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13. SUPPLEMENTARY NOTES

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). In the past year we have been exploring different approaches to expressing F protein trimers at the surface of yeast and have developed two novel strategies that utilize self-cleaving 2A peptides and reporter fluorescent proteins as our foundational constructs for F library generation and selection.

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 have 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.

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	75% 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	75% completed
Specific Aim 1 Milestones:		
Milestone 1	Month 4	In progress

Selection of best yeast expression clones for secretion of F proteins in yeast		
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	75% 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 yet started
Subtask 2: Transfect mutant libraries into yeast, propagate library, validate diversity and generate aliquots of library replicates for backup storage.	Month 8-10	Not yet started
Subtask 3: Conduct multiple rounds of library screening to enrich for clones that exhibit improved binding to conformation-specific antibodies	Month 10-14	Not yet started
Subtask 4: Isolate, sequence and analyze individual yeast clones from selection	Month 13-14	Not yet started
Specific Aim 2 Milestones:		
Milestone 1 Construct and validate PCR mutant library	Month 10	Not yet started
Milestone 2 Complete antibody-based selection of F mutants	Month 14	Not yet started
Milestone 3 Isolate individual F variants; analyze sequence	Month 14	Not yet started
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	Not yet started
Subtask 2: Purify F mutants, conduct immunoassays and electron microscopy assays	Month 16-18	Not yet started
Specific Aim 3 Milestones:		
Milestone 1 Produce mammalian expression constructs for F mutants	Month 16	Not yet started
Milestone 2 Complete antibody and EM analyses of F mutants	Month 18	Not yet started

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.*

During the past year, 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 have 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

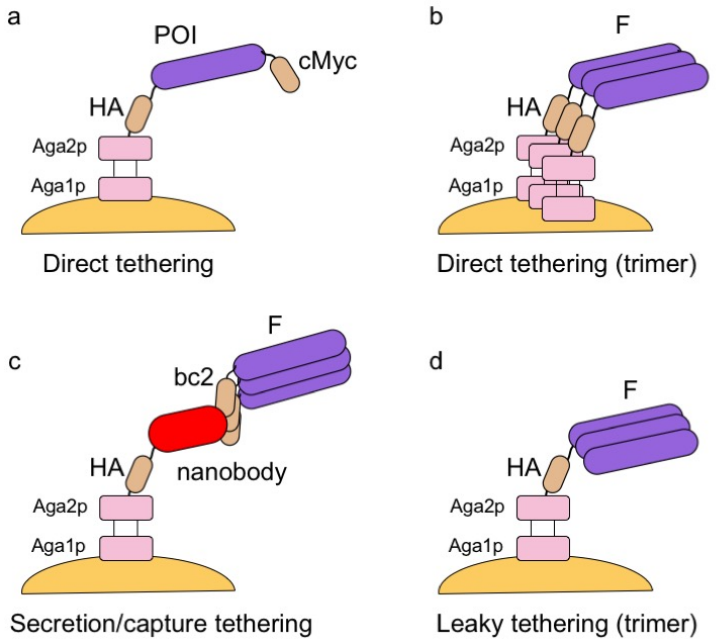

Figure 1. Strategies for expressing monomeric and oligomeric proteins of interest at the yeast cell surface. (a) Direct tethering of monomeric proteins through N- or C-terminal fusions to the Aga2p protein. Flanking epitope tags (e.g. HA or c-Myc) allow monitoring of surface expression. **(b)** Direct 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.

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 refeer 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.

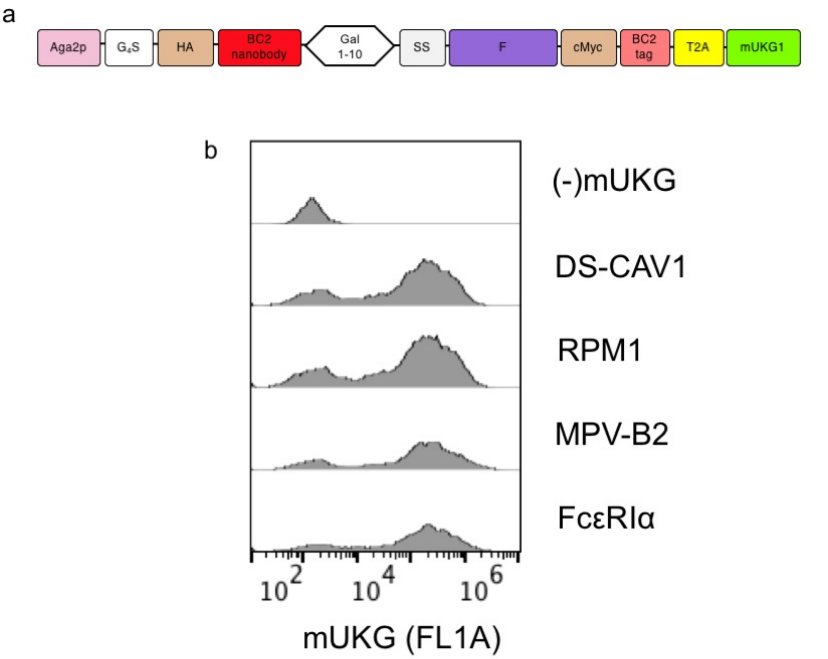

Figure 2. Design and validation of fluorescent reporter construct for F protein display. A. Construction of a fluorescent reporter and nanobody anchored display construct for proteins in yeast. The bi-directional Gal1-10 promoter drives the expression of both a Aga2p tethered BC2-nanobody and a secreted 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.

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 and believe that this is at least in part a result of a defect in BC2 nanobody expression and anchoring. Although we have independently validated the BC2 nanobody display in a different expression vector, we suspect that problems with the bidirectional Gal1-10 promoter or the specific BC2 nanobody construct in the dual expression vector is preventing the expression of nanobody. We are in the process of cloning our original BC2 nanobody construct into this dual expression vector, as the original construct contains a different epitope tag and other small changes which could impact expression. However, we feel that the mUKG1 expression is a good indicator that we are expressing proper F transcripts and that we are on track to using this system for F library display.

During the course of these experiments, we have decided to develop one additional approach to displaying F trimers, which may be more elegant and straightforward than the secretion/capture method. This fourth approach makes use of a modified self-cleaving 2A peptide, which results in incomplete peptide cleavage (Figure 1d). Using this "leaky" 2A peptide, we expect ~ 40% of the F protein to be produced fused to the Aga2p anchor with ~60% produced as soluble, secreted F. We are currently modifying our F constructs to append the "leaky" 2A peptide, followed by the Aga2p anchor, a robust 2A peptide and finally the fluorescent reporter mUKG1. Similar to the constructs shown in Figure 2b, production of mUKG1 will provide an independent monitor of transcription and translation. In this "leaky tethering" approach (Figure 1d), the expression of tethered and secreted F may allow for biosynthetic assembly of mixed oligomers, with tethering mediated by 1 subunit of the F trimer. This may allow for proper assembly and expression of the trimers at the yeast cell surface without requiring independent expression of the BC2 nanobody for capture of secreted protein. Both of these two systems should be amenable to library generation even in the absence of detectable F trimer expression, to overcome unanticipated blocks in the biosynthesis of these proteins.

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

We have not yet initiated the construction of PCR mutant libraries as we are still at the stage of developing and testing the best display strategy. We feel that the secretion/capture and leaky tethering approaches could both provide platforms for generating PCR mutant libraries, in particular since we have observed expression of our reporter fluorescent protein mUKG1. For the secretion/capture approach, we need to alter the vector construct to express functional BC2 protein and we have robust assays to monitor this, using anti-epitope tag antibodies and fluorescently labelled BC2 peptide. Once we have validated the BC2 nanobody expression and functionality, we will be in a position to generate F libraries, following mUKG1 expression and F-specific antibody binding. Similarly, once we have designed F expression construct for the leaky tethering approach, we will be able to test for F expression using the mUKG1 reporter, for surface expression using Aga2p-flanking epitope tags and anti-F antibodies. We will be in a position to create expression libraries once we have validated the expression of the mUKG1 reporter.

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

No milestones for Specific Aim 3 were set for Year 1 as these expression studies were anticipated to be initiated in the second year of the proposal.

Opportunities for training and professional development. The current research associated with this proposal is still too preliminary for formal presentations to outside groups. However, laboratory members make ~4-5 formal oral laboratory presentations on their research to the group, participate in weekly subgroup research meetings, attend and present 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. As described above, we are continuing with our studies as proposed and anticipate generating F protein libraries within the next 1-3 months.

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. We have adapted some of our constructs and approaches as described in more detail above, because of our initial observations that F protein expression was not detectable in a direct-tethering surface display approach. We have now incorporated the use of self-cleaving 2A peptides and a reporter fluorescent protein to verify that transcription and translation of our F constructs is occurring as an independent readout from F protein detection. However, the overall general approach remains the same.

Anticipated problems or delays. We do not anticipate any further problems of delays in our next stage of generating F libraries.

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	0.96 months
Contribution to project	Directed research

Funding support	DoD and NIH

Name	Luke Pennington
Project role	Graduate student
Researcher Identifier	
Nearest person month worked	2 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	12 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	6 months
Contribution to project	Contributing effort on Nipah and Hendra F proteins and antibody reagents
Funding support	DoD 7 NIH

Changes in other support.
The following grants have started:

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"

The following grants have ended:
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