)	Y	S	F	N	D	N

Figure 10 Receptor and viral determinants of SLCoV/ACE2 interactions (A) Sequence comparisons of ACE2 from different animal species In red are residues that are critical to the major species barriers between hosts for SARS-CoV infections In blue are residues that directly contact SARS-CoV Asterisks indicate positions which have a single, fully conserved residue Colons indicate positions which have strongly conserved residues Periods indicate positions which have weakly conserved residues (B) Sequence comparisons of S1-CTDs from different SARS-CoV strains and bat SLCoVs The strain names are in parenthesis Shown are six residue positions that are critical for ACE2 binding and are also prone to evolution

Experiment #3 Based on our knowledge about the RBD residues that play key roles in receptor binding (Fig 10B), we will introduce mutations into bat SLCoV RBDs corresponding to the residue differences between bat SLCoV RBDs and human, civet or mouse SARS-CoV RBDs We will then measure the binding between mutant bat SLCoV RBDs and ACE2 from different species

Experiment #4 Based on results from Experiments #1-3, we will introduce mutations into recombinant bat

SLCoVs using reverse genetics, and evaluate virus usage of ACE2 from different species in cell culture In parallel, we will passage bat SLCoVs in cell culture, using experimental evolution to select for novel receptor enhancing mutations in the bat SLCoVs

Response:

Experiments #1, #2, and #3: These experiments will all be carried out using either recombinant proteins or pseudoviruses The pseudoviruses consist of replication-deficient retroviruses that are pseudotyped with SLCoV spike proteins, and hence can only enter cells without replicating themselves No infectious SARS-CoV or SLCoV will be involved, and thus these experiments are not subject to the GOF pause

Experiment #4: This is the only experiment in the entire proposal that involves infectious SLCoVs

Rationale for Experiment #4: A growing body of evidence supports the hypothesis that a potentially large population of SARS-like bat coronaviruses (SLCoV) exist in nature that efficiently use the human, bat and civet ACE2 receptor, can cross the species barrier and replicate efficiently in human cells (PMC4801244, PMC4797993, PMC4810638, PMID 24172901) However, it remains uncertain whether these SLCoV will require additional adaptive mutations to efficiently utilize the human ACE2 receptor, especially in the context of primary airway epithelial cells Mutations which alter ACE2 interactions across species will likely forecast the potential for more serious human disease patterns, as seen during the SARS-CoV epidemic Therefore, identifying key mutations and mutation patterns that might forecast SLCoV emergence would i) assist policy makers in resource allocation decisions, ii) forecast disease patterns in human populations, and iii) inform human monoclonal antibody therapeutic efficacy While several groups have used phylogenetic approaches and some biochemistry to predict emerging coronavirus epidemic and disease severity patterns in human populations (PMC4810480, PMC4702133), these studies are highly speculative because they lack robust empirical support, potentially misinforming epidemic risk potential and outbreak management prioritizations We hypothesize that detailed studies on RBD mutation and evolution may provide critical information for informing the greater public health risk potential during an expanded outbreak Consequently, the experiments in this section are therefore of very high impact These experiments are designed to reveal fundamental mechanisms and patterns of SLCoV RBD mutation that regulate emergence (e g , civet, mouse, human), identify new strains with high epidemic risk potential in humans and animals based on sequence patterns, and inform early choice selection for immunotherapeutic control in an outbreak setting This information will potentially mitigate disease spread to health care workers, predict and reduce the severity of the expanded epidemic in human populations

List of mutations that will be introduced to bat SLCoVs using reverse genetics:

(i) For bat SLCoV rs3366 and bat SLCoV wiv1 Y442S, L472F, T487N (from strain Tor02), Y442S, L472F, K479N, S487N (from strain Sz02), Y442S, P472F, G480D, S487N (from strain Gd03), H436Y, Y442S, L472F, T487N (from mouse-adapted SARS-CoV)

(ii) For bat SLCoV shc014 Y442W, L472P, N479R, D480P, T487A (from strain Tor02), Y442W, L472P, K479R, D480P, S487A (from strain Sz02), Y442W, N479R, G480P, S487A (from strain Gd03), H436Y, Y442W, L472P, N479R, D480P, T487A (from mouse-adapted SARS-CoV)

Expected results from Experiment #4: When the residues from human SARS-CoV strain Tor02 are introduced to bat SLCoVs, the mutant bat SLCoVs may have enhanced binding affinity for human ACE2 because human SARS-CoV strain likely has been adapted to use human ACE2 more efficiently than bat

SLCoVs do Similarly, when the residues from civet or mouse SARS-CoV strains are introduced to bat SLCoVs, the mutant bat SLCoVs may have enhanced binding affinity for civet ACE2 or mouse ACE2, respectively In addition, passage of bat SLCoVs in human cell culture may lead to mutant bat SLCoVs with enhanced affinity for human ACE2 However, the enhanced affinity of SLCoVs for ACE2 doesn't necessarily lead to enhanced viral infectivity or transmissibility (see below)

GOF concerns over introducing mutations into SLCoVs using reverse genetics: The current paradigm argues that increased virus-receptor interactions correlate directly with increased virus growth and pathogenesis However, this has never been empirically tested It is possible that increased virus-receptor interactions attenuate viral infectivity by hindering viral release from infected cells It is also possible that RBD-receptor interactions are highly tuned to regulate associations and dissociations during entry, and as such, both lower and higher affinity RBD-ACE2 interactions may actually ablate receptor biding or downstream S protein conformational reprogramming events that are critical for activation of the fusion domain and entry Moreover, higher affinity interactions can also result in dominant negative effects on virus replication, e g , the production of defective "spike less" progeny virion because high affinity S glycoprotein-ACE2 complexes become tethered on membranes and S cannot participate in virion maturation and release A similar phenomena on virus maturation and release occurs when the receptor is over-expressed in cells (PMID 9123839) Finally, it is also important to consider that RBD interactions are dependent on backbone sequence context, which varies in rs3366, WIV1, SHC014 and SARS-MA, thus, mutations that enhance SARS-MA interaction with a particular ACE2 receptor may subtly enhance, attenuate or ablate interactions across different RBD sequence backbones Because WIV1, SHC014 and SARS-MA can already use the human, civet, mouse and bat ACE2, it is anticipated that most cross mammal responses will be subtle and less than 10 fold over the parental backbone, in either direction Thus, it cannot be reasonably expected that these mutations will result in increased pathogenesis or transmission in any mammalian host

Should any of these recombinants show evidence of enhanced virus growth >1 log in cells expressing the human, bat, mouse or civet ACE2 receptor over wildtype parental backbone SARS-CoV strain or grow more efficiently in primary human airway epithelial cells (HAE), we will immediately i) stop all experiments with the mutant, ii) inform program and the UNC IBC of these results and iii) participate in decision making trees to decide appropriate paths forward

GOF concerns over in vitro passage experiments: The gain of function documents are very clear and focus on experiments that are reasonably anticipated to increase transmissibility or pathogenesis in any mammal Cells in culture are not mammals and in fact in vitro passage has long been used as a strategy to attenuate virus pathogenesis, as viruses adapt to the in vitro environment, losing the capacity to replicate efficiently and produce disease in vivo We had proposed to passage viruses in primary human airway epithelial cell cultures (HAE) or in DBT cells expressing ACE2 receptors from different mammals (human, civet, mouse, bat) Recognizing that primary human cells may be pushing the envelope and the intent of the original GOF document, we will only passage recombinant viruses in mouse astraglial cells (DBT) expressing different ACE2 receptors We believe these experiments are safe as entry is governed by programmed interactions with receptors, entry proteases and other cellular components, which clearly will be different in mouse DBT cells and primary HAE cells, the latter being the normal target for virus infection Thus, entry protease and innate immune program differences in continuous astraglial cell types (as compared to HAE) would likely select for different adaptive mutations resulting in less fit experimentally evolved virus phenotypes when measured in primary HAE and in vivo After in vitro passage, we can evaluate the replication efficiently and perform fitness competition assays of wildtype and experimentally evolved viruses in HAE and DBT cells expressing various receptors, providing definitive information on the replication kinetics and fitness in vitro

Should any of these recombinants show evidence of enhanced virus growth >1 log in cells expressing the human, bat, mouse or civet ACE2 receptor over wildtype parental backbone SARS-CoV strain or grow more

efficiently in primary human airway epithelial cells (HAE), we will immediately i) stop all experiments with the mutant, ii) inform program and the UNC IBC of these results and iii) participate in decision making trees to decide appropriate paths forward

GOF concerns over the engineered SARS-CoV RBDs with "super binding affinity" for human or civet ACE2 receptor Previously we have engineered SARS-CoV RBDs with "super binding affinity" for human mouse or civet ACE2 receptor, respectively (PMID 22291007/ PMC3308800) The study was carried out in 2012 The initial study only involved recombinant proteins and pseudoviruses No infectious SARS-CoV was involved The study was mentioned as background information for subaim 2 1 Independently, the Baric laboratory obtained IBC approval for engineering the hPREDICT and mPREDICT recombinant viruses (see appended recombinant DNA documents) in 2012-2013 Shortly thereafter, the Baric group isolated recombinant hPREDICT and mPREDICT recombinant viruses, however these viruses have not demonstrated any substantial change in disease phenotype in mice or expanded growth phenotypes in Vero cells, as compared to the parental controls Recombinant virus growth kinetics have not been evaluated in primary human airway epithelial cells nor in DBT cells, expressing the civet. human or bat ACE2 receptor For safety considerations, the Baric laboratory is willing to evaluate the growth of these recombinants in HAE cultures and inform program of the results In relationship to this grant application, these experiments are not part of the proposal, and we have no plan to revisit these recombinant viruses encoding these mutant RBDs Thus there are no GOF concerns over these mutant RBDs with respect to this proposal

Alternative plans if NIAID still has concerns over these potential gain-of-function mutations that may enhance the binding affinity of SLCoVs for host ACE2, but may not enhance viral infectivity or transmissibility (we note that loss of function is not anywhere near as powerful a determination of causality as gain of function experiments, which provide more certain determinations of sequence and structure guided function)

(i) We could replace the potential gain-of-function mutations with loss-of-function mutations Specifically, because human SARS-CoV strain likely has been adapted to use human ACE2 more efficiently than bat SLCoVs, instead of introducing residues from human SARS-CoV strains into bat SLCoVs, we will introduce residues from bat SLCoVs into human SARS-CoV strains Correspondingly, instead of generating mutant bat SLCoV strains with enhanced affinity for human ACE2, we will generate mutant human SARS-CoV strains with reduced affinity for human ACE2 The new set of mutations are the reverse of the mutations listed previously in this response We will characterize the receptor-binding affinity of mutant viruses using biochemical assays before we introduce these mutations into infectious viruses

A

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ACE2   21          31          41
Human  IEEQAKTFLD  KFNHEAEDLF  YQSSLASWNY
Civet  TEELAKTFLE  TFNYEAQELS  YQSSVASWNY
Bat    TEDEAKMFLD  KFNTKAEDLS  HQSSLASWDY
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         *  ** **  .** .*..*  :*** *** *

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Mouse  AFYEEQSKTA  QSFSLQEIQT  PHMTQGFWAN  LGHGDFRIKM
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B

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Mouse (PMID 22072787)	H	Y	L	N	D	T
Bat (rs3366)	Y	S	F	N	D	N
Bat (shc014)	Y	W	P	R	P	A
Bat (wiv1)	Y	S	F	N	D	N
Bat (LYRall) (PMID 26719272)	F	S	E	S	T	V

Modified Figure 10 Receptor and viral determinants of SLCoV/ACE2 interactions (A) Sequence comparisons of ACE2 from different animal species In red are residues that are critical to the major species barriers between hosts for SARS-CoV infections In blue are residues that directly contact SARS-CoV Asterisks indicate positions which have a single, fully conserved residue Colons indicate positions which have strongly conserved residues Periods indicate positions which have weakly conserved residues (B) Sequence comparisons of S1-CTDs from different SARS-CoV strains and bat SLCoVs The strain names are in parenthesis Shown are six residue positions that are critical for ACE2 binding

(ii) Similarly, loss-of-function mutations will also be introduced into civet SARS-CoV strains and mouse-adapted SARS-CoV strains, respectively, to generate mutant civet SARS-CoV strains and mutant mouse-adapted SARS-CoV strains with reduced affinity for civet ACE2 and mouse ACE2, respectively The new sets of mutations are the reverse of the mutations listed previously in this response We will characterize the receptor-binding affinity of mutant viruses using biochemical assays before we introduce these mutations into infectious viruses

Although we expect these new sets of mutations to cause loss-of-function of human, civet, and mouse-adapted SARS-CoV strains, we will carefully monitor the receptor usage and cell infectivity of these mutant SARS-CoV strains on cell lines constitutively expressing the civet, human, bat and mouse ACE2 receptor Should any of these recombinants show evidence of enhanced virus growth >1 log in cells expressing the human, bat, mouse or civet ACE2 receptor over wildtype parental backbone strain or grow more efficiently in primary human airway epithelial cells (HAE), we will immediately i) stop all experiments with the mutant, ii) inform program and the UNC IBC of these results and iii) participate in decision making trees to decide appropriate paths forward

(iii) We could remove the experiment involving the passage of bat SLCoVs in cell culture However, we caution against this decision as virus passage very likely reveal novel and unanticipated mutational pathways to improved receptor usage, critical information for public health planning, epidemic monitoring and human antibody immunotherapeutic treatment regimen selection

(iv) We will expand Experiment #4 by incorporating a newly published bat SLCoV strain (LYRall) (PMID 26719272 / PMC4810638), which is listed in the modified Figure 10b Again, we will only study potentially loss-of-function mutations, and carefully monitor the changes of receptor usage and cell infectivity of any mutant SARS-CoV strains The potential loss-of-function mutations that will be introduced from bat SLCoV strain LYRall to other SARS-CoV strains include F436Y, S442Y, E472L, S479N, T480D, V487T (to strain Tor02), F436Y, S442Y, E472L, S479K, T480D, V487S (to strain Sz02), F436Y, S442Y, E472P, S479N, T480G, V487S (to strain Gd03), F436H, S442Y, E472L, S479N, T480D, V487T (to mouse-adapted SARS-CoV strain)

(v) We will also expand Experiment #4 by examining the interactions between wild-type bat SLCoVs and mutant ACE2 from human, civet, mouse, and bat (similar experiments will be done in Experiment #2 using recombinant proteins and pseudoviruses) The goal is to identify receptor determinants of the cross-species transmission of bat SLCoVs using wild-type infectious viruses To this end, mutations will be introduced to ACE2 molecules from non-bat species, the mutant ACE2 molecules will be expressed in cultured cells, and then the infectivity of bat SLCoVs in these cells will be measured *Because all of the mutations will be introduced to host ACE2, not viruses, these experiments are not subject to GOF pause*

In the event that the committee decides that our GOF determinations prove unexceptablethen in the modified Experiment #4 involving infectious viruses, we will focus on loss-of-function mutations in different SARS-CoV strains, expand the scope of the experiment to incorporate a newly published bat SLCoV strain, and also introduce mutations to host ACE2 receptors

Conclusion: We can either (1) maintain the originally proposed Experiment #4 through carefully monitoring any changes in viral infectivity or (2) modify Experiment #4 to remove all potential gain-of-function mutations in infectious viruses We prefer the former In either case, the budget for Experiment 4 in subaim 2 1 can be maintained

LSP ID 20157190
Sched G ID 11361

UNIVERSITY OF NORTH CAROLINA
LABORATORY SAFETY PLAN

Principal Investigator: RALPH S BARIC **Building: Hooker Research Center**

SCHEDULE G RECOMBINANT OR SYNTHETIC NUCLEIC ACID MOLECULES

CLASSIFICATION OF EXPERIMENTS ACCORDING TO NIH GUIDELINES:

You must provide this information to obtain IBC approval. Please consult the Classification Summary Page for NIH Guidelines to determine which section defines your experiments. Choose one category: III-D

DESCRIPTION OF RECOMBINANT OR SYNTHETIC NUCLEIC ACID MOLECULES RESEARCH (Section II)

1. Project Title: Generating a SARS-CoV Mutant to Improve Binding Efficiency for the Human ACE2 Receptor

2. If experiment involve a deliberate attempt to obtain an expression of foreign gene, identify what proteins will be produced:
No foreign genes will be expressed

To enhance the binding of the SARS-CoV attachment protein, spike, to the cellular receptor human angiotensin converting enzyme 2 (hACE), we would like to introduce two substitutions into the receptor binding domain

3. Will recombinant or synthetic nucleic acid molecules, recombinant RNA, virus particles or other micro-organisms containing recombinant or synthetic nucleic acid molecules or RNA, or cells containing recombinant or synthetic nucleic acid molecules or RNA be introduced into whole plants or animals (including insects)? NO

4. Will recombinant or synthetic nucleic acid molecules, recombinant RNA, virus particles or other micro-organisms containing recombinant or synthetic nucleic acid molecules or RNA, or cells containing recombinant or synthetic nucleic acid molecules or RNA be introduced into humans for clinical trial(s)? NO

5. What is the source/how was the DNA obtained? (Is it synthetic-for example synthesized de novo from sequence information by a core facility or company OR was it derived/amplified from a biological source?):

6. Containment conditions specified in the NIH Guidelines:

☐ **BSL 1** ☐ **BSL 2** ☑ **BSL 3**

7. IACUC # (or web ID):

8. Recipient organisms or cells to be used (e.g. E.coli, mouse, Arabidopsis, mouse primary liver cells, strain type of animal.): E coli to propagate fragments of the infectious clone, VeroE6 cells or murine delayed brain tumor cells expressing either the human or civet cat ACE2 molecules to propagate virus, in addition, we will infect primary airway epithelial cell cultures

9. Vectors to be used and source (e.g. pCMV plasmids, E1A deleted adenovirus): all of the infectious clone fragments are cloned into the E coli vector pSMART, the SARS-CoV infectious clone backbone will be used to introduce the changes into the receptor binding domain, fragments ordered from BioBasics will be shipped in pUC57

10. Nature of the inserted sequences (payload) and original source of the DNA (e.g. E.coli galactosidase driven by CMV-IE promoter, mus creating kinase promotor driving duck myosin gene, synthetic, hormone-responsive promotor drivingrat TGFa): we will purchase the DNA insert containing the desired mutations from BioBasics, which will then be ligated in place of the wild type receptor binding domain

11. Description of the Experiments: The "hPredict" for the human ACE2 mutant virus is a SARS-CoV variant incorporating two substitutions in the receptor binding domain (RBD) of the Spike glycoprotein designed to increase the specificity of binding for humanACE2 by reducing its affinity for the civet ACE2 These two substitutions, Y442F and L472F, are predicted to remove a potential steric clash with K31 of the human ACE2 and introduce a clash with T82 of the civet ACE2 (this residue is M82 in the human ACE2)

This construction will be used to test our ability to make functional predictions about the RBD-receptor interface The hPredict virus is predicted to increase the binding affinity for hACE2 while significantly reducing the affinity for the civet ACE2 We intend to assess replication on hACE2-DBT cells, cACE2-DBT cells, and human airway epithelial cells The hPredict RBD will be generated by synthesis of a 600bp cassette by Biobasic and then incorporated into the standard reverse genetics clone of the Urbani strain of SARS-CoV

12. Do these experiments use recombinant or synthetic nucleic acid molecules or RNA that encode microbial toxins? NO

13. Do these experiments involve more than 10 liters of culture at one time? NO

14. Do these experiments involve release of genetically modified organisms to the environment? NO

Life sciences research is essential to scientific advances that underpin improvements in public health and safety, agricultural crops and other plants, animals, the environment, materiel, and national security Despite its value and benefits, some research may provide knowledge, information, products, or technologies that could be misused for harmful purposes The updated Dual Use Policy, including the list of regulated agents and toxins can be found at
http //oba od nih gov/oba/biosecurity/pdf/united_states_government_policy_for_oversight_of_durc_final_version_032812 pdf

Research is not necessarily prohibited, however a risk analysis must be made to determine its Dual Use potential

LSP ID 20157190
Sched G ID 11361

15. Is the material a select agent/toxin or is it derived from a select agent or toxin? NO

16. Could the experiments proposed in this Schedule G change the select agent/toxin status of the material? NO

17. Do these experiments raise dual concern? NO

Categories of experiments that are regulated are those that
a) Enhance the harmful consequences of the agent or toxin,
b) Disrupt immunity or the effectiveness of an immunization against the agent or toxin without clinical or agricultural justification,
c) Confer to the agent or toxin resistance to clinically or agriculturally useful prophylactic or therapeutic interventions against that agent or toxin or facilitates their ability to evade detection methodologies,
d) Increase the stability, transmissibility, or the ability to disseminate the agent or toxin,
e) Alter the host range or tropism of the agent or toxin,
f) Enhance the susceptibility of a host population to the agent or toxin, or
g) Generate or reconstitute an eradicated or extinct agent or toxin listed in the link above

18. Do these experiments meet the definition of a Major Action?
"The deliberate transfer of a drug resistance trait to a microorganism, when such a transfer could compromise the use of the drug to control disease agents in humans, veterinary medicine, or agriculture, is a Major Action (see Section III-A-1-a of the NIH Guidelines). Because of the potential implications for public health, animals or agriculture such experiments warrant special review."NO

On behalf of the institution, the Principal Investigator is responsible for complying fully with UNC policies and NIH Guidelines in conducting any recombinant or synthetic nucleic acid molecules research.
Agreed and **Signed By:** AMY C SIMS

This registration was approved by IBC Chair, _____ on 12/06/2011

LSP ID 20157190
Sched G ID 11374

UNIVERSITY OF NORTH CAROLINA
LABORATORY SAFETY PLAN

Principal Investigator: RALPH S BARIC **Building: Hooker Research Center**

SCHEDULE G RECOMBINANT OR SYNTHETIC NUCLEIC ACID MOLECULES

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1. Project Title: Generating a SARS-CoV Mutant to Improve Binding Efficiency for the Mouse ACE2 Receptor

2. If experiment involve a deliberate attempt to obtain an expression of foreign gene, identify what proteins will be produced:

No foreign genes will be expressed

To enhance binding of the SARS-CoV attachment protein, spike to the cellular receptor murine angtiotensin converting enzyme 2, we would like to combine the mutations in the receptor binding domain (RBD) obtained from three viral passage/adaptation experiments SARS-CoV was intranasally inoculated into mice, allowed to replicate, the nares and lungs harvested and used to inoculate subsequent sets of mice Three separate point mutations in the spike RBD were obtained and we would like to engineer them into a single mutant construct

3. Will recombinant or synthetic nucleic acid molecules, recombinant RNA, virus particles or other micro-organisms containing recombinant or synthetic nucleic acid molecules or RNA, or cells containing recombinant or synthetic nucleic acid molecules or RNA be introduced into whole plants or animals (including insects)? YES

4. Will recombinant or synthetic nucleic acid molecules, recombinant RNA, virus particles or other micro-organisms containing recombinant or synthetic nucleic acid molecules or RNA, or cells containing recombinant or synthetic nucleic acid molecules or RNA be introduced into humans for clinical trial(s)? NO

5. What is the source/how was the DNA obtained? (Is it synthetic-for example synthesized de novo from sequence information by a core facility or company OR was it derived/amplified from a biological source?):

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8. Recipient organisms or cells to be used (e.g. E.coli, mouse, Arabidopsis, mouse primary liver cells, strain type of animal.):
E coli to propagate the pieces of the infectious clone, VeroE6 or murine delayed brain tumor cells expressing mouse ACE2 (mACE2-DBT), cells to propagate the virus, and mice

9. Vectors to be used and source (e.g. pCMV plasmids, E1A deleted adenovirus): all of the fragments in our infectious clone are in pSMART, the SARS-CoV infectious clone backbone will be used to introduce the changes into the RBD, fragments ordered from BioBasics will be shipped in pUC57

10. Nature of the inserted sequences (payload) and original source of the DNA (e.g. E.coli galactosidase driven by CMV-IE promoter, mus creating kinase promotor driving duck myosin gene, synthetic, hormone-responsive promotor drivingrat TGFa): we will purchase a DNA fragment from BioBasics containing the three desired point mutations which will then be ligated in place of the wild type receptor binding domain

11. Description of the Experiments: The mPredict virus is a SARS-CoV variant incorporating three substitutions in the receptor binding domain of the SARS Spike glycoprotein These three substitutions are designed to improve the efficiency of murine-ACE2 binding

Of the three independently derived mouse-adapted SARS-CoVs, each contain substitutions in the receptor binding domain (RBD) of the spike glycoprotein The substitutions are not identical, but there are conserved sites between the three mouse-adapted viruses Combining this sequence information with crystal structures and homology models of the SARS-RBD bound to the ACE2 receptor, we predict that a RBD combining three substitutions (Y442L, N479K, Y436H) will provide enhanced binding to the murine ACE2 receptor beyond that of the individual mouse-adapted viruses We predict that this enhanced binding will increase pathogenesis in the mouse model independent of mouse-adapted substitutions in viral proteins other than Spike

These three substitutions in spike will be generated by synthesis of a 1200bp cassette by Biobasic and then incorporated into the standard reverse genetics clone of the Urbani strain of SARS-CoV In addition, the cassette/substitutions will be combined with 1-5 additional mouse-adapted substitutions from the MA15 virus

Assessment of the mPredict virus will include both in vitro studies (i e growth curves on ACE2-limited DBT cell lines) and pathogenesis studies in mice Initial characterization will compare the mPredict virus to the MA15 and Urbani strains, including measures of mortality, weight loss, lung titer, and histology Future studies may assess the effectiveness of neutralizing antibodies against mPredict, both in vivo and in vitro, as we expect this virus to more effectively bind the murine ACE2 receptor, potentially altering neutralization profiles

12. Do these experiments use recombinant or synthetic nucleic acid molecules or RNA that encode microbial toxins? NO

LSP ID 20157190
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13. Do these experiments involve more than 10 liters of culture at one time? NO

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f) Enhance the susceptibility of a host population to the agent or toxin, or
g) Generate or reconstitute an eradicated or extinct agent or toxin listed in the link above

18. Do these experiments meet the definition of a Major Action?
"The deliberate transfer of a drug resistance trait to a microorganism, when such a transfer could compromise the use of the drug to control disease agents in humans, veterinary medicine, or agriculture, is a Major Action (see Section III-A-1-a of the NIH Guidelines). Because of the potential implications for public health, animals or agriculture such experiments warrant special review."NO

GENE TRANSFER EXPERIMENTS INVOLVING WHOLE ANIMALS OR PLANTS (Section III)

1. Provide a description of the vector. (Provide relevant literature citations, if available, or a preprint.): the SARS-CoV molecular clone published in 2003 Yount et al PNAS 100(22) 12995-13000

LSP ID 20157190
Sched G ID 11374

2. Provide a physical map of the vector: 20101706-704220087-20111024-570-45 pdf

3. Who constructed this vector? (Name, institution): Boyd Yount, Jr Damon Deming and Meagan Bolles at the University of North Carolina at Chapel Hill

4. List specific DNA or RNA segments from any human, veterinary, or plant pathogen included in the recombinant or synthetic nucleic acid molecules (include regulatory elements): SARS-CoV

5. How was the vector produced or packaged: Individual clones will be manipulated at BL2 but full length assembly and in vitro transcription will be performed in the BL3 facility In vitro transcripts will be electroporated in Vero cells and viruses isolated by plaque purification or blind serial passage

6. If a viral packaging or expression system is used, have experiments been done to show that replication competent viruses are excluded?: No

7. Which lab (Principal Investigator, Institution, Company, etc.) produced the DNA to be used in these experiments: Ralph Baric, University of North Carolina at Chapel Hill

8. Will attempts be made to insert recombinant or synthetic nucleic acid molecules into germ lines in order to establish a trangenic animal or plant line: No

On behalf of the institution, the Principal Investigator is responsible for complying fully with UNC policies and NIH Guidelines in conducting any recombinant or synthetic nucleic acid molecules research.

Agreed and **Signed By:** AMY C SIMS

This registration was approved by IBC Chair, _____ on 12/06/2011

Response to Summary Statement – 2R01AI 089728–06, Li (PI)/Baric (co-I)

On April 20, 2016, NIAID informed us that our grant application 2R01AI089728–06 needs to address two concerns from the summary statement (1) **BIOHAZARD COMMENT:** There are concerns that recombinant coronaviruses altered to enhance proteolytic cleavage or binding of the human angiotensin converting enzyme 2 (ACE 2) receptor may have novel and unexpected virulence phenotypes Therefore, biosafety level-3 (BSL-3) protections, training, and monitoring procedures should be considered unless otherwise indicated, (2) **BUDGETARY OVERLAP:** There is potential for budgetary overlap with the project R01AI110700 titled, "Mechanisms of MERS-CoV Entry, Cross-species Transmission and Pathogenesis" awarded to PI Ralph Baric (co-investigator on this proposal) Our response is as following

Response to BIOHAZARD COMMENT:

Biosafety level-3 (BSL-3) protections, training, and monitoring procedures will be used for any work related to SARS-CoV and bat SARS-like CoVs (SEE SECTION 11, APPENDED BELOW)

Response to BUDGETARY OVERLAP:

The project R01AI110700 titled "Mechanisms of MERS-CoV Entry, Cross-species Transmission and Pathogenesis" studies MERS-CoV and bat MERS-like CoVs such as HKU4 and HKU5 The current grant application 2R01AI089728–06 titled "Receptor recognition and cell entry of coronaviruses" proposes to study all of the other coronaviruses except MERS-CoV and bat MERS-like CoVs Therefore, there is no budgetary overlap between these two projects

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SECTION 11. Select Agent Research/Biohazards/Dual Use. SARS-CoV is a select agent, requires appropriate BSL3 facilities and UNC Chapel Hill has a license to work with this select agent pathogen Bat SARS-like Coronaviruses and civet SARS-like coronaviruses are not designated as select agents We recover and culture all SARS like viruses (Bt-SLCoV and Civet-SLCoV) under BSL3 conditions We are profoundly aware of the necessity for meeting the most rigorous standards for safe use of the viruses described in this proposal Dr Baric has certified and approved BSL3 laboratories dedicated for SARS-CoV research, which has been in use since 2003 Drs Baric has directed his own BSL3 laboratories for the past 12 years, primarily studying the SARS-CoV Detailed protocols for safe use of SARS-CoV, MERS-CoV and related BtSLCoV and viral derivitives, have been presented to and approved by the Institutional Biosafety Committee at UNC Detailed protocols were written, designed, and approved with the goal of ensuring the safety of the investigators and the general public, while allowing invaluable research to proceed If any BtSLCoV become select agents, we will register and abide by federal regulations, noting that our facilities are select agent certified to work with these agents In response to GOF requirements, we have responded to all noted expeirments of concerns in a timely fashion and provided these documents to NIH for review We have also provided alternative approaches, should certain experiments be deemed unexceptable During the course of this program, should experimental findings dictate the development of new recombinant viruses, we will notify the NIH of potential GOF concerns, while applying for approval from both the NIH and the local IBC

We recognize that experiments are proposed that might subtly alter the host range of the Bt SLCoV (WIV1, SHC014, etc) and discussed potential GOF concerns with the NIH and local IBC We have generated a molecular clone to SARS-CoV, WIV1 and SHC014 and isolated recombinant viruses Other emerging coronaviruses in the facilities are MERS-CoV and some other BtSARS-like coronaviruses HKU5-S and HKU3-BSRBD

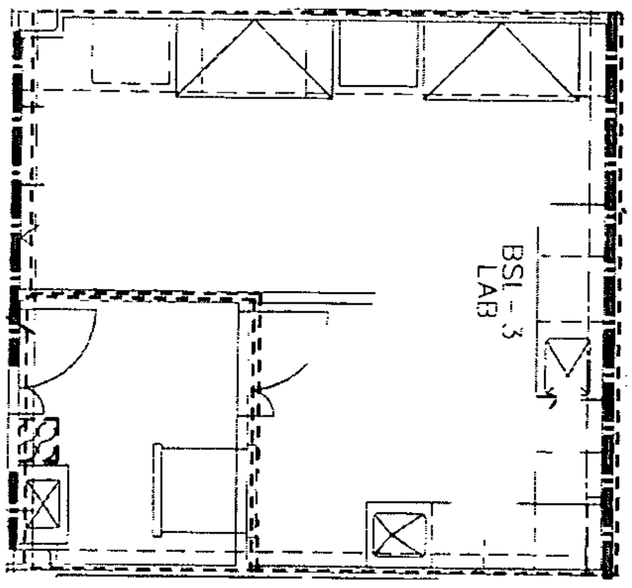
Biosafety:

- All work will be performed with approved standard operating procedures (SOPs) and safety conditions for wildtype and recombinant SARS-CoV, SHC014, WIV1, other BtSLCoV and derivative viruses For recovery

of SARS-CoV and SARS like CoV recombinant viruses, all transcription, electroporation and analysis will be performed at BSL3 as previously approved by UNC's institutional IBC

- Any derived viruses will be stored in -80 in the BSL3, as with any BSL3 recombinant virus isolate

Fig 12. MERS-CoV BSL3 Facility.



- Analysis will be by plaque assay, $TCID_{50}$, single cycle growth assays, protein detection by immunoblot and immunofluorescence or by RNA abundance using real time RT-PCR
- Biological samples will be inactivated prior to removal and shipment to Dr Li, using validated and approved SOPs Protocols have been approved for destroying full length viral genomes for SARS-CoV
- Any wildtype or recombinant BSL3 viruses will be shipped using FAA-approved protocols and permits from the CDC All laboratories have designated "shipping" trained personnel
- Some SARS-CoV variants required passage in cell culture to enhance adaptation to various civet, bat and/or human ACE2 receptors As in vitro passage oftentimes attenuates virus pathogenesis for the original host, we anticipate that these viruses are less pathogenic in humans, although direct evidence is lacking We have requested GOF approval for these experiments
- All experimental approaches, handling, and analysis will be based on the presumption that any recombinant or chimeric viruses will retain the potential for human infection and have the same safety risks as the parental strains SARS-CoV and btSLCoV isolates will be studied under BSL3
- Specifically, the transcription of genome RNA, electroporation of genome RNA into cells, infection of cells, analysis of virus growth, as well as all storage, will performed at BSL3 with full protections as described in approved SOPs
- Human antibodies are now available that neutralize SARS-CoV, and IFN cocktails may reduce virus growth significantly in some culture conditions Nucleoside analogues from Gildead Scientific have robust in vitro and in vivo activity against SARS and these SARS like CoV (Baric unpublished, manuscript in preparation)

Biosafety and Biocontainment. Baric Lab

Agent Information

Agent (Category C) Biosafety Level Diseases, Symptoms Caused by Agent

SARS-CoV-ARDS and fatal respiratory infection in humans BSL3

SHC014, WIV1 and other BtSLCoV- These viruses replicate in various primary human cells No infections have ever been reported in humans so their pathogenic potential in humans is unknown Recombinant viruses harboring mutations that enhance or attenuate ACE2 usage in various species may alter pathogenic outcomes, ranging from attenuation to potentially increased growth in primary human cells Should any mutants replicate 10 fold more efficiently in primary human airway epithelial cultures, we will halt experiments, notify our local IBC and NIH program officers

BSL3 SARS-CoV BSL3 Facility The Baric Labs BSL3 facilities have been designed to conform to the safety requirements recommended in Biosafety in Microbiological and Biomedical Laboratories (BMBL), U S Department of Health and Human Services, Public Health Service, CDC and National Institutes of Health Laboratory safety plans have been submitted and the facility has been approved for use by UNC Department of Environmental Health and Safety (EHS) Electronic card access will be required for entering into the facility All workers will be trained by EHS to safely use PAPR respirators, and appropriate work habits in a BSL3 facility and active medical surveillance plans are in place The Baric Hooker BSL3 facility (**Figure 12**) has been operational since 2004, contains redundant fans, emergency power to fans, biological safety cabinets and freezers, and accommodates two double-sided SealSafe mouse racks housing ~196 cages The Baric laboratory also has a second BSL3 suite in McGavran-Greenberg Research Hall, directly adjacent to the main BSL2 laboratory, which is about 800 sq feet, is supported by redundant fans, contains two biological safety cabinet and 2 Seal Safe Mouse Racks (~100 cages) (facility not shown) This facility is also registered as a Select Agent Facility for SARS-CoV research

BSL3 Materials Materials classified as BSL3 will consist of the wildtype and recombinant SARS-CoV, Civet and BtSLCoV (WIV1, SHC014, etc) and derivative viruses Within the BSL3 facility, experimentation with infectious virus, or with SARS-CoV RNA genomes over 2/3 length will be carried out in a certified Class II BSC All staff wear portal air breathing apparatus, tyvek suits and apron, booties, and hands are double gloved

Medical Monitoring and Exposure Management Plan
Laboratory Personnel Working with SARS-CoV and related BtSLCoV (Designed by discussion with the Baric laboratory, Department of Environmental Health and Safety(EHS), UEOHC (University Employee Occupational Health Clinic), UNC Division of Infectious Disease, UNC Division of Hospital Epidemiology) This monitoring plan is identical for SARS-CoV, MERS-CoV and BtSLCoV

General
All workers with potential exposure to SARS-CoV and BtSLCoV will be issued a thermometer and 2 surgical masks to keep at home IAV antivirals will be provided to research staff as well, noting that antivirals aren't available for the emerging coronaviruses They will be responsible to keep these items in working condition and in an accessible location The laboratory will maintain a supply of these items in the event that they are lost or damaged Serum samples from laboratory personnel who work in the BSL3 lab will be collected and analyzed on timely intervals to determine if subtle exposures resulting in sub-clinical infections have occurred Samples will be taken at the UEOHC and analyzed at an appropriate laboratory (UNC Hospitals, NC State Public Health Laboratories, or CDC laboratories)

Exposure Event
Definition: 1) animal bite, cut, or needle stick with a contaminated sharp, 2) mucous membrane of eyes, nose, or mouth exposure resulting from splash or contact with contaminated hands, 3) aerosol generating activity or event coinciding with failure primary containment such as of biological safety cabinet, PPE, and/or work practices
Procedure: Dr Ralph Baric and UEOHC are to be notified Worker confirms to UEOHC that they have surgical masks, antivirals and thermometer accessible at home or are provided replacements by before leaving work after appropriate decontamination procedures Worker will remain out of work for at least 10 days and is advised to minimize contacts outside of household Worker monitors temperature and symptoms Worker calls UEOHC daily to report on temperature and symptoms UEOHC asks standard question list for symptoms and advises worker regarding self-quarantine or need for medical evaluation
No Symptoms: If no temperature elevation above 100 degrees F or no symptoms consistent with SARS-CoV and BtSLCoV, which appear by day 10 after exposure, UEOHC will arrange to see employee to give final clearance to return to work without further monitoring Worker will be given specific appointment time and is to report directly to the designated location for the evaluation wearing a surgical mask This encounter may take place during normal working hours and in a place deemed to be appropriate for the circumstances by UEOHC This may be UEOHC itself or a negative pressure room in the Pulmonary Clinic of the ACC
Symptoms: If temperature elevates above 100 degrees F or if symptoms are consistent with SARS, BtSLCoV or MERS-CoV infection appear by day 10 after exposure, worker will be advised to self-quarantine (i e to minimize contacts) and to take precautions to minimize droplet generation including covering nose and mouth when coughing or sneezing UEOHC will arrange to have patient seen after hours in an appropriate location for clinical evaluation The site will likely be the ID clinic since the evaluation may need to include a physical examination, chest radiograph, and blood sampling and SARS/MERS-CoV laboratory test Depending on availability and clinical circumstances the evaluating MD may be the Medical Director of UEOHC, the attending from the ID consultation service or the attending for the General Medical Service on call for UEOHC after hours Disposition will depend on the outcome of the evaluation

Potential Exposure Event
Definition: A release of virus outside of primary containment with PPE intact Examples include 1) a spill in the open laboratory, 2) failure of biological safety cabinet during work with virus, 3) splash outside of a biological safety cabinet, 4) leakage of contaminated research material through gloves or other PPE, or 5) failure of secondary containment, e g negative pressure in lab, at the same time as a spill outside of the biological safety cabinet
Procedure: Dr Ralph Baric and UEOHC are to be notified Worker confirms to UEOHC they have surgical masks, and thermometer accessible at home or gets replacements at UEOHC before leaving work after appropriate decontamination procedures in laboratory Worker will monitor temperature and symptoms for 10 days Worker will communicate with UEOHC daily before work to report on temperature and symptoms UEOHC will ask a standard question list for symptoms and will advise worker whether they may report to work directly or should remain home
No Symptoms: If no temperature elevation above 100 degrees F or no symptoms consistent with SARS/MERS-CoV appears by day 10 after exposure, UEOHC will arrange to see employee to give final clearance to return to work without further monitoring Worker will be given specific appointment time and is to

report directly to the place for the evaluation wearing a surgical mask This encounter may take place during normal working hours and in a place deemed to be appropriate for the circumstances by UEOHC This may be UEOHC itself or a negative pressure room in the Pulmonary Clinic of the ACC

Symptoms: If temperature elevation above 100 degrees F or symptoms appears within days 10 after exposure, employee will remain away from work for a minimum of 72 hours after developing fever or symptoms They will be advised to self-quarantine (i e minimize contacts) and to take precautions to minimize droplet generation including covering nose and mouth when coughing or sneezing Worker will communicate with UEOHC daily to report temperature and symptoms

If temperature and symptoms are gone within 72 hours of appearance, UEOHC will arrange to see worker in clinic to give final approval to return to work Employee will be given specific appointment time and is to report directly to the place for the evaluation wearing a surgical mask This encounter may take place during normal working hours and in a place deemed to be appropriate for the circumstances by UEOHC This may be UEOHC itself or a negative pressure room in the Pulmonary Clinic of the ACC

If symptoms and /or fever are not gone after 72 hours, employee will continue to monitor himself/herself and report results to UEOHC by telephone Employee will be advised when/if arrangements for clinical evaluation (same as last step for high risk exposure management above) will be needed

Employee Symptoms with No Recognized Exposure

Definition: Employee develops respiratory symptoms or fever, but there has been no recognized exposure or laboratory accident involving breach of PPE or inadequate work practices, but employee develops respiratory symptoms or fever These procedures do not apply to personnel who have not worked in the SARS/MERS-CoV/BtSLCoV laboratory for 10 days or more prior to onset of symptoms They should, however, report their symptoms to the principal investigator and the UEOHC

Procedure: Employee notifies PI and UEOHC and confirms they have thermometer, and surgical mask available The Baric lab will make arrangements to replace these items if symptoms or fever are noted while employee is not at work If employee is at work, they will go home and take the required items with them from a supply maintained at the laboratory

Symptoms: Employee will remain away from work for a minimum of 72 hours after developing fever or symptoms Employee will be advised to self-quarantine (i e minimize contacts) and take precautions to minimize droplet generation including covering nose and mouth when coughing or sneezing Worker will communicate with UEOHC daily to report temperature and symptoms

If symptoms and fever are not gone after 72 hours, worker will continue to monitor his/her condition and will report results to UEOHC daily Employee will be advised when/if arrangements for clinical evaluation after hours will be needed

Resolution of Symptoms: If temperature and symptoms are gone within 72 hours of appearance, UEOHC will arrange to see worker to give final approval to return to work UEOHC will arrange to see employee to give final clearance to return to work without further monitoring and the supervisor will be notified Worker will be given specific appointment time and is to report directly to the location for the evaluation wearing a surgical mask This encounter may take place during normal working hours and in a place deemed to be appropriate for the circumstances by UEOHC This may be UEOHC itself or a negative pressure room in the Pulmonary Clinic of the ACC

Severe Illness: If employee develops a respiratory illness which may require specific medical treatment or evaluation for admission to hospital (including any degree of pneumonia diagnosed by a physician, PA, or NP) within 14 days of any direct contact with the laboratory or during any period of symptom monitoring required by UOEHC, the Orange County Health Department and the County Health Department in the county of residence of the worker, if different, must be notified Workers with a respiratory illness of severity, which may require specific medical treatment or hospitalization, will be evaluated by staff of the UNC Hospitals Emergency Department (ED) The patient will be instructed to wear a surgical mask when reporting to the ED and to come to a specified entrance, which minimizes contact with other patients and ED personnel Disposition will be determined by the evaluating physician with consultation with the ID consultation attending and UEOHC

Respiratory Disease Protection: For laboratory personnel, inactivated flu vaccines will be offered each year to minimize likelihood of severe respiratory disease unrelated to SARS-CoV, BtSLCoV or MERS-CoV exposure in the BSL3 laboratory Serum samples have been taken and stored at the University Employee Occupational Health Clinic on a twice yearly basis for future reference

SARS-CoV, BtSLCoV Identification The reverse genetic system developed for SARS-CoV, SHC014 and WIV1 results in recombinant viruses that contain unique marker mutations that allows the precise identification

of the source of virus responsible for any particular infection of laboratory personnel Similar markers exist for the other SLCoV studied in this proposal Primer sets are available in the laboratory to determine which particular recombinant virus was responsible for infection and to distinguish between naturally acquired (should SARS/MERS-CoV spreads) and laboratory acquired infections Primer sets are also available for distinguishing among the many different recombinant viruses generated in the laboratory

Fang Li, Ph D

April Coon, Assistant Director
Sponsored Projects Administration