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Current Opinion in Immunology

Review

# Principles and practical applications of structure-based vaccine design

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Viral proteins fold into a variety of structures as they perform their functions. Structure-based vaccine design aims to exploit knowledge of an antigen's architecture to stabilize it in a vulnerable conformation. We summarize the general principles of structure-based vaccine design, with a focus on the major types of sequence modifications: proline, disulfide, cavity-filling, electrostatic and hydrogen-bond substitution, as well as domain deletion. We then review recent applications of these principles to vaccine-design efforts across five viral families: *Coronaviridae, Orthomyxoviridae, Paramyxoviridae, Pneumoviridae*, and *Filoviridae*. Outstanding challenges include continued application of proven design principles to pathogens of interest, as well as development of new strategies for those pathogens that resist traditional techniques.

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**Current Opinion in Immunology** 2022, **77**:102209

This review comes from a themed issue on **Vaccines**

Edited by **Mariagrazia Pizza** and **Rino Rappuoli**

For complete overview of the section, please refer to the article collection, "Vaccines (August 2022)"

Available online 19th May 2022

https://doi.org/10.1016/j.coi.2022.102209



## Introduction

The past two years have witnessed an explosion of vaccine design in response to the coronavirus pandemic. Notwithstanding the focus on SARS-CoV-2, progress to develop vaccines against other viral pathogens has steadily continued. While all viruses exhibit unique characteristics, they also share certain fundamental similarities: viruses use biological macromolecules to perform essential functions such as host cell attachment, entry, replication, and exit. A wealth of high-resolution structures of viral proteins has shed light on the molecular underpinnings of essential viral protein functions at high resolution. Through a combination of rational design and experimental testing, researchers have converged on a common set of strategies for designing antigens.

A useful vaccine antigen must mimic some portion of a pathogen's structure to a sufficient degree such that it elicits a protective immune response. The relationship between antigen structure, stability, and immunogenicity is complex and must be determined on a case-by-case basis. The choice of the antigen itself therefore requires careful consideration of the pathogen's natural immunogenicity [1,2]. We eschew much discussion of antigen selection, since this topic has been recently reviewed both in this journal and elsewhere, though we emphasize the importance of this complementary aspect of vaccine design [3,4]. We also set aside detailed discussion of vaccine-delivery methods (e.g. protein subunits, nucleic acids in lipid nanoparticles, viral vectors, etc.), which have been reviewed extensively by others [5–7].

What remains constitutes the subject of this article: once an antigen is chosen, how does one ensure that it persists in a suitable form for presentation to the immune system? We divide our discourse into two parts. First, we summarize a set of general principles that can be applied to stabilize an antigen in a desired conformational shape, noting classic examples. Second, we describe recent applications of these principles to vaccine design and preview the outstanding challenges facing the field.

## Part 1: Principles of structure-based vaccine design

Structure-based vaccine design begins the way its name suggests: with an examination of the target antigen's structure. An antigen might adopt any number of folded and unfolded states, which comprise its conformational ensemble. Each state has an associated folding free energy ($\Delta G$), a measure of its thermodynamic stability. The rate of conversion between two given states constitutes kinetic stability. In the best case, the designer will have access to high-resolution structural models of one or more states, for example, prefusion and postfusion. An accurate homology model produced by deep-learning methods may serve as a useful starting point, especially when combined with low-resolution experimental data to guide model building [8–10]. If the antigen forms multiple structures, then comparing regions

that undergo conformational change can guide modifications aimed at stabilizing one conformation or another. In this way, successful structure-guided vaccine design flows from an appreciation of the forces that regulate protein folding: the hydrophobic effect, hydrogen bonding, van der Waals forces, electrostatic interactions, and the intrinsic propensities of individual amino acids to form secondary-structure elements [11].

### Amino acid substitution

In addition to their primary amino acid sequence, proteins form three other levels of structures [12]. Secondary structure refers to the arrangement of the peptide backbone over local distances (e.g. helices, sheets, and turns). Tertiary structure refers to the three-dimensional arrangement of secondary structures within a single polypeptide chain. Quaternary structure describes the joining of tertiary structures into oligomeric assemblies (e.g. trimers, tetramers, etc.). Each stratum of structure depends on the other, and the primary amino acid sequence ultimately specifies the ensemble of three-dimensional structures in solution [13–16]. For these reasons, amino acid substitutions constitute an essential element of the structure-based vaccine-design toolkit.

The first substitution type makes use of the intrinsic tendencies for individual amino acids to form secondary-structure elements, which can bias the antigen toward a desired tertiary/quaternary structure [17]. A common example of this type of substitution involves the placement of prolines at helix–turn–helix motifs that undergo conformational change (Figure 1, top-left panel). The unusual chemical structure of proline exerts two effects. Proline not only sterically restricts the range of possible secondary structures, but it also lacks an amide hydrogen, precluding its participation in the α-helical hydrogen-bonding network [18–20]. Furthermore, proline placement at the N-terminal end of a helix still allows participation of its backbone carbonyl in the desired α-helical structure. Proline has thus earned the moniker of 'helix breaker', however, a vaccine designer might sooner call it 'helix capper'. This strategy was first described by Judith White and colleagues, who discovered that proline's propensity for destabilizing helices could be used to disfavor formation of an extended coiled coil in the postfusion conformation of influenza hemagglutinin (HA), thereby stabilizing the prefusion conformation [21].

The second substitution type involves introducing pairs of cysteine substitutions that will form a disulfide bond in the folded state. Once formed, this covalent bond helps preserve a particular conformation by disfavoring the movement of secondary or tertiary structures away from each other. An early application of this design was used to achieve prefusion-stabilization of the respiratory syncytial virus fusion protein (RSV F) [22,23], and later influenza HA (Figure 1, bottom-left panel) and HIV-1 Env [24–26].

The third substitution type aims to stabilize a particular conformation by filling cavities located within the hydrophobic core of an antigen. An internal cavity refers to a region of space that is not occupied by protein backbone or sidechain atoms. Internal cavities destabilize a protein's folded conformation, whereas the introduction of larger hydrophobic side chains (e.g. valine, leucine, isoleucine, phenylalanine, and methionine) increases their stability [27–31]. This phenomenon, long known from the study of protein folding, was first applied to the engineering of thermostable enzymes before being incorporated into structure-based vaccine design. An early attempt to stabilize HIV gp120 with cavity-filling substitutions was followed by their application to stabilize prefusion RSV F (Figure 1, top-right panel) [22,23,32].

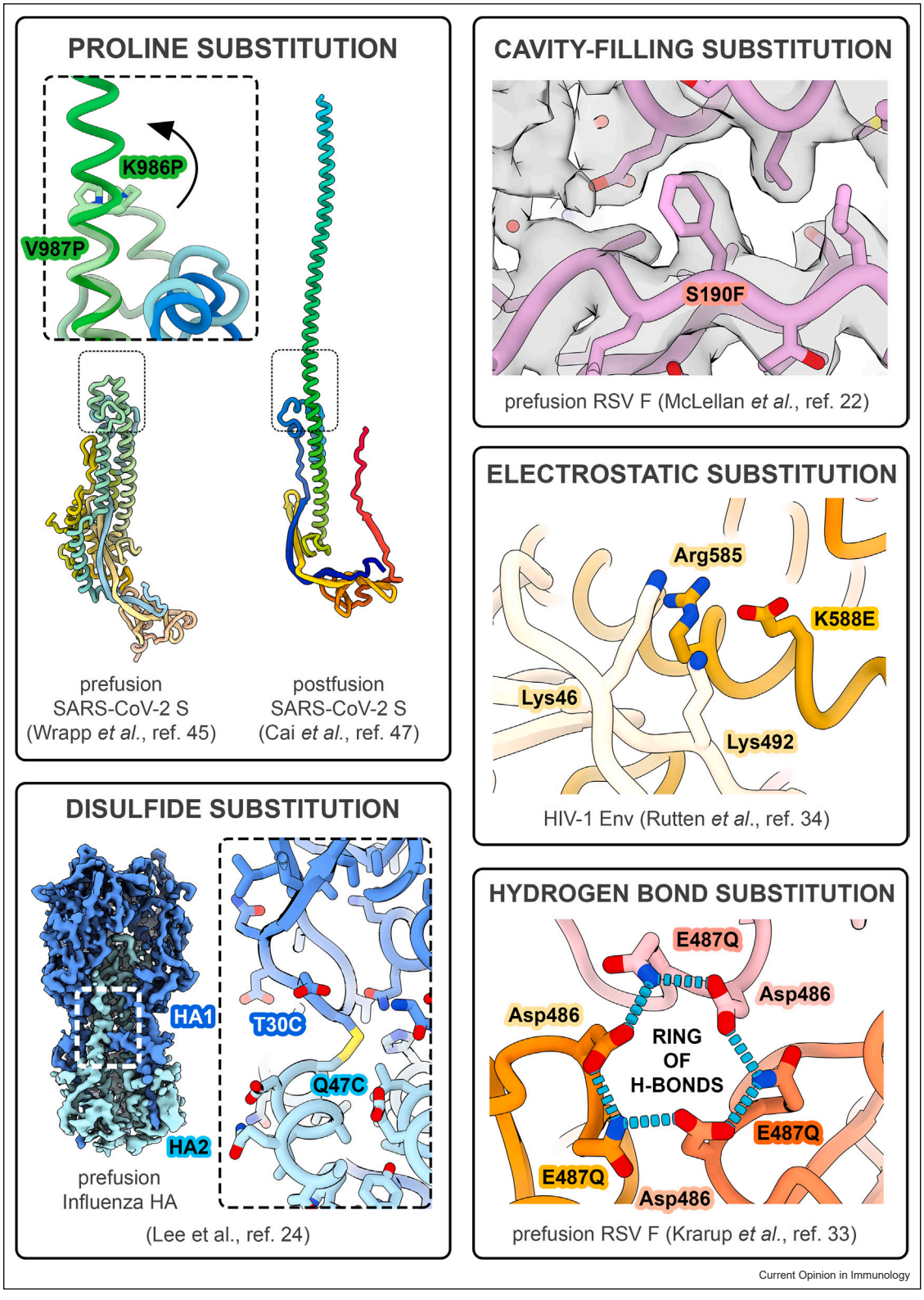
Additional substitution types include the manipulation of electrostatic interactions or hydrogen bonds (Figure 1) [33]. While these two forces make more modest contributions to overall protein stability [11], examples of electrostatic or hydrogen-bond-stabilizing substitutions abound in vaccine design, such as an engineered salt bridge that stabilizes the HIV-1 gp120–gp41 interface (Figure 1, middle-right and bottom panels) [34]. Finally, targeted addition of N-linked glycosylation sites has been successfully employed to mask certain epitopes and direct the immune response elsewhere on the antigen [35].

### Domain deletion, domain replacement, and domain combination

Vaccine designs for viral envelope proteins often employ some form of deletion. Common examples include modification of a proteolytic processing site or transmembrane (TM) domain. TM-domain deletion often pairs with addition of an exogenous oligomerization motif. Oligomerization motifs can be limited or extensive, such as those used for geometric antigen display on engineered nanoparticles. The homo-oligomeric nature of many viral glycoproteins (GPs) has led to their attachment at axes of nanoparticle symmetry, such as an influenza HA antigen displayed at a threefold symmetric axis on ferritin [36]. Other examples include antigen display on lumazine synthase or the computationally designed I53-50, both of which exhibit icosahedral symmetry [37,38].

Deletions can also improve an antigen's inherent immunogenicity. For instance, if one region within an antigen contains known neutralizing epitopes, while another region contains non-neutralizing epitopes, then the vaccine designer might want to target the vulnerable domain while avoiding the other altogether. Domain

**Figure 1**




Examples of stabilizing amino acid substitutions. Each panel shows structural models representing one type of amino acid substitution. Source references are listed within each panel. *Proline*: prefusion (pale rainbow) and postfusion (bright rainbow) SARS-CoV-2 S, colored from N-terminal (blue) to C-terminal (red). The inset shows a zoomed view of the K986P and V987P substitutions. *Disulfide*: prefusion influenza HA. Domains HA1 and HA2

are colored blue and cyan, respectively. The inset shows a zoomed view of the cysteine substitutions at positions 30 and 47, which covalently bond HA1 to HA2. *Cavity-filling*: prefusion RSV F (pink) with a S190F mutation. The experimental electron-density map (*2Fo–Fc*) is shown as transparent gray contours. *Electrostatic:* HIV-1 Env. Individual chains are shown as shades of yellow. *Hydrogen bond:* prefusion RSV F. A substitution near the C-terminus of the ectodomain forms a ring of hydrogen bonds, which are indicated as cyan dashed lines. Individual RSV F protomers are colored in shades of orange and red.

deletions can be combined to form chimeric antigens, such as the multicomponent design against *Neisseria meningitidis* serogroup B [39].

### The best designs are the ones that work

The complex and unpredictable nature of protein folding can lay waste to the best-laid rational designs. Successful application of these principles therefore depends on rigorous experimental testing, since stabilizing substitutions must ultimately preserve a particular protein conformation without disfiguring the antigen's surface. Indeed, many designs fail to achieve their intended effect. In Part 2, we highlight recent examples of vaccine design. In each case, the authors incorporated a set of structural and/or functional assays. For instance, electron microscopy can distinguish between prefusion and postfusion conformations. Measurement of binding to known ligands, such as host receptors or conformation-specific antibodies, can indicate the presence or absence of a particular conformation. Immunization of a model animal and characterization of antibodies can inform on an antigen's immunogenicity. While we focus on the structural aspects of these vaccine-design efforts in Part 2, we emphasize the indispensability of functional assays and refer the reader to individual papers for details. We note that Part 2 omits discussion of recent human immunodeficiency virus vaccine design, which has been thoroughly reviewed by others and remains an area of intense research [40,41].

## Part 2: Recent applications of structure-based vaccine-design principles

### SARS-CoV-2

Recent coronavirus vaccine design has focused on the SARS-CoV-2 spike (S) protein, although some efforts have begun to include epitopes outside the spike. As of this writing, the World Health Organization recommends five vaccine formulations for use against SARS-CoV-2, all of which involve presentation of the spike protein [42,43]. Of those five formulations, four employ prefusion-stabilizing tandem proline substitutions at positions 986 and 987 (K986P/V987P, known as S-2P), which were first designed at homologous positions in the MERS-CoV spike [44–46]. These two prolines reside within a loop between the heptad repeat 1 and the central helix, which changes its conformation to a coiled coil in the postfusion state [46,47]. The two proline substitutions thus exert their effect by destabilizing formation of the central coiled coil.

The fifth SARS-CoV-2 vaccine formulation, ChAdOx1-S, comprises a replication-deficient chimpanzee adenoviral vector displaying full-length wild-type spike protein. Despite the absence of prefusion-stabilizing mutations, spikes on the surface of ChAdOx1-S retain the trimeric prefusion conformation [48]. The ChAdOx1-S sequence includes the TM and C-terminal domains, thus, the viral membrane environment itself may play a role in prefusion-stabilization. Differences in delivery mechanism and manufacturing preclude direct assessment of the relationship between prefusion-stabilization (wild-type S or S-2P) and SARS-CoV-2 vaccine effectiveness, however, the MERS-CoV S-2P elicited higher titers of neutralizing antibodies than wild-type MERS-CoV S [44].

Additional efforts to design spike-based vaccines involve stabilizing the prefusion conformation of the spike ectodomain [49–55]. There are at least nine second-generation stabilized-spike sequences in development. Three of these designs [51,53,56] stabilize the three receptor-binding domains (RBDs) in the 'down' conformation, compared to the other designs, which exhibit a mixture of one or more RBDs in the 'up' conformation. Two such designs employ disulfide bonds between protomers, stabilizing a closed form of the spike timer and eliminating the need for an exogenous trimerization motif. Further development of these complementary candidates may shed light on how conformational heterogeneity, in particular heterogeneity within the RBDs, affects immunogenicity.

Additional spike vaccine candidates feature domain deletions and combinations to focus the immune response against the individual domains. Several designs consist of only the RBD, which exhibits attractive features: the RBD plays an essential role in binding to the host receptor angiotensin-converting enzyme 2 (ACE2), it elicits a strong neutralizing immune response, and it proves amenable to high-yield production in mammalian cells, insect cells, and yeast. Notable designs include two cavity-filling substitutions, I358F and F392W, which increased expression of the spike RBD [57,58]. Interestingly, these two mutations reside within a hydrophobic pocket located deep in the RBD, which has been shown to bind linoleic acid [59]. A complementary approach was applied to MERS-CoV, which deleted the entire S1 subunit leaving just the S2 stem region [60]. A dozen substitutions were incorporated to stabilize the MERS-CoV spike S2, comprising a mix of disulfide, cavity-filling, electrostatic, and polar substitutions. The

resulting stabilized-spike stem elicited antibodies that cross-reacted with coronavirus spike proteins. Another study created chimeric spikes by combining the N-terminal domains, RBDs, and S2 domains of several related coronaviruses, which elicited protection against challenge with a panel of sarbecoviruses [61]. Deletions within the spike protein, or combinations of deletions, thus present an attractive avenue for focusing the immune response to areas away from the immunodominant RBD.

### Influenza virus

Influenza vaccine design faces the challenge of seasonal antigenic drift, which can result in reduced vaccine effectiveness within a matter of months. A vaccine composed of chimeric hemagglutinin antigens aims at eliciting protection against various influenza strains [62,63]. The vaccine combines either H5 or H8 head domains with stalks from subtype H1. This strategy appears to focus the immune response to the HA stalk, which elicits high titers of broadly neutralizing antistalk antibodies [64,65]. Domain deletion achieves a similar immunofocusing effect, as demonstrated by efforts to engineer HA composed only of the HA2 stem domain (i.e. lacking the immunodominant HA1 head domain) [66,67]. Headless stem antigens elicit cross-reactive antibodies and confer partial protection in nonhuman primates. Stabilization of the stem required iterative domain deletions, replacements, disulfide mutations, and cavity-filling substitutions [68,69].

### Nipah virus

Recent *paramyxovirus* vaccine design has targeted both the fusion (F) and attachment (G) envelope GP [70–72]. Loomis and colleagues designed disulfide, cavity-filling, and proline substitutions that stabilized the Nipah virus (NiV) F protein in both the prefusion and the postfusion conformation. Vaccine formulations containing prefusion NiV F elicited higher titers of neutralizing antibodies than did those containing postfusion NiV F. A chimeric antigen of NiV F/G chimera stood out as a particularly potent antigen. A separate study took preexisting stabilizing substitutions from Hendra virus (HeV) F and applied them to homologous sites in NiV F. The resulting antigen was used to isolate neutralizing antibodies that protected against NiV and HeV infection [73,74].

### Human metapneumovirus F

Current *pneumovirus* vaccine design follows in the footsteps of structure-based RSV F antigen design. Stewart-Jones et al. engineered four pairs of cysteine substitutions to covalently stabilize the human metapneumovirus (hMPV) F protein in its prefusion conformation [75]. Two of the disulfide bonds form within a single protomer, whereas the other two bridge adjacent protomers to stabilize the closed trimeric F quaternary structure. Combinations of interprotomer and intraprotomer disulfides resulted in a prefusion hMPV F protein with enhanced expression yield and thermostability. The authors also designed disulfide substitutions to stabilize the postfusion conformation. In a separate approach, Hsieh et al. screened combinations of disulfide, proline, cavity-filling, and electrostatic substitutions to generate several prefusion-stabilized variants [76]. The most stable variant harbors four intraprotomer disulfide bonds, a cavity-filling substitution, and two electrostatic substitutions. Interestingly, this variant may adopt a mixture of open and closed states, resembling similar observations for RSV F [77]. Future comparisons of constitutively closed variants and heterogeneous open/closed variants may shed light on the relationship between conformational heterogeneity and immunogenicity.

### Ebola and Marburg viruses

The fusion GP of *filoviruses* is the only known target of neutralizing antibodies and is thus the primary target of vaccine development. Rutten et al. generated an engineered Ebola and Marburg GP ectodomain by combining a proline substitution at a helix hinge and a novel substitution that stabilized interfaces by introducing noncovalent interactions [78]. The authors identified target interfaces between individual protomers and systematically compared all 20 possible amino acids to see if any stabilized the GP trimer. The most favorable substitution replaced a partially buried interface lysine with phenylalanine (K588F). A separate effort by Fels and colleagues tested a library of GP functional variants from the literature [79]. They identified a single substitution, R64A, which increased GP thermostability and expression yield while rendering it resistant to proteolysis. The stabilization mechanism of R64A remains unclear, as it resides in a region of unknown function and does not fit with any of the classical stabilizing substitution types.

## Outstanding challenges

The outbreak of the coronavirus pandemic put the principles of vaccine design to the test. Swift application of prior innovations enabled rapid deployment of the various coronavirus vaccine formulations. Together with advances in vaccine delivery and antigen presentation, structure-based vaccine design harbors the potential to provide useful vaccines against a variety of viral families. Effective mitigation against future outbreaks will require the constant vigilance of circulating strains combined with adaptative antigen design. Design strategies have historically proceeded one substitution at a time, which has yielded reliable, if gradual, results. Computational pipelines that leverage phylogenetic relationships have led to the discovery of mutations that confer desirable properties to a variety of proteins, including the stabilization of the *Plasmodium falciparum* invasion protein RH5 [80–82].

Deep-learning approaches have led to the discovery of beneficial mutations for enzymes, which could also be applied to antigen stabilization [83]. Applications of new technologies, such as large-scale mutational scanning, offer exciting possibilities to mine the deepest reaches of sequence space [57]. This approach may prove useful for finding mutations that operate by allosteric mechanisms, especially in cases where the antigen's structural ensemble exhibits conformational heterogeneity or intrinsic disorder [84]. The uncharted domain of bacterial vaccine design also presents a conspicuous challenge, given the sheer complexity of bacterial genomes and the looming specter of antibiotic resistance. A marriage of methods, from the traditional to the unconventional, may yet reveal undiscovered strategies for antigen stabilization.

## Author contributions

Writing — original draft, P.O.B.; Writing — reviewing and editing, P.O.B and J.S.M.

## Conflict of interest statement

P.O.B. and J.S.M. are inventors on U.S. patent application no. 63/032,502 ["Engineered Coronavirus Spike (S) Protein and Methods of Use Thereof"]. J.S.M. is an inventor on U.S. patent application no. 63/089,978 ["Prefusion-stabilized hMPV F Proteins"]. J.S.M. is an inventor on U.S. patent application no. 62/972,886 ["2019-nCoV Vaccine"]. J.S.M. is an inventor on U.S. patent no. 10,960,070 ["Prefusion Coronavirus Spike Proteins and Their Use"]. J.S.M. is an inventor on U.S. patent nos. 9,738,689, 10,017,543, and 11,130,785 ["Prefusion RSV F Proteins and Their Use"]. J.S.M. is an inventor on U.S. patent application no. 62/714,230 ["Nipah virus Immunogens and Their Use"].

## Acknowledgements

We thank Ching-Lin Hsieh for helpful discussions during drafting and writing. We also thank Scott Rush and Madeline Sponholtz for a critical reading of the paper. This work was funded in part by Welch Foundation (USA)Grant number F-0003-19620604 (J.S.M.).

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- •• of outstanding interest

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