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Jillian Grace April 5, 2019

Characterization of Respiratory Synctial Virus (RSV) Fusion (F) Proteins on Viral-Like Particles

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Abstract

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The Respiratory Syncytial Virus (RSV) fusion (F) glycoprotein is an extracellular surface protein recognized by Th2 cells and expressed on the surface of RSV viruses. The F protein contains two variants that differ in conformation and antigenic sites available: the metastable prefusion conformation and the more stably expressed postfusion conformation that becomes stabilized after fusion of the virus with the host cell membrane. Artificially stabilized prefusion conformations possess antigenic sites that are critical to elicit neutralizing antibodies. This study is focused on generating RSV F prefusion variants expressed on virus-like particles (VLPs). VLPs are nanostructures that resemble viruses but do not contain the RNA viral genome and thus lack pathogenicity. We developed multiple VLP variants and characterized them by enzyme-linked immunosorbent assay (ELISA), immunoblotting, and electron microscopy (EM) analysis. Based on prior research evaluating anti-F antibodies in human sera, prefusion F protein elicits a significantly higher neutralization response compared to postfusion F due to availability of epitopes that are readily accessible and recognized by humoral immune cells. However, prefusion F protein is highly unstable and easily converts to postfusion F. We compared DS-Cav 1, SC-TM, and wildtype variants to determine which variant most stably produced prefusion F protein and was most readily recognized by anti-F prefusion-specific antibodies. We found DS-Cav 1 and SC-TM to have substantially greater recognition of anti-F antibodies via ELISA. This could indicate a need to create variants that contain mechanisms to prevent release of the fusion peptide, reduced charge repulsion in the refolding region 2 (RR2), and increased packing in the hydrophobic region of the prefusion conformation.

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Table of Contents

Introduction

Virus like-particles (VLPs) are self-assembling nanostructures that resemble the structures of viruses and generally contain one or more structural proteins arranged in single, double, or triple layers with a lipid outer envelope (1). VLPs are utilized in vaccine development since they have been proven to be an immunogenic and an efficient way to present viral antigens (2). VLP-based vaccines lack a viral genome, which prevents replication as well as reversion mutations or pathogenic infection (2). Examples of vaccines that utilize VLPS include vaccines for human papilloma virus (HPV) and hepatitis B virus. The efficacy of VLPs is due to recognition of repetitive subunits in host cell effector T cell antigens, which elicits a high cellular and humoral immune response (3). The additional benefit of VLP-based research is that they can be produced in a variety of systems, including bacterial, mammalian, yeast, and insect systems (3). Because VLPs are produced by the host cell, the choice of host cell to produce the VLP is important to ensure the VLP contents have the correct structure (3). One must consider a system that includes appropriate post-translational modification and protein-folding apparatuses. Mammalian cells are effective systems that have the capacity to produce more complex and accurate post-translational modifications, similar to what occurs in human infection. Mammalian post-translational modifications are essential for producing enveloped VLP complexes with structural proteins in several layers (3).

Respiratory Synctial Virus (RSV) is the primary cause of hospitalization in the first year of life for children in most parts of the world. Approximately, 100 percent of children in the United States are infected with RSV by 2 to 3 years of age (4). Groups most at-risk for severe RSV disease include premature babies born at <35 weeks of gestation, HIV-infected subjects, patients with underlying heart and lung disease, and patients with severely compromised immune

systems (4). Infection is restricted primarily to the ciliated epithelia of small bronchioles and type 1 pneumocytes in the alveoli thus only affects bronchopulmonary tree. However, some patients with T and B cell immunodeficiency may experience extrapulmonary disease. Given that infection with RSV causes sloughing off of epithelium in airways and the accumulation of inflammatory cell debris, fibrin, mucous, and lymphoid aggregates, airway hypersensitivity, restricted airflow, hypoxia, and wheezing results (5).

CD4-, CD8-, and Th2- specific immune responses are elicited upon infection with RSV. Immunoregulatory cytokines and chemokines are released in the upper respiratory tract, causing inflammation and subsequent wheezing and coughing (5). IgG responses include release of antibodies reactive with the fusion (F) and glycoprotein (G) RSV proteins (5). Additionally, RSV significantly induces substance P, a pro-inflammatory neuropeptide that acts on bronchiolar smooth muscle and results in a bronchial spasm. Infection reduces the density of inhibitory receptor NK1 on target smooth muscle cells, decreasing negative feedback which would result in decreased substance P. Furthermore, RSV has been shown to activate expression of nuclear factor (NF)-IL-6 and NF-κB, which activate the synthesis of multiple cytokines and chemokines with other important immunomodulating functions (5). These cytokines include TNF-α, IL-1β, IL-2, IL-6, GM-CSF, and G-CSF; adhesion molecules ICAM-1, VICAM-1, and E-selectin (4). Chemokines activated are IL-8, MIP-1α, MCP-1, and eotaxin. All of these molecules recruit immune cells to the site of infection and initiate an inflammatory cascade (4).

RSV is a cytoplasmic virus that consists of a single stranded, negative sense RNA genome with eight structural proteins and a lipid envelope. Viral proteins included in the viral genome and used to construct VLPs are the matrix (M) protein, F protein, G protein, and the phosphoprotein (P protein). There are two subtypes—RSV A and B—based on antigenic and

genetic differences among strains with the greatest differences being in the G and SH protein genes. The two proteins most important for inducing protective immunity are F and G. While G protein displays heterogeneity in antibody responses to A and B subgroups, F protein shows similar antibody responses regardless of whether the subgroup is A or B (6). Thus, infection with RSV A would produce neutralizing antibody responses to RSV F protein that could cross-react with group B viruses.

RSV F protein is a 574 amino acid class I fusion protein that consists of a trimer of F1 and F2 heterodimers. F1 is a 50 kilodalton (kDa) carboxy-terminal polypeptide, while F2 is a 20 kDa amino-terminal polypeptide (7). The F protein is distinguished by two furin cleavage sites that liberate a glycopeptide and expose the hydrophobic fusion peptide at the F1 amino terminus (7). There is also a cysteine-rich region in the F1 and two disulfide bonds connecting F1 and F2 (7). Only 25 amino acids in the F protein exposed to the extracellular matrix differ between RSV A and B subtypes (7). The F1 polypeptide contains sequences in the extracellular matrix domain that change folding patterns based on whether the fusion peptide has been released. RR1 is used to refold from the prefusion to postfusion conformation. It also includes the fusion peptide and heptad repeat A (HRA). HRA transforms from an assembly of secondary structures in the prefusion structure to form a long, continuous helix in the postfusion conformation. RR2 on F1 forms the C-terminal domain in the prefusion conformation and includes the heptad repeat B (HRB). HRB binds to HRA to form the six-helix bundle in prefusion and postfusion forms (2). Because F protein is relatively conserved between A and B subtypes and is strictly required for viral entry into a host cell, it has become an antigenic target for vaccine development. There are two known major antigenic sites on F associated with neutralizing activity: site II and site IV (6).

The atomic structure of antigenic sites II and IV were solved by crystalizing postfusion F protein in complex with prototypic antibodies (8).

Current prophylaxis therapy for RSV is Palivizumab, an injection administered to children during RSV season. Palivizumab is an IgG monoclonal antibody that targets an epitope on the RSV F protein (7). The first attempt at a vaccine for RSV was in 1960 using a formalin-inactivated, alum-precipitate, whole-virus vaccine administered to children between 2 months and 7 years of age. It caused 80% hospitalization in children vaccinated and 2 patients in the cohort died (7). The vaccine caused poor antibody function, given a reduced neutralization response despite high antibody titers, high levels of lymphoproliferative activity in peripheral blood mononuclear cells and tissue eosinophilia consistent with a Th2-biased immune response (7). Subsequent attempts have included F isolated from virions, a mixture of F, G, and N isolated from virions, a F/G fusion protein expressed in baculovirus (7).

We are concerned with developing a VLP that most effectively expresses prefusion F protein neutralizing epitopes. Previously, I have investigated the effects of utilizing different matrix proteins to enhance F and G protein expression in a human 293F cell line system. We transfected 293F cells with VP40 Ebola matrix protein, HIV GAG core protein, and RSV M protein with RSV F protein and G protein. The VP40 protein shares a similar tertiary and quaternary structure to the RSV M protein (9). Ultimately, we decided to continue experimentation using RSV VLPs with M protein plus P or M2-1, a protein also involved in virion formation, to form the VLP platform. The RSV VLPs entirely consists of RSV proteins that should better reflect the native structures of prefusion F protein and G protein.

Cell lines transfected with F assist the incorporation of G protein into RSV VLPs making it possible to develop VLPs with both F and G. The G protein has been shown to contain a CX3C

motif that binds to the CX3CR1 receptor in human airway epithelial cells that then facilitates F protein mediates subsequent membrane fusion and infection of the cell (10).

Previous efforts to design a variant of prefusion F that elicited the highest neutralizing response lead researchers to look for a "supersite" that is comprised of a collection of overlapping epitopes recognized by multiple antibodies. Three variants have been found in prior research that still retained binding capacity to the pre-F specific antibodies: DS, Cav 1, and DS-Cav 1 (6). DS was produced by substituting two serine amino acids with cysteines, resulting in a disulfide bond that connected terminal and central regions within the polypeptide F1 (3). Cav 1 is a "cavity-filling" variant with mutations that fills cavities in the F1 N-terminus to increase hydrophobic packing interactions and make exposure of hydrophobic residues to solvent unfavorable (3). DS-Cav1 contains both mutations. Antigenic site ∅ is present exclusively on prefusion F protein and the focus of designing a variant that stably expresses it because it is recognized by RSV-neutralizing antibodies that are 10-100 times more potent than palivizumab (3). By modifying regions that did not rearrange between pre- and post- fusion states, researchers did not stabilize antigenic site ∅. Moreover, although D25 stabilized the prefusion structure, its complex with prefusion F sterically occluded the ∅ target site for a neutralization response (3). The DS-Cav 1 variant combination appeared optimal in terms of trimer yield and physical stability in extremes of temperature, pH, osmolality, and freeze-thaw (9). Mice injected with DS-Cav 1 produced titers roughly 60-fold higher than those elicited by postfusion F against the RSV subtype A and more than 80-fold against RSV subtype B (9).

Regions termini of the F1 subunit show dramatic conformational changes between pre- and post- fusion F protein forms. The fusion peptide at the N-terminus of F1 with five secondary structural elements rearrange and fuse to form a long, extended postfusion helix (3). At the C-

terminus, the parallel β-strand unravels and allows the prefusion helix to move towards the extended α-helix created at the N-terminus upon fusion (3). Furthermore, the RSV F fusion peptide is located in the center of the trimer cavity and fuses with the host cell membrane independent of pH at the cell surface (3). This may indicate why the prefusion form can be expressed with a fibritin trimerization domain (10). This motif is not sufficient to stabilize the prefusion form, as it also requires the binding of D25 or a similar antibody. D25 binds the least conserved region of the F glycoprotein, or site ∅. There are six sequence variations in the region, all of which are located in the region bound by D25 (3). Thus, the D25 epitope may serve as the determinant of subtype-specific immunity. Additionally, antigenic site ∅-specific antibodies are distinct from neutralizing antibodies to other known antigenic sites on RSV F because of their exclusive recognition of the prefusion F structure and extremely high potency.

RSV G protein is a 90 kDa type II integral membrane protein and can mediate viral attachment to host cell membrane either through interaction with heparan sulfate on proteoglycans or a more specific but unknown receptor (7). Variability in the G protein is used to subtype RSV strains by sequence differences (7). Additionally, the ends contain extensive glycosylation, which may enable the virus to avoid host immunity (7). However, the central region of the G protein is relatively conserved between strains (7). This region contains a CX3C motif that interacts with the chemokine receptor, CX3CR1, influencing neutrophil migration (7). A phase I clinical trial using the central conserved region of G conjugated to a portion of the Streptococcal protein G was well-tolerated and increased NT activity two-fold (7). However, two individuals exhibited hypersensitivity (7). This could be due to the inflammatory side effects in the clinical studies with Streptococcal protein G conjugate component and should not completely rule out use of RSV G protein in future clinical applications (7).

In order to construct a VLP that stably expresses prefusion F protein, we analyzed the effects of utilizing different VLPs containing different structural proteins. The three structural proteins in RSV viruses and VLPs are RSV matrix (M) protein, RSV phosphoprotein (P protein), and RSV M2-1 protein. RSV M protein has been implicated with associating viral RNA, N (nucleocapsid) protein, and P protein with RSV envelope proteins before budding from the host cell membrane (11). However, the N-terminus of RSV M has been found to interact with M2-1 in order to join the "inclusion" complex with N and P proteins (11). The entire complex with N, P, M2-1, and M proteins forms a polymerase complex in which transcription of messenger RNA and genomic RNA take place (11). Likewise, the RSV P protein prevents degradation of viral RNA by forming the inclusion complex with N protein and subsequent polymerase complex (11).

Replication occurs via attachment to cell through G protein and subsequent fusion of the viral envelope with the plasma membrane through F protein. The nucleocapsid of the virus is then released into the cellular cytoplasm. Viral genomic RNA serves as a template for messenger RNA that serves as the template for translation of viral proteins. Viral proteins are usually detected after 9 hours in culture (5). This cytoplasmic replication occurs in granules comprising the nucleoprotein (N), the transcription processivity factor M2-1 protein, and the polymerase (L). Virus assembly occurs in a cholesterol-rich membrane microdomains where the virus buds as filopodia-like filaments that can fuse with adjacent cells.

We created multiple VLPs containing alternative constructs that display the same epitopes as prefusion F protein. SC-TM was evaluated by previous researchers to be an effective prefusion F variant based on DS-Cav 1. Specifically, researchers added a short linker between F1 and F2 subunits to prevent the release of fusion peptide (SC) (2). They then induced three mutations to

reduce charge repulsion in RR2 (2). Despite these changes, SC-TM only displays a slight deviation from DS-Cav 1 structurally (2). However, in immunologically challenged mice immunized with both DS-Cav 1 and SC-TM, SC-TM threefold greater concentrations of neutralizing titers and was stably expressed for up to 40 days, whereas DS-Cav 1 exhibited only approximately 50% prefusion conformation at 40 days (2).

Thus, we are interested in generating various RSV F protein constructs such as DS-Cav 1, wildtype prefusion F, and SC-TM together with RSV G protein on VLPs so that these can be evaluated as RSV vaccines.

Results

I. Evaluating the Presence of F Protein

We first transfected 293F cells in series using plasmids containing antibiotic resistance genes and genes that encode M, P, G, and F proteins. Antibiotic selection was utilized after each transfection to culture 293F cells stably expressing these proteins. We assessed 293F cells transfected with RSV M+P+G+F proteins using Western Blot to see if we could successfully form VLPs using these proteins. Samples showed a combination of F0 and F1 proteins. F0 is the premature polypeptide containing two cleavage sites flanking the F1 and F2 polypeptides. The presence of F0 and F1 indicates that cells successfully formed RSV F protein when probed with anti-F antibody motavizumab but potentially in an immature form (Fig 1).

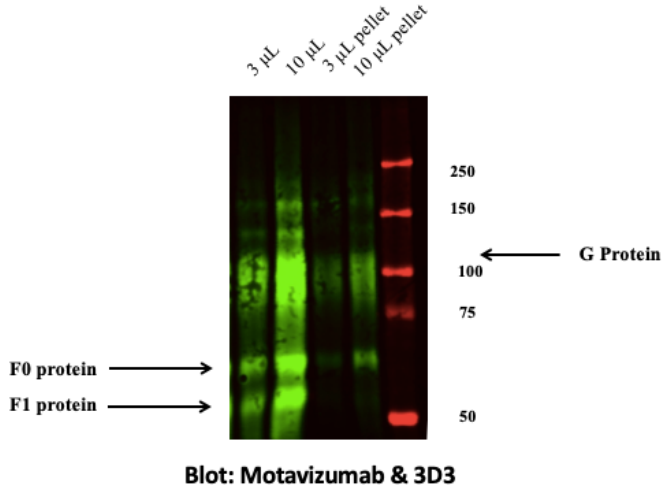

Figure 1. Western blot comparing expression of RSV F and G proteins between cell pellet and lysate to determine protocodl efficiency for purification of VLPs.

VLPs were purified using two rounds of centrifugation. After the first centrifugation, large cell debris is separated from VLPs, which are contained in the lysate. The lysate is then subjected to centrifugation at a higher speed and longer period of time. Additionally, a 20% sucrose solution

is added to separate smaller cellular proteins from viral proteins. Pellet and lysate samples of cells transfected with RSV proteins were analyzed to assess the efficacy of our purification methods. A noticeable signal was detected for pellet VLPs, indicating that VLPs may be trapped in the pellet after the initial centrifugation step. RSV G protein expression was analyzed using 3D3 anti-G antibody to assess the presence of another viral protein transfected in 293F cells and was likewise present in both pellet and lysate samples, indicating that VLPs were not completely purified after the initial round of centrifugation (Fig 1).

Additionally, we wanted to compare the effect of transfecting with RSV F protein on RSV G formation since F protein helps incorporate G into VLPs efficiently. This would also verify previous knowledge that F protein helps in the incorporation of G protein into RSV VLPs (--). ELISA was used as a quantification method of F recognition using anti-F motavizumab.

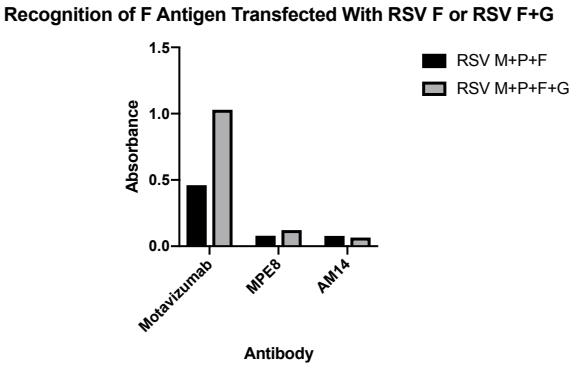

Figure 2. Comparison of RSV F recognition for RSV M+P+ either F or F+G VLPs

We compared VLPs harvested from cells transfected with M+P+F and M+P+G+F using ELISA. Motavizumab showed a significant increase in F antigen recognition with ELISA analysis of VLPs harvested with RSV G protein (Fig 2). Thus, RSV G protein assists in the formation of VLPs with RSV F protein. However, a lack of MPE8 and AM14 recognition indicates that the

form of RSV F formed in this experiment was likely postfusion RSV F. Therefore, a variant of RSV F that can stably express prefusion RSV F is needed.

II. Comparison of F antigen recognition for different variants of RSV prefusion F protein

Western blot was used again to assess the presence of F and G proteins in different VLP systems. These systems compared both F protein type (DS-Cav 1 versus wildtype F) and viral proteins critical to virion formation (P protein versus M2-1 protein). This blot shows the comparison between M+G+F+M2-1, M+G+P+F, and M+G+P+$F_{DS\text{-}Cav\ 1}$ VLPs.

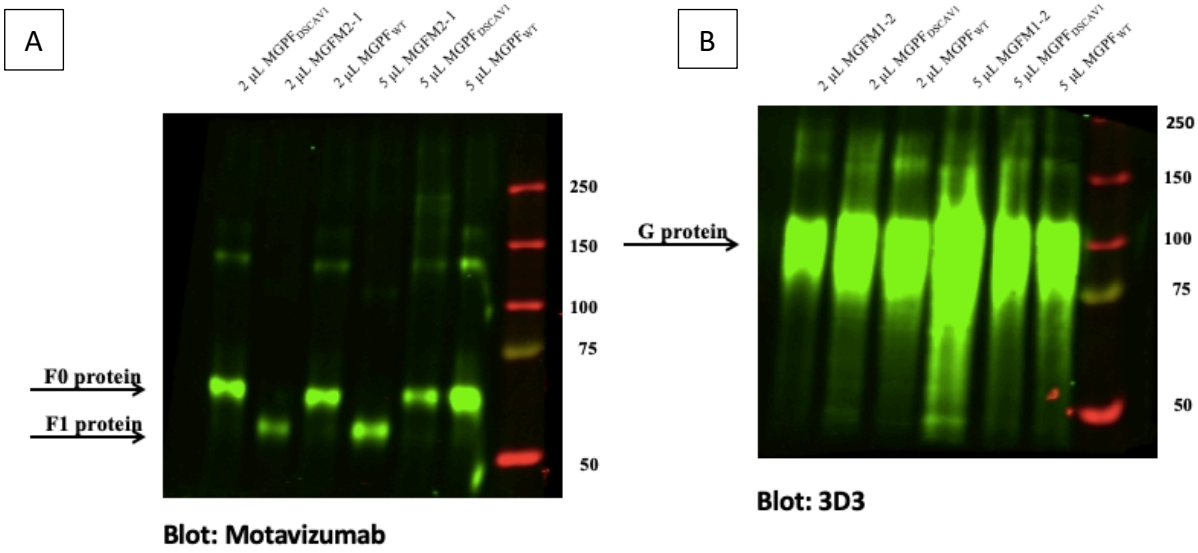

Figure 3. a) Comparison of F expression between M+G+F+M2-1, M+G+P+F, and M+G+P+$F_{DS\text{-}Cav\ 1}$ VLPs b) comparison of G expression between M+G+F+M2-1, M+G+P+F, and M+G+P+$F_{DS\text{-}Cav\ 1}$ VLPs.

Cleaved (F1) and non-cleaved (F0) versions of RSV F protein were expressed when probed with motavizumab. Both are able to be detected since they contain binding site II, the epitope where motavizumab is known to bind. Non-cleaved versions appear in M+G+F+M2-1 whereas cleaved versions appear in M+G+P+F and M+G+P+$F_{DS\text{-}Cav\ 1}$ VLPs. Both of these polypeptides can be

distinguished from one another based on molecular mass, with F0 containing additional sequences for F2 and p27 and is thus heavier. M2-1 was transfected in the place of P protein to test the efficacy of M2-1 plus M in producing prefusion F VLPs (10). However, these samples seemed to express F0 instead of F1, thus possibly impeding the cleaving of F0 into its constituent subunits.

ELISA was used again as a quantification of F protein recognition. Western blot provides evidence of the presence of VLPs. However, to compare which form of F protein is present and in what quantity is most effectively carried out using ELISA. Different antibodies were utilized to bind to different variants of prefusion F protein at different sites either inherent to the prefusion F protein or present in both the prefusion and postfusion conformations. D25 was used, which binds to site ∅ and spans two protomers at the apex of the prefusion F trimer (12). Motavizumab cross-competes with palivizumab, the previously described and currently utilized prophylactic therapy, for site II on F1. MPE8 recognizes an epitope slightly below site II at a different binding domain compared to D25. AM14 has shown to recognize prefusion F but a precise determination of where is unknown (8).

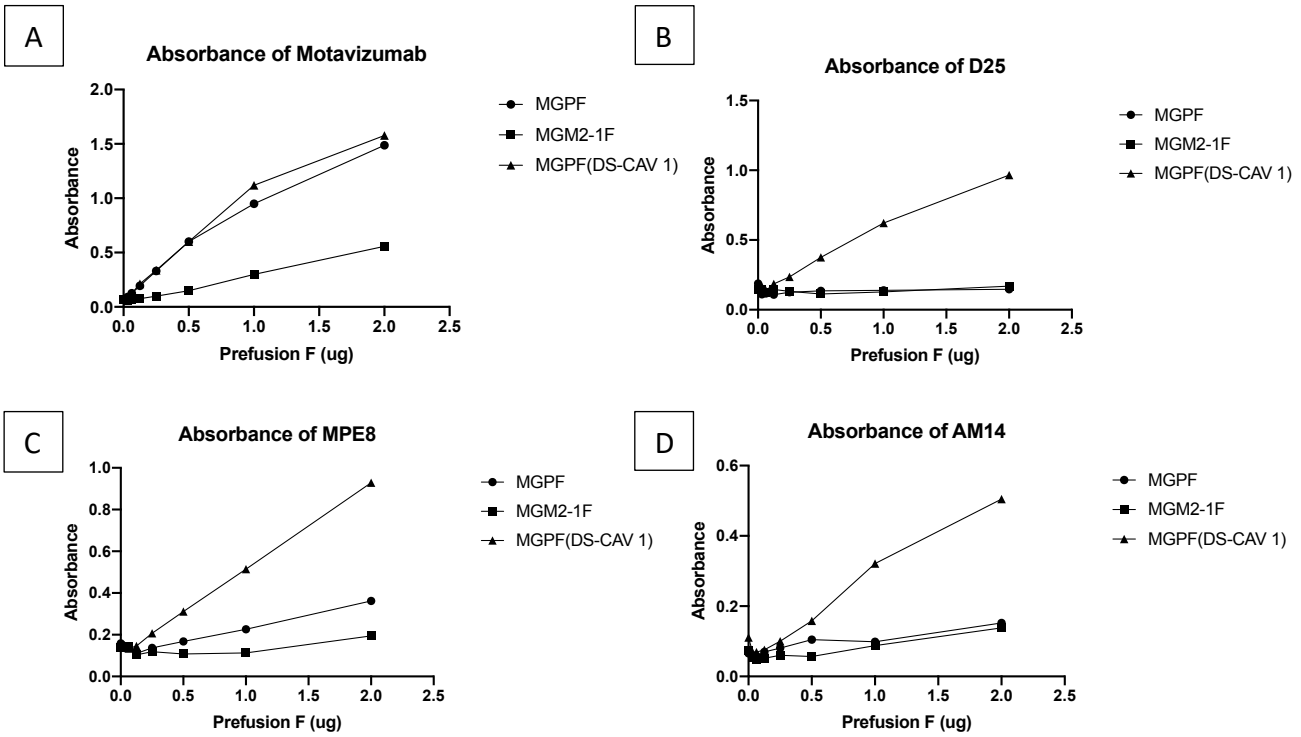

Figure 4. ELISA comparison of M+G+F+M2-1, M+G+P+F, and M+G+P+$F_{DS\text{-}Cav\ 1}$ VLPs to assess recognition of a) site II using anti-F motavizumab b) site ∅ on prefusion F using anti-F D25 c) below site II using anti-F MPE8 d) prefusion F using anti-F AM14

Clearly, the antibody with the highest recognition of F antigen was motavizumab. DS-Cav 1 was most significantly recognized for all antibodies tested. Given the high absorbance of D25 and MPE8 for DS-Cav 1, prefusion F was likely stably expressed in earlier transfected cells when the VLPs were harvested. Although DS- Cav 1 is recognized by AM14 more significantly than MGM2-1F and MGPF, the absorbance values for AM14 are much less than those of the other antibodies tested.

In order to see if any differences existed in density between DS-Cav 1 and wildtype prefusion F, we performed a sucrose density gradient. These blots display density-gradient in which the

"heavier" samples indicate samples with VLPs that were denser. Motavizumab was used to detect the presence of RSV F protein.

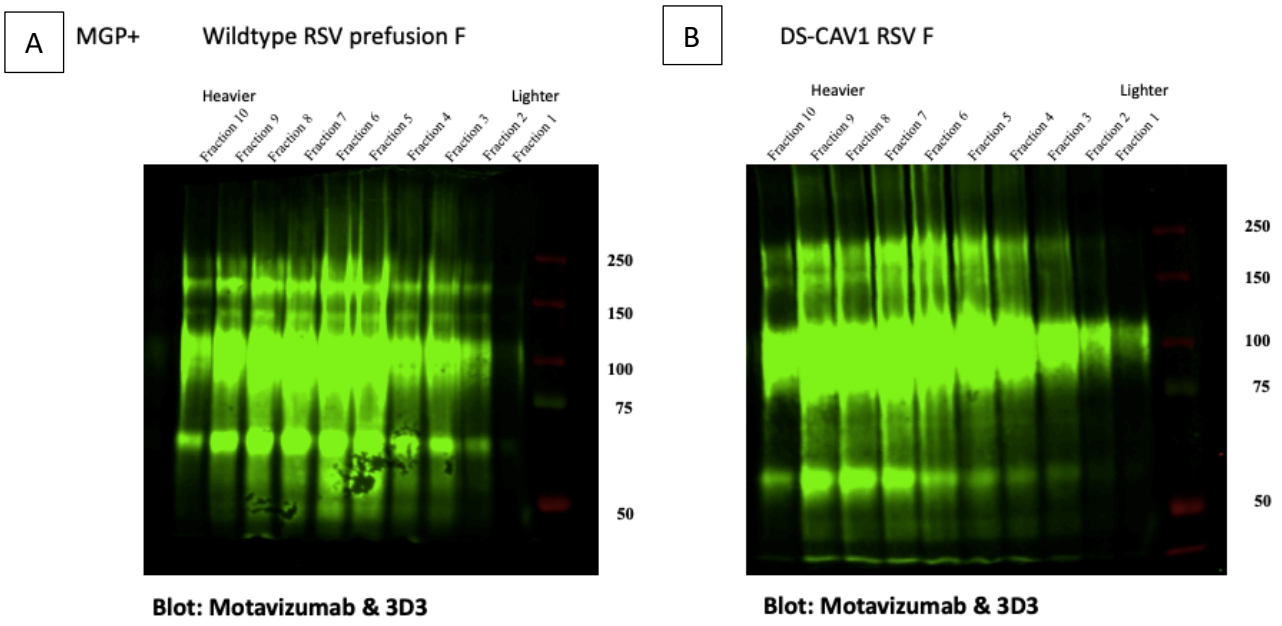

Figure 5. Sucrose gradient comparing density of a) wildtype prefusion F protein and b) DS-Cav 1 prefusion F protein.

Interestingly, wild-type RSV F is found most concentrated in the middle samples, at intermediate density. Whereas, DS-Cav 1 is located in the heavier samples. G protein has been shown to co-precipitate with F protein, thus resulting in similar patterns for detecting F and G in the sucrose gradient fractions (13). The G protein showed similar expression patterns between blots with DS-Cav 1 and wildtype prefusion F despite differences in F expression. Therefore, DS-Cav 1 is denser than wildtype prefusion F protein. G protein was expressed similarly, regardless of prefusion variant.

In order to assess how much of each sample generated is in the prefusion F conformation and compare the quantity present in each fraction, ELISA analysis should be carried out.

We assessed additional prefusion F protein variants that could potentially lead to more information regarding the formation of VLPs and stability of prefusion F protein. ELISA with utilization of different antibodies was carried out to quantify the conformation of the F protein expressed.

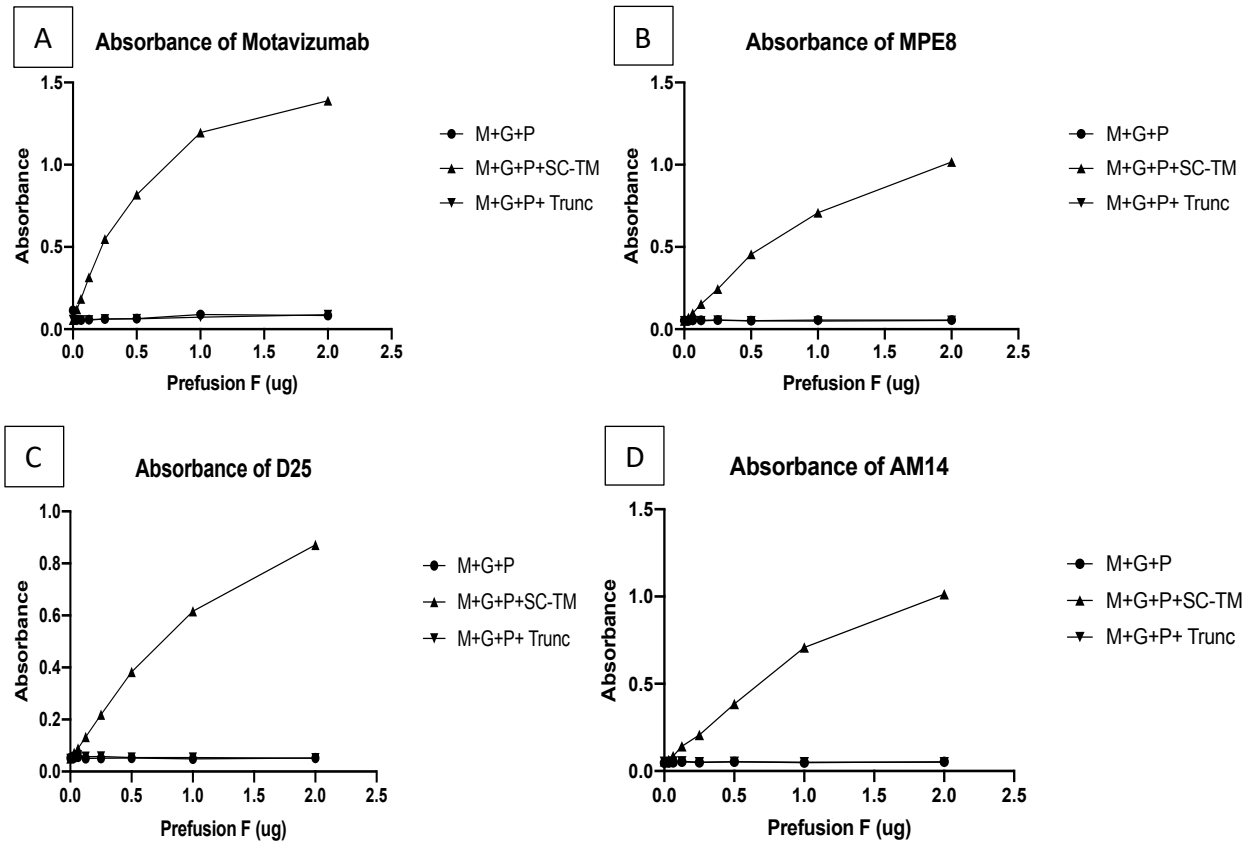

Figure 6. RSV F antigen recognition via ELISA analysis comparing M+G+P, M+G+P+$_{FSC-TM}$, and M+G+P+F_{Trunc} VLPs using a) motavizumab b) MPE8 c) D25 d) AM14

For MPE8, D25, and AM14, absorbance of SC-TM is the highest. SC-TM has similar binding sites as DS-Cav 1, thus explaining the high recognition of SC-TM by D25. MGP and Truncated F variants do not have F protein and thus should only show negligible recognition by all anti-F

antibodies tested. Motavizumab showed the highest recognition of M1-F and SC-TM. SC-TM thus has exposed site II binding sites.

The truncated form of F protein only contains transmembrane and cytoplasmic domains, unlike the other prefusion F protein forms. Additionally, RSV G protein assists in viral attachment to host membrane and thus plays an important role in attaching to primary human airway epithelial for subsequent injection of viral genome into the host cell. Thus, G is likely important in human RSV infection. As we have analyzed G protein recognition with DS-Cav 1 and wildtype F constructs, we used ELISA to detect presence of the G protein in VLPs with constructs of prefusion F protein, i.e. SC-TM and Truncated F.

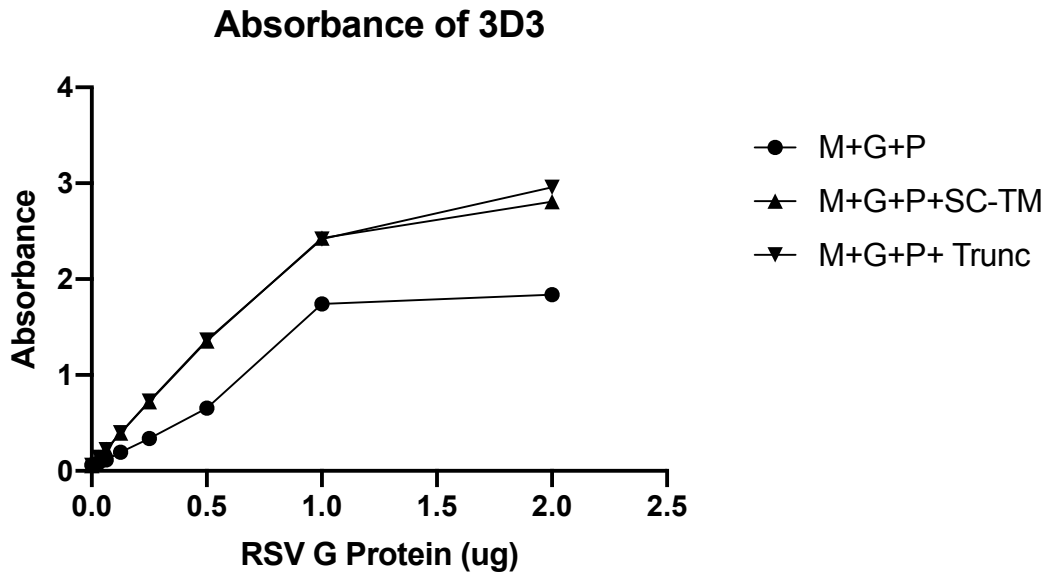

Figure 7. G antigen recognition via ELISA comparing M+G+P, M+G+P+$_{FSC-TM}$, and M+G+P+F_{Trunc} VLPs

G was present with all F protein variants but much less was detected without in MGP VLPs at 2 ug of RSV G protein (Fig 6). This is expected since RSV F protein assists in the incorporation of RSV G protein into VLPs. However, the highest recognition of G protein occurred with

truncated F. This indicates that the portion of F protein that assists in the incorporation of G protein into the VLP occurs using the cytoplasmic or transmembrane domains.

We performed a sucrose gradient on Truncated and SC-TM forms with recognition by anti-F motavizumab and anti-G 3D3 in order to determine whether these variants had similar densities. The truncated form is not recognized with anti-F antibodies, and it was not recognized in the ELISA. SC-TM is recognized by motavizumab.

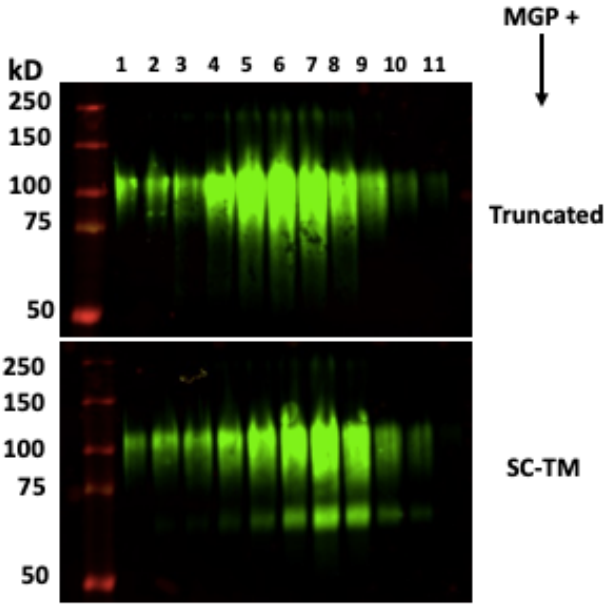

Figure 8. Sucrose gradient comparing density of M+G+P, M+G+P+$_{FSC-TM}$, and M+G+P+F$_{Trunc}$ VLPs expressing G and F proteins

All three express anti-G 3D3 recognition but not in a similar pattern. Truncated form show the highest density in fractions 5-7. However, SC-TM has the highest density of G protein in fractions 7-9. All three of these variants differ from the sucrose gradients for DS-Cav 1 and wildtype prefusion F protein.

As previously stated regarding the sucrose gradient in figure 5A-B, ELISA analysis comparing fractions should be carried out to ensure that each lane contains F protein in the prefusion conformation as recognized by MPE8, D25, and AM14.

Electron microscopy is a technique that enables visual proof of VLP formation and provides information regarding the structure of the VLP. Negative staining shows the VLP with all of its expressed extracellular glycoproteins.

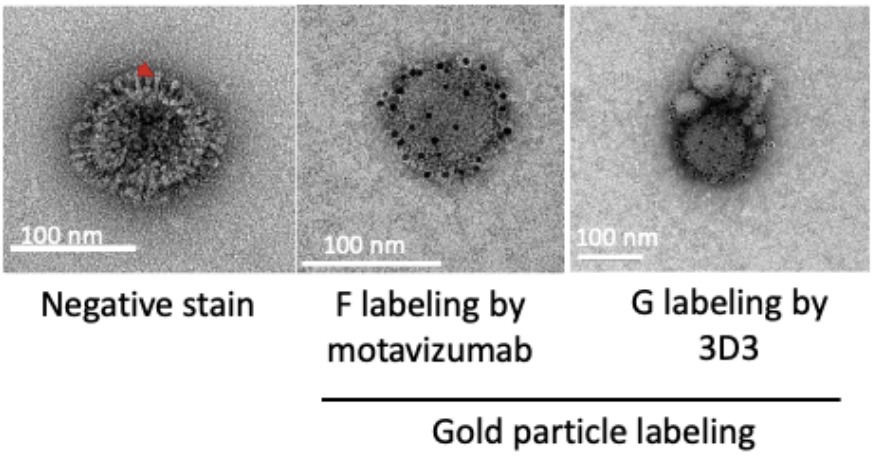

Figure 9. Electron microscopy image of VLPs using gold particle labeling by motavizumab and 3D3

Electron microscopy data of F and G proteins specifically confirms the presence of both VLPs and emphasizes the site II epitope recognized by motavizumab in F protein. The G labeling with 3D3 shows the conserved centrally conserved region between G variants in subtypes A and B.

Discussion

We found F protein to be expressed in transfected 293F cells based on Western blot analysis (fig 1). This result only indicates that F protein is present, not which form of RSV F. As previously stated, RSV F protein converts from prefusion to postfusion forms upon binding. RSV has two furin cleavage sites that flank the gene for p27 and are broken to create a dimer of F1 and F2 polypeptides arranged into a trimer.

G protein is useful to include on the plasmid with F protein, as G protein helps mediate viral attachment to the host cell membrane through interaction with heparan sulfate on proteoglycans (7). Thus, G expression is dependent on F protein. Hence, we used the truncated M+F +P+G VLPs. There was no problem expressing F alone. RSV F+G exhibited substantially more detection of F antigen with motavizumab than RSV F alone (Fig 2). The lack of substantial absorbance values for MPE8 and AM14 could be that the VLPs harvested for ELISA analysis did not stably express prefusion F protein and contained a mixture of prefusion and postfusion F protein. Regardless of conformation of F protein, RSV F + G is more effective in producing F antigen (Fig 2).

The prefusion form is characterized by specific epitopes not available in postfusion forms. Additionally, sites I, II, and IV are associated with neutralizing activity and are located on the postfusion and prefusion forms (7). However, motavizumab, the anti-F antibody used in this analysis, binds to an epitope at site II on F1. This Western blot shows two forms of F protein being detected based on the differing weights (Fig 3A). We hypothesize that RSV F0 was detected in addition to the RSV F1, with the non-cleaved F0 being heavier than RSV F1. F1 contains the site II binding site for motavizumab and is detected by motavizumab upon probing the Western blot.

M protein plus either P or M2-1 was transfected with F and G proteins to ensure VLP production. M plus either P or M2-1 is required for VLP formation (8). We compared using M+G+P+F and M+G+M2-1+F and M+G+P+$F_{DS\text{-}Cav\ 1}$ harvested VLPs (Fig 3A, B). We found that M+G+P+$F_{DS\text{-}Cav\ 1}$ and M+G+P+F seemed to express only the non-cleaved F0 variant whereas M+G+P+M2-1+F seemed to have expressed the F1 variant (Fig 3A). Given that F0 is the non-cleaved polypeptide precursor to F1 and F2, both F1 and F0 contain the motavizumab site II epitope. However, ELISA analysis of these same three samples showed that M+G+P+M2-1+F and M+G+P+F expressed F protein given absorbance values for the recognition of site II for motavizumab (Fig 4A-D). Only M+G+P+$F_{DS\text{-}Cav\ 1}$ seemed to be in the prefusion form since M+G+P+$F_{DS\text{-}Cav\ 1}$ contained high absorbance values for D25, MPE8, and AM14 (Fig 4A-D). Thus, M+G+P+M2-1+F could contain the postfusion F version while M+G+P+$F_{DS\text{-}Cav\ 1}$ expresses a stable version of prefusion F protein. The large mass of M+G+P+$F_{DS\text{-}Cav\ 1}$ coupled with the recognition of site ∅ via D25 and other prefusion-specific sites via MPE8 and AM14 may be due to cleaved and non-cleaved versions of prefusion F being present in the sample. The expression of M+G+P+F F protein at a heavier mass could also be due to both cleaved and non-cleaved versions of F protein being present in the sample. However, recognition of prefusion F epitopes via D25, MPE8, and AM14 in ELISA was threefold less than recognition with M+G+P+$F_{DS\text{-}Cav\ 1}$ and indicates that M+G+P+F did not purely express prefusion F protein (Fig 4A-D). This reinforces the necessity of developing a stably expressed version of prefusion F protein that exhibits site ∅.

A sucrose gradient of M+G+P+F and M+G+P+$F_{DS\text{-}Cav\ 1}$ was performed to compare the relative distributions of G and F between prefusion DS-Cav1 and wildtype F, which mainly contains postfusion (Fig 5). Wildtype F protein is expressed most strongly in fractions 5-8 (Fig

5). However, DS-Cav 1 showed strongest expression in fractions 7-9 (Fig 5). G protein expression was similar between both M+G+P+F and M+G+P+$F_{DS\text{-}Cav\ 1}$ sucrose gradients. Thus, DS-Cav 1 is likely denser than wildtype RSV F protein. This could be because DS-Cav 1 is designed with the hydrophobic cavity filled to reduce repulsion between amino groups, thus making it denser than wildtype F.

We also wanted to investigate the effect of other variants of prefusion F protein in addition to DS-Cav 1. M+G+P and M+G+P+ F_{Trunc} VLPs were used as controls for ELISA analysis (6A-D). The truncated version of F does not express any epitopes that the antibodies tested would bind. SC-TM also exhibits the site ∅ and binds to D25 (Fig 6C). SC-TM prefusion F was also recognized by AM14 and MPE8 and displayed the highest absorbance values for both (fig 6B, D). Motavizumab showed the highest absorbance for SC-TM, a novel prefusion F variant. Because SC-TM also showed absorbance of MPE8, D25, and AM14, SC-TM likely expresses the prefusion F form (Fig 6B, D). We followed up the comparison between prefusion F constructs with ELISA analysis of G antigen recognition via 3D3. We found that the M+G+P+ F_{Trunc} as well as M+G+P VLPs displayed lower absorbance values with increasing quantities of VLPs compared to VLPs with prefusion F construct, reinforcing the importance of utilizing VLPs with prefusion F protein to incorporate G protein in the VLP scaffold (Fig 7).

SC-TM and Truncated forms were compared via sucrose gradient to determine whether these prefusion F variants show differences in density. SC-TM shows greatest density in fractions 8 and 9 when probed by motavizumab. Truncated shows no recognition via motavizumab because of its absence of an ectodomain.

In the future, we would like to include further analysis of the difference between M+G+F+M2-1 VLPs in generating F1 compared to M+G+F+P and M+G+FDS-Cav 1 +P.

Additionally, adding M+G+P+F+M2-1 may increase the recognition of F antigen and increase the density of prefusion F on the VLP surface matrix. Additionally, we would like to compare SC-TM and DS-Cav 1 variants in generating prefusion F protein via ELISA to quantify prefusion F recognition.

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Methods

Cell culture:

frozen 293F cells expressing proteins that form virus-like particles (VLPs) were thawed and cultured at 37C, 8% CO_2 in the presence of blasticidin, zeocin, hygromycin, geneticin, and puromycin antibiotics. Cells were grown to a confluency of 3-4x10^6/ml before they were induced for 72h.

Purification of VLPs:

Induced 293F cells were centrifuged at 1,500 rpm for 15 minutes at 4ºC. The supernatant containing VLPs was collected and filtered through a 0.45 µm filter. 5 ml of 20% sucrose underlaid the supernatant and the tube was centrifuged at 12,000 RPM for 1h at 4ºC (Beckman Coulter Optimal L-90K, SW32 Ti rotor). After ultracentrifugation, the supernatant was removed and a small pellet containing VLPs was at the bottom of the flask. The pellet was resuspended in PBS. Samples were stored at -80ºC.

Detecting presence of VLPs and F protein:

To determine whether samples were effectively transfected and expressed VLPs with RSV F protein, samples of previously purified VLPs with F protein were subjected to Western Blot analysis. In brief, Laemmli sample buffer was added to samples and samples were boiled for 5 minutes at 100ºC. Samples were added to wells of a 8% agarose gel and run at 150 V for about 75 minutes. The gel with the samples were transferred to a nitrocellulose membrane by using 100 V for 90 minutes. VLP samples were probed for GAG (HIV matrix protein), M (RSV matrix protein), VP40 (Ebola matrix protein), and F protein using their respective antibody and Motavizumab (a monoclonal antibody against F protein).

Quantifying F protein concentration in VLPs:

To determine which F isoforms are expressed in VLPs, we used ELISA. ELISA plates were coated with samples at 2 ug/ml and diluted two-fold into each subsequent well until the final

sample concentration was 7.81x10-3 ug. Plates coated with samples were incubated overnight at 4ºC. F antibodies that recognize F protein isoforms (Motavizumab, MPE8, AM14, D25) or G antibody to recognizes G protein (3D3) were added at a concentration of 400 ng/mL (1:5000 dilution). HRP-conjugated secondary goat anti-human antibody was then used with addition of substrates to facilitate color development. A 4M H2SO4 solution was added to stop the reaction and the plate was read at 490nm.

Gel Electrophoresis and Western Blot:

10% Gels were made with Millipore H2O, 30% acrylamide mix, 1.5 M Tris (pH 8.8) (running gel), 10% SDS, 10% APS, TEMED, and 1.0 M Tris (pH 6.8) (stack gel). 2x running buffer was added 1:1 with the volume of the sample, and the sample mixture was heated at 100ºC for 5 minutes. The sample mixture was loaded into the wells and run at 100 V until the samples moved through the stack gel. Once through the stack, the voltage was increased to 150 V until the samples reached the bottom of the gel, about 1 hour. Cells were transferred to a nitrocellulose membrane at 20 V overnight in a cold room at 4ºC. After removing the membrane from the transfer apparatus, the membrane was blocked with 5% non-fat dry milk dissolved in TTBS for 1 hour. Primary antibody was then added to the membrane for 1 hour at room temperature. The membrane was washed three times for five minutes with TBS-tween. Secondary antibody was then added to the membrane for 30 minutes. The membrane was then washed three times again for five minutes with TBS-tween. The membrane was imaged using LI-COR imaging device.

Density Gradient Centrifugation:

VLPs were formed and purified as described above. In addition to purification via centrifugation at 12,000 rpm, the pellet containing the VLPs was resuspended in PBS. The solution was added to a column containing a sucrose gradient (20-60%). VLPs were spun at 8000 xg for 12 hours at

4ºC. after centrifugation, cells were divided into ten fractions (1,000 uL each) and then split evenly into two Eppendorf tubes (500 uL each). VLPs and sucrose were then further diluted with 1:3 1x PBS and spun at 12,000 rpm for 1 hour at 4ºC. The supernatant was discarded, and VLPs were resuspended in PBS. Analysis was carried out via gel electrophoresis and Western blot imaging as described above.

Electron Microscopy:

Images were contributed by Jae Yang (University of Wisconsin-Madison, Elizabeth Wright lab).