ENGINEERING VIRAL PROTEIN ANTIGENS THAT DIRECT THE HUMORAL IMMUNE RESPONSE TO CONSERVED EPITOPES

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The Academic Faculty

by

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ENGINEERING VIRAL PROTEIN ANTIGENS THAT DIRECT THE HUMORAL IMMUNE RESPONSE TO CONSERVED EPITOPES

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Dedicated to my parents.
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Figure 24 Characterization of S2mutS2’ and VLP-S2mutS2’. a) SDS-PAGE characterization of S2mutS2’ and VLP-S2mutS2’. S2mutS2’ was
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Figure 25 Protective efficacy of VLP-S2 and VLP-S2mutS2′. a) Schedule for hamster vaccination, serum collection, infection with SARS-CoV-2, and organ collection. b) Antibody endpoint titers of sera from hamsters immunized with either VLP-S2, VLP-S2mutS2′, or MS2-SA VLP against SARS-CoV-2 spike protein (geometric mean with geometric SD, n=6: two independent assays with sera from 3 hamsters). ns: not statistically significant, ****p < 0.0001, determined by a one-way analysis of variance (ANOVA) and Tukey post-hoc multiple comparison between groups (α = 0.05). c) Viral titer in the lungs of hamsters immunized with either VLP-S2, VLP-S2mutS2′, or MS2-SA VLP three days after infection with SARS-CoV-2 (geometric mean with geometric SD, n=3 hamsters). **p < 0.01, determined by a one-way analysis of variance (ANOVA) and Dunnett post-hoc multiple comparison between groups (α = 0.05). d) Viral titer in the nasal turbinates of hamsters immunized with either MS2-SA VLP, VLP-S2, or VLP-S2mutS2′ three days after SARS-CoV-2 infection (geometric mean with geometric SD, n=3 hamsters). **p < 0.01, determined by a one-way analysis of variance (ANOVA) and Dunnett post-hoc multiple comparison between groups (α = 0.05). e) Antibody endpoint titers of sera from hamsters immunized with either VLP-S2 (gray), VLP-S2mutS2′ (white), or MS2-SA VLP against spike proteins of the original Wuhan-Hu-1 SARS-CoV-2 (614D), the SARS-CoV-2 variant B.1.351, SARS-CoV-1, and the four endemic human coronaviruses HKU-1, OC43, NL63, and 229E (geometric mean with geometric SD, n=6 against SARS-CoV-2 614D S protein: two independent assays with sera from 3 hamsters; n=3 against all other S proteins: sera from 3 hamsters). ns: not statistically
significant, ****p < 0.0001, determined by a one-way analysis of variance (ANOVA) and Tukey post-hoc multiple comparison between groups (α = 0.05).
SUMMARY

Vaccines are considered to be one of the most cost-effective health interventions for infectious disease. A challenge in the development of an effective vaccine is the creation of an immunogen that elicits a broad immune response. This challenge stems from the variability of viral proteins across strains; when the immune system targets a variable region of a viral protein, the result is often an undesirable strain-specific response. Therefore, it may be beneficial to direct the immune response to conserved antigenic sites, which will likely result in a broad response that is protective against many different strains. In this dissertation, we have engineered antigens that direct the immune response to conserved areas of viral proteins. The immune responses to these engineered immunogens have provided us with significant insights that may have application in the design of broadly protective vaccines. We have found that a highly immunogenic yet variable antigenic site on the respiratory syncytial virus (RSV) fusion protein is not required to elicit a response that neutralizes across RSV subtypes, that the orientation of the influenza hemagglutinin protein on virus-like particles influences the elicitation of antibodies targeting the conserved hemagglutinin stalk domain, and that the multivalent display of the SARS-CoV-2 spike protein and its conserved S2 subunit is protective against SARS-CoV-2. Ultimately, these results may inform the design of broadly protective vaccines.
CHAPTER 1. INTRODUCTION

Vaccines are estimated to prevent 4 to 5 million deaths every year [1]. Vaccines prevent disease by training the immune system to recognize a pathogen such that it can prepare a defense against that pathogen. After a vaccine has been administered, the antigen that makes up the vaccine may eventually be bound by the receptor of a B cell [2]. Each B cell presents multiple copies of the same receptor on its surface. Once a B cell has bound to an antigen via these receptors, it may become activated. An activated B-cell may then eventually differentiate into a plasma cell or a memory B cell [2]. Plasma cells secrete antibodies that have the same specificity as the parent B cell. Memory B cells generally do not secrete antibodies. However, memory B cells do experience class switching and affinity maturation. Therefore, when memory B cells generated by vaccination are reactivated by infection, they have the potential to differentiate into plasma cells and generate a rapid and potent antibody-based response. Antibodies are soluble proteins that bind to antigens and provide protection through several different mechanisms [2]. One way in which an antibody can provide protection is through the neutralization of a pathogen’s function. For example, if a virus uses its surface protein to bind and enter host cells, an antibody can bind to that viral surface protein such that the viral surface protein is sterically inhibited from binding and therefore entering host cells. Another mechanism by which antibodies provide protection is opsonization. Opsonization occurs when cells of the immune system recognize the Fc domain of an antibody bound to an antigen. Often, these immune cells will be phagocytes that engulf and destroy the pathogen intracellularly. Other immune cells recognizing the Fc domain of an antibody may release lytic molecules
to destroy the pathogen. Finally, antibodies can activate the complement system. The activation of the complement system triggers a cascade of protein interactions that ultimately results in the destruction of the pathogen through lysis or complement-mediated opsonization. Collectively, these protective mechanisms associated with antibodies secreted from activated B cells constitute the humoral immune response [2]. While other protective mechanisms exist, this work will primarily focus on tuning the humoral immune response through the engineering of viral antigens.

There are different types of vaccines [3], and these types of vaccines continue to grow with advances in biotechnology. The earliest modern example of vaccination is generally considered to be the work of Edward Jenner in 1796 [4]. Jenner correctly reasoned that immunization with antigen derived from human sores containing cowpox virus would result in only mild symptoms while providing protection against the similar virus smallpox. This type of vaccine is a rudimentary example of a live attenuated virus vaccine. Live attenuated virus vaccines are made of the infectious virus they are intended to protect against, but the virus is weakened such that it does not cause serious illness. An advantage of live attenuated vaccines is that they emulate natural infection, so the immune response against the vaccine is typically effective. However, because these vaccines are made up of live virus there is the potential for pathogenic activity. Inactivated virus vaccines solve this problem, as the virus is not capable of replication after inactivation. However, a drawback of both live attenuated virus vaccines and inactivated virus vaccines is that they elicit an immune response to the entire virus. This can result in antibodies that are non-neutralizing. In fact, non-neutralizing antibodies may even result in enhanced disease [5]. For that reason, it may be beneficial to target a portion of the virus that elicits
potently neutralizing antibodies – this can be done with a subunit vaccine. A subunit vaccine contains a viral protein or proteins that elicit an effective immune response against the virus. Subunit vaccines can be produced by separating the molecules that make up the virus and then purifying the molecules of interest. Alternatively, these proteins can be recombinantly expressed by transfecting cells with the genetic code for the desired protein. One significant advantage of recombinant expression of subunit vaccines is that the genetic code can be altered to change and improve the immunogenicity of the antigen. In this manner, viral protein antigens can be engineered to generate broadly protective immune responses.

Eliciting a broadly protective immune response is a common challenge in the development of a vaccine, as viral proteins tend to vary from strain to strain of a virus. If a vaccine elicits an immune response that targets a variable region of a viral protein, the response will likely be strain specific. On the other hand, if the immune system targets a conserved region of a viral protein – a region that remains the same from strain to strain of a virus – the immune response will likely be protective against many different strains of the virus. Unfortunately, variable epitopes are often the favored targets of the immune system and are therefore said to be immunodominant. B cells specific to immunodominant epitopes – variable or not – are preferentially selected for activation, proliferation, and differentiation for reasons including B cell precursor frequency, epitope affinity, epitope valency, epitope accessibility, and strength of T cell help [6]. Therefore, to generate a broadly neutralizing antibody response it may be necessary to direct the immune response away from immunodominant variable regions of viral proteins and focus the response towards the more conserved regions.
Protein engineering strategies have been developed to direct immune response to conserved regions of viral antigens. For example, chimeric [7-9] and mosaic [10, 11] antigens have been created where variable regions change from antigen to antigen while the conserved region remains the same. These antigens are used for immunization in succession such that the response to the constant conserved area is boosted after each immunization, while the response to the varying variable regions remain relatively low. These approaches are based on original antigenic sin [12] – the observation that the immune system uses immunological memory to preferentially target antigenic sites that are shared with strains it has previously encountered in earlier infections. While this strategy has proven successful even in a phase 1 clinical trial [7], it requires multiple immunizations. Other methods that direct the immune response to conserved areas of viral proteins do not necessarily require multiple immunizations. For example, immune response can be directed towards conserved regions of viral proteins by using poorly immunogenic molecules such as glycans [13, 14] or PEG [15-17] to sterically inhibit B cell receptors from accessing variable epitopes. This method ideally ensures that B cells specific to the conserved regions of the antigen do not have to compete with B cells specific to the variable regions of the antigen. Consequently, the resulting immune response should strongly target the conserved regions of the antigen. Resurfacing is a similar method in which variable epitopes are substituted with residues that are less likely to interact with B cell receptors [18]. Unfortunately, both resurfaced epitopes and shielding molecules may themselves invoke an undesired immune response. In this case, it may be beneficial to completely remove certain variable regions of an antigen [19-24]. This strategy may involve a considerable amount of protein engineering to ensure that the truncated antigen
retains the conformation and immunogenicity it possesses in its native state. Similarly, individual conserved epitopes can be targeted through peptide-based vaccines [25, 26]. However, peptide-based vaccines are generally limited to the display of linear epitopes, which may not retain their native conformations [27]. To overcome these limitations, computational protein design strategies have been used to develop de novo proteins that mimic a single conformational epitope of a viral protein [28-30]. In this dissertation, we have similarly sought to direct the immune response to conserved areas of viral proteins.

The viral protein antigens we describe in this work are viral fusion proteins, which serve to fuse the viral membrane to a host cell membrane. This fusion of the viral membrane and host cell membrane allows viral genetic material to enter the host cell’s cytoplasm such that more copies of the virus can be made [31]. This vital role that the fusion proteins play in infection makes them an effective vaccine target, as antibodies that bind to the fusion machinery can inhibit fusion and ultimately prevent infection [32, 33]. The important function of the fusion protein also signifies that the fusion machinery within the protein is largely resistant to mutation, as mutations to the fusion machinery may prevent the fusion protein from effectively performing its function [34-36]. Therefore, the region of a fusion protein that contains the fusion machinery is often highly conserved and may serve as an effective target for broadly protective vaccines.

In this work, we have sought to develop broadly protective immunogens by directing the immune response to conserved areas of viral fusion proteins. We discuss our evaluation of a respiratory syncytial virus fusion (F) protein-based immunogen that uses glycans to shield the most variable region of the protein - site Ø. We found that this antigen with a shielded site Ø can generate an immune response to the more conserved parts of the
F protein, which neutralizes RSV of both subtypes. We also investigated how the orientation of the influenza hemagglutinin protein on virus-like particles affects the immune response against hemagglutinin’s conserved stalk domain. Our results suggest that inverting the orientation of hemagglutinin relative to how it appears naturally on the virus better elicits antibodies targeting the conserved stalk of hemagglutinin. Finally, we found that either the SARS-CoV-2 spike protein or its conserved S2 subunit displayed multivalently on virus-like particles can protect hamsters against SARS-CoV-2 infection. Ultimately, these results may inform the design of broadly protective vaccines.
CHAPTER 2. AN EVALUATION OF A RESPIRATORY SYNCYTIAL VIRUS FUSION PROTEIN-BASED IMMUNOGEN THAT SHIELDS THE VARIABLE ANTIGENIC SITE Ø

2.1 Introduction

Respiratory syncytial virus (RSV) is a respiratory pathogen that can cause severe disease in the young and the elderly. In fact, RSV is thought to be connected with 6.7 percent of the deaths of children aged 1 month to 1 year [37], and it infects nearly every child by 2 years of age [38]. Among the elderly, RSV creates a disease burden comparable to that of non-pandemic influenza A virus [39]. Despite this significant health impact, there is currently no licensed RSV vaccine. The RSV Fusion (F) protein is considered to be a promising vaccine target because it displays neutralizing epitopes and is generally conserved [40]. While the F protein is generally conserved, the most variable region of the protein has also been shown to elicit potent, neutralizing antibodies [41, 42]. The following study examines whether this variable site is necessary to generate an immune response that protects across RSV subtypes, which could inform the design of an effective and broadly protective RSV vaccine.

As the RSV fusion protein is a potential target for an RSV vaccine, its structure and immunogenicity has been studied extensively. For example, while the F protein exists in either a prefusion or postfusion conformation, researchers have found that the majority of

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RSV neutralizing activity in human sera is directed at the prefusion conformation [42]. It is interesting to note that the prefusion F protein displays several dominant, highly immunogenic sites - including site Ø [43]. Site Ø is located at the apex of the prefusion F (pre-F) protein. While the impact of site Ø-specific antibodies varies between individuals, antibodies specific to site Ø have been found to account for up to 22% of F-specific antibodies in human sera [43]. Moreover, a study by Ngwuta et al. found that site Ø-specific antibodies account for approximately 35% of RSV neutralizing activity in human sera [42]. These site Ø-specific antibodies can also be incredibly potent; some are 10 – 100 times more potent than the licensed monoclonal antibody Palivizumab, which is used for passive immunoprophylaxis to prevent RSV [41]. These insights that suggest our immune system naturally targets site Ø with potent, neutralizing antibodies have led researchers to conclude that inducing or boosting neutralizing activity by vaccination will be facilitated by using pre-F antigens that preserve site Ø [42].

However, it is important to consider that site Ø is the least conserved region on the F protein. One can inspect the sequence conservation of the RSV F ectodomain between the two subtypes of RSV (A and B) to find that only 77% of residues within site Ø are conserved whereas 96% of residues outside of site Ø are conserved [41]. This conserved region outside of site Ø has been found to elicit neutralizing antibodies to a variety of antigenic sites [44-46]. For example, the licensed monoclonal antibody, Palivizumab, binds to a highly conserved region on the RSV F protein outside of site Ø and has been shown to neutralize 57 strains of RSV from both the A and B subtypes [47]. Therefore, we hypothesize that directing the immune response away from site Ø and refocusing it towards the more conserved antigenic sites of the RSV F protein will serve to better elicit broadly
neutralizing antibodies. Here, as a first step, we have generated a stabilized prefusion RSV F protein variant with inserted glycans that shield site Ø and have demonstrated that sera from mice immunized with this protein can neutralize RSV across subtypes. This result may ultimately help to inform the design of a broadly protective RSV vaccine.

2.2 Methods

2.2.1 Expression and purification of RSV F

DNA encoding the prefusion stabilized SC-TM variant of the RSV F ectodomain, as reported by Krarup et al. [48], with a C-terminal T4 fibrin trimerization motif, SpyTag, and Strep-Tag II was cloned into pcDNA3.1 between the NcoI and XhoI restriction sites. Mutations analogous to those described in the work of Ngwuta et al. were made using a Q5 Site-Directed Mutagenesis kit (New England BioLabs) to create the RSV F ØKO DNA construct [42]. Specifically, these mutations include K65N, I67T, P205N, V207T, K209N, and S211T, and they result in the introduction of three N-glycan sequons per RSV F protomer. This DNA was transfected into Expi 293F cells using the ExpiFectamine Transfection Kit and protocol (Thermo Fisher Scientific). Five days after transfection, the cells were centrifuged at 5500g for 20 minutes, and the secreted RSV F in the supernatant media was purified using a StrepTrap HP 5 ml column (GE Healthcare) according to the manufacturer’s instructions. The RSV F proteins were further purified using SEC with a Superdex 200 Increase 10/300 column (GE Healthcare) to separate RSV F trimers from aggregates.

2.2.2 Expression and purification of antibodies
The variable regions of the heavy and light chains of D25, AM22, Motavizumab, MPE8 and 101F were cloned into TGEX-HC and TGEX-LC vectors (Antibody Design Labs), respectively, according to manufacturer’s instructions. This DNA was transfected into Expi 293F cells using the ExpiFectamine Transfection Kit and protocol (Thermo Fisher Scientific). Five days after transfection, the cells were centrifuged at 5500g for 20 minutes, and the secreted antibody in the supernatant media was purified using a 1 mL MabSelect SuRe column (GE Healthcare) per the manufacturer’s protocol. The purified antibody was buffer exchanged into 1x phosphate-buffered saline (PBS).

2.2.3 Synthesis of multivalent RSV F conjugates

The branched PEG scaffold was synthesized as previously described [15]. Briefly, a SpyCatcher containing an amber stop codon at residue 56 for incorporation of p-azido-L-phenylalanine was expressed in BL21(DE3) competent Escherichia coli (New England BioLabs). The cells were lysed and the SpyCatcher was purified using HisPur Ni-NTA resin (Thermo Scientific) followed by purification with a StrepTrap column (GE Healthcare). SpyCatcher was reacted in excess with a 4-arm-5kDa PEG-DBCO (Creative PEGworks) in the dark at 4°C overnight. Scaffolds functionalized with SpyCatchers were obtained by purification with a HiLoad Superdex 200 column. Excess RSV F containing a C-terminal SpyTag was reacted with the resulting scaffold at 4°C overnight and then run through a HiLoad Superdex 200 column to obtain purified multivalent RSV F conjugates.

2.2.4 SDS-PAGE

Protein samples were diluted in a 3:1 volume ratio in NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen). For reduced samples, β-mercaptoethanol was
diluted 10-fold and 1 µL of this diluted solution was added to the sample-LDS buffer mixture. PageRuler Plus Prestained Protein Ladder (Thermo Scientific) and protein samples were loaded onto a 4-12% Bis-Tris gel (Invitrogen), which was run in MES-SDS buffer at room temperature for 60 minutes at 120 volts. The gel was stained with SimplyBlue SafeStain (Invitrogen) and de-stained overnight. The gel was imaged using a ChemiDoc MP imaging system (Bio-Rad).

2.2.5 Dose-response ELISA

RSV F protein in PBS (0.1 µg per well) was coated on Nunc Maxisorp 96 well plates (Invitrogen) for 1 hour at room temperature. Plates were blocked with 5% bovine serum albumin (BSA)(EMD Millipore) in PBST (PBS with 0.05% Tween-20) for 45 minutes at room temperature. Plates were washed twice with PBST. Each antibody was diluted to a concentration of 1.4 x 10^{-7} M with 1% BSA in PBST, and 125 µl of the diluted antibody was added to the first well in a row. From this first well, 25 µL was then taken to make 5-fold serial dilutions down the remaining wells of the row such that each well was left with 100 µL of diluted sera. After incubation for an hour, plates were washed twice with PBST. HRP-conjugated goat anti-human IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) in 1% BSA in PBST was added to the wells. The plates were left to incubate at room temperature for an hour and then washed twice with PBST. Plates were developed with TMB substrate solution (Thermo Scientific) for 3 minutes, and then stop solution (0.16 M sulfuric acid) was added to the wells. Absorbance at 450 nm was read using a Spectramax i3x plate reader (Molecular Devices).

2.2.6 ELISA
RSV F protein in PBS (0.1 µg per well) was coated on Nunc Maxisorp 96 well plates (Invitrogen) for 1 hour at room temperature. Plates were blocked with 5% bovine serum albumin (BSA; EMD Millipore) in PBST (PBS with 0.05% Tween-20) for 45 minutes at room temperature. Plates were washed twice with PBST, and anti-RSV F antibody in 1% BSA in PBST was added to the appropriate wells. After incubation at room temperature for an hour, plates were washed twice with PBST. Subsequently, HRP-conjugated goat anti-human IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) in 1% BSA in PBST was added to the wells. The plates were left to incubate at room temperature for an hour and then washed twice with PBST. Plates were developed with TMB substrate solution (Thermo Scientific) for 5 minutes and stop solution (0.16 M sulfuric acid) was added to the wells. Absorbance at 450 nm was read using a Spectramax i3x plate reader (Molecular Devices).

2.2.7 Immunization

RSV F antigen solution for each mouse was prepared with 2.5 µg of RSVF in 50 µl of PBS. The amount of scaffold contained in this solution was added without RSV F to 50 µL of PBS for each mouse in the control group. These antigen solutions were mixed 1:1 v/v with AddaVax (InVivoGen) immediately before immunization. The 100 µl antigen/adjuvant solution was injected subcutaneously into 6-8 week old BALB/c mice (n=5 per group, 3 groups). Each mouse was bled and boosted with 100 µl of the same solution on days 14 and 35. The mice were terminally bled on day 42. These immunizations and bleeds were carried out by ProSci Incorporated (Poway, CA) within their USDA licensed, registered and NIH/OLAW assured animal facility. All the protocols that included experimental animal procedures were carried out in accordance with the US
Animal Welfare Act and approved by ProSci Incorporated’s Institutional Animal Care and Use Committee.

2.2.8 Antibody titer ELISA

RSV F protein in PBS (0.1 µg per well) was coated on Nunc Maxisorp 96 well plates (Invitrogen) for 1 hour at room temperature. Plates were blocked with 5% bovine serum albumin (BSA; EMD Millipore) in PBST (PBS with 0.05% Tween-20) for 45 minutes at room temperature. Plates were washed twice with PBST. Sera from a given mouse was diluted 1:1000 in 1% BSA in PBST, and 150 µl of the diluted sera was added to the first well in a row. From this first well, 50 µL was then taken to make 3-fold serial dilutions down the remaining wells of the row such that each well was left with 100 µL of diluted sera. After incubation for an hour, plates were washed twice with PBST. HRP-conjugated goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) in 1% BSA in PBST was added to the wells. The plates were left to incubate at room temperature for an hour and then washed twice with PBST. Plates were developed with TMB substrate solution (Thermo Scientific) for 5 minutes, and then stop solution (0.16 M sulfuric acid) was added to the wells. Absorbance at 450 nm was read using a Spectramax i3x plate reader (Molecular Devices). Titers were expressed as area under the curve (AUC). To express antibody titers as an AUC, a curve of absorbance values that represent antibody binding against log-transformed inverse sera dilutions was plotted. The curve’s baseline is defined as the mean absorbance resulting from the secondary, HRP-conjugated, anti-mouse antibody against BSA-blocked antigen plus three standard deviations. The area under the curve is the value that is reported as the titer AUC.
2.2.9 Microneutralization assay

The microneutralization assay was performed as previously described [49]. In brief, serum samples were heat inactivated (56 °C, 30 mins) and two-fold serially diluted starting at a dilution of 1:16. These dilutions were mixed with 50 fluorescent focus units (FFU) of mKate2-expressing virus for 1 hour at 37 °C, and the mixtures were subsequently spinoculated onto HEp-2 monolayers in 96 well plates at 2,900g for 30 mins at 4 °C. The monolayer was then overlaid with 0.75% methylcellulose in complete MEM. After 2 days of incubation at 37 °C, the FFUs in each well were counted, and EC$_{50}$ values were calculated as described [49].

2.2.10 Immunodepletion

High-capacity streptavidin agarose resin (Thermo Scientific) was washed twice with PBS. The resin was then incubated with RSV F ØKO containing a C-terminus Strep-Tag II such that streptavidin is in 4 times molar excess of the RSV F ØKO trimer. This mixture was incubated for 2 hours at 4°C. The resin displaying RSV F ØKO was washed 3 times in PBS and put into 1% BSA in PBST. This resin was then added to half of a serum sample that has been diluted in 1% BSA, such that there was 25 µg of RSV F ØKO for every µl of sera. The other half of the sera solution was mixed with an equal amount of streptavidin agarose resin that does not display RSV F ØKO – this half of the sera was considered not depleted. The sera solutions were incubated with the resin for at least 2 hours at 4°C. Afterwards, the mixture was centrifuged such that the depleted sera can be removed from the pelleted resin. This depletion procedure was repeated eight times before the sera is considered depleted.
2.2.11 Statistical analysis

All values were presented as mean ± SD. For Figure 4b, inverse sera dilutions were log-transformed and significance was determined using a Mann-Whitney test ($\alpha = 0.05$; $n = 5$). Significance was determined using a two-way analysis of variance (ANOVA) and Tukey’s post-hoc multiple comparison between groups ($\alpha = 0.05$; $n = 6$) for Figure 4c, and multiple Holm-Sidak corrected ($\alpha = 0.05$; $n = 3$) t-tests for Figure 4d. Statistical significance was indicated as *$p < 0.05$. Assumptions of normally distributed data were validated by the Shapiro-Wilk test. Assumptions of homogeneity of variance among groups were validated by Levene’s test. All statistical analysis was carried out using Prism 8 (GraphPad).

2.3 Results and Discussion

2.3.1 Design of a prefusion RSV F variant that shields site $\phi$

To demonstrate that prefusion F-based vaccines with a shielded site $\phi$ can be broadly neutralizing, we first needed a prefusion F protein that inhibits the binding of B-cell receptors to site $\phi$. To develop this modified F protein, we drew inspiration from a construct created by Ngwuta et al. \[42\]. These researchers mutated a stabilized prefusion F protein to introduce glycans that prevent antibody binding to site $\phi$. They used the resulting protein as a reagent to characterize the human immune response against RSV F. In contrast to this study, we propose the use of a similar prefusion F-based protein that incorporates glycans to shield site $\phi$ as an immunogen. This immunogen is intended to direct the immune response away from site $\phi$ and towards the more conserved sites of RSV F to elicit broadly neutralizing antibodies. Accordingly, we have mutated a different
stabilized prefusion F protein variant, reported by Krarup et al. [48], to introduce three glycans that shield site Ø per RSV F protomer. The locations of the mutations made to create the three N-glycan sequons were reported by Ngwuta et al. and include K65N, I67T, P205N, V207T, K209N, and S211T [42]. The resulting protein will be referred to as the Ø knock-out (ØKO) mutant.

2.3.2 Generation and in vitro characterization of RSV F WT and ØKO proteins

We created plasmids that would allow for the expression of wild type (WT) and ØKO versions of a stabilized prefusion RSV F protein derived from the RSV A2 strain, which is of the A subtype. These plasmids incorporated a C-terminal Strep-Tag II to facilitate purification by StrepTrap chromatography and a SpyTag to allow for conjugation to scaffolds. The WT and ØKO proteins were expressed in HEK 293F cells and purified using StrepTrap chromatography and size-exclusion chromatography in succession. A representative size-exclusion chromatography trace resulting from the purification of WT RSV F is shown in Figure 1a. The purified WT and ØKO proteins were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Figure 1b). Intense bands appear on the stained gel at about 65 kDa, which correspond to the approximate molecular weight of monomeric RSV F. As expected, the ØKO RSV F runs slightly higher than the WT. This shift is consistent with the addition of glycans incorporated to shield site Ø (Figure 1b). To ensure that the proteins had folded correctly, we generated ELISA dose-response curves by probing the binding of various antibodies in varying doses to different RSV F antigenic sites. Antibodies used in these ELISAs include two site Ø-directed antibodies D25 and AM22, as well as antibodies Motavizumab, MPE8 and 101F that bind to antigenic sites II, III and IV, respectively (Figure 1c) [41, 44-46].
As expected, all antibodies except for D25 and AM22 generate dose-response curves that indicate there is less than a 10 percent difference in the half maximal binding concentrations against RSV F WT and RSV F ØKO (Figure 1d). Since D25 and AM22 are antibodies specific to site Ø, their binding to the ØKO protein was inhibited while their binding to the WT protein was retained.

Figure 1. Characterization of recombinant RSV F proteins. a) Size exclusion chromatography trace resulting from purification of RSV F WT. b) SDS-
polyacrylamide gel electrophoresis (SDS-PAGE) characterization of RSV F proteins that have been reduced using β-mercaptoethanol. c) Binding sites of anti-RSV F antibodies used in ELISA. Antibodies D25 and AM22 bind to site Ø (blue), while Motavizumab, MPE8, and 101F bind to Site II (orange), Site III (purple), and site IV (red), respectively. [50] (PDB: 4JHW). d) ELISA dose-response curves to characterize the binding of RSV F-specific antibodies to RSV F WT (circles) and RSV F ØKO (squares) (mean ± SD, n = 6).

2.3.3 Multivalent display of RSV F WT and ØKO proteins

As multivalent display has been shown to enhance the immunogenicity of antigens [51], we next created a scaffold to allow the WT and ØKO proteins to be displayed multivalently. To generate this scaffold, we used a strain-promoted azide-alkyne cycloaddition click reaction to conjugate SpyCatcher proteins containing the unnatural amino acid \( p \)-azido-L-phenylalanine to a 4-arm-polyethylene glycol (PEG) molecule functionalized with dibenzocyclooctyne (DBCO) at the terminus of each arm (Figure 2a) [52]. SpyCatcher is a 12.3 kDa protein that forms an isopeptide bond with a 13 amino acid peptide known as SpyTag (Figure 2b) [53]. A SpyTag was incorporated at the C-terminus of the WT and ØKO RSV F proteins such that the RSV F proteins could be reacted with the branched PEG scaffold. This reaction results in multivalent RSV F conjugates (Figure 2c). The products of the reaction of RSV F with the branched PEG scaffold, after purification, were analyzed using SDS-PAGE (Figure 3a). The stained gel showed two high molecular weight bands representative of 3 or 4 RSV F protomers attached to the branched PEG scaffold. A third band appears at approximately 65 kDa, which corresponds to RSV F monomers that had dissociated from the RSV F protomers attached to the scaffold in the presence of SDS. To ensure that the structure of the RSV F proteins was maintained after reaction with scaffold, an ELISA was conducted to probe the binding of antibodies to different antigenic sites of the multivalent RSV F (Figure 3b). As expected, both the WT
and ØKO RSV F on scaffold generally behaved as they had before being conjugated to the scaffold. The binding of site Ø-directed antibodies D25 and AM22 was inhibited against scaffolds functionalized with the ØKO protein but retained to scaffolds functionalized with the WT protein. The other antibodies that bind outside of site Ø showed comparable binding to the WT and ØKO protein conjugates.

Figure 2. Synthesis of multivalent RSV F conjugates. a) Click reaction of SpyCatcher (gray) containing the unnatural amino acid p-azido-L-phenylalanine.
(pAzF) with 4-arm-PEG-DBCO (yellow). (PDB: 4MLI). b) Reaction of SpyCatcher (gray) and SpyTag (orange) through the formation of an isopeptide bond between the residues highlighted in cyan. (PDB: 4MLI). c) Formation of multivalent RSV F conjugates by the reaction of RSV F proteins (beige) incorporating a SpyTag (orange) at their C-terminus with a SpyCatcher-functionalized branched PEG scaffold. (PDB: 4JHW).

Figure 3. Characterization of multivalent RSV F conjugates. a) Characterization of the purified SpyCatcher-functionalized branched PEG scaffold and a multivalent RSV F ØKO conjugate by SDS-PAGE. b) Characterization of the binding of RSV F-specific antibodies to multivalent RSV F WT (black) and multivalent RSV F ØKO (white) by ELISA (mean ± SD, n = 3).

2.3.4 Characterization of immune response to multivalent RSV F WT and ØKO

After successfully characterizing the multivalent WT and ØKO proteins, we next sought to understand the difference in immune response elicited by WT RSV F and RSV F with a shielded site Ø (ØKO). Mice (n=5 per group; 3 groups) were immunized with either multivalent WT RSV F, multivalent ØKO RSV F, or scaffold alone. The mice were boosted twice and bled on day 42 (Figure 4a). Characterization of the resulting sera showed
that the multivalent WT and ØKO constructs generated comparable antibody titers against WT RSV F (Figure 4b). These antibody titers are expressed as the area under the curve (AUC). The neutralization potency of the sera was also tested against RSV strains of both the A and B subtype. The neutralization potencies of sera generated by the multivalent WT and multivalent ØKO constructs were comparable across subtypes (Figure 4c). Figure 4c shows that there is no statistically significant difference between the mean of the reciprocal half maximal effective concentration of sera from mice immunized with the multivalent WT construct and that of sera from mice immunized with the multivalent ØKO construct within or across the RSV strains tested. As a result, we have concluded that site Ø is dispensable for immunogenicity.

While site Ø may not be needed for protection across RSV subtypes, the remaining question is the following: should one choose to immunize with the ØKO RSV F if the WT RSV F generates a response with comparable neutralization potency across RSV subtypes? We think the ØKO construct may show an increased benefit in populations with pre-existing immunity. This is because these populations will already have immune memory to RSV that is likely site Ø dominant. In this context, the WT vaccine would serve to amplify this immune memory to site Ø whereas the ØKO vaccine would serve to redirect the existing site Ø response to the more conserved areas of the RSV F protein. We have conducted an immunodepletion experiment to demonstrate the ability of the ØKO vaccine to direct immune response away from site Ø (Figure 4d). Specifically, we probed the binding of sera from mice immunized with either RSV F WT or RSV F ØKO to RSV F WT before and after depletion with RSV F ØKO. While the two groups of sera showed comparable binding to RSV F WT before depletion, the sera from mice immunized with
RSV F WT showed significantly greater residual binding after depletion than did the sera from mice immunized with RSV F ØKO. This greater residual binding can likely be attributed to site Ø-specific antibodies, and suggests that the ØKO protein is capable of directing immune response away from site Ø.

Figure 4. Analysis of sera resulting from immunization with multivalent RSV F conjugates. a) Schedule for vaccination of mice and serum collection. b) Antibody titer of sera from mice immunized with the multivalent RSV F WT conjugate, multivalent RSV F ØKO conjugate, or scaffold alone measured against WT RSV F. Antibody titers are expressed as the area under the curve (AUC). (mean ± SD, n = 5). ns – not statistically significant, determined by the Mann-Whitney test (α = 0.05). c) Comparison of the neutralizing antibody titers of pooled sera from mice immunized with multivalent RSV F WT (black) and pooled sera from mice immunized with multivalent RSV F ØKO (white) against an A subtype and B subtype strain of RSV (mean ± SD, n = 6). The difference between any of the four EC₅₀ values is ns – not statistically significant, determined by a two-way analysis of variance (ANOVA) and Tukey’s post-hoc multiple comparison between groups (α =
0.05). d) Analysis of the binding of sera from mice immunized with either RSV F WT or RSV F ØKO to WT RSV F before and after depletion with RSV F ØKO. This analysis demonstrates the ability of RSV F ØKO to serve as an immunogen that directs immune response away from site Ø (mean ± SD, n = 3). ns – not statistically significant, *p < 0.05, determined by multiple Holm-Sidak corrected (α = 0.05) t-tests.

2.4 Conclusion and Future Directions

We have hypothesized that a vaccine with a shielded site Ø may be beneficial in directing pre-existing site Ø-dominant immune response away from site Ø and towards the more conserved sites of RSV F. This hypothesis has been supported by several recent studies that show the structural and sequence diversity of site Ø between subtypes may have an impact on antibody recognition and neutralization [50, 54, 55]. To demonstrate the viability of a prefusion F-based vaccine with a shielded site Ø, we have mutated a stabilized prefusion F protein to introduce glycans that restrict access to site Ø. These ØKO mutants were displayed multivalently on a branched PEG scaffold and were then injected in mice. The resulting sera was shown to neutralize RSV across subtypes, proving that an immune response to site Ø is not necessary for protection. Ultimately, this result may have application in the design of an effective and broadly protective RSV vaccine.

In future work we plan to further support our hypothesis that the ØKO vaccine may serve to better elicit broadly neutralizing antibodies by comparing the neutralizing breadth of antibodies that target site Ø to antibodies that target the regions of the RSV F prefusion protein outside of site Ø. We will use two constructs to elicit these two sets of antibodies. The ØKO vaccine can be used to elicit antibodies that target the regions of the RSV F prefusion protein outside of site Ø, but we will need to develop another construct to elicit antibodies that target only site Ø. To develop this construct, we will use the “protect,
modify, deprotect” method described by Weidenbacher et al. that allows for the development of a protein antigen with a single accessible epitope [17]. Specifically, we will immobilize a site $\varnothing$-specific antibody on resin. This resin can be added to a solution of RSV F such that the antibody “protects” site $\varnothing$. Then, the unprotected, exposed surface of the RSV F will be “modified” by allowing it to react with NHS-PEG, which reacts with surface-exposed lysine residues. After excess NHS-PEG is removed, the RSV F will be separated from the site $\varnothing$-specific antibody such that the RSV F will be “deprotected”. The result will be a PEGylated RSV F protein with a single exposed epitope – site $\varnothing$. We will then immunize mice with either this site $\varnothing$ antigen or the $\varnothing$KO antigen. The site $\varnothing$ antigen should elicit antibodies directed only at site $\varnothing$, whereas the $\varnothing$KO antigen should elicit antibodies specific to the regions outside of site $\varnothing$. We can then compare the neutralization potencies of these two sets of antibodies against various strains of RSV F. We expect that the antibodies targeting regions outside of site $\varnothing$ will have an increased breadth of neutralization compared to antibodies targeting site $\varnothing$. This result would suggest that using the $\varnothing$KO vaccine to direct immune response away from site $\varnothing$ and towards the more conserved areas of RSV F may serve to better elicit broadly neutralizing antibodies.

Recent literature supports the hypothesis that site $\varnothing$ antibodies are less likely to be broadly neutralizing than antibodies directed to the regions of the F protein outside of site $\varnothing$. For example, Joyce et al. noticed that immunization with a stabilized prefusion F protein of subtype A generated a response that resulted in 2-3-fold higher neutralizing titers against the homologous subtype A virus than against a subtype B virus [50]. Upon investigation of the molecular basis for this difference, they found that several site $\varnothing$-directed neutralizing antibodies showed over 100-fold reduction in recognition of subtype B RSV
when compared to recognition of subtype A RSV. Of particular interest is 5C4, an antibody that potently neutralizes RSV of subtype A, but does not bind to a strain of RSV B. The authors partially attributed these subtype-dependent differences in neutralization potency and antibody recognition to the structural and sequence diversity of site Ø between RSV subtypes. Other studies have led to similar conclusions [54, 55], which suggests that a prefusion F-based vaccine with a shielded site Ø may be useful to direct pre-existing site Ø immune memory to the other, more conserved areas of the F protein.
CHAPTER 3. THE EFFECT OF HEMAGGLUTININ ORIENTATION ON THE GENERATION OF BROADLY PROTECTIVE STALK-DIRECTED ANTIBODIES AGAINST INFLUENZA

3.1 Introduction

Influenza represents a major global health problem. Seasonal influenza virus epidemics have been estimated to cause as many as 250,000 to 500,000 deaths annually worldwide [56]. The spread of seasonal influenza virus can be prevented through vaccination. Such immunization (and natural infection) typically induces an immune response that primarily targets the globular head domain of the viral antigen hemagglutinin (HA) [57-59]. Unfortunately, the HA head domain rapidly acquires mutations – a phenomenon referred to as antigenic drift – which enables the virus to escape pre-existing immunity. For that reason, the influenza vaccine must be reformulated and readministered annually. However, there can be a mismatch between the strains selected for incorporation into seasonal vaccines and circulating viruses, resulting in a decrease in vaccine efficacy [60]. Another serious concern is that commercially available vaccines would provide little protection against novel influenza viruses and the consequences could be devastating. For example, the influenza pandemic of 1918 has been estimated to have claimed tens of millions of lives [61, 62].

In contrast to the highly plastic HA head domain [36], the stalk domain of HA is conserved and has a lower tolerance for mutations. The conserved nature of the stalk
domain makes it a prime target for broadly neutralizing antibodies. In fact, stalk-directed antibodies isolated from mice and humans are capable of neutralizing across influenza virus strains and subtypes [63-65]. Moreover, monoclonal antibodies that target the stalk domain have proven to be cross-protective in animal models [8, 63, 64, 66-68]. Unfortunately, these broadly protective stalk-specific antibodies are not commonly elicited by current influenza vaccines. However, broadly neutralizing vaccines that target the HA stalk domain are under development [69]. Examples of these vaccines include an HA molecule with a hyperglycosylated head domain that prevents B cells from accessing the HA head through steric shielding [14], a stalk-only HA construct in which the HA head domain has been removed [19-21, 23, 24], and chimeric HA molecules with conserved stalk domains that boost the stalk-directed response upon sequential immunization [7-9].

This work was motivated by a fundamental unresolved question: Why do natural infection and current vaccines elicit an immune response predominately targeting the HA head? Overcoming the natural immunodominance of the HA head may have important implications for the development of an effective broadly neutralizing influenza vaccine. While several possible explanations for the immunodominance of the HA head exist [70], one intriguing hypothesis suggests that the natural immunodominance of the HA head may result from its accessibility on the virion. Specifically, HA is positioned such that its globular head is displayed outwardly and is therefore easily accessible to head-specific B cell receptors [71, 72]. Meanwhile, the HA stalk is membrane proximal and its interaction with stalk-specific B cells may be sterically hindered. HA would have a similar orientation in live or inactivated viral vaccines as well as in currently available subunit vaccines, where HA molecules form rosettes clustering around the stalk and present the HA head
outwardly [73]. Therefore, in the case of both natural infection and vaccination, the accessibility of the stalk domain to stalk-specific B-cell receptors may be limited. This limited accessibility might inhibit the effective elicitation of broadly protective anti-stalk antibodies.

We reasoned that inverting the orientation of HA on virus-like particles would make the HA stalk domain more accessible to stalk specific B cells, which in turn should facilitate the elicitation of broadly protective anti-stalk antibodies. To test this hypothesis, we investigated how inverting HA on VLPs affects the protective anti-HA antibody response. Specifically, we created a VLP that displays multiple copies of an antigen binding fragment (Fab) that bind to the apex of the HA head such that the HA stalk is presented outwardly. We immunized mice with either this construct displaying multiple copies of HA in the inverted orientation or a construct in which HA is oriented on VLPs as it would naturally be oriented on the virus. The inverted HA construct generated higher antibody titers against the HA stalk. Furthermore, mice immunized with the inverted HA construct were better protected against an influenza virus displaying a chimeric hemagglutinin made up of the same stalk used for immunization but a different head. This suggests that the inverted HA construct generated enhanced stalk-based protection. Therefore, we have concluded that the orientation of HA affects the generation of anti-stalk antibodies. These results may inform the design of broadly protective influenza vaccines, as antibodies targeting the conserved stalk domain of HA have been found to be broadly protective. Furthermore, controlling antigen presentation using Fabs conjugated to VLPs may be a generally applicable strategy to enhance immune response against immunosubdominant viral epitopes.
3.2 Methods

3.2.1 Expression and purification of HA

DNA encoding both BirA and the ectodomain of HA (A/Puerto Rico/8/1934) with a C-terminal T4 fibritin trimerization domain, AviTag, and his-tag were cloned into pFastBac Dual (Gibco) to create the TT Avi HA DNA construct. Similarly, the ectodomain of HA (A/Puerto Rico/8/1934) with a C-terminal T4 fibritin trimerization domain and Strep-Tag II was cloned into pFastBac Dual (Gibco) to create the WT HA DNA construct. These plasmids were then transformed into DH10Bac cells (Gibco) according to the manufacturer’s instructions to generate recombinant bacmid. The transformation was plated on LB Agar plates and a blue/white screen was used to identify colonies containing the recombinant bacmid. A colony containing the recombinant bacmid was grown in 10 mL of LB media, which was incubated overnight at 37°C. The following morning, the cells of the culture were pelleted and bacmid was midiprepped using the PureLink HiPure Plasmid DNA Miniprep Kit (Invitrogen) and its associated protocol. The bacmid was then transfected into Sf9 cells using Cellfectin II (Gibco) to create P1 baculovirus stock. The baculovirus was amplified until at least a P3 baculovirus stock was made, as described by Margine et al. [74]. In brief, Sf9 cells were plated in T175 flasks at a density of 2 x 10^5 cells/cm^2. After a 20 minute incubation to allow for the cells to adhere to the flask, the media in the T175 flask was replaced with Grace’s Insect Medium (Gibco) supplemented with 3% FBS, 1% Pen-Strep and 0.1% Pluronic F68. Then 400 µL of the P1 baculovirus stock was added to the T175 flask. The flask was incubated at 28°C for 6 days, after which the media was harvested by centrifugation at 2,000xg for 5 minutes to give the P2 baculovirus stock. This procedure was repeated using the P2 stock to further amplify the
baculovirus and create subsequent stocks (P3, P4, P5 etc.). Ultimately, these amplified stocks were used to express the HA constructs using a procedure similar to that described by Margine et al. [74]. For a 100 mL expression, 30 million cells were centrifuged at 100xg for 7 minutes. The media was discarded, and the cell pellet was resuspended in 9 mL of a baculovirus suspension. After a 20-minute incubation, the baculovirus-cell suspension was added to a 250 mL Erlenmeyer flask containing 90 mL Express Five Serum Free Media (Gibco) supplemented with L-glutamine (Gibco) to a concentration of 18 mM. This culture was incubated for 3-4 days, after which the cells were pelleted at 5,500xg for 20 minutes. Resulting media containing secreted HA with a C-terminal Strep-Tag II was purified using a 5 mL StrepTrap HP column (Cytiva), which was operated according to the manufacturer’s instructions. The eluate was concentrated to approximately 1 mL using a 10 kDa MWCO centrifugal filter (Millipore Sigma) and run on a Superdex 200 Increase 10/300 column (Cytiva) to separate HA trimer from aggregate. Media containing secreted HA with a C-terminal his-tag was run through a gravity flow column (G-Biosciences) containing 1 mL of HisPur Ni-NTA resin (Thermo Scientific) that had been washed with 60 column volumes of DI water and pre-equilibrated with 30 column volumes of PBS. The column was then washed with 90 column volumes of wash buffer (42 mM sodium bicarbonate, 8 mM sodium carbonate, 300 mM NaCl, 20 mM imidazole) and the his-tagged protein was eluted by incubating the column in 3 mL of elution buffer (42 mM sodium bicarbonate, 8 mM sodium carbonate, 300 mM NaCl, 300 mM imidazole) for 5 minutes before collecting the eluate. This elution procedure was repeated three times to generate a total of 9 mL of eluate, which was buffer exchanged to 20 mM Tris, 20 mM NaCl, pH 8.0
to allow for in vitro biotinylation. The HA was then quantified using the BCA assay (Thermo Scientific).

3.2.2 Expression and purification of MS2

DNA encoding a single chain dimer of the MS2 coat protein with an AviTag inserted between the fourteenth and fifteenth residues of the second coat protein monomer was cloned into pET-28b between the NdeI and XhoI restriction sites by GenScript Biotech Corporation (Piscataway, NJ). This construct was expressed and purified as previously described [75]. The plasmid was co-transformed into BL21(DE3) E. coli (New England BioLabs) with a plasmid encoding pAcm-BirA (Avidity LLC). The transformed cells were added to 5 mL of 2xYT supplemented with kanamycin and chloramphenicol, and this culture was incubated overnight at 37°C. The next morning, the 5 mL culture was added to 1L of 2xYT supplemented with kanamycin and chloramphenicol. This larger culture was incubated at 37°C until reaching an optical density (OD) of 0.6. At this point, expression of MS2 and BirA was induced with IPTG (1 mM; GoldBio), 12.5 mg of biotin was added per L of culture, and the incubator temperature was reduced to 30°C. After overnight incubation, the culture was centrifuged at 7000xg for 7 minutes to pellet the cells. The resulting media was discarded and the cell pellet was resuspended in 25 mL of 20 mM Tris Base, pH 9.0, supplemented with lysozyme (0.5 mg/mL; Alfa Aesar), a protease inhibitor tablet (Sigma-Aldrich), and benzonase (125 units; EMD Millipore). This cell suspension was kept on ice and mixed occasionally for 20 minutes, after which sodium deoxycholate (Alfa Aesar) was added to a concentration of 0.1% (w/v). The cells were then sonicated on ice for 3 minutes with 3 second pulses at an amplitude of 35% (Sonifier S-450, Branson Ultrasonics). The lysate was allowed to cool on ice for 2 minutes and then
the sonication was repeated. The lysate was then centrifuged for 30 minutes at 27,000xg, and the resulting supernatant was centrifuged again for 15 minutes at 12,000xg. The supernatant resulting from the second centrifugation was then diluted 3-fold with 20 mM Tris, pH 8.0, and filtered using a 0.45 µm bottle-top filter. The MS2 was then purified from this diluted lysate using four HiScreen Capto Core 700 columns (Cytiva) in series according to the manufacturer’s operating instructions. Fractions resulting from the purification were run on an SDS-PAGE gel to determine purity and recovery of MS2. The desired fractions were then pooled, concentrated to approximately 1 mL using a 10 kDa MWCO centrifugal filter (Millipore Sigma), and further purified using a Superdex 200 increase 10/300 column (Cytiva). The purified MS2 was then buffer exchanged into 20 mM Tris, 20 mM NaCl, pH 8.0, in preparation for in vitro biotinylation. The MS2 was quantified using the BCA assay (Thermo Scientific).

3.2.3 Expression, refolding, and purification of streptavidin (SA)

Streptavidin was generally expressed, refolded, and purified as described previously [76, 77]. In brief, DNA encoding streptavidin (Addgene plasmid #46367, a gift from Mark Howarth) was transformed into BL21(DE3) E. coli and added to four culture tubes each containing 5 mL of 2xYT with ampicillin. These cultures were incubated overnight at 37°C. Then, each of the 5 mL cultures were added to shake flasks containing 1L of 2xYT and ampicillin. The 1L cultures were incubated at 37°C until the cultures reached an OD of 0.6. At this point, expression of inclusion bodies was induced with IPTG (1M; GoldBio) and the temperature of the incubator was reduced to 30°C. After overnight incubation, the cultures were centrifuged at 7,000xg for 7 minutes such that the cultures resulted in two cell pellets. The supernatant was discarded, and each cell pellet was
homogenized into 50 mL of resuspension buffer (50 mM Tris, 100 mM NaCl, pH 8.0) supplemented with lysozyme (1 mg/mL; Alfa Aesar) and benzonase (500 units; EMD Millipore). The resuspended cells were incubated at 4°C for an hour. The cells were further homogenized before adding sodium deoxycholate (Alfa Aesar) to a total concentration of 0.1% (w/v) and sonicating (Sonifier S-450, Branson Ultrasonics) for 3 minutes with 3 second pulses at 35% amplitude. The lysed cells were then centrifuged at 27,000xg for 15 minutes and the supernatant was discarded. This lysis procedure was repeated, but with a shorter 15-minute incubation at 4°C prior to sonication. The result was two inclusion body pellets that were then each suspended in 50 mL wash buffer (50 mM Tris, 100 mM NaCl, 100 mM EDTA, 0.5% (v/v) Triton X-100, pH 8.0), homogenized, sonicated for 30 seconds at an amplitude of 35%, and centrifuged at 27,000xg for 15 minutes. This step was repeated two more times. The resulting washed inclusion body pellets were then suspended in a second wash buffer (50 mM Tris, 10 mM EDTA, pH 8.0), homogenized, sonicated for 30 seconds at an amplitude of 35%, and centrifuged at 15,000xg for 15 minutes. This second wash step was repeated once more. The final washed inclusion body pellets were then unfolded by resuspension and homogenization in 10 mL of a 7.12 M guanidine hydrochloride solution, which was stirred vigorously at room temperature for an hour. The unfolded streptavidin was then centrifuged at 12,000xg for 10 minutes, and the supernatant was added dropwise at a rate of 30 mL/h to 1L of chilled PBS being stirred vigorously to refold the streptavidin. After stirring overnight at 4°C, the refolded streptavidin in PBS was centrifuged at 7,000xg for 10 minutes to remove insoluble protein. The supernatant was filtered with a 0.45 µm bottle-top filter, and ammonium sulfate was slowly added to the filtered solution to a concentration of 1.9 M in order to
precipitate out impurities. The resulting solution was stirred at 4°C for at least three hours. After three hours, the streptavidin solution with the precipitated impurities was centrifuged at 7,000xg for 10 minutes and the supernatant was filtered using an 0.45 µm bottle-top filter. Ammonium sulfate was added to the filtrate to bring the total concentration of ammonium sulfate to 3.68 M, which caused the precipitation of streptavidin. The resulting solution was stirred at 4°C for at least three hours, and was then centrifuged at 7,000xg for 20 minutes. The pellet was resuspended in 20 mL of Iminobiotin Affinity Chromatography (IBAC) binding buffer (50 mM Sodium Borate, 300 mM NaCl, pH 11.0) and allowed to flow through a gravity flow column (G-Biosciences) with 5 mL of Pierce Iminobiotin Agarose (Thermo Scientific) that had been washed with DI water and equilibrated with IBAC binding buffer. The column was then washed with 20 column volumes of IBAC binding buffer and the streptavidin was eluted with 6 column volumes of elution buffer (20 mM KH₂PO₄, pH 2.2). The eluate was dialyzed into PBS and concentrated using a 10 kDa MWCO centrifugal filter (Millipore Sigma). Streptavidin was quantified by measuring the UV absorption at 280 nm.

3.2.4 Expression and purification of H28-D14 Fab

DNA encoding the variable heavy and light chains of H28-D14 (sequence kindly provided by Dr. Jonathan Yewdell, National Institutes of Health [78]) were cloned into TGEX-FH and TGEX-LC (Antibody Design Labs), respectively, by Gene Universal, Inc. (Newark, DE). The TGEX-FH vector was modified to include a C-terminal AviTag immediately upstream of the 6xHis-Tag. These plasmids were transfected into Expi293F cells in a 2:1 light chain to heavy chain ratio using the ExpiFectamine 293 Transfection Kit (Gibco) and associated protocol. Four days after transfection, the culture was centrifuged
at 5,500xg for 20 minutes and the supernatant containing secreted H28-D14 Fab was dialyzed into PBS. The resulting H28-D14 in PBS was passed through a gravity flow column (G-Biosciences) with 1 mL of HisPur Ni-NTA resin (Thermo Scientific) that had been washed with 60 column volumes of DI water and equilibrated with 30 mL of PBS. The column was then washed with 90 column volumes of wash buffer (42 mM sodium bicarbonate, 8 mM sodium carbonate, 300 mM NaCl, 20 mM imidazole). To elute the Fab, the column was incubated in 3 mL of elution buffer (42 mM sodium bicarbonate, 8 mM sodium carbonate, 300 mM NaCl, 300 mM imidazole), which was collected after five minutes. This was repeated twice to create 9 mL of eluate. The eluate was buffer exchanged into 20 mM Tris, 20 mM NaCl, pH 8.0 to allow for in vitro biotinylation. H28-D14 Fab was then quantified by BCA assay (Thermo Scientific).

3.2.5 Expression and purification of H28-D14 and CR6261 antibodies

The DNA encoding the variable heavy chains and the variable light chains of the H28-D14 antibody [78] (sequence kindly provided by Dr. Jonathan Yewdell, National Institutes of Health) and the CR6261 antibody [68] was cloned into TGEX-HC and TGEX-LC (Antibody Design Labs), respectively, by Gene Universal, Inc. (Newark, DE). The resulting plasmids were transfected into Expi293F cells in a 2:1 light chain to heavy chain ratio using the ExpiFectamine 293 Transfection Kit (Gibco) and protocol. After 5 days, cells were pelleted by centrifugation for 20 minutes at 5,500xg. The supernatant containing secreted antibody was diluted in PBS and purified using a MabSelect SuRe (Cytiva) column according to the manufacturer’s protocol.

3.2.6 In vitro biotinylation of AviTagged HA, MS2, and H28-D14 Fab
AviTagged proteins were biotinylated *in vitro* using the BirA500 kit (Avidity LLC) essentially according to the manufacturer’s instructions. In brief, AviTagged protein was buffer exchanged into 20 mM Tris, 20 mM NaCl, pH 8.0 and its concentration was adjusted to 45 µM. BirA and Biomix B (a proprietary solution containing biotin, ATP, and magnesium acetate) were added to the protein solution as specified in the instructions. This solution was then shaken vigorously at 37°C for two hours. After the two-hour incubation, more Biomix B was added to the solution – the same volume that had been added previously – before incubating on a nutator overnight at 4°C. The biotinylated proteins were then purified using a Superdex 200 Increase 10/300 column (Cytiva) and quantified using the BCA assay (Thermo Scientific).

3.2.7 Assembly of VLP-HA<sub>Regular</sub>

One mL of biotinylated MS2 (~0.7 mg/mL) was added dropwise to a streptavidin solution (~50 mg/mL) that was in approximately 20 times molar excess and being stirred vigorously in a 5 mL glass vial. The solution was stirred for 30 minutes before purifying the MS2-SA from excess streptavidin using a Superdex 200 Increase 10/300 column (Cytiva). The MS2-SA was concentrated using a 10 kDa MWCO centrifugal filter (Millipore Sigma), and the amount of streptavidin in solution was quantified by running a boiled MS2-SA sample on an SDS-PAGE gel with lanes that included boiled streptavidin standards previously quantified by measuring the UV absorption at 280 nm. To determine appropriate stoichiometry between MS2-SA and biotinylated TT Avi HA, small-scale mixtures of MS2-SA and biotinylated TT Avi HA were run on an SDS-PAGE gel at 4°C in varying ratios. Once the appropriate stoichiometry had been determined, MS2-SA was added to a slight stoichiometric excess of biotinylated TT Avi HA. After incubating the
mixture on a nutator at room temperature for an hour, the resulting VLP-HA<sub>Regular</sub> construct was separated from excess biotinylated TT Avi HA using a Superdex 200 Increase 10/300 column (Cytiva). The amount of HA in the VLP-HA<sub>Regular</sub> solution was quantified by running a boiled VLP-HA<sub>Regular</sub> sample on an SDS-PAGE gel with BSA standards.

3.2.8 Assembly of VLP-HA<sub>Inverted</sub>

Approximately 1 mg of biotinylated H28-D14 Fab (~1 mg/mL) was added 5 µL at a time to a four-fold molar excess of streptavidin (~50 mg/mL) in a 1.5 mL Eppendorf tube. The mixture was vortexed between every 5 µL addition of biotinylated H28-D14 Fab. The streptavidin-Fab conjugate was purified from both excess streptavidin and Fab-streptavidin-Fab conjugates using a HiLoad Superdex 200 column (Cytiva). To determine the appropriate stoichiometry between MS2 biotin and the streptavidin-Fab conjugate, small scale mixtures of biotinylated MS2 and streptavidin-Fab conjugate were run on an SDS-PAGE gel at 4°C in varying ratios. Biotinylated MS2 was then added to the streptavidin-Fab conjugate in the appropriate stoichiometry and allowed to incubate on a nutator at room temperature for 45 minutes. Biotin in 10-fold molar excess of streptavidin was then added to occupy any biotin-binding sites that had not been occupied by the biotinylated MS2 or Fab. This mixture was incubated on a nutator at room temperature for 15 minutes before being added to a large excess of WT HA. This final mixture was incubated on a nutator at room temperature for an hour. The VLP-HA<sub>Inverted</sub> was then purified using a Superdex 200 Increase 10/300 column (Cytiva). The amount of HA in the VLP-HA<sub>Inverted</sub> solution was quantified by running a heated sample of VLP-HA<sub>Inverted</sub> on an SDS-PAGE gel with BSA standards.
3.2.9 SDS-PAGE

PageRuler Plus Prestained Protein Ladder (Thermo Scientific) and protein samples mixed with 5 µL of Nu-PAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) were pipetted into the wells of a 4-12% Bis-Tris gel (Invitrogen). Gels were cooled at 4°C while running in MES-SDS buffer for 60 minutes at 110 V. The gel was then stained with SimplyBlue SafeStain (Invitrogen), subsequently destained, and imaged using the ChemiDoc MP imaging system (Bio-Rad).

3.2.10 DLS

Protein was diluted in PBS to 100 µL and pipetted into a UVette (Eppendorf), which was loaded into a DynaPro NanoStar Dynamic Light Scattering detector (Wyatt Technology). Dynamics software (Wyatt Technology) allowed the temperature to equilibrate to 25°C before each measurement. Each measurement was the result of 10 acquisitions and was output as % Intensity vs. Radius. Radius and polydispersity (PD) index are presented as the mean ± SD of three measurements, while the plotted size distribution is the result of a single representative measurement.

3.2.11 ELISA

Antigen (0.1 µg HA in 100 µL of PBS per well) was coated onto Nunc MaxiSorp 96-well flat-bottom plates (Invitrogen) and allowed to incubate for an hour. The antigen solution was then discarded and 200 µL of 5% BSA (Millipore) in PBST (PBS with 0.05% Tween-20) was added to each well. The plate was incubated for 45 minutes. The wells of the plate were then emptied and washed three times with 200 µL of PBST. The first well
of each row was filled with 150 µL of antibody in 1% BSA in PBST solution, such that the molar ratio of antibody to hemagglutinin was 25:1, while all other wells in the row were filled with 100 µL of 1% BSA in PBST. Then, 50 µL of antibody solution was taken from the first well of each row and 3-fold serial dilutions were made across the row. These antibody solutions were incubated with the antigen for an hour before the wells were emptied and washed three times. Horseradish peroxidase-conjugated anti-human IgG Fc fragment antibody (MP Biomedicals; 1:5,000 dilution) in 1% BSA in PBST was then added to each well. After an hour incubation, the secondary antibody solution was discarded, and the plate was washed three times. To develop the plates, 100 µL of TMB substrate solution (Millipore) was added to each well and was stopped after 3 minutes using 100 µL of 0.16 M sulfuric acid. The absorbance of the wells at 450 nm was read using a Spectramax i3x plate reader (Molecular Devices).

3.2.12 Aggregation assay

VLP-HA (1 µg of HA) was diluted in PBS to 100 µL and its size was measured by DLS. Next, biotinylated MS2 was mixed with streptavidin-Fab conjugate in the appropriate stoichiometric ratio that was determined by running small scale mixtures of each in varying ratios on an SDS-PAGE gel to create VLP-Fab. The VLP-Fab was diluted in PBS to 50 µL and mixed with VLP-HA (1 µg of HA) diluted in PBS to 50 µL such that Fab was in 1.5x molar excess to HA. The 100 µL mixture was then pipetted into a Vvette (Eppendorf) and DLS measurements were made using the DynaPro NanoStar Dynamic Light Scattering detector (Wyatt Technology) and Dynamics software (Wyatt Technology).
3.2.13 Hemagglutination assay

One mL of a solution of Turkey Red Blood Cells (5%; Lampire) was pipetted into a 15 mL tube and mixed with 14 mL of PBS. The red blood cells were washed by centrifuging for 7 minutes at 100xg, decanting the PBS, and resuspending in fresh PBS. This washing procedure was repeated twice, and the washed red blood cell pellet was resuspended in 10 mL of PBS to create an 0.5% red blood cell solution. Next, wells of a 96 well plate were filled with antigen solution (VLP-HA\textsubscript{Regular}, VLP-HA\textsubscript{Inverted}, MS2-SA, inactivated virus (A/California/07/2009(H1N1))) that would be tested for the capability to agglutinate the red blood cells. The first well of each row was loaded with 100 µL of antigen containing 1.5 µg of HA, or an amount of MS2-SA VLP alone (the negative control) equivalent to the amount of MS2-SA VLP in the VLP-HA\textsubscript{Regular} sample. The remaining wells in each row were filled with 50 µL of PBS. Then, 50 µL of antigen solution was taken from the first well of each row and 2-fold serial dilutions were made across the row. Each well was then mixed with 50 µL of the 0.5% red blood cell solution, and the plate was incubated at room temperature for at least an hour. The plate was imaged using the ChemiDoc MP imaging system (Bio-Rad).

3.3 Results and Discussion

3.3.1 Design and generation of oriented HA constructs

To develop a virion-like scaffold that could be used to present HA in varying orientations (Figure 5), we generated streptavidin-coated virus like particles (VLPs) that can display biotinylated protein by exploiting the strong interaction between biotin and streptavidin (SA) [16, 75]. Specifically, we used BL21(DE3) competent \textit{Escherichia coli}
(E. coli) to express a single chain dimer of the MS2 bacteriophage coat protein [79, 80] with an inserted AviTag [16, 81], which allowed for the site-specific biotinylation of the VLPs. The inserted AviTag was placed in a surface loop that had previously been shown to tolerate mutations [82]. After the MS2-AviTag VLPs had been expressed, they were purified using both Capto Core 700 resin and size exclusion chromatography (SEC). Once pure, the VLPs were biotinylated in situ and subsequently separated from biotinylation reagents using SEC. Next, the resulting biotin-coated VLPs were added dropwise to an excess of streptavidin, which had been expressed in E. coli as inclusion bodies, refolded, and purified using iminobiotin affinity chromatography. The mixture was then run on an SEC column, which resulted in streptavidin-coated VLPs that could be used to display biotinylated protein such as hemagglutinin.

To display hemagglutinin on the streptavidin-coated MS2 VLPs in the orientation that HA naturally appears in on the influenza virion, we appended an AviTag to the C-terminus of the HA ectodomain from influenza strain A/Puerto Rico/8/1934. The biotinylation of this AviTag allowed for the conjugation of HA to the streptavidin-coated VLPs such that the HA head was displayed outwardly, and the stalk was proximal to the surface of the streptavidin-coated VLPs. The HA was expressed using the baculovirus-insect cell expression system, purified via a C-terminal his-tag using immobilized metal affinity chromatography (IMAC), and biotinylated in vitro. The biotinylated HA trimer was then separated from biotinylation reagents by using SEC. This purified, biotinylated HA was suitable for display on the streptavidin-coated MS2 VLPs. The approximate stoichiometric ratio of the biotinylated HA to MS2-SA VLP was determined by mixing the two constructs in varying ratios and running the resulting mixtures on an SDS-PAGE gel.
to characterize the amount of excess protein in each mixture. Once the appropriate ratio was determined, the MS2-SA VLPs were added to a slight excess of biotinylated HA. This mixture was run on an SEC column to purify the resulting VLPs displaying densely packed HA oriented in the same manner that HA would naturally be oriented on the influenza virus (Figure 5).

To present HA in the inverted orientation, a Fab was first displayed on VLPs. This Fab, called H28-D14, is specific to the Sb antigenic site of HA such that it binds the apex of the HA head [78], resulting in an inverted presentation of HA on VLPs with the HA stalk domain displayed outwardly (Figure 5). The H28-D14 Fab with a C-terminal AviTag and his-tag on the heavy chain was expressed in mammalian cells, purified via IMAC, biotinylated in vitro, and separated from biotinylation reagents by using SEC. The biotinylated H28-D14 Fab was then added dropwise to excess streptavidin, and SEC was used to purify the resulting SA-Fab conjugate. The SA-Fab conjugate could then be attached to biotin-coated MS2 VLPs by way of the streptavidin’s unoccupied biotin binding sites. To determine the appropriate stoichiometric ratio of SA-Fab conjugate to biotinylated MS2 VLP, varying ratios of the two constructs were mixed and run on an SDS-PAGE gel. The resulting gel was then analyzed to determine the ratio at which little to no excess protein remained. After the appropriate stoichiometry had been determined, the biotinylated MS2 VLPs and SA-Fab conjugate were mixed in the established ratio. After a short incubation, the VLPs displaying H28-D14 Fab were added to a large excess of wild type (WT) HA. This final mixture was run through an SEC column to purify the resulting VLPs displaying HA in the inverted orientation.
Figure 5. Assembly of VLP-HA<sub>Regular</sub> and VLP-HA<sub>Inverted</sub>. VLP-HA<sub>Regular</sub> was assembled by first mixing MS2 Biotin (yellow; PDB: 2MS2) with streptavidin (red; PDB: 3RY2) to create MS2-SA VLP. Then the MS2-SA VLP was mixed with TT HA, which is biotinylated at its C-terminus (head domain purple, stalk domain green; PDB: 1RU7). VLP-HA<sub>Inverted</sub> was assembled by first mixing MS2 Biotin with a Fab<sub>Sb</sub>-Streptavidin conjugate. Then the resulting VLP displaying the Fab<sub>Sb</sub> was mixed with WT HA (head domain purple, stalk domain green; PDB: 1RU7).

It should be noted that these oriented HA constructs use two different approaches to conjugate HA to VLP. The regularly oriented HA is conjugated to MS2-SA VLP through a C-terminal biotinylated AviTag, and the inverted HA binds to a head-specific Fab conjugated to VLP. While these two approaches are sufficient for initial tests to gauge the effect of HA orientation on the elicitation of stalk-specific antibodies, they result in different HA densities on the VLPs, which could contribute to the difference in the oriented constructs’ ability to elicit anti-stalk antibodies. In addition, complications may arise in animal models or humans with pre-existing immunity, where pre-existing serum antibodies specific to the apex of the HA head may compete with the VLP-conjugated Fab for binding.
to the WT HA. Therefore, in future work we will explore approaches to attach HA to VLP in both orientations using a uniform conjugation approach. For instance, we have considered inserting an AviTag peptide into the apex of the HA head for site-specific biotinylation and conjugation to the MS2-SA VLPs in the same manner the regularly oriented HA is conjugated to the MS2-SA VLP. However, we have found that this AviTag insertion disrupts antibody binding to the HA stalk. Similar strategies could be attempted that are less invasive than insertion of the AviTag peptide. For example, we could consider the insertion of an unnatural amino acid that can be covalently bound to a VLP through click chemistry [83, 84]. While this type of uniform conjugation method warrants further investigation, the preliminary work described herein should nevertheless provide important insights regarding the effect of HA orientation on the elicitation of anti-stalk antibodies.

3.3.2 In vitro characterization of oriented HA constructs

The HA displayed in both the regular and inverted orientations was characterized \textit{in vitro} using a variety of bioanalytical methods. The proteins used to make both oriented constructs were run on an SDS-PAGE gel, which confirmed that all proteins were pure and ran as expected according to their molecular weights (Figure 6a). Analytical SEC was used to further characterize HA on the VLPs in both orientations (Figure 6b). The UV traces corresponding to HA oriented on VLPs contain a single peak, which appears before the peak corresponding to HA alone. This characterization suggests that there is no unbound HA present in the oriented constructs. Finally, dynamic light scattering (DLS) was used to determine the approximate size of the VLP constructs (Figure 6c). The sizes of all the constructs are consistent with their expected sizes based on the theoretical sizes of the individual proteins. The regularly oriented HA construct is approximately 40 nm in radius,
while the inverted HA construct is approximately 45 nm in radius – the difference likely being due to the Fab present in the inverted HA construct.

Figure 6. *In vitro* characterization of VLP-HA\textsubscript{Regular} and VLP-HA\textsubscript{Inverted}. a) SDS-PAGE characterization of proteins used to assemble VLP-HA\textsubscript{Regular} and VLP-HA\textsubscript{Inverted}. b) Size exclusion chromatography traces for (i) VLP-HA\textsubscript{Regular} (solid line), TT HA (dashed line), (ii) VLP-HA\textsubscript{Inverted} (solid line), and WT HA (dashed line). c) Characterization of (i) intermediate VLPs, (ii) VLP-HA\textsubscript{Regular}, and (iii) VLP-HA\textsubscript{Inverted} by dynamic light scattering (mean ± SD; n=3).
It was also important to ensure that HA was oriented on the VLPs in the correct manner. To characterize the orientation of HA on the VLPs, we conducted two assays. The first assay is an “aggregation assay” in which we used DLS to measure the size of the oriented HA constructs in solution after mixing with VLPs displaying the H28-D14 Fab (Figure 7). We first mixed VLPs displaying H28-D14 Fab with HA regularly oriented on VLPs. We would expect that when HA is regularly oriented, the VLPs displaying the H28-D14 Fab can bind the HA and induce aggregation. Characterization of the resulting mixture by DLS confirmed the occurrence of this aggregation, consistent with HA being oriented on VLPs as it would be on the virus. Next, we mixed VLPs displaying H28-D14 Fab with HA in the inverted orientation. In this case, we would not expect aggregation because access to the apex of the HA head is likely sterically hindered. DLS confirmed that no large-scale aggregation had occurred. These results would suggest that both the regularly oriented HA and the inverted HA are displayed on the VLPs as expected. To further probe the orientation of the HAs on the VLPs, we conducted a hemagglutination assay (Figure 8). Here we probed whether each construct was capable of agglutinating red blood cells, which contain sialic acid on their surface. Hemagglutinin binds to sialic acid near the apex of its head domain, and so we would expect that the regularly oriented HA presenting the HA head domain outwardly would interact with and agglutinate the red blood cells. On the other hand, we would not expect the HA in the inverted orientation to interact with the sialic acid on the red blood cells. These expectations are consistent with the results of the hemagglutination assay (Figure 8). Therefore, we concluded that both the regularly oriented HA and the inverted HA are displayed on the VLPs as expected.
Figure 7. Characterization of HA orientation on VLP by aggregation assay. a) Cartoon and corresponding DLS measurement of (i) VLP-HA\textsubscript{Regular} alone and (ii) VLP-HA\textsubscript{Regular} mixed with VLP-Fab\textsubscript{Sb}. b) Cartoon and corresponding DLS measurement of (i) VLP-HA\textsubscript{Inverted} alone and (ii) VLP-HA\textsubscript{Inverted} mixed with VLP-Fab\textsubscript{Sb}. 
Figure 8. Characterization of HA orientation on VLP by hemagglutination assay.

3.3.3 Protective efficacy of oriented HA constructs

The in vivo efficacy of the constructs was next evaluated (Figure 9a). Mice were immunized with either the regularly oriented HA, the inverted HA, or MS2-SA VLPs alone. Mice immunized with the oriented HA constructs received 2.5 µg HA. Mice immunized with VLPs alone received the same amount of MS2-SA VLPs as is in a dose of the regularly oriented HA. After 28 days the mice were boosted. Sera was collected on day 42. An ELISA was conducted using the day 42 sera to measure antibody titers against cH6/1, which is a chimeric hemagglutinin that is made up of an H6 head and an H1 stalk. As the mice were immunized with H1 HA, the resulting antibodies should only be
capable of binding to the H1 stalk of the cH6/1 protein. Therefore, titer against cH6/1 is effectively a measure of anti-stalk titer. We found that sera from mice immunized with the inverted HA construct had significantly higher anti-cH6/1 titers than did sera from mice immunized with the regularly oriented HA construct, which suggests that the inverted HA construct elicits an enhanced stalk-directed immune response (Figure 9b). This result is exciting yet expected, as the HA in the inverted orientation presents the stalk outwardly such that it is accessible to stalk specific B cells, while the stalk of regularly oriented HA is membrane proximal, shielded by the globular HA head, and likely difficult for B cells to access. After this encouraging result, the vaccinated mice were challenged with a cH6/1 N5 influenza virus. This virus displays the cH6/1 protein, so we would expect that mice with higher anti-stalk titers would be better protected. In accordance with our expectation, mice immunized with the inverted HA lost very little bodyweight (Figure 9c) and all survived the duration of the challenge (Figure 9d). In contrast, mice immunized with the regularly oriented HA rapidly lost bodyweight (Figure 9c) and died by day 9 of the challenge (Figure 9d). This would suggest that the inverted HA generated a more protective stalk-directed response than did the regularly oriented HA. Again, this is consistent with expectations as the stalk should be more accessible to B cells when HA is inverted. Therefore, we have concluded that HA orientation influences the generation of anti-stalk antibodies. While this result is fundamentally interesting, it also indicates that an inverted HA construct could have application in the design of a broadly protective influenza vaccine.
Figure 9. Protective efficacy of oriented HA constructs. a) Schedule for vaccination of mice, serum collection, and viral challenge. b) Antibody titers generated by VLP-HA_{Regular} and VLP-HA_{Inverted}. Antibody titers are expressed as the area under the absorbance vs reciprocal sera dilution curve generated by ELISA (geometric mean with geometric standard deviation; n = 15). ****p < 0.0001 determined by Welch’s two-tailed t-test (α = 0.1). Assumptions of the normality of residuals were validated by the D’Agostino-Pearson normality test. c) Weight loss of mice immunized with VLP-HA_{Inverted} (blue) and VLP-HA_{Regular} (orange) and subsequently infected with cH6/1 N5 virus (mean ± SD; n=3). d) Survival of mice immunized with VLP-HA_{Inverted} (blue) and VLP-HA_{Regular} (orange) and subsequently infected with cH6/1 N5 virus (mean ± SD; n=3).

3.4 Conclusion and Future Directions

We are currently conducting studies to further analyze the immune response to the oriented HA constructs. To better characterize the protective efficacy of each construct, we will analyze the viral titers in the lungs of immunized mice after challenge with the
cH6/1 N5 virus. Based on the existing survival data, we expect that viral titers in the lungs of mice immunized with the inverted HA will be lower than the viral titers in the lungs of mice immunized with the regularly oriented HA. Along with the weight loss and survival data, this would suggest that the inverted HA better protects against challenge with the cH6/1 N5 virus than does the regularly oriented HA. While the protection afforded by the inverted HA is likely a result of stalk-specific antibodies, it is important to explicitly analyze the role that antibodies play in mediating this protection. To test the degree of protection conferred by antibodies, we will passively transfer sera from immunized mice to naive mice. These naive mice will then be challenged with the cH6/1 N5 virus. We expect the mice that received antibodies generated by the inverted HA construct will be protected against the cH6/1 N5 viral challenge, while the mice that received antibodies generated by the regularly oriented HA will not be protected. This result would confirm that the protection we had seen in mice immunized with the inverted HA is a result of antibodies that are predominately directed at the stalk. Finally, we will examine the breadth of reactivity of the antibodies elicited by the oriented HA constructs. As antibodies targeting the HA head domain are generally strain-specific [85], we do not expect antibodies elicited by the regularly oriented HA to be broadly reactive. In contrast, antibodies targeting the conserved HA stalk domain have been found to be broadly reactive [85]. Therefore, we expect that antibodies elicited by the inverted HA construct will be broadly reactive. These results would suggest that the orientation of HA influences the generation of anti-stalk antibodies that have the potential to be broadly protective. Consequently, the inverted HA construct could have application in the design of a broadly protective influenza vaccine.
CHAPTER 4. THE MULTIVALENT DISPLAY OF SARS-COV-2 SPIKE PROTEIN ON VIRUS-LIKE PARTICLES PROTECTS HAMSTERS AFTER A SINGLE DOSE

4.1 Introduction

SARS-CoV-2 is a novel coronavirus that has caused a global pandemic. The results of the pandemic have been devastating; millions have died and many more have been infected [86]. Without a vaccine, it was estimated that 40 million people could die from SARS-CoV-2 infection [87]. Therefore, there was a very urgent need for a vaccine; consequently, nucleic acid-based vaccines, viral vector-based vaccines, subunit vaccines, and inactivated vaccines designed to protect against SARS-CoV-2 are in various stages of clinical trials [88]. In addition, several vaccines have been authorized for use [89-92]. These vaccines target the spike (S) protein of SARS-CoV-2. The spike protein is regarded as an effective vaccine target because it facilitates viral entry by binding to the angiotensin-converting enzyme 2 (ACE2) receptor on the surface of host cells [93]. Therefore, neutralizing antibodies that target the spike protein could play a role in protecting against SARS-CoV-2 [94, 95].

Although the aforementioned vaccine platforms may provide the first generation of vaccines against SARS-CoV-2, nanotechnology [96] has the potential to offer improved vaccine platforms against diseases caused by emerging viruses including SARS-CoV-2.

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Nanoparticles such as virus-like particles (VLPs) are ideal scaffolds for antigen display, because they emulate many of the properties of natural viruses including their size and geometry [79, 96-98]. Moreover, the multivalent display of antigens from nanoscale scaffolds can result in the effective clustering of B cell receptors and greatly enhance their immunogenicity [51]. In fact, a recent report confirmed that the S protein displayed on a nanoscale scaffold was more immunogenic in mice than the S protein administered alone. However, this study used two sequential immunizations (prime + boost) and did not test protective efficacy in mice challenged with SARS-CoV-2 [99]. In the present study, our goal was to create a general platform for nanoparticle-based antigen display that could provide protection against SARS-CoV-2 after a single immunization.

4.2 Methods

4.2.1 Expression and purification of MS2

DNA encoding single chain MS2 coat protein dimer with an AviTag inserted between the fourteenth and fifteenth residues of the second coat protein monomer was cloned into pET-28b between the NdeI and XhoI restrictions sites by GenScript Biotech Corporation (Piscataway, NJ). The MS2 dimer with the inserted AviTag was cotransformed with pAcm-BirA (Avidity LLC) into BL21(DE3) competent E. coli (New England Biolabs) according to the manufacturer’s instructions. The transformation was added to 5 mL of 2xYT media and grown overnight at 37°C. The 5-mL starter culture was then added to 1 L of 2xYT media, which was incubated shaking at 37°C until induction with IPTG (1M; GoldBio) at an OD of 0.6. Immediately after induction, biotin (50 µM) was added to the culture and the incubator temperature was reduced to 30°C. After
overnight incubation, the culture was centrifuged for 7 minutes at 7,000xg and the supernatant was decanted. The cell pellet was homogenized into 25 mL of 20 mM Tris Base (pH 8.0) supplemented with lysozyme (0.5 mg/mL; Alfa Aesar), a protease inhibitor tablet (Sigma-Aldrich), and benzonase (125 units; EMD Millipore). The resuspended cells were then kept on ice and stirred intermittently for 20 minutes. Sodium deoxycholate (Alfa Aesar) was then added to a final concentration of 0.1% (w/v), and the mixture was sonicated for 3 minutes at 35% amplitude with a pulse of 3 seconds on and 3 seconds off (Sonifier S-450, Branson Ultrasonics). The sonication was repeated after allowing the lysate to cool on ice for two minutes. Next, the lysed cells were centrifuged for 30 minutes at 27,000xg. The supernatant was collected, and centrifuged again for 15 minutes at 12,000xg. The resulting supernatant was then diluted 3-fold in 20 mM Tris Base and filtered with a 0.45-µm bottle-top filter (VWR). Then, 25 mL of the diluted lysate was loaded onto four HiScreen Capto Core 700 columns (Cytiva) in series using an AKTA start system. The columns were washed with approximately 3 column volumes of 20 mM Tris Base while fractions were collected. Fractions were subsequently analyzed for purity and recovery of MS2 by using SDS-PAGE. Desirable fractions were pooled, concentrated by using a 10 kDa MWCO centrifugal filter (Millipore Sigma), and further purified by using a Superdex 200 Increase 10/300 column (Cytiva). MS2 was quantified by using a bicinchoninic acid assay (BCA) (Thermo Scientific).

4.2.2 Expression, refolding, and purification of streptavidin (SA)

SA was expressed, refolded, and purified essentially as previously described [76, 77]. Briefly, DNA encoding SA (Addgene plasmid #46367, a gift from Mark Howarth) [76] was transformed into BL21(DE3) cells (New England Biolabs) according
to the manufacturer’s protocol. The transformation was split among four culture tubes each containing 5 mL of 2xYT media, which were incubated overnight at 37°C. Each 5 mL culture was added to one of four 1 L flasks of 2xYT and grown at 37°C. Upon reaching an OD of 0.6, expression of inclusion bodies was induced using IPTG (1 M; GoldBio) and the temperature of the incubator was reduced to 30°C. After incubation overnight, the culture was centrifuged for 7 minutes at 7000xg such that 4 liters of culture resulted in two cell pellets. Each pellet was resuspended in 50 mL of resuspension buffer (50 mM Tris, 100 mM NaCl, pH 8.0) supplemented with lysozyme (1 mg/mL; Alfa Aesar) and benzonase (500 units; EMD Millipore) and was allowed to incubate at 4°C for 1 h with occasional mixing. These mixtures were then homogenized, brought to a concentration of 0.1% (w/v) sodium deoxycholate (Alfa Aesar), and sonicated (Sonifier S-450, Branson Ultrasonics) for 3 minutes at 35% amplitude with a pulse of 3 seconds on and 3 seconds off. The resulting lysate was then centrifuged for 15 minutes at 27,000xg. The supernatant was discarded, and the two pellets were each again resuspended in 50 mL of resuspension buffer supplemented with lysozyme (1 mg/mL; Alfa Aesar) and the lysis procedure was repeated. This procedure resulted in two inclusion body pellets, which were then washed. Each inclusion body pellet was resuspended in 50 mL of wash buffer #1 (50 mM Tris, 100 mM NaCl, 100 mM EDTA, 0.5% (v/v) Triton X-100, pH 8.0), homogenized, and sonicated for 30 seconds at an amplitude of 35%. Each mixture was then centrifuged at 27,000xg for 15 minutes and the supernatant was discarded. This wash was repeated twice. The two inclusion body pellets resulting from the third round of the initial wash were each resuspended in 50 mL of wash buffer #2 (50 mM Tris, 10 mM EDTA, pH 8.0), homogenized, and sonicated for 30 seconds at an amplitude of 35%. Each mixture was then
centrifuged at 15,000xg for 15 minutes. This wash was repeated once. The two resulting washed inclusion body pellets were then completely unfolded by resuspension in 10 mL of a 7.12 M guanidine hydrochloride solution. This mixture was stirred at room temperature for 1 h, and subsequently centrifuged at 12,000xg for 10 minutes. The supernatant was drawn into a syringe, which was loaded onto a syringe pump, and added at a rate of 30 mL/h to 1 L of chilled PBS that was being stirred rapidly. This solution of refolded protein was stirred continuously overnight at 4°C. Insoluble protein was then pelleted by centrifugation at 7,000xg for 15 minutes and discarded. The supernatant containing the folded SA was filtered by using a 0.45-µm bottle-top filter. The resulting filtrate was stirred vigorously, and ammonium sulfate was slowly added to a concentration of 1.9 M to precipitate out protein impurities. After being stirred for 3 h at 4°C, the precipitate was removed by centrifugation for 10 minutes at 7,000xg. The supernatant was then filtered by using a 0.45-µm bottle-top filter. The ammonium sulfate concentration of the resulting filtrate was brought up to a total concentration of 3.68 M and stirred for 3 h at 4°C to precipitate the SA. The SA precipitate was pelleted by centrifugation at 7,000xg for 20 minutes, and resuspended in 20 mL of Iminobiotin Affinity Chromatography (IBAC) binding buffer (50 mM Sodium Borate, 300 mM NaCl, pH 11.0). This SA solution was then passed through 5 mL of Pierce Iminobiotin Agarose (Thermo Scientific) in a gravity flow column (G-Biosciences) that had been pre-equilibrated with 5 column volumes of IBAC binding buffer. The IBAC column containing the bound SA was then washed with 20 column volumes of IBAC binding buffer. Then, 8 column volumes of elution buffer were passed through the column. The eluate was collected, dialyzed into PBS, and
concentrated using a 10 kDa MWCO centrifugal filter (Millipore Sigma). SA was quantified by measuring the UV absorption at 280 nm.

4.2.3 Assembly and purification of MS2-SA VLPs

Biotinylated MS2 was added dropwise to a molar excess of concentrated SA solution that was stirred vigorously in a 5-mL glass vial. After a 30-minute incubation, the MS2-SA VLP was separated from the excess SA through SEC with a Superdex 200 Increase 10/300 column (Cytiva). The MS2-SA VLP was quantified by boiling a small aliquot at 90°C in Nu-PAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) for 30 minutes and running the sample on a polyacrylamide gel. SA standards with known concentrations quantified by UV absorption at 280 nm were also run on the gel. Comparing the intensities of the bands resulting from the SA standards with the intensity of the band representing the SA from the MS2-SA allowed for quantification of the VLP.

4.2.4 Expression and purification of SARS-CoV-2 S proteins

DNA encoding the S-2P [100] and HexaPro [101] prefusion-stabilized versions of the SARS-CoV-2 S ectodomain (residues 1–1208) with a C-terminal T4 fibritin trimerization motif, AviTag, and a his-tag were cloned into pcDNA3.1 between the NcoI and XhoI restriction sites by Gene Universal Inc. (Newark, DE). These plasmids were transfected into Expi293F cells (Thermo Fisher Scientific) using the ExpiFectamine Transfection Kit and protocol (Thermo Fisher Scientific). Five days after transfection, the cells were pelleted by centrifugation for 20 minutes at 5500xg. The supernatant was dialyzed into PBS and passed through 1 mL of HisPur Ni-NTA resin (Thermo Fisher Scientific) in a gravity flow column (G-Biosciences). The column was then washed with
40 mL of wash buffer (42 mM sodium bicarbonate, 8 mM sodium carbonate, 300 mM NaCl, 20 mM imidazole). The S proteins were eluted from the column by incubating the Ni-NTA resin with 3 mL of elution buffer (42 mM sodium bicarbonate, 8 mM sodium carbonate, 300 mM NaCl, 300 mM imidazole) for 5 minutes before allowing for flow by gravity. This elution procedure was repeated twice, resulting in 9 mL of eluate. The eluate was concentrated by using a 10-kDa MWCO centrifugal filter (Millipore Sigma). S proteins were buffer exchanged into 20 mM Tris, 20 mM NaCl, pH 8.0 to allow for *in vitro* biotinylation and were quantified by using the BCA assay (Thermo Scientific).

### 4.2.5 *In vitro* biotinylation of AviTagged MS2 and SARS-CoV-2 S

Biotinylation was performed *in vitro* using a BirA biotin-protein ligase standard reaction kit (Avidity) following the manufacturer’s protocol. In brief, the protein solution (either MS2 or SARS-CoV-2 S) was buffer exchanged into a 20 mM Tris, 20 mM NaCl, pH 8.0 buffer and the protein concentration was adjusted to 45 µM. BirA and a proprietary mixture containing biotin, ATP, and magnesium acetate (Biomix B) was added to the protein solution. This solution was shaken vigorously at 37°C. After 2 h at 37°C, more Biomix B was added, and the solution was nutated at 4°C overnight. The proteins of interest were then purified through SEC with a Superdex 200 Increase 10/300 column (Cytiva) connected to an ÄKTA pure (Cytiva) and controlled by Unicorn 7.2 software (Cytiva). Biotinylated S proteins were quantified by using the BCA assay (Thermo Scientific).

### 4.2.6 Expression and purification of CR3022 and ACE2-Fc
The variable regions of the heavy and light chains of CR3022 [102] were cloned into the TGEX-HC and TGEX-LC vectors (Antibody Design Labs), respectively, according to the manufacturer’s protocol. Likewise, ACE2 (residues 1–615) was cloned into TGEX-HC. The DNA was then transfected into Expi293F cells (Thermo Fisher Scientific) by using the ExpiFectamine Transfection Kit (Thermo Fisher Scientific) following the provided protocol, and the cells were incubated in a humidified incubator at 37°C and 8% CO₂ for 5 days. The cells were then centrifuged at 5500xg for 20 minutes. The supernatant media was diluted 2-fold in PBS and run through a 1-mL MabSelect SuRe column (Cytiva) connected to an ÄKTA start (Cytiva) and controlled by Unicorn start 1.0 software (Cytiva) according to the manufacturer’s operation manual to purify the proteins. CR3022 and ACE2-Fc were quantified by using the BCA assay (Thermo Scientific).

4.2.7 SDS-PAGE

Protein samples were diluted 4-fold in Nu-PAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen). The samples were then boiled at 90°C for 30 minutes. PageRuler Plus Prestained Protein Ladder (Thermo Scientific) and protein samples were pipetted into the wells of a 4%–12% Bis-Tris gel (Invitrogen), which was run in MES-SDS buffer at 4°C for 1 h at 110 V. The gel was stained with SimplyBlue SafeStain (Invitrogen) and subsequently de-stained. Once sufficiently de-stained, the gel was imaged by using the ChemiDoc MP imaging system and Image Lab 5.2.1 software (Bio-Rad).

4.2.8 Preparation of VLP-S

MS2-SA and biotinylated S protein were mixed in a stoichiometric ratio found by using analytical SEC. We used analytical SEC to characterize mixtures consisting of 5 µg
of biotinylated S protein and varying amounts of MS2-SA VLP. The ratio of the mixture that contained the least MS2-SA VLP and also did not have excess S protein appear on the chromatogram was the stoichiometric ratio used to create the VLP-S. The concentration of the VLP-S was adjusted such that the solution contained 0.12 µg of S per µL. The VLP-S were further characterized by use of ELISA, SEC, and DLS as described below.

4.2.9 Characterization of S and VLP-S by ELISA

VLP-S and S protein in PBS were coated onto a Nunc Maxisorp 96-well plate such that each well contained 0.1 µg of S protein in 100 µL. After 1 h, the protein solutions were discarded from the wells and each well was blocked with 200 µL of 5% BSA (EMD Millipore) in PBST (PBS with 0.05% Tween-20) for 45 minutes. The plate was then washed twice with PBST, and CR3022 and ACE2-Fc in 1% BSA in PBST were added to the appropriate wells such that each well contained either one CR3022 or ACE2-Fc molecule per S trimer. One hour later, the wells were washed twice with PBST and a horseradish peroxidase-conjugated anti-human IgG Fc fragment goat antibody (MP Biomedicals; 1:5,000 dilution) in 1% BSA in PBST was added to each well and left to incubate for 1 h. Then, the plate was washed twice with PBST and developed with TMB substrate solution (Thermo Scientific) for 3 minutes; the reaction was then stopped with 0.16 M sulfuric acid. The absorbance of each well at 450 nm was read using a Spectramax i3x plate reader (Molecular Devices) and Gen5 2.07 software (BioTek).

4.2.10 Analytical SEC

A Superdex 200 Increase 10/300 column (Cytiva) connected to an ÄKTA pure (Cytiva) and controlled by Unicorn 7.2 software (Cytiva) was equilibrated with PBS. The
1-mL sample loop was washed with PBS and then 950 µl of either VLP-S solution or S alone was loaded into the sample loop. Each sample included 5 µg of S protein. The sample loop was then flushed with PBS such that the sample was directed through the column at a flowrate of 0.5 mL/min. One column volume of PBS was run through the column. Unicorn 7 (Cytiva) was used to control the system and to output a chromatogram of UV absorbance at 210 nm.

4.2.11 Dynamic light scattering

A UVette (Eppendorf) containing 100 µL of VLP-S at a concentration of approximately 0.05 µg S per µL was loaded into a DynaPro NanoStar Dynamic Light Scattering detector (Wyatt Technology). For each measurement, Dynamics software (Wyatt Technology) was used to allow the temperature to equilibrate to 25°C. Each measurement was the result of 10 acquisitions and was output as % Intensity vs. Radius. Radius and polydispersity (PD) index are presented as the mean ± SD of three measurements, while the plotted size distribution is the result of a single representative measurement.

4.2.12 Negative-stain transmission electron microscopy

Conventional negative-stain transmission electron microscopy (TEM) was performed, as described previously [103]. Briefly, 4 µl of the diluted samples was applied onto glow-discharged 300 mesh copper grids (CF300-Cu; Electron Microscopy Sciences, PA), washed with PBS (1X), and stained in droplets of 1% phosphotungstic acid (PTA, PH 6~7) for 1 min. The grids were then drained from the grid backside and air-dried inside a
petri dish for at least 30 min at room temperature to minimize the negative-stain artifacts of flattening and stacking [104].

4.2.13 **Plunge freezing and cryo-electron microscopy**

4 μl of the VLP suspension was added to a glow discharged copper grid (C-Flat 1.2/1.3, 400 mesh, Protochips). Grids were plunged frozen into liquid ethane by double-sided blotting using Vitrobot Mark IV (ThermoScientific) and stored in liquid nitrogen until imaging. Cryo-electron microscopy (cryo-EM) and cryo-electron tomography (cryo-ET) were performed as described previously on a Titan Krios (ThermoScientific Hillsboro, OR, USA) at 300 kV [105]. Images (defocus of -5 μm) were recorded on a post-GIF Gatan K3 camera in EFTEM mode (4.603 Å/pixel) with a 20-eV slit, CDS counting mode, using SerialEM 3.8 [106]. A total dose of 25-30 e/Å² was used and 34 frames were saved (1.14 e/Å² per frame). Frames were motion-corrected in MotionCor2 [107]. Images were low pass filtered to 10 Å for better visualization and contrast in EMAN2.2 [108].

4.2.14 **Virus and titration assays**

The virus isolate SARS-CoV-2/UT-NCGM02/Human/2020/Tokyo was used in this study and was previously characterized in Syrian hamsters [109].

Virus titrations were performed on Vero E6/TMPRSS2 cells that were obtained from the National Institute of Infectious Diseases, Japan [110]. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotic/antimycotic solution along with G418 (1 mg/ml).
To determine virus titers, confluent Vero E6/TMPRSS2 cells were infected with 100 µl of undiluted or 10-fold dilutions ($10^{-1}$ to $10^{-5}$) of clarified lung or nasal turbinate homogenates. After a 30-minute incubation, the inoculum was removed, the cells were washed once, and then overlaid with 1% methylcellulose solution in DMEM with 5% FBS. The plates were incubated for three days, and then the cells were fixed and stained with 20% methanol and crystal violet to count the plaques.

4.2.15 Hamster immunization study

The immunization study in hamsters was performed after approval by the Institutional Animal Care and Use Committee at the University of Wisconsin. Golden Syrian hamsters (4-week-old females) were immunized with either 60 µg of SARS-CoV-2 S protein presented on the MS2-SA VLP, an equal amount of MS2-SA VLP without the S protein, or an equal volume of sterile phosphate-buffered saline (PBS) by subcutaneous inoculation. Alhydrogel (2% solution; InvivoGen) added at an equal volume was thoroughly mixed with each vaccine preparation before inoculation. Animals were infected by intranasal inoculation with $10^3$ plaque-forming units (PFU) of SARS-CoV-2 while under isoflurane anesthesia. Animals were monitored daily for signs of illness and their body weights were recorded daily. Three days after infection, the animals were humanely sacrificed, and lung tissue and nasal turbinate samples were collected.

Serum was isolated from blood samples collected via the sublingual vein before the immunization and challenge with virus.

4.2.16 Antibody titer by ELISA
The ELISA was performed using a recombinant SASR-CoV-2 S RBD protein produced in Expi293F cells (Thermo Fisher Scientific) and then C-terminal his-tag purified by using TALON metal affinity resin. ELISA plates were coated overnight at 4°C with 50 µl of the RBD protein at a concentration of 2 µg/ml in PBS. After being blocked with PBS containing 0.1% Tween 20 (PBS-T) and 3% milk powder, the plates with incubated in duplicate with heat-inactivated serum diluted in PBS-T with 1% milk powder. Goat anti-hamster IgG secondary antibody conjugated with horseradish peroxidase (Invitrogen; 1:7,000 dilution) was used for detection. Plates were developed with SigmaFast o-phenylenediamine dihydrochloride solution (Sigma), and the reaction was stopped with the addition of 3M hydrochloric acid. The absorbance was measured at a wavelength of 490 nm (OD\text{490}). Background absorbance measurements from serum collected before immunization were subtracted from the absorbance measurements from plasma collected before challenge for each dilution. IgG antibody endpoint titers were defined as the highest plasma dilution with an OD\text{490} cut-off value of 0.15.

4.2.17 \textit{Neutralization assay}

Virus (\textsim 100 PFU) was incubated with the same volume of two-fold dilutions of heat-inactivated serum for 30 minutes at 37°C. The antibody/virus mixture was added to confluent Vero E6/TMPRSS2 cells that were plated at 30,000 cells per well the day prior in 96-well plates. The cells were incubated for 3 days at 37°C and then fixed and stained with 20% methanol and crystal violet solution. Virus neutralization titers were determined as the reciprocal of the highest serum dilution that completely prevented cytopathic effects.

4.2.18 \textit{Biosafety statement}
Research with SARS-CoV-2 was approved by the University of Wisconsin-Madison’s Institutional Biosafety Committee and performed under biosafety level 3 agriculture (BSL-3Ag) containment at the Influenza Research Institute, University of Wisconsin-Madison. The laboratory is approved for such use by the Centers for Disease Control and Prevention. The BSL-3Ag facility used was designed to exceed the standards outlined in *Biosafety in Microbiological and Biomedical Laboratories* (5th edition).

### 4.2.19 Statistics and reproducibility

*In vitro* characterizations of the binding of Fc-ACE2 and CR3022 to the VLP-S constructs using ELISA (Figure 14e and Figure 17f) were each conducted twice independently with three technical replicates for each condition. The data is presented as the mean ± SD. For *in vivo* characterization, there were four groups (receiving either VLP-S, VLP-S, MS2-SA, or PBS) each with three hamsters (n=3). To determine the resulting RBD IgG Endpoint Titers and Neutralizing Antibody titers (Table 1), three independent assays were conducted using sera from each hamster. The data is presented for each independent assay and also as the geometric mean with the geometric SD factor. Bodyweight after challenge with SARS-CoV-2 (Figure 18b) was presented as the mean ± SD and significance was determined by a one-way analysis of variance (ANOVA) and Dunnett post-hoc multiple comparison between groups (α = 0.1). Assumptions of the normality of residuals and homogeneity of variance were validated by the D’Agostino-Pearson test and the Brown-Forsythe test, respectively. Viral titers in the lungs and nasal turbinates of hamsters immunized with either PBS, MS2-SA VLP, VLP-S2Pro or VLP-S6Pro three days after SARS-CoV-2 infection (Figure 18c and Figure 18d) were presented as the geometric mean with geometric SD (n=3) and significance was determined by a one-way
analysis of variance (ANOVA) and Dunnett post-hoc multiple comparison between groups 
\((\alpha = 0.1)\). Assumptions of the normality of residuals and homogeneity of variance were validated by the Shapiro-Wilk test and the Brown-Forsythe test, respectively. All statistical analysis was carried out using Excel 2013 (Microsoft) and Prism 8 (GraphPad).

4.3 Results and Discussion

4.3.1 Generation of virus-like particles displaying the SARS-CoV-2 spike protein

We first sought to develop a general platform for the VLP-based multivalent display of the S protein of SARS-CoV-2. VLPs are comprised of coat proteins that self-assemble to form repetitive, dense arrays of antigen that emulate the size and geometry of natural viruses [97]. We generated VLPs coated with streptavidin (SA) that display biotinylated antigens, such as biotinylated SARS-CoV-2 S protein (Figure 10a), based on the very high affinity biotin-streptavidin interaction.

Specifically, we generated VLPs based on the coat protein of the RNA bacteriophage MS2 [79, 80]. MS2 consists of 180 monomeric coat proteins that self-assemble to form an icosahedral structure consisting of 90 homodimers. Peabody et al. generated a variant of the MS2 coat protein in which the two subunits of the dimer were genetically fused and found that a surface loop on this single-chain dimer could tolerate the insertion of a peptide [82]. Accordingly, we generated a single-chain MS2 coat protein dimer wherein the second monomer had an AviTag inserted in this surface loop. The inserted AviTag allows for site-specific biotinylation by the enzyme BirA. DNA encoding this MS2-AviTag construct was co-expressed with BirA in BL21(DE3) competent Escherichia coli (E. coli) cells. Following expression, the cells were lysed and the MS2-AviTag was purified by
using HiScreen Capto Core 700 columns and size exclusion chromatography (SEC). The purified MS2-AviTag was partially biotinylated due to its co-expression with BirA. A commercially available kit was then used to further biotinylate the MS2-AviTag in vitro, which resulted in near 100% biotinylation. The MS2-Biotin was then added dropwise to an excess of SA, which had been expressed as inclusion bodies, refolded, and purified using Iminobiotin Affinity Chromatography (IBAC). The resulting MS2-SA VLPs were separated from the excess SA through SEC. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine that each purified MS2-SA VLP contained approximately 72 streptavidin molecules (Figure 11). The purified MS2-SA VLPs were further characterized by analytical SEC (Figure 10b), dynamic light scattering (DLS; Figure 10c), and negative-stain transmission electron microscopy (NS-TEM; Figure 10d). Characterization by DLS indicated that the purified MS2-SA VLPs were approximately 50 nm in diameter, whereas characterization by NS-TEM indicated uniform particles with a diameter of approximately 30 nm. The larger average size indicated by DLS may arise because the scattering intensity is proportional to the sixth power of the radius, resulting in a disproportionately higher weighting to larger particles.
Figure 10. Assembly of VLP-S and characterization of MS2-SA VLP. a) Scheme illustrating assembly of VLP-S, where biotinylated MS2 (yellow, PDB: 2MS2) is added to streptavidin (red, PDB: 3RY2) to create the VLP. S (green, PDB: 6VSB) biotinylated at the C-terminus is mixed with the VLP to create VLP-S. b) Size exclusion chromatography trace for MS2-SA VLP. The column void volume is 7.2 mL. c) Characterization of the MS2-SA VLP by dynamic light scattering (mean ± SD; n=3). d) Negative-stain transmission electron micrograph of MS2-SA VLPs.
Figure 11. Characterization of MS2 to SA stoichiometry using SDS-PAGE. Amount of MS2 in heated MS2-SA VLP was compared to amount of MS2 in unheated MS2-SA VLP to determine that approximately 80 percent of MS2 is occupied by SA. Excess biotin was added to the unheated sample to occupy all unoccupied biotin binding sites prior to the addition of SDS. β-mercaptoethanol was added to all samples.

We next generated a biotinylated variant of the SARS-CoV-2 S protein that could be displayed on the MS2-SA VLPs. Wrapp et al. recently reported a prefusion-stabilized variant of the SARS-CoV-2 S protein, S-2P, which contains 2 proline substitutions [100]. To make a version of this variant that was compatible with display on the MS2-SA VLPs, we created plasmids encoding the stabilized prefusion S ectodomain with a C-terminal AviTag and a his-tag, which we termed S$_{2P}$Pro. The AviTag allows biotinylation and subsequent conjugation to the VLPs, whereas the his-tag allows purification by use of immobilized metal affinity chromatography (IMAC). We expressed the S$_{2P}$Pro protein in Expi293F cells and purified the secreted protein from the cell culture media by using IMAC. The protein was then biotinylated enzymatically in vitro by BirA. Finally, the
protein was separated from BirA and other impurities by using SEC and the purity was characterized by use of SDS-PAGE (Figure 12).

![SDS-PAGE gel of S\textsubscript{2Pro} before and after deglycosylation with PNGase F.](image)

**Figure 12.** SDS-PAGE gel of S\textsubscript{2Pro} before and after deglycosylation with PNGase F.

### 4.3.2 In vitro characterization of VLP-S constructs

The purified, biotinylated S\textsubscript{2Pro} protein was then mixed with the MS2-SA VLPs to form VLP-S\textsubscript{2Pro}. SDS-PAGE was used to determine that each purified VLP-S\textsubscript{2Pro} particle contained approximately 18 S\textsubscript{2Pro} molecules (Figure 13). Further SDS-PAGE analysis of the VLP-S\textsubscript{2Pro} revealed the expected three distinct bands (Figure 14a): the upper band runs alongside S protein alone and appears at \(\sim 140\) kDa, which corresponds to the approximate molecular weight of a single monomer of the S trimer; the middle band appears at the molecular weight of an MS2 coat protein dimer (\(\sim 29\) kDa); and the lower band corresponds to the molecular weight of a monomer of SA (\(\sim 14\) kDa). This characterization indicates
that the VLP-S_{2Pro} is pure and consists of only S_{2Pro}, SA, and MS2. The VLP-S_{2Pro} construct was also characterized by using analytical SEC (Figure 14b). The UV trace of the VLP-S_{2Pro} is represented by a solid line, which appears as a single peak with no trailing shoulder. The lack of a trailing shoulder suggests that there is little to no unbound S_{2Pro} protein in the VLP-S_{2Pro} solution, as the UV trace of the S_{2Pro} protein alone results is a single peak that slightly trails the peak of the VLP-S and is represented by a dashed line. Furthermore, the locations of the peaks are consistent with the constructs’ size relative to the size of the molecular weight standard thyroglobulin (660 kDa). The location at which thyroglobulin elutes is represented by a vertical gray line.

Figure 13. Characterization of MS2-SA VLP to S_{2Pro} stoichiometry using SDS-PAGE. Amount of unbound MS2-SA in VLP-S_{2Pro} was compared to MS2-SA VLP standards to determine approximately 25 percent of MS2-SA in VLP-S_{2Pro} was occupied by S_{2Pro}. A heated control was included to ensure the same amount of VLP was present within the VLP-S_{2Pro} and 100% VLP standard. Excess biotin was added to the unheated samples to occupy all unoccupied biotin binding sites prior to the addition of SDS. β-mercaptoethanol was added to all samples.
We characterized the VLP-$S_{2\text{Pro}}$ constructs by DLS (Figure 14c) and then by NS-TEM (Figure 14d) to confirm the presence and coating efficiency of biotinylated $S_{2\text{Pro}}$ on the VLP. Consistent with biochemical characterization, VLP-$S_{2\text{Pro}}$ displayed clear three-component layers, from outside to inside, prefusion-stabilized variants of $S_{2\text{Pro}}$, SA, and MS2 (Figure 14d). Compared to the naked MS2-SA, glycoprotein $S_{2\text{Pro}}$ decorates the exterior of VLP-$S_{2\text{Pro}}$ (Figure 14d, white arrowheads), forming a ~20 nm layer of a spike-containing protein shell. This result is consistent with expectations, as the S protein (with the trimerization domain and C-terminal AviTag) would theoretically be approximately 20 nm in length. Finally, to ensure that the S proteins remained properly folded after conjugation to the VLPs, we assessed the binding of ACE2-Fc and the receptor-binding domain (RBD)–binding monoclonal antibody CR3022 to $S_{2\text{Pro}}$ protein alone and to VLP-$S_{2\text{Pro}}$ (Figure 14e). ACE2 is the cellular receptor for SARS-CoV-2 and binds to the receptor binding motif of the S protein [111]. A common mechanism of SARS-CoV-2 neutralization is the inhibition of S protein binding to ACE2, so it is important to demonstrate that the ACE2 binding site is properly folded [112, 113]. CR3022 is an antibody that binds to the S protein RBD outside of the ACE2 binding site [102, 112]. ELISA showed that both ACE2-Fc and CR3022 can bind to the $S_{2\text{Pro}}$ protein alone and to VLP-$S_{2\text{Pro}}$. This analysis demonstrates that the protein epitopes needed to elicit a neutralizing immune response to SARS-CoV-2 are correctly folded and accessible.
Figure 14. Characterization of S<sub>2Pro</sub> and VLP-S<sub>2Pro</sub>. a) SDS-PAGE characterization of S<sub>2Pro</sub> and VLP- S<sub>2Pro</sub>. The VLP- S<sub>2Pro</sub> has been boiled to disrupt the streptavidin-biotin conjugation. b) Size exclusion chromatography traces for S<sub>2Pro</sub> (dashed line) and VLP- S<sub>2Pro</sub> (solid line). The vertical gray line represents the peak elution volume of the molecular weight standard thyroglobulin (660 kDa). The column void volume is 7.2 mL. c) Characterization of the VLP- S<sub>2Pro</sub> (solid line) by dynamic light scattering. d) Negative-stain transmission electron micrographs of S<sub>2Pro</sub> incorporated on the surface of MS2-SA VLPs. Arrowheads (white) indicate the S<sub>2Pro</sub> proteins on the VLP surface. e) Characterization of the binding of Fc-ACE2 (gray) and CR3022 (white) to S<sub>2Pro</sub> and VLP-S<sub>2Pro</sub> by ELISA (mean ± SD, n=6: two independent assays, each with three technical replicates).

We also generated VLPs displaying multiple copies of a second prefusion-stabilized variant of the S protein, called HexaPro, which was reported by Hsieh et al. to be more
stable than S-2P and give a higher expression yield [101]. We expressed a variant of HexaPro containing a C-terminal AviTag and a his-tag, which we termed S\textsubscript{6Pro} (Figure 15). VLP-S\textsubscript{6Pro} were generated and characterized (Figure 16; Figure 17) as described above for VLP-S\textsubscript{2Pro}. In addition, to preserve the sample’s native integrity, minimize conformational changes possibly introduced during the negative stain process, and further confirm the incorporation of spike proteins, we performed cryo-electron microscopy (cryo-EM) on the VLP-S\textsubscript{6Pro} constructs. The MS-SA core was an approximately icosahedral sphere 30 nm in diameter and S\textsubscript{6Pro} spikes were studded on the core and formed the outer shell (Figure 17e), the morphology of which was comparable to the previous reported structure of S\textsubscript{6Pro} (EMD: 22221 [101]).

![SDS-PAGE gel of S\textsubscript{6Pro} before and after deglycosylation with PNGase F.](image)

Figure 15. SDS-PAGE gel of S\textsubscript{6Pro} before and after deglycosylation with PNGase F.
Figure 16. Characterization of MS2-SA VLP to S$_6$Pro stoichiometry using SDS-PAGE. Amount of unbound MS2-SA in VLP-S$_6$Pro was compared to MS2-SA VLP standards to determine approximately 25 percent of MS2-SA in VLP-S$_6$Pro was occupied by S$_6$Pro. A heated control was included to ensure the same amount of VLP was present within the VLP-S$_6$Pro and 100% VLP standard. Excess biotin was added to the unheated samples to occupy all unoccupied biotin binding sites prior to the addition of SDS. β-mercaptoethanol was added to all samples.
Figure 17. Characterization of $S_{6Pro}$ and VLP-$S_{6Pro}$. a) SDS-PAGE characterization of $S_{6Pro}$ and VLP-$S_{6Pro}$. The VLP-$S_{6Pro}$ has been boiled to disrupt the streptavidin-biotin conjugation. b) Size exclusion chromatography traces for $S_{6Pro}$ (dashed line) and VLP-$S_{6Pro}$ (solid line). The vertical gray line represents the peak elution.
volume of the molecular weight standard thyroglobulin (660 kDa). The column void volume is 7.2 mL. c) Characterization of the VLP- S₆PrO (solid line) by dynamic light scattering. d) Negative-stain transmission electron micrographs of S₆PrO incorporated on the surface of MS2-SA VLPs. Arrowheads (white) indicate the S₆PrO proteins on the VLP surface. e) Cryo-EM of vitrified VLP-S₆PrO. The inset shows a low-pass filtered to 10 Å volume of HexaPr structure (EMD: 22221, reported previously) for comparison. Arrowheads (black) indicate the representative S₆PrO proteins on the VLP surface. f) Characterization of the binding of Fc-ACE2 (gray) and CR3022 (white) to S₆PrO and VLP-S₆PrO by ELISA (mean ± SD, n=6: two independent assays, each with three technical replicates).

4.3.3 Protective efficacy of a single dose of the VLP-S constructs

We next evaluated the antibody responses elicited by these nanoparticle-based vaccine candidates in Syrian hamsters. Our collaborators in the Kawaoka Lab [109] and others [114] have demonstrated that Syrian hamsters are highly susceptible to SARS-CoV-2 infection and present with pathological phenotypes similar to those of infected humans, making hamsters an ideal animal model to evaluate vaccine candidates. Hamsters (4 groups; 3 animals/group) were immunized with VLP-S₂PrO, VLP-S₆PrO, MS2-SA VLPs alone, or PBS along with Alhydrogel, an aluminum hydroxide base adjuvant. The hamsters were bled 28 days after immunization to characterize their antibody responses (Figure 18a). Hamsters immunized once with the VLP-S conjugates had appreciable levels of IgG antibodies against the RBD of the S protein as determined by ELISA, with endpoint titers ranging from 2.6x10⁴ to 8.2x10⁴ and high neutralizing antibody titers (representing the reciprocal of the highest dilution that completely prevented cytopathic effects) ranging from 320–640 (Table 1). In contrast, as expected, negligible anti-S antibodies were detected in hamsters immunized with the controls (VLPs alone or PBS).

Four weeks after immunization, the animals were intranasally inoculated with 10³ plaque-forming units of SARS-CoV-2/UT-NCGM02/Human/2020/Tokyo [109]. While
the animals in both control groups experienced significant weight loss, those immunized with VLP-S\textsubscript{2Pro} had recovered their average initial weight by day 3, and those immunized with VLP-S\textsubscript{6Pro} showed a slight increase in body weight over this 3-day period (Figure 18b).

Three days after virus challenge, which is when virus levels in the lungs peak [109], the animals were sacrificed and lung and nasal turbinate samples were collected. As expected, animals in both control groups (PBS and MS2-SA VLPs) had high viral loads in the lungs; however, in hamsters immunized with VLP-S\textsubscript{2Pro} or VLP-S\textsubscript{6Pro} no infectious virus was detected in the lungs (Figure 18c). The lack of infectious virus in the lungs was consistent with the differences observed in body weight change between the vaccine and control groups. Moreover, despite the intranasal mode of challenge with SARS-CoV-2, the hamsters immunized with VLP-S\textsubscript{2Pro} or VLP-S\textsubscript{6Pro} had less virus in their nasal turbinates (Figure 18d), with mean titers more than 150-fold lower (VLP-S\textsubscript{2Pro}) and more than 700-fold lower (VLP-S\textsubscript{6Pro}) relative to MS2-SA VLP controls. These results ultimately suggest that the multivalent, nanoparticle-based display of the SARS-CoV-2 spike protein could serve as an effective vaccine against SARS-CoV-2 after just a single dose. Moreover, this nanoparticle-based platform may be broadly applicable to the development of vaccines against other emerging pathogens.
Figure 18. Protective efficacy of VLP-S. a) Schedule for vaccination of hamsters, serum collection, infection, and organ collection. b) Body weight of hamsters immunized with a single dose of either VLP-S2Pro (---), VLP-S2Pro ( ), MS2-SA VLP (- - - - -), or PBS (- - - - - ) after SARS-CoV-2 infection (mean ± SD, n=3 hamsters). ns: not statistically significant, **p < 0.01, determined by a one-way analysis of variance (ANOVA) and Dunnett post-hoc multiple comparison between groups (α = 0.1). Assumptions of the normality of residuals and homogeneity of variance were validated by the D’Agostino-Pearson test and the Brown-Forsythe test, respectively. c) Viral titer in the lungs of hamsters immunized with either PBS, MS2-SA VLP, VLP-S2Pro or VLP-S6Pro three days after SARS-CoV-2 infection (geometric mean with geometric SD, n=3 hamsters). † - No infectious virus was detected in the lungs of hamsters immunized with VLP-S2Pro or VLP-S6Pro (detection limit 10 PFU/g). ns: not statistically significant, ****p < 0.0001, determined by a one-way analysis of variance (ANOVA) and Dunnett post-hoc multiple comparison between groups (α = 0.1). Assumptions of the normality of residuals and homogeneity of variance were validated by the Shapiro-Wilk test and the Brown-Forsythe test, respectively. d) Viral titer in the nasal turbinates of hamsters immunized with either PBS, MS2-SA VLP, VLP-S2Pro or VLP-S6Pro three
days after SARS-CoV-2 infection (geometric mean with geometric SD, n=3 hamsters). ns: not statistically significant, *p < 0.1, determined by a one-way analysis of variance (ANOVA) and Dunnett post-hoc multiple comparison between groups (α = 0.1). Assumptions of the normality of residuals and homogeneity of variance were validated by the Shapiro-Wilk test and the Brown-Forsythe test, respectively.

Table 1. Antibody responses to single immunization of VLP-S in Syrian hamsters

<table>
<thead>
<tr>
<th>Vaccine Group</th>
<th>Animal #</th>
<th>RBD IgG Endpoint Titer*</th>
<th>Neutralizing Antibody Titer#</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Replicate</td>
<td>Geometric Mean</td>
<td>Geometric SD Factor</td>
</tr>
<tr>
<td>PBS</td>
<td>1</td>
<td>&lt;10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&lt;10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&lt;10</td>
<td>-</td>
</tr>
<tr>
<td>MS2-SA VLP</td>
<td>1</td>
<td>&lt;10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&lt;10</td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>&lt;10</td>
<td>-</td>
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<td>1</td>
<td>20,480</td>
<td>35,113</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>81,920</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20,480</td>
<td>320</td>
</tr>
<tr>
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<td>81,920</td>
<td>70,225</td>
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<tr>
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<td>2</td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>40,960</td>
<td>320</td>
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</tbody>
</table>

* Viral antibody endpoint titers against the RBD (receptor-binding domain) from three independent assays (three animals in each group). Endpoint titers using 2-fold diluted sera were expressed as the reciprocal of the highest dilution with an optical density at 490 nm cutoff value >0.15; sera were collected on day 28 after immunization.

# Viral neutralization titers from three independent assays (three animals in each group). Endpoint titers using 2-fold diluted sera were expressed as the reciprocal of the highest dilution that completely prevented cytopathic effects; sera were collected on day 28 after immunization.

4.4 Conclusion and Future Directions

While we have demonstrated that VLP-S provides protection against an early isolate of SARS-CoV-2, spike-based vaccines have shown reduced efficacy against emerging SARS-CoV-2 variants [115-117]. Therefore, in future studies it will be
important to evaluate the protective efficacy of VLP-S against SARS-CoV-2 variants. In addition, it may be of interest to develop broadly protective coronavirus immunogens that better protect against SARS-CoV-2 variants. We have identified the conserved S2 subunit of the SARS-CoV-2 spike protein as an immunogen that may provide broad protection against not only SARS-CoV-2 variants, but also against other zoonotic coronaviruses. We discuss our assessment of the S2 subunit as a broadly protective coronavirus immunogen in Chapter 5.
CHAPTER 5.  AN ASSESSMENT OF THE S2 SUBUNIT OF THE SARS-COV-2 SPIKE PROTEIN AS A BROADLY PROTECTIVE IMMUNOGEN

5.1 Introduction

The novel coronavirus SARS-CoV-2 has had an astounding impact on world health since it was first identified in December 2019. In fact, over 3 million people worldwide have died as a result of contracting the virus, and many more have been infected [86]. A wide variety of SARS-CoV-2 vaccine candidates are being developed, including nucleic acid-based vaccines, viral vector-based vaccines, subunit vaccines, and inactivated vaccines [88]. In particular, a number of vaccines that target the SARS-CoV-2 spike (S) protein have been authorized for use [89-92]. The spike protein is a glycoprotein displayed on the surface of the SARS-CoV-2 virus that allows the virus to bind to host cells through its S1 subunit and fuse to the host cell membrane through its S2 subunit (Figure 19a). This key role in viral attachment and entry into cells has made the S protein an effective vaccine target and several S protein-based vaccines have been shown to successfully prevent SARS-CoV-2 infection [89-92]. However, the threat of emerging variants that may escape vaccine-mediated immunity is a cause for concern [115-117]. In addition, some zoonotic coronaviruses have been identified to have pandemic potential [118, 119] and others such as SARS-CoV-1 and MERS-CoV are already known to cause severe disease in humans. Therefore, a broadly protective coronavirus vaccine may prove useful.
The S2 subunit of the spike protein has been identified as a promising target for a broadly protective coronavirus vaccine, as it is considerably more conserved than the S1 subunit. In particular, the functionally important fusion peptide region in the S2 subunit may be an attractive target for cross-reactive antibodies [120, 121]. Antibodies targeting the S2 subunit have been isolated from convalescent COVID-19 patients and found to neutralize SARS-CoV-2 [122-126]. Even S2-specific antibodies that do not directly neutralize SARS-CoV-2 may mitigate pathological burden through Fc effector functions [127]. Furthermore, antibodies targeting the S2 subunit have been found to be cross-reactive among coronaviruses [128-130]. For instance, Wang et al. isolated two human monoclonal antibodies from immunized humanized mice that displayed cross-reactivity against the spike proteins of betacoronaviruses including SARS-CoV, SARS-CoV-2, MERS-CoV, and HCoV-OC43 [130]. Some cross-reactive S2-specific antibodies are also capable of neutralizing across coronavirus types [123-126, 131]. For example, Pinto et al. [125] described a human S2-specific monoclonal antibody that showed neutralization activity against not only authentic SARS-CoV-2 but also against viruses pseudotyped with SARS-CoV-1 S, Pangolin Guangdong 2019 S, MERS-CoV S, and OC43 S. Collectively, these findings indicate that S2-based vaccines may provide broad protection against coronaviruses.

Recently, an S2 immunogen was evaluated by Ravichandran et al. [132]. They found that compared to the spike ectodomain and other S1-based antigens, the S2 immunogen generated relatively low anti-spike antibody titers and weak SARS-CoV-2 neutralization titers. We hypothesized that the multivalent display of the S2 subunit with an appended C-terminal trimerization domain to promote its stability might help elicit a strong response
against S2, as the multivalent display of antigens has been shown to generate strong immune responses [51]. Moreover, we hypothesized that such an immunogen without the immunodominant S1 subunit would elicit a strong response targeting the S2 subunit that would have otherwise been directed towards the S1 subunit. In fact, antibodies to the S1 subunit’s receptor binding domain alone are found at much higher levels than S2-specific antibodies in SARS-CoV-2 infected individuals [133]. The immunosubdominance of the S2 subunit may be attributed to its extensive glycosylation [133]. To that end, we generated virus-like particles (VLPs) that multivalently displayed either the S2 subunit of the SARS-CoV-2 spike protein or an S2 variant designed to prevent potential proteolytic cleavage at the S2’ cut site. After characterizing the VLP-S2 constructs in vitro, we used them to vaccinate hamsters. The immunized hamsters showed significantly lower viral titers in the lungs and nasal turbinates after challenge with SARS-CoV-2 compared to control hamsters. Moreover, sera from the hamsters immunized with VLP-S2 showed substantial cross-reactivity to the spike proteins of the SARS-CoV-2 variant B.1.351, SARS-CoV-1, and the four endemic human coronaviruses. These results suggest that the S2 subunit of the SARS-CoV-2 spike protein may be an effective target for a broadly protective coronavirus vaccine.

5.2 Methods

5.2.1 Expression and purification of SARS-CoV-2 S2 and S2mutS2’ proteins

The gene encoding the S2 subunit of the SARS-COV-2 HexaPro [101] spike protein (residues 686 to 1208) with an N-terminal mouse Ig Kappa signal peptide and C-terminal T4 fibritin trimerization domain, AviTag, and his-tag was cloned into pcDNA3.1
between the NcoI and XhoI restriction sites by Gene Universal, Inc. (Newark DE). The S2\textsubscript{mutS2'} variant was created such that S2 residues 814 and 815 were mutated to glycine residues to eliminate the S2’ protease cut site. These plasmids were transfected into Expi293F cells using the ExpiFectamine Transfection Kit (Thermo Fisher Scientific) and associated protocol. The cells were incubated for 5 days, after which the cultures were centrifuged at 5,500\( \times \)g for 20 minutes. The supernatant was dialyzed into PBS and then was allowed to flow through 1 mL of of HisPur Ni-NTA resin (Thermo Scientific) in a gravity flow column (G-Biosciences) that had been washed with DI water and pre-equilibrated with phosphate-buffered saline (PBS). The column was then washed with 90 column volumes of wash buffer (42 mM sodium bicarbonate, 8 mM sodium carbonate, 300 mM NaCl, 20 mM imidazole). The protein was eluted by incubating the resin in 3 mL of elution buffer (42 mM sodium bicarbonate, 8 mM sodium carbonate, 300 mM NaCl, 300 mM imidazole) for 5 minutes before allowing the elution buffer to flow through the column. The eluate was collected. This elution procedure was repeated twice more such that a total of 9 mL of eluate was collected. The eluate was buffer exchanged into 20 mM Tris, 20 mM NaCl, pH 8.0, to prepare for \textit{in vitro} biotinylation. The concentration of the protein solutions was quantified using the BCA assay (Thermo Scientific).

5.2.2 Expression and purification of MS2

The following protocol regarding the expression and purification of MS2 has been previously described [134]. The DNA sequence corresponding to a single chain dimer of MS2 coat protein with an AviTag inserted between the fourteenth and fifteenth residues of the second coat protein monomer was cloned into pET-28b between the NdeI and XhoI restriction sites by GenScript Biotech Corporation (Piscataway, NJ). This plasmid and a
plasmid coding for pAcm-BirA (Avidity LLC) were co-transformed into BL21(DE3) *Escherichia coli* (*E. coli*) (New England BioLabs). The transformation was added to 5 mL of 2xYT that had been supplemented with kanamycin and chloramphenicol. This small culture was incubated in a shaking incubator overnight at 37°C. The following morning, the 5 mL culture was added to 1 L of 2xYT that had been supplemented with kanamycin and chloramphenicol. The 1 L culture was placed in a shaking incubator at 37°C. Once the culture’s optical density reached 0.6, expression of the MS2 and BirA was induced with IPTG (1 mM; GoldBio). The culture was also supplemented with approximately 12.5 µg of biotin, and remained shaking in the incubator overnight at 30°C. After the overnight expression, the culture was centrifuged at 7000 xg for 7 minutes to pellet the cells. The cell pellet was then homogenized into 25 mL of 20 mM Tris buffer (pH 9.0) supplemented with lysozyme (0.5 mg/mL; Alfa Aesar), a protease inhibitor tablet (Sigma-Aldrich), and benzonase (125 units; EMD Millipore). The resulting cell suspension was kept on ice for 20 minutes while occasionally mixing. Next, sodium deoxycholate was added to a final concentration of 0.1% (w/v). The cells were kept on ice and sonicated for 3 minutes at an amplitude of 35% with 3 second pulses (Sonifier S-450, Branson Ultrasonics). This sonication procedure was repeated after allowing the cells to cool on ice for at least 2 minutes. The resulting lysate was centrifuged at 27,000 xg for 30 minutes. The supernatant was collected and was centrifuged again at 12,000 xg for 15 minutes. The supernatant resulting from the second centrifugation was diluted 3-fold with 20 mM Tris, pH 8.0, and filtered using a 0.45 µm bottle-top filter. The filtrate was then run through four HiScreen Capto Core 700 columns (Cytiva) in parallel according to the manufacturer’s operating instructions, resulting in fractions that contained MS2. The fractions were run on an SDS-
PAGE gel to assess MS2 purity and recovery. Fractions containing pure MS2 were pooled, concentrated using a 10 kDa MWCO centrifugal filter (Millipore Sigma), and further purified using a Superdex 200 increase 10/300 SEC column (Cytiva). The SEC fractions containing MS2 were pooled and buffer exchanged into 20 mM Tris, 20 mM NaCl, pH 8.0, in preparation for in vitro biotinylation. MS2 was quantified using the BCA assay (Thermo Scientific).

5.2.3 Expression, refolding, and purification of streptavidin (SA)

The following protocol regarding the expression, refolding, and purification of SA has been previously described and was adapted from methods documented by Fairhead et al. and Howarth et al. [76, 77, 134]. A plasmid encoding SA (Addgene plasmid #46367, a gift from Mark Howarth) was transformed into BL21(DE3) E. coli. The transformation was added to 5 mL of 2xYT supplemented with ampicillin, and this small culture was grown overnight in a shaking incubator at 37°C. The next morning the culture was added to four, 1 L shake flasks of 2xYT supplemented with ampicillin. These larger cultures were placed in a shaking incubator at 37°C until the cultures’ OD reached 0.6, at which point the expression of streptavidin as inclusion bodies was induced with IPTG (1 mM; GoldBio), and the temperature of the incubator was reduced to 30°C. After overnight incubation, the cultures were centrifuged at 7,000 xg for 15 minutes such that there were two cell pellets. The two resulting cell pellets were each homogenized into 50 mL of resuspension buffer (50 mM Tris, 100 mM NaCl, pH 8.0) supplemented with lysozyme (1 mg/mL; Alfa Aesar) and benzonase (500 units; EMD Millipore). The homogenized cells were incubated at 4°C for at least 30 minutes. After this incubation step, the cells were further homogenized and sodium deoxycholate was added to a final concentration of 0.1%
(w/v) before sonicating (Sonifier S-450, Branson Ultrasonics) for 3 minutes with 3 second pulses at 35% amplitude. The lysed cells were then centrifuged at 27,000xg for 15 minutes. The supernatant was discarded, and the lysis procedure was repeated. When the lysis step was repeated the incubation time at 4°C prior to sonication was reduced to 15 minutes. After the lysis procedure had been performed twice, the two resulting inclusion body pellets were each suspended in 50 mL wash buffer (50 mM Tris, 100 mM NaCl, 100 mM EDTA, 0.5% (v/v) Triton X-100, pH 8.0), homogenized, sonicated for 30 seconds at an amplitude of 35%, and centrifuged at 27,000xg for 15 minutes. This wash procedure was repeated twice more. The resulting inclusion body pellets were then suspended in 50 mL of a second wash buffer (50 mM Tris, 10 mM EDTA, pH 8.0), homogenized, sonicated for 30 seconds at an amplitude of 35%, and centrifuged at 15,000xg for 15 minutes. This second wash step was performed twice. The washed inclusion body pellets were then unfolded by being homogenized into 10 mL of a 7.12 M guanidine hydrochloride solution. This solution of unfolded streptavidin in guanidine hydrochloride was stirred at room temperature for an hour, after which it was centrifuged at 12,000xg for 10 minutes. The supernatant was then added dropwise at a rate of 30 mL/h to 1L of chilled PBS that was being stirred vigorously. This rapid dilution of the streptavidin and guanidine hydrochloride allowed for the streptavidin to fold properly. The folded streptavidin in PBS was stirred overnight at 4°C, and was then centrifuged at 7,000xg for 15 minutes to remove insoluble protein. The supernatant was filtered using a 0.45 µm bottle-top filter, and was then stirred while ammonium sulfate was slowly added to a concentration of 1.9 M. This concentration of ammonium sulfate serves to precipitate out impurities. The solution was stirred for at least 3 h at 4°C, after which it was centrifuged at 7,000xg for 15 minutes to
pellet the precipitated impurities. The supernatant was filtered using a 0.45 µm bottle-top filter, and was then stirred while ammonium sulfate was added to a total concentration of 3.68 M. This concentration of ammonium sulfate precipitates the streptavidin. The solution was stirred for at least 3 h at 4°C before being centrifuged at 7,000xg for 20 minutes to pellet the streptavidin. The supernatant was discarded, and the pelleted streptavidin was suspended in 20 mL of Iminobiotin Affinity Chromatography (IBAC) binding buffer (50 mM Sodium Borate, 300 mM NaCl, pH 11.0). This streptavidin solution was then allowed to flow through 5 mL of Pierce Iminobiotin Agarose (Thermo Scientific) in a gravity flow column (G-Biosciences) that had been rinsed with DI water and pre-equilibrated with IBAC binding buffer. The column was next washed with 20 column volumes of IBAC binding buffer, and the streptavidin was eluted from the column with 6 column volumes of elution buffer (20 mM KH$_2$PO$_4$, pH 2.2). The eluate was collected, dialyzed into PBS, and concentrated using a 10 kDa MWCO centrifugal filter (Millipore Sigma). The concentration of streptavidin was quantified by measuring the UV absorption at 280 nm.

5.2.4 Expression and purification of 0304-3H3 antibody

The genes encoding the variable regions of the heavy chain and light chain of the 0304-3H3 antibody [122] were cloned into the TGEX-HC and TGEX-LC vectors (Antibody Design Labs), respectively, by Gene Universal, Inc. (Newark, DE). The plasmids were co-transfected in a 2:1 light chain to heavy chain ratio into Expi293F cells using the ExpiFectamine Transfection Kit (Thermo Fisher Scientific) and associated protocol. After a 4-day incubation, the culture was centrifuged at 5,500xg for 20 minutes. The supernatant was diluted in PBS and filtered before being purified by using a 1 mL
MabSelect SuRe column (Cytiva) according to the manufacturer’s protocol. The concentration of the purified 0304-3H3 antibody was quantified using the BCA assay (Thermo Scientific).

5.2.5 In vitro biotinylation of AviTagged proteins

The BirA-500 kit (Avidity LLC) and general protocol were used to biotinylate the AviTagged MS2 and S2 proteins. In brief, the proteins were buffer exchanged into 20 mM Tris, 20 mM NaCl, pH 8.0. The concentration of protein in solution was adjusted to either 45 µM for MS2 or 15 µM for S2 and S2mutS2' before adding the recommended amount of Biomix B (a proprietary mixture of biotin, ATP, and magnesium acetate). The recommended amount of BirA was added to the MS2 solution, while three times the recommended amount of BirA was added to the S2 solutions. These solutions were incubated at 37°C for 2 h while shaking vigorously. After the two-hour incubation, the solutions were moved to a nutator at 4°C for overnight incubation. Finally, the biotinylated proteins were separated from the biotinylation reagents using a Superdex 200 increase 10/300 column (Cytiva) and quantified by using the BCA assay (Thermo Scientific).

5.2.6 Assembly of MS2-SA VLP

The assembly of MS2-SA VLP has been previously described [134]. Approximately 1 mL of biotinylated MS2 at a concentration of about 0.7 mg/mL was added 2.5 µL at a time to stirred streptavidin that was in approximately 20-times molar excess and at a concentration of around 60 mg/mL. This mixture was stirred for 30 minutes at room temperature before the MS2-SA VLP was separated from excess streptavidin using a Superdex 200 increase 10/300 column (Cytiva). To quantify the purified MS2-SA VLP,
a small sample of the MS2-SA VLP in Nu-PAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) was heated at 90°C for at least 10 minutes and run on an SDS-PAGE gel with heated streptavidin standards of known mass.

5.2.7 Assembly of VLP-S2 and VLP-S2_mutS2⁺

MS2-SA and biotinylated S2 or S2_mutS2⁺ were mixed in a ratio determined using analytical SEC. Mixtures consisting of 5 µg of S2 or S2_mutS2⁺ and varying amounts of MS2-SA were run through a Superdex 200 increase 10/300 SEC column (Cytiva). The ratio of the mixture with the least amount of MS2-SA that resulted in a chromatogram without a peak corresponding to excess S2 or S2_mutS2⁺ was the stoichiometric ratio used to generate VLP-S2 and VLP-S2_mutS2⁺ for characterization and immunization.

5.2.8 SDS-PAGE

Protein samples were diluted with 5 µL of Nu-PAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen). These protein samples and PageRuler Plus Prestained Protein Ladder (Thermo Scientific) were loaded into the wells of a 4-12% Bis-Tris gel (Invitrogen). The gel was run in MES-SDS buffer at 110 V for 60 minutes while being chilled at 4°C. The gel was stained with SimplyBlue SafeStain (Invitrogen), destained, and imaged using the ChemiDoc MP imaging system (Bio-Rad).

5.2.9 Characterization of S2, S2_mutS2⁺, VLP-S2, and VLP-S2_mutS2⁺ by ELISA

Antigen (0.1 µg S2 and S2_mutS2⁺ – alone and on VLP) was coated onto Nunc MaxiSorp 96-well flat-bottom plates (Invitrogen). The antigen solution was incubated for 1 h, before the wells were emptied and 5% BSA (Millipore) in PBST (PBS with 0.05%
Tween-20) was added to the wells. This BSA solution remained in the wells for 45 minutes, after which it was discarded from the plate and each well was washed with 200 µL of PBST three times. Next, primary antibody (0304-3H3) was diluted in 1% BSA in PBST and a final volume of 100 µL was added to each well. The moles of antibody per well were equivalent to the moles of S2 trimer that had been coated in the well. The plate was left to incubate with the primary antibody for an hour, after which the plate was emptied, and each well was washed with 200 µL of PBST three times. Then 100 µL of the secondary antibody, horseradish peroxidase-conjugated anti-human IgG Fc fragment antibody (MP Biomedicals; 1:5,000 dilution) in 1% BSA in PBST was added to each well. The secondary antibody solution remained in the plate for 1 h, after which the solution was discarded, and the wells of the plate were washed with 200 µL of PBST three times. The plate was then developed by adding 100 µL of TMB substrate solution (Millipore) to each well. The reaction was stopped after three minutes by adding 0.16 M sulfuric acid to each well. The absorbance of each well was then read at 450 nm using a Spectramax i3x plate reader (Molecular Devices).

5.2.10 DLS

MS2-SA VLP was diluted in PBS to 100 µL such that there was 1 µg of SA in solution. VLP-S2 and VLP-S2mutS2’ were each diluted in PBS to 100 µL such that there was 5 µg of S2 in solution. Each 100 µL solution was then pipetted into a UVette (Eppendorf), which was inserted into a DynaPro NanoStar Dynamic Light Scattering detector (Wyatt Technology). Dynamics software (Wyatt Technology) brought the temperature of the measurement cell to 25°C. The detector then proceeded with the measurement. Each measurement was the result of 10 acquisitions and was output as %
Intensity vs. Radius. Radius and polydispersity (PD) index are presented as the mean ± SD of three measurements, while the plotted size distribution is the result of a single representative measurement.

5.2.11 Negative-stain transmission electron microscopy

Conventional native-stain transmission electron microscopy (TEM) was performed, as described previously [103]. Briefly, 4 µl of diluted samples were applied onto glow-discharged mesh copper grids (CF300-Cu; Electron Microscopy Science, PA), washed with PBS (1X), stained in droplets of 1% phosphotungstic acid (PTA, PH 6~7) for 1 min. The grids were then blotted from the grid backside and air-dried inside a petri dish for at least 30 min under room temperature to minimize the negative-stain artifacts of flattening and stacking [104]. The negative-stain grids were imaged in low-dose mode (50 e⁻/Å), using a Talos L120C transmission electron microscope (Thermo Fisher Scientific, previously FEI, Hillsboro, OR) at 120 kV, images were acquired on a 4k x 4k Ceta CMOS camera microscope (Thermo Fisher Scientific), using SerialEM 3.8 [106].

5.2.12 Plunge freezing and cryo-electron microscopy

4 µl of the VLP suspension was added to a glow discharged copper grid (C-Flat 1.2/1.3, 400mesh, Protochips) with an extra layer of carbon (~2 nm) on the holey carbon surface. Grids were plunged frozen using a Vitrobot Mark IV (ThermoScientific) and stored in liquid nitrogen until imaging. Cryo-electron microscopy (cryo-EM) imaging was performed on a Titan Krios (ThermoScientific Hillsboro, OR, USA) operated at 300 kV. Images (defocus of -2~5 µm) were recorded on a post-GIF Gatan K3 camera in EFTEM mode (2.176 Å/pixel) with a 20-eV slit, CDS counting mode, using SerialEM 3.8 [106]. A
total dose of 30–40 e/Å² was used and 40 frames were saved (~1.2 e/Å² per frame). Frames were motion-corrected in MotionCor2 [107]. Images were low pass filtered to 10 Å² for better visualization and contrast using EMAN2 [108].

5.2.13 Cells and virus

Vero E6/TMPRSS2 cells obtained from the National Institute of Infectious Diseases, Japan [109] were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotic/antimycotic solution along with G418 (1 mg/ml). SARS-CoV-2/UT-NCGM02/Human/2020/Tokyo (NCGM02) was amplified on Vero E6 TMPRSS2 cells and used as the challenge virus in the vaccinated hamster study.

5.2.14 Hamster study

Prior to its start, the animal study was approval by the Institutional Animal Care and Use Committee at the University of Wisconsin. The schedule of the hamster study is depicted in Figure 25a. Golden Syrian hamsters (females; 4-5 weeks old) were immunized with 20 µg of SARS-CoV-2 S2 protein presented on VLPs, a mutant S2 protein presented on VLPs, or VLP without the S protein, by subcutaneous inoculation. Addavax (InvivoGen) was added at an equal volume and thoroughly mixed with each vaccine preparation before inoculation. Animals were infected by intranasal inoculation with 10³ plaque-forming units of SARS-CoV-2 while under isoflurane anesthesia. Three days after infection, animals were humanely sacrificed and lung tissue and nasal turbinate samples were collected to measure amount of virus.
Virus titers in the tissues were determined on confluent Vero E6/TMPRSS2 cells by infecting cells with 100 µl of undiluted or 10-fold dilutions (10⁻¹ to 10⁻⁵) of clarified lung and nasal turbinate homogenates. After a 30-minute incubation, the inoculum was removed, the cells were washed once, and then overlaid with 1% methylcellulose solution in DMEM with 5% FBS. The plates were incubated for three days, and then the cells were fixed and stained with 20% methanol and crystal violet in order to count the plaques.

5.2.15 Antibody titer by ELISA

ELISAs were performed using recombinant spike SARS-CoV-2 proteins either produced in Expi293F cells (Thermo Fisher Scientific) and then C-terminal His-tag purified by using TALON metal affinity resin (Wuhan and B.1.351 spike antigens) or purchased from Sino Biological (229E, OC43, HKU-1, NL63, and CoV-1 strain Tor2 spike antigens). ELISA plates were coated overnight at 4 °C with 50 µl of spike antigen at a concentration of 2 µg/ml in PBS. After blocking with PBS containing 0.1% Tween 20 (PBS-T) and 3% milk powder, the plates with incubated in duplicate with heat-inactivated serum diluted in PBS-T with 1% milk powder. A hamster IgG secondary antibody conjugated with horseradish peroxidase (Invitrogen; 1:7,000 dilution) was used for detection. Plates were developed with SigmaFast o-phenylenediamine dihydrochloride solution (Sigma), and the reaction was stopped with the addition of 3M hydrochloric acid. The absorbance was measured at a wavelength of 490 nm (OD₄₉₀). Background absorbance measurements from serum collected before immunization was subtracted from serum collected before challenge for each dilution. IgG antibody endpoint titers were defined as the highest serum dilution with an OD₄₉₀ cut-off value of 0.15.
5.2.16 Neutralization assay

Virus (NCGM02; ~100 PFU) was incubated with the same volume of two-fold dilutions of heat-inactivated serum for 30 minutes at 37 °C. The antibody/virus mixture was added to confluent Vero E6/TMPRSS2 cells that were plated at 30,000 cells per well the day prior in 96-well plates. The cells were incubated for 3 days at 37 °C and then fixed and stained with 20% methanol and crystal violet solution. Virus neutralization titers were determined as the reciprocal of the highest serum dilution that completely prevented cytopathic effects.

5.2.17 Biosafety statement

Research with SARS-CoV-2 was performed under biosafety level 3 agriculture (BSL-3Ag) containment at the Influenza Research Institute with an approved protocol reviewed by approved the University of Wisconsin-Madison’s Institutional Biosafety Committee. The laboratory is designed to meet and exceed the standards outlined in *Biosafety in Microbiological and Biomedical Laboratories* (6th edition).

5.2.18 Statistics and reproducibility

*In vitro* characterizations of the binding of 0304-3H3 to the VLP-S2 and VLP-S2 using ELISA (Figs. 2e and 3e) were each conducted twice independently with three technical replicates for each condition. The data are presented as the mean ± SD. For *in vivo* characterization, there were three groups (receiving either VLP-S2, VLP-S2mutS2', or MS2-SA VLP) each with three hamsters (n=3). To determine the resulting endpoint titers against the SARS-CoV-2 spike protein (Figure 25b; Table 2), two independent assays were
conducted using sera from each hamster. Significance was determined by a one-way analysis of variance (ANOVA) and Tukey post-hoc multiple comparison between groups ($\alpha = 0.05$). All other endpoint titers and neutralizing titers (Figure 25e; Table 2) were determined by conducting an assay using sera from each of the three hamsters per group. The data are presented as the geometric mean with the geometric SD factor and significance was determined by a one-way analysis of variance (ANOVA) and Tukey post-hoc multiple comparison between groups ($\alpha = 0.05$). Viral titers in the lungs and nasal turbinates of hamsters immunized with either VLP-S2, VLP-S2$_{\text{mutS2'}}$, or MS2-SA VLP 3 days after SARS-CoV-2 infection (Figure 25c and Figure 25d) were presented as the geometric mean with geometric SD (n=3) and the significance was determined by a one-way analysis of variance (ANOVA) and Dunnett post-hoc multiple comparison between groups ($\alpha = 0.05$). For all tests of significance, assumptions of the normality of residuals and homogeneity of variance were validated by the D’Agostino-Pearson test and the Brown-Forsythe test, respectively. All statistical analysis was carried out using Prism 8 (GraphPad).

5.3 Results and Discussion

5.3.1 Generation of VLP-S2

We previously developed streptavidin-coated VLPs that we used to display biotinylated protein antigens such as the SARS-CoV-2 spike protein and DIII of the Zika virus envelope protein [16, 81]. In this study we have used these same VLPs to display the S2 subunit of the spike protein (Figure 19b). The VLPs are based on the bacteriophage MS2 coat protein [79]; 90 MS2 coat protein homodimers self-assemble into an icosahedral structure [80]. We used BL21(DE3) *Escherichia coli* (*E. Coli*) to express a single chain
dimer of the MS2 coat protein with an AviTag inserted in a surface loop that had been shown to tolerate peptide insertions [82]. The inserted AviTag allowed for site-specific biotinylation of each coat protein dimer. After expression, the VLPs were purified using Capto Core 700 resin and size exclusion chromatography (SEC). The VLPs were then biotinylated and subsequently separated from the biotinylation reagents using SEC. The biotinylated MS2 VLPs were added dropwise to a large excess of stirred streptavidin (SA), which had been expressed as inclusion bodies, refolded, and purified using iminobiotin affinity chromatography [16, 81]. The resulting MS2-SA VLPs were separated from excess streptavidin using size exclusion chromatography. Consistent with prior characterization [81], SDS-PAGE analysis of the MS2-SA VLPs indicated that there were approximately 70 streptavidin molecules bound to each MS2 biotin VLP (Figure 20). In addition, the MS2-SA VLPs were found to be pure and homogenous in size based on characterization by SEC (Figure 19c), dynamic light scattering (DLS; Figure 19d), negative-stain transmission electron microscopy (NS-TEM; Figure 19e) and cryo-electron microscopy (cryo-EM; Figure 19f).
Figure 19. Assembly of VLP-S2 and characterization of MS2-SA VLP. a) The SARS-CoV-2 spike ectodomain (PDB: 6XKL). The S1 subunit is highlighted in orange and the S2 subunit is highlighted in green. b) Scheme illustrating the assembly of VLP-S2, where biotinylated MS2 (yellow, PDB: 2MS2) is added to streptavidin to create the VLP. S2 biotinylated at the C-terminus (green; PDB: 6XKL) is mixed with the VLP to create the VLP-S2. c) Size exclusion chromatography trace for MS2-SA VLP. The vertical gray line represents the peak elution volume of the molecular weight standard thyroglobulin (660 kDa). The column void volume is 7.2 mL. d) Characterization of the MS2-SA VLP by dynamic light scattering (mean ± SD; n=3). e) Negative-stain transmission electron micrograph of MS2-SA VLPs. Scale bar = 50 nm. f) Cryo-EM of vitrified MS2-SA VLP. Scale bar = 50 nm.
Figure 20. Characterization of MS2 to SA stoichiometry using SDS-PAGE. Amount of MS2 in heated MS2-SA VLP was compared to amount of MS2 in unheated MS2-SA VLP to determine that approximately 78 percent of MS2 was bound to SA. Excess biotin was added to the unheated sample to occupy all unoccupied biotin binding sites prior to the addition of SDS. β-mercaptoethanol was added to all samples.

Biotinylated S2 was next produced such that it could be displayed on the MS2-SA VLPs. We used Expi293F mammalian cells to express the HexaPro [101] variant of the SARS-CoV-2 spike protein’s S2 subunit with an N-terminal signal peptide, a C-terminal trimerization domain to promote stability, a C-terminal AviTag for biotinylation, and a C-terminal his-tag for purification. The expressed S2 was purified using immobilized metal affinity chromatography (IMAC) and was then biotinylated in vitro. The biotinylated S2 was separated from biotinylation reagents using size exclusion chromatography and could then be displayed on the MS2-SA VLPs.

To determine the appropriate ratio of S2 to add to MS2-SA VLPs, analytical SEC was used. Mixtures of the two proteins were made that contained a constant amount of S2 and varying amounts of MS2-SA VLPs. The ratio of the mixture with the least amount of
MS2-SA VLPs that displayed no indication of excess S2 in an SEC chromatogram was determined to be the approximate stoichiometric ratio. Further analysis by SDS-PAGE indicated that this stoichiometric ratio resulted in approximately 30 S2 molecules conjugated to each MS2-SA VLP (Figure 21). The MS2-SA and biotinylated S2 were mixed in this ratio to create the VLP-S2 immunogen.

![Figure 21. Characterization of MS2-SA to S2 stoichiometry using SDS-PAGE. The intensity of bands corresponding to S2 and MS2 in VLP-S2 was compared to BSA standards and quantified to determine that approximately 30 S2 molecules were displayed on each MS2-SA VLP. S2 was deglycosylated with PNGase F and all samples were heated with β-mercaptoethanol and LDS sample buffer.](image)

5.3.2 In vitro characterization of VLP-S2 and VLP-S2mutS2

The VLP-S2 immunogen was characterized in vitro using several different bioanalytical techniques. First, the proteins that made up VLP-S2 were characterized by SDS polyacrylamide gel electrophoresis (SDS-PAGE), which indicated that the proteins were pure (Figure 22a). In addition, comparison of the molecular weight ladder to the bands representing deglycosylated S2 (~63 kDa), biotinylated MS2 (~29 kDa), and
monomeric streptavidin (~15 kDa) demonstrated that these proteins aligned as expected with molecular weight standards. The VLP-S2 was also analyzed using analytical SEC, where chromatograms were generated for VLP-S2, S2 alone, and the molecular weight standard thyroglobulin (Figure 22b). The resulting UV trace corresponding to the VLP-S2 contained a single peak that appeared before the peak for S2 alone. Therefore, the VLP-S2 was free of excess S2 and was generally uniform in size. To obtain a direct size measurement of the VLP-S2, we used Dynamic Light Scattering (DLS) (Figure 22c), NS-TEM (Figure 22d), and cryo-EM (Figure 22e). The DLS measurements indicated that the VLP-S2 construct was approximately 90 nm in diameter. Characterization of the VLP-S2 by NS-TEM and cryo-EM confirmed the presence and coating of the S2 protein on the surface of the MS2-SA VLP. NS-TEM analysis suggested that VLP-S2 was ~65 nm in diameter on average (n=300). The larger size indicated by DLS may be a result of the fact that scattering intensity is proportional to the sixth power of the radius, giving rise to a disproportionately higher weighting of larger particles. We next used ELISA to probe the binding of the anti-S2 monoclonal antibody 0304-3H3 [122] to S2 and VLP-S2 (Figure 22f). This antibody bound to both the S2 and VLP-S2, suggesting that S2 retained its bioreactivity after conjugation to VLPS.
Figure 22. Characterization of S2 and VLP-S2. a) SDS-PAGE characterization of S2 and VLP-S2. S2 was deglycosylated with PNGase F. The samples were heated with β-mercaptoethanol and LDS sample buffer. b) Size exclusion chromatography traces for S2 (dashed line) and VLP-S2 (solid line). The vertical gray line represents the peak elution volume of the molecular weight standard thyroglobulin (660 kDa). The column void volume is 7.2 mL. c) Characterization of the VLP-S2 by dynamic light scattering (mean ± SD; n=3). d) Negative-stain transmission electron micrograph of VLP-S2. Arrowheads (white) indicate the S2 protein on the VLP surface. Scale bars = 50 nm. e) Cryo-EM of vitrified VLP-S2. Arrowheads (white) indicate the S2 protein on the VLP surface. Scale bars = 50 nm. f) Characterization of the binding of anti-S2 antibody 0304-3H3 to S2 and VLP-S2 by ELISA. (mean ± SD, n=6: two independent assays, each with three technical replicates).
In addition to the VLP-S2, we generated VLP-S2\textsubscript{mutS2'} particles. The VLP-S2\textsubscript{mutS2'} displayed an S2 variant (S2\textsubscript{mutS2'}) that contained S2’ cut site residues that had been mutated to glycine residues. The purpose of this mutation was to prevent potential proteolytic cleavage of the S2 immunogen at the S2’ cut site. The VLP-S2\textsubscript{mutS2'} was generated and characterized using the same procedures described above for the VLP-S2 (Figure 23; Figure 24).

**Figure 23.** Characterization of MS2-SA to S2\textsubscript{mutS2'} stoichiometry using SDS-PAGE. The intensity of bands corresponding to S2\textsubscript{mutS2'} and MS2 in the VLP-S2\textsubscript{mutS2'} was compared to BSA standards and quantified to determine that approximately 30 S2\textsubscript{mutS2'} molecules were displayed on each MS2-SA VLP. S2\textsubscript{mutS2'} was deglycosylated with PNGase F and all samples were heated with β-mercaptoethanol and LDS sample buffer.
Figure 24. Characterization of S$_{2mutS2}$' and VLP-S$_{2mutS2}$'. a) SDS-PAGE characterization of S$_{2mutS2}$' and VLP-S$_{2mutS2}$'. S$_{2mutS2}$' was deglycosylated with PNGase F. The samples were heated with β-mercaptoethanol and LDS sample buffer. b) Size exclusion chromatography traces for S$_{2mutS2}$' (dashed line) and VLP-S$_{2mutS2}$' (solid line). The vertical gray line represents the peak elution volume of the molecular weight standard thyroglobulin (660 kDa). The column void volume is 7.2 mL. c) Characterization of the VLP-S$_{2mutS2}$' by dynamic light scattering (mean ± SD; n=3). d) Negative-stain transmission electron micrograph of VLP-S$_{2mutS2}$'. Arrowheads (white) indicate the S$_{2mutS2}$' protein on the VLP surface. Scale bars = 50 nm. e) Cryo-EM of vitrified VLP-S$_{2mutS2}$'. Arrowheads (white) indicate the S$_{2mutS2}$' protein on the VLP surface. Scale bars = 50 nm. f) Characterization of the binding of anti-S2 antibody 0304-3H3 to S2 and VLP-S2 by ELISA. (mean ± SD, n=6: two independent assays, each with three technical replicates).

5.3.3 Protective efficacy of VLP-S2 and VLP-S$_{2mutS2}$'
The *in vivo* efficacy of the VLP-S2 and VLP-S2\textsubscript{mutS2'} was next assessed (Figure 25a). Hamsters were immunized with either VLP-S2, VLP-S2\textsubscript{mutS2'}, or MS2-SA VLP alone and were boosted 28 days later. Hamsters immunized with the VLP-S2 and VLP-S2\textsubscript{mutS2'} generated high antibody titers against the S ectodomain (Figure 25b; Table 2). To gauge whether immunization with VLP-S2 and VLP-S2\textsubscript{mutS2'} was protective, the vaccinated hamsters were intranasally inoculated with $10^3$ plaque-forming units of SARS-CoV-2/UT-NCGM02/Human/2020/Tokyo (an early isolate that contains 614D) [109] 51 days after the initial immunization. The hamsters were then sacrificed 3 days after infection and viral titers in their lungs and nasal turbinates were quantified. The geometric mean viral titer in the lungs of hamsters immunized with VLP-S2 was nearly 100-fold lower than that of the hamsters immunized with MS2-SA VLP (Figure 25c). The geometric mean viral titer in the lungs of hamsters immunized with VLP-S2\textsubscript{mutS2'} was more than 7,000-fold lower than that of hamsters immunized with MS2-SA VLP – a statistically significant difference (Figure 25c). These results demonstrate that immunization with the S2-based immunogens VLP-S2 and VLP-S2\textsubscript{mutS2'} provides protection against SARS-CoV-2. Characterization of viral titers in the nasal turbinates of the immunized hamsters also indicated that the multivalent S2 constructs provided protection against SARS-CoV-2 (Figure 25d). The geometric mean viral titers in the nasal turbinates of hamsters immunized with VLP-S2 and VLP-S2\textsubscript{mutS2'} were respectively 3- and 36-fold lower than that of hamsters immunized with MS2-SA VLP.

5.3.4 *Breadth of response generated by VLP-S2 and VLP-S2\textsubscript{mutS2'}*

Next, the breadth of the humoral immune response generated by VLP-S2 and VLP-S2\textsubscript{mutS2'} was evaluated using ELISA. Immunization with the multivalent S2 constructs
elicited high antibody titers against the spike protein of not only the original Wuhan-Hu-1 SARS-CoV-2 (614D) [135], but also against the spike proteins of the SARS-CoV-2 variant B.1.351, SARS-CoV-1, and the four endemic human coronaviruses HKU-1, OC43, NL63, and 229E (Figure 25e; Table 2). This substantial cross-reactivity suggests that immunization with multivalent S2-based immunogens may be a promising strategy for eliciting a broadly protective response against coronaviruses.

Interestingly, despite this protection against a viral challenge, high antibody titers (Figure 25b), and broad cross-reactivity (Figure 25e; Table 2), sera from hamsters immunized with VLP-S2 and VLP-S2mutS2' did not show neutralization activity in vitro against SARS-CoV-2 (614D) or the variants B.1.351 and B.1.617.2 (Table 2). This result suggests that the protection afforded to the hamsters through immunization with the multivalent S2 constructs might arise from other mechanisms, such as Fc effector functions. Fc effector functions have previously been identified as a mechanism by which S2-specific antibodies provide protection [127]. In addition, antibodies targeting the S2-analogous region of the influenza protein hemagglutinin (the stalk domain) are known to provide protection through Fc effector functions [136]. While further studies will be needed to elucidate the exact mechanism of protection imparted by VLP-S2 and VLP-S2mutS2', our results demonstrate that the multivalent S2 constructs are capable of eliciting a broadly cross-reactive immune response that protects against SARS-CoV-2. Therefore, the S2 subunit should be strongly considered in the development of next-generation coronavirus vaccines designed to protect against emerging SARS-CoV-2 variants and other zoonotic coronaviruses with pandemic potential.
Figure 25. Protective efficacy of VLP-S2 and VLP-S2mutS2'. a) Schedule for hamster vaccination, serum collection, infection with SARS-CoV-2, and organ collection. b) Antibody endpoint titers of sera from hamsters immunized with either VLP-S2, VLP-S2mutS2, or MS2-SA VLP against SARS-CoV-2 spike protein (geometric mean
with geometric SD, n=6: two independent assays with sera from 3 hamsters). ns: not statistically significant, ****p < 0.0001, determined by a one-way analysis of variance (ANOVA) and Tukey post-hoc multiple comparison between groups (α = 0.05). c) Viral titer in the lungs of hamsters immunized with either VLP-S2, VLP-S2mutS2', or MS2-SA VLP three days after infection with SARS-CoV-2 (geometric mean with geometric SD, n=3 hamsters). **p < 0.01, determined by a one-way analysis of variance (ANOVA) and Dunnett post-hoc multiple comparison between groups (α = 0.05). d) Viral titer in the nasal turbinates of hamsters immunized with either MS2-SA VLP, VLP-S2, or VLP-S2mutS2 three days after SARS-CoV-2 infection (geometric mean with geometric SD, n=3 hamsters). **p < 0.01, determined by a one-way analysis of variance (ANOVA) and Dunnett post-hoc multiple comparison between groups (α = 0.05). e) Antibody endpoint titers of sera from hamsters immunized with either VLP-S2 (gray), VLP-S2mutS2 (white), or MS2-SA VLP against spike proteins of the original Wuhan-Hu-1 SARS-CoV-2 (614D), the SARS-CoV-2 variant B.1.351, SARS-CoV-1, and the four endemic human coronaviruses HKU-1, OC43, NL63, and 229E (geometric mean with geometric SD, n=6 against SARS-CoV-2 614D S protein: two independent assays with sera from 3 hamsters; n=3 against all other S proteins: sera from 3 hamsters). ns: not statistically significant, ****p < 0.0001, determined by a one-way analysis of variance (ANOVA) and Tukey post-hoc multiple comparison between groups (α = 0.05).
Table 2. Antibody responses to VLP-S2 and VLP-S2\textsubscript{mutS2'} after prime and boost in Syrian hamsters

<table>
<thead>
<tr>
<th>Vaccine Group</th>
<th>Spike IgG Endpoint Titer\textsuperscript{a}</th>
<th>Neutralizing Antibody Titer\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SARS-CoV-2</td>
<td>SARS-CoV-1</td>
</tr>
<tr>
<td></td>
<td>S14D Geometric Mean</td>
<td>S14D Geometric SD Factor</td>
</tr>
<tr>
<td>S14D</td>
<td>Geometric Mean</td>
<td>Geometric SD Factor</td>
</tr>
<tr>
<td>MS2-SA VLP</td>
<td>&lt;20</td>
<td>-</td>
</tr>
<tr>
<td>VLP-S2</td>
<td>292,867</td>
<td>1.98</td>
</tr>
<tr>
<td>VLP-S2\textsubscript{mutS2'}</td>
<td>291,930</td>
<td>1.33</td>
</tr>
<tr>
<td>S14D</td>
<td>Geometric Mean</td>
<td>Geometric SD Factor</td>
</tr>
<tr>
<td>MS2-SA VLP</td>
<td>&lt;20</td>
<td>&lt;20</td>
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<tr>
<td>VLP-S2</td>
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<tr>
<td>VLP-S2\textsubscript{mutS2'}</td>
<td>&lt;20</td>
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</table>

\textsuperscript{a} Viral antibody endpoint titers against the SARS-CoV-2 spike (three animals in each group). Endpoint titers using 2-fold diluted sera were expressed as the reciprocal of the highest dilution with an optical density at 490 nm cutoff value >0.15; sera were collected on day 42 after the initial immunization.

\textsuperscript{b} Viral neutralization titers (three animals in each group). Endpoint titers using 2-fold diluted sera were expressed as the reciprocal of the highest dilution that completely prevented cytopathic effects; sera were collected on day 42 after immunization.
5.4 Conclusion and Future Directions

In future studies we will further evaluate the immune response to the VLP-S2 construct. In particular, we will more thoroughly examine the breadth of protection that VLP-S2 provides by challenging VLP-S2-immunized hamsters with SARS-CoV-2 variants and zoonotic coronaviruses. In addition to exploring the protective breadth of the immune response against VLP-S2, we also plan to elucidate the mechanism by which the response to VLP-S2 provides protection against SARS-CoV-2. As the antibodies generated by the VLP-S2 did not show neutralizing activity in \emph{in vitro} neutralization assays, protection may instead be conferred through anti-S2 antibody Fc effector functions. It is reasonable to hypothesize that the elicited anti-S2 antibodies may afford protection through Fc effector functions, because both S2-specific antibodies and antibodies targeting the S2-analogous stalk of influenza hemagglutinin have been found to confer protection through Fc effector functions [127, 136]. Therefore, we will perform antibody dependent cellular cytotoxicity, phagocytosis and complement deposition (ADCC, ADCP, and ADCD) assays to determine whether the anti-S2 antibodies provide protection through Fc effector functions. These results will allow us to better understand the immune response generated by VLP-S2, and whether this immune response is broadly protective.

While our preliminary results indicate that the S2 subunit may be fit for the development of a broadly protective coronavirus vaccine, the stability of the S2 antigen is a point of concern. We have noticed that the S2 antigen aggregates and precipitates out of solution over time. This aggregation may be a result of exposed hydrophobic residues that are normally shielded by the S1 subunit. Therefore, these exposed hydrophobic residues should be identified and mutated to either hydrophilic or neutral amino acids. Similar
mutations were made for an analogous stalk-only hemagglutinin (HA) construct called mini-HA [19]. The mini-HA construct was further stabilized using intramolecular disulfide bridges [19]. We can explore the effect of analogous mutations on the stability of the S2 antigen; however, HexaPro mutations have been incorporated into the S2 subunit antigen [101], which may be sufficient to stabilize the S2 antigen in its prefusion conformation.

An alternative to this unstable S2 subunit antigen may be an inverted spike construct – similar to the inverted HA construct discussed in Chapter 3. To invert the orientation of the spike protein, we will use MS2-SA VLPs to display a biotinylated nanobody. We have identified the VHH E nanobody described by Koenig et al. as a promising candidate, as it binds to the RBD of the spike protein with nanomolar affinity [137]. The MS2-SA-VHH E construct would then be mixed with SARS-CoV-2 spike protein such that the S1 subunit is membrane proximal and the S2 subunit is displayed outwardly and easily accessible to B cells. This orientation approach should elicit a strong immune response against the S2 subunit, while better maintaining the stability of the S2 subunit.
CHAPTER 6. CONCLUSION AND FUTURE DIRECTIONS

In this work we have engineered viral protein antigens to elicit broadly neutralizing antibodies. The immune response to these engineered antigens may provide valuable insights to guide the design of broadly protective vaccines. Specifically, we have evaluated a prefusion RSV F-based antigen that shields the highly immunogenic yet variable site Ø and found that this antigenic site is not required for immunogenicity. This result could have important implications for the design of an RSV vaccine, especially for populations with pre-existing immunity to RSV. We have also investigated how the orientation of the influenza HA protein on virus-like particles affects the humoral immune response. We found that inverting HA on virus-like particles influences the generation of antibodies targeting the conserved hemagglutinin stalk domain. This is fundamentally a very interesting result and may also have application in the development of a broadly protective influenza vaccine. We also assessed the multivalent display of the SARS-CoV-2 spike protein and its conserved S2 subunit on VLPs. We found that the VLP-S construct provides protection after a single dose, and the VLP-S2 construct generates protective antibodies that cross-react with spike proteins from a SARS-CoV-2 variant of concern, SARS-CoV-1, and the four endemic human coronaviruses. While these results are exciting, there is the opportunity to conduct future studies that could significantly enhance the quality of this work as a whole. For example, the immune response to each vaccine should be more fully characterized by examining the cellular response mediated by T cells. In addition, the vaccine scaffolds we have used in these studies would likely require modification for use in humans.
We have primarily analyzed the humoral immune response to our vaccines, as our goal was to engineer viral antigens that generate broadly neutralizing antibodies. However, we have not assessed the cellular response mediated by T cells. To fully characterize the immune response to our vaccines, the T cell response should be examined. In fact, the T cell response directly affects the humoral response, as helper T cells are often required for B cell activation [2]. Similarly, these helper T cells can recruit and activate other cells of the immune system to promote phagocytic, inflammatory, allergic, and mucosal immunity in response to a pathogen [2]. In addition, cytotoxic T cells can secrete cytotoxic proteins to directly kill pathogen-infected cells [2]. To evaluate the extent of the cellular response to our vaccines, we can harvest the splenocytes of vaccinated mice and probe for cell-secreted cytokines such as IFN-γ, IL-2, and IL-4 using an ELISpot assay [138]. To determine the protection afforded by the T cell response, we can deplete T cells from immunized mice and compare their weight loss and survival after viral challenge to that of immunized mice that have not been depleted of T cells [25]. This analysis of the cellular immune response will provide a more comprehensive evaluation of the immune response to our vaccines.

We will also need to further consider the scaffold we are using to present our engineered antigens. The 4-arm-PEG-spycatcher and MS2-SA VLP scaffolds served the purpose of multivalently displaying recombinant proteins for the initial experiments documented in this dissertation. These scaffolds are especially useful for these initial experiments because they can be used to display nearly any recombinant protein in a “plug-and-display” manner [139, 140]. However, there are drawbacks to these scaffolds that could make them undesirable in later-stage testing. For example, there is likely a
significant immune response to the scaffold itself that may weaken the response to the intended targets – the viral proteins. PEG and SA have been previously utilized in human therapies [141, 142]. Therefore, certain populations may have pre-existing antibodies and memory immune cells that will eradicate the 4-arm-PEG-spycatcher and MS2-SA VLPs before a response can be developed against the viral protein antigen that these scaffolds display. To mitigate this problem, there are methods we could explore to reduce the immune response to the proteins that make up these scaffolds. For example, non- or poorly immunogenic molecules such as glycans, PEG, XTEN and PAS have been used to shield immunogenic epitopes [143]. While this shielding strategy may reduce the immune response to the scaffold itself, there is still potential for an undesired immune response to the shielding molecule. An alternative strategy that has been used to deimmunize proteins is the identification and deletion of B cell and T cell epitopes [143]. These epitopes can be predicted computationally, and key epitope residues are then mutated to deimmunize the epitope. While this strategy can be effective, it is important to consider that these mutations may affect the structure and function of the protein scaffold.

In addition to exploring methods to deimmunize our existing scaffolds, we can also investigate alternative methods to display our engineered antigens multivalently. For instance, certain vaccines such as the influenza vaccine FluBlok retain the hydrophobic transmembrane domain of hemagglutinin at its C-terminus [144]. FluBlok’s hemagglutinin aggregates around its hydrophobic transmembrane domain, creating a rosette of hemagglutinin [73]. This approach would eliminate the potential for an immune response to anything other than the viral protein antigen, and such a method could be applied to many of the vaccines presented in this work. Elastin-like polypeptide (ELPs)
are another appendage that we could potentially fuse to the C-terminus of our engineered viral protein antigens. ELP diblock polypeptides consisting of a hydrophobic region and a hydrophilic region with repeating sequence Gly-Xaa-Gly-Val-Pro (Xaa is any amino acid) have been designed to assemble into nanoparticles at physiological conditions [145]. Given that ELPs are based on human elastin, they are generally regarded as safe and unlikely to elicit a significant immune response [146]. In fact, ELP fusion protein therapeutics have entered clinical trials [147]. Another significant advantage to both transmembrane domain and ELP-based scaffolds is the relatively simple production process as compared to that of the 4-arm-PEG-spycatcher scaffold and the MS2-SA VLP. To produce the multivalent antigens discussed in this work on a large-scale, this simplified production process will be necessary.

Despite this works’ limitations, we have successfully developed viral antigens that direct immune response to conserved areas of viral protein antigens. These engineered immunogens have provided valuable insights into the immune response against RSV F, the hemagglutinin protein of influenza, and the SARS-CoV-2 spike protein. The results documented in this dissertation may ultimately inform the design of broadly protective vaccines.
REFERENCES


