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14. ABSTRACT
This research proposal focuses on the development of a novel library screening approach to engineering highly stabilized subunit vaccine candidates for major pathogens within the paramyxovirus family. The research addresses the PRMRP topic areas related to vaccine development for infectious pathogens as well as addressing the topic concerning emergent viruses. The paramyxoviruses encompass many pathogens that cause disease in humans and this proposal focuses on four viruses that fall into two subclasses within the broader family, respiratory syncytial virus (RSV), human metapneumovirus (HMPV), Nipah virus (NiV) and Hendra Virus (HeV).

15. SUBJECT TERMS
Respiratory viruses, Emerging infectious disease, vaccine engineering, yeast surface display

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1. INTRODUCTION:

Paramyxoviruses are responsible for many human diseases, such as mumps and measles, as well as many other diseases for which vaccines are currently not available. Two respiratory viruses within this family, respiratory syncytial virus (RSV) and human metapneumovirus (HMPV), are responsible for widespread infections in humans, with especially significant morbidity in children and the elderly. Two other emergent viruses, Nipah virus (NiV) and Hendra virus (HeV), can infect humans with highly deadly outcomes, with over 60% fatality rates. There are no vaccines available for these four viruses, although significant industrial and academic efforts are pushing RSV vaccine development forward with promising results. The paramyxovirus fusion (F) protein is a major target of the neutralizing antibody response, but it exists in two distinct conformations (pre- and post-fusion), which display distinct epitopes. The metastable prefusion F is a more potent protective antigen and significant efforts have been made to engineer RSV F in a stabilized form suitable for vaccine development.

In this proposal, we are pursuing an alternative method for F protein antigen design, based on yeast surface display library methods. We seek to use large libraries to isolate highly stabilized F proteins that retain their authentic prefusion fold, using conformation sensitive antibody reagents for selection. Our initial goal has been to engineer suitable F protein constructs that display well folded F at the yeast surface. During the first year of this award we focused on different approaches to expressing F proteins in yeast, using direct tethering the yeast Aga2p, a secretion-capture approach based on a nanobody-peptide tag and a leaky self-cleaving peptide anchoring approach. During the remaining 6 months of the award, we examined underlying blocks to expressing stable F proteins in yeast and pursued an alternative approach to generating F mutant libraries in mammalian cells, as well as evaluated computational approaches to predicting stabilizing F mutants using structural information.

2. KEYWORDS: Paramyxovirus, Fusion protein, vaccine development, infectious disease, yeast surface display, Henipavirus, RSV, HMPV

3. ACCOMPLISHMENTS:

Major goals and accomplishments

	Overview of proposed task and current accomplishments	
Specific Aim 1: Cell surface display of RSV, HMPV and Nipah virus F proteins.	**Proposed timeframes**	**Current Status (12 months)**
Major Task 1: Create recombinant yeast expressing F proteins		
Subtask 1: Generate and analyze a set of secreted constructs for four F proteins with codon optimized and mutated variants. Generate additional variants to further optimize F secretion in yeast	1-4	Completed
Subtask 2: Express and/or obtain samples of conformation specific antibodies for detection of RSV, HMPV. Nipah and Hendra virus F proteins, including D25, MPE8, 5B4	1-4	Completed
Subtask 3: Generate recombinant yeast and evaluate yeast surface display expression of F constructs using anti-tag and anti-F antibodies	4-6	Completed
Specific Aim 1 Milestones:		

Milestone 1 Selection of best yeast expression clones for secretion of F proteins in yeast	Month 4	Not completed
Milestone 2 Obtain antibody reagents necessary for conformational screening of yeast clones	Month 4-5	Completed
Milestone 3 Analyze surface expression of F constructs and select best strategy/approach for library production	Month 6	Not completed
Specific Aim 2: Generation and screening of F protein mutant libraries for stabilized, prefusion F proteins.		
Major Task 2: Screening of F mutant libraries for stabilized conformational variants		
Subtask 1: Generate 4 libraries of F sequence variants using PCR-based random mutagenesis approaches. Validate the library diversity by DNA sequencing.	Month 6-8	Not completed
Subtask 2: Transfect mutant libraries into yeast, propagate library, validate diversity and generate aliquots of library replicates for backup storage.	Month 8-10	Not completed
Subtask 3: Conduct multiple rounds of library screening to enrich for clones that exhibit improved binding to conformation-specific antibodies	Month 10-14	Not completed
Subtask 4: Isolate, sequence and analyze individual yeast clones from selection	Month 13-14	Not completed
Specific Aim 2 Milestones:		
Milestone 1 Construct and validate PCR mutant library	Month 10	Not completed
Milestone 2 Complete antibody-based selection of F mutants	Month 14	Not completed
Milestone 3 Isolate individual F variants; analyze sequence	Month 14	Not completed
Specific Aim 3: Validation of the stability and conformational states of candidate stabilized F antigens.		

Major Task 3: Express, purify and analyze top candidate F mutants for each target virus		
Subtask 1: Subclone a selected panel of F mutants into mammalian expression vectors	Month 14-16	Partially completed
Subtask 2: Purify F mutants, conduct immunoassays and electron microscopy assays	Month 16-18	Partially completed
Specific Aim 3 Milestones:		
Milestone 1 Produce mammalian expression constructs for F mutants	Month 16	Partially completed
Milestone 2 Complete antibody and EM analyses of F mutants	Month 18	Partially completed

Specific Aim 1: Cell surface display of RSV, HMPV and Nipah virus F proteins.

Major Task 1: Create recombinant yeast expressing F proteins
Year 1 Milestones:

1. *Selection of best yeast expression clones for secretion of F proteins in yeast.*
2. *Obtain antibody reagents necessary for conformational screening of yeast clones*
3. *Analyze surface expression of F constructs and select best strategy/approach for library production.*

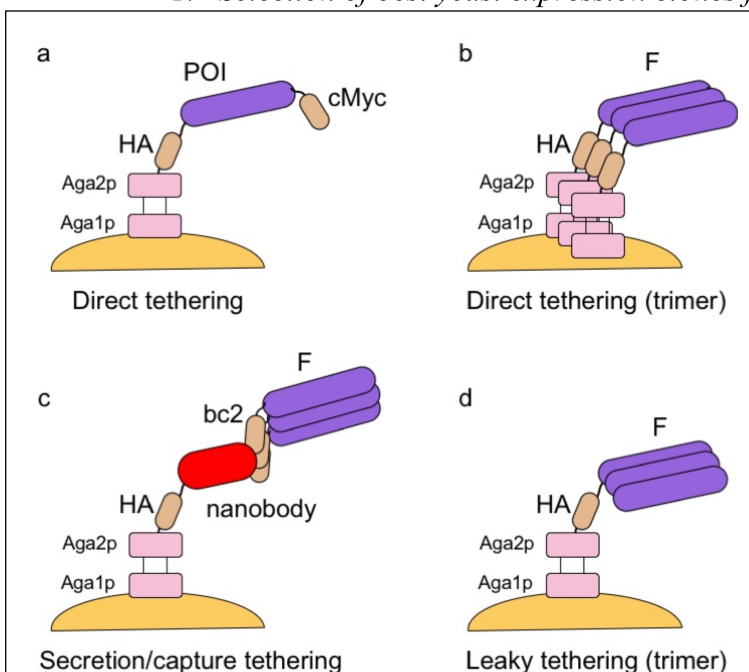

Figure 1. Strategies for expressing monomeric and oligomeric proteins of interest at the yeast cell surface. (a) Direct tethering of monomeric proteins through N- or C-terminal fusions to the Aga2p protein. Flanking epitope tags (e.g. HA or c-Myc) allow monitoring of surface expression. **(b)** Direct tethering of F trimers results in multiple Aga2p attachment points per oligomer, which may interfere with display of functional F. **(c)** A secretion/capture approach using the BC2 nanobody and BC2-peptide-tagged F. **(d)** A leaky tethering approach using a F-Aga2p fusion linked by a self-cleaving 2A peptide with incomplete cleavage, potentially mixed trimers of anchored and secreted subunits.

During the term of this exploratory grant, our primary focus has been on reaching Aim 1 milestones using different strategies for displaying our F trimers at the yeast surface. For Milestone 2, we accumulated the necessary antibody reagents to conduct our yeast screening, including a collection of monoclonal antibodies that are specific for RSV F (motavizumab), prefusion RSV and MPV F (MPE8), MPV F (monoclonals 4G14, 2J6, 4I3, 5L4 and 10C11) and prefusion Nipah/Hendra F (5B4). Additional antibodies (e.g. D25) are commercially available if needed. We have also synthesized codon optimized DNA expression constructs for displaying our F proteins in yeast.

The standard approach to displaying proteins at the yeast surface is shown in Figure 1a. In this approach the protein of interest (POI) is fused directly to the yeast anchoring protein Aga2p. The fusion can be either at the N- or the C-terminus of the POI and the POI can be flanked with epitope tags (e.g. HA and c-myc peptide tags) to monitor POI expression independent of POI-

specific antibodies or functional binding partners. The Aga2p protein forms covalent interactions with Aga1p, leading to the stable anchoring of the POI at the yeast cell surface.

We have tested the simplest approach to displaying our F protein trimers, which consists of fusing our F protein ectodomain constructs to Aga2p directly, with Aga2p located at the C-terminal end of the F protein (Figure 1b). The direct tethering of oligomeric proteins through Aga2p fusions is not always successful, potentially because the multiple distinct attachment points of the oligomer through Aga2p may interfere with proper folding or assembly. Our constructs of directly tethered F trimers did not yield detectable surface expression of protein, although we could not rule out issues with transcription and translation of our F constructs. We therefore sought to modify our approach to provide an independent monitor of F transcription and translation in transfected yeast, as described below.

The display of protein oligomers, such as IgG antibody dimers, has been achieved in yeast using a secretion/capture tethering approach (Figure 1c). In the case of IgG dimer expression a high affinity IgG-specific binding protein was fused to Aga1p. Using a dual expression vector, the IgG antibody was expressed in a secreted form and captured to the yeast cell surface non-covalently. We refer to this approach as the secretion/capture method and have adapted this strategy to use a more general tag/capture system in place of the IgG-specific binding protein. For our F protein experiments, we have expressed a peptide-specific nanobody that has high affinity for a peptide tag (the BC2 nanobody). In independent experiments, we have shown that functional nanobody can be expressed at the yeast surface and is active in binding exogenously added fluorescently labelled BC2 peptide. We therefore moved forward with the construction of a dual expression yeast vector (Figure 2a), which is designed to express the tethered BC2 nanobody along with the secreted, BC2-peptide-tagged F protein. In this approach, secreted F protein would be able to properly fold into trimers with BC2 peptide tags at the C-terminus allowing high affinity capture to the cell surface.

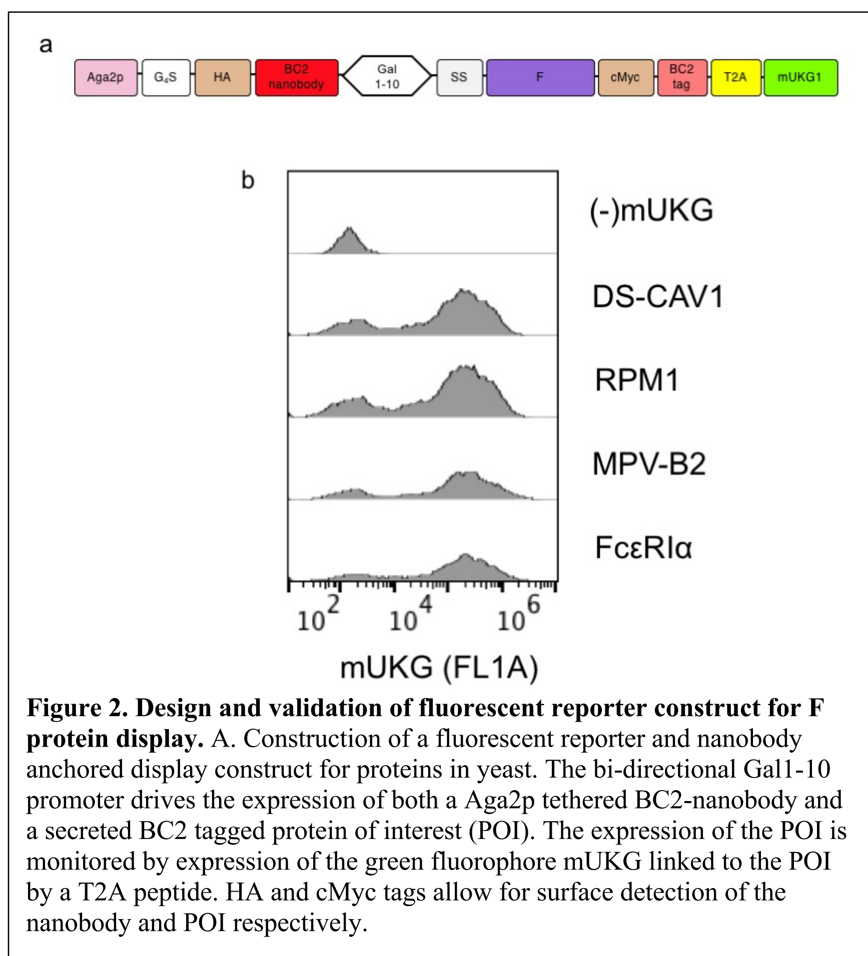

Figure 2. Design and validation of fluorescent reporter construct for F protein display. A. Construction of a fluorescent reporter and nanobody anchored display construct for proteins in yeast. The bi-directional Gal1-10 promoter drives the expression of both a Aga2p tethered BC2-nanobody and a secreted BC2 tagged protein of interest (POI). The expression of the POI is monitored by expression of the green fluorophore mUKG linked to the POI by a T2A peptide. HA and cMyc tags allow for surface detection of the nanobody and POI respectively.

Because we did not observe expression of the F protein in our initial experiments with the direct tethering approach with epitope tags, we further engineered this expression system to provide a reporter fluorophore that would directly result from transcription and translation of the F genes. As shown in Figure 2a, after the C-terminal BC2 peptide tag, we appended a self-cleaving peptide sequence (T2A) followed by a green fluorescent protein variant - monomeric Umikinoko-green 1 (mUKG1). The self-cleaving peptide allows production of two independent proteins from the same mRNA, by inducing a ribosomal break in the polypeptide chain at the C-terminus of the T2A peptide sequence. The expression of mUKG1 is therefore linked to transcription and translation of the F gene, but results in secreted F with separate and cytosolically located mUKG1 as a cell-specific reporter.

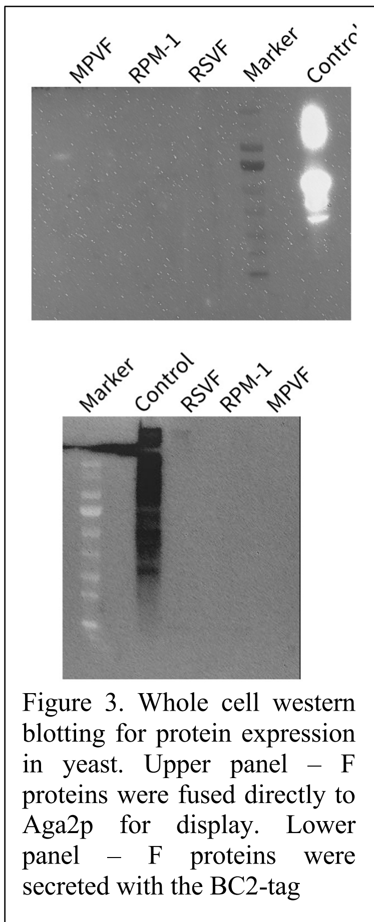

Figure 3. Whole cell western blotting for protein expression in yeast. Upper panel – F proteins were fused directly to Aga2p for display. Lower panel – F proteins were secreted with the BC2-tag

As can be seen in Figure 2b, constructs for three of our F proteins (RSV DS-CAV1, MPV RPM1 and MPV B2) as well as a control protein construct (FcεRIα), result in robust expression of mUKG1. These data indicate that the F genes are transcribed and translated. This approach provides a readout for selecting yeast that express our manipulated F genes, which can be used for cell sorting and monitoring relative mRNA production independently of F protein expression. However, we were not able to detect F protein at the yeast surface. In order to check whether F protein was expressed at detectable levels in yeast cells, we conducted Western blots of our original and BC2-tag constructs, using a yeast strain expressing a single chain antibody Fv fragment (scFv) as a control. While robust expression of the control scFv was detected in Western blots, none of the F samples showed any protein accumulation, except for a very weak band for the MPV F construct (Figure 3). These data indicated that our RSV and MPV F constructs, although transcribed and able to produce mUKG1 protein, did not yield any appreciable F protein. These data indicate that the F protein stability and folding in the yeast may be compromised.

As a further test of the yeast system, we generated full length Measles virus F protein constructs containing either a C-terminal mCherry or GFP tag (Figure 4) for expression in both yeast and mammalian cells. When this protein is expressed in yeast, we observed degradation of the intact F into smaller fragments detectable in Western blots, potentially consistent with cleavage by the yeast furin-like Kex2 protease. In contrast, when the F protein is expressed in mammalian cells, we do not observe this degradation pattern and instead observe primarily cleavage of F into F1 and F2 fragments (Figure 4). These observations suggest significant differences in the ability of yeast and mammalian cells to properly fold RSV, MPV and measles virus F proteins. Differences in yeast ER chaperones, proteases and glycosylation may all impact F stability and folding. We concluded that further studies of the F protein expression in yeast should explore expression in alternate yeast strains that have more mammalian-like glycosylation, or that express key mammalian ER chaperones, or potentially should be conducted with mutant proteins resistant to Kex2-like proteolysis.

Within the remaining period of the grant, we decided to pursue additional approaches to stabilizing the F proteins. For one of these approaches, we investigated the possibility of generating mutant F libraries directly in 293 cells, using commercially available FLP-in cells. This system relies on FLP recombinase mediated integration of a single clone into a single engineered site in the 293-Flp-in cells. This approach has been used previously for antibody display and selection and provides a mechanism to generate cells expressing single clones for phenotypic expression. We used our mCherry-tagged Measles virus F protein construct cloned into the pcDNA5/FRT expression vector (Figure 5). We generated a mutant library using error prone PCR mutagenesis with primers targeting the 5’ and 3’ ends of the F protein gene (Figure 5). The library was cloned into the expression vector and cells expressing integrated F clones were selected using Hygromycin B. While this approach

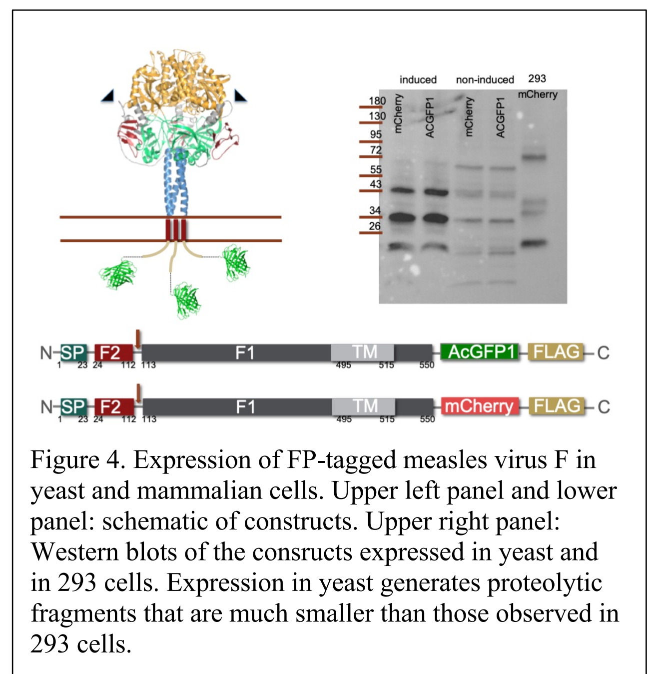

Figure 4. Expression of FP-tagged measles virus F in yeast and mammalian cells. Upper left panel and lower panel: schematic of constructs. Upper right panel: Western blots of the consructs expressed in yeast and in 293 cells. Expression in yeast generates proteolytic fragments that are much smaller than those observed in 293 cells.

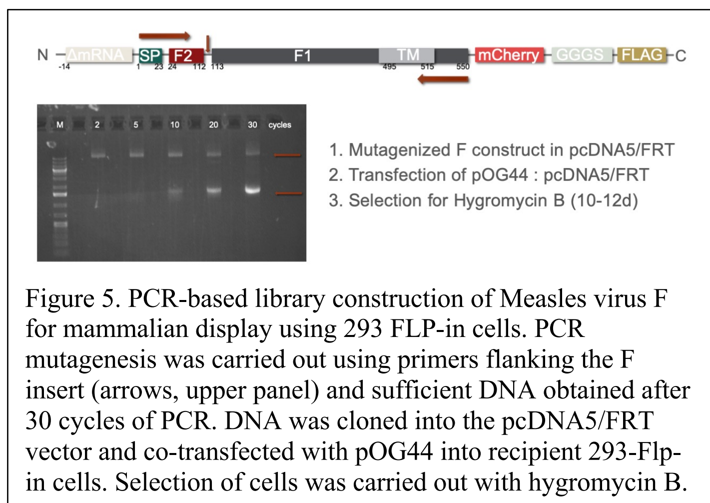

Figure 5. PCR-based library construction of Measles virus F for mammalian display using 293 FLP-in cells. PCR mutagenesis was carried out using primers flanking the F insert (arrows, upper panel) and sufficient DNA obtained after 30 cycles of PCR. DNA was cloned into the pcDNA5/FRT vector and co-transfected with pOG44 into recipient 293-Flp-in cells. Selection of cells was carried out with hygromycin B.

yielded some resistant clones, it was clear that significant optimization would be necessary in order to produce libraries of sufficient diversity for our needs.

In addition to pursuing these experimental approaches to selecting stabilized F variants, we also took a computational approach to stabilizing the MPV F protein, based on the recently published crystal structure of the prefusion protein. We used the Schrodinger suite of computational tools to predict sties for cysteine mutation within the F ectodomain, which would yield stabilized prefusion F. A set of disulfide mutants were made and analysis of these mutants is ongoing.

Specific Aim 2: Generation and screening of F protein mutant libraries for stabilized, prefusion F proteins.

Major Task 2: Screening of F mutant libraries for stabilized conformational variants
Year 1 Milestones:

a. Construct and validate PCR mutant library

As indicated above, we generated a PCR mutant library for the Measles Virus F protein for expression in 293 Flp-in cells. We did not screen for stabilized conformational variants, based on the limited library size that we were able to generate using this approach.

Specific Aim 3: Validation of the stability and conformational states of candidate stabilized F antigens.

Major Task 3: Express, purify and analyze top candidate F mutants for each target virus
Year 1 Milestones: n/a

We generated constructs for computationally designed MPV F variants, using methods implemented in the Schrodinger package for designing stabilizing disulfide bond mutants. Preliminary studies of three of these mutants have been carried out, indicating that these may be only partially expressed in the prefusion conformation.

Opportunities for training and professional development. The current research associated with this proposal remained too preliminary for formal presentations to outside groups. However, laboratory members involved in the project made ~4-5 formal oral laboratory presentations on their research to the group, participated in weekly subgroup research meetings, attended and presented at the Structural Biology annual retreat and have attended other scientific meetings.

How were the results disseminated to communities of interest. Results have not yet been disseminated.

Plans for the next reporting period. Not applicable

4. IMPACT:
Impact on the principal discipline. Nothing to report.

Impact on other disciplines. Nothing to report.

Impact on society. Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach. As indicated above, we encountered significant barriers to expressing F proteins in yeast for the proposed display studies. Additional approaches to overcoming these barriers may include using alternative yeast strains that express mammalian-like glycosylation pathways or chaperones. It may be possible to recover F protein expression using random mutagenesis approaches. However, the most promising approach appears to be to pursue library expression in mammalian cells, using a virus-based delivery approach.

Anticipated problems or delays. Nothing to report.

Changes in human subjects, vertebrate animals, biohazards and/or select agents. Nothing to report.

6. PRODUCTS:

Publications. Nothing to report.

Website or other internet sites. Nothing to report.

Technologies or techniques. Nothing to report.

Inventions, patent applications and/or licenses. Nothing to report.

Other products. Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals working on the project.

Name	Theodore Jardetzky
Project role	PI
Researcher Identifier	
Nearest person month worked	1.44 months
Contribution to project	Directed research
Funding support	DoD and NIH

Name	Luke Pennington
Project role	Graduate student
Researcher Identifier	
Nearest person month worked	0.9 months
Contribution to project	Contributing expertise on yeast display experiments
Funding support	NIH fellowship support

Name	Xiaolin Wen
Project role	Research Associate
Researcher Identifier	
Nearest person month worked	9 months
Contribution to project	Contributing effort on RSV and HMPV F expression and RSV/MPV antibody reagents
Funding support	DoD

Name	Joyce Wong
Project role	Research Associate
Researcher Identifier	
Nearest person month worked	7.2 months
Contribution to project	Contributing effort on Measles, Nipah and Hendra F proteins and antibody reagents
Funding support	DoD 7 NIH

Name	Silke Kleinboelting
Project role	Postdoctoral fellow
Researcher Identifier	
Nearest person month worked	(not charged while on independent fellowship)
Contribution to project	Contributing effort on Measles virus F proteins and antibody reagents
Funding support	DoD

Changes in other support.
The following grants started during this award:

1. NIH/NIAID R01DK113504 Hsieh (PI) 5/5/2017-4/30/2022
"Therapeutic Exploitation of IPSE, a Urogenital Parasite-Derived Host Modulatory Protein, for Bladder Hypersensitivity Syndromes"

2. NIH/NIAID R21 AI133192 Jardetzky (PI) 7/1/2017-6/30/2019
"Structure and function of HCMV gHgL complexes"; The specific aims focus on investigation of the structure and inhibition of HCMV gHgL complexes.

3. NIH/NIAID R01 AI137267 Longnecker, Jardetzky, Zhou, co-PIs 4/1/2018-3/31/2023
"Structure and function of EBV protein complexes that trigger epithelial cell entry"

4. NIH/NIAID R01 HL141493 Jardetzky (PI) 4/1/2018-3/31/2022
"Suppression of basophil activation by IgE glycovariants"

5. NIH/NIAID R01 AI137523 Jardetzky (PI) 9/6/2018-8/31/2022
"Repertoire studies of human antibodies to RSV and MPV F". The specific aims are focused on studying human monoclonal and repertoire antibody responses to RSV and HMPV F proteins.

The following grants ended during this award:

1. NIH/NIAID R01 AI076183 Longnecker & Jardetzky (Co-PIs) 4/1/2008-3/31/2018
"Structural and Functional Studies of gp42 and HLA Class II in EBV Entry"

2. DoD PR130130 Jardetzky (PI) 9/1/2014-9/29/2017
"Novel IgE Inhibitors for the Treatment of Food Allergies"

3. NIH/NIAID R21 AI119480 Jardetzky, Longnecker, Zhou – co-PIs 7/1/2015-6/30/2017
"Structure and function of EBV protein complexes that trigger epithelial cell entry"

4. NIH/NCI R01 CA117794 Jardetzky, PI; Longnecker, Co-PI 7/31/2012-5/31/2017

“Inhibitors of the Epstein-Barr Virus Entry Machinery”

Other organizations. Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS:

Nothing to report.

9. APPENDICES: n/a