

From: Murphy, Joseph P Maj USMC DARPA DIRO (USA) <[REDACTED]>

Sent:

To:

Cc:

Subject:

Capt xxxxx,

Thanks for responding.

I'm reaching out to communicate some information relative to COVID that I don't believe xxxxx or your director is aware of. You probably saw earlier this week that more official documents linking NIH and EcoHealth Alliance to the Wuhan Institute of Virology were published by The Intercept. I came across additional incriminating documents and produced an analysis shortly after leaving DARPA last month. This report was routed to the DOD IG office.

I'm unsure whether the significance of what I communicated is understood by those that received the report. Decisions with regards to the vaccines do not appear to be informed by analysis of the documents. The main points being that SARS-CoV-2 matches the SARS vaccine variants the NIH-EcoHealth program was making in Wuhan; that the DOD rejected the program proposal because vaccines would be ineffective and because the spike proteins being inserted into the variants were deemed too dangerous (gain-of-function); and that the DOD now mandates vaccines that copy the spike protein previously deemed too dangerous. To me, and to those who informed my analysis, this situation meets no-go or abort criteria with regards to the vaccines until the toxicity of the spike protein can be investigated. There's also information within the documents about which drugs effectively treat the program's SARS-CoVs.

Thus why I'm reaching out. I'm trying to help aid leadership grapple with the vaccines and the mandate with as much information as is available. I wanted to push this information your way.

Several of the documents referenced in the IG report have since been downgraded.

Please reach out to me with questions.

V/R,

Major Joe Murphy USMC
Marine Program Liaison
Code 34 & 35

Office of Naval Research

Work: [REDACTED]

Cell: [REDACTED]
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[REDACTED]
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DEFENSE ADVANCED RESEARCH PROJECTS AGENCY
675 NORTH RANDOLPH STREET
ARLINGTON, VA 22203-2114

13 Aug 21

From: COMMANDANT OF THE MARINE CORPS FELLOW, DARPA
To: INSPECTOR GENERAL

Subj: SARS-CoV-2 ORIGINS INVESTIGATION WITH US GOVERNMENT PROGRAM
UNDISCLOSED DOCUMENT ANALYSIS

- Ref: (1) Executive Slide HR00118S0017 EcoHealth Alliance DEFUSE
(2) HR00118S0017-PREEMPT-FP-019-PM Summary (Selectable - Not Recommended)
(3) PREEMPT Volume 1 no ESS HR00118S0017 EcoHealth Alliance DEFUSE
(4) PREEMPT Volume 2 EHA Final HR00118S0017 EcoHealth Alliance DEFUSE
(5) SF424_2_0-V2.0 HR00118S0017 EcoHealth Alliance DEFUSE
(6) WIV Budget packet HR00118S0017 EcoHealth Alliance DEFUSE
(7) WS00094394-RR_KeyPersonExpanded_2_0-V2.0 HR00118S0017 EcoHealth Alliance DEFUSE
(8) WS00094394-RR_PersonalData_1_2-V1.2 HR00118S0017 EcoHealth Alliance DEFUSE

1. SARS-CoV-2 is an American-created recombinant bat vaccine, or its precursor virus. It was created by an EcoHealth Alliance program at the Wuhan Institute of Virology (WIV), as suggested by the reporting surrounding the lab leak hypothesis. The details of this program have been concealed since the pandemic began. These details can be found in the EcoHealth Alliance proposal response to the DARPA PREEMPT¹¹ program Broad Agency Announcement (BAA) HR00118S0017, dated March 2018¹¹ - a document not yet publicly disclosed.

The contents of the proposed program are extremely detailed. Peter Daszak lays out step-by-step what the organization intends to do by phase and by location. The primary scientists involved, their roles, and their institutions are indicated. The funding plan for the WIV work is its own document. The reasons why nonpharmaceutical interventions like masks and medical countermeasures like the mRNA vaccines do not work well can be extrapolated from the details. The reasons why the early treatment protocols work as curatives are apparent.

SARS-CoV-2's form as it emerged is likely as a precursor, deliberately virulent, humanized recombinant SARSr-CoV that was to be reverse engineered into a live attenuated SARSr-Cov bat vaccine. Its nature can be determined from analysis of its genome with the context provided by the EcoHealth Alliance proposal. Joining this analysis with US intelligence collections on Wuhan will aid this determination.

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When synthesized with the EcoHealth Alliance proposal, US collections confirm EcoHealth Alliance was performing the work proposed. The analysts produce their reports in a vacuum, absent the context the proposal provides. As a fellow at DARPA, I could see both, and can do the synthesis. For instance, WIV personnel identified in intelligence reports are named in the proposal, these people use the lexicon of the proposal in the collections, and the virus variants proposed for experimentation are identical to those gleaned by collections. Moreover, I am also privy to information obtained by congressional office investigators and by DRASTIC^{iv}, which further corroborates that the program detailed in the BAA response was conducted until it was shut down in April 2020.

The purpose of the EcoHealth program, called DEFUSE^v in the proposal, was to inoculate bats in the Yunnan, China caves where confirmed SARS-CoVs were found. Ostensibly, doing this would prevent another SARS-CoV pandemic; the bats' immune systems would be reinforced to prevent a deadly SARS-CoV from emerging. The specific language used is "inoculate bats with novel chimeric polyvalent spike proteins to enhance their adaptive immune memory against specific high-risk viruses."^{vi} Being defense-related, it makes sense that EcoHealth submitted the proposal first to the Department of Defense, before it settled with NIH/NIAID. The BAA response is dated March 2018 and was submitted by Peter Daszak, president of EcoHealth Alliance.

DARPA rejected the proposal because the work was too close to violating the gain-of-function (GoF) moratorium,^{vii} despite what Peter Daszak says in the proposal (that the work would not^{viii}). As is known, Dr. Fauci with NIAID did not reject the proposal. The work took place at the WIV and at several sites in the US, identified in detail in the proposal.^{ix}

The EcoHealth Alliance response to the PREEMPT BAA is placed along with other proposal documents in the PREEMPT folder on the DARPA Biological Technologies Office JWICS (top secret) share drive, address: Network/filer/BTO/CI Folder/PREEMPT

This folder was empty for a year. The files, completely unmarked with classification or distribution data, were placed in this folder in July 2021, which conspicuously aligns with media reporting, my probing, and Senator Paul's inquiry into NIH/NIAID gain-of-function programs. The unmarked nature combined with the timing signals that the documents were being hidden. No files at DARPA go unmarked in classification or distribution, including proprietary documents. Furthermore, PREEMPT is an unclassified program.

The files are also now held by Marine Corps Intelligence Activity (MCIA). They are identified in the reference block above.

2. SARS-CoV-2, hereafter referred to as SARSr-CoV-WIV, is a synthetic spike protein chimera engineered to attach to human ACE2 receptors and

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inserted into a recombinant bat SARSr-CoV backbone. It is likely a live vaccine not yet engineered to a more attenuated state that the program sought to create with its final version. It leaked and spread rapidly because it was aerosolized so it could efficiently infect bats in caves, but it was not ready to infect bats yet, which is why it does not appear to infect bats. The reason the disease is so confusing is because it is less a virus than it is engineered spike proteins hitch-hiking a ride on a SARSr-CoV quasispecies swarm. The closer it is to the final live attenuated vaccine form, the more likely that it has been deattenuating since initial escape in August 2019.

The utility of certain countermeasures can be extrapolated from the documents:

- The team selected for SARSr-CoVs that were most monoclonal antibody and vaccine resistant.
- It is not practical to inoculate bats directly with shots, nor can bats get respiratory infections from droplets, so the team developed an aerosol to deliver the inoculations directly into the caves. To ensure it worked well, they developed the aerosol against masked civets.
- The proposal notes that interferon, Remdesivir, and chloroquine phosphate inhibit SARSr-CoV viral replication.

Because of its (now) known nature, the SARSr-CoV-WIV's illness is readily resolved with early treatment that inhibits the viral replication that spreads the spike proteins around the body (which induce a harmful overactive immune response as the body tries to clear the spikes from the ACE2 receptors). Many of the early treatment protocols ignored by the authorities work because they inhibit viral replication or modulate the immune response to the spike proteins, which makes sense within the context of what EcoHealth was creating. Some of these treatment protocols also inhibit the action of the engineered spike protein. For instance, Ivermectin (identified as curative in April 2020) works throughout all phases of illness because it both inhibits viral replication and modulates the immune response. Of note, chloroquine phosphate (Hydroxychloroquine, identified April 2020 as curative) is identified in the proposal as a SARSr-CoV inhibitor, as is interferon (identified May 2020 as curative).

The gene-encoded, or "mRNA," vaccines work poorly because they are synthetic replications of the already-synthetic SARSr-CoV-WIV spike proteins and possess no other epitopes. The mRNA instructs the cells to produce synthetic copies of the SARSr-CoV-WIV synthetic spike protein directly into the bloodstream, wherein they spread and produce the same ACE2 immune storm that the recombinant vaccine does. Many doctors in the country have identified that the symptoms of vaccine reactions mirror the symptoms of the disease, which corroborates with the similar synthetic nature and function of the respective spike proteins. The vaccine recipient has no defense against the bloodstream entry, but their nose protects them from the recombinant spike protein quasispecies during "natural infection" (better termed as aerosolized inoculation).

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Furthermore, the EcoHealth proposal states that a "vaccine approach lacks sufficient epitope coverage to protect against quasispecies of coronavirus." Consequently, they were trying to make vaccines work by "targeted immune boosting via vaccine inoculators using chimeric polyvalent recombinant spike proteins."^{x1} The nature of using a spike protein vaccine with one epitope against a spike protein vaccine with a quasispecies may explain the unusual (and potentially detrimental) antibody response amongst the vaccinated to the new COVID variants.^{x11} Fundamentally, the knowledge the proposal provides signals that the risk of Antibody Dependent Enhancement (ADE) from vaccination should be evaluated with high priority, on top of the reality that single-epitope vaccines will have little effect against SARSr-CoV-WIV, as indicated in the proposal.

The potential for SARSr-CoV-WIV to deattenuate requires immediate attention. Live vaccines have been found to deattenuate in the past. If this is the case with SARSr-CoV-WIV, then the mass vaccination campaign actually performs an accelerated gain-of-function for it. Since it is designed for bats off of a human-susceptible SARS-CoV, vaccinating humans against it actually gains its function back towards a more deattenuated human-susceptible form. Improving the SARSr-CoV-WIV spike protein to gain robustness against monoclonal vaccines is one of the steps of the DEFUSE program. The mechanism to improve the SARSr-CoV-WIV spike protein (other than direct engineering) is to challenge it against animals that have spike protein-only antibodies. The attenuated virus will either die or adapt its form to neutralize the spike protein-only antibodies. The intent was to perform this task against humanized mice and then "batified" mice. Instead, it was done with the world's population.

SARSr-CoV-WIV is not meant to kill the bats, but to immunize them. This nature may explain its general harmlessness to most people, and its harmfulness to the old and comorbid, who are in general more susceptible to vaccine reactions. The asymptomatic nature is also explained by the bat vaccine-intention of its creators (a good vaccine does not generate symptoms). Such effects would be expected of an immature vaccine, or a vaccine being reverse engineered from a more virulent form into an attenuated form. The spike protein effect on ACE2 receptors exacerbates the harmfulness in accordance with age and comorbidity. The nature of SARSr-CoV-WIV's deattenuation will also indicate future virulence, though knowing its nature at last neutralizes the threat as effective treatments can be applied with confidence.

3. DRASTIC and other scientists will clean up my description of SARSr-CoV-WIV's nature and progression within the DEFUSE program. This information is sufficient for an investigative report and more than enough to correct the existing pandemic strategy. Previously, the nation did not know itself, nor the adversary in the pandemic conflict. Now it knows both. The problem can be framed appropriately and specifically against a confirmed hypothesis. Limiting disease transmission can be dropped as the implied strategic end, as it is not the actual problem,

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nor is it actually feasible. The strategy will then align early treatment protocols and prophylaxis with the known curatives as ways and means. This course of action will achieve the strategic end of clinical resolution for those that are susceptible to the adverse effects from SARS-CoV-WIV inoculation.

4. I will inevitably be asked how I figured this out and how I discovered the documents. The pandemic response became the predominant focus of my fellowship efforts. DARPA worked a number of pandemic innovations and much of its team was familiar with biodefense. I had the opportunity to "sit in the back row" per se and observe and listen-in on the government's efforts. My obligation-light fellowship also allowed me to observe and read the field. This observation grew in scope to the point that it became a series of reports, like a military scout would prepare when tasked to investigate a problem.

These reports served as iterative thinking against the problem over many months. Eventually, I arrived at a hypothesis that what leaked from the WIV could be a bat vaccine or its precursor. It was feasible that the US would try to avoid a SARS-CoV outbreak by stopping it at its source, not by halting its infections amongst people, but by halting the infections amongst the bats. Americans are creative, even if imprudent, and technologically confident enough to try it. This concept seemed to fit within the PREEMPT program construct as well, and DRASTIC had discovered that some earlier specimens within the USAID PREDICT program were obtained in Africa and sent to the WIV. Moreover, the unusual nature and pathology of the virus hinted that it could be a vaccine or be vaccine-like.

A technological challenge as difficult as inoculating bats in China would be tried at DARPA first. The massive, "Manhattan Project"-level of information suppression executed by the government and the Trusted News Initiative indicates that it would be covered-up if something had happened. The lab-leak hypothesis and squabbling between Senator Paul and Dr. Fauci indicated that the cover up was more localized. Further, an actual cover-up would be more disciplined with its paperwork. So I presumed that unclassified files would be concealed on a higher network and found them where I expected them to be. I understood what they were and their content, pushed the files off-site, and compiled this report.

8/13/2021

X *J. Murphy*

Joseph Murphy
Major, US Marine Corps
Signed by: MURPHY.JOSEPH.PATRICK.1275023554

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ⁱ DARPA: Defense Advanced Research Projects Agency

ⁱⁱ PREEMPT: Preventing Emerging Pathogenic Threats

^{iv} DRASTIC: Decentralized Radical Autonomous Search Team Investigating COVID-19. This collection of scientists and sleuths broke open the lab leak hypothesis into the mainstream and has picked apart Chinese and World Health Organization (WHO) reports on SARS-CoV-2's origins in Wuhan.

^v DEFUSE: Defusing Threat of Bat-borne Coronavirus

^{vi} PREEMPT Volume 1 no ESS HR00118S0017 EcoHealth Alliance DEFUSE. Another description used: "We will develop recombinant chimera spike proteins from known SARSr-CoVs, and those characterized by DEFUSE, using details of SARS S protein structure and host cell binding, we will sequence, reconstruct, and characterize spike trimmers and RBDs of SARSr-CoVs, incorporate them into nanoparticles or raccoon poxvirus vectors for delivery to bats."

^{vii} Dr. James Gimbert, DARPA Program Manager states: "team's approach does potentially involve GoF/DURC research (they aim to synthesize spike glycoproteins that may bind to human cell receptors and insert them into SARS-CoV backbones to assess capacity to cause SARS-like disease.)"

^{viii} "We will commercially synthesize SARSr-CoV S glycoprotein genes, designed for insertion into SHC014 or WIV16 molecular clone backbones (88% and 97% S protein identity to epidemic SARS-Urbani). These are BSL-3, not select agents or subject to P3CO" (they use bat SARSr-CoV backbones which are exempt)"

^{ix} Duke NUS Medical School, UNC, USGS National Wildlife Health Center, Palo Alto Research Center, Kunning, Signapore, and Madison, WI.

^x PREEMPT Volume 1 no ESS HR00118S0017 EcoHealth Alliance DEFUSE

^{xi} PREEMPT Volume 1 no ESS HR00118S0017 EcoHealth Alliance DEFUSE

^{xii} "For Delta, neutralizing antibodies have a decreased affinity for spike protein, while facilitating antibodies have a "strikingly increased" affinity for spike protein." Yahi, et al. "Infection-enhancing anti-SARS-CoV-2 antibodies recognize both the original Wuhan/D614G strain and Delta variants. A potential risk for mass vaccination?" *Journal of Infection*. August 9, 2021. [https://www.journalofinfection.com/article/S0163-4453\(21\)00392-3/fulltext](https://www.journalofinfection.com/article/S0163-4453(21)00392-3/fulltext)

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

PM SUMMARY SHEET
SOURCE SELECTION SENSITIVE

Solicitation Number: HR001118S0017
Solicitation Title: PREventing EMerging Pathogenic Threats (PREEMPT)
PM Name: James Gimlett
Proposer: EcoHealth Alliance
Proposal Title: Project DEFUSE: Defusing the Threat of Bat-borne Coronaviruses
Proposal Identifier: HR001118S0017-PREEMPT-FP-019

I have reviewed the attached proposal and Evaluation Reports and find that this proposal is selectable based on the evaluation criteria included in the BAA. However, I am not recommending funding at this time based on the rationale provided below.

Funding Requested (by proposer):

Phase I	Phase II	Total
\$8,411,546	\$5,797,699	\$14,209,245

This proposal aims to identify and model spillover risk of novel, pandemic-potential SARS-related coronaviruses (SARSr-CoVs) in Asia, focusing specifically on known hotspot bat caves in China. In prior work under USAID Predict, the team identified high risk of SARSr-CoVs in specific caves in Asia. The project has a good running start since the hotspot caves already test positive, with high prevalence, for several SARSr viruses so the team won't be looking for needles in haystacks. The team will build on past surveillance work as well as some impressive work in developing geo-based risk maps of zoonotic hotspots based on past spillovers and ecological data. Two approaches are proposed to preempt zoonotic spillover through reduction of viral shedding in the bat caves: 1) innate immune boosting to downregulate viral regulation; 2) targeted immune boosting via vaccine inoculations using chimeric polyvalent recombinant spike proteins to protect against specific high risk viruses.

Two of three reviewers marked this proposal as Selectable. Key strengths are the experienced team and the selected coronavirus hotspot caves that show high prevalence for novel bat coronaviruses. Experimental in vivo and in vitro work is logically thought out and will be used to validate molecular and evolutionary models. Proposed preemption approaches, while somewhat conventional, have the advantage of a fast timeline for validation on bat or "batenized" mouse models. Multiple vaccine delivery mechanisms are proposed, including aerosolized spray, transdermal nanoparticle application, and edible adhesive gels. However, several weaknesses to the proposal were also noted. These include a lack of detail regarding data, statistical analyses and model development and how prior work will be leveraged and extended. Proposal also lacks clear decision points to assess the deployment and validation of TA2 preemption methods in the

SUMMARY OF PROPOSED COSTS

Wuhan Institute of Virology (WIV)

DARPA-BAA-HR001418S0017

	PHASE 1		PHASE 2		
	BASE 1 12/1/2018 Through 11/30/2019	BASE 2 12/1/2019 Through 11/30/2020	OPTION 1 12/1/2020 Through 11/30/2021	OPTION 2 12/1/2021 Through 5/31/2022	PROJECT TOTAL
Direct Labor - Senior and Key Personnel	37,875	37,875	37,875	22,153	136,078
Direct Labor - Other Personnel	37,027	40,824	40,824	18,987	137,662
Fringe Benefits	22,500	23,639	23,639	12,341	82,119
Total Direct Labor & Fringe Benefits	97,502	102,438	102,438	53,481	355,859
Materials and Supplies	167,661	198,167	210,887	56,597	643,113
Travel	16,739	7,282	15,823	8,027	47,571
Equipment	0	0	0	0	0
Other Direct Costs	8,200	6,200	6,200	8,200	28,800
Total Other Direct Costs	192,600	211,649	232,410	82,824	719,484
Subtotal: Direct Labor, Fringe, Overhead & Other Direct Co	290,102	314,087	334,848	136,305	1,075,343
Exclusion(e) From Base For F&A	290,102.25	314,087.15	334,848.25	136,305.25	1,075,342.90
Adjusted Base for F&A	29,010.00	31,409.00	33,485.00	13,631.00	107,535.00
F&A	10.0%	10.0%	10.0%	10.0%	10.0%
Total Proposed Cost	319,112.25	345,496.15	368,333.25	149,936.25	1,182,877.90

DIRECT LABOR BREAKDOWN											
PHASE ONE - BASE PERIOD (24 months)											
PROJECT DEFUSE	BASE 1			BASE 2			Total Salary				
	Hourly Rate	# Months	# Hours	Hourly Rate	# Months	# Hours	Amount Y1	Amount Y2	Amount Y3	Amount Y4	Amount Y5
Personnel											
Investigator	\$25.56	3.00	528	\$25.56	3.00	528	\$13,488	\$13,488	\$13,488	\$13,488	\$13,488
Dr. Peng Zhou (Senior Scientist)	\$18.26	6.00	1056	\$18.26	6.00	1056	\$19,284	\$19,284	\$19,284	\$19,284	\$19,284
Dr. Ben Hu (Research Fellow)	\$10.95	3.00	528	\$10.95	3.00	528	\$5,784	\$5,784	\$5,784	\$5,784	\$5,784
Associate Professor	\$13.69	6.00	1056	\$13.69	6.00	1056	\$14,460	\$14,460	\$14,460	\$14,460	\$14,460
Senior Technician	\$10.95	6.00	1056	\$10.95	6.00	1056	\$11,568	\$11,568	\$11,568	\$11,568	\$11,568
Technician 1	\$7.30	9.00	1584	\$7.30	9.00	1584	\$11,568	\$11,568	\$11,568	\$11,568	\$11,568
Technician 2	\$7.30	6.00	1056	\$7.30	6.00	1056	\$7,712	\$7,712	\$7,712	\$7,712	\$7,712
TOTAL DIRECT LABOR							\$76,156	\$76,156	\$76,156	\$76,156	\$76,156
FRINGE BENEFITS							\$22,846.84	\$22,846.84	\$22,846.84	\$22,846.84	\$22,846.84
Fringe)							\$99,002.97	\$99,002.97	\$99,002.97	\$99,002.97	\$99,002.97

Total Labor	summary	e
\$358,179.69	355,859	#####

PHASE TWO - OPTION PERIOD (18 months)											
PERSONNEL	OPTION 1			OPTION 2			Total Salary				
	Hourly Rate	# Months	# Hours	Hourly Rate	# Months	# Hours	Amount Y3	Amount Y4	Amount Y5	Amount Y6	Amount Y7
Dr. Zhengli Shi (Co-Investigator)	\$25.56	3.00	528	\$25.56	2.00	352	\$13,488	\$13,488	\$13,488	\$13,488	\$13,488
Dr. Peng Zhou (Senior Scientist)	\$18.26	6.00	1056	\$18.26	3.00	528	\$19,284	\$19,284	\$19,284	\$19,284	\$19,284
Dr. Ben Hu (Research Fellow)	\$10.95	3.00	528	\$10.95	2.00	352	\$5,782	\$5,782	\$5,782	\$5,782	\$5,782
Associate Professor	\$13.69	6.00	1056	\$13.69	3.00	528	\$14,457	\$14,457	\$14,457	\$14,457	\$14,457
Senior Technician	\$10.95	6.00	1056	\$10.95	3.00	528	\$11,563	\$11,563	\$11,563	\$11,563	\$11,563
Technician 1	\$7.30	6.00	1056	\$7.30	3.00	528	\$7,709	\$7,709	\$7,709	\$7,709	\$7,709
Technician 2	\$7.30	6.00	1056	\$7.30	3.00	528	\$7,709	\$7,709	\$7,709	\$7,709	\$7,709
TOTAL DIRECT LABOR							\$79,997	\$79,997	\$79,997	\$79,997	\$79,997
FRINGE BENEFITS							\$23,999.16	\$23,999.16	\$23,999.16	\$23,999.16	\$23,999.16
TOTAL LABOR (Salary + Fringe)							\$103,996.48	\$103,996.48	\$103,996.48	\$103,996.48	\$103,996.48

Total Travel	\$47,571.00
Base 1	\$18,739.00
Base 2	\$7,282.00
Option 1	\$15,523.00
Option 2	\$5,027.00

Wuhan Institute of Virology - TRAVEL BREAKDOWN									
WIV	DAIPA BAA- HR001118S0017	Y1	Y2	Location: Arlington, VA	Contract Period:	Base 1	Base 2	Option 1	Option 2
Purpose: DAIPA Kick-off Meeting (21st key personnel)		Days: 4		Per Diem	Leaving	Other	Total		
Unlimited Expenses for "Other"		# of People: 2		\$60.00	\$14.00	\$212.00	\$418.00		
Description		Amount							
Transportation within Wuhan airport		\$111.00							
Transportation within Wuhan airport		\$102.00							
Total		\$213.00							
Y1		Y2		Location: New York, NY	Contract Period:	Base 1			
Purpose: Annual Meeting (21st key personnel)		Days: 6		Per Diem	Leaving	Other	Total		
Unlimited Expenses for "Other"		# of People: 2		\$74.00	\$291.00	\$122.00	\$1,017.00		
Description		Amount							
Transportation within Wuhan airport		\$168.00							
Transportation within NYC airport		\$254.00							
Total		\$422.00							
Y1		Y2		Location: Lake City, UT	Contract Period:	Base 2			
Purpose: Ecological Study of America Conference (21st key personnel)		Days: 4		Per Diem	Leaving	Other	Total		
Unlimited Expenses for "Other"		# of People: 2		\$74.00	\$113.00	\$102.00	\$389.00		
Description		Amount							
Transportation within Wuhan airport		\$112.00							
Transportation within NYC airport		\$196.00							
Total		\$308.00							
Y1		Y2		Location: New York, NY	Contract Period:	Option 1			
Purpose: Annual Meeting in NYC (21st key personnel)		Days: 4		Per Diem	Leaving	Other	Total		
Unlimited Expenses for "Other"		# of People: 2		\$74.00	\$101.00	\$168.00	\$343.00		
Description		Amount							
Transportation within Wuhan airport		\$168.00							
Transportation within NYC airport		\$254.00							
Total		\$422.00							
Y1		Y2		Location: Atlanta, GA	Contract Period:	Option 2			
Purpose: International Conference on Emerging Infectious Diseases (21st key personnel)		Days: 4		Per Diem	Leaving	Other	Total		
Unlimited Expenses for "Other"		# of People: 2		\$74.00	\$144.00	\$122.00	\$440.00		
Description		Amount							
Transportation within Wuhan airport		\$111.00							
Transportation within NYC airport		\$111.00							
Total		\$222.00							
Y1		Y2		Location: New York, NY	Contract Period:	Option 3			
Purpose: Annual Meeting (21st key personnel)		Days: 3		Per Diem	Leaving	Other	Total		
Unlimited Expenses for "Other"		# of People: 2		\$74.00	\$291.00	\$122.00	\$1,017.00		
Description		Amount							
Transportation within Wuhan airport		\$168.00							
Transportation within NYC airport		\$254.00							
Total		\$422.00							
Y1		Y2		Location: New York, NY	Contract Period:	Option 4			
Purpose: Annual Meeting (21st key personnel)		Days: 4		Per Diem	Leaving	Other	Total		
Unlimited Expenses for "Other"		# of People: 2		\$74.00	\$144.00	\$122.00	\$440.00		
Description		Amount							
Transportation within Wuhan airport		\$111.00							
Transportation within NYC airport		\$111.00							
Total		\$222.00							

NOTE: Dr. SHI WILL STAY AN ADDITIONAL DAY FOR PROJECT CLOSOUT

NOTE: Dr. SHI WILL STAY AN ADDITIONAL DAY FOR PROJECT CLOSOUT

WIV
DARPA-8AA-HRQ01118S0017

Task #		PROJECT DEFUSE	TECHNICAL AREA / TASK	PHASE 1			PHASE 2		TASK TOTAL
				Base 1	Base 2	Option 1	Option 2		
								12/1/18 - 11/30/19	
TA1-PI-TB.1	PCR screening of longitudinal specimens from target bat species	\$	35,455.92	\$	38,388.46	\$	-	\$	73,844.38
TA1-PI-TB.2	Genetically sequence SARS-CoV spike proteins from PCR-positive samples	\$	35,455.92	\$	38,388.46	\$	-	\$	73,844.38
TA1-PI-TB.1	Design Luciferase Immunoprecipitation system (LIPS) assays to high- and low- jump risk SARS-CoV QSO we have characterized	\$	35,455.92	\$	38,388.46	\$	-	\$	73,844.38
TA1-PI-TB.2	Determine specificity of LIPS assays by recombinant protein or attenuated virus inoculation into rabbits	\$	35,455.92	\$	38,388.46	\$	-	\$	73,844.38
TA1-PI-TB.3	Validate LIPS assays using positive serum samples, spike protein based LIPS and viral neutralization	\$	35,455.92	\$	38,388.46	\$	-	\$	73,844.38
TA1-PI-TB.4	Test previously-collected human sera from Yunnan Province to assess SARS-CoV QS spillover	\$	35,455.92	\$	38,388.46	\$	-	\$	73,844.38
TA2-PI-TB.5	Test targeted immune boosting in wild-caught captive Rhinolophus spp.	\$	35,455.92	\$	38,388.46	\$	-	\$	73,844.38
TA2-PH-TB.1	Develop chimeric SARS-CoV S immunogens	\$	35,455.92	\$	38,388.46	\$	-	\$	73,844.38
TA2-PH-TB.2	Design and test 2nd generation chimeric S glycoprotein immunogens in humanized mice	\$	35,455.92	\$	38,388.46	\$	-	\$	73,844.38
TA2-PI-TB.5	Test targeted immune boosting in wild-caught captive Rhinolophus spp.	\$	-	\$	-	\$	61,388.88	\$	24,989.38
TA2-P2-TS.1	Identify specific sites (entry, exit points), identify FEA automatic aerosolization points, fine-tune deployment plan	\$	-	\$	-	\$	61,388.88	\$	24,989.38
TA2-P2-TS.2	Conduct bat viral surveillance of one test-site cave and two control caves at our cave complex to assess baseline data for 4 months before deployment proof-of-concept experiment (EHA Consultant)	\$	-	\$	-	\$	61,388.88	\$	24,989.38
TA2-P2-TS.2	Zhu, Wivi	\$	-	\$	-	\$	61,388.88	\$	24,989.38
TA2-P2-TS.3	Run deployment experiment of most effective immune boosting molecules and delivery techniques via FEA aerosolization mechanism at one test and two control bat cave sites in Yunnan, China	\$	-	\$	-	\$	61,388.88	\$	24,989.38
TA2-P2-TS.4	Conduct bat viral surveillance of one test-site cave and two control caves at our cave complex to assess baseline data for 4 months after deployment proof-of-concept experiment	\$	-	\$	-	\$	61,388.88	\$	24,989.38
TA2-P2-TS.5	Assess efficacy of proof-of-concept trial.	\$	-	\$	-	\$	61,388.88	\$	24,989.38

WIV DARPA-BAA-HR001118S0017

SUMMARY COST BUILDUP BY PHASE				
	Phase I:		Phase II:	
	24 MONTHS		18 MONTHS	
	12/1/18 - 11/30/20		12/1/20 - 05/30/22	
Personnel	\$	153,601	\$	119,939
Fringe Benefits	\$	48,139	\$	35,980
Supplies	\$	365,828	\$	277,285
Travel	\$	24,021	\$	23,550
Other Direct Costs	\$	14,400	\$	14,400
Indirect Costs	\$	60,419	\$	47,116
TOTAL	\$	664,608	\$	518,270

SUMMARY COST BUILDUP BY YEAR				
	Year 1		Year 2	
	12/1/18 - 11/30/20		12/1/20 - 05/30/22	
Personnel	\$	75,002	\$	78,799
Fringe Benefits	\$	22,500	\$	23,639
Supplies	\$	167,661	\$	198,167
Travel	\$	16,739	\$	7,282
Other Direct Costs	\$	8,200	\$	6,200
Indirect Costs	\$	29,010	\$	31,409
TOTAL	\$	319,112	\$	345,496

SUMMARY COST BUILDUP BY YEAR				
	Year 3		Year 4	
	12/1/22 - 11/30/23		12/1/23 - 11/30/24	
Personnel	\$	78,799	\$	41,140
Fringe Benefits	\$	23,639	\$	12,341
Supplies	\$	210,687	\$	66,597
Travel	\$	15,523	\$	8,027
Other Direct Costs	\$	6,200	\$	8,200
Indirect Costs	\$	33,485	\$	13,631
TOTAL	\$	368,333	\$	149,936

TOTAL PROJECT				
Personnel	\$	273,740	\$	273,740
Fringe Benefits	\$	82,119	\$	82,119
Supplies	\$	843,113	\$	843,113
Travel	\$	47,571	\$	47,571
Other Direct Costs	\$	28,800	\$	28,800
Indirect Costs	\$	107,535	\$	107,535
TOTAL	\$	1,182,878	\$	1,182,878

Wuhan Institute of Virology - SUMMARY COST BUILDUP BY TASK

WIV
DARPA-BAA-HR001118S0017
PROJECT DEFUSE

PROJECT DEFUSE		PHASE 1				PHASE 2		TASK TOTAL
		Base 1	Base 2	Option 1	Option 2			
Task #	TECHNICAL AREA / TASK	12/1/18 - 11/30/19	12/1/19 - 11/30/20	12/1/20 - 11/30/21	12/1/21 - 5/31/22			
TA1-PI-T3.1	PCR screening of longitudinal specimens from target bat species	\$	\$	\$	\$	\$	\$	\$
TA1-PI-T3.2	Genetically sequence SARS-CoV spike proteins from PCR-positive samples	\$	\$	\$	\$	\$	\$	\$
TA1-PI-T6.1	Design Luciferase Immunoprecipitation system (LIPS) assays to high- and low-risk SARS-CoV QSD we have characterized	\$	\$	\$	\$	\$	\$	\$
TA1-PI-T6.2	Determine specificity of LIPS assays by recombinant protein or attenuated virus inoculation into rabbits	\$	\$	\$	\$	\$	\$	\$
TA1-PI-T6.3	Validate LIPS assays using positive serum samples, spike protein based LIPS and viral neutralization	\$	\$	\$	\$	\$	\$	\$
TA1-PI-T6.4	Test previously-collected human sera from Yunnan Province to assess SARS-CoV Q5 spillover	\$	\$	\$	\$	\$	\$	\$
TA2-PI-T7.5	Test targeted immune boosting in wild-caught captive Rhinolophus spp.	\$	\$	\$	\$	\$	\$	\$
TA2-PI-T8.1	Develop chimeric SARS-CoV S immunogens	\$	\$	\$	\$	\$	\$	\$
TA2-PI-T8.2	Design and test 2nd generation chimeric S glycoprotein immunogens in humanized mice	\$	\$	\$	\$	\$	\$	\$
TA2-PI-T8.5	Test targeted immune boosting in wild-caught captive Rhinolophus spp.	\$	\$	\$	\$	\$	\$	\$
TA2-P2-T5.1	Identify specific sites (entry, exit points), identify FEA automatic aerosolization points, fine-tune deployment plan Conduct bat viral surveillance of one test-site cave and two control caves at our cave complex to assess baseline data for 4 months before deployment proof-of-concept experiment (EHA Consultant Zhu, WIV)	\$	\$	\$	\$	\$	\$	\$
TA2-P2-T5.2	Run deployment experiment of most effective immune boosting molecules and delivery techniques via FEA aerosolization mechanism at one test and two control bat cave sites in Yunnan, China	\$	\$	\$	\$	\$	\$	\$
TA2-P2-T5.3	Conduct bat viral surveillance of one test-site cave and two control caves at our cave complex to assess baseline data for 4 months after deployment proof-of-concept experiment	\$	\$	\$	\$	\$	\$	\$
TA2-P2-T5.5	Assess efficacy of proof-of-concept trial.	\$	\$	\$	\$	\$	\$	\$
								\$

24 March 2018

Dear Committee for DARPA Preventing Emerging Pathogenic Threats (PREEMPT),

Please accept the following proposal to the PREEMPT-Merging Pathogenic Threats (PREEMPT-M, HR00111850017) program. The PI for this project is:

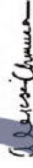
Dr. Peter Daszak
President, EcoHealth Alliance
460 W. 34th Street, 17th Floor
New York, NY 10001
212-380-4474

Title: Project Defuse: Defusing the Threat of Bat-Borne Coronaviruses

Amount of the Requested Proposal: \$14,209,245

Thank you for your time, and I look forward to hearing from you. If you have any questions, do not hesitate to call or email me.

Yours sincerely,



Aleksel Chimura
Chief of Staff, EcoHealth Alliance
460 W. 34th Street, 17th Floor
New York, NY 10001
212-380-4473

Project DEFUSE: Defusing the Threat of Bat-borne Coronaviruses



Principal Investigator and Technical Point of Contact

Peter Daszak, PhD
EcoHealth Alliance
460 West 34th Street, 17th Floor
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Administrative Point of Contact

Lalio Hamel
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460 West 34th Street, 17th Floor
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(p) 646-960-4709
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(f) 212-380-4465

Identifying Number: HR00111850017-4-DEFUSE-4A-001

Award Instrument Requested: Grant

Places and Periods of Performance: 12/1/18 - 5/31/22; Palo Alto, CA; Kunming and Wuhan, China; Chapel Hill, NC; New York, NY; Singapore; Madison, WI

Total funds requested: \$14,209,245

Proposal validity period: 6 months

Date proposal submitted: 3/27/18

Section II A. EXECUTIVE SUMMARY

Technical Approach: Our goal is to defuse the potential for spillover of novel bat-origin high-zoonotic risk SARS-related coronaviruses in Asia. In TA1 we will intensively sample bats at our field sites where we have identified high spillover risk SARS-CoVs. We will sequence their spike proteins, reverse engineer them to conduct binding assays, and insert them into bat SARS-CoV (WIV1, SHC014) backbones (these use bat-SARS-CoV backbones, not SARS-CoV, and are exempt from dual-use and gain of function concerns) to infect humanized mice and assess capacity to cause SARS-like disease. Our modeling team will use these data to build machine-learning genotype-phenotype models of viral evolution and spillover risk. We will uniquely validate these with serology from previously-collected human samples via LIPS assays that assess which spike proteins allow spillover into people. We will build host-pathogen spatial models to predict the bat species composition of caves across Southeast Asia, parameterized with a full inventory of host-virus distribution at our field test sites, three caves in Yunnan Province, China, and a series of unique global datasets on bat host-viral relationships. By the end of Y1, we will create a prototype app for the warfighter that identifies the likelihood of bats harboring dangerous viral pathogens at any site across Asia.

In TA2, we will evaluate two approaches to reduce SARS-CoV shedding in cave bats: (1) Broadscale immune boosting, in which we will inoculate bats with immune modulators to upregulate their innate immune response and downregulate viral replication; (2) Targeted Immune boosting, in which we will inoculate bats with novel chimeric polyvalent recombinant spike proteins plus the immune modulator to enhance innate immunity against specific, high-risk viruses. We will trial inoculum delivery methods on captive bats including a novel automated aerosolization system, transdermal nanoparticle application and edible adhesive gels. We will use stochastic simulation modeling informed by field and experimental data to characterize viral dynamics in our cave test sites, maximize timing, inoculation protocol, delivery method and efficacy of viral suppression. The most effective biologicals will be trialed in our test cave sites in Yunnan Province, with reduction in viral shedding as proof-of-concept. **Management Approach:** Members of our collaborative group have worked together on bats and their viruses for over 15 years. The lead organization, EcoHealth Alliance, will oversee all work. EHA staff will develop models to evaluate the probability of specific SARS-related CoV spillover, and identify the most effective strategy for delivery of both immune boosting and immune targeting inocula. Specific work will be subcontracted to the following organizations:

- Prof. Baric, Univ. N. Carolina, will lead targeted immune boosting work, building on his two-decade track record of reverse-engineering CoV and other virus spike proteins.
- Prof. Wang, Duke-NUS, Univ. Singapore, will lead work on broadscale immune boosting, building on his group's pioneering work on bat immunity.
- Dr. Shi, Wuhan Institute of Virology will conduct viral testing on all collected samples, binding assays and some humanized mouse work.
- Dr. Rocks, USGS National Wildlife Health Center will optimize delivery of immune modulating biologics, building on her vaccine delivery work in wildlife, including bats.
- Dr. Unidat, Palo Alto Research Center will lead development of novel delivery automated aerosolization mechanism for immune boosting molecules.

We are requesting \$14,209,245 total funds for this project across 3.5 project years.

Section II

C. GOALS AND IMPACT

Overview: The overarching goals of DEFUSE are to:

- Identify and model spillover risk of novel SARS-related coronaviruses (SARS-CoVs) in Asia.
- Design and demonstrate proof-of-concept that upregulating the naturally low innate immunity of bats (broadscale immune boosting) and targeting high risk SARS-CoVs in particular (targeted immune boosting) will transiently reduce spillover risk.

Our strategy to reduce risk of viral emergence from bats will protect the warfighter within USPACOM, and will be scalable to other regions and viruses (Ebola, Hantavirus, rabies).

Innovation and uniqueness:

Bats harbor more emerging zoonoses than any other group of mammals, are ubiquitous, abundant, and often overlooked. However, other than PPE, there is no available technology to reduce exposure risk to novel CoVs from bats, and no effective therapeutics or countermeasures. SARS-CoVs are

endemic in Asian, African, and European bats¹ that roost in caves but forage widely at night, shedding virus in their feces and urine. We have now published direct evidence of spillover of novel SARS-CoVs into people in Yunnan Province, China, close to a cave complex where we have isolated strains that produce SARS-like disease in humanized mice but don't respond to antibody treatment or vaccination. These viruses are a clear-and-present danger to our military and to global health security because of their circulation and evolution in bats and periodic spillover into humans.

EcoHealth Alliance (EHA) leads the world in predictive models of viral emergence. We will use machine-learning models of spillover hotspot, host-pathogen spatial and genotype-phenotype mapping, and unique datasets to validate and refine hotspot risk maps of viral emergence. We have shown that dampened innate immunity in bats allows them to carry otherwise lethal viruses, likely as an adaptation to the physiologic stress of flight. We will design strategies like small molecule RIG-like receptor (RLR) or Toll-like receptor (TLR) agonists, to upregulate bat immunity, and suppress viral replication, thereby significantly reducing viral shedding and spillover (broadscale immune boosting). We will complement this by coupling agonists treatments with SARS-CoV recombinant spike proteins to boost pre-existing adaptive immune response adult bats against specific, high-risk SARS-CoVs (targeted immune boosting). We will design novel delivery and automated application methods, based on our previous work on wildlife vaccines, to reduce hazard during deployment.

Technical Area 1

PROGRAM ADMINISTRATION

Principal Investigator: **P. Daszak**

Program Management:

Program Manager:

Program Manager:

Program Manager:

Program Manager:

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Our strategy begins by a complete inventory of bats and their SARS-CoV strains at our intervention test site cave complex in Yunnan, China that harbors bats with high-risk SARS-CoV. We will collect data from three caves in that system (one is our intervention test site and two control sites) on: monthly bat abundance and diversity; viral prevalence and diversity; individual bat viral load and host physiological markers; genomic characterization of low- and high-risk SARS-CoV strains among bat species, sexes, and age classes; satellite telemetry and mark-recapture data on bat home range and inter-cave movement; and monitoring of daily, weekly and seasonal changes in bat populations. We will use stochastic neural networks to build joint species distribution models (SDM) to predict bat species composition of caves, and high-risk SARS-CoV diversity across S. China, South and SE Asia. These will be parameterized with BHA's database of bat host-viral relationships and estimates of zoonotic viral richness per bat species²; biological inventory data on all bat caves in Southern China; the full SARS-CoV inventory from our cave test sites in Yunnan; and species distribution data for all bats. We will test and validate viral diversity predictions using data from >10,000 previously collected bat samples from 6 Asian countries under our USAID-funded PREDICT project. We will produce a prototype app for the warfighter to identify the risk of bats harboring dangerous viruses at a site. This 'spatial viral spillover risk' app will be field-deployable and updated real-time with surveillance data, to ground-truth and fine-tune predictions.

To characterize spillover risk of SARS-CoV zoonotic species (QS), the Wuhan Institute of Virology team (WIV) will test bat fecal, oral, and blood samples for SARS-CoV by PCR. We will collect viral load data from fresh fecal pellets. SARS-CoV spike proteins will be sequenced, viral recombination events identified, and isolates used to identify strains that can replicate in human cells. The Univ. N. Carolina (UNC) team will reverse-engineer spike proteins of a large sample of high- and low-risk viruses for further characterization. This will effectively freeze the QS we analyze at t=0. These QSs strain viral spike glycoproteins will be synthesized, and those binding to human cell receptor ACE2 will be inserted into SARS-CoV backbone (non-DURC, non-Gof), and inoculated into humanized mice to assess capacity to cause SARS-like disease, efficacy of monoclonal therapies, the inhibitor GS-5734⁴ or vectines against SARS-CoV^{4,11}.

We will use these data to build machine-learning genotype-to-phenotype Bayesian network models of viral evolution and host jump risk. These will predict the capacity of QSs strains to infect human cells based on genetic traits and experimental assays above. Using data on diversity of spike proteins, recombinant CoVs, and flow of genes via bat movement and migration, we will estimate evolutionary rates, rates of recombination, and capacity to generate novel strains capable of human infection. Finally, virus-host relationship and bat home range data will be used to estimate spillover potential - extending models well beyond our field sites. We will validate model predictions of host jump risk by 1) conducting further spike

protein-based binding and cell culture experiments, and 2) identifying whether designated high-risk SARS-CoV strains have already spilled over into people near our bat cave sites. Our preliminary work shows ~35% seroprevalence to bat SARS-CoVs in people at this site¹⁴. We will test these previously collected human sera (>2000) for presence of antibodies to the hN- and hM-antigen SARS-CoVs identified by our modeling, using Luciferase immunoprecipitation system (LIPS) assays we design against the SARS-CoVs identified in this project¹⁴.

Technical Area 2

In TA2, we will develop scalable approaches to suppress SARS-CoVs within bat reservoir species, to reduce the likelihood of virus transmission into humans. We will evaluate two approaches to defuse spillover potential: 1) Broad-scale immune boosting: we will apply immune modulators like bat interferon and TLR agonists to up-regulate bat innate immunity and suppress viral replication and shedding; 2) Targeted immune boosting: we will apply polyvalent chimeric recombinant SARS-CoV spike proteins in the presence of broad-scale immune boosting treatments to boost immune memory and suppress specific SARS-CoVs.

Both TA2 lines of work will run parallel beginning Yr 1. Prof. Wang (Duke-NUS, Univ. Singapore) - Duke-NUS will lead the broad-scale immune boosting work, building on his pioneering work on bat immunity¹⁵. Including identifying weakened functionality of innate immunity factors like STING, a central DNA-interferon (IFN) sensing molecule, that may allow bats to maintain an effective, but not over-response to viruses¹⁶, and IFNA, which is constitutively expressed without stimulation¹⁷. We will trial the following, concurrently and competitively, for efficacy and scalability: i) Activating TLR/RLR pathways to induce IFN induction, e.g. poly(I:C) or 5'ppp-dsRNA. A similar strategy has been demonstrated in a mouse model for SARS-CoV^{18,19}; ii) Universal bat interferon. Interferon has been used clinically in people, e.g. against filoviruses²⁰, and replication of SARS-CoV is sensitive to interferon²¹; iii) Boosting bat IFN by blocking negative regulators. Bat IFNα is constitutively expressed but cannot be induced to a high level²². We will use CRISPRi to identify potential negative regulators and screen for compounds targeting this gene; iv) Activating dampened IFN production pathways via DNA-STING-dependent and siRNA-TLR7-dependent pathways. Mutant bat STING restores antiviral functionality, suggesting these pathways are important in bat-viral coexistence¹⁸. We will directly activate the pathways downstream of STING/TLR7, to promote viral clearance; v) Inoculating crude CoV fragments to upregulate innate immune responses to specific CoVs - a partial step towards the targeted immune boosting work below.

Prof. Baric (UNC) will lead the targeted immune boosting work. We will develop recombinant chimeric spike proteins²³ from known SARS-CoVs, and those characterized by DEFUSE. Using details of SARS S protein structure and host cell binding²⁴, we will sequence, reconstruct and characterize spike trimers and receptor binding domains of SARS-CoVs, incorporate them into nanoparticles or racoon poxvirus-vectors for delivery to bats^{25,26,27}. In combination with immune-boosting small molecules, we will use these to boost immune memory in adult bats previously exposed to SARS-CoVs, taking the best candidate forward for field-testing. Recombinant S glycoprotein-based constructs with immunogenic blocks from across group 2B SARS-CoVs should induce broadscale adaptive immune responses that reduce heterogeneous virus burden in bats and transmission risk to people^{28,29}. Innate immune damping is highly conserved in all bat species tested so far. We will use the unique Duke-NUS

Asian cave bat (*Eonycteris spelaea*) breeding colony to conduct initial proof-of-concept tests, extended to small groups of wild-caught *Rhinolophus sinicus* bats at WY.

A novel delivery method for our immune boosting molecules will be developed and implemented by Dr. Rocke at the USGS National Wildlife Health Center (NWHC) who has previously developed an oral vaccine through to licensure¹⁵. Using locally acquired insectivorous bats^{16,17}, we will assess delivery vehicles and methods including: 1) transdermally applied nanoparticles; 2) highly soluble gels that bats mutually groom and consume; 3) aerosolized spray triggered by timers and movement detectors at critical cave entry points. We have extensive preliminary data on these techniques for wildlife, including vaccinating bats against rabies in the lab¹⁸, successful delivery, consumption and spread in wild vampire bats. We will use the NWHC captive bat colony and wild bats in US caves to trial delivery vehicles using the biomarker rhodamine B (which fluorescently marks hair on consumption) to assess uptake. The most optimal deployment approaches will be tested on wild bats at our test cave sites in Yunnan, using the most effective immune modulation preparations. Bat populations from experimental and control caves will be surveyed longitudinally for viral load before and after deployment trials. ENA has had unique access to these sites for ~10 years. In DEFUSE Y1, we will seek permission for experimental trials from collaborators at the Yunnan Forestry Department and Center for Disease Control, following our proven track record of rapidly obtaining IACUC and DOD/ACURO approval for animal research. We will model optimal strategies to maximize treatment efficacy for TAZ, using stochastic simulation modeling of viral circulation dynamics at our sites, informed by field and experimental data. We will estimate frequency and population coverage required for our intervention, and model the time period of viral suppression, until re-colonization or evolution leads to return of a high-risk SARS-CoV.

Deliverables

- Open source models and App identifying geographical and host-specific risk of spillover for novel SARS-CoVs
- Experimentally validated genotype-phenotype models of spillover for viral strains.
- Proven technology to modulating bat innate immunity to reduce viral shedding
- Tested and validated delivery mechanism for bat cave usage including vaccines in other bat host-pathogen systems (e.g. rabies, WNS).
- Proof-of-concept approach to transiently reducing viral shedding in wild bats that can be adapted for other systems including Ebola virus.

Section III: D. TECHNICAL PLAN

Technical Area II

Choice of site and model host-virus system. For the past 14 years, our team has conducted CoV surveillance in bat populations across S. China, resulting in >180 unique SARS-CoVs in ~10,000 samples (>5% prevalence, including multiple individuals harboring the same viral strain)^{2,23,24} and a per-bat species prevalence up to 10.9%. Bat SARS-CoVs are genetically diverse, especially in the S gene, and most are highly divergent from SARS-CoV. However, our test cave site in Yunnan Province, harbors a quasispecies (QS) population assemblage that contains all the genetic components of epidemic SARS-CoV²⁴. We have isolated three strains there (WV1,

WV16 and SHCQ34) that unlike other SARS-CoVs, do not contain two deletions in the receptor-binding domain (RBD) of the spike, have a higher sequence identity to SARS-CoV (Fig. 3). Use human ACE2 receptor for cell entry, as SARS-CoV does (Fig. 2), and replicate efficiently in various animal and human cells^{25,26,27}, including primary human lung alveolar cells, similar to epidemic SARS-CoV^{28,29}. Chimeras (recombinants) with these SARS-CoV S genes inserted into a SARS-CoV backbone, and synthetically reconstructed full length SHCQ34 and WV1 cause SARS-like illness in humanized mice (mice expressing human ACE2), with clinical signs that are not reduced by SARS-CoV monoclonal antibody therapy or vaccination^{13,30}. People living up to 6 kilometers from our test cave have SARS-CoV antibodies (~3% seroprevalence)³¹, suggesting active spillover. These data, phylogeography of SARS-CoVs, and evolutionary analysis of bats and their CoVs (unpublished), suggest that bat caves in SW China, and *Rhinolophus* spp. bats are the likely origin of the SARS-CoV clade, and are a clear-and-present danger for the emergence of a SARS-CoV from the current QS. The *Rhinolophus* spp. bats that harbor these viruses occur across Asia, Europe, and Africa. Thus, while DEFUSE fieldwork will focus on high-risk sites in S. China, our approach to reduce the risk of these viruses spilling over is broadly applicable across four combatant command regions (PACOM, CENTCOM, EUCOM, AFRICOM).

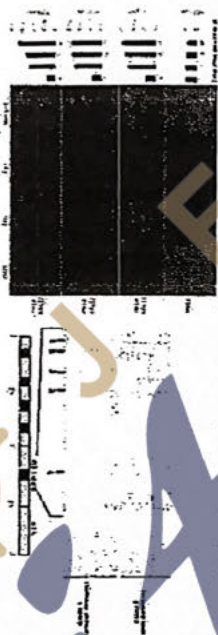


Fig. 3 (top left) alignment of amino acid sequences of the spike protein receptor-binding motif of SARS-CoVs. SARS-CoV-2 (Wuhan) and SARS-CoV-2 (Guangdong) are responsible for human ACE2²⁵. Fig. 3 (top right): Bat SARS-CoV WY1 replicates efficiently in mice cells expressing human ACE2 and bat ACE2.

Full inventory of bat SARS-CoV Q23 at our test cave sites, Yunnan, China. To provide data to train and validate our modeling, and as baseline for our immune modulation trials (TA2), DEFUSE fieldwork will target the high-risk cave site in Yunnan Province, SW China (Fig. 4, red triangle) where we will conduct our field trial, and where we have previously identified and isolated high-risk SARS-CoVs^{23,24}. At three cave sites (one designated for our trial, two as controls), we will determine the baseline QS risk of SARS-CoV spillover. We will conduct longitudinal surveillance of bat populations to detect and isolate SARS-CoVs, determine changes in viral prevalence over time, and measure bat population demographics and movement, definitively characterizing their SARS-CoV host-viral dynamics. Field data will allow us to test the accuracy of our model predictions and compare efficacy of lab animal models with field trials. Our preliminary data (Table 2) demonstrate that *R. sinicus*, *R. ferrumequinum*, and *R. affinis* (which co-roost at our test site) are primary reservoirs of SARS-CoV and the only reservoirs of three high-risk strains (WV1, WV16, SHCQ34), with *Hipposideros* and *Myotis* spp. playing an

insignificant (<1% prevalence) role in viral dynamics. We will capture *Rhinolophus* spp. bats using harp traps and mist nets during evening flights, collect rectal, oral, and whole blood samples (<2 per bat) using sterile technique to avoid cross-contamination, and take 2-mm wing tissue punch biopsies for host DNA bar-coding, host ACE2 receptor gene sequencing (interface site ~ 3 individuals per species), and copy-sequencing analyses. Bats will be subcutaneously microchipped (PIT tag), and morphological and physiological data (age class, body weight, reproductive status etc.).

In Phase I we will sample 60 bats each of *R. sinicus*, *R. ferrumequinum*, and *R. affinis*, (180 bats per cave) every three months non-destructively for 18 months from our three cave sites. Given ~6-9% prevalence (n=3,304) of SARS-CoVs in *Rhinolophus* spp. at our sites, this sample size would allow detection of 10% fluctuation in viral prevalence among sampling periods and caves. During the 2 months per quarter without physical bat trapping we will collect fresh fecal pellets by placing clean zip polyethylene sheets beneath roosting bats³⁴. *Rhinolophus* spp. have a 7-week gestation period, spring birthing, and aggregate during mating periods. Our monthly sampling strategy will collect adequate data to parameterize stochastic simulation models, and cover two mating and gestation periods to assess life-history driven changes in viral prevalence and immune marker (e.g. interferon) levels. We will conduct pre- and post-intervention sampling (biweekly fecal pellet sampling for 4 months, and 10 male and 10 female bats per species tested every 2 weeks post-intervention for 4 months, prior to- and post-deployment) to monitor SARS-CoV Q5 and bat immune status changes in test and control site bats during Phase II (TA2). Immune status can be followed in individual bats due to the relatively small roost sites in these caves and our individual marking of captured bats. We will be assessing immune status using nanosizing immune profiling panels validated during captive bat studies at Duke NUS. We will use infrared spotlights and digital infrared imaging to record the number and species of bats above each plastic sheet and fecal pellets will be genetically bar-coded to confirm species identification³⁵.

Species	n	SARS-CoV prevalence
<i>Rhinolophus sinicus</i>	1036	3.09%
<i>R. ferrumequinum</i>	191	6.3%
<i>R. affinis</i>	518	3.2%

Samples will be preserved in viral transport medium, immediately frozen in liquid nitrogen dry shippers, and transported to partner laboratories with maintained cold chain and under strict biosafety protocols. PIT tag readers and weatherproof thermal imaging IR cameras mounted at each cave entrance will passively monitor temporal roost site fidelity, rates of inter-cave movement, and daily fluctuation in bat population³⁶. ICARUS satellite transmitters (2g) will be attached to 12 *Rhinolophus* spp. bats from each study roost (36 bats total) to determine nightly foraging dispersal patterns (<https://www.icecube.wisc.edu/>). Telemetry and PIT tag data will be used to calculate home range, degree of mixing among roosts, and parameterize dynamic models. Study caves will be surveyed using portable LIDAR technology⁴⁰⁻⁴², to give a 3-D image of roost areas and data on species composition for targeting of immune modulation treatments in TA2 (Fig. 3). Sampling quotas will be adjusted based on lab and model results to optimize viral detection.



Fig. 3. Light Detection and Ranging (LIDAR) scanning to characterize caves and quantify number of individual bats roosting in clusters. A) LIDAR system takes a 360° omnidirectional photo of clustered bats. B)

photo converted to 3-D point cloud. C) Non-bat points, based on laser return intensity removed. D) automated counting algorithm counts individual bats. Figure from⁴³.

Our team has more than 50 years collective experience in safe and humane handling of bats for biological sampling. This project will operate under appropriate IACUC/ACURO and PPE guidelines. EHA has several ongoing DTRA-supported projects, has obtained ACURO approval for animal research from the DoD, and currently maintains IACUC protocols through Tufts University (EHA staff are adjunct faculty), which we will use for DEFUSE IACUCs. IACUCs already approved for lab/field work at Duke-NUS, UNC, NWHC, and WIV, will be modified for DEFUSE.

Predictive models of high-risk sites and bat species across Asia. We will build models that predict bat and viral diversity and spillover risk across Asia to enable warfighters and planners to assess risk and necessity for intervention deployment (TA2). We will combine regional-scale joint species distribution models (JSDM), machine-learning host-virus association models, and non-parametric viral richness estimators to respectively predict the composition of bat communities in caves across Asia, host range for key viral clades, and as-of-yet unsampled viral diversity. We will use a stochastic feedforward neural network to implement JSDMs that are effective at multiple scales with incomplete observations (as occurs for bats and their viruses), and that account for bat-species co-occurrences driven by environment or evolution⁴⁴. We will fit our JSDM to biological inventory data on over 200 caves in the region⁴⁵, to physiologically relevant bioclimatic variables (BIOTCLIM)⁴⁶, open source topographic data, and proxies for subterranean habitat such as ruggedness and habitat heterogeneity. As in previous work⁴⁷, we will refine these models with regional-scale environmental variables (land-use, distance to roads, etc.) and cave-specific variables (cave length, availability of roosting area, entrance dimensions, cave complexity, etc.). We will validate them using independent bat occurrence estimates and observations⁴⁸, and use EHA's unique database of all known host-virus relationships to extend predictions of bat CoV diversity and host range⁴⁹ (Fig. 4). We will use generalized additive host trait predictive models and machine-learning algorithms (BRT, random forest)⁵⁰ with non-parametric estimators to predict SARS-CoV diversity in the OS of each bat species⁵¹, and assess viral discovery rates in real time through sampling (Fig. 5).

Fig. 4. Predictive map of endemic viral diversity in bats for China and SE Asia. Yellow-shaded areas, based on all known mammal host-virus relationships. Our human test case site is labeled (red asterisk). Fig. 5. CoV diversity estimates (dashed line with 95% confidence intervals) based on PREDICT sampling data (solid line) for four bat genera.

To extend the geographic scope of predictive models, we will include data from >1800 viral detections (CoVs and others) from >10,000 individual bat samples in 6 Asian countries (NIAID- and USAID PREDICT-funded). For species composition and viral presence predictions, we will validate models against a 20% validation subset of data, and field data.

Protocaps app for the warfighter. Drawing on experience building applications for data collection and analysis for DoD (e.g.,

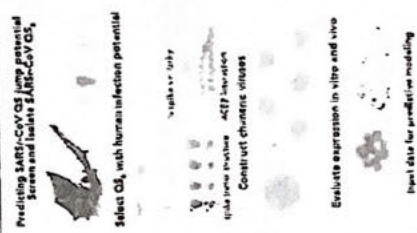
we will produce a prototype 'spatial viral spillover risk' app for the

warfighter that identifies probability of dangerous viral pathogens spilling over from bats at a site. We will use outputs from our spatial risk modeling, observed and predicted crowd-sourced associations, open-source species and pathogen ontologies, and app-directed crowd-sourced echolocation data to ground-truth and fine-tune its predictive capacity. This app will be updated in Y2 and Y3 to incorporate additional risk data from host-virus binding assays and SARS-CoV surveys. We will use EHA's risk-ranking algorithm (https://github.com/DEFUSE/SARS-CoV) to display critical areas of high risk based on geolocation features, recency of information, host and pathogen characteristics. This app will collect user GPS location data and pre-load bat species distribution and community composition estimates from our JSOKs. There will be refined with real-time surveillance data collected without the need to enter cave sites using mobile phone-enabled high-frequency microphones for bat detection⁴³, validated and trained with reference acoustic calls using convolutional neural networks⁴⁴. Identified bat species will be automatically linked with viral diversity data from EHA's host-pathogen database and SARS-CoV data from DEFUSE to deliver high-risk pathogen lists, displayed as pathogen-centric, bat-centric, or map-centric views, with proactive alerts when critical information is received. All code modules will be available and documented on GitHub (https://github.com/DEFUSE/SARS-CoV). This technology will improve over all situational awareness of existing and novel infectious agents found in bats, allowing DoD personnel to quickly identify areas high spillover risk steps and rapidly deploy resources to respond to and mitigate their impact preemptively when necessary.

SARS-CoV OS detection, sequencing, and recovery. We will screen samples for SARS-CoV nucleic acid using our pan-CoV consensus one-step hemi-nested RT-PCR assay targeting a 440-nt fragment in the RNA-dependent RNA polymerase gene (RdRp) of all known α - and β -CoV¹⁴³ and specific assays for known SARS-CoV^{21,131,134}. PCR products will be gel purified, sequenced and qPCR performed on SARS-CoV-positive samples to determine viral load. Full-length genomes or S genes of all SARS-CoV¹⁴³ will be high-throughput sequenced followed by genome walking^{23,134}. We will analyze the S gene for its ability to bind human ACE2 by Biocore or virus entry assay. **Synthesis of chimeric novel SARS-CoV OS.** We will commercially synthesize SARS-CoV S glycoprotein genes, designed for insertion into 3xCMV4 or WNV16 molecular clone backbones (88% and 97% S-protein identity to epidemic SARS-Urbani). These are 8S1-3, 90S backbones (88% and 97% S-protein identity to epidemic SARS-Urbani). These are 8S1-3, 90S select agents or subject to P3CO (they use bat SARS-CoV backbones which are exempt) and are pathogenic to hACE2 transgenic mice. Different backbone strains increase recovery of viable viruses identification of barriers for RNA recombination-mediated gene transfer between human, bat or chimeric ACE2 receptors to support cultivation of viruses with a weaker RBD-human ACE2 interface. **Recovery of full length SARS-CoV.** We will compile sequence/RNAseq data from a panel of closely related strains (<5% nucleotide variation) and compare full length genomes, scanning for unique SNPs representing sequencing errors¹⁴⁴. Consensus candidates genomes will be synthesized commercially (e.g. BioBasic), using established techniques and genome-length RNA and electroporation to recover recombinant viruses¹⁴⁵.

Predicting strain-specific SARS-CoV spillover risk. We will combine detailed experimental characterization of OS at our test cave sites with state-of-the-art phenotype-phenotype Bayesian network models. This will enable us to predict the jump probability of future OS that emerge with unique genetic recombinations. Our models will be parameterized with experimental data from a series of assays on the S genes of bat SARS-CoVs (Fig. 6, right), with experimental and modeling work flowing together in iterative steps. Our prior data will act as baseline to parameterize spillover risk modeling^{143,146}. This will be supplemented by characterization of isolated viruses under DEFUSE (at WIV), approximately 15-20 bat SARS-CoV spike proteins/year (at UNC, WIV), and >180 bat SARS-CoV strains sequenced in our prior work and not yet examined for spillover potential. All experiments will be performed in triplicate and data fed to models in real time.

Experimental assays of SARS-CoV OS jump potential (Fig. 6, right). Pre-screening via structural protein modeling. Is the major species restriction preventing spillover of SARS-CoV¹⁴³. To select OS for further characterization we will first use structural modeling of SARS-CoV S protein RBD^{143,147}, and host protease proteolytic processing of the S glycoprotein¹⁴⁸ regulate SARS-CoV cell entry and cross-species infectivity. Mismatches in the S-RBD-ACE2 molecules or S proteolytic processing will prevent cell entry of SARS-CoV¹⁴³ and will be deprioritized. Single amino acid variations could dramatically alter these phenotypes and we will evaluate the impact of low abundant, high consequence mutations in the RBD using RNAseq to identify low abundant OS variants encoding mutations relevant to ACE2 binding. We will conduct *in vitro* pseudovirus binding assays, using established techniques¹⁴⁹, and live virus binding assays (at WIV to prevent delays and unnecessary dissemination of viral cultures) for isolated strains. Initial model predictions based on these data inputs will be used to guide strain selection for further characterization. ***In vitro* testing of chimeric viruses.** All chimeric viruses will be sequence verified and evaluated for: i) ACE2 receptor usage across species *in vitro*, ii) growth in primary HAE, iii) sensitivity to broadly cross neutralizing human monoclonal antibodies that recognize unique epitopes in the RBD^{144,150}. Should some isolates prove highly resistant to our mAb panel, we will evaluate cross neutralization against a limited number of human SARS-CoV serum samples from the Toronto outbreak. Chimeric viruses that encode novel S genes with spillover potential will be used to identify SARS-CoV strains for recovery as full genome length viable viruses. ***In vivo* pathogenesis.** Groups of 10 animals will be infected intranasally with 1.0×10^6 PFU of each vSARS-CoV, clinical signs (weight loss, respiratory function, mortality, etc.) followed for 6 days



pL, and sacrificed at day 2 or 6 p.i. for virologic analysis, histopathology and immunohistochemistry of the lung and for 22-segment complete blood count (CBC) and bronchoalveolar lavage (BAL). Validation with full-length genome Q5. We will validate results from chimeric viruses by re-characterizing full-length genome versions, testing whether backbone genome sequence alters full length SARS-CoV spillover potential. Q5 for full-genome characterization will be selected to reflect strain differences in antigenicity, receptor usage, growth in human cells and pathogenesis. We will test growth in primary HAE cultures and in who in HACE2 transgenic mice. We anticipate recovering ~3-5 full length genome viruses/yr.

Testing Synthetic Modifications: We will synthesize Q5 with novel combinations of mutations to determine the effects of specific genetic traits and the jump potential of future and unknown recombinants. **RBD deletions:** Small deletions at specific sites in the SARS-CoV RBD alter risk of human infection. We will analyze the functional consequences of these RBD deletions on SARS-CoV HACE2 receptor usage, growth in HAE cultures and *in vivo* pathogenesis. First, we will delete these regions, sequentially and in combination, in SHC05a and SARS-CoV Urban, anticipating that the introduction of deletions will prevent virus growth in Vero cells and HAE²³. In parallel, we will evaluate whether RBD deletion repair restores the ability of low risk strains to use human ACE2 and grow in human cells. **S2 Proteolytic Cleavage and Glycosylation Sites:** After receptor binding, a variety of cell surface or endosomal proteases^{24,25} cleave the SARS-CoV S glycoprotein causing massive changes in S structure²⁶ and activating fusin-mediated entry^{27,28}. We will analyze all SARS-CoV S gene sequences for appropriately conserved proteolytic cleavage sites in S2 and for the presence of potential furin cleavage sites^{29,30}. SARS-CoV S with mismatches in proteolytic cleavage sites can be activated by exogenous trypsin or cathepsin L. Where clear mismatches occur, we will introduce appropriate human-specific cleavage sites and evaluate growth potential in Vero cells and HAE cultures. In SARS-CoV, we will ablate several of these sites based on pseudotyped particle studies and evaluate the impact of select SARS-CoV S changes on virus replication and pathogenesis. We will also review deep sequence data for low abundant high risk SARS-CoV that encode functional proteolytic cleavage sites, and if so, introduce these changes into the appropriate high abundant, low risk parental strain. **N-linked glycosylation:** Some glycosylation events regulate SARS-CoV particle binding DC-SIGN^{31,32}, alternative receptors for SARS-CoV entry into macrophages or monocytes³³. Mutations that introduced two new N-linked glycosylation sites may have been involved in the emergence of human SARS-CoV from civet and raccoon dogs³⁴. While the sites are absent from civet and raccoon dog strains and clade 2 SARS-CoV, they are present in WNV1, WNV16 and SHC014, supporting a potential role for these sites in host jumping. To evaluate this, we will sequentially introduce clade 2 disrupting residues of SARS-CoV and SHC014 and evaluate virus growth in Vero cells, nonpermissive cells ectopically expressing DC-SIGN, and in human monocytes and macrophages anticipating reduced virus growth efficiency. We will introduce the clade 1 mutations that result in N-linked glycosylation in rs4237 RBD deletion repaired strains, evaluating virus growth efficiency in HAE, Vero cells, or nonpermissive cells ± ectopic DC-SIGN expression³⁵. *In vivo*, we will evaluate pathogenesis in transgenic HACE2 mice. **Low abundance micro-variations:** We will structurally model and identify highly variable residue changes in the SARS-CoV S RBD, use commercial gene blocks to introduce these changes singly and in combination into the S glycoprotein gene of the low risk, parental strain and test ACE2 receptor usage, growth in HAE and *in vivo* pathogenesis.



Fig. 7: A simplified directed graph of a Bayesian network model representing the causal relationships between input data, model processes, and outputs.

Network machine-learning to predict spillover potential of high-risk SARS-CoV strains. We will use experimental data from above to build genotype-phenotype models of bat SARS-CoV spillover potential. We will use Bayesian Network Models (BNM), fit via MCMC methods³⁶ to predict spillover risk based on bat SARS-CoV genotype data (presence of deletions in RBD, proteolytic binding and glycosylation sites etc.) and the ecological traits of hosts - integrating data on multiple, interacting processes and Q5 spillover potential to generate overall spillover probabilities. The Bayesian approach will allow us to update our models iteratively as new data is acquired, and use interim model predictions to guide which experiments to prioritize to maximize predictive ability³⁷. We will control for experimental conditions (assays on live viral isolates, full-genome or synthetic chimeric viruses, and the molecular backbone of the latter). Traits will be used as inputs to BNM's causal graph, to predict latent variables representing interconnected processes that contribute to SARS-CoV Q5 infection in new hosts: receptor binding, cell entry, immune system interaction, and intracellular growth, all measured by our lab assays. There, in turn will act as predictors for the ultimate outcomes of host pathogenesis and host jumping potential (Fig. 7). We will use published work on these genetic traits to put informative priors on strength and direction of interactions in the causal graph. We will use prior-knowledge model simulations to select target sequences from our sampling for characterization and genome-sequencing, to collect data that maximally enhances the predictive power of our model and update these simulations iteratively throughout the experimental phase to continually guide Q5 selection. We will use regularizing priors to reduce over-fitting and select the most predictive variables in the final model.

Model validation using SARS-CoV serology from previously-collected human samples and surveillance data. Active spillover of SARS-CoV in our study region enables us to measure actual spillover risk to validate our models of Q5 jump potential. We will gather data on viral Q5 antibodies found in the local human population using LIPS assays on >2,000 previously-collected human sera (NAID, Duszak P1) from people living close to our test cave sites in Yunnan Province, a sub-sample of which showed 2.7% seropositivity to bat SARS-CoVs³⁸. The IRB for

this work is current and covers proposed DEFUSE testing. We will design UPS assays targeting high- and low-spillover risk SARS-CoV-2 OS, as done previously for SARS-CoV-2^{13,14} and the novel SARS-CoV-2¹⁵. We will: 1) insert different high- and low-risk SARS-CoV-2 N gene into pGEN-2 vector (UPS vector), firstpassing N gene similarity to determine their potential cross-reactivity in a UPS assay; 2) determine UPS assay specificity by producing polyclonal sera via infection of recombinant protein or attenuated virus into rabbits; 3) validate UPS assays by incubating antigen with their respective positive serum samples and the antigen antibody complex eluted using protein A/G beads; 4) validate UPS positive sera results by spike protein based UPS and viral neutralization assay. As a confirmatory test, the positive samples from UPS will be validated by virus neutralization assay. We will use these UPS assays to test serum samples for presence of antibodies to high- and low-risk SARS-CoV-2 OS. We will validate predictions of jump potential and extend the BNMs to predict actual spillover probabilities by modeling bat-human contact rates with bats. We will use ecological data on bat hosts and human behavioral survey data collected previously from these individuals to estimate wildlife contact in predicting exposure measured by our UPS assays.

Evolutionary modelling and simulation to predict potential strains. Our Bayesian network modelling will generate predictions of the spillover risk of OS sequences we identify. To examine risk associated with the total viral population, we will model and simulate evolutionary processes to identify likely viral OS that our sampling has not captured, and viral OS likely to arise in the future ("OS_f"). We will use a large dataset of S protein sequences and full-length genomes generated from prior work and DEFUSE fieldwork to estimate SARS-CoV-2 substitution rates and its genome-wide variation using coalescent and molecular clock models within a Bayesian MCMC framework¹⁶. We will estimate SARS-CoV-2 recombination rates at the cave population level using these data and Bayesian Inference^{17,18}. We will apply RDP¹⁹, similarity plots, and bootscan to identify recombination breakpoints and hotspots within the SARS-CoV-2 genome as done previously²⁰, now extended to the full genome. Using these estimates we will simulate the evolution of the SARS-CoV-2 OS genome using a forward-time approach implemented in simulators that model specific RNA virus functions (e.g., VIRAPOPS²¹). We will predict the rate at which new combinations of genetic traits can spread in viral populations and compare recombination rates among caves and bat communities. Our forward-simulated results will provide a pool of likely unknown and future OS_f species. Using these and our SEM model for spillover risk, we will predict the OS_f most likely to arise and have spillover and pathogenic potential. We will use evolutionary simulation results to iteratively improve our Bayesian network model. The number of genetic traits with potential for prediction of pathogenicity is large, so we will perform variable reduction using tree-based clustering, treating highly co-occurring traits as joint clusters for prediction. We will generate these clusters from all SARS-CoV-2 sequences from DEFUSE fieldwork and prior work. As trait clusters may be modified through recombination, we will use our forward-evolutionary modelling to predict how well trait clusters will be conserved, retaining only those unlikely to arise in unknown or OS_f genomes. This will enable a trade-off between increased predictive power based on current samples and generalizability to future strains that have not yet evolved.

Teshale Alemu 2

Immune modulation approach to reducing bat SARS-CoV-2 spillover risk. Our work shows that the following unique immunological features of bats may explain their capacity to harbor high viral loads with minimal clinical signs: a) bats maintain constitutively high expression of IFN α that may respond to and restrict viral infection²²; b) several interferon activation pathways are dampened, e.g. STING (a central cytosolic DNA-sensor molecule to induce interferon) dependent and TLR7 dependent pathways²³; c) the NLRP3 dependent inflammatory pathway is dampened, and key inflammation response genes like AIM2 are not present in bats²⁴. These traits may be due to bat immune-sensing pathway adaptation as a fitness cost of flight²⁵. We hypothesize that bat virus replication will likely be restricted quickly by constitutively expressed IFN α . In bats, resulting in lower B/T cell stimulation due to lower viral stimuli. Second, dampened interferon and inflammation responses will result in lower cytokine responses that are required to trigger T79 cell dependent adaptive immunity (e.g. antibody response), ultimately resulting in suppression of viral replication and shedding. We and others have demonstrated proof-of-concept of this phenomenon: Experimental Marburg virus infection of Egyptian fruit bats, a natural reservoir host, resulted in widespread tissue distribution with low viral load, brief viremia, low per conversion and a low antibody titer that waned quickly, suggesting no long-term protection is established^{26,27}. Poor neutralizing antibody responses occur after experimental infection of bats with Tacariba virus²⁸, and in our studies of experimental infection of bats with SARS-CoV (Wang, unpubl.). We also successfully showed that bat interferon can inhibit bat SARS-CoV²⁹. We hypothesize that use of immune modulators that target the naturally low innate immunity of bats to their viruses, will transiently suppress viral replication and shedding, reducing the host jump risk. We further hypothesize that because *Rhinolophus* bats are long-lived (20+ yrs in the wild), most bats in a population will have been exposed to a range of SARS-CoV OS at our sites. Specifically targeting suppression of their adaptive immunity (immune memory) to high-risk viral strains may lead to heightened clearance of high-risk strains. We will evaluate two immune modulation approaches to reduce spillover of SARS-CoVs from bats to humans: 1) Broad-scale immune boosting strategies (Wang, Duke-NUS); we will apply immune modulators like TLR-ligands, small molecule RIG-like receptor (RLR) agonists or bat interferon in live bats, to up-regulate their innate immunity and suppress viral replication and shedding. 2) Targeted immune boosting (Batis UNCC). The broad-scale immune boosting approach will be applied in the presence of chimeric immunogens to activate immune memory in adult bats and boost clearance of high-risk SARS-CoVs. We will use novel chimeric polyvalent recombinant proteins in microparticle encapsulated gels for oral delivery and/or virus-adjuvanted immune boosting strategies where chimeric recombinant SARS-CoV-2 are expressed by raccoon-poxvirus. Both lines of work will begin in Year 1 and run parallel, be assessed competitively for efficiency, cost, and scalability, and successful candidates from captive animal trials will be used in live bat trials at our test cave in Yunnan. The finding of low innate immunity across bats suggest that immune boosting could be broadly applicable to bat genera and viral families.

Broad-scale immune boosting (Duke-NUS). We will work on the following key leads to identify the most effective approach to up-regulate innate immunity and suppress viral loads. *Toll-like*

receptors (TLR/NLR-like Receptor (RLR) ligands): Our work indicates a robust response in live bats to TLR-stimuli like poly(I:C) as measured by transcriptomics on spleen tissue (Fig. 8), liver, lung and lymph node, with matched proteomics to characterize immune activation *in vivo*. These activation profiles will be used to assess bat immune response to different stimuli and identify those which lower viral load in our experimental system at Duke-NUS (below).

Fig. 8: Pathway analysis from Ingenuity Pathway Analysis (IPA) of whole spleen NGS after stimulation with either poly(I:C) or poly(I:C). Z-score increase over control bats is indicated on per scale, and suggests strong activation of many pathways.

We will also stimulate the RIG-I pathway with 5'pppRNA, a mimetic of the natural RIG-I stimulant that will activate functional bat IFN production pathways, as shown in a mouse model that cleared SARS-CoV, IAV and HAV^{14,15}

Universal bat interferon: We will design a conserved universal bat interferon protein sequence with artificial gene synthesis and produce recombinant protein by cleavable-affinity-tagged purification of supernatant from over-expressing bat cells, as used previously for recombinant *Pteropus alecto* IFN^{17,18} and CSF-2/IL-4. Utilization of a universal IFN for bats will overcome species-dependent response to the ligand, allowing the use of IFN throughout broad geographical and ecological environments and across many bat species. We have already produced recombinant non-universal, tagged, bat IFN that induce appropriate immune activation (Fig. 9). This ligand has been shown to reduce viral titers in humans, ferrets and mouse models intranasally and orally^{19,20}. Interferon has been used clinically in humans as an effective countermeasure when antiviral drugs are unavailable, e.g. against filoviruses²⁰. Interferon is known to be toxic, therefore we will carefully examine dose tolerance in bats and assess clinical effects of the treatment. We have shown that replication of SARS-CoV is sensitive to IFN treatments²¹. The successful delivery, immune activation and outcome on the host will be characterized thoroughly to optimize rapid immune activation.

Fig. 9: Bat IFNs are sensitive to IFN treatment. A) Recombinant bat SARS-related coronavirus (SARSr-CoV) replication was inhibited by human IFN α in a dose dependent manner in Vero cells. B) Bat (SARSr-CoV) PIV2B replication was inhibited by recombinant bat IFN α in a dose dependent manner in bat P41203 cells.

Boosting bat IFN by blocking bat-specific IFN negative regulators: Uniquely, bat IFN α is naturally constitutively expressed but cannot be induced to a high level, indicating a negative regulatory factor in the bat interferon production pathway^{17,22}. We will use a *Pteropus alecto* CRISPRi library pool that we have created covering multiple RNA targets in the *P. alecto* genome (Wang, unpubl.

data). Genes affecting influenza replication in bat cells have already been identified using this library. Using CRISPRi we will identify negative regulator genes and screen for compounds targeting them to boost the inducibility of the IFN system in a shorter time-frame. Based on previous work^{23,24}, it is highly likely this will be a conserved pathway across all bats. **Activating dampened bat-specific innate immune pathways which include DNA-STING-dependent *endTLR-dependent pathways:*** We have shown that mutant bat STING or reconstitution of AIM2 and functional NLRP3 homologs restores antiviral functionality, suggesting these pathways are important in bat-viral coexistence. By identifying small molecules to directly activate pathways downstream of STING or TLR/RLRs, such as TBK1 activation, we will activate bat innate defense by interferons, promote viral clearance and, we hypothesize, significantly reduce viral load in bats. **Validating *Ina* bat-mouse model:** Various CoVs show efficient infection and replication inside the human host but exhibit defective entry and replication using mouse as a host due in part to differences in DPP4 and ACE2 receptors.

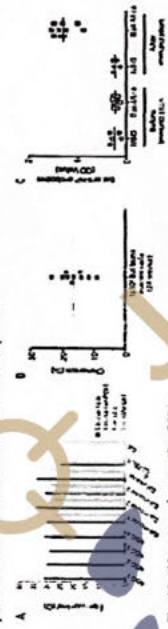


Fig. 10: A) Presence of an-specific qPCR in reconstituted mice after 12 weeks. B) Chimeric ratio of bat-mouse cells in circulation after 24 weeks. C) Specific antibody response to a H4N2-virus antigen generated by bat-reconstituted mice.

We have shown efficient reconstitution of irradiated mice using bat bone marrow from multiple species, including *E. spallans* (Fig. 10), including reconstitution of bat PBMCs in the mouse, presence of circulating bat cells and generation of bat-specific antibodies in mice incapable of producing an antibody response. This 'batified' mouse model can be utilized for both circulating infection of SARS-CoV (in the immune compartment only) and as a model for generating bat-specific antibodies against CoV proteins. Efficient validation of infection into bat cells will be used to validate the infectivity of the viruses and generation of bat antibodies will facilitate validation of the best proteins/peptide to elicit an effective immune response.

Targeted immune boosting (UNC). To boost targeted adaptive immunity (immune memory) in wild bats chronically exposed to circulating SARS-CoV CoS, we will inoculate with chimeric S glycoproteins in the presence of the broadscale immune boosting agonists above. We have developed novel group 2B SARS-CoV chimeric S glycoproteins that encode neutralizing domains from phylogenetically distant strains (e.g. Urbani, HKU3, BtCoV 279, ~25% diversity). The chimeric S programs efficient expression when introduced in the HKU3 backbone full length genome, and elicits protective immunity against multiple group 2B strains. We will develop robust expression systems for SARS-CoV chimeric S using ectopic expression *in vitro*. We will work with Dr. Alnalla (UNC-Pharmacy) who has developed novel microparticle delivery systems and dry powders for aerosol release that encapsulate recombinant proteins and adjuvants (innate immune agonists) that we will use for parallel broadscale immune boosting strategies