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DESCRIPTION

Description

RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Application No. 61/780,910, filed March 13, 2013, No. 61/798,389, filed March 15, 2013, No. 61/857,613, filed July 23, 2013, and No. 61/863,909, filed August 9, 2013.

FIELD

[0002] This disclosure relates to polypeptides, polynucleotides, compositions, and methods of their use, for elicitation and detection of an immune response to respiratory syncytial virus (RSV).

BACKGROUND

[0003] Respiratory syncytial virus (RSV) is an enveloped non-segmented negative-strand RNA virus in the family Paramyxoviridae, genus *Pneumovirus*. It is the most common cause of bronchiolitis and pneumonia among children in their first year of life. RSV also causes repeated infections including severe lower respiratory tract disease, which may occur at any age, especially among the elderly or those with compromised cardiac, pulmonary, or immune systems. Passive immunization currently is used to prevent severe illness caused by RSV infection, especially in infants with prematurity, bronchopulmonary dysplasia, or congenital heart disease. Current treatment includes administration of a RSV-neutralizing antibody, Palivizumab (SYNAGIS®; MedImmune, Inc.), which binds a 24-amino acid, linear, conformational epitope on the RSV Fusion (F) protein.

[0004] In nature, the RSV F protein is initially expressed as a single polypeptide precursor, designated F_0. F_0 trimerizes in the endoplasmic reticulum and is processed by a cellular furin-like protease at two conserved sites, generating, F_1, F_2 and Pep27 polypeptides. The Pep27 polypeptide is excised and does not form part of the mature F protein. The F_2 polypeptide originates from the N-terminal portion of the F_0 precursor and links to the F_1 polypeptide via two disulfide bonds. The F_1 polypeptide originates from the C-terminal portion of the F_0 precursor and anchors the mature F protein in the membrane via a transmembrane domain, which is linked to an ~24 amino acid cytoplasmic tail. Three protomers of the F_2-F_1 heterodimer assemble to form a mature F protein, which adopts a metastable prefusion conformation that is triggered to undergo a conformational change that fuses the viral and target-cell membranes. Due to its obligatory role in RSV entry, the RSV F protein is the target of neutralizing antibodies and the subject of vaccine development; however, like other RSV antigens, prior efforts to develop an RSV F protein-based vaccine have proven unsuccessful.

[0005] Margo et al (2012) PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol 109x, no. 8, p3089-3094 discloses antibodies that bind the human RSV protein. McLellan, et al (2013) SCIENCE, Vol, 340, no. 6136, p1113-1117 disclose the structure of the prefusion RSV F protein.

SUMMARY

[0006] The invention is defined in the claims. The embodiments and/or examples of the following description which are not covered by the appended claims are considered as not being part of the present invention. Any references in the description to methods of treatment refer to the compounds, pharmaceutical compositions and medicaments of the present invention for use in a method for treatment of the human or animal body by therapy or for diagnosis.

[0007] As described herein, the three-dimensional structure of RSV F protein in its pre-fusion conformation was elucidated. The disclosure reveals for the first time the atomic level details of the prefusion conformation of RSV F, which presents a unique antigenic site ("antigenic site Ø") at its membrane distal apex. Using the three-dimensional structure of prefusion F as a guide, stabilized forms of prefusion F ("PreF" antigens) were engineered and constructed, and used to generate RSV neutralizing immune responses many fold greater than that achieved with prior RSV F protein-based immunogens, and which provide protection against RSV challenge in animal models. The PreF antigens can be used, for example, as both potential vaccines for RSV and as diagnostic molecules.

[0008] Isolated recombinant RSV F proteins that are stabilized in a prefusion conformation, as well as nucleic acid molecules encoding the recombinant RSV F proteins are disclosed. In several embodiments, the recombinant RSV F proteins are stabilized in a prefusion conformation by S155C, S290C, S190F, and V207L substitutions (compared to a native RSV F protein as set forth as onse of SEQ ID Nos: 1-184) such that the RSV F proteins can specifically bind to the prefusion-specific antibody D25. In several embodiments, the recombinant RSV F protein comprises an antigenic site ø comprising residues 62-69 and 196-209 of a RSV F protein sequence, such as SEQ ID NO: 370. In some embodiments, the immunogen can specifically bind to the antibody after the immunogen is incubated at 20°C in phosphate buffered saline at physiological pH for at least 24 hours in the absence of the antibody. In further embodiments, the immunogen can form a homogeneous population when dissolved in aqueous solution, wherein at least 90% of the immunogen in the population can specifically bind to the prefusion-specific antibody.

[0009] In some embodiments, the F_2 and F_1 polypeptides comprise RSV F positions 62-69 and 196-209, respectively, and the F_2 polypeptide comprise or consists of 8-84 residues of RSV F positions 26-109, and the F_1 polypeptides comprise or consists of 14-393 residues of RSV F positions 137-529, wherein the RSV F positions correspond to the amino acid sequence of a reference F_0 polypeptide set forth as SEQ ID NO: 124.

[0010] In several embodiments, the recombinant RSV F protein includes S155C, S290C, S 190F, and V207L amino acid substitutions that stabilize the protein in the prefusion conformation, for example, that stabilize the membrane distal portion of the F protein (including the N-terminal region of the F1 polypeptide) in the prefusion conformation. For example, the amino acid substitution can introduce a non-natural disulfide bond or can be a cavity-filling amino acid substitution. In several embodiments, the recombinant RSV F protein includes S155C and S290C substitutions that form a non-natural disulfide bond that stabilizes the protein in a prefusion conformation; that is, in a conformation that specifically binds to one or more pre-fusion specification antibodies, and/or presents an antigenic site, such as antigenic site Ø, that is present on the pre- but not post-fusion conformation of RSV F protein. In further embodiments, the recombinant RSV F protein can further include a F, L, W, Y, H, or M substitution at position 190, position 207, or positions 190 and 207. In one non-limiting example, the recombinant RSV F protein includes

S155C, S290C, S190F, and V207L substitutions (referred to herein as "DSCav1").

[0011] In additional embodiments, the recombinant RSV F protein can include one or more modifications to the C-terminus of the F1 polypeptide (such as truncations and amino acid substitutions) that, together with the modifications that stabilize the membrane distal region of the F polypeptide, can increase stabilization of the recombinant F protein in the prefusion conformation. Exemplary modifications include linkage of the F_1 polypeptide to a trimerization domain (such as a foldon domain) or introduction of one or more cysteine residues in the C-terminal region of the F1 polypeptide (for example, at positions 512 and 513) that can form inter-protomer disulfide bonds.

[0012] The PreF antigen can be included on a protein nanoparticle, or on a viral-like particle. Nucleic acid molecules encoding the PreF antigens are also disclosed. In some embodiments, the PreF antigen includes a recombinant RSV F protein that is a single chain RSV F protein.

[0013] Additional embodiments include an epitope-scaffold protein including RSV F positions 62-69 and 196-209, or a circular permutant thereof, linked to a heterologous scaffold protein, wherein the epitope scaffold protein specifically binds to a prefusion-specific antibody.

[0014] Compositions including the PreF antigens, protein nanoparticle, nucleic acid molecule or vector are also provided. The composition may be a pharmaceutical composition suitable for administration to a subject, and may also be contained in a unit dosage form. The compositions can further include an adjuvant.

[0015] Immunogenic compositions for use in methods for generating an immune response in a subject in need thereof are disclosed, as are immunogenic compositions for use in methods of treating, inhibiting or preventing a RSV infection in a subject in need thereof. In some embodiments of the methods, a subject, such as a human or bovine subject, is administered an effective amount of a disclosed antigen and/or a nucleic acid molecule encoding a disclosed antigen. In some embodiments, the methods include administration of an immunogenic composition including an adjuvant selected to elicit a Th1 biased immune response in a subject. In additional embodiments, the methods include a prime boost immunization, using human subtype A and human subtype B RSV F proteins stabilized in a prefusion conformation with the modifications disclosed herein. Methods for detecting or isolating an RSV binding antibody in a subject infected with RSV are disclosed. In some embodiments, the recombinant RSV F proteins can be used to detect and quantify target antibodies in a polyclonal serum response.

[0016] The foregoing and other objects, features, and advantages of the embodiments will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

[0017]

FIGs. 1A-1C are a set of graphs and an diagram illustrating RSV neutralization, F glycoprotein recognition, and the crystal structure of human antibody D25 in complex with the prefusion RSV F trimer. The prefusion conformation of RSV F is metastable, and when expressed in a soluble form readily adopts the postfusion state; a number of potent antibodies, including D25, bind to a newly revealed antigenic site at the top of the prefusion F glycoprotein. (**A**) RSV neutralization by antibodies including palivizumab, the FDA-approved prophylactic antibody to prevent severe RSV disease. (**B**) Enzyme linked immunosorbant assay (ELISA) measuring antibody binding to postfusion F glycoprotein. (**C**) D25-RSV F trimer structure in ribbon and molecular surface representations. One protomer of the F glycoprotein trimer is shown as ribbons. Molecular surfaces are shown for the other two F protomers. The D25 Fab bound to the F protomer shown in ribbons is also displayed in ribbon representation, with heavy chain shaded dark grey and light chain shaded light grey. The other D25 Fabs are shaded same, but shown in surface representation.

FIGs. 2A and 2B are a set of diagrams and a sequence aligned with RSV secondary structure illustrating the structural rearrangement of RSV F. To mediate virus-cell entry, the RSV F glycoprotein transitions from a metastable prefusion conformation to a stable postfusion conformation. (A) Prefusion and postfusion structures. Outer images display prefusion (left) and postfusion (right) trimeric structures, shaded the same as in FIG. 1C. A complex glycan, shown as sticks, is modeled at each of the three N-linked glycosylation sites found in the mature protein. Inner images display a single RSV F protomer in ribbon representation. (**B**) RSV F sequence and secondary structure. Sites of N-linked glycosylation are highlighted by black triangles, antigenic sites are labeled, and downward arrows indicate the position of furin cleavage sites. Secondary structures are shown below the sequence (SEQ ID NO: 370), with cylinders representing α-helices and arrows representing β-strands. Disordered or missing residues are indicated by an "X"; residues that move over 5 Å between prefusion and postfusion conformations shown with grey shadow and are boxed.

FIGs. 3A-3C show a set of diagrams and a sequence alignment illustrating the RSV F interface with D25. Antibody D25 binds a quaternary epitope spanning two protomers at the apex of the prefusion F trimer. **(A)** Close-up of the interface between D25 and RSV F. Side chains of F residues interacting with D25 are labeled and shown as sticks. Oxygen atoms are shaded light grey and nitrogen atoms are shaded dark grey. Hydrogen bonds are depicted as dotted lines. The two images are related by a 90° rotation about the vertical axis. (**B**) Position and conformation of the D25 epitope on the prefusion and postfusion F molecules. RSV F residues at the D25 interface are shown. Polarity of α4 and $\alpha 5_{post}$ indicated with arrows, with fragment N- and C-termini indicated. (**C**) Sequence conservation of F residues in regions recognized by D25. Amino acids in human RSV subtype B (hRSV/B) or in bovine RSV (bRSV) that differ from hRSV/A are underlined. Ectodomain is defined as F residues 26-109 and 137-524.

FIGs. 4A-4D are series of graphs and digital images concerning antigenic site Ø. Highly effective RSV-neutralizing antibodies target a site at the membrane-distal apex of the prefusion F trimer. (**A**) The ability of antibodies to block D25 binding to RSV-infected cells was measured as a function of antibody concentration. (**B**) Analysis of RSV F/Fab complexes by negative stain electron microscopy: (Left) Reprojection of a 12 Å slice through the crystal structure of RSV F + D25 Fab filtered to 10 Å resolution and sliced to include the F-trimer cavity. (Middle) Aligned average of 263 particles of RSV F + D25 Fab. (Right) Aligned average of 550 particles of RSV F + AM22 Fab. Scale bar in middle panel is 50 Å. (**C**) Fusion inhibition and (**D**) attachment inhibition activity for antibodies targeting antigenic site QJ and F-specific antibodies targeting other antigenic sites. For the attachment-inhibition assay, heparin was used as a positive control.

FIG. 5 shows a schematic diagram illustrating the methods used to express complexes of RSV F and D25. Plasmids expressing RSV F(+) Fd (F circle), the D25 light chain (L circle), and the D25 heavy chain (with or without a stop codon in the hinge region, H circle) were simultaneously transfected into HEK293 cells in suspension. Alternatively, the RSV F(+) Fd plasmid could be transfected, with purified D25 Fab or IgG added to the cells 3 hours post-transfection. The best yields were obtained by simultaneously expressing F and D25 Fab (~1.0 mg of purified complex per liter of cells).

FIG. 6 shows a set of ribbon diagrams illustrating the comparison of D25-bound RSV F to prefusion PIV5 F. Ribbon representation of D25-bound RSV F (+) Fd (left) and PIV5 F-GCNt (right). There is excellent agreement of secondary structure elements between the two proteins, despite having only -12% sequence identity. One of the most striking differences is the location of the fusion peptide (N-terminus of F_1 subunit), also shown in FIG. 7. The PIV5 F structure was described as consisting of three domains: I, II and III (Yin et al., Nature, 439, 38 (2006)). Domain III termed the membrane distal lobe, whereas domains I and II encompass the central barrel and membrane proximal lobe. The cleaved PIV5 structure shown here was generated from PDB ID: 4GIP (Welch et al., Proc. Natl. Acad. Sci., U.S.A. 109, 16672 (2012)).

FIG. 7 shows a series of diagrams illustrating Type I prefusion viral glycoproteins. Prefusion structures of RSV F, PIV5 F (PDB ID: 4GIP (Welch et al., Proc. Natl. Acad. Sci., U.S.A. 109, 16672 (2012)), influenza HA (PDB ID: 2HMG; Wilson et al., Nature, 289, 366 (1981)) and Ebola GP (PDB ID: 3CSY; Lee et al., Nature, 454, 177 (2008)) are shown as molecular surfaces, with each protomer colored differently. On the bottom row, a sphere is shown for the C-terminal residue of F_2 (RSV and PIV5) or HA_1 (Flu), and a sphere is show for the N-terminal residue of the fusion peptide. The RSV and PIV5 are both paramyxoviruses and their F proteins share ~12% sequence identity. Although Ebola GP is a type I fusion protein, it lacks a free N-terminal fusion peptide on GP2, and instead contains an internal fusion loop that is commonly seen in type II and type III fusion proteins. Thus, the Ebola GP was omitted from the fusion peptide comparison.

FIG. 8 is a set of graphs concerning RSV neutralization by IgG and Fab. D25, AM22 and Motavizumab neutralize RSV equally well as IgG or Fab. Note that the x-axis for the Motavizumab plot is different than the others.

FIGs. 9A and 9B are a series of diagrams and graphs illustrating properties of antigenic sites on the RSV F glycoprotein. Only antibodies directed to antigenic site Ø bind specifically to the prefusion conformation and have exceptional neutralization potency. (A) For site Ø, an image of a single D25 Fab binding to the prefusion RSV F trimer is shown, along with neutralization curves for AM22 and D25. For site I, arrows point to Pro389, a known escape mutation (Lopez et al., J. Virol., 72, 6922 (1998)). A neutralization curve is shown for antibody 131-2a. Like antibody 2F (Magro et al., J. Virol., 84, 7970 (2010)), antibody 131-2a only neutralizes ~ 50% of the virus. (B) For antigenic sites II and IV, models of Motavizumab (site II) and 101F (site IV) binding to the prefusion and postfusion (McLellan et al., J. Virol., 85, 7788 (2011)) F structures were made using the coordinates of antibody-peptide structures (McLellan et al., J. Virol., 84, 12236 (2010); McLellan et al., Nat. Struct. Mol. Biol., 17, 248 (2010)).

FIG. 10 shows an image of a polyacrylamide gel illustrating expression of the recombinant RSV F protein construct with S155C and S290C amino acid substitutions and a Foldon domain linked to the C-terminus of F_1, and a set of diagrams illustrating that the disulfide bond between S155C and S290C can only form in the prefusion conformation of RSV F protein.

FIG. 11 is a set of graphs showing results from ELISA and gel filtration assays using the recombinant RSV F protein construct with S155C and S290C amino acid substitutions and a Foldon domain linked to the C-terminus of F_1. The ELISA data indicate that the S155C/S290C construct is specifically bound by RSV F prefusion specific antibodies. The gel filtration profiles show that the S155C/S290C construct exists solely as a trimer, whereas aggregates and rosettes form in solution with a control RSV F construct lacking the S155C/S290C substitutions.

FIG. 12 shows negative-stain electron microscopy images of recombinant RSV F protein construct with S155C and S290C amino acid substitutions and a Foldon domain linked to the C-terminus of F1. The images below the large panel are 2D averages of individual particles. The results indicate that the S155C/S290C construct is stabilized in the prefusion conformation.

FIGs. 13-14 show a set of graphs illustrating the neutralizing antibody response of mice administered native RSV (RSV), formalin inactivated RSV (FI-RSV), the recombinant RSV F protein construct with S155C and S290C amino acid substitutions and a Foldon domain linked to the C-terminus of F_1 (prefusion F), or a RSV F protein construct stabilized in the postfusion conformation (postfusion RSV). The antibody response at 5 weeks (FIG. 13) and 7 weeks (FIG. 14) post-initial immunization is shown.

FIG. 15 shows digital images of the crystals of a soluble recombinant RSV F protein stabilized in a prefusion conformation by S155C and S290C substitutions. Left, standard light images; Right, ultraviolet images, indicative of proteins. The formation of crystals from aqueous buffered solutions demonstrates that this protein is substantially homogeneous in solution.

FIG. 16 shows the design of a RSV F protein based antigen (RSV_A F(+)FdTHS) stabilized by engineered disulfide bond mutations S155C and S290C ("DS"), cavity-filling mutations S190F and V207L ("Cav1"), and appended C-terminal heterologous trimerization domain (Fd). The D25-bound RSV F structure is shown with two of the protomers displayed as a molecular surface colored pink and tan, and the third protomer displayed as ribbons. The N- and C-terminal residues of F_1 that move more than 5Å between the pre and postfusion conformations are shown. Insets show the engineered disulfide bond between residues S155C and S290C (named "DS"), as well as the space-filling cavity mutations S190F and V207L (named "CavI"). A model of the T4 phage fibritin trimerization domain is shown at the base of the prefusion trimer. The RSV F protein including the S155C and S290C, and S190F and V207L substitutions in human RSV subtype A, and the appended C-terminal heterologous Foldon domain, is termed RSV_A F(+)FdTHS DSCav1. Mutations compatible with D25 recognition, but insufficiently stable to allow purification as a homogenous trimer, are labeled and shown in black stick representation.

FIG. 17 shows the antigenic characterization of RSV_A F(+)FdTHS DSCav1. The association and dissociation rates of soluble D25, AM22, 5C4, 101F, Motavizumab, and Palivizumab Fab interaction with immobilized RSV_A F(+)FdTHS DSCav1 were measured using an OctetRED 384™ instrument (ForteBio, Melno Park, CA). Equilibrium dissociation constants for each antibody are provided.

FIG. 18 shows size exclusion chromatography of RSV_A F(+)FdTHS DSCav1. Purified protein, after thrombin cleavage to remove the tags, was passed over a 16/70 Superose 6 size exclusion column. The elution volume is consistent with a glycosylated trimer.

FIG. 19 shows a table listing antigenic and physical characteristics of RSV_A F(+)FdTHS variants stabilized by DS, Cav1 or DSCav1 alterations. The left most column defines the RSV F variant, and the rest of the columns provide variant properties, including yield from transiently expressed plasmids, antigenicity against various antigenic sites, and the retention of D25-binding (provided as a fractional amount) after 1 hour of incubation at various temperatures, pHs, and osmolality, or to 10 cycles of freeze-thaw. The DSCav1 variant retains antigenic site Ø recognition, with improved physical stability, as judged by higher retention of D25-reactivity after exposure to extremes of temperature, pH, osmolality and freeze-thaw, then either DS or CavI variants.

FIG. 20 shows a ribbon representation of the 3.1 Å crystal structure of RSV_A F(+)FdTHS DSCav1. Thicker ribbons correspond to increasing B-factors. Despite stabilizing mutations, antigenic site Ø, at the trimer apex, retains significant flexibility.

FIG. 21 shows comparison of RSV_A F(+)FdTHS DSCav1 to D25-bound RSV F. Ribbon representation of RSV_A F(+)FdTHS DSCav1, superposed with a ribbon representation of D25-bound RSV F colored white (PDB ID 4JHW). The images are related by a 90° rotation about the vertical axis.

FIG. 22 shows stabilizing mutations in RSV_A F(+)FdTHS DSCav1 structure. Ball-and-stick representation of RSV_A F(+)FdTHS DSCav1 crystal structure with $2F_o$-F_c electron density contoured at 1σ is shown as a mesh. These images indicate that electron density corresponding to the disulfide bond between cysteine residues 155 and 290 (left), as well as the cavity-filling Phe190 residue (right), is observed.

FIG. 23 shows mouse immunogenicity of RSV_A F(+)FdTHS DSCav1. Ten CB6 mice per group were immunized with 10 µg of RSV_A F(+)FdTHS DSCav1 protein mixed with 50 µg of poly I:C adjuvant. Immunizations occurred at 0 and 3 weeks, and sera from week 5 and week 7 were tested for neutralization of RSV subtype A (RSV_A) and B (RSV_B). Mean values are indicated by horizontal lines.

FIG. 24 shows non-human primate (NHP) immunogenicity of RSV_A F(+)FdTHS DSCav1. Four RSV-naïve rhesus macaques per group were immunized with 50 µg of RSV_A F(+)FdTHS DSCav1 protein mixed with 500 µg of poly I:C adjuvant. Immunizations occurred at 0 and 4 weeks, and sera from week 6 were tested for neutralization of RSV subtype A (left) and B (right). Mean values are indicated by horizontal lines.

FIGs. 25A-25C show plasmid maps of expression vectors. **(A)** A map of the RSV_A F(+)FdTHS DSCav1 paH expression vector (SEQ ID NO: 384) for expressing recombinant RSV F protein from human subtype A including S155C, S290C, S190F and V207L amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II. **(B)** A map of the RSV_B (B1) F(+)FdTHS DSCav1 paH expression vector (SEQ ID NO: 386) for expressing recombinant RSV F protein from human subtype B (strain B1) including S155C, S290C, S190F and V207L amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II. **(C)** A map of the RSV_B (18537) F(+)FdTHS DSCav1 paH expression vector (SEQ ID NO: 388) for expressing recombinant RSV F protein from human subtype B (strain 18537) including S155C, S290C, S190F and V207L amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II.

FIGs. 26A-26D illustrate structure-based vaccine design for RSV: a supersite paradigm. (A) Natural infection by RSV elicits diverse antibodies, with a range of viral neutralization potencies. (B) A cluster of epitopes for naturally elicited, highly potent antibodies defines a supersite of viral vulnerability. Shown are antigen-binding fragments of the potently neutralizing antibody D25 recognizing an epitope at the apex of the RSV F trimer. Spatially overlapping epitopes at the trimer apex are also recognized by the AM22 and 5C4 antibodies, which share the same desired neutralization characteristics as D25. These overlapping epitopes define antigenic site Ø as a supersite of RSV vulnerability. (C) After selection of a target supersite, an iterative process of design, characterization of antigenic and physical properties, atomic-level structure determination, and assessment of immunogenicity allows for the structure-based optimization of vaccine antigens encoding the target supersite. (D) Because the supersite of viral vulnerability naturally elicits highly protective antibodies, immunization with "supersite immunogens" more easily elicits protective response than immunogens based on viral regions recognized by subdominant or non-potently neutralizing antibodies.

FIG. 27 shows design of soluble trimeric site Ø-stabilized RSV Fs. Over 100 variants of RSV F containing the T4 fibritin-trimerization domain (foldon) were designed to more stably retain antigenic site Ø. Shown here is the structure of the RSV F trimer in its D25-bound conformation with modeled foldon. The trimer is displayed with two protomers as molecular surfaces shaded light grey tan and pink, and the third promoter as ribbons. The ribbon is shaded white in regions where it is relatively fixed between pre- and postfusion, while the N- and C-terminal residues that move more than 5 Å between pre- and postfusion conformations are shaded darker grey. Mutations compatible with expression and initial D25 recognition, but insufficiently stable to allow purification as a homogenous trimer are labeled and shown in black stick representation. Insets show close-ups of stabilizing mutations in stick representation for DS, Cavl and TriC variants, all of which stably retain antigenic site Ø (FIG. 31).

FIGs. 28A-28C show structures of RSV F trimers, engineered to preserve antigenic site Ø. (A-C) Six structures for RSV F variants are shown, labeled by stabilizing mutation (DS, Cav1, DS-Cav1, and DS-Cav1-TriC) and by the lattice (cubic and tetragonal) and crystallization pH. (A) RSV F trimers are displayed in Cα-worm representation, colored according to atomic mobility factor. Missing regions are shown as dotted lines. These occur at the C-terminal membrane-proximal region, where the foldon motif is not seen, except in the DS-Cav1-TriC structure (far right). In the DS structure, two loops in the head region are also disordered. (B) Antigenic site Ø of a RSV F protomer is displayed in ribbon diagram, with the structure of D25-bound RSV F in gray and different variants indicated. Stabilizing mutations are labeled and shown in stick representation. (C) Atomic-level details are shown in stick representation, with regions of RSV F that change conformation between prefusion and postfusion conformation in dark grey, and those that remain constant in lighter gray. Stabilizing carbon atoms for stabilizing mutations are indicated. In Cavl (pH5.5) and in DS-Cav1 (pH5.5) novel features were observed involving the interaction of the C-terminus of the F_2 peptide with a sulfate ion and the fusion peptide. In the DS-Cav 1- TriC structure, the D486HE487Q-F488W-D489H mutations interact with the two neighboring protomers around the trimer axis.

FIGs. 29A-29B show results concerning immunogenicity of engineered RSV F trimers. RSV F proteins engineered to stably display antigenic site Ø elicit neutralizing titers significantly higher than those elicited by postfusion F. (A) Neutralization titers of sera from mice immunized with 10 μg of RSV F (left). Postfusion F, as well as RSV F bound by antibodies AM22 or D25, were immunized at 20 μg per mouse (right). Geometric mean is indicated by a horizontal line. (B) Neutralization titers of sera from rhesus macaques immunized with 50 μg of RSV F protein variants. Geometric mean is indicated by a horizontal line. Protective threshold is indicated by a dotted line, and *p*-value provided for postfusion versus DS-Cav1.

FIGs. 30A-30D show how physical, structural, and antigenic properties of antigenic site Ø-stabilized RSV F correlate with immunogenicity. (A) Physical stability of site Ø versus immunogenicity. Inset shows information transFer Physical stability as determined by 7 measurements of D25 retention of activity in FIG. 31 were averaged (horizontal axis) and compared to elicited RSV-protective titers from Fig. 29 (vertical axis). (B) Structural mimicry of site Ø versus immunogenicity. Inset shows information transfer. Structural mimicry (horizontal axis) is the rmsd between different structures (FIG. 28) and D25-bound RSV F for all atoms within 10 Å of D25. This is compare to elicited RSV-protective titers from Fig. 29 (vertical axis). (C) Antigenic analysis of sera from immunized macaques. Binding of sera to immobilized DS-Cav1 (left) or postfusion F (right) was measured directly (Blank, black bars) or after incubation with excess postfusion F (dark grey bars) or DS-Cav1 (light grey bars). The mean response of the four macaque sera is graphed, with error bars for the standard deviation. (D) Correlation of immunogenicity and antigenicity of NHP sera. The mean neutralization titers of the four macaque sera in each group are plotted against the ratio of binding responses to DS-Cav1 and postfusion F.

FIGs. 31A-31B are a table showing the results of antigenic and physical characterization of RSV F protein immunogens. [#]Defined for trimeric state, but if no trimeric state could be purified, then the oligomeric state of the dominant oligomeric species. If total yield is <0.1 mg/l, then oligomeric state is not determined (N.D.). *Yield is shown for specific oligomeric state. >1000 nM = no binding at 1 μM Fab concentration. N/A = not applicable.

FIG. 32 shows the location of S155 and S290 in the pre- and postfusion RSV F structures. The β-carbons of serine residues 155 and 290 are 4.4 Å apart in the D25-bound RSV F structure and 124.2 Å apart in the postfusion structure. The mutations S155C and S290C (called "DS") restrained the structure in the prefusion conformation.

FIGs. 33A-33C shows negative staining of stabilized F-protein. A) and B) show representative fields of negatively stained specimens for DS and DS-Cav1. The proteins are highly homogenous with <1% and <0.1% of post-F conformations observed in DS and DSCav1, respectively. Examples of post-F conformations are indicated by black arrows. Bar = 50 nm. 2D particle averages are shown as insets in the top right corner at twice the magnification. Bar = 5 nm. C) shows a comparison of the 2D averages with the average of F+D25 complex (McLellan et al. 2013). Bar = 5 nm.

FIG. 34 shows the antibody D25 based ELISA of the crude culture supernatants is correlated (Spearman R = 0.7752 and a P value=0.0041) to the yield of purified oligomeric RSV F glycoprotein variants. RSV F glycoprotein production by 293 Expi cells was determined by D25 ELISA of the crude culture supernatants at 4°C one week after harvesting and found to correlate with the yield of pure oligomeric RSV F glycoprotein variants (Table 1).

FIG. 35 shows the antibody motavizumab based ELISA of the crude culture supernatants versus the yield of purified RSV F glycoprotein variants. (A) RSV F glycoprotein production by 293 Expi cells was determined by motavizumab ELISA of the crude culture supernatants at 4°C immediately upon harvest and (B) one week after harvesting. ELISA data is plotted versus the yield of RSV F glycoprotein variants after streptactin affinity. Interestingly, three proteins, RSV F(+) Fd and two variants F137W-F140W and T357C-N371C were detected as high expressers by motavizumab ELISA but low yields were obtained after large scale purification (points shown along the ordinate).

FIG. 36 shows the characterization of engineered RSV F glycoproteins using size exclusion chromatography. RSV F variants, a: Cav1; b: Cav1-TriC; c: DS-Cav1-TriC; d: F488W; e: DS-Cav1; f: TriC, g: DS-TriC; h: DS; exhibit elution profiles characteristic of a globular trimeric protein, whereas RSV F variants i: S190F-V296F; j: K87F-V90L; k: V207L-V220L; 1: V178N; m: S403C-T420C; n: I506K; o: V185E; p: F137W-F140W-F488W; q: D486H-E487Q-D489H exhibit elution profiles characteristic of higher oligomeric species. Protein standards of known molecular weight are labeled on the base of the chromatogram.

FIG. 37 shows antigenic site Ø shown from above. The regions of DS which are not visible are represented by dotted lines.

FIGs. 38A-38B shows results concerning antigenic characterization of immunogen-adjuvant complexes for non-human primate immunization. (A) RSV F post fusion, DS and DS-Cavlsample reactivity was assessed against 1 µM D25 antigen-binding fragment less than 3 h following immunogen formulation with Poly I:C and NHP immunizations at day 0 and (B) week 4.

FIGs. 39A-39B shows antigenic analysis of sera from immunized mice and rhesus macaques. A) Sera from mice immunized with multiple stabilized RSV F variants was assessed for binding to immobilized DS-Cav1 was measured directly or DS-Cav1 after incubation with excess D25 or motavizumab antigen-binding fragments to assess the site Ø or site II responses . B) Sera from rhesus macaques was assessed for binding to immobilized DS-Cav1 or postfusion RSV F variants also blocked with D25 or motavizumab antigen-binding fragments. The mean response of the animal sera is shown, with error bars for the standard deviation.

FIG. 40 shows crystallographic data collection and refinement statistics.

FIG. 41 shows the effect of using RSV subtype B constructs with the DS substitutions and that adjuvants including TLR4 agonists can work with the stabilized F protein. CB6F1/J mice were immunized with 10 µg of the DS S155C/S290C version of stabilized prefusion F formulated with 50 µl of Ribi (Ribi adjuvant system, Sigma). Mice were inoculated at 0 and 3 weeks with either the subtype A construct (SEQ ID NO: 185), the subtype B construct (SEQ ID NO: 1479), or both (10 µg of each). At the 5 week time point (2 weeks after the second injection), serum was obtained for neutralization assays. The two major findings from this experiment were that, 1) $preF_A$-DS and $preF_B$-DS induce equal levels of neutralizing activity against RSV subtype A, while $preF_B$-DS induced a higher level of neutralizing activity than $preF_B$-DS against RSV subtype B. This suggests that using the RSV subtype B constructs may have better cross-neutralizing potential than subtype A constructs or that hybrid versions of RSV F that include elements from both subtypes may be preferred. 2) The Ribi adjuvant is an oil-in-water emulsion containing monophosphoryl lipid A, which is a TLR4 agonist and representative of some of commercial adjuvants. These data show that in addition to polyI:C (a TLR3 agonist), adjuvants that include TLR4 agonists function with the stabilized prefusion F protein as a vaccine antigen.

FIG. 42 shows that the stabilized prefusion F can be formulated in alum as well as polyI:C and retain immunogenicity conferred by antibody responses to antigenic site Ø. BALB/c mice were immunized with 20 µg of the DS S155C/S290C version of stabilized prefusion F derived from subtype A and formulated with alum (aluminum hydroxide gel 10 mg/ml, Brenntag, Frederikssund, Denmark) or polyI:C. Mice were inoculated at 0 and 3 weeks, and at the 5 week time point (2 weeks after the second injection), serum was obtained for neutralization assays.

FIG. 43 is a schematic diagram illustrating an exemplary design scheme for prefusion-stabilized single-chain RSV F (scF) antigens, including the variables that are involved with several different RSV scF designs. Design elements that pertain to RSV scF no. 9 (BZGJ9 DS-Cav1; SEQ ID NO: 669 are outlined in dark grey.

FIGs. 44A and 44B illustrate the design of single-chain RSV F construct no. 9 (scF no. 9; BZGJ9 DSCav1; SEQ ID NO: 669). Numbering indicates residue locations of the various components described below. (A) Schematic representations of furin-cleaved RSV F(+) glycoprotein as shown in FIG. 44B (top), and RSV scF no. 9 design (bottom), showing the foldon trimerization domain (grey oval), and the artificial linker (grey square) bridging the polypeptide backbones of F_2 (left) and F_1 (right). (B) Structural basis for RSV scF no. 9 design using a prefusion-stabilized RSV F(+) structure as a model (PBD ID: 4MMV). RSV F(+) is shown in cartoon representation and the foldon trimerization domain shown in sphere representation. Shown on the left is the prefusion-stabilized RSV F(+) trimer, with the three protomers colored black, gray, and white. Shown on the right is a single RSV F(+) protomer showing F_1 (medium gray), F_2 (dark grey), the fusion peptide (indicated), and the foldon trimerization domain (light grey, indicated). The inset shows the fusion peptide in stick representation, and the location of the flexible linker sequence (dashed line) joining residues 104 and 147.

FIG. 45 shows a table concerning the design, oligomeric state, and production yield of engineered single-chain RSV F constructs expressed in HEK293-F cells. RSV F construct no. 9 DSCav1 (scF no. 9; BZGJ9 DSCav1; SEQ ID NO: 669), RSV F construct no. 10 DSCav1 (scF no. 10; BZGJ10 DSCav1; SEQ ID NO: 670) RSV F construct no. 11 DSCav1 (scF no. 11; BZGJ11 DSCav1; SEQ ID NO: 671) are indicated. The provided linker sequences include GSGNIGLGG (SEQ ID NO: 364), GSGGNGIGLGG (SEQ ID NO: 359), GSGNVLGG (SEQ ID NO: 361), and GSGNVGLGG (SEQ ID NO: 362). (%) Prefusion stabilizing mutations include the following: S155C and S290C (DS); S190F and V207L (Cavl); no additional mutations (a). All variants contain the point mutation L373R. (==) Trimerization domains include the following: L512C and L513C (CC); D486C, E487P, and F489C (CPC). (#) Variants were often observed to exist in a mixture of oligomer states on size chromatography. If a measureable trimeric fraction was observed, then then oligomeric state is listed as "Trimer". If no trimeric fraction was observed, then the oligomeric state of the dominant species is provided. If the total yield prior to size chromatography was <0.1 mg/L , then oligomeric state is listed as not determined (N.D.). If oligomeric state was indistinguishable by size exclusion chromatography, oligomeric state is listed as "Aggregate". (*) Yield shown was calculated post-StrepTag purification and is listed for the specified oligomeric state. (Φ) HEK 293F yield is estimated based on observed ratio between Expi293F expression yield and Freestyle293F expression yield seen in scF constructs (~2:1).

FIGs. 46A and 46B are a set of graphs illustrating characterization of the engineered single-chain RSV F glycoproteins by size-exclusion chromatography. **(A)** Size-exclusion profiles of RSV scF variants (scF no. 3, 4, 6, 8 though 11) and RSV F(+) containing DS-Cav1 stabilizing mutations. Single-chain constructs were expressed in HEK293F cells and F(+) DS-Cav1 was expressed in Expi 293-F cells. F(+) DS-Cav1 and scF no. 3 DS-Cav1, no. 4 DS-Cav1, no. 6 DS-Cav1, and no. 9 DS-Cav1 exhibit elution profiles characteristic of a globular trimeric protein, whereas scF no. 11 DSCav1 exhibits an elution profile characteristic of a globular monomeric protein. RSV scF no. 8 DS-Cav1 and scF no. 10 DS-Cav1 exhibit elution profiles suggesting a heterogeneous mixture of both monomeric and trimeric species. **(B)** Size-exclusion profiles of RSV F(+) DS-Cav1 and RSV scF no. 9 containing different stabilizing mutations. F(+) DS-Cav1, and scF no. 9 Cav1 were expressed in Expi 293-F cells and the remaining scF no. 9 variants were expressed in HEK293F cells. The slight deviation in the elution profiles of scF no 9 variants suggest that scF no. 9 runs at a higher molecular weight than trimeric F(+). An asterisk indicates that purification tags were cleaved prior to gel filtration.

FIG. 47 is a table summarizing the results of antigenic characterization of RSV scF no. 9 DS-Cav1.

FIG. 48 is a table showing the crystallographic data and refinement statistics for the three dimensional structure of RSV scF no. 9 DS-Cav1.

FIGs. 49A and 49B show a series of diagrams concerning the crystal structure of RSV scF no. 9 DS-Cav1 trimer. The orientation of the protomer displayed in cartoon representation (dark grey) is kept constant. Thick dotted lines represent the C-terminal foldon motif located at the membrane-proximal region, which is not visible in the crystal structure. **(A)** RSV scF no. 9 DS-Cav1 trimer displayed with protomers in cartoon representation and ribbon representation (dark grey), and molecular surface representation (light grey). Inset shows enlargement of the "GS" scF no. 9 linker loop (indicated) and the adjacent protomer (dark grey), both in stick representation. **(B)** Prefusion stabilizing mutations in the RSV scF no. 9 DS-Cav1 structure. DS and Cav1 prefusion stabilizing mutations are indicated and shown in stick representation.

FIG. 50 is a diagram illustrating the structural alignment of RSV scF no. 9 DS-Cav1 (medium grey) with the F(+) DS-Cav1 structure (light gray; rmsd = 0.839 Å) and with the D25-bound F(+) structure (dark gray; rmsd = 0.534 Å), all displayed in cartoon representation. Inset shows a close-up of the scF no. 9 linker loop and the fusion peptides of the F(+) DS-Cav1 structure, and the D25-bound F(+) structure.

FIG. 51 shows diagrams illustrating the comparison of RSV scF designs no. 3, 4, 6, 8-11 using the crystal structure of RSV scF no. 9 DS-Cav1. Thick dotted lines represent the C-terminal foldon motif. RSV scF no. 9 DS-Cavl protomer displayed in cartoon representation (dark grey). Inset shows enlargement of the "GS" scF no. 9 linker loop in stick representation joining residues 105 (F_2) and 145 (F_1). The predicted locations of the flexible linker sequences for scF designs no. 3, 4, 6 and 8 (thin dotted line) joining residues 97 (F_2) and 150 (F_1) are mapped onto the scF no. 9 DS-Cav1 crystal structure. The predicted locations of the linker sequences for scF designs no. 3, 4, 6 and 8 (thin dotted lines) are mapped onto the scF no. 9 DS-Cav1 trimer structure. Linker end point residue locations are approximated.

FIG. 52 shows a set of digital images concerning characterization of the engineered single-chain RSV F glycoproteins characterized by SDS-PAGE gel electrophoresis post *Strep*Tag purification. RSV scF constructs were expressed in HEK293F cells and purified by His_6-tag and *Strep*Tag affinity chromatography.

FIG. 53 is a set of graphs and a table providing week 5 neutralization data for the indicated constructs (10 animals/group). Immunizations at Week 0 and Week 3 with 10 µg protein + 50 µg Poly I:C per animal.

FIG. 54 is a ribbon and stick diagram highlighting the Proline residue at RSV F position 101 in the three-dimensional structure of RSV scF no. 9 (SEQ ID NO: 669). The structure indicates that the single chain linker region may be improved by removing Proline 101 or shortening/mutating the linker residues and adjacent residues.

FIG. 55 is a graph and a sequence alignment illustrating modification of the scF no. 9 construct (SEQ ID NO: 669) to generate the BZG J9-1 to BZG J9-10 constructs. The sequence alignment shows BZG J9-1 to BZG J9-10 sequences corresponding to RSV F residues 97-159 of SEQ ID NOs: 698-707, respectively. These constructs were expressed in Expi cells and assessed by gel filtration (left).

FIG. 56 is a series of graphs and schematic diagrams illustrating a ferritin nanoparticle including the scF no. 9 protein, which was generated by linking the C-terminus of the F1 polypeptide in scF no.9 to a ferritin subunit. This construct is termed "BZGJ9-DS-Cav1-LongLink-Ferritin" and provided as SEQ ID NO: 1429.

FIG. 57 is a set of graphs illustrating the physical stability of BZGJ9-DS-Cav1-LongLink-Ferritin compared to RSV F DS-Cav1.

FIGs. 58A-58C are a set of graphs and a table illustrating the immunogenicity of different prefusion stabilized RSV F proteins. The three constructs tested were RSV F DSCav1 (SEQ ID NO: 371), BZGJ9-DS-Cav1-LongLink-Ferritin (SEQ ID NO: 1429), and scF no. 9 (also termed BZGJ9 DS-Cav1, SEQ ID NO: 669). Macaca mulatta animals of Indian origin weighing 8.26-11.34 kg were intramuscularly injected with immunogens at week 0 with 50 µg protein + 500 µg Ribi per animal, Boost at Week 4 with 50 µg protein + 500 µg Ribi per animal; immunogenicity was assessed at week 3.5.

FIGs. 59A and 59B are a set of diagrams illustrating the three-dimensional structure of the RSV F protein from the B 18537 strain with the DSCav1 mutations (SEQ ID NO: 372) (A) Cartoon representation of a protomer of RSV F. (B) Trimeric form of the fusion glycoprotein with the additional protomers shown in surface and ribbon representations.

FIGs. 60A-60D are a set of images illustrating the atomic level details of the RSV B18537 F glycoprotein with DSCav1 substitutions, and showing that the DSCav1 substitutions can be introduced into a RSV F glycoprotein B subtype to stabilize antigenic site Ø. (A) DS-Cav1 mutations are highlighted. (B) Antigenic site Ø located at the apex of the trimer is shown in stick representation in dark grey. (C) The interaction between the fusion peptide and β strands 15, 16 and 19 to form and inter-protomeric elongated sheet. (D) Interaction between the F2 C-terminus and the fusion peptide.

FIGs. 61A and 61B are a set of graphs and digital images illustrating antigenic characterization of RSV B18537 Fusion glycoprotein with DSCav1 substitutions. (A) Biolayer Interferometry measurements of prototypic site-specific antibodies were carried out by serial dilution of each Fab molecule and the association and dissociation rates to immobilized B18537 F DSCav1 proteins measured. (B) Structural comparison of antigenic site Ø from strain B18537 and A2. Surface exposed residues that differ between the two strains are labelled.

FIGs. 62A and 62B are graphs illustrating purification of the RSV strain B18537 F protein with DSCav1. A. SDS-PAGE of the elution fraction (reduced and non-reduced) and flowthrough fraction after StrepTagII affinity purification. B. Gel filtration of RSV B18537 F glycoprotein in GFB buffer on a 120 ml Superdex-200 size-exclusion column.

FIGs. 63-68 illustrate design and production of trimeric recombinant RSV F proteins stabilized in a prefusion conformation without a C-terminal trimerization domain to maintain stability of the membrane proximal lobe of RSV F. In place of the C-terminal trimerization domain, a ring of disulfide bonds is introduced into the C-terminus of the F1 polypeptide by substituting cysteine residues for amino acids of the α10 helix.

FIGs. 69A-69E are a set of tables showing ELISA data for the indicated recombinant RSV F variants. Expression and antigenic stability of RSV F variants (SEQ ID NOs: 859-1018). DNA encoding these RSV F variants was transfect into cell in the 96-well format under conditions where the recombinant RSV F proteins are secreted from the cells into the cell media. Each construct contains a leader sequence that causes the protein to enter the secretory system and be secreted. The medium was then centrifuged and the supernatant used for antigenicity testing for binding to the Site Ø specific antibody D25 and the Site II specific antibody Motavizumab ("Mota", FIGs. 69A-69E). The conditions tested include D25 and Mota binding on day 0 (conditions 1 and 2), D25 and Mota binding on day 0 after incubation at 70°C for one hour (conditions 3 and 4), and D25 and Mota binding after 1 week at 4°C (conditions 5 and 6). The control is the DSCav1 construct with a foldon domain. Specific antigenicity data for each construct is provided in FIGs. 69A-69E, with the conditions tested are noted in the header rows.

FIGs. 70A-70E are a set of schematic diagrams illustrating different design strategies to generate RSV F Antigenic Site Ø Immunogens. Antigenic site Ø includes the D25 recognition site on the outer surface of pre-fusion RSV F helix α4 and the loop just N-terminal to helix α1 of each protomer. Five methods were used to present isolated Site Ø epitopes on the surface of an immunogen: A) circular permutation (i.e. altering secondary structure linkers to alter the connectivity of site Ø segments for reasons of design ease and stability), B) incorporation of site Ø into a small scaffold protein, C) trimerization of circular permutations or scaffolded site Ø to match the native site Ø trimerization observed in the pre-fusion RSV F context (as in the left panel), D) including all of domain III for added stability of the site Ø fold and E) incorporation of A-D onto a nanoparticle platform for added immunogenicity.

FIG. 71 is a summary of the minimal site ø immunogens that were designed, produced and tested for antigenicity to the site Ø specific antibodies D25, AN22 and 5C4 by ELISA under the indicated conditions. The table shows the number of site Ø immunogens that fall within each design category, and which produced an ELISA result of at least 1.5.

FIGs. 72A-72F are a set of tables showing ELISA data for the indicated minimal site ø constructs binding to D25, AN22 or 5C4 antibody. The conditions tested include D25 binding after 0 and 1 week at 4°C (condition 1) and 2), D25 binding after 1 hr. at 60°C (condition 3), 70°C (condition 4), 80°C (condition 5), 90°C (condition 6), or 100°C (condition 7), AM22 binding after two weeks at 4°C (condition 8), 5C4 binding at week 0 (condition 9). The average of D25, AM22, and D25 binding after 1 hour at 70°C is also shown (condition 10). ELISA scores of ≥1.5 are highlighted in dark grey; scores of 0.5-1.5 are highlighted in light grey.

FIG. 73 is a set of graphs illustrating that immunization with DS version of stabilized prefusion F subtype A or B or both is induces neutralizing activity against both subtypes

FIG. 74 is a set of graphs illustrating that DSCav1 antibody response is durable in mice after two doses with immunization at weeks 0 and 4.

FIG. 75 is a set of graphs illustrating that DS immunization can prevent RSV infection in mice.

FIG. 76 is a set of graphs illustrating that DS immunization does not induce Type 2 cytokine responses in mice post-challenge.

FIG. 77 is a set of graphs illustrating that the neutralizing immune response to DSCav1 is boosted and sustained after a 3rd dose in non-human primates, which have been previously immunized with DS-Cav1 or DS at weeks 0 and 4.

FIG. 78 is a graph illustrating that DS-CAV1 can be effectively formulated in alum and retain immunogenicity.

FIG. 79 is a set of graphs illustrating that alum is an effective adjuvant for DSCav1 in non-human primates.

FIG. 80 is a graph illustrating that DS-CAV1 is immunogenic when expressed from a gene-based vector either alone or as priming for a protein boost.

FIG. 81 is a set of graphs and a table illustrating that DS-Cav1 RSV F Subtype A or B can boost a prime immunization using gene base delivery of wildtype F protein in non-human primates.

FIG. 82 is a set of graphs illustrating that DS-Cav1 RSV F Subtype A or B can boosts rAd-F(A)WT-primed non-human primate.

FIG. 83 is a set of graphs illustrating that immunization with the DS version of stabilized prefusion F subtype A or B or both is induces neutralizing activity against both subtypes of RSV.

FIG. 84 is a graph illustrating that altering glycosylation reduces immunogenicity of stabilized prefusion F.

SEQUENCE LISTING

[0018] The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file in the form of the file named "Sequence.txt" (~3.2 MB), which was created on March 12, 2014. In the accompanying Sequence Listing:

SEQ ID NOs: 1-128 are the amino acid sequences of native RSV F proteins from RSV type A.

SEQ ID NOs: 129-177 are the amino acid sequences of native RSV F proteins from RSV type B.

SEQ ID NOs: 178-184 are the amino acid sequences of native RSV F proteins from bovine RSV.

SEQ ID NOs: 185-350 are the amino acid sequences of recombinant RSV F proteins.

SEQ ID NO: 351 is the amino acid sequence of a T4 fibritin Foldon domain.

SEQ ID NO: 352 and 355-365 are amino acid sequences of peptide linkers.

SEQ ID NO: 353 is the amino acid sequence of a *Helicobacter pylori* ferritin protein (GENBANK® Accession No. EJB64322.1,as present in the database on February 28, 2013).

SEQ ID NO: 354 is the amino acid sequence of an encapsulin protein (GENBANK® Accession No. YP_001738186.1, as present in the database on February 28, 2013).

SEQ ID NOs: 366 and **367** are the V_H and V_L amino acid sequences of the AM22 mAb, respectively.

SEQ ID NO: 368 and 369 are the V_H and V_L amino acid sequences of the D25 mAb, respectively.

SEQ ID NO: 370 is a recombinant RSV F_0 protein variant amino acid sequence of the prototypical A2 strain (GENBANK accession No. P03420, as present in the database on February 28, 2012), including P102A, I379V, and M447V substitutions compared to the P03420 sequence.

SEQ ID NO: 371 is the amino acid sequence of a recombinant RSV F protein from human subtype A including S155C, S290C, S190F and V207L amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II. The four mutated residues, and the C-terminal appendage are underlined.

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MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCNGTDAKVKL
IKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNYTLNNAKKTNVTLSKKRKRRFLGFLLGVGSAIASGVAVCK
VLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTFKVLDLKNYIDKQLLPILNKQSCSISNIETVIEFQQKNNRLLEIT
REFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMCIIKEEVLAYVVQLPLYGVIDTP
CWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPK
YDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNKQEGKSLY
VKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGEWVLLSTFLGG
LVPRGSHHHHHHSAWSHPQFEK (RSV_A F(+)FdTHS DSCav1)
```

SEQ ID NO: 372 is the amino acid sequence of a recombinant RSV F protein from human subtype B including S155C, S290C, S190F and V207L amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II. The four mutated residues, and the C-terminal appendage are underlined.

```
MELLIHRLSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIELSNIKETKCNGTDTKVKL
IKQELDKYKNAVTELQLLMQNTPAANNRARREAPQYMNYTINTTKNLNVSISKKRKRRFLGFLLGVGSAIASGIAVCK
VLHLEGEVNKIKNALLSTNKAVVSLSNGVSVLTFKVLDLKNYINNQLLPILNQQSCRISNIETVIEFQQKNSRLLEIN
REFSVNAGVTTPLSTYMLTNSELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMCIIKEEVLAYVVQLPIYGVIDTP
CWKLHTSPLCTTNIKEGSNICLTRTDRGWYCDNAGSVSFFPQADTCKVQSNRVFCDTMNSLTLPSEVSLCNTDIFNSK
YDCKIMTSKTDISSSVITSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNKLEGKNLY
VKGEPIINYYDPLVFPSDEFDASISQVNEKINQSLAFIRRSDELLSAIGGYIPEAPRDGQAYVRKDGEWVLLSTFLGG
LVPRGSHHHHHHSAWSHPQFEK  (RSV B F(+)FdTHS DSCav1)
```

SEQ ID NO: 373 is the amino acid sequence of a recombinant RSV F protein from bovine RSV including S155C, S290C, S190F and V207L amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II. The four mutated residues, and the C-terminal appendage are underlined.

```
MAATAMRMIISIIFISTYMTHITLCQNITEEFYQSTCSAVSRGYLSALRTGWYTSVVTIELSKIQKNVCKSTDSKVKL
IKQELERYNNAVIELQSLMQNEPASFSRAKRGIPELIHYTRNSTKRFYGLMGKKRKRRFLGFLLGIGSAIASGVAVCK
VLHLEGEVNKIKNALLSTNKAVVSLSNGVSVLTFKVLDLKNYIDKELLPKLNNHDCRISNIETVIEFQQKNNRLLEIA
REFSVNAGITTPLSTYMLTNSELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMCVVKEEVIAYVVQLPIYGVIDTP
CWKLHTSPLCTTDNKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPTDVNLCNTDIFNTK
YDCKIMTSKTDISSSVITSIGAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNKLEGKALY
IKGEPIINYYDPLVFPSDEFDASIAQVNAKINQSLAFIRRSDELLSAIGGYIPEAPRDGQAYVRKDGEWVLLSTFLGG
LVPRGSHHHHHHSAWSHPQFEK  (bRSV F(+)FdTHS DSCav1)
```

SEQ ID NO: 374 is the amino acid sequence of a recombinant RSV F protein from human subtype A including S155C, S290C, and S190F amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II. The three mutated residues, and the C-terminal appendage are underlined.

SEQ ID NO: 375 is the amino acid sequence of a recombinant RSV F protein from human subtype B including S155C, S290C, and S190F amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II (RSV_B F(+)FdTHS DSS190F)

SEQ ID NO: 376 is the amino acid sequence of a recombinant RSV F protein from bovine RSV including S155C, S290C, and S190F amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II.. (bRSV F(+)FdTHS DSS190F)

SEQ ID NO: 377 is the amino acid sequence of a recombinant RSV F protein from RSV A including S155C, S290C, S190F, V207L amino acid substitutions, fused to a C-terminal ferritin domain. _(RSV_A F(+)FdTHS DSCav1 Ferritin)

SEQ ID NO: 378 is the amino acid sequence of a recombinant RSV F protein from RSV B including S155C, S290C, S190F, V207L amino acid substitutions, fused to a C-terminal ferritin domain. (RSV_B F(+)FdTHS DSCav1 ferritin)

SEQ ID NO: 379 is the amino acid sequence of a recombinant RSV F protein from bRSV including S155C, S290C, S190F, V207L amino acid substitutions, fused to a C-terminal ferritin domain. (bRSV F(+)FdTHS DSCav1 ferritin)

SEQ ID NO: 380 is the amino acid sequence of a recombinant RSV F protein from RSV A including S155C, S290C, S190F amino acid substitutions, fused to a C-terminal ferritin domain. (RSV_A F(+)FdTHS DSS190F Ferritin)

SEQ ID NO: 381 is the amino acid sequence of a recombinant RSV F protein from RSV B including S155C, S290C, S190F amino acid substitutions, fused to a C-terminal ferritin domain. (RSV_B F(+)FdTHS DSS190F ferritin)

SEQ ID NO: 382 is the amino acid sequence of a recombinant RSV F protein from bRSV including S155C, S290C, S190F amino acid substitutions, fused to a C-terminal ferritin domain. (bRSV F(+)FdTHS DSS190F ferritin)

SEQ ID NO: 383 is an exemplary nucleotide sequence encoding a recombinant RSV F protein from human subtype A including S155C, S290C, S190F and V207L amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II (DNA encoding RSV_A F(+)FdTHS DSCav1 expressed from VRC3798).

SEQ ID NO: 384 is a nucleotide sequence of an expression vector for expressing recombinant RSV F protein from human subtype A including S155C, S290C, S190F and V207L amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II (RSV_A F(+)FdTHS DSCav1 paH vector; VRC3798).

SEQ ID NO: 385 is an exemplary nucleotide sequence encoding a recombinant RSV F protein from human subtype B (strain B1) including S155C, S290C, S190F and V207L amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II (DNA encoding RSV_B (B1) F(+)FdTHS DSCav1; expressed from VRC3764).

SEQ ID NO: 386 is a nucleotide sequence of an expression vector for expressing recombinant RSV F protein from human subtype B (strainB1) including S155C, S290C, S190F and V207L amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II (RSV_B (B1) F(+)FdTHS DSCav1 paH vector; VRC3764).

SEQ ID NO: 387 is an exemplary nucleotide sequence encoding a recombinant RSV F protein from human subtype B (Strain 18537) including S155C, S290C, S190F and V207L amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II (DNA encoding RSV_B F(+)FdTHS DSCav1; expressed from VRC3799).

SEQ ID NO: 388 is a nucleotide sequence of an expression vector for expressing recombinant RSV F protein from human subtype B (Strain 18537) including S 155C, S290C, S190F and V207L amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II (RSV_B F(+)FdTHS DSCav1 paH vector; VRC3799).

SEQ ID NOs: 389-693 are the amino acid sequences of recombinant RSV F proteins stabilized in a prefusion conformation.

SEQ ID NOs: 694-697 are the amino acid sequences of modified Foldon domain polypeptides.

SEQ ID NOs: 698-697 are the amino acid sequences of modified Foldon domain polypeptides.

SEQ ID NOs: 698-828, 1429-1442 and 1474-1478 are the amino acid sequences of single chain recombinant RSV F proteins.

SEQ ID NOs: 829-1025 and 1456-1468 are the amino acid sequences of recombinant RSV F proteins linked to a cleavable foldon domain, or not linked to a foldon domain.

SEQ ID NO: 1026 is the amino acid sequence of a RSV F protein without prefusion-stabilizing substitutions.

SEQ ID NOs: 901-968 are the amino acid sequences of recombinant RSV F proteins stabilized in a prefusion conformation.

SEQ ID NOs: 1027-1088 and 1099-1428 are the amino acid sequences of minimal site ø immunogens that are described in Example 14.

STRUCTURAL COORDINATES

[0019] The atomic coordinates of the crystal structure of RSV F protein bound by D25 Fab are recited in Table 1 of U.S. Provisional Application No. 61/780,910, filed March 13, 2013. These atomic coordinates of the crystal structure of RSV F protein bound by D25 Fab are also deposited as Protein Data Bank Accession No. 4JHW, as present in that database on May 1, 2013.

DETAILED DESCRIPTION

[0020] The invention is defined in the claims. The RSV F glycoprotein it is a type I fusion protein that facilitates fusion of viral and cellular membranes (Walsh and Hruska, J. Virol., 47, 171 (1983)). After initial synthesis, RSV F adopts a metastable prefusion conformation that stores folding energy, which is released during a structural rearrangement to a highly stable postfusion conformation after contact with host cell membranes. Three antigenic sites (I, II, and IV) on RSV F protein have been found to elicit neutralizing activity (Arbiza et al., J. Gen. Virol., 73, 2225 (1992); Lopez et al., J. Virol., 72, 6922 (1998); Lopez et al., J. Virol., 64, 927 (1990)), and all exist on the postfusion form of RSV F protein as determined by structural and biophysical studies (McLellan et al., J. Virol., 85, 7788 (2011); Swanson et al., Proc. Natl. Acad. Sci. U.S.A., 108, 9619 (2011)). Absorption of human sera with postfusion RSV F, however, fails to remove the majority of F-specific neutralizing activity, suggesting that the prefusion form of RSV F harbors novel neutralizing antigenic sites (Magro et al., Proc. Natl. Acad. Sci. U.S.A., 109, 3089 (2012)).

[0021] Prior to the work disclosed herein, a homogeneous preparation of soluble prefusion RSV F protein was unavailable, precluding determination of the prefusion F structure and identification of novel prefusion F-specific antigenic sites. As described herein, RSV F protein specific antibodies were identified that neutralize RSV, but do not specifically bind to postfusion RSV F, and the three-dimensional structure of prefusion F, recognized by these antibodies, was obtained. The results provided herein reveal for the first time the prefusion conformation of RSV F and the mechanism of neutralization for a category of remarkably potent RSV prefusion F neutralizing antibodies. Using the three-dimensional structure of prefusion F as a guide, stabilized forms of prefusion F ("PreF" antigens) were constructed and used to generate RSV neutralizing immune responses many fold greater than that achieved with prior RSV F protein-based immunogens.

I. Terms

[0022] Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology can be found in Benjamin Lewin, Genes VII, published by Oxford University Press, 1999; Kendrew et al. (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994; and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995; and other similar references.

[0023] As used herein, the singular forms "a," "an," and "the," refer to both the singular as well as plural, unless the context clearly indicates otherwise. For example, the term "an antigen" includes single or plural antigens and can be considered equivalent to the phrase "at least one antigen." As used herein, the term "comprises" means "includes." Thus, "comprising an antigen" means "including an antigen" without excluding other elements. It is further to be understood that any and all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for descriptive purposes, unless otherwise indicated. Although many methods and materials similar or equivalent to those described herein can be used, particular suitable methods and materials are described herein. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. To facilitate review of the various embodiments, the following explanations of terms are provided:

5C4: A neutralizing monoclonal antibody that specifically binds to the prefusion conformation of the RSV F protein, but not to the post fusion conformation of RSV F protein. The 5C4 antibody include heavy and light chain variable regions with the amino acid sequences set forth as SEQ ID NOs: 1470 and 1471, respectively. As described in McLellan et al., Science, 340(6136):1113-7, 2013, 5C4 specifically binds to a quaternary epitope found on the RSV F protein in its prefusion conformation, but not the post fusion conformation. In several embodiments, antibody 5C4 specifically binds to the PreF antigens disclosed herein.

5C4 Heavy Chain Variable Domain:
EVQLQQSGAELVKPGASVKLSCTASGFNIKDTFFHWVKQRPEQGLEWIGRIDPADGHTKYDPKFQGKATIT
ADTSSNTAFLQLSSLTSVDTAVYYCATTITAVVPTPYNAMDYWGQGTSVTVSS (SEQ ID NO: 1470)

5C4 Kappa Light Chain Variable Domain:
DIVLTQSPASLAVSLGQRTTISCRASESVDSFDNSFIHWYQQKPGQPPKLLIFLASSLESGVPARFSGSGSRTD
FTLTIDPVEADDAATYYCQQSNEDPFTFGSGTKLEIK (SEQ ID NO: 1471)

[0024] Adjuvant: A vehicle used to enhance antigenicity. Adjuvants include a suspension of minerals (alum, aluminum hydroxide, or phosphate) on which antigen is adsorbed; or water-in-oil emulsion, for example, in which antigen solution is emulsified in mineral oil (Freund incomplete adjuvant), sometimes with the inclusion of killed mycobacteria (Freund's complete adjuvant) to further enhance antigenicity (inhibits degradation of antigen and/or causes influx of macrophages). Immunostimulatory oligonucleotides (such as those including a CpG motif) can also be used as adjuvants. Adjuvants include biological molecules (a "biological adjuvant"), such as costimulatory molecules. Exemplary adjuvants include IL-2, RANTES, GM-CSF, TNF-α, IFN-γ, G-CSF, LFA-3, CD72, B7-1, B7-2, OX-40L, 4-1BBL and toll-like receptor (TLR) agonists, such as TLR-9 agonists. The person of ordinary skill in the art is familiar with adjuvants (see, *e.g.*, Singh (ed.) Vaccine Adjuvants and Delivery Systems. Wiley-Interscience, 2007). Adjuvants can be used in combination with the disclosed PreF antigens.

[0025] Administration: The introduction of a composition into a subject by a chosen route. Administration can be local or systemic. For example, if the chosen route is intravenous, the composition (such as a composition including a disclosed immunogen) is administered by introducing the composition into a vein of the subject.

[0026] Agent: Any substance or any combination of substances that is useful for achieving an end or result; for example, a substance or combination of substances useful for inhibiting RSV infection in a subject. Agents include proteins, nucleic acid molecules, compounds, small molecules, organic compounds, inorganic compounds, or other molecules of interest, such as viruses, such as recombinant viruses. An agent can include a therapeutic agent (such as an anti-RSV agent), a diagnostic agent or a pharmaceutical agent. In some embodiments, the agent is a polypeptide agent (such as an immunogenic RSV polypeptide), or an anti-viral agent. The skilled artisan will understand that particular agents may be useful to achieve more than one result.

[0027] AM22: A neutralizing monoclonal antibody that specifically binds to the prefusion conformation of the RSV F protein, but not the post fusion conformation of RSV F protein. AM22 protein and nucleic acid sequences are known, for example, the heavy and light chain amino acid sequences of the AM22 antibody are set forth in U.S. Pat. App. Pub. No. 2012/0070446). As described in Example 1, AM22 specifically binds to an epitope (included on antigenic site Ø) including positions found on the RSV F protein in its prefusion conformation, but not the post fusion conformation. This epitope is included within RSV F positions 62-69 and 196-209, and located at the membrane distal apex of the RSV F protein in the prefusion conformation (see, *e.g.*, FIGs. 2B and 9A). Prior to this disclosure it was not known that AM22 was specific for the prefusion conformation. In several embodiments, antibody AM22 specifically binds to the PreF antigens disclosed herein.

[0028] Amino acid substitutions: The replacement of one amino acid in an antigen with a different amino acid or a deletion of an amino acid. In some examples, an amino acid in an antigen is substituted with an amino acid from a homologous protein.

[0029] Animal: A living multi-cellular vertebrate or invertebrate organism, a category that includes, for example, mammals. The term mammal includes both human and non-human mammals. Similarly, the term **"subject"** includes both human and veterinary subjects, such as non-human primates. Thus, administration to a subject can include administration to a human subject. Non-limiting examples of veterinary subjects include domesticated animals (such as cats and dogs), livestock (for example, cattle, horses, pigs, sheep, and goats), and laboratory animals (for example, mice, rabbits, rats, gerbils, guinea pigs, and non-human primates).

[0030] Antibody: A polypeptide that in nature is substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, which specifically binds and recognizes an analyte (such as an antigen or immunogen) such as a RSV F protein or antigenic fragment thereof. Immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. The term "antibody," as used herein, includes antibody fragments produced, for example, by the modification of whole antibodies and by de novo synthesis using recombinant DNA methodologies.

[0031] Antibodies exist, for example, as intact immunoglobulins and as a number of well characterized antibody fragments. For instance, Fabs, Fvs, and single-chain Fvs (SCFvs) that bind to RSV F protein, would be RSV F protein-specific binding agents. This includes intact immunoglobulins and the variants and portions of them well known in the art, such as Fab' fragments, F(ab)'$_2$ fragments, single chain Fv proteins ("scFv"), and disulfide stabilized Fv proteins ("dsFv"). A scFv protein is a fusion protein in which a light chain variable region of an immunoglobulin and a heavy chain variable region of an immunoglobulin are bound by a linker, while in dsFvs, the chains have been mutated to introduce a disulfide bond to stabilize the association of the chains. The term also includes genetically engineered forms such as chimeric antibodies (such as humanized murine antibodies), heteroconjugate antibodies (such as bispecific antibodies). See also, Pierce Catalog and Handbook, 1994-1995 (Pierce Chemical Co., Rockford, IL); Kuby, J., Immunology, 3rd Ed., W.H. Freeman & Co., New York, 1997.

[0032] Antibody fragments are defined as follows: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) (Fab')2, the fragment of the antibody obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; (4) F(ab')2, a dimer of two Fab' fragments held together by two disulfide bonds; (5) Fv, a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (6) single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

[0033] Typically, a naturally occurring immunoglobulin has heavy (H) chains and light (L) chains interconnected by disulfide bonds. There are two types of light chain, lambda (λ) and kappa (κ). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE. The disclosed antibodies can be class switched.

[0034] Each heavy and light chain contains a constant region and a variable region, (the regions are also known as "domains"). In several embodiments, the heavy and the light chain variable domains combine to specifically bind the antigen. In additional embodiments, only the heavy chain variable domain is required. For example, naturally occurring camelid antibodies consisting of a heavy chain only are functional and stable in the absence of light chain (see, *e.g.*, Hamers-Casterman et al., Nature, 363:446-448, 1993; Sheriff et al., Nat. Struct. Biol., 3:733-736, 1996). Light and heavy chain variable domains contain a "framework" region interrupted by three hypervariable regions, also called "complementarity-determining regions" or "CDRs" (see, e.g., Kabat et al., Sequences of Proteins of Immunological Interest, U.S. Department of Health and Human Services, 1991). The sequences of the framework regions of different light or heavy chains are relatively conserved within a species. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs in three-dimensional space.

[0035] The CDRs are primarily responsible for binding to an epitope of an antigen. The amino acid sequence boundaries of a given CDR can be readily determined using any of a number of well-known schemes, including those described by Kabat et al. ("Sequences of Proteins of Immunological Interest," 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991; "Kabat" numbering scheme), Al-Lazikani et al., (JMB 273,927-948, 1997; "Chothia" numbering scheme), and Lefranc, et al. ("IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains," Dev. Comp. Immunol., 27:55-77, 2003; "IMGT" numbering scheme).

[0036] The CDRs of each chain are typically referred to as CDR1, CDR2, and CDR3 (from the N-terminus to C-terminus), and are also typically identified by the chain in which the particular CDR is located. Thus, a V_H CDR3 is located in the variable domain of the heavy chain of the antibody in which it is found, whereas a V_L CDR1 is the CDR1 from the variable domain of the light chain of the antibody in which it is found. Light chain CDRs are sometimes referred to as CDR L1, CDR L2, and CDR L3. Heavy chain CDRs are sometimes referred to as CDR H1, CDR H2, and CDR H3.

[0037] Antigen: A compound, composition, or substance that can stimulate the production of antibodies or a T cell response in an animal, including compositions that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous antigens, such as the disclosed recombinant RSV F proteins.

[0038] Examples of antigens include, but are not limited to, polypeptides, peptides, lipids, polysaccharides, combinations thereof (such as glycopeptides) and nucleic acids containing antigenic determinants, such as those recognized by an immune cell. In some examples, antigens include peptides derived from a pathogen of interest, such as RSV. In specific examples, an antigen is derived from RSV, such as an antigen including a modified RSV F protein stabilized in a prefusion conformation. "Epitope" or "antigenic determinant" refers to the region of an antigen to which B and/or T cells respond.

[0039] Anti-RSV agent: An agent that specifically inhibits RSV from replicating or infecting cells. Non-limiting examples of anti-RSV agents include the monoclonal antibody palivizumab (SYNAGIS®; Medimmune, Inc.) and the small molecule anti-viral drug ribavirin (manufactured by many sources, e.g., Warrick Pharmaceuticals, Inc.).

[0040] Atomic Coordinates or Structure coordinates: Mathematical coordinates derived from mathematical equations related to the patterns obtained on diffraction of a monochromatic beam of X-rays by the atoms (scattering centers) such as an antigen, or an antigen in complex with an antibody. In some examples that antigen can be RSV F protein (for example stabilized in a prefusion conformation by binding to a prefusion -specific antibody, or by introduction of stabilizing modifications) in a crystal. The diffraction data are used to calculate an electron density map of the repeating unit of the crystal. The electron density maps are used to establish the positions of the individual atoms within the unit cell of the crystal. In one example, the term "structure coordinates" refers to Cartesian coordinates derived from mathematical equations related to the patterns obtained on diffraction of a monochromatic beam of X-rays, such as by the atoms of a RSV F protein in crystal form.

[0041] Those of ordinary skill in the art understand that a set of structure coordinates determined by X-ray crystallography is not without standard error. For the purpose of this disclosure, any set of structure coordinates that have a root mean square deviation of protein backbone atoms (N, Cα, C and O) of less than about 1.0 Angstroms when superimposed, such as about 0.75, or about 0.5, or about 0.25 Angstroms, using backbone atoms, shall (in the absence of an explicit statement to the contrary) be considered identical.

[0042] Cavity-filling amino acid substitution: An amino acid substitution that fills a cavity within the protein core of the RSV F protein, for example a cavity present in a protomer of the RSV F protein, or a cavity between protomers of the RSV F protein. Cavities are essentially voids within a folded protein where amino acids or amino acid side chains are not present. In several embodiments, a cavity filling amino acid substitution is introduced to fill a cavity in the RSV F protein core present in the RSV F protein prefusion conformation that collapse (e.g., have reduced volume) after transition to the postfusion conformation.

[0043] Circular Permutant: A modified recombinant protein in which the connections between different regions of a protein tertiary structure is modified, so that the relative order of different regions in the primary sequence is altered, but the placement of the regions in the tertiary structure is preserved. For example, with a 4-stranded antiparallel sheet, with strand A, B, C and D, which has the following N and C termini and connectivity,

Nterm - strand A - linker - strand B - linker - strand C -linker - strand D - Cterm, circular permutants of the 4 strands, A, B, C and D by altering linker connection between strands would include Permutation with N- and C- termini altered:

Nterm - strand C - linker - strand D - linker - strand A - linker - strand B - Cterm

Permutation with N terminus preserved:

Nterm - strand A - linker - strand D - linker - strand C - linker - strand B - C term

Permutation with C terminus preserved:

Nterm - strand C - linker - strand B - linker - strand A - linker - strand D - C term.

[0044] Contacting: Placement in direct physical association; includes both in solid and liquid form. Contacting includes contact between one molecule and another molecule, for example the amino acid on the surface of one polypeptide, such as an antigen, that contact another polypeptide, such as an antibody. Contacting also includes administration, such as administration of a disclosed antigen to a subject by a chosen route.

[0045] Control: A reference standard. In some embodiments, the control is a negative control sample obtained from a healthy patient. In other embodiments, the control is a positive control sample obtained from a patient diagnosed with RSV infection. In still other embodiments, the control is a historical control or standard reference value or range of values (such as a previously tested control sample, such as a group of RSV patients with known prognosis or outcome, or group of samples that represent baseline or normal values).

[0046] A difference between a test sample and a control can be an increase or conversely a decrease. The difference can be a qualitative difference or a quantitative difference, for example a statistically significant difference. In some examples, a difference is an increase or decrease, relative to a control, of at least about 5%, such as at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 150%, at least about 200%, at least about 250%, at least about 300%, at least about 350%, at least about 400%, at least about 500%, or greater than 500%.

[0047] D25: A neutralizing monoclonal antibody that specifically binds to the prefusion conformation of the RSV F protein, but not the post fusion conformation of RSV F protein. D25 protein and nucleic acid sequences are known, for example, the heavy and light chain amino acid sequences of the D25 antibody are set forth in U.S. Pat. App. Pub. No. 2010/0239593; see also, Kwakkenbos et al., Nat. Med., 16:123-128, 2009). As described in Example 1, D25 specifically binds to a quaternary epitope (included on antigenic site Ø) found on the RSV F protein in its prefusion conformation, but not the post fusion conformation. This epitope is included within RSV F positions 62-69 and 196-209, and located at the membrane distal apex of the RSV F protein in the prefusion conformation (see, *e.g.*, FIGs. 2B and 9A). Prior to this disclosure it was not known that D25 was specific for the prefusion conformation of RSV F protein). In several embodiments, antibody D25 specifically binds to the PreF antigens disclosed herein.

[0048] Degenerate variant and conservative variant: A polynucleotide encoding a polypeptide that includes a sequence that is degenerate as a result of the genetic code. For example, a polynucleotide encoding a disclosed antigen, or an antibody that specifically binds a disclosed antigen, that includes a sequence that is degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included as long as the amino acid sequence of the antigen or antibody that binds the antigen encoded by the nucleotide sequence is unchanged. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance, the codons CGU, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified within a protein encoding sequence, the codon can be altered to any of the corresponding codons described without altering the encoded protein. Such nucleic acid variations are "silent variations," which are one species of conservative variations. Each nucleic acid sequence herein that encodes a polypeptide also describes every possible silent variation. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

[0049] One of ordinary skill will recognize that individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (for instance less than 5%, in some embodiments less than 1%) in an encoded sequence are conservative variations where the alterations result in the substitution of an amino acid with a chemically similar amino acid.

[0050] Conservative amino acid substitutions providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another:

1. 1) Alanine (A), Serine (S), Threonine (T);
2. 2) Aspartic acid (D), Glutamic acid (E);
3. 3) Asparagine (N), Glutamine (Q);
4. 4) Arginine (R), Lysine (K);
5. 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6. 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

[0051] Not all residue positions within a protein will tolerate an otherwise "conservative" substitution. For instance, if an amino acid residue is essential for a function of the protein, even an otherwise conservative substitution may disrupt that activity, for example the specific binding of an antibody to a target epitope may be disrupted by a conservative mutation in the target epitope.

[0052] Epitope: An antigenic determinant. These are particular chemical groups or peptide sequences on a molecule that are antigenic, such that they elicit a specific immune response, for example, an epitope is the region of an antigen to which B and/or T cells respond. An antibody binds a particular antigenic epitope, such as an epitope of a RSV F protein, for example, a D25 or AM22 epitope present on the prefusion conformation of the RSV F protein.

[0053] Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5, about 9, or about 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and nuclear magnetic resonance. Epitopes can also include post-translation modification of amino acids, such as N-linked glycosylation.

[0054] In one embodiment, T cells respond to the epitope, when the epitope is presented in conjunction with an MHC molecule. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5, about 9, or about 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and nuclear magnetic resonance.

[0055] A "target epitope" is a particular epitope on an antigen that specifically binds an antibody of interest, such as a monoclonal antibody. In some examples, a target epitope includes the amino acid residues that contact the antibody of interest, such that the target epitope can be selected by the amino acid residues determined to be in contact with the antibody of interest.

[0056] Effective amount: An amount of agent, such as a PreF antigen or nucleic acid encoding a PreF antigen or other agent that is sufficient to generate a desired response, such as an immune response to RSV F protein, or a reduction or elimination of a sign or symptom of a condition or disease, such as RSV infection. For instance, this can be the amount necessary to inhibit viral replication or to measurably alter outward symptoms of the viral infection. In general, this amount will be sufficient to measurably inhibit virus (for example, RSV) replication or infectivity. When administered to a subject, a dosage will generally be used that will achieve target tissue concentrations (for example, in respiratory tissue) that has been shown to achieve *in vitro* inhibition of viral replication. In some examples, an "effective amount" is one that treats (including prophylaxis) one or more symptoms and/or underlying causes of any of a disorder or disease, for example to treat RSV infection. In one example, an effective amount is a therapeutically effective amount. In one example, an effective amount is an amount that prevents one or more signs or symptoms of a particular disease or condition from developing, such as one or more signs or symptoms associated with RSV infection.

[0057] Expression: Translation of a nucleic acid into a protein. Proteins may be expressed and remain intracellular, become a component of the cell surface membrane, or be secreted into the extracellular matrix or medium.

[0058] Expression Control Sequences: Nucleic acid sequences that regulate the expression of a heterologous nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (ATG) in front of a protein-encoding gene, splicing signal for introns, maintenance of the correct reading frame of that gene to permit proper translation of mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter.

[0059] A promoter is a minimal sequence sufficient to direct transcription. Also included are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the gene. Both constitutive and inducible promoters are included (see for example, Bitter et al., Methods in Enzymology 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage lambda, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. In one embodiment, when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (such as metallothionein promoter) or from mammalian viruses (such as the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) can be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences.

[0060] A polynucleotide can be inserted into an expression vector that contains a promoter sequence, which facilitates the efficient transcription of the inserted genetic sequence of the host. The expression vector typically contains an origin of replication, a promoter, as well as specific nucleic acid sequences that allow phenotypic selection of the transformed cells.

[0061] Ferritin: A protein that stores iron and releases it in a controlled fashion. The protein is produced by almost all living organisms. Ferritin assembles into a globular protein complex that in some cases consists of 24 protein subunits. In some examples, ferritin is used to form a nanoparticle presenting antigens on its surface, for example an RSV antigen, such as the disclosed RSV F protein antigens stabilized in a prefusion conformation.

[0062] Foldon domain: An amino acid sequence that naturally forms a trimeric structure. In some examples, a Foldon domain can be included in the amino acid sequence of a disclosed RSV F protein antigen stabilized in a prefusion conformation so that the antigen will form a trimer. In one example, a Foldon domain is the T4 Foldon domain set forth as SEQ ID NO: 351 (GYIPEAPRDGQAYVRKDGEWVLLSTF). Several embodiments include a Foldon domain that can be cleaved from a purified protein, for example by incorporation of a thrombin cleave site adjacent to the Foldon domain that can be used for cleavage purposes.

[0063] Glycoprotein (gp): A protein that contains oligosaccharide chains (glycans) covalently attached to polypeptide side-chains. The carbohydrate is attached to the protein in a cotranslational or posttranslational modification. This process is known as glycosylation. In proteins that have segments extending extracellularly, the extracellular segments are often glycosylated. Glycoproteins are often important integral membrane proteins, where they play a role in cell-cell interactions. In some examples a glycoprotein is an RSV glycoprotein, such as a RSV F protein antigen stabilized in a prefusion conformation or an immunogenic fragment thereof.

[0064] Glycosylation site: An amino acid sequence on the surface of a polypeptide, such as a protein, which accommodates the attachment of a glycan. An N-linked glycosylation site is triplet sequence of NX(S/T) in which N is asparagine, X is any residues except proline, and (S/T) is a serine or threonine residue. A glycan is a polysaccharide or oligosaccharide. Glycan may also be used to refer to the carbohydrate portion of a glycoconjugate, such as a glycoprotein, glycolipid, or a proteoglycan.

[0065] Homologous proteins: Proteins that have a similar structure and function, for example, proteins from two or more species or viral strains that have similar structure and function in the two or more species or viral strains. For example a RSV F protein from RSV A is a homologous protein to a RSV F protein from bovine RSV. Homologous proteins share similar protein folding characteristics and can be considered structural homologs.

[0066] Homologous proteins typically share a high degree of sequence conservation, such as at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, or at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence conservation, and a high degree of sequence identity, such as at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, or at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity.

[0067] Host cells: Cells in which a vector can be propagated and its DNA expressed. The cell may be prokaryotic or eukaryotic. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used.

[0068] Immunogen: A protein or a portion thereof that is capable of inducing an immune response in a mammal, such as a mammal infected or at risk of infection with a pathogen. Administration of an immunogen can lead to protective immunity and/or proactive immunity against a pathogen of interest. In some examples, an immunogen includes a disclosed PreF antigen.

[0069] Immune response: A response of a cell of the immune system, such as a B cell, T cell, or monocyte, to a stimulus. In one embodiment, the response is specific for a particular antigen (an "antigen-specific response"). In one embodiment, an immune response is a T cell response, such as a CD4+ response or a CD8+ response. In another embodiment, the response is a B cell response, and results in the production of specific antibodies.

[0070] A "Th1" biased immune response is characterized by the presence of $CD4^+$ T helper cells that produce IL-2 and IFN-γ, and thus, by the secretion or presence of IL-2 and IFN-γ. In contrast, a "Th2" biased immune response is characterized by a preponderance of $CD4^+$ helper cells that produce IL-4, IL-5, and IL-13.

[0071] Immunogenic composition: A composition comprising an antigen that induces an immune response, such as a measurable CTL response against virus expressing the antigen, or a measurable B cell response (such as production of antibodies) against the antigen. As such, an immunogenic composition includes one or more antigens (for example, polypeptide antigens) or antigenic epitopes. An immunogenic composition can also include one or more additional components capable of eliciting or enhancing an immune response, such as an excipient, carrier, and/or adjuvant. In certain instances, immunogenic compositions are administered to elicit an immune response that protects the subject against symptoms or conditions induced by a pathogen. In some cases, symptoms or disease caused by a pathogen is prevented (or reduced or ameliorated) by inhibiting replication of the pathogen (e.g., RSV) following exposure of the subject to the pathogen. In one example, an "immunogenic composition" includes a recombinant RSV F protein stabilized in a prefusion conformation, that induces a measurable CTL response against virus expressing RSV F protein, or induces a measurable B cell response (such as production of antibodies) against RSV F protein. It further refers to isolated nucleic acids encoding an antigen, such as a nucleic acid that can be used to express the antigen (and thus be used to elicit an immune response against this polypeptide).

[0072] For *in vitro* use, an immunogenic composition may include an antigen or nucleic acid encoding an antigen. For *in vivo* use, the immunogenic composition will typically include the protein, immunogenic peptide or nucleic acid in pharmaceutically acceptable carriers, and/or other agents. Any particular peptide, such as a disclosed RSV F protein stabilized in a prefusion conformation or a nucleic acid encoding a disclosed RSV F protein stabilized in a prefusion conformation, can be readily tested for its ability to induce a CTL or B cell response by art-recognized assays. Immunogenic compositions can include adjuvants, which are well known to one of skill in the art.

[0073] Immunologically reactive conditions: Includes reference to conditions which allow an antibody raised against a particular epitope to bind to that epitope to a detectably greater degree than, and/or to the substantial exclusion of, binding to substantially all other epitopes. Immunologically reactive conditions are dependent upon the format of the antibody binding reaction and typically are those utilized in immunoassay protocols or those conditions encountered *in vivo*. The immunologically reactive conditions employed in the methods are "physiological conditions" which include reference to conditions (such as temperature, osmolarity, pH) that are typical inside a living mammal or a mammalian cell. While it is recognized that some organs are subject to extreme conditions, the intra-organismal and intracellular environment is normally about pH 7 (such as from pH 6.0 to pH 8.0, more typically pH 6.5 to 7.5), contains water as the predominant solvent, and exists at a temperature above 0°C and below 50°C. Osmolarity is within the range that is supportive of cell viability and proliferation.

[0074] Immunological probe: A molecule that can be used for selection of antibodies from sera which are directed against a specific epitope or antigen, including from human patient sera. In some examples, the disclosed RSV F proteins stabilized in a prefusion conformation can be used as immunological probes in both positive and negative selection of antibodies specific for RSV F protein in a prefusion conformation.

[0075] Immunogenic surface: A surface of a molecule, for example RSV F protein, capable of eliciting an immune response. An immunogenic surface includes the defining features of that surface, for example the three-dimensional shape and the surface charge. In some examples, an immunogenic surface is defined by the amino acids on the surface of a protein or peptide that are in contact with an antibody, such as a neutralizing antibody, when the protein and the antibody are bound together. A target epitope includes an immunogenic surface. Immunogenic surface is synonymous with antigenic surface.

[0076] Inhibiting or treating a disease: Inhibiting the full development of a disease or condition, for example, in a subject who is at risk for a disease, such as RSV infection. "Treatment" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop. The term "ameliorating," with reference to a disease or pathological condition, refers to any observable beneficial effect of the treatment. The beneficial effect can be evidenced, for example, by a delayed onset of clinical symptoms of the disease in a susceptible subject, a reduction in severity of some or all clinical symptoms of the disease, a slower progression of the disease, an improvement in the overall health or well-being of the subject, or by other parameters well known in the art that are specific to the particular disease. A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs for the purpose of decreasing the risk of developing pathology.

[0077] The term "reduces" is a relative term, such that an agent reduces a response or condition if the response or condition is quantitatively diminished following administration of the agent, or if it is diminished following administration of the agent, as compared to a reference agent. Similarly, the term "prevents" does not necessarily mean that an agent completely eliminates the response or condition, so long as at least one characteristic of the response or condition is eliminated. Thus, an immunogenic composition that reduces or prevents an infection or a response, such as a pathological response, *e.g.*, vaccine enhanced viral disease, can, but does not necessarily completely eliminate such an infection or response, so long as the infection or response is measurably diminished, for example, by at least about 50%, such as by at least about 70%, or about 80%, or even by about 90% of (that is to 10% or less than) the infection or response in the absence of the agent, or in comparison to a reference agent.

[0078] Isolated: An "isolated" biological component (such as a protein, for example a disclosed PreF antigen or nucleic acid encoding such an antigen) has been substantially separated or purified away from other biological components, such as other biological components in which the component naturally occurs, such as other chromosomal and extrachromosomal DNA, RNA, and proteins. Proteins, peptides and nucleic acids that have been "isolated" include proteins purified by standard purification methods. The term also embraces proteins or peptides prepared by recombinant expression in a host cell as well as chemically synthesized proteins, peptides and nucleic acid molecules. Isolated does not require absolute purity, and can include protein, peptide, or nucleic acid molecules that are at least 50% isolated, such as at least 75%, 80%, 90%, 95%, 98%, 99%, or even 99.9% isolated. The PreF antigens disclosed herein (for example, an isolated recombinant RSV F protein stabilized in a prefusion conformation) are isolated from RSV F proteins in a post-fusion conformation, for example, are at least 80% isolated, at least 90%, 95%, 98%, 99%, or even 99.9% isolated from RSV F proteins in a postfusion conformation. In several embodiments, the PreF antigen is substantially separated from RSV F proteins that do not include antigen site ø and/or are not specifically bound by a prefusion specific monoclonal antibody (such as D25 or AM22), for example, the PreF antigen may be at least 80% isolated, at least 90%, 95%, 98%, 99%, or even 99.9% isolated from RSV F proteins that do not include antigen site ø and/or are not specifically bound by a prefusion specific monoclonal antibody, such as D25 or AM22.

[0079] K_d: The dissociation constant for a given interaction, such as a polypeptide-ligand interaction or an antibody-antigen interaction. For example, for the bimolecular interaction of an antibody (such as D25) and an antigen (such as RSV F protein), it is the concentration of the individual components of the bimolecular interaction divided by the concentration of the complex. Methods of determining the K_d of an antibody:antigen interaction are familiar to the person of ordinary skill in the art.

[0080] Label: A detectable compound or composition that is conjugated directly or indirectly to another molecule to facilitate detection of that molecule. Specific, non-limiting examples of labels include fluorescent tags, enzymatic linkages, and radioactive isotopes. In some examples, a disclosed PreF antigen is labeled with a detectable label. In some examples, label is attached to a disclosed antigen or nucleic acid encoding such an antigen.

[0081] Linker: A bi-functional molecule that can be used to link two or more molecules into one contiguous molecule, for example, to link a carrier molecule to a immunogenic polypeptide. Non-limiting examples of peptide linkers include a $(G_4S)_1$, $(G_4S)_2$, or a $(G_4S)_3$ peptide linker.

[0082] The terms "conjugating," "joining," "bonding," or "linking" can refer to making two molecules into one contiguous molecule; for example, linking two other polypeptides into one contiguous polypeptide, or covalently attaching a carrier molecule or other molecule to an immunogenic polypeptide, such as an recombinant RSV F protein as disclosed herein. The linkage can be either by chemical or recombinant means. "Chemical means" refers to a reaction, for example, between the immunogenic polypeptide moiety and the carrier molecule such that there is a covalent bond formed between the two molecules to form one molecule.

[0083] MPE8: A neutralizing monoclonal antibody that specifically binds to the prefusion conformation of the RSV F protein, but not to the post fusion conformation of RSV F protein. As described in Corti et al. (Nature, 501(7467)439-443, 2013) the MPE8 antibody binds to an epitope found on the pre-, but not post-, fusion conformations of the RSV F protein. The MPE8 epitope is not part of antigenic site Ø. The heavy and light chain variable region sequences are set forth as SEQ ID NOs: 1472 and 1473, respectively.

[0084] Native antigen or native sequence: An antigen or sequence that has not been modified by selective mutation, for example, selective mutation to focus the antigenicity of the antigen to a target epitope. Native antigen or native sequence are also referred to as wild-type antigen or wild-type sequence.

[0085] Nucleic acid: A polymer composed of nucleotide units (ribonucleotides, deoxyribonucleotides, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof) linked via phosphodiester bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Thus, the term includes nucleotide polymers in which the nucleotides and the linkages between them include non-naturally occurring synthetic analogs, such as, for example and without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs), and the like. Such polynucleotides can be synthesized, for example, using an automated DNA synthesizer. The term "oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (*i.e.*, A, T, G, C), this also includes an RNA sequence (*i.e.*, A, U, G, C) in which "U" replaces "T."

[0086] "Nucleotide" includes, but is not limited to, a monomer that includes a base linked to a sugar, such as a pyrimidine, purine or synthetic analogs thereof, or a base linked to an amino acid, as in a peptide nucleic acid (PNA). A nucleotide is one monomer in a polynucleotide. A nucleotide sequence refers to the sequence of bases in a polynucleotide.

[0087] Conventional notation is used herein to describe nucleotide sequences: the left-hand end of a single-stranded nucleotide sequence is the 5'-end; the left-hand direction of a double-stranded nucleotide sequence is referred to as the 5'-direction. The direction of 5' to 3' addition of nucleotides to nascent RNA transcripts is referred to as the transcription direction. The DNA strand having the same sequence as an mRNA is referred to as the "coding strand;" sequences on the DNA strand having the same sequence as an mRNA transcribed from that DNA and which are located 5' to the 5'-end of the RNA transcript are referred to as "upstream sequences;" sequences on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the coding RNA transcript are referred to as "downstream sequences."

[0088] "cDNA" refers to a DNA that is complementary or identical to an mRNA, in either single stranded or double stranded form.

[0089] "Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (for example, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA produced by that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and non-coding strand, used as the template for transcription, of a gene or cDNA can be referred to as encoding the protein or other product of that gene or cDNA. Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns. In some examples, a nucleic acid encodes a disclosed PreF antigen.

[0090] Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

[0091] Prefusion-specific antibody: An antibody that specifically binds to the RSV F protein in a prefusion conformation, but does not specifically binds to the RSV F protein in a post-fusion conformation. Exemplary prefusion specific antibodies include the D25, AM22, 5C4 and MPE8 antibodies

[0092] Polypeptide: Any chain of amino acids, regardless of length or post-translational modification (such as glycosylation or phosphorylation). "Polypeptide" applies to amino acid polymers including naturally occurring amino acid polymers and non-naturally occurring amino acid polymer as well as in which one or more amino acid residue is a non-natural amino acid, for example an artificial chemical mimetic of a corresponding naturally occurring amino acid. A "residue" refers to an amino acid or amino acid mimetic incorporated in a polypeptide by an amide bond or amide bond mimetic. A polypeptide has an amino terminal (N-terminal) end and a carboxy terminal (C-terminal) end. "Polypeptide" is used interchangeably with peptide or protein, and is used interchangeably herein to refer to a polymer of amino acid residues.

[0093] A single contiguous polypeptide chain of amino acid residues can include multiple polypeptides. For example, the RSV F_0 polypeptide includes a N-terminal signal peptide, a F_2 polypeptide, a pep27 polypeptide, and a F_1 polypeptide including the F_1 extracellular domain, transmembrane domain and cytosolic tail. Further, in some embodiments a recombinant RSV F protein is a single chain RSV F protein including a RSV F_2 polypeptide linked to a RSV F_1 polypeptide by a peptide linker.

[0094] In many instances, a polypeptide folds into a specific three-dimensional structure, and can include surface-exposed amino acid residues and non-surface-exposed amino acid residues. In some instances a protein can include multiple polypeptides that fold together into a functional unit. For example, the RSV F protein is composed of F_1/F_2 heterodimers that trimerize in to a multimeric protein.

[0095] "Surface-exposed amino acid residues" are those amino acids that have some degree of exposure on the surface of the protein, for example such that they can contact the solvent when the protein is in solution. In contrast, non-surface-exposed amino acids are those amino acid residues that are not exposed on the surface of the protein, such that they do not contact solution when the protein is in solution. In some examples, the non-surface-exposed amino acid residues are part of the protein core.

[0096] A **"protein core"** is the interior of a folded protein, which is substantially free of solvent exposure, such as solvent in the form of water molecules in solution. Typically, the protein core is predominately composed of hydrophobic or apolar amino acids. In some examples, a protein core may contain charged amino acids, for example aspartic acid, glutamic acid, arginine, and/or lysine. The inclusion of uncompensated charged amino acids (a compensated charged amino can be in the form of a salt bridge) in the protein core can lead to a destabilized protein. That is, a protein with a lower T_m then a similar protein without an uncompensated charged amino acid in the protein core. In other examples, a protein core may have a cavity within the protein core. Cavities are essentially voids within a folded protein where amino acids or amino acid side chains are not present. Such cavities can also destabilize a protein relative to a similar protein without a cavity. Thus, when creating a stabilized form of a protein, it may be advantageous to substitute amino acid residues within the core in order to fill cavities present in the wild-type protein.

[0097] Amino acids in a peptide, polypeptide or protein generally are chemically bound together via amide linkages (CONH). Additionally, amino acids may be bound together by other chemical bonds. For example, linkages for amino acids or amino acid analogs can include CH_2NH-, -CH_2S-, -CH_2-CH_2 -, -CH=CH-- (cis and trans), -$COCH_2$ --, -CH(OH)CH_2-, and -CHH_2SO- (These and others can be found in Spatola, in Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3, Peptide Backbone Modifications (general review); Morley, Trends Pharm Sci pp. 463-468, 1980; Hudson, et al., Int JPept ProtRes 14:177-185, 1979; Spatola et al. Life Sci 38:1243-1249, 1986; Harm J. Chem. Soc Perkin Trans. 1307-314, 1982; Almquist et al. J. Med. Chem. 23:1392-1398, 1980; Jennings-White et al. Tetrahedron Lett 23:2533, 1982; Holladay et al. Tetrahedron. Lett 24:4401-4404, 1983; and Hruby Life Sci 31:189-199, 1982.

[0098] Peptide modifications: Peptides, such as the disclosed RSV F proteins stabilized in a prefusion conformation can be modified, for example to include an amino acid substitution compared to a Native RSV protein sequence, or by a variety of chemical techniques to produce derivatives having essentially the same activity and conformation as the unmodified peptides, and optionally having other desirable properties. For example, carboxylic acid groups of the protein, whether carboxyl-terminal or side chain, may be provided in the form of a salt of a pharmaceutically-acceptable cation or esterified to form a C_1-C_{16} ester, or converted to an amide of formula NR_1R_2 wherein R_1 and R_2 are each independently H or C_1-C_{16} alkyl, or combined to form a heterocyclic ring, such as a 5- or 6- membered ring. Amino groups of the peptide, whether amino-terminal or side chain, may be in the form of a pharmaceutically-acceptable acid addition salt, such as the HCl, HBr, acetic, benzoic, toluene sulfonic, maleic, tartaric and other organic salts, or may be modified to C_1-C_{16} alkyl or dialkyl amino or further converted to an amide.

[0099] Hydroxyl groups of the peptide side chains can be converted to C_1-C_{16} alkoxy or to a C_1-C_{16} ester using well-recognized techniques. Phenyl and phenolic rings of the peptide side chains can be substituted with one or more halogen atoms, such as F, Cl, Br or I, or with C_1-C_{16} alkyl, C_1-C_{16} alkoxy, carboxylic acids and esters thereof, or amides of such carboxylic acids. Methylene groups of the peptide side chains can be extended to homologous C_2-C_4 alkylenes. Thiols can be protected with any one of a number of well-recognized protecting groups, such as acetamide groups.

[0100] Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers of use are conventional. Remington's Pharmaceutical Sciences, by E. W. Martin, Mack Publishing Co., Easton, PA, 19th Edition, 1995, describes compositions and formulations suitable for pharmaceutical delivery of the disclosed immunogens.

[0101] In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (e.g., powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate. In particular embodiments, suitable for administration to a subject the carrier may be sterile, and/or suspended or otherwise contained in a unit dosage form containing one or more measured doses of the composition suitable to induce the desired anti-RSV immune response. It may also be accompanied by medications for its use for treatment purposes. The unit dosage form may be, for example, in a sealed vial that contains sterile contents or a syringe for injection into a subject, or lyophilized for subsequent solubilization and administration or in a solid or controlled release dosage.

[0102] Prime-boost vaccination: An immunotherapy including administration of a first immunogenic composition (the primer vaccine) followed by administration of a second immunogenic composition (the booster vaccine) to a subject to induce an immune response. The primer vaccine and/or the booster vaccine include a vector (such as a viral vector, RNA, or DNA vector) expressing the antigen to which the immune response is directed. The booster vaccine is administered to the subject after the primer vaccine; the skilled artisan will understand a suitable time interval between administration of the primer vaccine and the booster vaccine, and examples of such timeframes are disclosed herein. In some embodiments, the primer vaccine, the booster vaccine, or both primer vaccine and the booster vaccine additionally include an adjuvant. In one non-limiting example, the primer vaccine is a DNA-based vaccine (or other vaccine based on gene delivery), and the booster vaccine is a protein subunit or protein nanoparticle based vaccine.

[0103] Protein nanoparticle: A multi-subunit, protein-based polyhedron shaped structure. The subunits are each composed of proteins or polypeptides (for example a glycosylated polypeptide), and, optionally of single or multiple features of the following: nucleic acids, prosthetic groups, organic and inorganic compounds. Non-limiting examples of protein nanoparticles include ferritin nanoparticles (see, *e.g.*, Zhang, Y. Int. J. Mol. Sci., 12:5406-5421, 2011,), encapsulin nanoparticles (see, *e.g.*, Sutter et al., Nature Struct. and Mol. Biol., 15:939-947, 2008), Sulfur Oxygenase Reductase (SOR) nanoparticles (see, *e.g.*, Urich et al., Science, 311:996-1000, 2006), lumazine synthase nanoparticles (see, *e.g.*, Zhang et al., J. Mol. Biol., 306: 1099-1114, 2001) or pyruvate dehydrogenase nanoparticles (see, *e.g.*, Izard et al., PNAS 96: 1240-1245, 1999). Ferritin, encapsulin, SOR, lumazine synthase, and pyruvate dehydrogenase are monomeric proteins that self-assemble into a globular protein complexes that in some cases consists of 24, 60, 24, 60, and 60 protein subunits, respectively. In some examples, ferritin, encapsulin, SOR, lumazine synthase, or pyruvate dehydrogenase monomers are linked to a disclosed antigen (for example, a recombinant RSV F protein stabilized in a prefusion conformation) and self-assembled into a protein nanoparticle presenting the disclosed antigens on its surface, which can be administered to a subject to stimulate an immune response to the antigen.

[0104] Recombinant: A recombinant nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination can be accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, for example, by genetic engineering techniques. A recombinant protein is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. In several embodiments, a recombinant protein is encoded by a heterologous (for example, recombinant) nucleic acid that has been introduced into a host cell, such as a bacterial or eukaryotic cell. The nucleic acid can be introduced, for example, on an expression vector having signals capable of expressing the protein encoded by the introduced nucleic acid or the nucleic acid can be integrated into the host cell chromosome.

[0105] Repacking amino acid substitution: An amino acid substitution that increases the interactions of neighboring residues in a protein, for example, by enhancing hydrophobic interactions or hydrogen-bond formation, or by reducing unfavorable or repulsive interactions of neighboring residues, for example, by eliminating clusters of similarly charged residues. In several embodiments, a repacking amino acid substitution is introduced to increase the interactions of neighboring residues in the RSV F protein prefusion conformation, that are not in close proximity in the RSV F postfusion conformation. Typically, introduction of a repacking amino acid substitution will increase the T_m of the prefusion conformation of the RSV F protein, and lower the T_m of the postfusion conformation of the RSV F protein.

[0106] Respiratory Syncytial Virus (RSV): An enveloped non-segmented negative-sense single-stranded RNA virus of the family *Paramyxoviridae.* It is the most common cause of bronchiolitis and pneumonia among children in their first year of life and infects nearly all children by 3 years of age. RSV also causes repeated infections including severe lower respiratory tract disease, which may occur at any age, especially among the elderly or those with compromised cardiac, pulmonary, or immune systems. In the United States, RSV bronchiolitis is the leading cause of hospitalization in infants and a major cause of asthma and wheezing throughout childhood (Shay et al., JAMA, 282, 1440 (1999); Hall et al., N. Engl. J. Med., 360, 588 (2009)). Globally, RSV is responsible for 66,000-199,000 deaths each year for children younger than five years of age (Nair et al., Lancet, 375, 1545 (2010)), and accounts for 6.7% of deaths among infants one month to one year old-more than any other single pathogen except malaria (Lozano et al., Lancet, 380, 2095 (2013)).

[0107] The RSV genome is ~15,000 nucleotides in length and includes 10 genes encoding 11 proteins, including the glycoproteins SH, G and F. The F protein mediates fusion, allowing entry of the virus into the cell cytoplasm and also promoting the formation of syncytia. Two subtypes of human RSV strains have been described, the A and B subtypes, based on differences in the antigenicity of the G glycoprotein. RSV strains for other species are also known, including bovine RSV. Exemplary RSV strain sequences are known to the person of ordinary skill in the art. Further, several models of human RSV infection are available, including model organisms infected with hRSV, as well as model organisms infected with species specific RSV, such as use of bRSV infection in cattle (see, *e.g.,* Bern et al., Am J, Physiol. Lung CellMol. Physiol., 301: L148-L156, 2011).

[0108] Several methods of diagnosing RSV infection are known, including use of Direct Fluorescent Antibody detection (DFA), Chromatographic rapid antigen detection, and detection of viral RNA using RT PCR. Quantification of viral load can be determined, for example, by Plaque Assay, antigen capture enzyme immunoassay (EIA), or PCR. Quantification of antibody levels can be performed by subtype-specific Neutralization assay or ELISA. Current RSV treatment is passive administration of the monoclonal antibody palivizumab (SYNAGIS®), which recognizes the RSV F protein (Johnson et al., J. Infect. Dis., 176, 1215 (1997); Beeler and van Wyke Coelingh, J. Virol., 63, 2941 (1989)) and reduces incidence of severe disease (The IMpact-RSV Study Group, Pediatrics, 102, 531 (1998)). (Also see, *e.g.,* Nam and Kun (Eds.). Respiratory Syncytial Virus: Prevention, Diagnosis and Treatment. Nova Biomedical Nova Science Publisher, 2011; and Cane (Ed.) Respiratory Syncytial Virus. Elsevier Science, 2007.)

[0109] There are several subtypes of RSV, including human subtype A, human subtype B, and bovine subtype. Within the subtypes of RSV, there are individual strains of each subtype. For example, SEQ ID NOs: 1-128 provided herein include RSV F protein sequences for many strains of subtype A RSV, which (as shown in Table 3 below) are highly homologous.

[0110] RSV Fusion (F) protein: An RSV envelope glycoprotein that facilitates fusion of viral and cellular membranes. In nature, the RSV F protein is initially synthesized as a single polypeptide precursor approximately 574 amino acids in length, designated F_0. F_0 includes an N-terminal signal peptide that directs localization to the endoplasmic reticulum, where the signal peptide (approximately the first 25 residues of F_0) is proteolytically cleaved. The remaining F_0 residues oligomerize to form a trimer which is again proteolytically processed by a cellular protease at two conserved furin consensus cleavage sequences (approximately F_0 positions 109 and 136; for example, $RARR_{109}$ (SEQ ID NO: 124, residues 106-109) and $RKRR_{136}$ (SEQ ID NO: 124, residues 133-136) to generate two disulfide-linked fragments, F_1 and F_2. The smaller of these fragments, F_2, originates from the N-terminal portion of the F_0 precursor and includes approximately residues 26-109 of F_0. The larger of these fragments, F_1, includes the C-terminal portion of the F_0 precursor (approximately residues 137-574) including an extracellular/lumenal region (~ residues 137-524), a transmembrane domain (-residues 525-550), and a cytoplasmic domain (-residues 551-574) at the C-terminus.

[0111] Three F_2-F_1 protomers oligomerize in the mature F protein, which adopts a metastable "prefusion" conformation that is triggered to undergo a conformational change (to a "postfusion" conformation) upon contact with a target cell membrane. This conformational change exposes a hydrophobic sequence, known as the fusion peptide, which is located at the N-terminus of the F_1 polypeptide, and which associates with the host cell membrane and promotes fusion of the membrane of the virus, or an infected cell, with the target cell membrane.

[0112] A number of neutralizing antibodies that specifically bind to antigenic sites on RSV F protein have been identified. These include monoclonal antibodies 131-2a and 2F, which bind to antigenic site I (centered around residue P389); monoclonal antibodies palivizumab and motavizumab, which bind to antigenic site II (centered around residues 254-277); and monoclonal antibodies 101F and mAb19, which bind to antigenic site IV (centered around residues 429-437).

[0113] Single chain RSV F protein: A recombinant RSV F protein that is expressed as a single polypeptide chain including the RSV F_1 polypeptide and the RSV F_2 polypeptide. The single chain RSV F protein trimerizes to form a RSV F protein ectodomain. A single chain RSV F protein does not include the furin cleavage sites flanking the pep27 polypeptide of RSV F protein; therefore, when produced in cells, the F_0 polypeptide is not cleaved into separate F_1 and F_2 polypeptides. In some embodiments, a single chain RSV F protein includes deletion of the two furin cleavage sites, the pep27 polypeptide, and the fusion peptide. In one embodiment, position 103 or 105 is linked to position 145 of the RSV protein to generate the single chain construction. In several embodiments, the remaining portions of the F_1 and F_2 polypeptides are joined by a linker, such as a peptide linker.

[0114] RSV F_0 polypeptide (F_0): The precursor of the RSV F protein, including the amino acids of a N-terminal signal peptide, a F_2 polypeptide, a pep27 polypeptide, and a F_1 polypeptide including the F_1 extracellular domain, transmembrane domain and cytosolic tail. The native F_0 polypeptide is proteolytically processed at a signal sequence cleavage site, and two furin cleavage sites (approximately F_0 positions 109 and 136; for example, $RARR_{109}$ (SEQ ID NO: 124, residues 106-109) and $RKRR_{136}$ (SEQ ID NO: 124, residues 133-136), resulting in the F_1 and F_2 fragments. Examples of F_0 polypeptides from many different RSV subgroups are known, including from the A, B and bovine subgroups, examples of which are set forth herein as SEQ ID NOs: 1-128, 129-177, and 178-184, respectively.

[0115] RSV F_1 polypeptide (F_1): A peptide chain of the RSV F protein. As used herein, "F_1 polypeptide" refers to both native F_1 polypeptides and F_1 polypeptides including modifications (e.g., amino acid substitutions, insertions, or deletion) from the native sequence, for example, modifications designed to stabilize a recombinant F protein (including the modified F_1 polypeptide) in a RSV F protein prefusion conformation. Native F_1 includes approximately residues 137-574 of the RSV F_0 precursor, and includes (from N- to C-terminus) an extracellular/lumenal region (~ residues 137-524), a transmembrane domain (-residues 525-550), and a cytoplasmic domain (-residues 551-574). Several embodiments include an F_1 polypeptide modified from a native F_1 sequence, for example an F_1 polypeptide that lacks the transmembrane and cytosolic domain, and/or includes one or more amino acid substitutions that stabilize a recombinant F protein (containing the F_1 polypeptide) in a prefusion conformation. In one example, a disclosed RSV F protein includes a F_1 polypeptide with deletion of the transmembrane and cytosolic domains, and cysteine substitutions at positions 155 and 290. In another example, a disclosed RSV F protein includes a F_1 polypeptide with deletion of the transmembrane and cytosolic domains, cysteine substitutions at positions 155 and 290, and a phenylalanine substitution at position 190. In another example, a disclosed RSV F protein includes a F_1 polypeptide with deletion of the transmembrane and cytosolic domains, cysteine substitutions at positions 155 and 290, a phenylalanine substitution at position 190, and a leucine substitution at position 207. In several embodiments, the F1 polypeptide includes a C-terminal linkage to a trimerization domain. Many examples of native F_1 sequences are known which are provided herein as approximately positions 137-524 of SEQ ID NOs: 1-184.

[0116] RSV F_2 polypeptide (F_2): A polypeptide chain of the RSV F protein. As used herein, "F_2 polypeptide" refers to both native F_2 polypeptides and F_2 polypeptides including modifications (e.g., amino acid substitutions) from the native sequence, for example, modifications designed to stabilize a recombinant F protein (including the modified F_2 polypeptide) in a RSV F protein prefusion conformation. Native F_2 includes approximately residues 26-109 of the RSV F_0 precursor. In native RSV F protein, the F_2 polypeptide is linked to the F_1 polypeptide by two disulfide bonds. Many examples of native F_2 sequences are known which are provided herein as approximately positions 26-109 of SEQ ID NOs: 1-184.

[0117] RSV pep27 polypeptide (pep27): A 27 amino acid polypeptide that is excised from the F_0 precursor during maturation of the RSV F protein. pep27 is flanked by two furin cleavage sites that are cleaved by a cellular protease during F protein maturation to generate the F_1 and F_2 polypeptide. Examples of native pep27 sequences are known which are provided herein as positions 110-136 of SEQ ID NOs: 1-184.

[0118] RSV F protein prefusion conformation: A structural conformation adopted by the RSV F protein prior to triggering of the fusogenic event that leads to transition of RSV F to the postfusion conformation and following processing into a mature RSV F protein in the secretory system. The three-dimensional structure of an exemplary RSV F protein in a prefusion conformation is disclosed herein (see Example 1) and the structural coordinates of the exemplary RSV F protein in a prefusion conformation bound by the prefusion-specific antibody D25 are provided in Table 1. As shown herein, the prefusion conformation of RSV F is similar in overall structure to the prefusion conformation of other paramyxoviruses (such as PIV, see FIG. 7), though with some significant differences. In the prefusion state, the RSV F protein includes an antigenic site at the membrane distal apex ("**antigenic site** QJ," see Example 1), that includes RSV F residues 62-69 and 196-209, and also includes the epitopes of the D25 and AM22 antibodies. As used herein, a recombinant RSV F protein stabilized in a prefusion conformation can be specifically bound by an antibody that is specific for the prefusion conformation of the RSV F protein, such as an antibody that specifically binds to an epitope within antigenic site Ø, for example, the D25 or AM22 antibody. Additional prefusion specific antibodies include the 5C4 and MPE8 antibodies.

[0119] RSV F protein postfusion conformation: A structural conformation adopted by the RSV F protein that is not the prefusion conformation, and in which the N- and C- termini of the RSV F protein are proximal in a stable coil-coil. The post fusion conformation of RSV F protein has been described at the atomic level (see, *e.g.*, McLellan et al., J. Virol., 85, 7788, 2011; Swanson et al., Proc. Natl. Acad. Sci. U.S.A., 108, 9619, 2011; and structural coordinates deposited PDB Accession No. 3RRR). The post-fusion conformation of RSV F protein is similar to that known for other paramyxovirus glycoproteins, including the PIV5 F protein. In the postfusion conformation, the RSV F protein does not include antigenic site Ø, and therefore does not include the D25 epitope and is not specifically bound by D25 or AM22. The RSV postfusion conformation occurs, for example, following fusion of the F protein with the cell membrane. The sequence of a RSV F protein that when expressed, can fold into a post-fusion conformation, is provided as SEQ ID NO: 1469.

[0120] Resurfaced antigen or resurfaced immunogen: A polypeptide immunogen derived from a wild-type antigen in which amino acid residues outside or exterior to a target epitope are mutated in a systematic way to focus the immunogenicity of the antigen to the selected target epitope. In some examples a resurfaced antigen is referred to as an antigenically-cloaked immunogen or antigenically-cloaked antigen.

[0121] Root mean square deviation (RMSD): The square root of the arithmetic mean of the squares of the deviations from the mean. In several embodiments, RMSD is used as a way of expressing deviation or variation from the structural coordinates of a reference three dimensional structure. This number is typically calculated after optimal superposition of two structures, as the square root of the mean square distances between equivalent C_α atoms. In some embodiments, the reference three-dimensional structure includes the structural coordinates of the RSV F protein bound to monoclonal antibody D25, set forth herein in Table 1.

[0122] Sequence identity/similarity: The identity/similarity between two or more nucleic acid sequences, or two or more amino acid sequences, is expressed in terms of the identity or similarity between the sequences. Sequence identity can be measured in terms of percentage identity; the higher the percentage, the more identical the sequences are. Homologs or orthologs of nucleic acid or amino acid sequences possess a relatively high degree of sequence identity/similarity when aligned using standard methods.

[0123] Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, Adv. Appl. Math. 2:482, 1981; Needleman & Wunsch, J. Mol. Biol. 48:443, 1970; Pearson & Lipman, Proc. Natl. Acad. Sci. USA 85:2444, 1988; Higgins & Sharp, Gene, 73:237-44, 1988; Higgins & Sharp, CABIOS 5:151-3, 1989; Corpet et al., Nuc. Acids Res. 16:10881-90, 1988; Huang et al. Computer Appls. in the Biosciences 8, 155-65, 1992; and Pearson et al., Meth. Mol. Bio. 24:307-31, 1994. Altschul et al., J. Mol. Biol. 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

[0124] Once aligned, the number of matches is determined by counting the number of positions where an identical nucleotide or amino acid residue is present in both sequences. The percent sequence identity is determined by dividing the number of matches either by the length of the sequence set forth in the identified sequence, or by an articulated length (such as 100 consecutive nucleotides or amino acid residues from a sequence set forth in an identified sequence), followed by multiplying the resulting value by 100. For example, a peptide sequence that has 1166 matches when aligned with a test sequence having 1554 amino acids is 75.0 percent identical to the test sequence (1166-1554* 100=75.0). The percent sequence identity value is rounded to the nearest tenth. For example, 75.11, 75.12, 75.13, and 75.14 are rounded down to 75.1, while 75.15, 75.16, 75.17, 75.18, and 75.19 are rounded up to 75.2. The length value will always be an integer.

[0125] The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., J. Mol. Biol. 215:403, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. A description of how to determine sequence identity using this program is available on the NCBI website on the internet.

[0126] Homologs and variants of a polypeptide are typically characterized by possession of at least about 75%, for example at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity counted over the full length alignment with the amino acid sequence of interest. Proteins with even greater similarity to the reference sequences will show increasing percentage identities when assessed by this method, such as at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% sequence identity. When less than the entire sequence is being compared for sequence identity, homologs and variants will typically possess at least 80% sequence identity over short windows of 10-20 amino acids, and may possess sequence identities of at least 85% or at least 90% or 95% depending on their similarity to the reference sequence. Methods for determining sequence identity over such short windows are available at the NCBI website on the internet. One of skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it is entirely possible that strongly significant homologs could be obtained that fall outside of the ranges provided.

[0127] For sequence comparison of nucleic acid sequences, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters are used. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482, 1981, by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443, 1970, by the search for similarity method of Pearson & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, e.g., Sambrook et al. (Molecular Cloning: A Laboratory Manual, 4th ed, Cold Spring Harbor, New York, 2012) and Ausubel et al. (In Current Protocols in Molecular Biology, John Wiley & Sons, New York, through supplement 104, 2013). One example of a useful algorithm is PILEUP. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, J. Mol. Evol. 35:351-360, 1987. The method used is similar to the method described by Higgins & Sharp, CABIOS 5:151-153, 1989. Using PILEUP, a reference sequence is compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG sequence analysis software package, e.g., version 7.0 (Devereaux et al., Nuc. Acids Res. 12:387-395, 1984.

[0128] Another example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and the BLAST 2.0 algorithm, which are described in Altschul et al., J. Mol. Biol. 215:403-410, 1990 and Altschul et al., Nucleic Acids Res. 25:3389-3402, 1977. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (ncbi.nlm.nih.gov). The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands. The BLASTP program (for amino acid sequences) uses as defaults a word length (W) of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915, 1989). An oligonucleotide is a linear polynucleotide sequence of up to about 100 nucleotide bases in length.

[0129] Another indicia of sequence similarity between two nucleic acids is the ability to hybridize. The more similar are the sequences of the two nucleic acids, the more stringent the conditions at which they will hybridize. The stringency of hybridization conditions are sequence-dependent and are different under different environmental parameters. Thus, hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method of choice and the composition and length of the hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength (especially the Na^+ and/or Mg^{++} concentration) of the hybridization buffer will determine the stringency of hybridization, though wash times also influence stringency. Generally, stringent conditions are selected to be about 5°C to 20°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Conditions for nucleic acid hybridization and calculation of stringencies can be found, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001; Tijssen, Hybridization With Nucleic Acid Probes, Part I: Theory and Nucleic Acid Preparation, Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Ltd., NY, NY, 1993; and Ausubel et al. Short Protocols in Molecular Biology, 4th ed., John Wiley & Sons, Inc., 1999.

[0130] As used herein, reference to "at least 80% identity" refers to "at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even 100% identity" to a specified reference sequence.

[0131] Signal Peptide: A short amino acid sequence (e.g., approximately 18-25 amino acids in length) that directs newly synthesized secretory or membrane proteins to and through membranes (for example, the endoplasmic reticulum membrane). Signal peptides are typically located at the N-terminus of a polypeptide and are removed by signal peptidases after the polypeptide has crossed the membrane. Signal peptide sequences typically contain three common structural features: an N-terminal polar basic region (n-region), a hydrophobic core, and a hydrophilic c-region). Exemplary signal peptide sequences are set forth as residues 1-25 of SEQ ID NOs: 1-182 (RSV F protein signal peptides from A, B, and bovine RSV).

[0132] Specifically bind: When referring to the formation of an antibody:antigen protein complex, refers to a binding reaction which determines the presence of a target protein, peptide, or polysaccharide (for example a glycoprotein), in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated conditions, an antibody binds preferentially to a particular target protein, peptide or polysaccharide (such as an antigen present on the surface of a pathogen, for example RSV F) and does not bind in a significant amount to other proteins or polysaccharides present in the sample or subject. An antibody that specifically binds to the prefusion conformation of RSV F protein (e.g., and antibody that specifically binds to antigenic site Ø) does not specifically bind to the postfusion conformation of RSV F protein. Specific binding can be determined by methods known in the art. With reference to an antibody:antigen or Fab:antigen complex, specific binding of the antigen and antibody has a K_d (or apparent K_d) of less than about 10^{-6} Molar, such as less than about 10^{-7} Molar, 10^{-8} Molar, 10^{-9}, or even less than about 10^{-10} Molar.

[0133] Soluble protein: A protein capable of dissolving in aqueous liquid at room temperature and remaining dissolved. The solubility of a protein may change depending on the concentration of the protein in the water-based liquid, the buffering condition of the liquid, the concentration of other solutes in the liquid, for example salt and protein concentrations, and the heat of the liquid. In several embodiments, a soluble protein is one that dissolves to a concentration of at least 0.5 mg/ml in phosphate buffered saline (pH 7.4) at room temperature and remains dissolved for at least 48 hours.

[0134] Therapeutic agent: A chemical compound, small molecule, or other composition, such as nucleic acid molecule, capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject.

[0135] Therapeutically effective amount of effective amount: The amount of agent, such as a disclosed antigen or immunogenic composition containing a disclosed antigen, that is sufficient to prevent, treat (including prophylaxis), reduce and/or ameliorate the symptoms and/or underlying causes of any of a disorder or disease, for example to prevent, inhibit, and/or treat RSV infection. In some embodiments, a therapeutically effective amount is sufficient to reduce or eliminate a symptom of a disease, such as RSV infection. For instance, this can be the amount necessary to inhibit viral replication or to measurably alter outward symptoms of the viral infection. In general, this amount will be sufficient to measurably inhibit virus (for example, RSV) replication or infectivity. When administered to a subject, a dosage will generally be used that will achieve target tissue concentrations that has been shown to achieve *in vitro* inhibition of viral replication. It is understood that to obtain a protective immune response against a pathogen can require multiple administrations of the immunogenic composition. Thus, a therapeutically effective amount encompasses a fractional dose that contributes in combination with previous or subsequent administrations to attaining a protective immune response.

[0136] Transmembrane domain: An amino acid sequence that inserts into a lipid bilayer, such as the lipid bilayer of a cell or virus or virus-like particle. A transmembrane domain can be used to anchor an antigen to a membrane. In some examples a transmembrane domain is a RSV F protein transmembrane domain. Exemplary RSV F transmembrane domains are familiar to the person of ordinary skill in the art, and provided herein. For example, the amino acid sequences of exemplary RSV F transmembrane domains are provided as approximately positions 525-550 of SEQ ID NOs: 1-183.

[0137] Transformed: A transformed cell is a cell into which a nucleic acid molecule has been introduced by molecular biology techniques. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including transfection with viral vectors, transformation with plasmid vectors, and introduction of DNA by electroporation, lipofection, and particle gun acceleration.

[0138] Vaccine: A pharmaceutical composition that elicits a prophylactic or therapeutic immune response in a subject. In some cases, the immune response is a protective immune response. Typically, a vaccine elicits an antigen-specific immune response to an antigen of a pathogen, for example a viral pathogen, or to a cellular constituent correlated with a pathological condition. A vaccine may include a polynucleotide (such as a nucleic acid encoding a disclosed antigen), a peptide or polypeptide (such as a disclosed antigen), a virus, a cell or one or more cellular constituents.

[0139] Vector: A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. Recombinant DNA vectors are vectors having recombinant DNA. A vector can include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. A vector can also include one or more selectable marker genes and other genetic elements known in the art. Viral vectors are recombinant DNA vectors having at least some nucleic acid sequences derived from one or more viruses.

[0140] A replication deficient viral vector that requires complementation of one or more regions of the viral genome required for replication, as a result of, for example a deficiency in at least one replication-essential gene function. For example, such that the viral vector does not replicate in typical host cells, especially those in a human patient that could be infected by the viral vector in the course of a therapeutic method. Examples of replication-deficient viral vectors and systems for their use are known in the art and include; for example replication-deficient LCMV vectors (see, *e.g.,* U.S. Pat. Pub. No. 2010/0297172) and replication deficient adenoviral vectors (see, *e.g.,* PCT App. Pub. No. WO2000/00628).

[0141] Virus: A virus consists essentially of a core of nucleic acid surrounded by a protein coat, and has the ability to replicate only inside a living cell. "Viral replication" is the production of additional virus by the occurrence of at least one viral life cycle. A virus may subvert the host cells' normal functions, causing the cell to behave in a manner determined by the virus. For example, a viral infection may result in a cell producing a cytokine, or responding to a cytokine, when the uninfected cell does not normally do so. In some examples, a virus is a pathogen.

[0142] Virus-like particle (VLP): A non-replicating, viral shell, derived from any of several viruses. VLPs are generally composed of one or more viral proteins, such as, but not limited to, those proteins referred to as capsid, coat, shell, surface and/or envelope proteins, or particle-forming polypeptides derived from these proteins. VLPs can form spontaneously upon recombinant expression of the protein in an appropriate expression system. Methods for producing particular VLPs are known in the art. The presence of VLPs following recombinant expression of viral proteins can be detected using conventional techniques known in the art, such as by electron microscopy, biophysical characterization, and the like. Further, VLPs can be isolated by known techniques, *e.g.*, density gradient centrifugation and identified by characteristic density banding. See, for example, Baker et al. (1991) Biophys. J. 60:1445-1456; and Hagensee et al. (1994) J. Virol. 68:4503-4505; Vincente, J Invertebr Pathol., 2011; Schneider-Ohrum and Ross, Curr. Top. Microbiol. Immunol., 354: 53073, 2012).

II. Description of several embodiments

[0143] It is disclosed herein that the RSV F protein undergoes a dramatic structural rearrangement between its pre-and postfusion conformations (see Example 1, below). As shown in FIG. 2B, the N-terminal region of the F_1 polypeptide in the prefusion conformation (corresponding in part to the membrane distal lobe shown in FIG. 2A) includes the indicated α2, α3, β3, β4, and α4 helical and beta sheet structures, whereas the corresponding region of the N-terminus of the F_1 polypeptide in the postfusion structure includes an extended oc5 helical structure. Further, the C-terminal region of the F_1 polypeptide in the prefusion conformation (corresponding in part to the membrane proximal lobe shown in FIG. 2A) includes the indicated β22, α9, and β23 beta sheet and helical structures, whereas the corresponding C-terminal region of the of the F_1 polypeptide in the postfusion conformation structure includes an extended α10 helical structure. Thus, the membrane distal and membrane proximal lobes of the RSV F protein in its prefusion conformation include several distinct structural elements that are absent from the corresponding regions of the RSV F protein in its postfusion conformation. Amino acid positions (and sequences) corresponding to these regions are highlighted in grey in FIG. 2, including positions 137-216, and 461-513 of the F_1 polypeptide.

[0144] RSV F protein antigens are provided that are stabilized or "locked" in a prefusion conformation, termed "PreF antigens." Using structure-guided design, positions of the RSV F_1 and F_2 polypeptides are targeted for modification (e.g., amino acid substitution) to hinder or prevent transition of the RSV F protein from a pre- to postfusion conformation. Such antigens have utility, for example, as immunogens to induce a neutralizing response to RSV F protein.

A. Native RSV F Proteins

[0145] Native RSV F proteins from different RSV groups, as well as nucleic acid sequences encoding such proteins and methods, are known. For example, the sequence of several subtype A, B and bovine precursor RSV F_0 proteins provided as SEQ ID NOs: 1-184. The GenInfo Identifier (gi) and corresponding accession number for each of these sequences, as well as the corresponding RSV group are provided in Table 3:

Table 3. Exemplary Subtype A, B and bovine RSV F protein sequences

SEQ ID	Subtype	Accession	SEQ ID	Subtype	Accession
1	A	>gi|113472470|gb|ABI35685.1	93	A	>gi|346683047|gb|AEO45919.1
2	A	>gi|46405966|gb|AAS93651.1	94	A	>gi|46405974|gb|AAS93655.1
3	A	>gi|346682949|gb|AEO45830.1	95	A	>gi|46405976|gb|AAS93656.1
4	A	>gi|392301680|gb|AFM55244.1	96	A	>gi|346683069|gb|AEO45939.1
5	A	>gi|392301896|gb|AFM55442.1	97	A	>gi|1353201|sp|P11209.2
6	A	>gi|392301692|gb|AFM55255.1	98	A	>gi|1912295|gb|AAC57027.1
7	A	>gi|392301728|gb|AFM55288.1	99	A	>gi|9629375|ref]NP_044596.1
8	A	>gi|392976459|gb|AFM95385.1	100	A	>gi|21263086|gb|AAM44851.1
9	A	>gi|392976475|gb|AFM95400.1	101	A	>gi|417346951|gb|AFX60127.1
10	A	>gi|21689583|gb|AAM68157.1	102	A	>gi|417347009|gb|AFX60156.1
11	A	>gi|21689587|gb|AAM68160.1	103	A	>gi|29290043|gb|AAO72325.1
12	A	>gi|346682981|gb|AEO45859.1	104	A	>gi|138252|sp|P12568.1
13	A	>gi|352962949|gb|AEQ63444.1	105	A	>gi|226438|prf||1512372A
14	A	>gi|353441614|gb|AEQ98752.1	106	A	>gi|37674744|gb|AAQ97026.1

SEQ ID	Subtype	Accession	SEQ ID	Subtype	Accession
15	A	>gi|392301740|gb|AFM55299.1	107	A	>gi|37674754|gb|AAQ97031.1
16	A	>gi|346682971|gb|AEO45850.1	108	A	>gi|37674746|gb|AAQ97027.1
17	A	>gi|346682992|gb|AEO45869.1	109	A	>gi|37674748|gb|AAQ97028.1
18	A	>gi|346683003|gb|AEO45879.1	110	A	>gi|37674750|gb|AAQ97029.1
19	A	>gi|346683036|gb|AEO45909.1	111	A	>gi|37674752|gb|AAQ97030.1
20	A	>gi|21689579|gb|AAM68154.1	112	A	>gi|146738079|gb|ABQ42594.1
21	A	>gi|326578296|gb|ADZ95777.1	113	A	>gi|403379|emb|CAA81295.1
22	A	>gi|330470871|gb|AEC32087.1	114	A	>gi|226838116|gb|ACQ83302.1
23	A	>gi|346683058|gb|AEO45929.1	115	A	>gi|326578304|gb|ADZ95781.1
24	A	>gi|392301644|gb|AFM55211.1	116	A	>gi|326578306|gb|ADZ95782.1
25	A	>gi|392301656|gb|AFM55222.1	117	A	>gi|326578308|gb|ADZ95783.1
26	A	>gi|392301776|gb|AFM55332.1	118	A	>gi|326578310|gb|ADZ95784.1
27	A	>gi|46405962|gb|AAS93649.1	119	A	>gi|326578312|gb|ADZ95785.1
28	A	>gi|326578298|gb|ADZ95778.1	120	A	>gi|60549171|gb|AAX23994.1
29	A	>gi|392301872|gb|AFM55420.1	121	A	>gi|226838109|gb|ACO83297.1
30	A	>gi|346682960|gb|AEO45840.1	122	A	>gi|352962877|gb|AEQ63378.1
31	A	>gi|346683080|gb|AEO45949.1	123	A	>gi|346683014|gb|AEO45889.1
32	A	>gi|227299|prf||1701388A/1-574	124	A	>gi|138251|sp|P03420.11
33	A	>gi|352962996|gb|AEQ63487.1	125	A	>gi|1695263|gb|AAC55970.1
34	A	>gi|352963032|gb|AEQ63520.1	126	A	>gi|61211|emb|CAA26143.1
35	A	>gi|46405970|gb|AAS93653.1	127	A	>gi|226838114|gb|ACO83301.1
36	A	>gi|392976437|gb|AFM95365.1	128	A	>gi|352963080|gb|AEQ63564.1
37	A	>gi|392976449|gb|AFM95376.1	129	B	>gi|109689536|dbj|BAE96918.1
38	A	>gi|352962805|gb|AEQ63312.1	130	B	>gi|380235900|gb|AFD34266.1
39	A	>gi|346340362|gb|AEO23051.1	131	B	>gi|401712638|gb|AFP99059.1
40	A	>gi|352962829|gb|AEQ63334.1	132	B	>gi|401712648|gb|AFP99064.1
41	A	>gi|352962865|gb|AEQ63367.1	133	B	>gi|380235886|gb|AFD34259.1
42	A	>gi|392302028|gb|AFM55563.1	134	B	>gi|326578302|gb|ADZ95780.1
43	A	>gi|392302016|gb|AFM55552.1	135	B	>gi|326578294|gb|ADZ95776.1
44	A	>gi|417346971|gb|AFX60137.1	136	B	>gi|326578300|gb|ADZ95779.1
45	A	>gi|417347051|gb|AFX60173.1	137	B	>gi|380235892|gb|AFD34262.1
46	A	>gi|392301812|gb|AFM55365.1	138	B	>gi|46405984|gb|AAS93660.1
47	A	>gi|29290039|gb|AAO72323.1	139	B	>gi|46405986|gb|AAS93661.1
48	A	>gi|29290041|gb|AAO72324.1	140	B	>gi|46405990|gb|AAS93663.1
49	A	>gi|262479010|gb|ACY68435.1	141	B	>gi|46405992|gb|AAS93664.1
50	A	>gi|330470867|gb|AEC32085.1	142	B	>gi|345121421|gb|AEN74946.1
51	A	>gi|392301704|gb|AFM55266.1	143	B	>gi|417347137|gb|AFX60215.1
52	A	>gi|392301716|gb|AFM55277.1	144	B	>gi|380235888|gb|AFD34260.1
53	A	>gi|392301800|gb|AFM55354.1	145	B	>gi|346340378|gb|AEO23054.1
54	A	>gi|345548062|gb|AEO12131.1	146	B	>gi|384872848|gb|AFI25262.1
55	A	>gi|346340367|gb|AEO23052.1	147	B	>gi|380235890|gb|AFD34261.1
56	A	>gi|352962889|gb|AEQ63389.1	148	B	>gi|46405978|gb|AAS93657.1
57	A	>gi|353441606|gb|AEQ98748.1	149	B	>gi|46405982|gb|AAS93659.1
58	A	>gi|353441604|gb|AEQ98747.1	150	B	>gi|352963104|gb|AEQ63586.1
59	A	>gi|353441608|gb|AEQ98749.1	151	B	>gi|352963128|gb|AEQ63608.1
60	A	>gi|353441616|gb|AEQ98753.1	152	B	>gi|352963164|gb|AEQ63641.1
61	A	>gi|353441620|gb|AEQ98755.1	153	B	>gi|46405996|gb|AAS93666.1
62	A	>gi|353441624|gb|AEQ98757.1	154	B	>gi|417347131|gb|AFX60212.1
63	A	>gi|409905594|gb|AFV46409.1	155	B	>gi|417347135|gb|AFX60214.1
64	A	>gi|409905610|gb|AFV46417.1	156	B	>gi|417347145|gb|AFX60219.1
65	A	>gi|417346953|gb|AFX60128.1	157	B	>gi|380235898|gb|AFD34265.1
66	A	>gi|417347079|gb|AFX60187.1	158	B	>gi|352963116|gb|AEQ63597.1
67	A	>gi|417346955|gb|AFX60129.1	159	B	>gi|401712640|gb|AFP99060.1
68	A	>gi|417346967|gb|AFX60135.1	160	B	>gi|352963152|gb|AEQ63630.1
69	A	>gi|417346979|gb|AFX60141.1	161	B	>gi|401712642|gb|AFP99061.1
70	A	>gi|417346993|gb|AFX60148.1	162	B	>gi|417347133|gb|AFX60213.1
71	A	>gi|417346999|gb|AFX60151.1	163	B	>gi|417347147|gb|AFX60220.1
72	A	>gi|417347043|gb|AFX60169.1	164	B	>gi|417347151|gb|AFX60222.1
73	A	>gi|417347105|gb|AFX60200.1	165	B	>gi|417347169|gb|AFX60231.1
74	A	>gi|417347107|gb|AFX60201.1	166	B	>gi|417347171|gb|AFX60232.1
75	A	>gi|392301788|gb|AFM55343.1	167	B	>gi|417347175|gb|AFX60234.1

SEQ ID	Subtype	Accession	SEQ ID	Subtype	Accession
76	A	>gi|409905578|gb|AFV46401.1	168	B	>gi|46405988|gb|AAS93662.1
77	A	>gi|409905596|gb|AFV46410.1	169	B	>gi|138250|sp|P13843.1
78	A	>gi|353441622|gb|AEQ98756.1	170	B	>gi|2582041|gb|AAB82446.1
79	A	>gi|409905582|gb|AFV46403.1	171	B	>gi|9629206|ref|NP_056863.1
80	A	>gi|417347109|gb|AFX60202.1	172	B	>gi|38230490|gb|AAR14266.1
81	A	>gi|409905602|gb|AFV46413.1	173	B	>gi|326578292|gb|ADZ95775.1
82	A	>gi|409905604|gb|AFV46414.1	174	B	>gi|345121416|gb|AEN74944.1
83	A	>gi|417347121|gb|AFX60208.1	175	B	>gi|345121418|gb|AEN74945.1
84	A	>gi|409905614|gb|AFV46419.1	176	B	>gi|46405994|gb|AAS93665.1
85	A	>gi|409905616|gb|AFV46420.1	177	B	>gi|380235896|gb|AFD34264.1
86	A	>gi|417346973|gb|AFX60138.1	178	Bovine	>gi|138247|sp|P22167.1
87	A	>gi|417346997|gb|AFX60150.1	179	Bovine	>gi|3451386|emb|CAA76980.1
88	A	>gi|417347021|gb|AFX60162.1	180	Bovine	>gi|17939990|gb|AAL49399.1
89	A	>gi|417347085|gb|AFX60190.1	181	Bovine	>gi|9631275|ref|NP_048055.1
90	A	>gi|425706126|gb|AFX95851.1	182	Bovine	>gi|94384139|emb|CAI96787.1
91	A	>gi|392301836|gb|AFM55387.1	183	Bovine	>gi|425678|gb|AAB28458.1
92	A	>gi|392301992|gb|AFM55530.1	184	Bovine	>gi|17940002|gb|AAL49410.1

[0146] The RSV F protein exhibits remarkable sequence conservation across RSV subtypes (see Table 3, which shows average pairwise sequence identity across subtypes and F protein segments). For example, RSV subtypes A and B share 90% sequence identity, and RSV subtypes A and B each share 81% sequence identify with bRSV F protein, across the F_0 precursor molecule. Within RSV subtypes the F_0 sequence identity is even greater; for example within each of RSV A, B, and bovine subtypes, the RSV F_0 precursor protein has -98% sequence identity. Nearly all identified RSV F_0 precursor proteins are approximately 574 amino acids in length, with minor differences in length typically due to the length of the C-terminal cytoplasmic tail. Sequence identity across RSV F proteins is illustrated in Table 4:

Table 4. RSV F protein sequence identity

RSV subtype	hRSV A (SEQ NOs: 1-128)	hRSVB (SEQ NOs: 129-177)	bRSV (SEQ NOs: 178-184)
F_0 (positions 1-574)			
hRSV A (SEQ NOs: 1-128)	98%	-	
RSV B (SEQ NOs: 129-177)	90%	99%	-
Bovine RSV (SEQ NOs: 178-184)	81%	81%	98%
F_2 (positions 26-109)			
hRSV A (SEQ NO: 1-128)	98%	-	-
hRSV B (SEQ NO: 129-177)	93%	99%	-
Bovine RSV	77%	77%	98%
(SEQ NOs: 178-184)			
F_1 (positions 137-513)			
hRSV A (SEQ NOs: 1-128)	99%	-	-
hRSV B (SEQ NOs: 129-177)	95%	>99%	-
Bovine RSV (SEQ NOs: 178-184)	91%	92%	99%

[0147] In view of the conservation of RSV F sequences, the person of ordinary skill in the art can easily compare amino acid positions between different native RSV F sequences, to identify corresponding RSV F amino acid positions between different RSV strains and subtypes. For example, across nearly all identified native RSV F_0 precursor proteins, the furin cleavage sites fall in the same amino acid positions. Thus, the conservation of RSV F protein sequences across strains and subtypes allows use of a reference RSV F sequence for comparison of amino acids at particular positions in the RSV F protein. For the purposes of this disclosure (unless context indicates otherwise), RSV F protein amino acid positions are given with reference to the reference F_0 protein precursor polypeptide set forth as SEQ ID NO: 124 (corresponding to GENBANK® Acc. No. P03420, as present in GENBANK® on February 28, 2013).

B. PreF Antigens

[0148] Isolated antigens are disclosed herein that include a recombinant RSV F protein stabilized in a prefusion conformation ("PreF antigens") by S155C, S290C, S190F, and V207L substitutions (compared to a native RSV F protein as set forth as one of SEQ ID Nos: 1-184). The PreF antigens contain a recombinant RSV F protein or fragment thereof that has been modified from a native form to increase immunogenicity. For example, the disclosed recombinant RSV F proteins have been modified from the native RSV sequence to be stabilized in a prefusion conformation. The person of ordinary skill in the art will appreciate that the disclosed PreF antigens are useful to induce immunogenic responses in vertebrate animals (such as mammals, for example, humans and cattle) to RSV (for example RSV A, RSV B, or bovine RSV). Thus, in several embodiments, the disclosed antigens are immunogens.

[0149] The D25 antibody recognizes a quaternary epitope including multiple protomers of the RSV F protein. This epitope is contained within an antigenic site ("Antigenic site Ø") located on the membrane-distal apex of the RSV F glycoprotein (see, *e.g.*, FIG. 1C), when it is in a prefusion conformation. While the secondary structural elements of this epitope remain mostly unchanged between pre- and post-fusion F conformations, their relative orientation changes substantially, with the α4-helix pivoting -180° relative to strand β2 in pre- and post-fusion conformations (see, *e.g.*, FIG. 3B). The conformational changes in the structure of the RSV F protein between the pre- and post-fusion conformations determine the presence of the D25 epitope on the RSV F protein. Accordingly, in several embodiments, a PreF antigen including a recombinant RSV F protein stabilized in a prefusion conformation can be identified by determining the specific binding of the D25 monoclonal antibody to the antigen. The person of ordinary skill in the art will appreciate that other antibodies that specifically bind to antigenic site Ø of the RSV F protein (such as the AM22 antibody or 5C4 antibody), or other antibodies that are pre-fusion specific, but do not bind antigenic site Ø (such as MPE8) can also be used to identify a PreF antigen including a RSV F protein stabilized in a prefusion conformation.

[0150] Thus, the PreF antigens disclosed herein are specifically bound by an antibody that is specific for the RSV F prefusion conformation but not the post-fusion conformation. In several embodiments, the PreF antigen is specifically bound by the D25 and /or AM22 antibody, which (as disclosed herein) are antibodies specific for the pre-but not post-fusion conformation of the RSV F protein. In several examples, the prefusion-specific antibody (such as D25 or AM22) specifically binds to the PreF antigen with a dissociation constant of less than about 10^{-6} Molar, such as less than about 10^{-7} Molar, 10^{-8} Molar, or less than 10^{-9} Molar. Specific binding can be determined by methods known in the art. The determination of specific binding may readily be made by using or adapting routine procedures, such as ELISA, immunocompetition, surface plasmon resonance, or other immunosorbant assays (described in many standard texts, including Harlow and Lane, Using Antibodies: A Laboratory Manual, CSHL, New York, 1999).

[0151] In further embodiments, the PreF antigen is not specifically bound by an antibody that binds the postfusion conformation of the RSV F protein. For example, an antibody specific for the six helix bundle found only in the postfusion conformation of RSV F protein (*e.g.*, as described in Magro et al., Proc. Nat'l. Acad. Sci. U.S.A., 109:3089-3094, 2012). In several examples, the dissociation constant for the RSV F postfusion specific antibody binding to the PreF antigen is greater than 10^{-5} Molar, such as at least 10^{-5} Molar, 10^{-4} Molar, or 10^{-3}.

[0152] In several embodiments, any of the PreF antigens includes a RSV F protein prefusion epitope (such as a D25 or AM22 epitope) in a RSV F protein prefusion-specific antibody-bound conformation (such as a D25 or AM22 bound conformation). For example, in several embodiments, any of the PreF antigens includes an epitope in a D25 or AM22 epitope-bound confirmation (e.g., the conformation defined by the structural coordinates provided in Table 1) when the PreF antigen is not bound by D25 or AM22, that is, the PreF antigen is stabilized in the D25- or AM22-bound conformation. Methods of determining if a disclosed PreF antigen includes a RSV F protein prefusion epitope (such as a D25 or AM22 epitope) in a RSV F protein prefusion specific monoclonal antibody-bound conformation (such as a D25 or AM22 bound conformation) are known to the person of ordinary skill in the art and further disclosed herein (see, for example, McLellan et al., Nature, 480:336-343, 2011; and U.S. Patent Application Publication No. 2010/0068217). For example, the disclosed three-dimensional structure of the D25 Fab fragment in complex with the RSV F protein can be compared with three-dimensional structure of any of the disclosed PreF antigens.

[0153] The person of ordinary skill in the art will appreciate that a disclosed PreF antigen can include an epitope in a prefusion specific monoclonal antibody-bound conformation even though the structural coordinates of antigen are not strictly identical to those of the prefusion F protein as disclosed herein. For example, in several embodiments, any of the disclosed PreF antigens include a RSV F prefusion-specific epitope (such as a D25 or AM22 epitope) that in the absence of the RSV F prefusion specific monoclonal antibody can be structurally superimposed onto the corresponding epitope in complex with the RSV F prefusion specific monoclonal antibody with a root mean square deviation (RMSD) of their coordinates of less than 1.0, 0.75, 0.5, 0.45, 0.4, 0.35, 0.3 or 0.25 Å/residue, wherein the RMSD is measured over the polypeptide backbone atoms N, Cα, C, O, for at least three consecutive amino acids.

[0154] In several embodiments, the PreF antigen is soluble in aqueous solution. For example, in some embodiments, the PreF antigen is soluble in a solution that lacks detergent. In some embodiments, the PreF antigen dissolves to a concentration of at least 0.5 mg/ml (such as at least 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml, 3.0 mg/ml, 4.0 mg/ml or at least 5.0 mg/ml) in phosphate buffered saline (pH 7.4) at room temperature (e.g., 20-22 degrees Celsius) and remains dissolved for at least for at least 12 hours (such as at least 24 hours, at least 48 hours, at least one week, at least two weeks, or more time). In one embodiment, the phosphate buffered saline includes NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (10 mM), KH_2PO_4 (1.8 mM) at pH 7.4. In some embodiments, the phosphate buffered saline further includes $CaCl_2$ (1 mM) and $MgCl_2$ (0.5 mM). The person of skill in the art is familiar with methods of determining if a protein remains in solution over time. For example, the concentration of the protein dissolved in a aqueous solution can be tested over time using standard methods.

[0155] In several embodiments, any of the disclosed PreF antigens can be used to induce an immune response to RSV in a subject. In several such embodiments, induction of the immune response includes production of neutralizing antibodies to RSV. Methods to assay for neutralization activity are known to the person of ordinary skill in the art and further described herein, and include, but are not limited to, plaque reduction neutralization (PRNT) assays, micro neutralization assays (see *e.g.*, Anderson et al., J. Clin. Microbiol., 22: 1050-1052, 1985), or flow cytometry based assays (see, *e.g.*, Chen et al., J. Immunol. Methods., 362:180-184, 2010). Additional neutralization assays are described herein, and familiar to the person of ordinary skill in the art.

[0156] In some embodiments, the PreF antigen includes a recombinant RSV F protein that, when dissolved in an aqueous solution, forms a population of recombinant RSV F proteins stabilized in a prefusion conformation. The aqueous solution can be, for example, phosphate buffered saline at physiological pH, such as pH 7.4. In some embodiments, the population is a homogeneous population including one or more recombinant RSV F proteins that are, for example, all stabilized in a prefusion conformation. In some embodiments, at least about 90% of the recombinant RSV F proteins (such as at least about 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.9% of the RSV F proteins) in the homogeneous population are stabilized in the prefusion conformation. In some embodiments, at least about 90% of the recombinant RSV F proteins (such as at least about 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.9% of the RSV F proteins) in the homogeneous population are specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or include a RSV F prefusion specific conformation (such as antigenic site Ø). It will be understood that a homogeneous population of RSV F proteins in a particular conformation can include variations (such as protein modification variations, e.g., glycosylation state), that do not alter the conformational state of the RSV F protein. In several embodiments, the population of recombinant RSV F protein remains homogeneous over time. For example, the PreF antigen can include a recombinant RSV F protein that, when dissolved in aqueous solution, forms a population of recombinant RSV F proteins that is stabilized in a prefusion conformation for at least 12 hours, such as at least 24 hours, at least 48 hours, at least one week, at least two weeks, or more.

[0157] In several embodiments, the isolated PreF antigens are substantially separated from RSV F proteins in a post-fusion conformation. Thus, the PreF antigen can be, for example, at least 80% isolated, at least 90%, 95%, 98%, 99%, or even 99.9% separated from RSV F proteins in a postfusion conformation. In several embodiments, the PreF antigens are also separated from RSV F proteins that do not include antigen site ø and/or are not specifically bound by a prefusion specific monoclonal antibody (such as D25 or AM22). For example, the PreF antigen can be at least 80% isolated, at least 90%, 95%, 98%, 99%, or even 99.9% separated from RSV F proteins that do not include antigen site ø and/or are not specifically bound by a prefusion specific monoclonal antibody (such as D25 or AM22).

[0158] In some embodiments, the PreF antigen includes a recombinant RSV F protein that, when incubated in an aqueous solution, forms a population of recombinant RSV F proteins stabilized in a prefusion conformation, wherein at least 70% (such as at least 80%, or at least 90% or at least 95% or at least 98%) of the isolated antigens in the population specifically bind to a RSV F protein prefusion-specific antibody (such as D25 or AM22) after

(a) incubation for one hour in 350 mM NaCl pH 7.0, at 50°C;

(b) incubation for one hour in 350 mM NaCl pH 3.5, at 25°C;

(c) incubation for one hour in 350 mM NaCl pH 10, at 25°C;

(d) incubation for one hour in 10 mM osmolarity, pH 7.0, at 25°C;

(e) incubation for one hour in 3000 mM osmolarity, pH 7.0, at 25°C;

(g) a combination of two or more of (a)-(e); or

a combination of (a) and (b); (a) and (c); (a) and (d); (a) and (e); (b) and (d); (b) and (e); (c) and (d); (c) and (e); (a), (b), and (d); (a), (c), and (d); (a), (b), and (e); or (a), (c), and (e)

[0159] In further embodiments, the PreF antigen includes a recombinant RSV F protein that, when incubated in an aqueous solution, forms a population of recombinant RSV F proteins stabilized in a prefusion conformation, wherein at least 60% (such as at least 70%, at least 80%, or at least 90%) of the isolated antigens in the population specifically bind to the prefusion-specific antibody after ten freeze-thaw cycles in 350 mM NaCl pH 7.0.

[0160] In some embodiments, the PreF antigens are provided as a homogenous population that does not include detectable RSV F protein in a post-fusion conformation. RSV F protein is detectable by negative stain electron microscope and/or specific binding by a postfusion antibody.

1. Recombinant RSV F proteins stabilized in a prefusion conformation

[0161] The PreF antigens disclosed herein include a recombinant RSV F protein stabilized in a prefusion conformation by S155C, S290C, S190F, and V207L substitutions (compared to a native RSV F protein as set forth as one of SEQ ID NOs: 1-184) and include an F_1 polypeptide and a F_2 polypeptide. The F_1 polypeptide, F_2 polypeptide, or both, can include at least one additional modification (e.g., an amino acid substitution) that further stabilizes the recombinant RSV F protein in its prefusion conformation. In several embodiments, the F_2 polypeptide and the F_1 polypeptide are linked by a peptide linker (for example, in embodiments including a single chain RSV F protein). Stabilization of the recombinant RSV F protein in the prefusion conformation preserves at least one prefusion-specific epitope (*i.e.*, an epitope present in the pre- (but not post-) fusion conformation of the RSV F protein) that specifically binds to a RSV F prefusion-specific monoclonal antibody (*i.e.*, an antibody that specifically binds to the RSV F protein in a prefusion conformation, but not a post fusion conformation). Thus, the disclosed PreF antigens are specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0162] In some examples, the PreF antigen includes a recombinant RSV F protein including a F_1 and/or F_2 polypeptide from a RSV A virus, for example, a F_1 and/or F_2 polypeptide from a RSV F_0 protein provided as one of SEQ ID NOs: 1-128, or 370, that is modified to include S155C, S290C, S190F, and V207L substitutions that stabilize the recombinant RSV F protein in a prefusion conformation. In some examples, the PreF antigen includes a recombinant RSV F protein including a F_1 and/or F_2 polypeptide from a RSV B virus, for example, a F_1 and/or F_2 polypeptide from a RSV F_0 protein provided as one of SEQ ID NOs: 129-177, that is modified to stabilize the recombinant RSV F protein in a prefusion conformation. In some examples, the PreF antigen includes a recombinant RSV F protein including a F_1 and/or F_2 polypeptide from a RSV bovine virus, for example, a F_1 and/or F_2 polypeptide from a RSV F_0 protein provided as one of SEQ ID NOs: 178-184, that is modified to S155C, S290C, S190F, and V207L substitutions that stabilize the recombinant RSV F protein in a prefusion conformation. F_1 and/or F_2 polypeptides from other RSV subtypes can also be used. The recombinant RSV F protein can include further modifications of the native RSV sequences, such as amino acid substitutions, deletions or insertions, glycosylation and/or covalent linkage to unrelated proteins (e.g., a protein tag), as long as the PreF antigen retains the recombinant RSV F protein stabilized in a prefusion conformation by the S155C, S290C, S190F, and V207L substitutions. RSV F proteins from the different RSV subgroups, as well as nucleic acid sequences encoding such proteins and methods for the manipulation and insertion of such nucleic acid sequences into vectors, are disclosed herein and known in the art (see, *e.g.*, Tan et al., PLOS one, 7: e51439, 2011; Sambrook et al., Molecular Cloning, a Laboratory Manual, 2d edition, Cold Spring Harbor Press, Cold Spring Harbor, N. Y. (1989); Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and John Wiley & Sons, New York, N. Y. (1994)).

[0163] In some embodiments, the recombinant RSV F protein comprises or consists of a F_2 polypeptide and a F_1 polypeptide comprising amino acid sequences at least 80% identical to amino acids 26-103 and 145-310, respectively, of a native RSV F protein sequence set forth as any one of SEQ ID NOs: 1-184, such as SEQ ID NO: 124.

[0164] In some embodiments, the recombinant RSV F protein comprises or consists of a F_2 polypeptide and a F_1 polypeptide comprising amino acid sequences at least 80% (such as at least 90%, at least 95% or at least 98%) identical to amino acids 26-103 and 145-513, respectively, of a native RSV F protein sequence set forth as any one of SEQ ID NOs: 1-184, such as SEQ ID NO: 124.

[0165] In some embodiments, the recombinant RSV F protein comprises or consists of a F_2 polypeptide and a F_1 polypeptide comprising amino acid sequences at least 80% (such as at least 90%, at least 95% or at least 98%) identical to amino acids 26-103 and 145-529, respectively, of a native RSV F protein sequence set forth as any one of SEQ ID NOs: 1-184, such as SEQ ID NO: 124.

[0166] In some embodiments, the recombinant RSV F protein comprises or consists of a F_2 polypeptide and a F_1 polypeptide comprising amino acid sequences at least 80% (such as at least 90%, at least 95% or at least 98%) identical to amino acids 26-103 and 145-551, respectively, of a native RSV F protein sequence set forth as any one of SEQ ID NOs: 1-184, such as SEQ ID NO: 124.

[0167] In some examples, the PreF antigen includes a recombinant RSV F protein including a F_1 and/or F_2 polypeptide including a polypeptide sequences having at least 75% (for example at least 85%, 90%, 95%, 96%, 97%, 98% or 99%) sequence identity with a RSV F_1 and/or F_2 polypeptide from a RSV A virus, for example, a F_1 and/or F_2 polypeptide from a RSV F_0 protein provided as one of SEQ ID NOs: 1-128 or 370. In further examples, the PreF antigen includes a recombinant RSV F protein including a F_1 and/or F_2 polypeptide including a polypeptide sequences having at least 75% (for example at least 85%, 90%, 95%, 96%, 97%, 98% or 99%) sequence identity with a RSV F_1 and/or F_2 polypeptide from a RSV B virus, for example, a F_1 and/or F_2 polypeptide from a RSV F_0 protein provided as one of SEQ ID NOs: 129-177. In further examples, the PreF antigen includes a recombinant RSV F protein including a F_1 and/or F_2 polypeptide including a polypeptide sequences having at least 75% (for example at least 85%, 90%, 95%, 96%, 97%, 98% or 99%) sequence identity with a RSV F_1 and/or F_2 polypeptide from a RSV bovine virus, for example, a F_1 and/or F_2 polypeptide from a RSV F_0 protein provided as one of SEQ ID NOs: 178-184.

[0168] In several embodiments, the PreF antigen includes a recombinant RSV F protein including a F_1 polypeptide including or consisting of at least 300 consecutive amino acids (such as at least 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, or 430 consecutive amino acids) from a native F_1 polypeptide sequence, such as positions 137-513 of one of SEQ ID NOs: 1-184 or 370, including any polypeptide sequences having at least 75% (for example at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%) sequence identity to a native F_1 polypeptide sequence, such as positions 137-513 of any one of SEQ ID NOs: 1-184 or 370. For example, in some embodiments, the PreF antigen includes a recombinant F protein includes a F_1 polypeptide including or consisting of positions 137-513, 137-481, 137-491, or position 137 to the C-terminus, or positions 137-to the transmembrane domain, of any one of SEQ ID NOs: 1-184 or 370, including any polypeptide sequences having at least 75% (for example at least 85%, 90%, 95%, 96%, 97%, 98% or 99%) sequence identity to a native F_1 polypeptide sequence, such as positions 137-513, or position 137 to the C-terminus, or positions 137-to the transmembrane domain, any one of SEQ ID NOs: 1-184 or 370. The person of ordinary skill in the art will appreciate that the PreF antigen including the recombinant RSV F protein can include a F1 polypeptide with N- or C-terminal truncations (for example, deletion of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 or more amino acids) compared to extracellular region of a native F1 polypeptide (for example, positions 137-524), as long as the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0169] In some embodiments, the PreF antigen includes a F_1 polypeptide including a maximum length, for example no more than 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, or no more than 440 amino acids in length. The F_1 polypeptide may include, consist or consist essentially of the disclosed sequences. The disclosed contiguous F_1 polypeptide sequences may also be joined at either end to other unrelated sequences (for examiner, non-RSV F_1 protein sequences, non-RSV F protein sequences, non-RSV, non-viral envelope, or non-viral protein sequences)

[0170] In several embodiments, the PreF antigen includes a recombinant RSV F protein including a F_2 polypeptide including or consisting of at least 60 consecutive amino acids (such as at least 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108 or 109 consecutive amino acids) from a native F_2 polypeptide sequence, such as positions 26-109 of any one of SEQ ID NOs: 1-184 or 370, including a polypeptide sequences having at least 75% (for example at least 85%, 90%, 95%, 96%, 97%, 98% or 99%) sequence identity to a native F_1 polypeptide sequence, such as positions 26-109 any one of SEQ ID NOs: 1-184 or 370. For example, in some embodiments, the PreF antigen includes a recombinant F protein including a F_2 polypeptide including or consisting of 70-109 consecutive amino acids (such as 60-100, 75-95, 80-90, 75-85, 80-95, 81-89, 82-88, 83-87, 83-84, or 84-85 consecutive amino acids) from a native F_2 polypeptide sequence, such as positions 26-109 any one of SEQ ID NOs: 1-184 or 370, including any polypeptide sequences having at least 75% (for example at least 85%, 90%, 95%, 96%, 97%, 98% or 99%) sequence identity to a native F_2 polypeptide sequence, such as positions 137-513 any one of SEQ ID NOs: 1-184 or 370.

[0171] In some embodiments, the PreF antigen includes a F_2 polypeptide is also of a maximum length, for example no more than 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87,. 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 amino acids in length. The F_2 polypeptide may include, consist or consist essentially of the disclosed sequences. The disclosed contiguous F_2 polypeptide sequences may also be joined at either end to other unrelated sequences (for examiner, non-RSV F_2 protein sequences, non-RSV F protein sequences, non-RSV, non-viral envelope, or non-viral protein sequences).

[0172] In some embodiments, the PreF antigen includes a recombinant RSV F protein including a F_2 polypeptide including or consisting of at least 60 consecutive amino acids (such as at least 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87,. 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108 or 109 consecutive amino acids) from a native F_2 polypeptide sequence, such as positions 26-109 of any one of SEQ ID NOs: 1-184 or 370, including polypeptide sequences having at least 75% (for example at least 85%, 90%, 95%, 96%, 97%, 98% or 99%) sequence identity to a native F_2 polypeptide sequence, such as amino acids 26-109 any one of SEQ ID NOs: 1-184 or 370, and further includes a F_1 polypeptide including or consisting of at least 300 consecutive amino acids (such as at least 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, or 430 consecutive amino acids) from a native F_1 polypeptide sequence, such as positions 137-513 of one of SEQ ID NOs: 1-184 or 370, including any polypeptide sequences having at least 75% (for example at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%) sequence identity to a native F_1 polypeptide sequence, such as positions 137-513 of any one of SEQ ID NOs: 1-184 or 370.

[0173] In one non-limiting example, the PreF antigen includes a recombinant RSV F protein including a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, including polypeptide sequences having at least 75% (for example at least 85%, 90%, 95%, 96%, 97%, 98% or 99%) sequence identity to a positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370.

[0174] As noted above, the RSV F protein is initially synthesized as a F_0 precursor protein and is cleaved at multiple sites (including two conserved furin cleavage sites) during maturation in eukaryotic cells. Thus, the native RSV F protein lacks the N-terminal signal peptide and the pep27 peptide (or a portion thereof) of the F_0 precursor protein. In several embodiments, the disclosed recombinant RSV F proteins stabilized in the prefusion conformation do not include the signal peptide (or a portion thereof) and/or do not include the pep27 peptide (or a portion thereof). The person of ordinary skill in the art will appreciate that recombinant RSV F proteins lacking the RSV F signal peptide and/or pep27 peptide can be generated by expressing the recombinant F_0 polypeptide in cells where the signal peptide and the pep27 peptide will be excised from the F_0 precursor by cellular proteases.

[0175] Several embodiments include a PreF antigen including a multimer of any of the disclosed recombinant RSV F proteins, for example, a multimer including 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more of the disclosed recombinant RSV F proteins. In several examples, any of the disclosed recombinant RSV F proteins can be linked (*e.g.*, via a peptide linker) to another of the recombinant RSV F proteins to form the multimer.

[0176] It is understood in the art that some variations can be made in the amino acid sequence of a protein without affecting the activity of the protein. Such variations include insertion of amino acid residues, deletions of amino acid residues, and substitutions of amino acid residues. These variations in sequence can be naturally occurring variations or they can be engineered through the use of genetic engineering technique known to those skilled in the art. Examples of such techniques are found in Sambrook J, Fritsch E F, Maniatis T et al., in Molecular Cloning-A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989, pp. 9.31-9.57), or in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Thus, in some embodiments, the PreF antigen includes a F_1 polypeptide, a F_2 polypeptide, or both a F_1 and F_2 polypeptide, that include one or more amino acid substitutions compared to the corresponding native RSV sequence. For example, in some embodiments, the F_1 polypeptide, F_2 polypeptide, or both the F_1 polypeptide and the F_2 polypeptide, include up to 20 (such as up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19) amino acid substitutions compared to a native F_1 polypeptide sequence, such as a native RSV sequence set forth as any one of SEQ ID NOs: 1-184 or 370, wherein the PreF antigen is specifically bound by a RSV F prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). In additional embodiments, the F_1 polypeptide, F_2 polypeptide, or both the F_1 polypeptide and the F_2 polypeptide, include up to 20 (such as up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19) conservative amino acid substitutions compared to a native F_1 polypeptide sequence, such as a native RSV sequence set forth as any one of SEQ ID NOs: 1-184 or 370, wherein the PreF antigen is specifically bound by a RSV F prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). For example, in some embodiments, the PreF antigen includes a recombinant RSV F protein in a prefusion conformation that is modified to increase expression of the protein for protein productions purposes, *e.g.*, by elimination of one or more nuclear localization signals present on the RSV F protein. Manipulation of the nucleotide sequence encoding the F_1 or F_2 polypeptide sequence (such as a nucleotide sequence encoding the F_0 polypeptide including the F_1 and F_2 polypeptides) using standard procedures, including in one specific, non-limiting, embodiment, site-directed mutagenesis or in another specific, non-limiting, embodiment, PCR, can be used to produce such variants. Alternatively, the F_1 and F_2 polypeptides can be synthesized using standard methods. The simplest modifications involve the substitution of one or more amino acids for amino acids having similar biochemical properties. These so-called conservative substitutions are likely to have minimal impact on the activity of the resultant protein.

a. **Membrane distal stabilizing modifications**

[0177] As disclosed herein, the RSV F protein undergoes a structural rearrangement between its pre-and post-fusion conformations. As shown in FIG. 2B, the N-terminal region of the F_1 polypeptide in the prefusion conformation (corresponding in part to the membrane distal lobe shown in FIG. 2A) includes the indicated α2, α3, β3, β4, and α4 helical and beta sheet structures, whereas the corresponding region of the N-terminus of the F_1 polypeptide in the postfusion structure includes an extended oc5 helical structure - the α2, α3, β3, β4, and α4 helical and beta sheet structures are absent. Further, the C-terminal region of the F_1 polypeptide in the prefusion conformation (corresponding in part to the membrane proximal lobe shown in FIG. 2A) includes the indicated β22, α9, and β23 beta sheet and helical structures, whereas the corresponding C-terminal region of the F_1 polypeptide in the postfusion conformation structure includes an extended α10 helical structure and extended coil - the β22, α9, and β23 beta sheet and helical structures are absent. Thus, the membrane distal and membrane proximal lobes of the RSV F protein in its prefusion conformation include several distinct structural elements that are absent from the corresponding regions of the RSV F protein in its postfusion conformation.

[0178] Guided by the structural features identified in the pre- and post-fusion conformations of the RSV F protein, several modes of stabilizing the RSV F protein in a prefusion conformation are available, including amino acid substitutions that introduce one or more non-natural disulfide bonds, fill cavities within the RSV F protein, alter the packing of residues in the RSV F protein, introduce N-linked glycosylation sites, and combinations thereof. The stabilize modifications provided herein are targeted modifications that stabilize the recombinant RSV F protein in the prefusion conformation. In several embodiments, the RSV F protein is not stabilized by non-specific cross-linking, such as glutaraldehyde crosslinking, for example glutaraldehyde crosslinking of membrane bound RSV F trimers.

[0179] In some non-limiting embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation by introduction of a disulfide bond, wherein the recombinant RSV F protein includes S155C and S290C; G151C and I288C; A153C and K461C; A149C and Y458C; G143C and S404S substitutions; or Y33C and V469C amino acid substitutions. Non-limiting examples of precursor proteins of such recombinant RSV F proteins (including a Foldon domain linked to the C-terminus of the F1 polypeptide) are set forth herein as SEQ ID NO: 185, SEQ ID NO: 189, SEQ ID NO: 205, SEQ ID NO: 207, SEQ ID NO: 209, and SEQ ID NO: 211. In further non-limiting embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation by introduction of a disulfide bond and one or more cavity filling substitutions, wherein the recombinant RSV F protein includes S155C, S290C substitutions, and a large hydrophobic residue at position 190, and/or position 207 (e.g., a S190F, S190W, or S190L substation, and/or a V207L, V207F, or V207W substitution). Non-limiting examples of precursor proteins of such recombinant RSV F precursor proteins (including a foldon domain linked to the C-terminus of the F1 polypeptide) are set forth herein as SEQ ID NO: 371, SEQ ID NO: 372, SEQ ID NO: 373, SEQ ID NO: 374, SEQ ID NO: 375, and SEQ ID NO: 376.

[0180] Many of the sequences of recombinant RSV F proteins disclosed herein include the sequence of protease cleavage sites (such as thrombin sites), protein tags (such as a His tag, a Strep Tag II, a Avi tag, etc., that are not essential for the function of the RSV F protein, such as for induction of an immune response in a subject. The person of ordinary skill in the art will recognize such sequences, and when appropriate, understand that these tags or protease cleavage sites are not included in a disclosed recombinant RSV F protein.

i. Non-natural Disulfide Bonds

[0181] In several embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation by at least one non-natural disulfide bond including a pair of cross-linked cysteine residues. A non-natural disulfide bond is one that does not occur in a native RSV F protein, and is introduced by protein engineering (e.g., by including one or more substituted cysteine residues that form the non-natural disulfide bond). For example, in some embodiments, any of the disclosed recombinant RSV F protein is stabilized in a prefusion conformation by any one of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 disulfide bonds including a pair of cross-linked cysteine residues. In one specific non-limiting example, the recombinant RSV F protein is stabilized in a prefusion conformation by a single pair of cross-linked cysteine residues. In another non-limiting example, any of the disclosed recombinant RSV F protein is stabilized in a prefusion conformation by two pairs of crosslinked cysteine residues.

[0182] The cysteine residues that form the disulfide bond can be introduced into native RSV F protein sequence by one or more amino acid substitutions. For example, in some embodiments, a single amino acid substitution introduces a cysteine that forms a disulfide bond with a cysteine residue present in the native RSV F protein sequence. In additional embodiments, two cysteine residues are introduced into a native RSV sequence to form the disulfide bond. The location of the cysteine (or cysteines) of a disulfide bond to stabilize the RSV F protein in a prefusion conformation can readily be determined by the person of ordinary skill in the art using the disclosed structure of RSV F protein in its prefusion conformation, and the previously identified structure of RSV F protein in its post fusion conformation.

[0183] For example, the amino acid positions of the cysteines are typically within a sufficiently close distance for formation of a disulfide bond in the prefusion conformation of the RSV F protein. Methods of using three-dimensional structure data to determine if two residues are within a sufficiently close distance to one another for disulfide bond formation are known (see, e.g., Peterson et al., Protein engineering, 12:535-548, 1999 and Dombkowski, Bioinformatics, 19:1852-1853, 3002 (disclosing DISULFIDE BY DESIGN™). For example, residues can be selected manually, based on the three dimensional structure of RSV F protein in a prefusion conformation provided herein, or a software, such as DISULFIDEBYDESIGN™, can be used. Without being bound by theory, ideal distances for formation of a disulfide bond are generally considered to be about ~5.6Å for Cα-Cα distance, ~2.02 Å for Sy-Sy distance, and 3.5-4.25Å for Cβ-Cβ distance (using the optimal rotomer). The person of ordinary skill in the art will appreciate that variations from these distances are included when selecting residues in a three dimensional structure that can be substituted for cysteines for introduction of a disulfide bond. For example, in some embodiments the selected residues have a Cα-Cα distance of less than 7.0 Å and/or a Cβ-Cβ distance of less than 4.7Å. In some embodiments the selected residues have a Cα-Cα distance of from 2.0-8.0 Å and/or a Cβ-Cβ distance of from 2.0-5.5 Å. In several embodiments, the amino acid positions of the cysteines are within a sufficiently close distance for formation of a disulfide bond in the prefusion, but not post-fusion, conformation of the RSV F protein.

[0184] The person of ordinary skill in the art can readily determine the relative position of a particular amino acid between the pre- and post-fusion conformations of the RSV F protein, for example by comparing the prefusion structures defined herein by the structural coordinates provided in Table 1, with the previously identified postfusion structure described in McLellan et al., J. Virol., 85, 7788, 2011, with structural coordinates deposited as PDB Accession No. 3RRR). Methods of determining relative position of a particular amino acid between the two protein structures (e.g., between the three dimensional structures pre- and post-fusion RSV F protein) are known. For example the person of ordinary skill in the art can use known superimposition methods to compare the two structures (e.g., methods using the LSQKAB program (Kabsch W. Acta. Cryst. A32 922-923 (1976)). In one example, the pre-and postfusion structures can be superimposed by using LSQKAB to align F protein positions 26-60, 77-97, 220-322, and 332-459 defined by the structural coordinates provided in Table 1, with the F protein positions 26-60, 77-97, 220-322, and 332-459 defined by the structural coordinates deposited as PDB Accession No. 3RRR, and comparing the distance between the Cα atom for each residue in the pre- and post-fusion structures to identify the deviation of particular residues between the two structures.

[0185] In several embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation by a disulfide bond between a cysteine introduced into an amino acid position that changes conformation, and a cysteine introduced into an amino acid position that does not change conformation, between the pre- and post-fusion structures, respectively. For example, in some embodiments, the PreF antigen includes a recombinant RSV F protein including amino acid substitutions introducing a pair of cysteines, wherein the first cysteine is in an amino acid position of the RSV F protein that has a root mean square deviation of at least 5 (such as at least 6, at least 7, at least 8, at least 9 or at least 10) angstroms between the three-dimensional structure of the RSV F protein pre- and post-fusion conformations, and the second cysteine is in an amino acid position of the RSV F protein that has a root mean square deviation of less than 4 (such as less than 3, 2, or 1) angstroms between the three-dimensional structure of the RSV F protein pre- and post-fusion conformations, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0186] Based on a comparison of the pre- and post-fusion RSV F structures, there are at least two regions that undergo large conformational changes, located at the N- and C-termini of the F_1 subunit (residues 137-216 and 461-513, respectively). For example, as illustrated in FIG. 2B, the positions 137-216 and 461-513 of the F_1 polypeptide undergo structural rearrangement between the Pre-and Post- F protein conformations, whereas positions 217-460 of the F_1 polypeptide remain relatively unchanged. Thus, in some embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation by a disulfide bond between a first cysteine in one of positions 137-216 or 461-513 of the F_1 polypeptide, and a second cysteine in one of positions 217-460 of the F_1 polypeptide. In further embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation by a disulfide bond between a first cysteine in one of positions 137-216 or 461-513 of the F_1 polypeptide, and a second cysteine in a position of the F2 polypeptide, such as one of positions 26-109 (for example, one of positions 26-61 or 77-97) of the F_2 polypeptide.

[0187] In additional embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation by a disulfide bond between cysteines that are introduced into amino acid positions that change conformation between the pre- and post-fusion structures, respectively. For example, in some embodiments, the PreF antigen includes a recombinant RSV F protein including amino acid substitutions introducing a pair of cysteines, wherein the first cysteine and the second cysteine is in an amino acid position of the RSV F protein that has a root mean square deviation of at least 5 (such as at least 6, at least 7, at least 8, at least 9 or at least 10) angstroms between the three-dimensional structure of the RSV F protein pre- and post-fusion conformations, wherein the PreF antigen includes specific binding activity to an RSV F prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific epitope (e.g., a D25 or AM22 epitope). In some such embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation by a disulfide bond between a the first cysteine and the second cysteine in positions 137-216 of the F_1 polypeptide. In additional embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation by a disulfide bond between the first cysteine and the second cysteine in positions 461-513 of the F_1 polypeptide. In further embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation by a disulfide bond between the first cysteine and the second cysteine in positions 137-216 and 461-513, respectively, of the F_1 polypeptide.

[0188] The person of ordinary skill in the art can readily determine the location of a particular amino acid in the pre- and post-fusion conformations of the RSV F protein (and any difference in a position between the two conformations) using the structural coordinates of the three-dimensional structure the RSV F protein in the prefusion conformation (which are set forth in Table 1), and the structural coordinates of the three-dimensional structure of the RSV F protein in the postfusion conformation (which are set forth in Protein Databank Accession No. 3RRR). For example, such comparison methods are described in Example 1. Table 5 provides examples of cysteine pairs and amino acid substitutions that can be used to stabilize a RSV F protein in a prefusion conformation.

Table 5. Exemplary Cysteine Pairs for Disulfide Bond Stabilization

	F protein Residue Pair(s) for Cysteine Substitution	Substitutions corresponding to SEQ ID NO: 124	SEQ ID NO
F_1 substitutions - Intra-Protomer Disulfide Bond			
1	155 and 290	S155C and S290C	185
2	151 and 288	G151C and I288C	189
3	137 and 337	F137C and T337C	213
4	397 and 487	T397C and E487C	247
5	138 and 353	L138C and P353C	257
6	341 and 352	W341C and F352C	267
7	403 and 420	S403C and T420C	268
8	319 and 413	S319C and I413C	269
9	401 and 417	D401C and Y417C	270
10	381 and 388	L381C and N388C	271
11	320 and 415	P320C and S415C	272
12	319 and 415	S319C and S415C	273
13	331 and 401	N331C and D401C	274
14	320 and 335	P320C and T335C	275
15	406 and 413	V406C and I413C	277
16	381 and 391	L381C and Y391C	278
17	357 and 371	T357C and N371C	279
18	403 and 417	S403C and Y417C	280
19	321 and 334	L321C and L334C	281
20	338 and 394	D338C and K394C	282
21	288 and 300	I288C and V300C	284
F_2 and F_1 Substitutions - Intra-Protomer Disulfide Bond			
22	60 and 194	E60C and D194C	190
23	33 and 469	Y33C and V469C	211
24	54 and 154	T54C and V154C	212
25	59 and 192	I59C and V192C	246
26	46 and 311	S46C and T311C	276
27	48 and 308	L48C and V308C	283
28	30 and 410	E30C and L410C	285

F_2 and F_1 Substitutions - Intra-Protomer Disulfide Bond			
F_1 substitutions - Inter-Protomer Disulfide Bond			
29	400 and 489	T400C and D489C	201
30	144 and 406	V144C and V406C	202
31	153 and 461	A153C and K461C	205
32	149 and 458	A149C and Y458C	207
33	143 and 404	G143C and S404S	209
34	346 and 454	S346C and N454C	244
35	399 and 494	K399C and Q494C	245
36	146 and 407	S146C and I407C	264
37	374 and 454	T374C and N454C	265
38	369 and 455	T369C and T455C	266
39	402 and 141	V402C and L141C	302
F_2 and F_1 Substitutions - Inter-Protomer Disulfide Bond			
40	74 and 218	| A74C and E218C	243
Amino acid insertions to orient the Disulfide bond			
41	145 and 460 (Inter), AA insertion between positions 146 and 147	S145C and 460C; AA insertion between positions 146/147	338
42	183 and 423 (Inter), AAA insertion between positions 182 and 183	N183C and K423C; AAA insertion between positions 182/183	339
43	330 and 430 (Inter); CAA insertion between positions 329 and 330	A329C and S430C; and a CAA insertion between positions 329 and 330	340
Combinations			
44	155 and 290 (Intra); and 402 and 141 (Inter)	S155C and S290C; and V402C and L141C	303
45	155 and 290(Intra); and 74 and 218	S155C and S290C; and A74C and E218C	263
46	155 and 290 (Intra); and 146 and 460 (Inter); G insertion between position 460 and 461	S155C and S290C; and S146C and N460C; G insertion between position 460 and 461	258
47	155 and 290 (Intra); and 345 and 454(Inter); C	S155C and S290C; and N345C and N454G; C	259
	insertion between positions 453 and 454	insertion between positions 453 and 454	
48	155 and 290 (Intra); and 374 and 454(Inter); C insertion between positions 453 and 454	S155C and S290C; and T374C and N454G; C insertion between positions 453 and 454	260
49	155 and 290 (Intra); and 239 and 279(Inter); C insertion between positions 238 and 239	S155C and S290C; and S238G and Q279C; C insertion between positions 238 and 239	261
50	155 and 290 (Intra); and 493 paired with C insertion between positions 329 and 330	S155C and S290C; and S493C paired with a C insertion between positions 329 and 330	262
51	183 and 428 (Inter), G insertion between positions 182 and 183	N183C and N428C; G insertion between positions 182 and 183	296
52	183 and 428 (Inter), C insertion between positions 427 and 428	N183C and N427G; C insertion between positions 427 and 428	297
53	155 and 290 (Intra); and 183 and 428(Inter); G insertion between positions 182 and 183	S155C and S290C; and N183C and N428C; G insertion between positions 182 and 183	298
54	155 and 290 (Intra); and 183 and 428(Inter); C insertion between positions 427 and 428	S155C and S290C; and N183C and N427G; C insertion between positions 427 and 428	299

[0189] In some embodiments, the PreF antigen includes a recombinant RSV F protein including one or more (such as 2, 3, 4, 5, 6, 7, 8, 9 or 10) disulfide bonds, including disulfide bond between cysteine residues located at the RSV F positions listed in one or more of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, or 54 of column 2 of Table 5, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0190] In further embodiments, the PreF antigen includes a recombinant RSV F protein including one or more (such as 2, 3, 4, 5, 6, 7, 8, 9 or 10) disulfide bonds, including disulfide bonds between cysteine residues that are introduced by the cysteine amino acid substitutions listed in one or more of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, or 54 of column 3 of Table 5, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0191] The SEQ ID NOs listed in column 4 of Table 5 set forth amino acid sequences including the indicated substitutions, as well as, a signal peptide, F_2 polypeptide (positions 26-109), a pep27 polypeptide (positions 110-136), a F_1 polypeptide (positions 137-513), a trimerization domain (a Foldon domain) and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))).

[0192] Thus, in additional embodiments, the PreF antigen includes a RSV F protein including a F_1 polypeptide and a F_2 polypeptide as set forth in any one of the SEQ ID NOs listed in column 4 of Table 5, such as a SEQ ID NO listed in one of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, or 54 of column 4 of Table 5, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). For example, the PreF antigen can include a RSV F protein including a F_1 polypeptide and a F_2 polypeptide, wherein the F_2 and the F_1 polypeptide include the amino acid sequence set forth as positions 26-109 and 137-513, respectively, of any one of the SEQ ID NOs listed in column 4 of Table 5, such as a SEQ ID NO listed in one of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, or 54 of column 4 of Table 5, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0193] In further embodiments, the PreF antigen includes a recombinant RSV F protein including one or more (such as 2, 3, 4, 5, 6, 7, 8, 9 or 10) intra-protomer disulfide bonds, including disulfide bond between cysteine residues located at the RSV F positions of the F_1 polypeptide listed in of one or more of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 of column 2 Table 5. For example, the PreF antigen can include a recombinant RSV F protein including one or more (such as 2, 3, 4, 5, 6, 7, 8, 9 or 10) intra-protomer disulfide bonds, including disulfide bonds between cysteine residues that are introduced by the F_1 polypeptide amino acid substitutions listed in of one or more of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 of column 3 of Table 5. In any of these embodiments, the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0194] In further embodiments, the PreF antigen includes a recombinant RSV F protein including one or more (such as 2, 3, 4, 5, 6, or 7) intra-protomer disulfide bonds, including disulfide bond between cysteine residues located at the RSV F positions of the F_2 and F_1 polypeptides listed in of one or more of rows 22, 23, 24, 25, 26, 27, or 28 of column 2 of Table 5. For example, the PreF antigen can include a recombinant RSV F protein including one or more (such as 2, 3, 4, 5, 6, or 7) intra-protomer disulfide bonds, including disulfide bond between cysteine residues that are introduced by the F_2 and F_1 polypeptide amino acid substitutions listed in of one or more of rows 22, 23, 24, 25, 26, 27, or 28 of column 3 of Table 5. In any of these embodiments, the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0195] In further embodiments, the PreF antigen includes a recombinant RSV F protein including one or more (such as 2, 3, 4, 5, 6, 7, 8, 9 or 10) inter-protomer disulfide bonds, including disulfide bond between cysteine residues located at the RSV F positions of the F_1 polypeptide listed in one or more of rows 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, or 39 of column 2 of Table 5. For example, the PreF antigen can include a recombinant RSV F protein including one or more (such as 2, 3, 4, 5, 6, 7, 8, 9 or 10) inter-protomer disulfide bonds, including disulfide bond between cysteine residues that are introduced by the F_1 polypeptide amino acid substitutions listed in of one or more of rows 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, or 39 of column 3 of Table 5. In any of these embodiments, the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0196] In further embodiments, the PreF antigen includes a recombinant RSV F protein including an inter-protomer disulfide bond between cysteine residues located at the RSV F positions of the F_2 and F_1 polypeptides listed in column 2 of row 40 of Table 5. In further embodiments, the PreF antigen includes a recombinant RSV F protein including an inter-protomer disulfide bond between cysteine residues that are introduced by the amino acid substitutions in the F_2 and F_1 polypeptide listed in column 3 of row 40 of Table 5. In any of these embodiments, the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0197] In some embodiments, amino acids can be inserted (or deleted) from the F protein sequence to adjust the alignment of residues in the F protein structure, such that particular residue pairs are within a sufficiently close distance to form an intra- or inter-protomer disulfide bond in the prefusion, but not postfusion, conformation. In several such embodiments, the PreF antigen includes a recombinant RSV F protein including a disulfide bond between cysteine residues located at the RSV F positions of the F_1 polypeptide, as well as the amino acid insertion, listed in one or more of rows 41, 42, or, 43 of column 2 of Table 5. In further embodiments, the PreF antigen includes a recombinant RSV F protein including a disulfide bond between cysteine residues that are introduced by the F_1 polypeptide amino acid substitutions, as well as the amino acid insertion, listed in of one or more of rows 41, 42, or, 43 of column 3 of Table 5.

[0198] In one example, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation includes a disulfide bond between cysteines at F1 positions 155 and 290, such as a recombinant F1 polypeptide protein with S155C and S290C substitutions.

[0199] In some embodiments, the PreF antigen includes a recombinant RSV F protein including a combination of two or more of the disulfide bonds between cysteine residues listed in Table 5 or Table 5b, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). It is understood that some combinations will not result in a RSV F protein stabilized in a prefusion conformation; such combinations can be identified by methods disclosed herein, for example by confirming that the antigen containing such a polypeptide is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø)

In further embodiments, the PreF antigen includes a recombinant RSV F protein including a non-natural disulfide bond stabilizing the F protein in a prefusion conformation, wherein the F protein includes the substitutions listed in one of row 1, 2, 3, 4, 5, 6, 7, 8 , 9, 10, 11, 12, 13, 14, 15, or 16 of column 3 of Table 5b, wherein cysteine residues are inserted in the F protein for formation of the non-natural disulfide bond. In any of these embodiments, the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0200] The SEQ ID NOs listed in column 4 of Table 5b set forth amino acid sequences including the indicated substitutions, as well as, a signal peptide, F_2 polypeptide (positions 26-109), a pep27 polypeptide (positions 110-136), a F_1 polypeptide (positions 137-513), a trimerization domain (a Foldon domain) and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))). Thus, in additional embodiments, the PreF antigen includes a RSV F protein including a F_1 polypeptide and a F_2 polypeptide as set forth in any one of the SEQ ID NOs listed in column 4 of Table 5b, such as a SEQ ID NO listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of column 4 of Table 5b, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). For example, the PreF antigen can include a RSV F protein including a F_1 polypeptide and a F_2 polypeptide, wherein the F_2 and the F_1 polypeptide include the amino acid sequence set forth as positions 26-109 and 137-513, respectively, of any one of the SEQ ID NOs listed in column 4 of Table 5b, such as a SEQ ID NO listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 of column 4 of Table 5b, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

Table 5b. Exemplary stabilized F protein substitutions and sequences

	Description	Substitutions	SEQ ID NO:
1	Intrachain disulfide	S238C, E92C	421
2	Intrachain disulfide	L193C, I59C	422
3	Intrachain disulfide	I59C, L297C	423
4	Intrachain disulfide	L297C, I292C	424
5	Intrachain disulfide	K176C, S190C	425
6	Intrachain disulfide	T189C, A177C	426
7	Intrachain disulfide	T58C, K191C	427

	Description	Substitutions	SEQ ID NO:
8	Intrachain disulfide	A424C, V450C	428
9	Intrachain disulfide	L171C, K191C	429
10	Intrachain disulfide	K176C, S190C	430
11	Interchain disulfide	K77C, I217C	431
12	Intrachain disulfide	K427C, D448C	434
13	Intrachain disulfide	G151C, N302C	435
14	Intrachain disulfide	G151C, V300C	436
15	Intrachain disulfide	T189C, V56C	437
16	Intrachain disulfide	L171C, K191C	438

ii. Cavity Filling Amino Acid Substitutions

[0201] Comparison of the structure of the prefusion conformation of the RSV F protein (*e.g.,* in complex with D25 Fab as disclosed herein) to the structure of the postfusion RSV F protein (disclosed, *e.g.,* in as disclosed in McLellan et al., J. Virol., 85, 7788, 2011) identifies several internal cavities or pockets in the prefusion conformation that must collapse for F to transition to the postfusion conformation. These cavities include those listed in Table 6.

[0202] Accordingly, in several embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation by one or more amino acid substitutions that introduce an amino acid that reduces the volume of an internal cavity that collapses in the postfusion conformation of RSV F protein. For example, cavities are filled by substituting amino acids with large side chains for those with small side chains. The cavities can be intra-protomer cavities, or inter-protomer cavities. One example of a RSV F cavity filling amino acid substitution to stabilize the RSV protein in its prefusion conformation a RSV F protein with S190F and V207L substitutions. In another embodiment, the cavity filling amino acid substitution to stabilize the RSV protein in its prefusion conformation a RSV F protein includes a S190F, S190L, S190W, S190H, S190M, or S190Y substitution.

[0203] The person of ordinary skill in the art can use methods provided herein to compare the structures of the pre-and post- fusion conformations of the RSV F protein to identify suitable cavities, and amino acid substitutions for filling the identified cavities. Exemplary cavities and amino acid substitutions for reducing the volume of these cavities are provided in Table 6.

Table 6. Exemplarity cavity-filling amino acid substitutions

Row	Cavity/Cavities	A.A. Substitutions	SEQ ID NO:
1	Ser190 and Val207	190F and 207L	191
2	Val207	207L and 220L	193
3	Ser190 and Val296	296F and 190F	196
4	Ala153 and Val207	220L and 153W	197
5	Val207	203W	248
6	Ser190 and Val207	83W and 260W	192
7	Val296	58W and 298L	195
8	Val90	87F and 90L	194
9	Ser190	190F, 190L, 190W, 190H, 190M, or 190Y	

[0204] The indicated cavities are referred to by a small residue abutting the cavity that can be mutated to a larger residue to fill the cavity. It will be understood that other residues (besides the one the cavity is named after) could also be mutated to fill the same cavity.

[0205] Thus, in some embodiments, the PreF antigen includes a recombinant RSV F protein including one or more amino acid substitutions that reduce the volume of one or more of the cavities listed in column 2 of Table 6, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). In additional embodiments, the PreF antigen includes a recombinant RSV F protein including one or more of the amino acid substitutions listed in of row 1, 2, 3, 4, 5, 6, 7, 8, or 9 of column 3 of Table 6, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0206] The SEQ ID NOs listed in Table 6 set forth amino acid sequences including the indicated substitutions, as well as, a signal peptide, F_2 polypeptide (positions 26-109), a pep27 polypeptide (positions 110-136), a F_1 polypeptide (positions 137-513), a trimerization domain (a Foldon domain) and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))). Thus, in additional embodiments, the PreF antigen includes a recombinant RSV F protein including a F_1 polypeptide and a F_2 polypeptide as set forth in any one of the SEQ ID NOs listed in of row 1, 2, 3, 4, 5, 6,7 or 8 of column 4 of Table 6, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). For example, the PreF antigen can include a recombinant RSV F protein including a F_1 polypeptide and a F_2 polypeptide as set forth as positions 26-109 and 137-513, respectively, as set forth in any one of the SEQ ID NOs listed in of row 1, 2, 3, 4, 5, 6, 7, or 8 of column 4 of Table 6, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0207] In additional embodiments, the PreF antigen includes a recombinant RSV F protein including the amino acid substitutions listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83 or 84 of column 3 of Table 6b, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0208] The SEQ ID NOs listed in Table 6a set forth amino acid sequences including the indicated substitutions a signal peptide, F_2 polypeptide (positions 26-109), a pep27 polypeptide (positions 110-136), a F_1 polypeptide (positions 137-513), a trimerization domain (a Foldon domain) and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions

559-568 of SEQ ID NO: 185))). Thus, in additional embodiments, the PreF antigen includes a recombinant RSV F protein including a F_1 polypeptide and a F_2 polypeptide as set forth in any one of the SEQ ID NOs listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, or 84 of column 4 of Table 6b, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). For example, the PreF antigen can include a recombinant RSV F protein including a F_1 polypeptide and a F_2 polypeptide as set forth as positions 26-109 and 137-513, respectively, as set forth in any one of the SEQ ID NOs listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, or 84 of column 4 of Table 6b, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

Table 6b. Exemplarity cavity-filling amino acid substitution

	Description	Mutations	SEQ ID NO
1	Cavity filling	L230F	391
2	Cavity filling	L158F	392
3	Cavity filling	L230F, L158F	393
4	Cavity filling	L203F	395
5	Cavity filling	V187F	396
6	Cavity filling	Y198F	397
7	Cavity filling	Y198W	398
8	Cavity filling	L204F	399
9	Cavity filling	Y53F, L188F	400
10	Cavity filling	V187F, L203F	401
11	Cavity filling	Y198F, L203F	402
12	Cavity filling	L141W	403
13	Cavity filling	L142F	404
14	Cavity filling	L142W	405
15	Cavity filling	V144F	406
16	Cavity filling	V144W	407
17	Cavity filling	V90F	408
18	Cavity filling	L83F	409
19	Cavity filling	V185F, T54A	410
20	Cavity filling	I395F	411
21	Cavity filling	V90F, V185F, T54A	412
22	Cavity filling	L83F, V90F	413
23	Cavity filling	L83F, V185F, T54A	414
24	Cavity filling	L230F, V90F, I395F	415
25	Cavity filling	I395F, V185F, T54A	416
26	Cavity filling	L203F, V90F, L230F, L158F, S509F, I395F, V185F, T54A	417
27	Cavity filling	I221Y	419
28	cavity filling	F140W	439
29	cavity filling	F137W	440
30	cavity filling	S190L, V192L	441
31	cavity filling	V187F, S190L, V192L	442
32	cavity filling	V187L, S190L, V192L	443
33	cavity filling	V185F V187L S190L V192L	444
34	cavity filling	V154L, V157L, V185L, V187L	445
35	cavity filling	V154L, V185L, V187L	446
36	cavity filling	V187F	447
37	cavity filling	T58L A298L	448
38	cavity filling	T58L V154L V185L V187L A298L	449
39	cavity filling	Y458W	450
40	cavity filling	L158F, I167A	451
41	cavity filling	L158W, I167A	452
42	cavity filling	L158F	453
	cavity filling	L158W	454
43	cavity filling	V56L, I167L, A298L	455
44	cavity filling	V56L, I167L, A298M	456
45	cavity filling	V56L, A167L	457
46	cavity filling	I167F	458
47	cavity filling	I167M	459
48	cavity filling	V154F	460
49	cavity filling	V56L, I167L, A298L, V154F	461
50	cavity filling	I199L, L203F	462
51	cavity filling	I199L, L203F, P205Q, I206T	463

	Description	Mutations	SEQ ID NO
52	cavity filling	I199L, L203F, P205E, I206K	464
53	cavity filling	I199L, L203F, V207F	465
54	cavity filling	I199L, L203F, P205Q, I206T, V207F	466
55	cavity filling	I199L, L203F, P205E, I206K, V207F	467
56	cavity filling	I199L, L203F, L83F	468
57	cavity filling	I199L, L203F, P205Q, I206T, L83F	469
58	cavity filling	I199L, L203F, P205E, I206K, L83F	470
59	cavity filling	I199L, L203F, S190L, V192L	471
60	cavity filling	I199L, L203F, P205Q, I206T, V187F, S190L, V192L	472
61	cavity filling	S55A, S190M, L203F, V207I, V296I	473
62	cavity filling	Y53F, S55A, K176I, S190L, V207I, S259L, D263L, V296I	474
63	cavity filling	L158F, V207M, V296I	475
64	cavity filling	V56L, V207M, V296I	476
65	cavity filling	V56L, V207I, V296I	477
66	cavity filling	V56I, V207M, V296I	478
67	cavity filling	V154L, V207M, V296I	479
68	cavity filling	Y198F, V207I, T219W, V296I	480
69	cavity filling	Y198F, V207I, T219I, V296I	481
70	cavity filling	Y198F, V207M, T219W, V296I	482
71	cavity filling	Y198F, V207M, T219I, V296I	483
72	cavity filling	Y198F, V207M, T219L, V296I	484
73	Cavity filling	S190Y	432
74	Cavity filling	S190W	433
75	cavity filling	I206F, V207M, T219V, V296I	487
76	cavity filling	Y198F, V207M, T219L, K226M	488
77	cavity filling	Y198F, V207M, T219L, K226W	489
78	cavity filling	Y198F, V207M, T219L, K226L	490
79	cavity filling	L158F, L203F, V207I, V296I	497
80	cavity filling	F488W	498
81	Cavity filling	F488R	499
82	Cavity filling test 207L	V207L	500
83	Cavity filling test 207L	S190F	501
84	Cavity filling	S190M	502

iii. **Repacking Substitutions**

[0209] In some embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation by one or more repacking amino acid substitutions. Repacking substitutions increase attractive interactions (such as hydrophobic interactions or hydrogen-bond formation), or decrease repulsive interactions (such as repulsive forces between clusters of similarly charged residues), between amino acids in a protein.

[0210] The person of ordinary skill in the art can use methods provided herein to compare the structures of the pre-and post-fusion conformations of the RSV F protein to identify suitable sites of repulsive and/or attractive interactions between RSV F protein residues, and amino acid substitutions for reducing or increasing these interactions, respectively. For example, by identifying repulsive interactions in the structure of the RSV F protein in the prefusion conformation provided herein, and introducing substitutions that reduce these repulsive interactions. Alternatively, the RSV F protein can include substitutions that increase attractive interactions between RSV F protein residues in the prefusion conformation of the RSV F protein, but not the postfusion conformation of the RSV F protein. Exemplary amino acid substitutions are provided in Table 7.

Table 7. Repacking Amino Acid Substitutions

Row	Substitutions	SEQ ID NO
1	I64L, I79V, Y86W, L193V, L195F, Y198F, I199F, L203F, V207L, I214L	227
2	I64L, I79L, Y86W, L193V, L195F, Y198F, I199F, L203F, I214L	228
3	I64W, I79V, Y86W, L193V, L195F, Y198F, I199F, L203F, V207L, I214L	229
4	I79V, Y86F, L193V, L195F, Y198F, I199F, L203F, V207L, I214L	230
5	I64V, I79V, Y86W, L193V, L195F, Y198F, 1199Y, L203F, V207L, I214L	231
6	I64F, I79V, Y86W, L193V, L195F, Y198F, I199F, L203F, V207L, I214L	232
7	I64L, I79V, Y86W, L193V, L195F, I199F, L203F, V207L, I214L	233
8	V56I, T58I, V164I, L171I, V179L, L181F, V1871, 1291V, V296I, A298I	234
9	V56I, T58I, V164I, V179L, T189F, 1291V, V296I, A298I	235
10	V56L, T58I, L158W, V164L, I167V, L171I, V179L, L181F, V1871, 1291V, V296L	236
11	V56L, T58I, L158Y, V164L, I167V, V187I, T189F, 1291V, V296L	237
12	V56I, T58W, V164I, 1167F, L171I, V179L, L181V, V1871, 1291V, V296I	238
13	V56I, T58I, I64L, I79V, Y86W, V164I, V179L, T189F, L193V, L195F, Y198F, I199F, L203F, V207L, I214L, I291V, V296I, A298I	239

Row	Substitutions	SEQ ID NO
14	V56I, T58I, I79V, Y86F, V164I, V179L, T189F, L193V, L195F, Y198F, I199F, L203F, V207L, I214L, I291V, V296I, A298I	240
15	V56I, T58W, I64L, I79V, Y86W, V164I, I167F, L171I, V179L, L181V, V187I, L193V, L195F, Y198F, I199F, L203F, V207L, I214L, 1291V, V296I	241
16	V56I, T58W, I79V, Y86F, V164I, I167F, L171I, V179L, L181V, V187I, L193V, L195F, Y198F, I199F, L203F, V207L, I214L, 1291V, V296I	242
17	D486N, E487Q, D489N, and S491A	249
18	D486H, E487Q, and D489H	250
19	T400V, D486L, E487L, and D489L	251
20	T400V, D486I, E487L, and D489I,	252
21	T400V, S485I, D486L, E487L, D489L, Q494L, and K498L	253
23	T400V, S485I, D486I, E487L, D489I, Q494L, and K498L	254
24	K399I, T400V, S485I, D486L, E487L, D489L, Q494L, E497L, and K498L	255
25	K399I, T400V, S485I, D486I, E487L, D489I, Q494L, E497L, and K498L	256
26	L375W, Y391F, and K394M	286
27	L375W, Y391F, and K394W	287
28	L375W, Y391F, K394M, D486N, E487Q, D489N, and S491A	288
29	L375W, Y391F, K394M, D486H, E487Q, and D489H	289
30	L375W, Y391F, K394W, D486N, E487Q, D489N, and S491A	290
31	L375W, Y391F, K394W, D486H, E487Q, and D489H	291
32	L375W, Y391F, K394M, T400V, D486L, E487L, D489L, Q494L, and K498M	292
33	L375W, Y391F, K394M, T400V, D486I, E487L, D489I, Q494L, and K498M	293
34	L375W, Y391F, K394W, T400V, D486L, E487L, D489L, Q494L, and K498M	294
35	L375W, Y391F, K394W, T400V, D486I, E487L, D489I, Q494L, and K498M	295
36	F137W and R339M	326
37	F137W and F140W	327
38	F137W, F140W, and F488W	328
39	D486N, E487Q, D489N, S491A, and F488W	329
40	D486H, E487Q, D489H, and F488W	330
41	T400V, D486L, E487L, D489L, and F488W	331
42	T400V, D486I, E487L, D489I, and F488W	332
43	D486N, E487Q, D489N, S491A, F137W, and F140W	333
44	D486H, E487Q, D489H, F137W, and F140W	334
45	T400V, D486L, E487L, D489L, F137W, and F140W	335
46	L375W, Y391F, K394M, F137W, and F140W	336
47	L375W, Y391F, K394M, F137W, F140W, and R339M	337

[0211] Thus, in some embodiments, the PreF antigen includes a recombinant RSV F protein including the amino acid substitutions listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, or 47 of column 2 of Table 7, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0212] The SEQ ID NOs listed in Table 7 set forth amino acid sequences including the indicated substitutions, as well as, a signal peptide, F_2 polypeptide (positions 26-109), a pep27 polypeptide (positions 110-136), a F_1 polypeptide (positions 137-513), a trimerization domain (a Foldon domain) and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))). Thus, in additional embodiments, the PreF antigen includes a recombinant RSV F protein including a F_1 polypeptide and a F_2 polypeptide as set forth in one of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, or 47 of column 3 of Table 7, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). For example, the PreF antigen can include a recombinant RSV F protein including a F_1 polypeptide and a F_2 polypeptide as set forth as positions 26-109 and 137-513, respectively, as set forth in any one of the SEQ ID NOs listed in one of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, or 47 of column 3 of Table 7, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0213] Several embodiments include combinations of the amino acid substitutions listed above.

iv. N-Linked Glycosylation Sites

[0214] Comparison of the structure of the prefusion conformation of the RSV F protein (*e.g.*, in complex with D25 or AM22 as disclosed herein) to the structure of the postfusion RSV F protein (disclosed, *e.g.*, in as disclosed in McLellan et al., J. Virol., 85, 7788, 2011) identifies several regions of the RSV F protein that are solvent-accessible in the prefusion RSV F conformation described herein, but solvent-inaccessible in the postfusion RSV F conformation (as disclosed in McLellan et al., J. Virol., 85, 7788, 2011).

[0215] Thus, in some embodiments, the PreF antigen includes a recombinant RSV F protein including an amino acid substitution that introduces an N-linked glycosylation site at a position that is solvent-accessible in the prefusion RSV F conformation described herein, but solvent-inaccessible in the postfusion RSV F conformation (as disclosed in McLellan et al., J. Virol., 85, 7788, 2011). These amino acid substitutions stabilize the recombinant RSV F protein in the prefusion conformation by increasing the energy required for the protein to adopt the postfusion state.

[0216] To create an N-linked glycosylation site, the sequence Asn-X-Ser/Thr (where X is any amino acid except Pro) needs to be introduced. This can be accomplished by substitution of a Ser/Thr amino acid two residues C-terminal to a native Asn residue, or by substitution of an Asn amino acid two residues N-terminal to a native Ser/Thr residue, or by substitution of both an Asn and Ser/Thr residue separated by one non-proline amino acid. Thus, in several embodiments, any of the disclosed recombinant RSV F proteins are glycosylated. For example, the RSV F protein includes an amino acid substitution that introduces a N-linked glycosylation site in the RSV F protein that is solvent-accessible in the prefusion RSV F conformation disclosed herein but solvent-inaccessible in the postfusion conformation of RSV F as disclosed in McLellan et al., J. Virol., 85, 7788, 2011). Exemplary N-linked glycosylation site modifications are provided in Table 8.

Table 8. Exemplary N-linked glycosylation

Row	N-linked glycosylation site position	Exemplary substitutions	Exemplary SEQ ID NO
1	506	I506N and K508T	198
2	175	A177S	199
3	178	V178N	200
4	276	V278T	203
5	476	Y478T	204
6	185	V185N and V187T	214
7	160	L160N and G162S	215
8	503	L503N and a F505S	216
9	157	V157N	217

[0217] In some embodiments, a PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation by a N-linked glycosylation site at one or more of (such as 2, 3, 4, 5, 6, 7, 8, or 9 of) positions 506, 175, 178, 276, 476, 185, 160, 503, or 157 of the F_1 polypeptide, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). For example, the F_1 polypeptide can include an amino acid substitution that introduces an N-linked glycosylation site at one or more of (such as 2, 3, 4, 5, 6, 7, 8, or 9 of) positions 506, 175, 178, 276, 476, 185, 160, 503, or 157 of the F_1 polypeptide.

[0218] The SEQ ID NOs listed in Table 8 set forth amino acid sequences including the indicated substitutions, as well as, a signal peptide, F_2 polypeptide (positions 26-109), a pep27 polypeptide (positions 110-136), a F_1 polypeptide (positions 137-513), a trimerization domain (a Foldon domain) and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))). In some embodiments, the PreF antigen includes a F_1 polypeptide including I506N and K508T substitutions to introduce a N-linked glycosylation site at position 506. In some embodiments, the PreF antigen includes a F_1 polypeptide including an A177S substitution to introduce a N-linked glycosylation site at position 175. In some embodiments, the PreF antigen includes a F_1 polypeptide including a V178N substitution to introduce a N-linked glycosylation site at position 178. In some embodiments, the PreF antigen includes a F_1 polypeptide including a V278T substitution to introduce a N-linked glycosylation site at position 276. In some embodiments, the PreF antigen includes a F_1 polypeptide including a Y478T substitution to introduce a N-linked glycosylation site at position 476. In some embodiments, the PreF antigen includes a F_1 polypeptide including V185N and V187T substitutions to introduce a N-linked glycosylation site at position 185. In some embodiments, the PreF antigen includes a F_1 polypeptide including L160N and G162S substitutions to introduce a N-linked glycosylation site at position 160. In some embodiments, the PreF antigen includes a F_1 polypeptide including L503N and F505S substitutions to introduce a N-linked glycosylation site at position 503. In some embodiments, the PreF antigen includes a F_1 polypeptide including a V157N substitution to introduce a N-linked glycosylation site at position 157. In any of these embodiments, the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø)

In additional embodiments, the F_1 polypeptide comprises residues 137-513 of SEQ ID NO: 198 (N-linked glycosylation site at position 506); SEQ ID NO: 199 (N-linked glycosylation site at position 175); SEQ ID NO: 200 (N-linked glycosylation site at position 178); SEQ ID NO: 203 (N-linked glycosylation site at position 276); SEQ ID NO: 204 (N-linked glycosylation site at position 476); SEQ ID NO: 214 (N-linked glycosylation site at position 185); SEQ ID NO: 215 (N-linked glycosylation site at position 160); SEQ ID NO: 216 (N-linked glycosylation site at position 503); or SEQ ID NO: 217 (N-linked glycosylation site at position 157), wherein the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0219] Methods of making glycosylated polypeptides are disclosed herein and are familiar to the person of ordinary skill in the art. For example, such methods are described in U.S. Patent Application Pub. No. 2007/0224211, U.S. Pat. No. 7,029,872; 7,834,159, 7,807,405, Wang and Lomino, ACS Chem. Biol., 7:110-122, 2011, and Nettleship et al., Methods Mol. Biol, 498:245-263, 2009. In some embodiments, glycosylated PreF antigens are produced by expressing the recombinant RSV F protein in mammalian cells, such as HEK293 cells or derivatives thereof, such as GnTI$^{-/-}$ cells (ATCC® No. CRL-3022). In some embodiments, the RSV F protein antigens are produced by expression the RSV F protein antigens in mammalian cells, such as HEK293 cells or derivatives thereof, with swainsonine added to the media in order to inhibit certain aspects of the glycosylation machinery, for example to promote production of hybrid glycans.

[0220] In several embodiments, the F1 polypeptide includes two or more of the N-linked glycosylation sites listed in Table 8.

v. <u>Exemplary stabilizing modifications</u>

[0221] The person of skill in the art will appreciate that the PreF antigen can include a recombinant RSV F protein stabilized in a prefusion conformation by S155C, S290C, S190F, and V207L substitutions (compared to a native RSV F protein as set forth as one of SEQ ID NOs: 1-184) and combinations of one or more further stabilizing amino acid substitutions described herein, such as a combination of amino acid substitutions that introduce one or more disulfide bonds, fill cavities within the RSV F protein, alter the packing of residues in the RSV F protein, introduce N-linked glycosylation sites. For example, in several embodiments, recombinant RSV F protein includes amino acid substitutions that introduce a disulfide bond, and that fill cavities within the RSV F protein.

[0222] In some embodiments, a recombinant RSV F protein stabilized in a prefusion conformation includes a disulfide bond between a pair of cysteines at positions 155 and 290, and a cavity-filling amino acid substitution at position 190; or a disulfide bond between a pair of cysteines at positions 155 and 290, a cavity-filling amino acid substitution at position 190, and a cavity-filling amino acid substitution at position 207. For example, the cavity filling substitution at position 190 and/or position 207 can be a large aromatic or hydrophobic amino acid substitution (such as tyrosine, leucine, phenylalanine, histidine, or tryptophan).

[0223] In some embodiments, the F1 polypeptide of the recombinant RSV F protein includes S155C, S290C, and S190F amino acid substitutions. In some embodiments, the F1 polypeptide of the recombinant RSV F protein includes S155C, S290C, and S190W amino acid substitutions. In some embodiments, the F1 polypeptide of the recombinant RSV F protein includes S155C, S290C, and S190L amino acid substitutions

[0224] In some embodiments, the F1 polypeptide of the recombinant RSV F protein includes S155C, S290C, S190F, and V207L amino acid substitutions. In some embodiments, the F1 polypeptide of the recombinant RSV F protein includes S155C, S290C, S190W, and V207L amino acid substitutions. In some embodiments, the F1 polypeptide of the recombinant RSV F protein includes S155C, S290C, S190L, and V207L amino acid substitutions. In some embodiments, the F1 polypeptide of the recombinant RSV F protein includes S155C, S290C, S190F, and V207F amino acid substitutions. In some embodiments, the F1 polypeptide of the recombinant RSV F protein includes S155C, S290C, S190W, and V207F amino acid substitutions. In some embodiments, the F1 polypeptide of the recombinant RSV F protein includes S155C, S290C, S190L, and V207F amino acid substitutions. In some embodiments, the F1 polypeptide of the recombinant RSV F protein includes S155C, S290C, S190F, and V207W amino acid substitutions. In some embodiments, the F1 polypeptide of the recombinant RSV F protein includes S155C, S290C, S190W, and V207W amino acid substitutions. In some embodiments, the F1 polypeptide of the recombinant RSV F protein includes S155C, S290C, S190L, and V207W amino acid substitutions.

[0225] In several embodiments, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_1 polypeptide and a F_2 polypeptide from a human RSV A subtype, a human RSV B subtype, or a bovine RSV, wherein the F_1 polypeptide includes including one of the above combinations of stabilizing substitutions.

[0226] In some aspects of the disclosure not forming part of the invention, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, and S190F amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, and S190W amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, and S190L amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, and S190H amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, and S190M amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, and S190Y amino acid substitutions.

[0227] In some embodiments, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190F, and V207L amino acid substitutions. In some embodiments, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190W, and V207L amino acid substitutions. In some embodiments, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190L, and V207L amino acid substitutions. In some embodiments, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190H, and V207L amino acid substitutions. In some embodiments, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190M, and V207L amino acid substitutions. In some embodiments, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190Y, and V207L amino acid substitutions.

[0228] In some aspects of the disclosure not forming part of the invention, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190F, and V207F amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190W, and V207F amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190L, and V207F amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190H, and V207F amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190M, and V207F amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190Y, and V207F amino acid substitutions.

[0229] In some aspects of the disclosure not forming part of the invention, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190F, and V207W amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190W, and V207W amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190L, and V207W amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190H, and V207W amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190M, and V207W amino acid substitutions. In some aspects, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2

polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190Y, and V207W amino acid substitutions.

[0230] In some embodiments, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190F, V207L, and F488W amino acid substitutions. In some embodiments, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of any one of SEQ ID NOs: 1-184 or 370, and further includes S155C, S290C, S190F, and F488W amino acid substitutions.

[0231] In some embodiments, the recombinant RSV F protein stabilized in a prefusion conformation includes a F_2 polypeptide and a F_1 polypeptide including positions 26-109 and 137-513, respectively, of SEQ ID NO: 371 (RSV A with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 372 (RSV B with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 373 (bovine RSV with S155C, S290C, S190F and V207L substitutions). Other aspects of the disclosure not forming part of the invention include: SEQ ID NO: 374 (RSV A with S155C, S290C, and S190F substitutions), SEQ ID NO: 375 (RSV B with S155C, S290C, and S190F substitutions); or SEQ ID NO: 376 (bovine RSV with S155C, S290C, and S190F substitutions).

[0232] In some embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation that includes the amino acid substitutions listed in one of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, or 54 of column 3 of Table 8b. The amino acid substitutions stated in rows 25-40 do not form part of the claimed invention. The stabilized RSV F protein can be specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

Table 8b: Exemplary recombinant RSV F protein substitutions and sequences with and without a C-terminal thrombin-cleavable Foldon domain

	Description	Mutations	Without Foldon domain SEQ ID NO	With Thrombin-Cleavable Foldon domain SEQ ID NO
1	DSCav1 + Cavity filling + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + L503E/I506K	503	552
2	DSCav1 + Cavity filling + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + L503E/I506K/F505W	504	553
3	DSCav1 + Cavity filling + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + L503E/I506K/L230F/L158F	505	554
4	DSCav1 + Cavity filling + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + L503E/I506K/S509F/F505W/L230F/L158F	506	555
5	DSCav1 + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + L160K/V178T/L258K/V384T/I431S/L467Q /	507	556
6	DSCav1 + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + F477K/L481Q/V482K/L503Q/I506K	508	557
7	DSCav1 + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + L160K/V178T/L258K/V384T/I431S/L467Q /F477K/L481Q/V482K/L503Q/I506K	509	558
8	DSCav1 + ds	(S155C, S290, S190F, V207L) + (L512C/L513C)	510	559
9	DSCav1 + ds + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + (L512C/L513C) + L 160K/V 178T/L258K/V3 84T/I431 S/L467Q /	511	560
10	DSCav1 + ds + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + (L512C/L513C) + F477K/L481Q/V482K/L503Q/I506K	512	561
11	DSCav1 + ds + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + (L512C/L513C) + L 160K/V 178T/L258K/V3 84T/I431 S/L467Q /F477K/L481Q/V482K/L503Q/I506K	513	562
12	DSCav1 + cavity filling	(S155C, S290, S190F, V207L) + F505W	514	563
13	DSCav1 + cavity filling + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + F505W + L160K/V178T/L258K/V384T/I431S/L467Q /	515	564
14	DSCav1 + cavity filling + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + F505W + F477K/L481Q/V482K/L503Q/I506K	516	565
15	DSCav1 + cavity filling + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + F505W + L160K/V178T/L258K/V384T/I431S/L467Q /F477K/L481Q/V482K/L503Q/I506K	517	566
16	DSCav1 + ds + cavity filling	(S155C, S290, S190F, V207L) + L512C/L513C + F505W	518	567
17	DSCav1 + ds + cavity filling + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + L512C/L513C + F505W + L160K/V178T/L258K/V384T/I431S/L467Q /	519	568
18	DSCav1 + ds + cavity filling + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + L512C/L513C + F505W +	520	569
		F477K/L481Q/V482K/L503Q/I506K		
19	DSCav1 + ds + cavity filling + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + L512C/L513C + F505W + L160K/V178T/L258K/V384T/I431S/L467Q /F477K/L481Q/V482K/L503Q/I506K	521	570
20	DSCav1 + Cavity filling + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + I506K/S509F/L83F/V90F	522	571
21	DSCav1 + Cavity filling + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + I506K/S509F/L83F/V90F/L230F/L158F	523	572
22	DSCav1 + Cavity filling + replace exposed hydrophobic residues	(S155C, S290, S190F, V207L) + I506K/S509F/F505W/L83F/V90F/L230F/V 185F/T54A	524	573
23	DSCav1 + Cavity filling	(S155C, S290, S190F, V207L) + L83F/V90F/L230F/I395F	525	574
24	DSCav1 + Cavity filling + replace	(S155C, S290, S190F, V207L) +	526	575

	Description	Mutations	Without Foldon domain SEQ ID NO	With Thrombin-Cleavable Foldon domain SEQ ID NO
	exposed hydrophobic residues	I506K/S509F/F505W/L83F/V90F/L230F/L158F/I395F/V185F/T54A		
25	DS + S190F + Disulfide stabilization of C-term plus more mutations	S190F, S155C, S290C, F488W, L513C, A514E, I515C	527	576
26	DS + S190F + F488W + Disulfide stabilization of C-term plus more mutations	S190F, S155C, S290C, F488W, L513C, A514E, G515E, 516C	528	577
27	DS + S190F + F488W + Disulfide stabilization of C-term plus more mutations	S190F, S155C, S290C, F488W, L512C, L513E, A514C	529	578
28	DS + S190F + F488W + Disulfide stabilization of C-term plus more mutations	S190F, S155C, S290C, F488W, L512C, L513E, A514E, G515C	530	579
29	DS + S190F + F488W + Disulfide stabilization of C-term plus more mutations plus 2 extra intrachain disulfides	S190F, S155C, S290C, A424C, V450C, L171C, K191C, F488W, L513C, A514E, I515C	531	580
30	DS + S190F + F488W + Disulfide stabilization of C-term plus more mutations plus 2 extra intrachain disulfides	S190F, S155C, S290C, A424C, V450C, L171C, K191C, F488W, L513C, A514E, G515E, 516C	532	581
31	DS + S190F + F488W + Disulfide stabilization of C-term plus more mutations plus 2 extra intrachain disulfides	S190F, S155C, S290C, A424C, V450C, L171C, K191C, F488W, L512C, L513E, A514C	533	582
32	DS + S190F + F488W + Disulfide stabilization of C-term plus more mutations plus 2 extra intrachain disulfides	S190F, S155C, S290C, A424C, V450C, L171C, K191C, F488W, L512C, L513E, A514E, G515C	534	583
33	DS + S190F + F488W + Disulfide stabilization of C-term plus more mutations plus 2 extra intrachain disulfide and 1 extra interchain disulfide	K77C, I217C, S190F, S155C, S290C, A424C, V450C, L171C, K191C, F488W, L513C, L514E, A515C	535	584
34	DS + S190F + F488W + Disulfide stabilization of C-term plus more mutations plus 2 extra intrachain disulfide and 1 extra interchain disulfide	K77C, I217C, S190F, S155C, S290C, A424C, V450C, L171C, K191C, F488W, L513C, L514E, A515E, G516C	536	585
35	DS + S190F + F488W + Disulfide stabilization of C-term plus more mutations plus 2 extra intrachain disulfide and 1 extra interchain disulfide	K77C, I217C, S190F, S155C, S290C, A424C, V450C, L171C, K191C, F488W, L512C, L513E, A514C	537	586
36	DS + S190F + F488W + Disulfide stabilization of C-term plus more mutations plus 2 extra intrachain disulfide and 1 extra interchain disulfide	K77C, I217C, S190F, S155C, S290C, A424C, V450C, L171C, K191C, F488W, L512C, L513E, A514E, G515C	538	587
37	DS + C-term stabilization cysteine ring	(S155C, S290C) + L513C, 514E, 515C	539	588
38	DS + C-term stabilization cysteine ring	(S155C, S290C) + L513C, 514E, 515E, 516C	540	589
39	DS + C-term stabilization cysteine ring	(S155C, S290C) + L512C, 513E, 514C	541	590
40	DS + C-term stabilization cysteine ring	(S155C, S290C) + L512C, 513E, 514E, 515C	542	591
41	DSCav1 + 512/513ds + end at residue 513	(S155C, S290C, S190F, V207L) + (L512C/L513C)	543	592
42	DSCav1 + end at residue 492	(S155C, S290C, S190F, V207L) + 486DEF to CPC	544	593
43	DSCav1	(S155C, S290C, S190F, V207L)		601
44	DSCav1 with C-terminal cavity filling mutations	S155C, S290C, S190F, V207L + L512F	672	683
45	DSCav1 with C-terminal cavity filling mutations	S155C, S290C, S190F, V207L + L513F	673	684
46	DSCav1 with C-terminal cavity filling mutations	S155C, S290C, S190F, V207L + L512F, L513F	674	685
47	DSCav1 with C-terminal cavity filling mutations	S155C, S290C, S190F, V207L + L512Y, L513Y	675	686
48	DSCav1 with C-terminal cavity filling mutations	S155C, S290C, S190F, V207L + L512F, L513Y	676	687
49	DSCav1 with C-terminal cavity filling mutations	S155C, S290C, S190F, V207L + L512W, L513W	677	688
50	DSCav1 with C-terminal cavity filling mutations	S155C, S290C, S190F, V207L + L5132W, L513Y	678	689
51	DSCav1 with C-terminal cavity filling mutations	S155C, S290C, S190F, V207L + S509W	679	690
52	DSCav1 with C-terminal cavity filling mutations	S155C , S290C, S190F, V207L + S509F	680	691
53	DSCav1 with C-terminal cavity filling	S155C, S290C, S190F, V207L + S509W, L512F	681	692

	Description	Mutations	Without Foldon domain SEQ ID NO	With Thrombin-Cleavable Foldon domain SEQ ID NO
	mutations			
54	DSCav1 with C-terminal cavity filling mutations	S155C, S290C, S190F, V207L + S509W, L512F, L513F	682	693

[0233] The SEQ ID NOs listed in Table 8b set forth amino acid sequences including the indicated substitutions, a signal peptide, F_2 polypeptide (positions 26-109), a pep27 polypeptide (positions 110-136), a F_1 polypeptide (positions 137-513), and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))) or a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)), a trimerization domain (a Foldon domain), and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))). Thus, in some embodiments, the PreF antigen includes a recombinant RSV F protein including a F_1 polypeptide (e.g., approx. positions 137-513) and a F_2 polypeptide (e.g., approx. positions 26-109) as set forth in the SEQ ID NO of one of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 59, 50, 51, 52, 53, or 54 of column 4 (without Foldon domain) or column 5 (with cleavable Foldon domain) of Table 8b. SEQ ID NOs of rows 25-40 do not form part of the claimed invention.

[0234] In some aspects of the disclosure not forming part of the invention, the PreF antigen includes a recombinant RSV F protein stabilized in a prefusion conformation that includes the amino acid substitutions listed in one of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13, of column 3 of Table 8c. The stabilized RSV F protein can be specifically bound by a prefusion-specific antibody (*e.g.,* D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0235] The SEQ ID NOs listed in Table 8c set forth amino acid sequences including the indicated substitutions, a signal peptide, F_2 polypeptide (positions 26-109), a pep27 polypeptide (positions 110-136), a F_1 polypeptide (positions 137-513), a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)), and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))). Thus, in additional aspects, the PreF antigen includes a recombinant RSV F protein including a F_1 polypeptide (e.g., approx. positions 137-513) and a F_2 polypeptide (e.g., approx. positions 26-109) as set forth in the SEQ ID NO of one of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 of column 4 of Table 8c.

Table 8c: Exemplary recombinant RSV F protein substitutions and sequences

	Description	Substitutions	SEQ ID NO
1	Cavity filling + replace exposed hydrophobic residues	L503E/I506K/S509F	389
2	Cavity filling + replace exposed hydrophobic residues	L503E/I506K/S509F/F505W	390
3	Cavity filling + replace exposed hydrophobic residues	L503E/I506K/S509F/L230F/L158F	394
4	Interchain disulfide	Q279C, S238C	418
5	Cavity filling/hydrophobic patch	Q501F	420
6	cavity filling + replace hydrophilic	E82V/V207M/N227L/V296I	485
7	cavity filling + replace hydrophilic	E82V/V207I/N227L/V296I	486
8	cavity filling + prevent helix formation	L158F/Y198F/V207M/S215G/N216P/T219L	491
9	cavity filling + prevent helix formation	L158F/Y198F/V207M/S213G/S215G/T219L	492
10	cavity filling + replace hydrophilic	V56L/E82V/L203F/V207M/N227L/L230F/V296I	493
11	cavity filling + replace hydrophilic	E82V/L158F/L203F/V207M/N227L/L230F/V296I	494
12	cavity filling + replace hydrophilic	E82V/L203F/V207M/K226M/N227L/L230F/V296I	495
13	Disulfide + cavity filling	L203F/V207I/S180C/S186C/V296I	496

b. Membrane proximal stabilizing modifications

[0236] In several embodiments, the PreF antigen includes a membrane anchored form of the recombinant RSV F protein (e.g., with a transmembrane domain). In other embodiments, the PreF antigen includes a soluble form of the recombinant RSV F protein (e.g., without a transmembrane domain or other membrane anchor). It will be understood that there are several different approaches for generating a soluble or membrane anchored recombinant RSV F protein, including those discussed below. Examples include introduction of a trimerization domain, introduction of cysteine pairs that can form a disulfide bond that stabilizes the C-terminal region of F_1, and introduction of a transmembrane domain (*e.g.*, for applications including a membrane-anchored PreF antigen).

[0237] Further, as disclosed herein, the structure of the RSV F protein in complex with D25 Fab (*i.e.*, in a prefusion conformation) compared to the structure of the postfusion RSV F protein (disclosed, *e.g.*, in McLellan et al., J. Virol., 85, 7788, 2011, with coordinates deposited as PDB Accession No. 3RRR) show structural rearrangements between pre- and post-fusion conformations in both the membrane-proximal and membrane-distal lobes. Several embodiments include a modification targeted for stabilization of the membrane proximal lobe of the RSV F protein prefusion conformation. It will be understood that these modifications are not strictly necessary to stabilize a recombinant RSV F protein in a prefusion conformation, but that, in some instances, they are combined with other prefusion stabilizing modifications, such as those described above.

i. Trimerization Domain

[0238] In several embodiments, the PreF antigen is linked to a trimerization domain, for example the PreF antigen can include a recombinant RSV F protein including an F1 polypeptide with a trimerization domain linked to its C-terminus. In some embodiments, the trimerization domain promotes trimerization of the three F1/F2 monomers in the recombinant RSV F protein. Several exogenous multimerization domains promote stable trimers of soluble recombinant proteins: the GCN4 leucine zipper (Harbury et al. 1993 Science 262:1401-1407), the trimerization motif from the lung surfactant protein (Hoppe et al. 1994 FEBS Lett 344:191-195), collagen (McAlinden et al. 2003 J Bial Chem 278:42200-42207), and the phage T4 fibritin Foldon (Miroshnikov et al. 1998 Protein Eng 11:329-414), any of which can be linked to the F1 polypeptide in the PreF antigen to promote trimerization of the recombinant F protein, as long as the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.,* D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0239] In some examples, the PreF antigen can be linked to a GCN4 leucine zipper domain, for example the PreF antigen can include a recombinant RSV F protein including an F1 polypeptide with a GCN4 leucine zipper domain linked to its C-terminus. In specific examples, GCN4 leucine zipper domain is provided in the CSGJ series of constructs described herein.

[0240] In some examples, the PreF antigen can be linked to a Foldon domain, for example, the PreF antigen can include a recombinant RSV F protein including an F1 polypeptide with a Foldon domain linked to its C-terminus. In specific examples, the Foldon domain is a T4 fibritin Foldon domain such as the amino acid sequence GYIPEAPRDGQAYVRKDGEWVLLSTF (SEQ ID NO: 351), which adopts a β-propeller conformation, and can fold and trimerize in an autonomous way (Tao et al. 1997 Structure 5:789-798).

[0241] In some specific examples, the PreF antigen includes a recombinant RSV F protein linked to a T4 fibritin Foldon domain, includes a F_2 polypeptide and an F_1 polypeptide linked to a Foldon domain as set forth in one of SEQ ID NOs: 185, 189-303, or 371-376. Typically, the heterologous multimerization motif is positioned C-terminal to the F_1 domain. Optionally, the multimerization domain is connected to the F_1 polypeptide via a linker, such as an amino acid linker, such as the sequence GG. The linker can also be a longer linker (for example, including the sequence GG, such as the amino acid sequence: GGSGGSGGS; SEQ ID NO: 352). Numerous conformationally neutral linkers are known in the art that can be used in this context without disrupting the conformation of the PreF antigen. Some embodiments include a protease cleavage site for removing the Foldon domain from the F1 polypeptide, such as, but not limited to, a thrombin site between the F1 polypeptide and the Foldon domain.

[0242] In some embodiments, the PreF antigen includes a recombinant RSV F protein including any of the trimerization domain modifications listed above combined with any of the modifications listed in section II.B.1.a. For example, in some embodiments, the PreF antigen includes a recombinant RSV F protein including any of the trimerization domain modifications listed above in combination with one or more of the disulfide bond modification listed in one of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or 51 of Table 5, and/or one or more of the cavity filling modifications listed in one of rows 1, 2, 3, 4, 5, 6, 7, or 8 of Table 6, and/or one or more of the repacking modifications listed in one of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, or 47 of Table 7, and/or one or more of the glycosylation modifications listed in one or rows 1, 2, 3, 4, 5, 6, 7, 8, or 9 of Table 8, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0243] In some embodiments, the PreF antigen includes a recombinant RSV F protein including any of the trimerization domain modifications listed above linked to an F1 polypeptide including a disulfide bond between a pair of cysteines at positions 155 and 290, a cavity-filling amino acid substitution at position 190, and a cavity-filling amino acid substitution at position 207.

[0244] In some embodiments, the PreF antigen includes a recombinant RSV F protein including any of the trimerization domain modifications listed above linked to an F1 polypeptide including S155C, S290C, S190F, and V207L amino acid substitutions.

[0245] For example, in some aspects of the disclosure, the PreF antigen includes a recombinant RSV F protein stabilized in a RSV F protein prefusion conformation, wherein the F_2 polypeptide and the F_1 polypeptide linked to the foldon domain include the amino acid sequence set forth as positions 26-109 and 137-544, respectively, of any one of SEQ ID NO: 185, SEQ ID NO: 189, SEQ ID NO: 201, SEQ ID NO: 202, SEQ ID NO: 205, SEQ ID NO: 207, SEQ ID NO: 209, SEQ ID NO: 213, SEQ ID NO: 244, SEQ ID NO: 245, SEQ ID NO: 247, SEQ ID NO: 257, SEQ ID NO: 264, SEQ ID NO: 265, SEQ ID NO: 266, SEQ ID NO: 267, SEQ ID NO: 268, SEQ ID NO: 269, SEQ ID NO: 270, SEQ ID NO: 271, SEQ ID NO: 272, SEQ ID NO: 273, SEQ ID NO: 274, SEQ ID NO: 275, SEQ ID NO: 277, SEQ ID NO: 278, SEQ ID NO: 279, SEQ ID NO: 280, SEQ ID NO: 281, SEQ ID NO: 282, SEQ ID NO: 284, SEQ ID NO: 302, SEQ ID NO: 303, SEQ ID NO: 190, SEQ ID NO: 211, SEQ ID NO: 212, SEQ ID NO: 243, SEQ ID NO: 246, SEQ ID NO: 276, SEQ ID NO: 283, SEQ ID NO: 285, or SEQ ID NO: 263; or positions 26-109 and 137-545, respectively, of any one of SEQ ID NO: 258, SEQ ID NO: 259, SEQ ID NO: 260, SEQ ID NO: 261, SEQ ID NO: 262, SEQ ID NO: 296, SEQ ID NO: 297, SEQ ID NO: 298, or SEQ ID NO: 299, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.,* D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0246] In some embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a RSV F protein prefusion conformation, wherein the F_2 polypeptide and the F_1 polypeptide linked to the foldon domain include the amino acid sequence set forth as positions 26-109 and 137-544, respectively, of any one of SEQ ID NO: 371 (RSV A with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 372 (RSV B with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 373 (bovine RSV with S155C, S290C, S190F and V207L substitutions) wherein the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.,* D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). Other SEQ ID NOs not forming part of the invention include: SEQ ID NO: 374 (RSV A with S155C, S290C, and S190F substitutions), SEQ ID NO: 375 (RSV B with S155C, S290C, and S190F substitutions); or SEQ ID NO: 376 (bovine RSV with S155C, S290C, and S190F substitutions), wherein the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.,* D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0247] In some embodiments, the PreF antigen includes a recombinant RSV F protein including a F_1 polypeptide and a F_2 polypeptide from a human RSV A subtype, a human RSV B subtype, or a bovine RSV, wherein the F_1 polypeptide is linked to any of the trimerization domain modifications listed above, and the F1 polypeptide further includes the S155C, S290C, S190F, and V207L stabilizing modifications described herein.

[0248] In some embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a RSV F protein prefusion conformation, and includes one or more cavity-filling amino acid substitution and a Foldon domain, wherein the F_2 polypeptide and the F_1 polypeptide linked to the Foldon domain include the amino acid sequence set forth as positions 26-109 and 137-544, respectively, of any one of SEQ ID NO: 191, SEQ ID NO: 193 , SEQ ID NO: 196, SEQ ID NO: 197, SEQ ID NO: 248, SEQ ID NO: 192, SEQ ID NO: 195, or SEQ ID NO: 194; wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0249] In some embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a RSV F protein prefusion conformation, and includes one or more repacking amino acid substitutions and a foldon domain, wherein the F_2 polypeptide and the F_1 polypeptide linked to the foldon domain include the amino acid sequence set forth as positions 26-109 and 137-544, respectively, of any one of SEQ ID NO: 249, SEQ ID NO: 250, SEQ ID NO: 251, SEQ ID NO: 252, SEQ ID NO: 253, SEQ ID NO: 254, SEQ ID NO: 255, SEQ ID NO: 256, SEQ ID NO: 288, SEQ ID NO: 289, SEQ ID NO: 290, SEQ ID NO: 291, SEQ ID NO: 292, SEQ ID NO: 293, SEQ ID NO: 294, SEQ ID NO: 295, SEQ ID NO: 296, SEQ ID NO: 297, SEQ ID NO: 326, SEQ ID NO: 327, SEQ ID NO: 328, SEQ ID NO: 329, SEQ ID NO: 330, SEQ ID NO: 331, SEQ ID NO: 332, SEQ ID NO: 333, SEQ ID NO: 334, SEQ ID NO: 335, SEQ ID NO: 336, or SEQ ID NO: 337; wherein the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.,* D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0250] In some embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a RSV F protein prefusion conformation, and includes one or more N-linked glycosylation sites and a Foldon domain, wherein the F_2 polypeptide and the F_1 polypeptide linked to the Foldon domain include the amino acid sequence set forth as positions 26-109 and 137-544, respectively, of any one of SEQ ID NOs selected from the group consisting of SEQ ID NO: 198, SEQ ID NO: 199, SEQ ID NO: 200, SEQ ID NO: 203, SEQ ID NO: 204, SEQ ID NO: 214, SEQ ID NO: 215, SEQ ID NO: 216, or SEQ ID NO: 217; wherein the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0251] In some embodiments, the PreF antigen includes a recombinant RSV F protein including the amino acid substitutions listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of column 3 of Table 5b, wherein the F1 polypeptide of the recombinant RSV F protein is linked to a Foldon domain. Some embodiments include a protease cleavage site for removing the Foldon domain from the F1 polypeptide, for example a thrombin cleavage site.

[0252] In some embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a RSV F protein prefusion conformation, including a F_2 polypeptide and a F_1 polypeptide linked to a Foldon domain, wherein the F_2 polypeptide and the F_1 polypeptide linked to the Foldon domain include the amino acid sequence set forth as positions 26-109 and 137-544, respectively, of one of the SEQ ID NOs listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of column 4 of Table 5b,. In several embodiments, the F_1 polypeptide linked to the Foldon domain further includes a protease cleavage site, such as, but not limited to, a thrombin site, between the F1 polypeptide and the Foldon domain.

[0253] In some embodiments, the PreF antigen includes a recombinant RSV F protein including the amino acid substitutions listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, or 84 of column 3 of Table 6b, wherein the F1 polypeptide of the recombinant RSV F protein is linked to a Foldon domain.

[0254] In some embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a RSV F protein prefusion conformation, including a F_2 polypeptide and a F_1 polypeptide linked to a Foldon domain, wherein the F_2 polypeptide and the F_1 polypeptide linked to the Foldon domain include the amino acid sequence set forth as positions 26-109 and 137-544, respectively, of one of the SEQ ID NOs listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, or 84 of column 4 of Table 6b.

[0255] In further embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a RSV F protein prefusion conformation, wherein the recombinant RSV F protein includes the amino acid substitutions listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 59, 50, 51, 52, 53, or 54 of column 3 of Table 8b,wherein the F1 polypeptide of the recombinant RSV F protein is linked to a Foldon domain. The amino acid subsitutions listed in rows 25-40 do not form part of the invention. In some embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a RSV F protein prefusion conformation, including a F_2 polypeptide and a F_1 polypeptide linked to a Foldon domain, wherein the F_2 polypeptide and the F_1 polypeptide linked to the Foldon domain include the amino acid sequence set forth as positions 26-109 and 137-544, respectively, of one of the SEQ ID NOs listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, or 54 of column 5 of Table 8b. The SEQ ID NOs listed in rows 25-40 do not form part of the invention. These sequences include a thrombin cleavage site between the F1 polypeptide and the Foldon domain.

[0256] In further embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a RSV F protein prefusion conformation, wherein the recombinant RSV F protein includes the amino acid substitutions listed in row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 of column 3 of Table 8c, wherein the F1 polypeptide of the recombinant RSV F protein is linked to a Foldon domain. In some embodiments, the PreF antigen includes a recombinant RSV F protein stabilized in a RSV F protein prefusion conformation, including a F_2 polypeptide and a F_1 polypeptide linked to a Foldon domain, wherein the F_2 polypeptide and the F_1 polypeptide linked to the Foldon domain include the amino acid sequence set forth as positions 26-109 and 137-544, respectively, of the SEQ ID NO listed in row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 of column 4 of Table 8c.

[0257] Modified Foldon domains can also be used, such as a Foldon domain including an amino acid sequence set forth as GYIPEAPRDGQCYVRCDGEWVLLSTF (SEQ ID NO: 694), GYIPECPRDGQAYVCKDGEWVLLSTF (SEQ ID NO: 695), GYIPEAPRDGQCYCRKDGEWVLLSTF (SEQ ID NO: 696), or GYIPEAPRDGQACVRKDGECVLLSTF (SEQ ID NO: 697). These modified Foldon domains include amino acid substitutions that add two cysteine residues for formation of stabilizing disulfide bonds. Exemplary RSV F protein sequences including the DSCav1 amino acid substitutions linked to the modified Foldon domains include those set forth as SEQ ID NO: 651, SEQ ID NO: 652, SEQ ID NO: 653, and SEQ ID NO: 654. In some embodiments, any of the disclosed recombinant RSV F proteins can be linked to a modified Foldon domain as described herein.

ii. Disulfide Bonds

[0258] In some embodiments, the PreF antigen includes a recombinant RSV F protein including a F1 polypeptide including one or more disulfide bonds that are used to stabilize the membrane proximal lobe of the recombinant RSV F protein. The cysteine residues that form the disulfide bond can be introduced into the recombinant RSV F protein by the relevant S155C, S290C, S190F, and V207L amino acid substitutions.

[0259] The location of the cysteine (or cysteines) of a disulfide bond to stabilize the membrane proximal lobe of the RSV F protein in a prefusion conformation can readily be determined by the person of ordinary skill in the art using methods described herein and familiar to the skilled artisan. In some embodiments, a ring of disulfide bonds is introduced into the C-terminus of the F1 polypeptide by substituting cysteine residues for amino acids of the α10 helix. The three α10 helixes of the RSV F Ectodomain for a coil-coil that stabilized the membrane proximal portion of the protein. When expressed in cells, inter-protomer disulfide bonds form between the cysteines introduced into the α10 helix, thereby "locking" the three α10 helix's in close proximity and preventing movement of the membrane proximal domain from the pre-to the post-fusion conformation. The α10 helix of the RSV F protein includes residues 492 to the transmembrane domain (residue 529).

[0260] In some embodiments, the PreF antigen includes a recombinant RSV F protein including a disulfide bond between cysteine residues located at RSV F positions 486 and 487, or between cysteine residues located at RSV F positions 512 and 513, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.,* D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). In some such embodiments, the F_1 polypeptide includes D486C and E487C substitutions, L512C and L513C substitutions, or D486C, E487C, L512C, and L513C substitutions respectively.

[0261] In some embodiments, amino acids can be inserted (or deleted) from the F protein sequence to adjust the alignment of residues in the F protein structure, such that particular residue pairs are within a sufficiently close distance to form an disulfide bond. In some such embodiments, the PreF antigen includes a recombinant RSV F protein including a disulfide bond between cysteine residues located at 486 and 487; with a proline insertion between positions 486 and 487, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.,* D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). In some such embodiments, the F_1 polypeptide includes D486C and E487C substitutions, and a proline insertion between positions 486 and 487.

[0262] In additional embodiments, the PreF antigen includes a recombinant RSV F protein including a disulfide bond between a cysteine residue located at position 493 and a cysteine residue inserted between positions 329 and 330, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). In some such embodiments, the F_1 polypeptide includes S493C substitution, and a cysteine residue inserted between positions 329 and 330.

[0263] In additional embodiments, the PreF antigen includes a recombinant RSV F protein including a disulfide bond between a cysteine residue located at position 493 and a cysteine residue inserted between positions 329 and 330, and further includes a glycine insertion between residues 492 and 493, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). In some such embodiments, the F_1 polypeptide includes S493C substitution, a cysteine residue inserted between positions 329 and 330, and a glycine insertion between residues 492 and 493

[0264] In additional embodiments, the recombinant RSV F protein includes cysteine substitutions in the α10 helix at positions 525 and 526, 512 and 513, and/or 519 and 520, which can form interprotomer disulfide bonds to stabilize the C-terminal region of the F1 polypeptide. For example, in some embodiments, the recombinant RSV F protein includes any of the "motifs" listed in Table 23. In additional embodiments, the recombinant RSV F protein includes an amino acid sequence at least 80% (such as at least 90%, at least 95% or at least 98% identical) to the amino acid sequence set forth as any one of SEQ ID NOs: 829-1025 or1456-1468, optionally without including the purification tags or trimerization domains included in these sequences.

[0265] In some embodiments, the recombinant RSV F protein includes, extending C-terminal from position 512, the amino acid sequence set forth as one of CCHNVNAGKSTTN (residues 512-524 of SEQ ID NO: 844) or CCHNVNACCSTTN (residues X-Y of SEQ ID NO: 849); or CCHNVNACCSTTNICCTT (residues 512-529 of SEQ ID NO: 853).

[0266] In some embodiments, the PreF antigen includes a recombinant RSV F protein including any of the above disulfide bond modifications for stabilizing the membrane proximal lobe of the RSV F protein, combined with any of the stabilization modifications listed in section II.B.1.a. In some embodiments, the PreF antigen includes a recombinant RSV F protein including any of the disulfide bond modifications for stabilizing the membrane proximal lobe of the RSV F protein listed above in combination with the disulfide bond substitutions listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or 51 of Table 5, or row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 of Table 5b, or the cavity filling substitutions listed in one of row 1, 2, 3, 4, 5, 6, 7, or 8 of Table 6, or one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83 or 84 of column 3 of Table 6b, or the repacking substitutions listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, or 47 of Table 7, or the glycosylation modifications listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, or 9 of Table 8, or the substitutions listed in row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 59, 50, 51, 52, 53, or 54 of column 3 of Table 8b, or the substitutions listed in row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 of column 3 of Table 8c, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0267] In some embodiments, the PreF antigen includes a recombinant RSV F protein including any of the disulfide bond modifications for stabilizing the membrane proximal lobe of the RSV F protein listed above and further includes a F1 polypeptide including a disulfide bond between a pair of cysteines at positions 155 and 290, and a cavity-filling amino acid substitution at position 190; or a disulfide bond between a pair of cysteines at positions 155 and 290, a cavity-filling amino acid substitution at position 190, and a cavity-filling amino acid substitution at position 207.

[0268] In some embodiments, the PreF antigen includes a recombinant RSV F protein including any of the disulfide bond modifications for stabilizing the membrane proximal lobe of the RSV F protein listed above and further includes a F1 polypeptide including S155C, S290C, and S190F amino acid substitutions, S155C, S290C, and S190W amino acid substitutions, or S155C, S290C, and S190L amino acid substitutions. In further embodiments, the PreF antigen includes a recombinant RSV F protein including any of the disulfide bond modifications for stabilizing the membrane proximal lobe of the RSV F protein listed above and further includes a F1 polypeptide including S155C, S290C, S190F, and V207L amino acid substitutions, S155C, S290C, S190W, and V207L amino acid substitutions, S155C, S290C, S190L, and V207L amino acid substitutions, S155C, S290C, S190F, and V207F amino acid substitutions, S155C, S290C, S190W, and V207F amino acid substitutions, S155C, S290C, S190L, and V207F amino acid substitutions, S155C, S290C, S190F, and V207W amino acid substitutions, S155C, S290C, S190W, and V207W amino acid substitutions, or S155C, S290C, S190L, and V207W amino acid substitutions.

[0269] In some embodiments, the PreF antigen includes a recombinant RSV F protein including a F_2 polypeptide and a F_1 polypeptide including the amino acid sequence set forth as positions 26-109 and 137-513, respectively, of any one of SEQ ID NO: 371 (RSV A with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 372 (RSV B with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 373 (bovine RSV with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 374 (RSV A with S155C, S290C, and S190F substitutions), SEQ ID NO: 375 (RSVB with S155C, S290C, and S190F substitutions); or SEQ ID NO: 376 (bovine RSV with S155C, S290C, and S190F substitutions), wherein the recombinant RSV F protein further includes any of the disulfide bond modifications for stabilizing the membrane proximal lobe of the RSV F protein listed above, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). SEQ ID NOs: 374-376 not forming part of the claimed invention.

[0270] In several embodiments, the PreF antigen includes a recombinant RSV F protein including a F_1 polypeptide and a F_2 polypeptide from a human RSV A subtype, a human RSV B subtype, or a bovine RSV, wherein the recombinant RSV F protein further includes any of the disulfide bond modifications for stabilizing the membrane proximal lobe of the RSV F protein listed above, and wherein the F1 polypeptide further includes the stabilizing modifications S155C, S290C, S190F, and V207L.

iii. <u>Transmembrane Domains</u>

[0271] In some embodiments, the recombinant RSV F protein includes a transmembrane domain linked to the F_1 polypeptide, for example, for an application including a membrane anchored PreF antigen). For example, the presence of the transmembrane sequences is useful for expression as a transmembrane protein for membrane vesicle preparation. The transmembrane domain can be linked to a F_1 protein containing the stabilizing mutations S155C, S290C, S190F, and V207L provided herein, for example, those described above, such as a F_1 protein with S155C, S290C, S190F, and V207L substitutions. Additionally, the transmembrane domain can be further linked to a RSV F_1 cytosolic tail. Examples including a signal peptide, F_2 polypeptide (positions 26-109), pep27 polypeptide (positions 110-136), F_1 polypeptide (positions 137-513), a RSV transmembrane domain are provided as SEQ ID NO: 323 (without a cytosolic domain) and SEQ ID NO: 324 (with a cytosolic domain).

[0272] In some embodiments, the PreF antigen includes a recombinant RSV F protein including an F1 polypeptide linked to a transmembrane domain, combined with any of the stabilization modifications listed in section II.B.1a. For example, in some embodiments, the PreF antigen includes a recombinant RSV F protein including an F1 polypeptide linked to a transmembrane domain, and further includes the disulfide bond substitutions listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or 51 of Table 5, or row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 of Table 5b, or the cavity filling substitutions listed in one of row 1, 2, 3, 4, 5, 6, 7, or 8 of Table 6, or one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83 or 84 of column 3 of Table 6b, or the repacking substitutions listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, or 47 of Table 7, or the glycosylation modifications listed in one of row 1, 2, 3, 4, 5, 6, 7, 8, or 9 of Table 8, or the substitutions listed in row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 59, 50, 51, 52, 53, or 54 of column 3 of Table 8b, or the substitutions listed in row 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 of column 3 of Table 8c, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0273] In some embodiments, the PreF antigen includes a recombinant RSV F protein including an F1 polypeptide linked to a transmembrane domain, wherein the F1 polypeptide includes a disulfide bond between a pair of cysteines at positions 155 and 290, and a cavity-filling amino acid substitution at position 190; and a cavity-filling amino acid substitution at position 207.

[0274] In some embodiments, the PreF antigen includes a recombinant RSV F protein including an F1 polypeptide linked to a transmembrane domain, wherein the F1 polypeptide includes S155C, S290C, S190F and V207L amino acid substitutions, further amino acid substitutions can include: S155C, S290C, and S190W amino acid substitutions, or S155C, S290C, and S190L amino acid substitutions. In further embodiments, the PreF antigen includes a recombinant RSV F protein including an F1 polypeptide linked to a transmembrane domain, wherein the F1 polypeptide further includes S155C, S290C, S190F, and V207L amino acid substitutions, further amino acid substitutions can include: S155C, S290C, S190W, and V207L amino acid substitutions, S155C, S290C, S190L, and V207L amino acid substitutions, S155C, S290C, S190F, and V207F amino acid substitutions, S155C, S290C, S190W, and V207F amino acid substitutions, S155C, S290C, S190L, and V207F amino acid substitutions, S155C, S290C, S190F, and V207W amino acid substitutions, S155C, S290C, S190W, and V207W amino acid substitutions, or S155C, S290C, S190L, and V207W amino acid substitutions.

[0275] In some embodiments, the PreF antigen includes a recombinant RSV F protein including a F_2 polypeptide and a F1 polypeptide linked to a transmembrane domain, wherein the F_2 polypeptide and the F_1 polypeptide linked to the transmembrane domain include the amino acid sequence set forth as positions 26-109 and 137-513, respectively, of any one of SEQ ID NO: 371 (RSV A with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 372 (RSV B with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 373 (bovine RSV with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 374 (RSV A with S155C, S290C, and S190F substitutions), SEQ ID NO: 375 (RSV B with S155C, S290C, and S190F substitutions); or SEQ ID NO: 376 (bovine RSV with S155C, S290C, and S190F substitutions), wherein the PreF antigen is specifically bound by a prefusion-specific antibody *(e.g.,* D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). SEQ ID NOs 374-376 not forming part of the claimed invention.

[0276] In several embodiments, the PreF antigen includes a recombinant RSV F protein including a F_1 polypeptide and a F_2 polypeptide from a human RSV A subtype, a human RSV B subtype, or a bovine RSV, wherein the F_1 polypeptide is linked to any of the transmembrane domains listed above, and the F1 polypeptide includes the stabilizing modifications S155C, S290C, S190F, and V207L.

iv. cavity filling substitutions

[0277] In some embodiments, the PreF antigen includes a recombinant RSV F protein including a F1 polypeptide including one or more cavity filling substitutions that are used to stabilize the membrane proximal lobe of the recombinant RSV F protein. In some embodiments, the PreF antigen includes a recombinant RSV F protein including a F1 polypeptide with V207L and L512F; L513F; L512F and L513F; L512Y and L513Y; L512F and L513Y; L512W and L513W; L5132W and L513Y; S509W; S509F; S509W and L512F; or S509W, L512F and L513F substitutions, wherein the PreF antigen is specifically bound by a prefusion-specific antibody (e.g., D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). Exemplary sequences with such substitutions include SEQ ID NOs: 672-682.

c. Antigenic sites

[0278] In some embodiments, the PreF antigen includes a recombinant RSV F protein that is stabilized in a prefusion conformation and includes further modification to eliminate a known antigenic site other than antigenic site Ø. For example, the recombinant RSV F protein can include a modification that disrupts antigenic site I, II or IV. Such modifications can be identified, for example, by binding of antibodies specific for these sites.

[0279] In some embodiments, the antigens are provided that include a recombinant RSV F protein that includes modification to eliminate antigenic site Ø. Such antigens are useful, for example, as control reagents.

[0280] Exemplary modifications for removing antigenic site Ø and/or antigenic site II are listed in Table 8c1.

Table 8c1: Exemplary recombinant RSV F protein substitutions and sequences

	Description	Substitutions	SEQ ID NO
1	knock out site Ø binding	K65N/N67T, P205N/V207T, K209N/S211T +Avi-tag	655
2	knock out site II binding	Q270T +Avi-tag	656
3	knock out site II binding	N268R/K272E +Avitag	657
4	knock out site Ø binding	K65N/N67T, P205N/V207T, K209N/S211T +Avi-tag	658
5	knock out site II binding	Q270T +Avi-tag	659
6	knock out site II binding	N268R/K272E +Avitag	660
7	knock out site Ø and II binding	K65N/N67T, P205N/V207T, K209N/S211T, Q270T+Avi-tag	661
8	knock out site Ø and II binding	K65N/N67T, P205N/V207T, K209N/S211T,N268R/K272E+Avi-tag	662
9	knock out site Ø and II binding	K65N/N67T, P205N/V207T, K209N/S211T, Q270T+Avi-tag	663

	Description	Substitutions	SEQ ID NO
10	knock out site Ø and II binding	K65N/N67T, P205N/V207T, K209N/S211T,N268R/K272E+Avi-tag	664

d. Single Chain RSV F proteins

[0281] In some embodiments, the recombinant RSV F protein is a single chain RSV F protein, which includes a single polypeptide chain including the RSV F_1 polypeptide and the RSV F_2 polypeptide. The disclosed single chain RSV F proteins do not include the furin cleavage sites flanking the pep27 polypeptide of RSV F protein; therefore, when produced in cells, the F polypeptide is not cleaved into separate F1 and F2 polypeptides. In several embodiments, the remaining portions of the F_1 and F_2 polypeptides are joined by a linker, such as a peptide linker.

[0282] In several embodiments, a single polypeptide chain including the F_2, pep27, and F_1 sequences is produced. The single chain RSV F proteins can include the pep27 sequence, or this sequence can be deleted. Further, in examples wherein the pep27 sequence is deleted, a linker (such as a peptide linker) optionally can be placed between the F_2 and F_1 polypeptides in the recombinant single chain RSV F protein. In some embodiments, a single chain RSV F protein includes deletion of RSV F positions 98-149 or 106-149 which removes the two furin cleavage sites, the pep27 polypeptide, and the fusion peptide. In some embodiments, a single chain RSV F protein includes deletion of RSV F positions 98-136, 98-144, 98-149, 106-136, 104-144, or 106-144.

[0283] In several embodiments, the stabilizing mutations disclosed herein (for example, in sections (B. 1.a) through (B.1.c) above are included in the single chain RSV F protein. For example, in some embodiments, the single chain RSV F protein includes S155C, S290C, S190F, and V207L substitutions. In some embodiments, the PreF antigen includes a recombinant RSV F protein in single chain format stabilized in a prefusion conformation that further includes the amino acid substitutions listed in one of rows 1, 2, 3, 4, 5, 6, or 7 of column 3 of Table 8d. The stabilized RSV F protein can be specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0284] Exemplary sequences are listed in Table 8d. The SEQ ID NOs listed in Table 8d set forth amino acid sequences including the indicated substitutions, a signal peptide, F_2 polypeptide (positions 26-109), a pep27 polypeptide (positions 110-136), a F_1 polypeptide (positions 137-513), and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))) or a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)), a trimerization domain (a Foldon domain), and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))). Thus, in additional embodiments, the PreF antigen includes a recombinant RSV F protein including a F_1 polypeptide (e.g., approx. positions 137-513) and a F_2 polypeptide (e.g., approx. positions 26-109) as set forth in the SEQ ID NO listed in of one of rows 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 of column 4 (without Foldon domain) or column 5 (with cleavable Foldon domain) of Table 8d. Additional exemplary single chain RSV F protein mutations and sequences are described herein, for example as disclosed in Table 8e (e.g., rows 34-43) and Table 18.

Table 8d. Single chain recombinant RSV F proteins

	Description	Mutations	Without Foldon domain SEQ ID NO	With Thrombin-Cleavable Foldon domain SEQ ID NO
1	Single chain DS	S155C, S290C	545	594
2	Single chain Cav1	S190F, V207L	546	595
3	Single chain F488W	F4 8 8W	547	596
4	Single chain DSCav1	(S155C, S290, S190F, V207L)	548	597
5	Single chain DS + F488W	(S155C, S290C) + F488W	549	598
6	Single chain Cav1 + F488W	(S190F, V207L) + F488W	550	599
7	Single chain DSCavl + F488W	(S155C, S290, S190F, V207L) + F488W	551	600
8	add cav1 to SEQ ID NO: 320 single chain	add cav1 to SEQ ID NO: 320 single chain	665	
9	add cav1, F488W to SEQ ID NO: 320] single chain	add cav1, F488W to SEQ ID NO: 320] single chain	666	
10	add cav1 to SEQ ID NO: 319 single chain	add cav1 to SEQ ID NO: 319 single chain	667	
11	add cav1 F488W to SEQ ID NO: 319 single chain	add cav1 F488W to SEQ ID NO: 319 single chain	668	
12	single chain with improved linker	155C, S290C, S190F, V207L, GS linker between 105/145		669 (not a cleavable Foldon)
13	single chain with improved linker	155C, S290C, GS linker between residue 105 to 145	670	
14	single chain with improved linker	155C, S290C, GS linker between residue 105 to 145	671	

Sequences of additional single chain RSV f proteins that are stabilized in a prefusion confirmation are provided in Table 19, including single chain RSV F proteins with non-cleavable Foldon domains, cleavable Foldon domains, and linked to protein nanoparticle subunits.

2. Minimal Site Ø Immunogens

[0285] The site Ø epitope of RSV F is located on the apex of the trimer spike and includes the region recognized by the three neutralizing antibodies D25, AM22 and 5C4. More specifically, as delineated by the crystal structure of the RSV F/D25 complex, this epitope comprises the outer surface of helix a4 (residues 196-209) and the adjacent loop (residues 63-68) between β2 and α1. Provided herein are immunogens that include these minimal aspects of the RSV F protein and which are useful, for example, for inducing an immune response to RSV, and also for specific binding to RSV F protein antibodies, for example as probes to identify or detect such antibodies.

[0286] Accordingly, in some embodiments, the recombinant RSV F protein includes the minimal region necessary to stimulate an immune response to RSV. In some embodiments, the RSV F protein includes or consists of an amino acid sequence at least 80% identical to a sequence set forth in Table 20. In additional embodiments, the recombinant RSV F protein comprises circular permutation of antigenic site Ø as set forth in Table 20, such as as set forth in SEQ ID NOs: 1027-1052.

[0287] The minimal epitope region can be linked to a scaffold protein to stabilize the epitope in an antigenic conformation. For example, any of the minimal site Ø antigen listed herein can be linked to a 2KNO, 2A90, 2W59, 3U2E, 2VJ1, 1CHD, 1PQZ, or a 2MOE scaffold protein. These are the reference identifiers for specific sequences located in the PDB database, as present in the data base on March 11, 2014. Sepcific examples of minoal site Ø antigen ilinked to a scaffold protein are provided herein in Table 20.

[0288] Any of the minimal site Ø antigen can be linked to a protein nanoparticle subunit, for example a ferritin subunit or a lumazine synthase subunit, to generate a protein nanoparticle. Specifc examples of minimal site Ø antigens linked to a protein nanoparticle subunit are provided herein in the Table 21.

[0289] In several embodiments, the PreF antigen includes an epitope-scaffold protein including a RSV F protein prefusion specific epitope in a prefusion specific conformation. In some examples, the epitope scaffold protein includes any of the recombinant RSV F proteins stabilized in a prefusion conformation as disclosed herein. The prefusion specific epitope can be placed anywhere in the scaffold protein (for example, on the N-terminus, C-terminus, or an internal loop), as long as the PreF antigen including the epitope scaffold protein is specifically bound by a prefusion-specific antibody (*e.g.,* D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

[0290] Methods for identifying and selecting scaffolds are disclosed herein and known to the person of ordinary skill in the art. For example, methods for superposition, grafting and de novo design of epitope-scaffolds are disclosed in U.S. Patent Application Publication No. 2010/0068217.

[0291] "Superposition" epitope-scaffolds are based on scaffold proteins having an exposed segment with similar conformation as the target epitope--the backbone atoms in this "superposition-region" can be structurally superposed onto the target epitope with minimal root mean square deviation (RMSD) of their coordinates. Suitable scaffolds are identified by computationally searching through a library of protein crystal structures; epitope-scaffolds are designed by putting the epitope residues in the superposition region and making additional mutations on the surrounding surface of the scaffold to prevent clash or other interactions with the antibody.

[0292] "Grafting" epitope-scaffolds utilize scaffold proteins that can accommodate replacement of an exposed segment with the crystallized conformation of the target epitope. For each suitable scaffold identified by computationally searching through all protein crystal structures, an exposed segment is replaced by the target epitope and the surrounding sidechains are redesigned (mutated) to accommodate and stabilize the inserted epitope. Finally, as with superposition epitope-scaffolds, mutations are made on the surface of the scaffold and outside the epitope, to prevent clash or other interactions with the antibody. Grafting scaffolds require that the replaced segment and inserted epitope have similar translation and rotation transformations between their N- and C-termini, and that the surrounding peptide backbone does not clash with the inserted epitope. One difference between grafting and superposition is that grafting attempts to mimic the epitope conformation exactly, whereas superposition allows for small structural deviations.

[0293] "De novo" epitope-scaffolds are computationally designed from scratch to optimally present the crystallized conformation of the epitope. This method is based on computational design of a novel fold (Kuhlman, B. et al. 2003 Science 302:1364-1368). The de novo allows design of immunogens that are both minimal in size, so they do not present unwanted epitopes, and also highly stable against thermal or chemical denaturation.

[0294] The scaffold can be a heterologous scaffold. In several embodiments, the native scaffold protein (without epitope insertion) is not a viral envelope protein. In additional embodiments, the scaffold protein is not a RSV protein. In still further embodiments, the scaffold protein is not a viral protein.

[0295] In additional embodiments, the epitope-scaffold protein includes the amino acid sequence set forth as any one of SEQ ID NOs: 341-343, or a polypeptide with at least 80% sequence identity (such as at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity) to any one of SEQ ID NOs: 341-343, and wherein the epitope-scaffold protein is specifically bound by a prefusion-specific antibody (*e.g.,* D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). In additional embodiments, the RSV F protein is any one of SEQ ID NOs: 341-343, wherein the amino acid sequence of the RSV F protein has up to 20 amino acid substitutions, and wherein the epitope scaffold protein is specifically bound by a prefusion-specific antibody (*e.g.,* D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø), in the absence of binding by the corresponding prefusion-specific antibody (*e.g.,* D25 or AM22 antibody). Alternatively, the polypeptide can have none, or up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19 amino acid substitutions.

[0296] The recombinant RSV F protein stabilized in a prefusion conformation can be placed anywhere in the scaffold, as long as the resulting epitope-scaffold protein is specifically bound by a prefusion-specific antibody (*e.g.,* D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø), in the absence of binding by the corresponding prefusion-specific antibody (*e.g.,* D25 or AM22 antibody). Methods for determining if a particular epitope-scaffold protein is specifically bound by a prefusion-specific antibody (*e.g.,* D25 or AM22 antibody) are disclosed herein and known to the person of ordinary skill in the art (see, for example, International Application Pub. Nos. WO 2006/091455 and WO 2005/111621). In addition, the formation of an antibody-antigen complex can be assayed using a number of well-defined diagnostic assays including conventional immunoassay formats to detect and/or quantitate antigen-specific antibodies. Such assays include, for example, enzyme immunoassays, *e.g.*, ELISA, cell-based assays, flow cytometry, radioimmunoassays, and immunohistochemical staining. Numerous competitive and non-competitive protein binding assays are known in the art and many are commercially available. Methods for determining if a particular epitope-scaffold protein includes a RSV F prefusion specific conformation (such as antigenic site Ø), in the absence of binding by the corresponding prefusion-specific antibody (*e.g.,* D25 or AM22 antibody) are also described herein and further known to the person of ordinary skill in the art.

3. <u>Virus-Like Particles</u>

[0297] In some embodiments, a virus-like particle (VLP) is provided that includes a disclosed recombinant RSV F protein stabilized in a prefusion conformation. VLPs lack the viral components that are required for virus replication and thus represent a highly attenuated form of a virus. The VLP can display a polypeptide (*e.g.,* a recombinant RSV F protein stabilized in a prefusion conformation) that is capable of eliciting an immune response to RSV when administered to a subject. Virus like particles and methods of their production are known and familiar to the person of ordinary skill in the art, and viral proteins from several viruses are known to form VLPs, including human papillomavirus, HIV (Kang et al., Biol. Chem. 380: 353-64 (1999)), Semliki-Forest virus (Notka et al., Biol. Chem. 380: 341-52 (1999)), human polyomavirus (Goldmann et al., J. Virol. 73: 4465-9 (1999)), rotavirus (Jiang et al., Vaccine 17: 1005-13 (1999)), parvovirus (Casal, Biotechnology and Applied Biochemistry, Vol 29, Part 2, pp 141-150 (1999)), canine parvovirus (Hurtado et al., J. Virol. 70: 5422-9 (1996)), hepatitis E virus (Li et al., J. Virol. 71: 7207-13 (1997)), and Newcastle disease virus. For example, a chimeric VLP containing a RSV antigen and can be a Newcastle disease virus-based VLP. Newcastle disease based VLPs have previously been shown to elicit a neutralizing immune response to RSV in mice. The formation of such VLPs can be detected by any suitable technique. Examples of suitable techniques known in the art for detection of VLPs in a medium include, *e.g.*, electron microscopy techniques, dynamic light scattering (DLS), selective chromatographic separation (e.g., ion exchange, hydrophobic interaction, and/or size exclusion chromatographic separation of the VLPs) and density gradient centrifugation.

[0298] In some embodiments, the virus like particle includes a recombinant RSV F protein including an F2 polypeptide and a F1 polypeptide (such as an F1 polypeptide linked to a transmembrane domain), wherein the F1 polypeptide includes a disulfide bond between a pair of cysteines at positions 155 and 290, and a cavity-filling amino acid substitution at position 190; and a cavity-filling amino acid substitution at position 207.

[0299] In some embodiments, the virus like particle includes a recombinant RSV F protein including an F2 polypeptide and a F1 polypeptide (such as an F1 polypeptide linked to a transmembrane domain), wherein the F1 polypeptide includes S155C, S290C, S190F, and V207L amino acid substitutions and may further include: S155C, S290C, S190W, and V207L amino acid substitutions, S155C, S290C, S190L, and V207L amino acid substitutions, S155C, S290C, S190F, and V207F amino acid substitutions, S155C, S290C, S190W, and V207F amino acid substitutions, S155C, S290C, S190L, and V207F amino acid substitutions, S155C, S290C, S190F, and V207W amino acid substitutions, S155C, S290C, S190W, and V207W amino acid substitutions, or S155C, S290C, S190L, and V207W amino acid substitutions.

[0300] In some embodiments, the virus like particle includes a recombinant RSV F protein including an F2 polypeptide and a F1 polypeptide (such as an F1 polypeptide linked to a transmembrane domain), wherein the F_2 polypeptide and the F_1 polypeptide include the amino acid sequence set forth as positions 26-109 and 137-513, respectively, of any one of SEQ ID NO: 371 (RSV A with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 372 (RSV B with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 373 (bovine RSV with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 374 (RSV A with S155C, S290C, and S190F substitutions), SEQ ID NO: 375 (RSV B with S155C, S290C, and S190F substitutions); or SEQ ID NO: 376 (bovine RSV with S155C, S290C, and S190F substitutions). SEQ ID NOs: 374-376 not forming part of the claimed invention.

[0301] In several embodiments, the virus like particle includes a recombinant RSV F protein including a F_1 polypeptide (such as an F1 polypeptide linked to a transmembrane domain) and a F_2 polypeptide from a human RSV A subtype, a human RSV B subtype, or a bovine RSV, wherein the F1 polypeptide includes the stabilizing modifications S155C, S290C, S190F, and V207L.

4. Protein Nanoparticles

[0302] In some embodiments a protein nanoparticle is provided that includes one or more of any of the disclosed recombinant RSV F protein stabilized in a prefusion conformation, wherein the protein nanoparticle is specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). Non-limiting example of nanoparticles include ferritin nanoparticles, an encapsulin nanoparticles and Sulfur Oxygenase Reductase (SOR) nanoparticles, which are comprised of an assembly of monomeric subunits including ferritin proteins, encapsulin proteins and SOR proteins, respectively. To construct protein nanoparticles including the disclosed recombinant RSV F protein stabilized in a prefusion conformation, the antigen is linked to a subunit of the protein nanoparticle (such as a ferritin protein, an encapsulin protein or a SOR protein). The fusion protein self-assembles into a nanoparticle under appropriate conditions.

[0303] Ferritin nanoparticles and their use for immunization purposes (e.g., for immunization against influenza antigens) has been disclosed in the art (see, e.g., Kanekiyo et al., Nature, 499:102-106, 2013).

[0304] In some embodiments, any of the disclosed recombinant RSV F proteins stabilized in a prefusion conformation are linked to a ferritin polypeptide or hybrid of different ferritin polypeptides to construct a ferritin protein nanoparticle, wherein the ferritin nanoparticle is specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). Ferritin is a globular protein that is found in all animals, bacteria, and plants, and which acts primarily to control the rate and location of polynuclear $Fe(III)_2O_3$ formation through the transportation of hydrated iron ions and protons to and from a mineralized core. The globular form of ferritin is made up of monomeric subunits, which are polypeptides having a molecule weight of approximately 17-20 kDa. An example of the sequence of one such monomeric subunit is represented by SEQ ID NO: 353. Each monomeric subunit has the topology of a helix bundle which includes a four antiparallel helix motif, with a fifth shorter helix (the c-terminal helix) lying roughly perpendicular to the long axis of the 4 helix bundle. According to convention, the helices are labeled 'A, B, C, D & E' from the N-terminus respectively. The N-terminal sequence lies adjacent to the capsid three-fold axis and extends to the surface, while the E helices pack together at the four-fold axis with the C-terminus extending into the capsid core. The consequence of this packing creates two pores on the capsid surface. It is expected that one or both of these pores represent the point by which the hydrated iron diffuses into and out of the capsid. Following production, these monomeric subunit proteins self-assemble into the globular ferritin protein. Thus, the globular form of ferritin comprises 24 monomeric, subunit proteins, and has a capsid-like structure having 432 symmetry. Methods of constructing ferritin nanoparticles are known to the person of ordinary skill in the art and are further described herein (see, *e.g.*, Zhang, Int. J. Mol. Sci., 12:5406-5421, 2011).

[0305] In specific examples, the ferritin polypeptide is *E. coli* ferritin, *Helicobacter pylori* ferritin, human light chain ferritin, bullfrog ferritin or a hybrid thereof, such as *E. coli*-human hybrid ferritin, *E. coli*-bullfrog hybrid ferritin, or human-bullfrog hybrid ferritin. Exemplary amino acid sequences of ferritin polypeptides and nucleic acid sequences encoding ferritin polypeptides for use in the disclosed RSV F protein antigens stabilized in a prefusion conformation can be found in GENBANK®, for example at accession numbers ZP_03085328, ZP_06990637, EJB64322.1, AAA35832, NP_000137 AAA49532, AAA49525, AAA49524 and AAA49523, as available February 28, 2013. In one embodiment, any of the disclosed recombinant RSV F proteins stabilized in a prefusion conformation is linked to a ferritin protein including an amino acid sequence at least 80% (such as at least 85%, at least 90%, at least 95%, or at least 97%) identical to amino acid sequence set forth as SEQ ID NO: 353. A specific example of the disclosed recombinant RSV F proteins stabilized in a prefusion conformation linked to a ferritin protein include the amino acid sequence set forth as SEQ ID NO: 350.

[0306] In some embodiments, the ferritin polypeptide is a *Helicobacter pylori* ferritin (such as a ferritin polypeptide set forth as SEQ ID NO: 353) and includes a substitution of the cysteine residue at position 31, such as a C31S, C31A or C31V substitution. Any of the disclosed recombinant RSV F proteins (e.g., a RSV F polypeptide with S155C, S290C, and S190F substitutions, or with S155C, S290C, S190F and V207L substitutions) can be linked to a *Helicobacter pylori* ferritin (such as a ferritin polypeptide set forth as SEQ ID NO: 353) that further includes a substitution of the cysteine residue at position 31 of the ferritin polypeptide, such as a C31S, C31A or C31V substitution,.

[0307] In some embodiments, the ferritin protein nanoparticle includes a recombinant RSV F protein including an F2 polypeptide and a F1 polypeptide, wherein the F1 polypeptide is linked to the ferritin protein, and wherein the F1 polypeptide includes a disulfide bond between a pair of cysteines at positions 155 and 290, and a cavity-filling amino acid substitution at position 190 and a cavity-filling amino acid substitution at position 207.

[0308] In some embodiments, the ferritin protein nanoparticle includes a recombinant RSV F protein including an F2 polypeptide and a F1 polypeptide, wherein the F1 polypeptide is linked to the ferritin protein, and wherein the F1 polypeptide includes S155C, S290C, S190F, and V207L amino acid substitutions and may further include: S155C, S290C, S190W, and V207L amino acid substitutions, S155C, S290C, S190L, and V207L amino acid substitutions, S155C, S290C, S190F, and V207F amino acid substitutions, S155C, S290C, S190W, and V207F amino acid substitutions, S155C, S290C, S190L, and V207F amino acid substitutions, S155C, S290C, S190F, and V207W amino acid substitutions, S155C, S290C, S190W, and V207W amino acid substitutions, or S155C, S290C, S190L, and V207W amino acid substitutions.

[0309] The RSV F protein included on the ferritin nanoparticle can be a human subtype A, human subtype B or bovine RSV F protein include the substitutions disclosed herein for prefusion stabilization.

[0310] In some embodiments, the ferritin protein nanoparticle includes a recombinant RSV F protein including an F2 polypeptide and a F1 polypeptide, wherein the F1 polypeptide is linked to the ferritin protein, and wherein the F_2 polypeptide and the F_1 polypeptide include the amino acid sequence set forth as positions 26-109 and 137-513, respectively, of any one of SEQ ID NO: 371 (RSV A with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 372 (RSV B with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 373 (bovine RSV with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 374 (RSV A with S155C, S290C, and S190F substitutions), SEQ ID NO: 375 (RSV B with S155C, S290C, and S190F substitutions); or SEQ ID NO: 376 (bovine RSV with S155C, S290C, and S190F substitutions). SEQ ID NOs: 374-376 not forming part of the claimed invention.

[0311] In several embodiments, the ferritin protein nanoparticle includes a recombinant RSV F protein including a F_1 polypeptide and a F_2 polypeptide from a human RSV A subtype, a human RSV B subtype, or a bovine RSV, wherein the F1 polypeptide includes any of the stabilizing modifications S155C, S290C, S190F, and V207L.

[0312] In some embodiments the ferritin nanoparticle includes a recombinant RSV F protein including an F2 polypeptide and a F1 polypeptide, wherein the F1 polypeptide is linked to the ferritin protein, and wherein the F_2 polypeptide and the F_1 polypeptide linked to the ferritin protein include the amino acid sequence set forth as positions 26-109 and 137-679, respectively of SEQ ID NO: 377 (RSV A including S155C, S290C, S190F, V207L amino acid substitutions, with C-terminal ferritin domain), or SEQ ID NOs: 378-382.

[0313] In some embodiments the ferritin nanoparticle includes a recombinant RSV F protein including an F2 polypeptide and a F1 polypeptide, wherein the F1 polypeptide is linked to ferritin, and wherein the F_2 polypeptide and the F_1 polypeptide linked to ferritin include the amino acid substitutions S155C, S290C, S190F, V207L and any additional substitutions listed in row 1, 2, 3, 4, 5, 6, 7,8 , 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, or 49 of column 3 of Table 8e. In some embodiments the ferritin nanoparticle includes a recombinant RSV F protein including an F2 polypeptide and a F1 polypeptide, wherein the F1 polypeptide is linked to the ferritin protein, and wherein the F_2 polypeptide and the F_1 polypeptide linked to the ferritin protein include the amino acid sequence of the F1 and F2 polypeptide set forth in the SEQ ID NO listed in row 1, 2, 3, 4, 5, 6, 7,8 , 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, or 49 of column 4 of Table 8e. It will be appreciated that SEQ ID NOs. 602-617 and 620-634 and 645-650 listed in Table 8e include signal sequence and pep27 polypeptide sequences, which are removed by proteolytic processing when the corresponding F protein is made in eukaryotic cells, as well as C-terminal protein tags.

Table 8e: Exemplary RSV F protein mutations and sequences for production of ferritin nanoparticles

row	Description	Substitutions/modifications	SEQ ID NO
1	Cavity filling	(S155C, S290C, S190F, V207L) + L230F	602
2	Cavity filling	(S155C, S290C, S190F, V207L) + L158F	603
3	Cavity filling	(S155C, S290C, S190F, V207L) + L230F/L158F	604
4	DSCav1 + replace exposed hydrophobic residues	(S155C, S290C, S190F, V207L) + L160K/V178T/L258K/V384T/I431S/L467Q/	605
5	DSCav1+ replace exposed hydrophobic residues	(S155C, S290C, S190F, V207L) + F477K/L481Q/V482K/L503Q/I506K	606
6	DSCav1 + replace exposed hydrophobic residues	(S155C, S290C, S190F, V207L) + L160K/V178T/L258K/V384T/I431S/L467Q/F477K/L481Q/V482K/L503Q/I506K	607
7	Cavity filling + replace exposed hydrophobic residues	(S155C, S290C, S190F, V207L) + L158F/L230F/L83F/V90F/I506K/I395F/V185F/T54A	608
8	Cavity filling + replace exposed hydrophobic residues	(S155C, S290C, S190F, V207L) + L83F/V90F/I506K	609
9	DS-S190F with C-terminal	S190F, S155C, S290C, F488W, L513C,	610
	Cys ring1	A514E, I515C	
10	DS-S190F with C-terminal Cys ring2	S190F, S155C, S290C, F488W, L513C, A514E, G515E, 516C	611
11	DS-S190F with C-terminal Cys ring3	S190F, S155C, S290C, F488W, L512C, L513E, A514C	612
12	DS-S190F with C-terminal Cys ring4	S190F, S155C, S290C, F488W, L512C, L513E, A514E, G515C	613
13	DS-S190F with Foldon	S190F, S155C, S290C, F488W, Foldon	614
14	DS-S190F with 1 extra disulfide bridge with Foldon	S190F, S155C, S290C, L171C, K191C, F488W, Foldon	615
15	DS-S190F with 2 extra disulfide bridges with Foldon	S190F, S155C, S290C, A424C, V450C, L171C, K191C, F488W, Foldon	616
16	DS-S190F with 3 extra disulfide bridges with Foldon	K77C, I217C, S190F, S155C, S290C, A424C, V450C, L171C, K191C, F488W, Foldon	617
17	Single chain and shorten F protein to end at residue 513	Single chain F with (S155C, S290C, S190F, V207L)	618
18	Single chain and shorten F protein to end at residue 492	Single chain F with(S155C, S290C, S190F, V207L)	619
19	Disulfide	ferritin: S29C/C31S/V68C	620
20	Disulfide	ferritin: C31S/A115C/H128C	621
21	Disulfide + cavity filling	L158F/L203F/V296I; Ferritin: S29C/C31S/V68C/A115C/H128C	622
22	Disulfide + cavity filling	Y198F/T219L/K226M; ferritin: C31S/A115C/H128C	623
23	Disulfide + cavity filling	E82V/K226M/N227L/V296I; ferritin: C31S/A115C/H128C	624
24	Improved purification of DSCav1 Ferritin particles	(S155C, S290C, S190F, V207L) + and DYKDDDDKGG insertion at N-terminus of F	625
25	Improved purification of DSCav1 Ferritin particles	(S155C, S290C, S190F, V207L) + and QHHHHHHGG insertion at N-terminus F	626

row	Description	Substitutions/modifications	SEQ ID NO
26	Improved purification of DSCav1 Ferritin particles	(S155C, S290C, S190F, V207L) + and QHHHHHHHHGG insertion at N-terminus F	627
27	Improved purification of DSCav1 Ferritin particles	(S155C, S290C, S190F, V207L) + and GGHHHHHHGG insertion at residue 327 of F	628
28	Improved purification of DSCav1 Ferritin particles	(S155C, S290C, S190F, V207L) + and GGHHHHHHHHGG insertion at residue 327 of F	629
29	Improved purification of DSCav1 Ferritin particles	(S155C, S290C, S190F, V207L) + and HHHHH insertion at residue 323 of F	630
30	Improved purification of DSCav1 Ferritin particles	(S155C, S290C, S190F, V207L) + and QSAWSHPQFEKHHHHHHGGLVPRGSGG insertion at N-terminus of F	631
31	Improved purification of DSCav1 Ferritin particles	(S155C, S290C, S190F, V207L) + and QSAWSHPQFEKHHHHHHGGLVPRGSGG insertion at N-terminus of F	632
32	Longer linker between RSV F DSCav1 and Ferritin	(S155C, S290C, S190F, V207L) + 10 aa linker to Ferritin	633
33	Longer linker between RSV F DSCav1 and Ferritin	(S155C, S290C, S190F, V207L) + N500Q + 10 aa linker to Ferritin	634
34	single chain end at residue 513, longer linker	DS-Cav1 single chain with longer linker	635
35	single chain end at residue 492 longer linker	DS-Cav1 single chain with longer linker	636
36	single chain end at residue 513, N500Q remove glycan	DS-Cav1 single chain with N500Q	637
37	single chain end at residue 513, longer linker N500Q remove glycan	DS-Cav1 single chain with longer linker N500Q	638
38	single chain RSV F DS-Cav1 and Ferritin	S155C, S290C, S190F, V207L single chain N105-G145 linkGS	639
39	single chain RSV F DS-Cav1 and Ferritin end at 492	S155C, S290C, S190F, V207L, N500Q single chain end at 492 N105-G145 linkGS	640
40	single chain RSV F DS-Cav1 and Ferritin longer linker	S155C, S290C, S190F, V207L single chain N105-G145 + 10 aa linker to Ferritin	641
41	single chain RSV F DS-Cav1 and Ferritin end at 492 longer linker	S155C, S290C, S190F, V207L, N500Q single chain end at 492 N105-G145 + 10 aa linker to Ferritin	642
42	single chain RSV F DS-Cav1 and Ferritin and remove N500 glycan	S155C, S290C, S190F, V207L, N500Q single chain N105-G145 linkGS	643
43	single chain RSV F DS-Cav1 and Ferritin longer linker and remove N500 glycan	S155C, S290C, S190F, V207L, N500Q single chain N105-G145 + 10 aa linker to Ferritin	644
44	DS-cav1 + exposed hydrophobic + 10 aa linker	DS-cav1 + L160K/V178T/L258K/V384T/I431S/L467Q/ + 10 aa linker	645
45	DS-cav1 + exposed hydrophobic + 10 aa linker	DS-cav1 + F477K/L481Q/V482K/L503Q/I506K + 10 aa linker	646
46	DS-cav1 + exposed hydrophobic + 10 aa linker	DS-cav1 + L160K/V178T/L258K/V384T/I431S/L467Q/F47 7K/L481Q/V482K/L503Q/I506K + 10 aa linker	647
47	DS-cav1 + exposed hydrophobic + 10 aa linker +N500 glycan removal	DS-cav1 + L160K/V178T/L258K/V384T/I431S/L467Q/ + 10 aa linker +N500Q	648
48	DS-cav1 + exposed hydrophobic + 10 aa linker +N500 glycan removal	DS-cav1 + F477K/L481Q/V482K/L503Q/I506K + 10 aa linker +N500Q	649
49	DS-cav1 + exposed hydrophobic + 10 aa linker +N500 glycan removal	DS-cav1 + L160K/V178T/L258K/V384T/I431S/L467Q/F47 7K/L481Q/V482K/L503Q/I506K + 10 aa linker +N500Q	650

[0314] In additional embodiments, any of the disclosed RSV F protein antigens stabilized in a prefusion conformation are linked to an encapsulin polypeptide to construct an encapsulin nanoparticle, wherein the encapsulin nanoparticle is specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). Encapsulin proteins are a conserved family of bacterial proteins also known as linocin-like proteins that form large protein assemblies that function as a minimal compartment to package enzymes. The encapsulin assembly is made up of monomeric subunits, which are polypeptides having a molecule weight of approximately 30 kDa. An example of the sequence of one such monomeric subunit is provided as SEQ ID NO: 354. Following production, the monomeric subunits self-assemble into the globular encapsulin assembly including 60 monomeric subunits. Methods of constructing encapsulin nanoparticles are known to the person of ordinary skill in the art, and further described herein (see, for example, Sutter et al., Nature Struct. and Mol. Biol., 15:939-947, 2008). In specific examples, the encapsulin polypeptide is bacterial encapsulin, such as *E. coli* or *Thermotoga maritime* encapsulin. An exemplary encapsulin sequence for use with the disclosed RSV F protein antigens stabilized in a prefusion conformation is set forth as SEQ ID NO: 354.

[0315] In additional embodiments, any of the disclosed recombinant RSV F proteins stabilized in a prefusion conformation are linked to a Sulfer Oxygenase Reductase (SOR) polypeptide to construct a SOR nanoparticle, wherein the SOR nanoparticle is specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).. SOR proteins are microbial proteins (for example from the thermoacidophilic archaeon *Acidianus ambivalens* that form 24 subunit protein assemblies. Methods of constructing SOR nanoparticles are known to the person of ordinary skill in the art (see, *e.g.*, Urich et al., Science, 311:996-1000, 2006). Specific examples of the disclosed recombinant RSV F proteins stabilized in a prefusion conformation linked to a SOR protein include the amino acid sequences set forth as SEQ ID NO: 344 and SEQ ID NO: 345.

[0316] In additional embodiments, any of the disclosed recombinant RSV F proteins stabilized in a prefusion conformation are linked to a Lumazine synthase polypeptide to construct a Lumazine synthase nanoparticle, wherein the Lumazine synthase nanoparticle is specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). Specific examples of the disclosed recombinant RSV F proteins stabilized in a prefusion conformation linked to a Lumazine synthase protein include the amino acid sequences set forth as SEQ ID NOs: 346-348.

[0317] In additional embodiments, any of the disclosed recombinant RSV F proteins stabilized in a prefusion conformation are linked to a pyruvate dehydrogenase polypeptide to construct a pyruvate dehydrogenase nanoparticle, wherein the pyruvate dehydrogenase nanoparticle is specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø). A specific example of the disclosed recombinant RSV F proteins stabilized in a prefusion conformation linked to a pyruvate dehydrogenase protein include the amino acid sequence set forth as SEQ ID NO: 349.

[0318] In some examples, any of the disclosed recombinant RSV F proteins stabilized in a prefusion conformation is linked to the N- or C-terminus of a ferritin, encapsulin, SOR, lumazine synthase or pyruvate dehydrogenase protein, for example with a linker, such as a Ser-Gly linker. When the constructs have been made in HEK 293 Freestyle cells, the fusion proteins are secreted from the cells and self-assembled into nanoparticles. The nanoparticles can be purified using known techniques, for example by a few different chromatography procedures, *e.g.* Mono Q (anion exchange) followed by size exclusion (SUPEROSE® 6) chromatography.

[0319] Several embodiments include a monomeric subunit of a ferritin, encapsulin, SOR, lumazine synthase or pyruvate dehydrogenase protein, or any portion thereof which is capable of directing self-assembly of monomeric subunits into the globular form of the protein. Amino acid sequences from monomeric subunits of any known ferritin, encapsulin, SOR, lumazine synthase or pyruvate dehydrogenase protein can be used to produce fusion proteins with the disclosed recombinant RSV F proteins stabilized in a prefusion conformation, so long as the monomeric subunit is capable of self-assembling into a nanoparticle displaying the recombinant RSV F proteins stabilized in a prefusion conformation on its surface.

[0320] The fusion proteins need not comprise the full-length sequence of a monomeric subunit polypeptide of a ferritin, encapsulin, SOR, lumazine synthase or pyruvate dehydrogenase protein. Portions, or regions, of the monomeric subunit polypeptide can be utilized so long as the portion comprises amino acid sequences that direct self-assembly of monomeric subunits into the globular form of the protein.

[0321] In some embodiments, it may be useful to engineer mutations into the amino acid sequence of the monomeric ferritin, encapsulin, SOR, lumazine synthase or pyruvate dehydrogenase subunits. For example, it may be useful to alter sites such as enzyme recognition sites or glycosylation sites in order to give the fusion protein beneficial properties (*e.g.*, half-life).

[0322] It will be understood by those skilled in the art that fusion of any of the disclosed recombinant RSV F proteins stabilized in a prefusion conformation to the ferritin, encapsulin, SOR, lumazine synthase or pyruvate dehydrogenase protein should be done such that the disclosed recombinant RSV F proteins stabilized in a prefusion conformation portion of the fusion protein does not interfere with self-assembly of the monomeric ferritin, encapsulin, SOR, lumazine synthase or pyruvate dehydrogenase subunits into the globular protein, and that the ferritin, encapsulin, SOR, lumazine synthase or pyruvate dehydrogenase protein portion of the fusion protein does not interfere with the ability of the disclosed recombinant RSV F protein antigen stabilized in a prefusion conformation to elicit an immune response to RSV. In some embodiments, the ferritin, encapsulin, SOR, lumazine synthase or pyruvate dehydrogenase protein and disclosed recombinant RSV F protein stabilized in a prefusion conformation can be joined together directly without affecting the activity of either portion. In other embodiments, the ferritin, encapsulin, SOR, lumazine synthase or pyruvate dehydrogenase protein and the recombinant RSV F protein stabilized in a prefusion conformation are joined using a linker (also referred to as a spacer) sequence. The linker sequence is designed to position the ferritin, encapsulin, SOR, lumazine synthase or pyruvate dehydrogenase portion of the fusion protein and the disclosed recombinant RSV F protein stabilized in a prefusion conformation portion of the fusion protein, with regard to one another, such that the fusion protein maintains the ability to assemble into nanoparticles, and also elicit an immune response to RSV. In several embodiments, the linker sequences comprise amino acids. Preferable amino acids to use are those having small side chains and/or those which are not charged. Such amino acids are less likely to interfere with proper folding and activity of the fusion protein. Accordingly, preferred amino acids to use in linker sequences, either alone or in combination are serine, glycine and alanine. One example of such a linker sequence is SGG. Amino acids can be added or subtracted as needed. Those skilled in the art are capable of determining appropriate linker sequences for construction of protein nanoparticles.

[0323] In certain embodiments, the protein nanoparticles have a molecular weight of from 100 to 5000 kDa, such as approximately 500 to 4600 kDa. In some embodiments, a Ferritin nanoparticle has an approximate molecular weight of 650 kDa, an Encapsulin nanoparticle has an approximate molecular weight of 2100 kDa, a SOR nanoparticle has an approximate molecular weight of 1000 kDa, a lumazine synthase nanoparticle has an approximate molecular weight of 4000 kDa, and a pyruvate dehydrogenase nanoparticle has an approximate molecular weight of 4600 kDa, when the protein nanoparticle include a recombinant RSV F protein stabilized in a prefusion conformation.

[0324] The disclosed recombinant RSV F proteins stabilized in a prefusion conformation linked to ferritin, encapsulin, SOR, lumazine synthase or pyruvate dehydrogenase proteins can self-assemble into multi-subunit protein nanoparticles, termed ferritin nanoparticles, encapsulin nanoparticles, SOR nanoparticles, lumazine synthase nanoparticles, and pyruvate dehydrogenase nanoparticles, respectively. The nanoparticles include the disclosed recombinant RSV F proteins stabilized in a prefusion conformation have substantially the same structural characteristics as the native ferritin, encapsulin, SOR, lumazine synthase or pyruvate dehydrogenase nanoparticles that do not include the disclosed recombinant RSV F proteins stabilized in a prefusion conformation. That is, they contain 24, 60, 24, 60, or 60 subunits (respectively) and have similar corresponding symmetry. In the case of nanoparticles constructed of monomer subunits including a disclosed recombinant RSV F protein stabilized in a prefusion conformation, such nanoparticles are specifically bound by a prefusion-specific antibody (*e.g.*, D25 or AM22 antibody), and/or includes a RSV F prefusion specific conformation (such as antigenic site Ø).

C. Polynucleotides Encoding Antigens

[0325] Polynucleotides encoding the disclosed PreF antigens (*e.g.*, a recombinant RSV F protein stabilized in a prefusion conformation, or epitope-scaffold protein, or virus-like particle or protein nanoparticle containing such proteins) are also provided. These polynucleotides include DNA, cDNA and RNA sequences which encode the antigen.

[0326] In some embodiments, the nucleic acid molecule encodes a precursor F_0 polypeptide that, when expressed in an appropriate cell, is processed into a disclosed PreF antigen. In some embodiments, the nucleic acid molecule encodes a precursor F_0 polypeptide that, when expressed in an appropriate cell, is processed into a disclosed PreF antigen, wherein the precursor F_0 polypeptide includes, from N- to C-terminus, a signal peptide, a F_2 polypeptide, a Pep27 polypeptide, and a F_1 polypeptide. In some embodiments, the Pep27 polypeptide includes the amino acid sequence set forth as positions 110-136 of any one SEQ ID NOs: 1-184 or 370, wherein the amino acid positions correspond to the amino acid sequence of a reference F_0 polypeptide set forth as SEQ ID NO: 124. In some embodiments, the signal peptide includes the amino acid sequence set forth as positions 1-25 of any one SEQ ID NOs: 1-184 or 370, wherein the amino acid positions correspond to the amino acid sequence of a reference F_0 polypeptide set forth as SEQ ID NO: 124.

[0327] In some embodiments, the nucleic acid molecule encodes a precursor F_0 polypeptide that, when expressed in an appropriate cell, is processed into a disclosed PreF antigen, wherein the precursor F_0 polypeptide includes the amino acid sequence set forth as any one of SEQ ID NOs: 185, or 189-303. In some embodiments, the nucleic acid molecule encodes a precursor F_0 polypeptide that, when expressed in an appropriate cell, is processed into a disclosed PreF antigen, wherein the precursor F_0 polypeptide includes the amino acid sequence set forth as residues 1-513 of any one of SEQ ID NOs: 185, or 189-303.

[0328] In some embodiments, the nucleic acid molecule encodes a precursor F_0 polypeptide that, when expressed in an appropriate cell, is processed into a disclosed PreF antigen including a recombinant RSV F protein including an F2 polypeptide and a F1 polypeptide, and wherein the F1 polypeptide includes a disulfide bond between a pair of cysteines at positions 155 and 290, and a cavity-filling amino acid substitution at position 190; or a disulfide bond between a pair of cysteines at positions 155 and 290, a cavity-filling amino acid substitution at position 190, and a cavity-filling amino acid substitution at position 207.

[0329] In some embodiments, the nucleic acid molecule encodes a precursor F_0 polypeptide that, when expressed in an appropriate cell, is processed into a disclosed PreF antigen including a recombinant RSV F protein including an F2 polypeptide and a F1 polypeptide, and wherein the F1 polypeptide includes S155C, S290C, S190F, and V207L amino acid substitutions and may include further substitutions including:, S155C, S290C, S190W, and V207L amino acid substitutions, S155C, S290C, S190L, and V207L amino acid substitutions, S155C, S290C, S190F, and V207F amino acid substitutions, S155C, S290C, S190W, and V207F amino acid substitutions, S155C, S290C, S190L, and V207F amino acid substitutions, S155C, S290C, S190F, and V207W amino acid substitutions, S155C, S290C, S190W, and V207W amino acid substitutions, or S155C, S290C, S190L, and V207W amino acid substitutions.

[0330] In some embodiments, the nucleic acid molecule encodes a precursor F_0 polypeptide that, when expressed in an appropriate cell, is processed into a disclosed PreF antigen including a recombinant RSV F protein including an F2 polypeptide and a F1 polypeptide, wherein the F_2 polypeptide and the F_1 polypeptide include the amino acid sequence set forth as positions 26-109 and 137-513, respectively, of any one of SEQ ID NO: 371 (RSV A with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 372 (RSV B with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 373 (bovine RSV with S155C, S290C, S190F and V207L substitutions), SEQ ID NO: 374 (RSV A with S155C, S290C, and S190F substitutions), SEQ ID NO: 375 (RSV B with S155C, S290C, and S190F substitutions); or SEQ ID NO: 376 (bovine RSV with S155C, S290C, and S190F substitutions). SEQ ID NOs 374-376 not forming part of the clamed invention.

[0331] In several embodiments, the nucleic acid molecule encodes a precursor F_0 polypeptide that, when expressed in an appropriate cell, is processed into a disclosed PreF antigen including a recombinant RSV F protein including an F2 polypeptide and a F1 polypeptide from a human RSV A subtype, a human RSV B subtype, or a bovine RSV, wherein the F1 polypeptide includes the stabilizing modifications S155C, S290C, S190F, and V207L.

[0332] In one non-limiting example the nucleic acid molecule encodes a precursor F_0 polypeptide that, when expressed in an appropriate cell, is processed into a disclosed PreF antigen including a recombinant RSV F protein including an F2 polypeptide and a F1 polypeptide, wherein the F1 polypeptide is linked to a ferritin protein, and wherein the F_2 polypeptide and the F_1 polypeptide linked to the ferritin protein include the amino acid sequence set forth as positions 26-109 and 137-679, respectively of SEQ ID NO: 377 (RSV A including S155C, S290C, S190F, V207L amino acid substitutions, with C-terminal ferritin domain), or SEQ ID NOs: 378-382.

[0333] In one non-limiting example, the nucleic acid molecule includes the sequence set forth as SEQ ID NO: 383 (RSV F protein from human subtype A including S155C, S290C, S190F and V207L amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II).

[0334] In another non-limiting example, the nucleic acid molecule is an expression vector, and includes the sequence set forth as SEQ ID NO: 384 (RSV F protein from human subtype A including S155C, S290C, S190F and V207L amino acid substitutions, fused to a C-terminal Foldon domain, thrombin cleavage site, 6xHis tag and a StrepTag II).

[0335] Methods for the manipulation and insertion of the nucleic acids of this disclosure into vectors are well known in the art (see for example, Sambrook et al., Molecular Cloning, a Laboratory Manual, 2d edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 1989, and Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and John Wiley & Sons, New York, N.Y., 1994).

[0336] A nucleic acid encoding PreF antigens (*e.g.*, a recombinant RSV F protein stabilized in a prefusion conformation, or epitope-scaffold protein, or virus-like particle or protein nanoparticle containing such proteins) can be cloned or amplified by *in vitro* methods, such as the polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), the self-sustained sequence replication system (3SR) and the Qβ replicase amplification system (QB). For example, a polynucleotide encoding the protein can be isolated by polymerase chain reaction of cDNA using primers based on the DNA sequence of the molecule. A wide variety of cloning and *in vitro* amplification methodologies are well known to persons skilled in the art. PCR methods are described in, for example, U.S. Patent No. 4,683,195; Mullis et al., Cold Spring Harbor Symp. Quant. Biol. 51:263, 1987; and Erlich, ed., PCR Technology, (Stockton Press, NY, 1989). Polynucleotides also can be isolated by screening genomic or cDNA libraries with probes selected from the sequences of the desired polynucleotide under stringent hybridization conditions.

[0337] The polynucleotides encoding PreF antigens (*e.g.*, a recombinant RSV F protein stabilized in a prefusion conformation, or epitope-scaffold protein, or virus-like particle or protein nanoparticle containing such proteins) include a recombinant DNA which is incorporated into a vector into an autonomously replicating plasmid or virus or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (such as a cDNA) independent of other sequences. The nucleotides can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. The term includes single and double forms of DNA.

[0338] DNA sequences encoding PreF antigens (*e.g.*, a recombinant RSV F protein stabilized in a prefusion conformation, or epitope-scaffold protein, or virus-like particle or protein nanoparticle containing such proteins) can be expressed *in vitro* by DNA transfer into a suitable host cell. The cell may be prokaryotic or eukaryotic. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

[0339] Polynucleotide sequences encoding PreF antigens (*e.g.*, a recombinant RSV F protein stabilized in a prefusion conformation, or epitope-scaffold protein, or virus-like particle or protein nanoparticle containing such proteins) can be operatively linked to expression control sequences. An expression control sequence operatively linked to a coding sequence is ligated such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. The expression control sequences include, but are not limited to, appropriate promoters, enhancers, transcription terminators, a start codon (*i.e.*, ATG) in front of a protein-encoding gene, splicing signal for introns, maintenance of the correct reading frame of that gene to permit proper translation of mRNA, and stop codons.

[0340] Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Non-limiting examples of suitable host cells include bacteria, archea, insect, fungi (for example, yeast), plant, and animal cells (for example, mammalian cells, such as human). Exemplary cells of use include *Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae, Salmonella typhimurium,* SF9 cells, C129 cells, 293 cells, *Neurospora,* and immortalized mammalian myeloid and lymphoid cell lines. Techniques for the propagation of mammalian cells in culture are well-known (see, Jakoby and Pastan (eds), 1979, Cell Culture. Methods in Enzymology, volume 58, Academic Press, Inc., Harcourt Brace Jovanovich, N.Y.). Examples of commonly used mammalian host cell lines are VERO and HeLa cells, CHO cells, and WI38, BHK, and COS cell lines, although cell lines may be used, such as cells designed to provide higher expression, desirable glycosylation patterns, or other features. In some embodiments, the host cells include HEK293 cells or derivatives thereof, such as GnTI$^{-/-}$ cells (ATCC® No. CRL-3022).

[0341] Transformation of a host cell with recombinant DNA can be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as, but not limited to, *E. coli,* competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method using procedures well known in the art. Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell if desired, or by electroporation.

[0342] When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate coprecipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or viral vectors can be used. Eukaryotic cells can also be co-transformed with polynucleotide sequences encoding a disclosed antigen, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein (see for example, Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

D. Viral Vectors

[0343] The nucleic acid molecules encoding a recombinant RSV F protein stabilized in a prefusion conformation can be included in a viral vector, for example for expression of the antigen in a host cell, or for immunization of a subject as disclosed herein. In some embodiments, the viral vectors are administered to a subject as part of a prime-boost vaccination. In several embodiments, the viral vectors are included in a vaccine, such as a primer vaccine or a booster vaccine for use in a prime-boost vaccination.

[0344] In several examples, the viral vector encoding the recombinant RSV F protein stabilized in a prefusion conformation can be replication-competent. For example, the viral vector can have a mutation (e.g., insertion of nucleic acid encoding the PreF antigen) in the viral genome that does not inhibit viral replication in host cells. The viral vector also can be conditionally replication-competent. In other examples, the viral vector is replication-deficient in host cells.

[0345] In several embodiments, the recombinant RSV F protein stabilized in a prefusion conformation is expressed by a viral vector that can be delivered via the respiratory tract. For example, a paramyxovirus (PIV) vector, such as bovine parainfluenza virus (BPIV) vector (*e.g.,* a BPIV-1, BPIV-2, or BPV-3 vector) or human PIV vector, a metapneumovirus (MPV) vector, a Sendia virus vector, or a measles virus vector, is used to express a disclosed antigen. A BPIV3 viral vector expressing the RSV F and the hPIV F proteins (MEDI-534) is currently in clinical trials as a RSV vaccine. Examples of paramyxovirus (PIV) vector for expressing antigens are known to the person of skill in the art (see, *e.g.,* U.S. Pat. App. Pubs. 2012/0045471, 2011/0212488, 2010/0297730, 2010/0278813, 2010/0167270, 2010/0119547, 2009/0263883, 2009/0017517, 2009/0004722, 2008/0096263, 2006/0216700, 2005/0147623, 2005/0142148, 2005/0019891, 2004/0208895, 2004/0005545, 2003/0232061, 2003/0095987, and 2003/0072773). In another example, a Newcastle disease viral vector is used to express a disclosed antigen (see, *e.g.,* McGinnes et al., J. Virol., 85: 366-377, 2011, describing RSV F and G proteins expressed on Newcastle disease like particles). In another example, a Sendai virus vector is used to express a disclosed antigen (see, *e.g.,* Jones et al., Vaccine, 30:959-968, 2012, which discloses use of a Sendai virus-based RSV vaccine to induce an immune response in primates).

[0346] Additional viral vectors are also available for expression of the disclosed antigens, including polyoma, *i.e.,* SV40 (Madzak et al., 1992, J. Gen. Virol., 73:15331536), adenovirus (Berkner, 1992, Cur. Top. Microbial. Immunol., 158:39-6; Berliner et al., 1988, Bio Techniques, 6:616-629; Gorziglia et al., 1992, J. Virol., 66:4407-4412; Quantin et al., 1992, Proc. Natl. Acad. Sci. USA, 89:2581-2584; Rosenfeld et al., 1992, Cell, 68:143-155; Wilkinson et al., 1992, Nucl. Acids Res., 20:2233-2239; Stratford-Perricaudet et al., 1990, Hum. Gene Ther., 1:241-256), vaccinia virus (Mackett et al., 1992, Biotechnology, 24:495-499), adeno-associated virus (Muzyczka, 1992, Curr. Top. Microbiol. Immunol., 158:91-123; On et al., 1990, Gene, 89:279-282), herpes viruses including HSV and EBV and CMV (Margolskee, 1992, Curr. Top. Microbiol. Immunol., 158:67-90; Johnson et al., 1992, J. Virol., 66:29522965; Fink et al., 1992, Hum. Gene Ther. 3:11-19; Breakfield et al., 1987, Mol. Neurobiol., 1:337-371; Fresse et al., 1990, Biochem. Pharmacol., 40:2189-2199), Sindbis viruses (H. Herweijer et al., 1995, Human Gene Therapy 6:1161-1167; U.S. Pat. Nos. 5,091,309 and 5,2217,879), alphaviruses (S. Schlesinger, 1993, Trends Biotechnol. 11:18-22; I. Frolov et al., 1996, Proc. Natl. Acad. Sci. USA 93:11371-11377) and retroviruses of avian (Brandyopadhyay et al., 1984, Mol. Cell Biol., 4:749-754; Petropouplos et al., 1992, J. Virol., 66:3391-3397), murine (Miller, 1992, Curr. Top. Microbiol. Immunol., 158:1-24; Miller et al., 1985, Mol. Cell Biol., 5:431-437; Sorge et al., 1984, Mol. Cell Biol., 4:1730-1737; Mann et al., 1985, J. Virol., 54:401-407), and human origin (Page et al., 1990, J. Virol., 64:5370-5276; Buchschalcher et al., 1992, J. Virol., 66:2731-2739). Baculovirus (Autographa californica multinuclear polyhedrosis virus; AcNMPV) vectors are also known in the art, and may be obtained from commercial sources (such as PharMingen, San Diego, Calif.; Protein Sciences Corp., Meriden, Conn.; Stratagene, La Jolla, Calif.). Additional viral vectors are familiar to the person of ordinary skill in the art.

[0347] In several embodiments, the methods and compositions disclosed herein include an adenoviral vector that expresses a recombinant RSV F protein stabilized in a prefusion conformation. Adenovirus from various origins, subtypes, or mixture of subtypes can be used as the source of the viral genome for the adenoviral vector. Non-human adenovirus (*e.g.,* simian, chimpanzee, gorilla, avian, canine, ovine, or bovine adenoviruses) can be used to generate the adenoviral vector. For example, a simian adenovirus can be used as the source of the viral genome of the adenoviral vector. A simian adenovirus can be of serotype 1, 3, 7, 11, 16, 18, 19, 20, 27, 33, 38, 39, 48, 49, 50, or any other simian adenoviral serotype. A simian adenovirus can be referred to by using any suitable abbreviation known in the art, such as, for example, SV, SAdV, SAV or sAV. In some examples, a simian adenoviral vector is a simian adenoviral vector of serotype 3, 7, 11, 16, 18, 19, 20, 27, 33, 38, or 39. In one example, a chimpanzee serotype C Ad3 vector is used (see, *e.g.,* Peruzzi et al., Vaccine, 27:1293-1300, 2009). Human adenovirus can be used as the source of the viral genome for the adenoviral vector. Human adenovirus can be of various subgroups or serotypes. For instance, an adenovirus can be of subgroup A (e.g., serotypes 12, 18, and 31), subgroup B (*e.g.,* serotypes 3, 7, 11, 14, 16, 21, 34, 35, and 50), subgroup C *(e.g.,* serotypes 1, 2, 5, and 6), subgroup D (*e.g.,* serotypes 8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 36-39, and 42-48), subgroup E (*e.g.,* serotype 4), subgroup F (*e.g.,* serotypes 40 and 41), an unclassified serogroup (e.g., serotypes 49 and 51), or any other adenoviral serotype. The person of ordinary skill in the art is familiar with replication competent and deficient adenoviral vectors (including singly and multiply replication deficient adenoviral vectors). Examples of replication-deficient adenoviral vectors, including multiply replication-deficient adenoviral vectors, are disclosed in U.S. Patent Nos. 5,837,51 1 ; 5,851 ,806; 5,994,106; 6,127,175; 6,482,616; and 7,195,896, and International Patent Application Nos. WO 94/28152, WO 95/02697, WO 95/16772, WO 95/34671, WO 96/22378, WO 97/12986, WO 97/21826, and WO 03/02231 1.

E. Compositions

[0348] The disclosed PreF antigens, viral vectors, and nucleic acid molecules can be included in a pharmaceutical composition, including therapeutic and prophylactic formulations, and can be combined together with one or more adjuvants and, optionally, other therapeutic ingredients, such as antiviral drugs. In several embodiments, compositions including one or more of the disclosed PreF antigens, viral vectors, or nucleic acid molecules are immunogenic compositions. The composition can include any of the PreF antigens including a recombinant RSV F protein as disclosed herein, (such as a protein nanoparticle including any of the recombinant RSV F proteins as disclosed herein), a virus-like particle including any of the recombinant RSV F proteins as disclosed herein, a nucleic acid molecule encoding any of the recombinant RSV F proteins as disclosed herein, or a vector encoding or including any of the recombinant RSV F proteins as disclosed herein.

[0349] In some embodiments, the composition includes a first isolated antigen including a recombinant RSV F protein stabilized in a prefusion conformation by the substitutions S155C, S290C, S190F, and V207L, wherein the stabilized RSV F protein is based on a subtype A RSV F protein, and a second isolated antigen including a recombinant RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions), wherein the stabilized RSV F protein is based on a subtype B RSV F protein.

[0350] In some embodiments, the composition includes a first protein nanoparticle including a recombinant RSV F protein stabilized in a prefusion conformation by the substitutions S155C, S290C, S190F, and V207L, wherein the stabilized RSV F protein is based on a subtype A RSV F protein, and a second protein nanoparticle including a recombinant RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions), wherein the stabilized RSV F protein is based on a subtype B RSV F protein.

[0351] In some embodiments, the composition includes a first viral vector including a recombinant RSV F protein stabilized in a prefusion conformation by the substitutions S155C, S290C, S190F, and V207L, wherein the stabilized RSV F protein is based on a subtype A RSV F protein, and a second viral vector including a recombinant RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions), wherein the stabilized RSV F protein is based on a subtype B RSV F protein.

[0352] In some embodiments, the composition includes a first virus-like particle including a recombinant RSV F protein stabilized in a prefusion conformation by the substitutions S155C, S290C, S190F, and V207L, wherein the stabilized RSV F protein is based on a subtype A RSV F protein, and a second virus-like particle including a recombinant RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions), wherein the stabilized RSV F protein is based on a subtype B RSV F protein.

[0353] In some embodiments, the composition includes a first nucleic acid molecule (such as an expression vector) encoding a recombinant RSV F protein stabilized in a prefusion conformation by the substitutions S155C, S290C, S190F, and V207L, wherein the stabilized RSV F protein is based on a subtype A RSV F protein, and a second nucleic acid molecule (such as an expression vector) including a recombinant RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions), wherein the stabilized RSV F protein is based on a subtype B RSV F protein.

[0354] Such pharmaceutical compositions can be administered to subjects by a variety of administration modes known to the person of ordinary skill in the art, for example, nasal, pulmonary, intramuscular, subcutaneous, intravenous, intraperitoneal, or parenteral routes.

[0355] To formulate the compositions, the disclosed PreF antigens, viral vectors, or nucleic acid molecules can be combined with various pharmaceutically acceptable additives, as well as a base or vehicle for dispersion of the conjugate. Desired additives include, but are not limited to, pH control agents, such as arginine, sodium hydroxide, glycine, hydrochloric acid, citric acid, and the like. In addition, local anesthetics (for example, benzyl alcohol), isotonizing agents (for example, sodium chloride, mannitol, sorbitol), adsorption inhibitors (for example, TWEEN® 80), solubility enhancing agents (for example, cyclodextrins and derivatives thereof), stabilizers (for example, serum albumin), and reducing agents (for example, glutathione) can be included. Adjuvants, such as aluminum hydroxide (ALHYDROGEL®, available from Brenntag Biosector, Copenhagen, Denmark and AMPHOGEL®, Wyeth Laboratories, Madison, NJ), Freund's adjuvant, MPL™ (3-O-deacylated monophosphoryl lipid A; Corixa, Hamilton, IN), IL-12 (Genetics Institute, Cambridge, MA) TLR agonists (such as TLR-9 agonists), among many other suitable adjuvants well known in the art, can be included in the compositions.

[0356] When the composition is a liquid, the tonicity of the formulation, as measured with reference to the tonicity of 0.9% (w/v) physiological saline solution taken as unity, is typically adjusted to a value at which no substantial, irreversible tissue damage will be induced at the site of administration. Generally, the tonicity of the solution is adjusted to a value of about 0.3 to about 3.0, such as about 0.5 to about 2.0, or about 0.8 to about 1.7.

[0357] The disclosed PreF antigens, viral vectors, or nucleic acid molecules can be dispersed in a base or vehicle, which can include a hydrophilic compound having a capacity to disperse the antigens, and any desired additives. The base can be selected from a wide range of suitable compounds, including but not limited to, copolymers of polycarboxylic acids or salts thereof, carboxylic anhydrides (for example, maleic anhydride) with other monomers (for example, methyl (meth)acrylate, acrylic acid and the like), hydrophilic vinyl polymers, such as polyvinyl acetate, polyvinyl alcohol, polyvinylpyrrolidone, cellulose derivatives, such as hydroxymethylcellulose, hydroxypropylcellulose and the like, and natural polymers, such as chitosan, collagen, sodium alginate, gelatin, hyaluronic acid, and nontoxic metal salts thereof. Often, a biodegradable polymer is selected as a base or vehicle, for example, polylactic acid, poly(lactic acid-glycolic acid) copolymer, polyhydroxybutyric acid, poly(hydroxybutyric acid-glycolic acid) copolymer and mixtures thereof. Alternatively or additionally, synthetic fatty acid esters such as poly glycerin fatty acid esters, sucrose fatty acid esters and the like can be employed as vehicles. Hydrophilic polymers and other vehicles can be used alone or in combination, and enhanced structural integrity can be imparted to the vehicle by partial crystallization, ionic bonding, cross-linking and the like. The vehicle can be provided in a variety of forms, including fluid or viscous solutions, gels, pastes, powders, microspheres and films, for examples for direct application to a mucosal surface.

[0358] The disclosed PreF antigens, viral vectors, or nucleic acid molecules can be combined with the base or vehicle according to a variety of methods, and release of the antigens can be by diffusion, disintegration of the vehicle, or associated formation of water channels. In some circumstances, the disclosed antigens, or a nucleic acid or a viral vector encoding, expressing or including the antigen, is dispersed in microcapsules (microspheres) or nanocapsules (nanospheres) prepared from a suitable polymer, for example, isobutyl 2-cyanoacrylate (see, for example, Michael et al., J. Pharmacy Pharmacol. 43:1-5, 1991), and dispersed in a biocompatible dispersing medium, which yields sustained delivery and biological activity over a protracted time.

[0359] The pharmaceutical compositions can contain as pharmaceutically acceptable vehicles substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, and triethanolamine oleate. For solid compositions, conventional nontoxic pharmaceutically acceptable vehicles can be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like.

[0360] Pharmaceutical compositions for administering the disclosed PreF antigens, viral vectors, or nucleic acid molecules can also be formulated as a solution, microemulsion, or other ordered structure suitable for high concentration of active ingredients. The vehicle can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), and suitable mixtures thereof. Proper fluidity for solutions can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of a desired particle size in the case of dispersible formulations, and by the use of surfactants. In many cases, it will be desirable to include isotonic agents, for example, sugars, polyalcohols, such as mannitol and sorbitol, or sodium chloride in the composition. Prolonged absorption of the disclosed antigens can be brought about by including in the composition an agent which delays absorption, for example, monostearate salts and gelatin.

[0361] In certain embodiments, the disclosed PreF antigens, viral vectors, or nucleic acid molecules can be administered in a time-release formulation, for example in a composition that includes a slow release polymer. These compositions can be prepared with vehicles that will protect against rapid release, for example a controlled release vehicle such as a polymer, microencapsulated delivery system or bioadhesive gel. Prolonged delivery in various compositions of the disclosure can be brought about by including in the composition agents that delay absorption, for example, aluminum monostearate hydrogels and gelatin. When controlled release formulations are desired, controlled release binders suitable for use in accordance with the disclosure include any biocompatible controlled release material which is inert to the active agent and which is capable of incorporating the disclosed antigen and/or other biologically active agent. Numerous such materials are known in the art. Useful controlled-release binders are materials that are metabolized slowly under physiological conditions following their delivery (for example, at a mucosal surface, or in the presence of bodily fluids). Appropriate binders include, but are not limited to, biocompatible polymers and copolymers well known in the art for use in sustained release formulations. Such biocompatible compounds are non-toxic and inert to surrounding tissues, and do not trigger significant adverse side effects, such as nasal irritation, immune response, inflammation, or the like. They are metabolized into metabolic products that are also biocompatible and easily eliminated from the body. Numerous systems for controlled delivery of therapeutic proteins are known (*e.g.*, U.S. Patent No. 5,055,303; U.S. Patent No. 5,188,837; U.S. Patent No. 4,235,871; U.S. Patent No. 4,501,728; U.S. Patent No. 4,837,028; U.S. Patent No. 4,957,735; and U.S. Patent No. 5,019,369; U.S. Patent No. 5,055,303; U.S. Patent No. 5,514,670; U.S. Patent No. 5,413,797; U.S. Patent No. 5,268,164; U.S. Patent No. 5,004,697; U.S. Patent No. 4,902,505; U.S. Patent No. 5,506,206; U.S. Patent No. 5,271,961; U.S. Patent No. 5,254,342; and U.S. Patent No. 5,534,496).

[0362] Exemplary polymeric materials for use include, but are not limited to, polymeric matrices derived from copolymeric and homopolymeric polyesters having hydrolyzable ester linkages. A number of these are known in the art to be biodegradable and to lead to degradation products having no or low toxicity. Exemplary polymers include polyglycolic acids and polylactic acids, poly(DL-lactic acid-co-glycolic acid), poly(D-lactic acid-co-glycolic acid), and poly(L-lactic acid-co-glycolic acid). Other useful biodegradable or bioerodable polymers include, but are not limited to, such polymers as poly(epsilon-caprolactone), poly(epsilon-aprolactone-CO-lactic acid), poly(epsilon.-aprolactone-CO-glycolic acid), poly(beta-hydroxy butyric acid), poly(alkyl-2-cyanoacrilate), hydrogels, such as poly(hydroxyethyl methacrylate), polyamides, poly(amino acids) (for example, L-leucine, glutamic acid, L-aspartic acid and the like), poly(ester urea), poly(2-hydroxyethyl DL-aspartamide), polyacetal polymers, polyorthoesters, polycarbonate, polymaleamides, polysaccharides, and copolymers thereof. Many methods for preparing such formulations are well known to those skilled in the art (see, for example, Sustained and Controlled Release Drug Delivery Systems, J. R. Robinson, ed., Marcel Dekker, Inc., New York, 1978). Other useful formulations include controlled-release microcapsules (U.S. Patent Nos. 4,652,441 and 4,917,893), lactic acid-glycolic acid copolymers useful in making microcapsules and other formulations (U.S. Patent Nos. 4,677,191 and 4,728,721) and sustained-release compositions for water-soluble peptides (U.S. Patent No. 4,675,189).

[0363] Pharmaceutical compositions typically are sterile and stable under conditions of manufacture, storage and use. Sterile solutions can be prepared by incorporating the disclosed PreF antigens, viral vectors, or nucleic acid molecules in the required amount in an appropriate solvent with one or a combination of ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the disclosed antigen and/or other biologically active agent into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated herein. In the case of sterile powders, methods of preparation include vacuum drying and freeze-drying which yields a powder of the disclosed antigen plus any additional desired ingredient from a previously sterile-filtered solution thereof. The prevention of the action of microorganisms can be accomplished by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like.

[0364] Actual methods for preparing administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as Remingtons Pharmaceutical Sciences, 19th Ed., Mack Publishing Company, Easton, Pennsylvania, 1995.

[0365] In several embodiments, the compositions include an adjuvant. The person of ordinary skill in the art is familiar with adjuvants, for example, those that can be included in an immunogenic composition. In several embodiments, the adjuvant is selected to elicit a Th1 biased immune response in a subject administered an immunogenic composition containing the adjuvant and a disclosed antigens, or a nucleic acid or a viral vector encoding, expressing or including the antigen,.

[0366] One suitable adjuvant is a non-toxic bacterial lipopolysaccharide derivative. An example of a suitable non-toxic derivative of lipid A, is monophosphoryl lipid A or more particularly 3-Deacylated monophoshoryl lipid A (3D-MPL). See, for example, U.S. Pat. Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094. 3D-MPL primarily promotes CD4+ T cell responses with an IFN-γ (Th1) phenotype. 3D-MPL can be produced according to the methods disclosed in GB2220211 A. Chemically it is a mixture of 3-deacylated monophosphoryl lipid A with 3, 4, 5 or 6 acylated chains. In the compositions, small particle 3D-MPL can be used. Small particle 3D-MPL has a particle size such that it can be sterile-filtered through a 0.22 μm filter. Such preparations are described in WO94/21292.

[0367] In other embodiments, the lipopolysaccharide can be a β(1-6) glucosamine disaccharide, as described in U.S. Pat. No. 6,005,099 and EP Patent No. 0 729 473 B1. One of skill in the art would be readily able to produce various lipopolysaccharides, such as 3D-MPL, based on the teachings of these references. In addition to the aforementioned immunostimulants (that are similar in structure to that of LPS or MPL or 3D-MPL), acylated monosaccharide and disaccharide derivatives that are a sub-portion to the above structure of MPL are also suitable adjuvants.

[0368] In several embodiments, a Toll-like receptor (TLR) agonist is used as an adjuvant. For example a disclosed PreF antigen can be combined with a TLR agonist in an immunogenic composition used for elicitation of a neutralizing immune response to RSV. For example, the TLR agonist can be a TLR-4 agonist such as a synthetic derivative of lipid A (see, *e.g.*, WO 95/14026, and WO 01/46127) an alkyl Glucosaminide phosphate (AGP; see, *e.g.*, WO 98/50399 or U.S. Pat. No. 6,303,347; 6,764,840). Other suitable TLR-4 ligands, capable of causing a signaling response through TLR-4 are, for example, lipopolysaccharide from gram-negative bacteria and its derivatives, or fragments thereof, in particular a non-toxic derivative of LPS (such as 3D-MPL). Other suitable TLR agonists are: heat shock protein (HSP) 10, 60, 65, 70, 75 or 90; surfactant Protein A, hyaluronan oligosaccharides, heparan sulphate fragments, fibronectin fragments, fibrinogen peptides and β-defensin-2, and muramyl dipeptide (MDP). In one embodiment the TLR agonist is HSP 60, 70 or 90. Other suitable TLR-4 ligands are as described in WO 2003/011223 and in WO 2003/099195.

[0369] Additional TLR agonists (such as an agent that is capable of causing a signaling response through a TLR signaling pathway) are also useful as adjuvants, such as agonists for TLR2, TLR3, TLR7, TLR8 and/or TLR9. Accordingly, in one embodiment, the composition further includes an adjuvant which is selected from the group consisting of: a TLR-1 agonist, a TLR-2 agonist, TLR-3 agonist, a TLR-4 agonist, TLR-5 agonist, a TLR-6 agonist, TLR-7 agonist, a TLR-8 agonist, TLR-9 agonist, or a combination thereof .

[0370] In one embodiment, a TLR agonist is used that is capable of causing a signaling response through TLR-1, for example one or more of from: Tri-acylated lipopeptides (LPs); phenol-soluble modulin; Mycobacterium tuberculosis LP; S-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-L-ys(4)--OH, trihydrochloride (Pam3Cys) LP which mimics the acetylated amino terminus of a bacterial lipoprotein and OspA LP from Borrelia burgdorferi. In another embodiment, a TLR agonist is used that is capable of causing a signaling response through TLR-2, such as one or more of a lipoprotein, a peptidoglycan, a bacterial lipopeptide from M tuberculosis, B burgdorferi or T pallidum; peptidoglycans from species including Staphylococcus aureus; lipoteichoic acids, mannuronic acids, Neisseria porins, bacterial fimbriae, Yersina virulence factors, CMV virions, measles haemagglutinin, and zymosan from yeast. In some embodiments, a TLR agonist is used that is capable of causing a signaling response through TLR-3, such as one or more of double stranded RNA (dsRNA), or polyinosinicpolycytidylic acid (Poly IC), a molecular nucleic acid pattern associated with viral infection. In further embodiments, a TLR agonist is used that is capable of causing a signaling response through TLR-5, such as bacterial flagellin. In additional embodiments, a TLR agonist is used that is capable of causing a signaling response through TLR-6, such as one or more of mycobacterial lipoprotein, di-acylated LP, and phenol-soluble modulin. Additional TLR6 agonists are described in WO 2003/043572. In an embodiment, a

TLR agonist is used that is capable of causing a signaling response through TLR-7, such as one or more of a single stranded RNA (ssRNA), loxoribine, a guanosine analogue at positions N7 and C8, or an imidazoquinoline compound, or derivative thereof. In one embodiment, the TLR agonist is imiquimod. Further TLR7 agonists are described in WO 2002/085905. In some embodiments, a TLR agonist is used that is capable of causing a signaling response through TLR-8. Suitably, the TLR agonist capable of causing a signaling response through TLR-8 is a single stranded RNA (ssRNA), an imidazoquinoline molecule with anti-viral activity, for example resiquimod (R848); resiquimod is also capable of recognition by TLR-7. Other TLR-8 agonists which can be used include those described in WO 2004/071459.

[0371] In further embodiments, an adjuvant includes a TLR agonist capable of inducing a signaling response through TLR-9. For example, the adjuvant can include HSP90, bacterial or viral DNA, and/or DNA containing unmethylated CpG nucleotides (*e.g.*, a CpG oligonucleotide). For example, CpG-containing oligonucleotides induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 95/26204, WO 96/02555, WO 99/33488 and U.S. Pat. Nos. 5,278,302, 5,666,153, and. 6,008,200 and 5,856,462. Accordingly, oligonucleotides for use as adjuvants in the disclosed compositions include CpG containing oligonucleotides, for example, containing two or more dinucleotide CpG motifs. Also included are oligonucleotides with mixed internucleotide linkages.

[0372] Other adjuvants that can be used in immunogenic compositions with the antigens, or a nucleic acid or a viral vector encoding, expressing or including an antigen, *e.g.*, on their own or in combination with 3D-MPL, or another adjuvant described herein, are saponins, such as QS21. In some examples, saponins are used as an adjuvant, *e.g.*, for systemic administration of a PreF antigen. Use of saponins (e.g., use of Quil A, derived from the bark of the South American tree Quillaja Saponaria Molina) as adjuvants is familiar to the person of ordinary skill in the art (see, *e.g.*, US 5,057,540 and EP 0 362 279 B1. EP 0 109 942 B1; WO 96/11711; WO 96/33739). The haemolytic saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in U.S. Pat. No. 5,057,540 and EP 0 362 279 B1.

[0373] The adjuvant can also include mineral salts such as an aluminum or calcium salts, in particular aluminum hydroxide, aluminum phosphate and calcium phosphate.

[0374] Another class of suitable Th1 biasing adjuvants for use in compositions includes outer membrane proteins (OMP)-based immunostimulatory compositions. OMP-based immunostimulatory compositions are particularly suitable as mucosal adjuvants, *e.g.*, for intranasal administration. OMP-based immunostimulatory compositions are a genus of preparations of (OMPs, including some porins) from Gram-negative bacteria, *e.g.*, Neisseria species, which are useful as a carrier or in compositions for immunogens, such as bacterial or viral antigens (see, *e.g.*, U.S. Pat. No. 5,726,292; U.S. Pat. No. 4,707,543). Further, proteosomes have the capability to auto-assemble into vesicle or vesicle-like OMP clusters of about 20 nm to about 800 nm, and to noncovalently incorporate, coordinate, associate (*e.g.*, electrostatically or hydrophobically), or otherwise cooperate with protein antigens (Ags), particularly antigens that have a hydrophobic moiety. Proteosomes can be prepared, for example, as described in the art (see, *e.g.*, U.S. Pat. No. 5,726,292 or U.S. Pat. No. 5,985,284; 2003/0044425.).

[0375] Proteosomes are composed primarily of chemically extracted outer membrane proteins (OMPs) from Neisseria meningitidis (mostly porins A and B as well as class 4 OMP), maintained in solution by detergent (Lowell G H. Proteosomes for Improved Nasal, Oral, or Injectable Vaccines. In: Levine M M, Woodrow G C, Kaper J B, Cobon G S, eds, New Generation Vaccines. New York: Marcel Dekker, Inc. 1997; 193-206). Proteosomes can be formulated with a variety of antigens such as purified or recombinant proteins derived from viral sources, including the PreF polypeptides disclosed herein. The gradual removal of detergent allows the formation of particulate hydrophobic complexes of approximately 100-200 nm in diameter (Lowell G H. Proteosomes for Improved Nasal, Oral, or Injectable Vaccines. In: Levine M M, Woodrow G C, Kaper J B, Cobon G S, eds, New Generation Vaccines. New York: Marcel Dekker, Inc. 1997; 193-206).

[0376] Combinations of different adjuvants can also be used in compositions with the disclosed PreF antigens, viral vectors, or nucleic acid molecules in the composition. For example, as already noted, QS21 can be formulated together with 3D-MPL. The ratio of QS21:3D-MPL will typically be in the order of 1:10 to 10:1; such as 1:5 to 5:1, and often substantially 1:1. Typically, the ratio is in the range of 2.5:1 to 1:1 3D-MPL:QS21 (such as AS01 (GlaxoSmithKline). Another combination adjuvant formulation includes 3D-MPL and an aluminum salt, such as aluminum hydroxide (such as AS04 (GlaxoSmithKline). When formulated in combination, this combination can enhance an antigen-specific Th1 immune response.

[0377] In some instances, the adjuvant formulation a mineral salt, such as a calcium or aluminum (alum) salt, for example calcium phosphate, aluminum phosphate or aluminum hydroxide. In some embodiments, the adjuvant includes an oil and water emulsion, *e.g.*, an oil-in-water emulsion (such as MF59 (Novartis) or AS03 (GlaxoSmithKline). One example of an oil-in-water emulsion comprises a metabolisable oil, such as squalene, a tocol such as a tocopherol, *e.g.*, alpha-tocopherol, and a surfactant, such as sorbitan trioleate (Span 85) or polyoxyethylene sorbitan monooleate (Tween 80), in an aqueous carrier.

[0378] The pharmaceutical composition typically contains a therapeutically effective amount of a disclosed PreF antigen, viral vector, or nucleic acid molecule and can be prepared by conventional techniques. Preparation of immunogenic compositions, including those for administration to human subjects, is generally described in Pharmaceutical Biotechnology, Vol.61 Vaccine Design-the subunit and adjuvant approach, edited by Powell and Newman, Plenum Press, 1995. New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Pat. No. 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Pat. No. 4,372,945 and by Armor et al., U.S. Pat. No. 4,474,757. Typically, the amount of antigen in each dose of the immunogenic composition is selected as an amount which induces an immune response without significant, adverse side effects.

[0379] The amount of the disclosed PreF antigen, viral vector, or nucleic acid molecule can vary depending upon the specific antigen employed, the route and protocol of administration, and the target population, for example. Typically, each human dose will comprise 1-1000 µg of protein, such as from about 1 µg to about 100 µg, for example, from about 1 µg to about 50 µg, such as about 1 µg, about 2 µg, about 5 µg, about 10 µg, about 15 µg, about 20 µg, about 25 µg, about 30 µg, about 40 µg, or about 50 µg. The amount utilized in an immunogenic composition is selected based on the subject population (*e.g.*, infant or elderly). An optimal amount for a particular composition can be ascertained by standard studies involving observation of antibody titers and other responses in subjects. It is understood that a therapeutically effective amount of an antigen in a immunogenic composition can include an amount that is ineffective at eliciting an immune response by administration of a single dose, but that is effective upon administration of multiple dosages, for example in a prime-boost administration protocol.

[0380] In several examples, pharmaceutical compositions for eliciting an immune response against RSV in humans include a therapeutically effective amount of a disclosed PreF antigens, viral vectors, or nucleic acid molecules for administration to infants (*e.g.*, infants between birth and 1 year, such as between 0 and 6 months, at the age of initial dose) or elderly patients subject (such as a subject greater than 65 years of age). It will be appreciated that the choice of adjuvant can be different in these different applications, and the optimal adjuvant and concentration for each situation can be determined empirically by those of skill in the art.

[0381] In certain embodiments, the pharmaceutical compositions are vaccines that reduce or prevent infection with RSV. In some embodiments, the immunogenic compositions are vaccines that reduce or prevent a pathological response following infection with RSV. Optionally, the pharmaceutical compositions containing the disclosed PreF antigen, viral vector, or nucleic acid molecule are formulated with at least one additional antigen of a pathogenic organism other than RSV. For example, the pathogenic organism can be a pathogen of the respiratory tract (such as a virus or bacterium that causes a respiratory infection). In certain cases, the pharmaceutical composition contains an antigen derived from a pathogenic virus other than RSV, such as a virus that causes an infection of the respiratory tract, such as influenza or parainfluenza. In other embodiments, the additional antigens are selected to facilitate administration or reduce the number of inoculations required to protect a subject against a plurality of infectious organisms. For example, the antigen can be derived from any one or more of influenza, hepatitis B, diphtheria, tetanus, pertussis, Hemophilus influenza, poliovirus, Streptococcus or Pneumococcus, among others.

F. Compositions for use in Methods of Treatment

[0382] Reference to methods of treatment refer to substances/composisitons for use in said methods of treatment.

[0383] In several embodiments, the disclosed PreF antigens, or a nucleic acid or a viral vector encoding, expressing or including a PreF antigen are used to induce an immune response to RSV in a subject. Thus, in several embodiments, a therapeutically effective amount of an immunogenic composition including one or more of the disclosed PreF antigens, or a nucleic acid or a viral vector encoding, expressing or including the antigen, can be administered to a subject in order to generate an immune response to RSV.

[0384] In accordance with the disclosure herein, a prophylactically or therapeutically effective amount of a immunogenic composition including a PreF antigen, or a nucleic acid or a viral vector encoding, expressing or including the antigen, is administered to a subject in need of such treatment for a time and under conditions sufficient to prevent, inhibit, and/or ameliorate a RSV infection in a subject. The immunogenic composition is administered in an amount sufficient to elicit an immune response against an RSV antigen, such as RSV F protein, in the subject.

[0385] In some embodiments, the composition administered to the subject includes (or encodes) a first recombinant RSV F protein that is a subtype A RSV F protein stabilized in a prefusion conformation, and a second recombinant RSV F protein that is a subtype B RSV F protein stabilized in a prefusion conformation. In several embodiments, the composition administered to the subject includes a mixture (such as about a 1:1, 1:2, 2:1, 2:3, 3:2, 1:3, 3:1, 1:4, 4:1, 3:5, 5:3, 1:5, 5:1, 5:7, 7:5 mixture), of a first recombinant RSV F protein that is a subtype A RSV F protein stabilized in a prefusion conformation, and a second recombinant RSV F protein that is a subtype B RSV F protein stabilized in a prefusion conformation.

[0386] In some embodiments the composition administered to the subject includes a first protein nanoparticle including a recombinant RSV F protein stabilized in a prefusion conformation by the substitutions S155C, S290C, S190F, and V207L, wherein the stabilized RSV F protein is based on a subtype A RSV F protein, and a second protein nanoparticle including a recombinant RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions), wherein the stabilized RSV F protein is based on a subtype B RSV F protein.

[0387] In some embodiments the composition administered to the subject includes a first nucleic acid molecule (such as an expression vector) encoding a recombinant RSV F protein stabilized in a prefusion conformation by the substitutions S155C, S290C, S190F, and V207L, wherein the stabilized RSV F protein is based on a subtype A RSV F protein, and a second nucleic acid molecule (such as an expression vector) including a recombinant RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions), wherein the stabilized RSV F protein is based on a subtype B RSV F protein.

[0388] In some embodiments, a composition including ferritin nanoparticles including the recombinant RSV F protein stabilized in a prefusion conformation by the substitutions S155C, S290C, S190F, and V207L is administered to a subject. In some embodiments the composition administered to the subject includes a first ferritin nanoparticle including a recombinant RSV F protein stabilized in a prefusion conformation by the substitutions S155C, S290C, S190F, and V207L, wherein the stabilized RSV F protein is based on a subtype A RSV F protein, and a second ferritin nanoparticle including a recombinant RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions), wherein the stabilized RSV F protein is based on a subtype B RSV F protein. Methods of making ferritin nanoparticles including a viral antigen and their use for immunization purposes (e.g., for immunization against influenza antigens) have been disclosed in the art (see, e.g., Kanekiyo et al., Nature, 499:102-106, 2013).

[0389] In some embodiments, a subject is selected for treatment that has, or is at risk for developing, an RSV infection, for example, because of exposure or the possibility of exposure to RSV. Following administration of a therapeutically effective amount of the disclosed therapeutic compositions, the subject can be monitored for RSV infection, symptoms associated with RSV infection, or both. Because nearly all humans are infected with RSV by the age of 3, the entire birth cohort is included as a relevant population for immunization. This could be done, for example, by beginning an immunization regimen anytime from birth to 6 months of age, from 6 months of age to 5 years of age, in pregnant women (or women of child-bearing age) to protect their infants by passive transfer of antibody, family members of newborn infants or those still in utero, and subjects greater than 50 years of age.

[0390] Subjects at greatest risk of RSV infection with severe symptoms (e.g. requiring hospitalization) include children with prematurity, bronchopulmonary dysplasia, and congenital heart disease are most susceptible to severe disease. Atopy or a family history of atopy has also been associated with severe disease in infancy. During childhood and adulthood, disease is milder but can be associated with lower airway disease and is commonly complicated by sinusitis. Disease severity increases in the institutionalized elderly (e.g., humans over 65 years old). Severe disease also occurs in persons with severe combined immunodeficiency disease or following bone marrow or lung transplantation. (See, *e.g.,* Shay et al., JAMA,282:1440-6, 1999; Hall et al., N Engl J Med. 2009;360:588-598; Glezen et al., Am J Dis Child., 1986;140:543-546; and Graham, Immunol. Rev., 239:149-166, 2011). Thus, these subjects can be selected for administration of the disclosed PreF antigens, or a nucleic acid or a viral vector encoding, expressing or including a PreF antigen.

[0391] Typical subjects intended for treatment with the compositions and methods of the present disclosure include humans, as well as non-human primates and other animals, such as cattle. To identify subjects for prophylaxis or treatment according to the methods of the disclosure, screening methods employed to determine risk factors associated with a targeted or suspected disease or condition, or to determine the status of an existing disease or condition in a subject. These screening methods include, for example, conventional work-ups to determine environmental, familial, occupational, and other such risk factors that may be associated with the targeted or suspected disease or condition, as well as diagnostic methods, such as various ELISA and other immunoassay methods, which are available and well known in the art to detect and/or characterize RSV infection. These and other routine methods allow the clinician to select patients in need of therapy using the methods and pharmaceutical compositions of the disclosure. An immunogenic composition can be administered as an independent prophylaxis or treatment program, or as a follow-up, adjunct or coordinate treatment regimen to other treatments.

[0392] The immunogenic composition can be used in coordinate vaccination protocols or combinatorial formulations. In certain embodiments, combinatorial immunogenic compositions and coordinate immunization protocols employ separate immunogens or formulations, each directed toward eliciting an immune response to an RSV antigen, such as an immune response to RSV F protein. Separate immunogenic compositions that elicit the immune response to the RSV antigen can be combined in a polyvalent immunogenic composition administered to a subject in a single immunization step, or they can be administered separately (in monovalent immunogenic compositions) in a coordinate immunization protocol.

[0393] The administration of the immunogenic compositions can be for either prophylactic or therapeutic purpose. When provided prophylactically, the immunogenic composition is provided in advance of any symptom, for example in advance of infection. The prophylactic administration of the immunogenic compositions serves to prevent or ameliorate any subsequent infection. When provided therapeutically, the immunogenic composition is provided at or after the onset of a symptom of disease or infection, for example after development of a symptom of RSV infection, or after diagnosis of RSV infection. The immunogenic composition can thus be provided prior to the anticipated exposure to RSV so as to attenuate the anticipated severity, duration or extent of an infection and/or associated disease symptoms, after exposure or suspected exposure to the virus, or after the actual initiation of an infection.

[0394] Administration induces a sufficient immune response to treat or prevent the pathogenic infection, for example, to inhibit the infection and/or reduce the signs and/or symptoms of the infection. Amounts effective for this use will depend upon the severity of the disease, the general state of the subject's health, and the robustness of the subject's immune system. A therapeutically effective amount of the disclosed immunogenic compositions is that which provides either subjective relief of a symptom(s) or an objectively identifiable improvement as noted by the clinician or other qualified observer.

[0395] For prophylactic and therapeutic purposes, the immunogenic composition can be administered to the subject in a single bolus delivery, via continuous delivery (for example, continuous transdermal, mucosal or intravenous delivery) over an extended time period, or in a repeated administration protocol (for example, by an hourly, daily or weekly, repeated administration protocol). The therapeutically effective dosage of the immunogenic composition can be provided as repeated doses within a prolonged prophylaxis or treatment regimen that will yield clinically significant results to alleviate one or more symptoms or detectable conditions associated with a targeted disease or condition as set forth herein. Determination of effective dosages in this context is typically based on animal model studies followed up by human clinical trials and is guided by administration protocols that significantly reduce the occurrence or severity of targeted disease symptoms or conditions in the subject. Suitable models in this regard include, for example, murine, rat, porcine, feline, ferret, non-human primate, and other accepted animal model subjects known in the art. Alternatively, effective dosages can be determined using *in vitro* models (for example, immunologic and histopathologic assays). Using such models, only ordinary calculations and adjustments are required to determine an appropriate concentration and dose to administer a therapeutically effective amount of the immunogenic composition (for example, amounts that are effective to elicit a desired immune response or alleviate one or more symptoms of a targeted disease). In alternative embodiments, an effective amount or effective dose of the immunogenic composition may simply inhibit or enhance one or more selected biological activities correlated with a disease or condition, as set forth herein, for either therapeutic or diagnostic purposes.

[0396] In one embodiment, a suitable immunization regimen includes at least three separate inoculations with one or more immunogenic compositions, with a second inoculation being administered more than about two, about three to eight, or about four, weeks following the first inoculation. Generally, the third inoculation is administered several months after the second inoculation, and in specific embodiments, more than about five months after the first inoculation, more than about six months to about two years after the first inoculation, or about eight months to about one year after the first inoculation. Periodic inoculations beyond the third are also desirable to enhance the subject's "immune memory." The adequacy of the vaccination parameters chosen, e.g., formulation, dose, regimen and the like, can be determined by taking aliquots of serum from the subject and assaying antibody titers during the course of the immunization program. If such monitoring indicates that vaccination is sub-optimal, the subject can be boosted with an additional dose of immunogenic composition, and the vaccination parameters can be modified in a fashion expected to potentiate the immune response. It is contemplated that there can be several boosts, and that each boost can include the same or a different PreF antigen.

[0397] For prime-boost protocols, the prime can be administered as a single dose or multiple doses, for example two doses, three doses, four doses, five doses, six doses or more can be administered to a subject over days, weeks or months. The boost can be administered as a single dose or multiple doses, for example two to six doses, or more can be administered to a subject over a day, a week or months. Multiple boosts can also be given, such one to five, or more. Different dosages can be used in a series of sequential inoculations. For example a relatively large dose in a primary inoculation and then a boost with relatively smaller doses. The immune response against the selected antigenic surface can be generated by one or more inoculations of a subject with an immunogenic composition disclosed herein.

[0398] In some embodiments, the prime composition administered to the subject includes (or encodes) a recombinant RSV F protein that is a subtype A RSV F protein stabilized in a prefusion conformation, and the boost composition administered to the subject includes (or encodes) a recombinant RSV F protein that is a subtype B RSV F protein stabilized in a prefusion conformation. In some embodiments, the prime composition administered to the subject includes (or encodes) a recombinant RSV F protein that is a subtype B RSV F protein stabilized in a prefusion conformation, and the boost composition administered to the subject includes (or encodes) a recombinant RSV F protein that is a subtype A RSV F protein stabilized in a prefusion conformation.

[0399] In some embodiments, the methods include administering a composition including a recombinant subtype A RSV F protein stabilized in a prefusion conformation and a recombinant subtype B RSV F protein stabilized in a prefusion conformation once, or more than one (such as in a prime-boost protocol) as a series of injections.

[0400] In some embodiments, the methods include administering a composition including a ferritin nanoparticle including a recombinant subtype A RSV F protein stabilized in a prefusion conformation and ferritin nanoparticle including a recombinant subtype B RSV F protein stabilized in a prefusion conformation, once, or more than one (such as in a prime-boost protocol) as a series of injections.

[0401] In some embodiments, the methods include administering a composition including a vector encoding a recombinant subtype A RSV F protein stabilized in a prefusion conformation and vector encoding a recombinant subtype B RSV F protein stabilized in a prefusion conformation, once, or more than one (such as in a prime-boost protocol) as a series of injections. In some embodiments, the method can further include administration of a composition including recombinant subtype A RSV F protein stabilized in a prefusion conformation and recombinant subtype B RSV F protein stabilized in a prefusion conformation, and/or a composition including a ferritin nanoparticle including a recombinant subtype A RSV F protein stabilized in a prefusion conformation and ferritin nanoparticle including a recombinant subtype B RSV F protein stabilized in a prefusion conformation.

[0402] In some embodiments, the methods include administering a composition including a nucleic acid molecule encoding a recombinant subtype A RSV F protein stabilized in a prefusion conformation and nucleic acid molecule encoding a recombinant subtype B RSV F protein stabilized in a prefusion conformation once, or more than one (such as in a prime-boost protocol) as a series of injections. In some embodiments, the method can further include administration of a composition including recombinant subtype A RSV F protein stabilized in a prefusion conformation and recombinant subtype B RSV F protein stabilized in a prefusion conformation, and/or a composition including a ferritin nanoparticle including a recombinant subtype A RSV F protein stabilized in a prefusion conformation and ferritin nanoparticle including a recombinant subtype B RSV F protein stabilized in a prefusion conformation.

[0403] In some embodiments, the prime and boost compositions administered to the subject each include (or encode) a first recombinant RSV F protein that is a subtype A RSV F protein stabilized in a prefusion conformation, and a second recombinant RSV F protein that is a subtype B RSV F protein stabilized in a prefusion conformation. In several embodiments, the prime and boost compositions administered to the subject each include (or encode) a mixture (such as about a 1:1, 1:2, 2:1, 2:3, 3:2, 1:3, 3:1, 1:4, 4:1, 3:5, 5:3, 1:5, 5:1, 5:7, 7:5 mixture), of a first recombinant RSV F protein that is a subtype A RSV F protein stabilized in a prefusion conformation, and a second recombinant RSV F protein that is a subtype B RSV F protein stabilized in a prefusion conformation.

[0404] In some embodiments the prime and boost compositions administered to the subject each include a recombinant RSV F protein that is a subtype A RSV F protein stabilized in a prefusion conformation by the substitutions S155C, S290C, S190F, and V207L, and a second recombinant RSV F protein that is a subtype B

RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions).

[0405] In some embodiments the prime and boost compositions administered to the subject each include a nucleic acid molecule encoding a recombinant RSV F protein that is a subtype A RSV F protein stabilized in a prefusion conformation by the substitutions S155C, S290C, S190F, and V207L, and a nucleic acid molecule encoding a recombinant RSV F protein that is a subtype B RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions).

[0406] In some embodiments the prime and boost compositions administered to the subject each include a first protein nanoparticle (such as a ferritin nanoparticle) including a recombinant RSV F protein that is a subtype A RSV F protein stabilized in a prefusion conformation by the substitutions S155C, S290C, S190F, and V207L, and a second protein nanoparticle (such as a ferritin nanoparticle) including a recombinant RSV F protein that is a subtype B RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions).

[0407] In some embodiments the prime and boost compositions administered to the subject each include a vector including or encoding a recombinant RSV F protein that is a subtype A RSV F protein stabilized in a prefusion conformation by the substitutions S155C, S290C, S190F, and V207L, and a vector including or encoding a recombinant RSV F protein that is a subtype B RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions).

[0408] In some embodiments the prime composition administered to the subject includes a first nucleic acid molecule (such as a DNA plasmid expression vector) encoding a recombinant RSV F protein that is a subtype A RSV F protein stabilized in a prefusion conformation by the substitutions S155C, S290C, S190F, and V207L substitutions), and a second nucleic acid molecule (such as an expression vector) including a recombinant RSV F protein that is a subtype B RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions), and the boost composition administered to the subject includes a first protein nanoparticle (such as a ferritin nanoparticle) including a recombinant RSV F protein that is a subtype A RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions), and a second protein nanoparticle (such as a ferritin nanoparticle) including a recombinant RSV F protein that is a subtype B RSV F protein stabilized in a prefusion conformation by any of the substitutions disclosed herein (such as S155C, S290C, and S190F substitutions or S155C, S290C, S190F, and V207L substitutions).

[0409] Immunization protocols using a DNA plasmid prime and ferritin nanoparticle boost are known to the person of ordinary skill in the art (see, e.g., Wei et al., Science, 329(5995): 1060-4, 2010).

[0410] The actual dosage of the immunogenic composition will vary according to factors such as the disease indication and particular status of the subject (for example, the subject's age, size, fitness, extent of symptoms, susceptibility factors, and the like), time and route of administration, other drugs or treatments being administered concurrently, as well as the specific pharmacology of the immunogenic composition for eliciting the desired activity or biological response in the subject. Dosage regimens can be adjusted to provide an optimum prophylactic or therapeutic response. As described above in the forgoing listing of terms, an effective amount is also one in which any toxic or detrimental side effects of the disclosed antigen and/or other biologically active agent is outweighed in clinical terms by therapeutically beneficial effects.

[0411] A non-limiting range for a therapeutically effective amount of the disclosed PreF antigens within the methods and immunogenic compositions of the disclosure is about 0.0001 mg/kg body weight to about 10 mg/kg body weight, such as about 0.01 mg/kg, about 0.02 mg/kg, about 0.03 mg/kg, about 0.04 mg/kg, about 0.05 mg/kg, about 0.06 mg/kg, about 0.07 mg/kg, about 0.08 mg/kg, about 0.09 mg/kg, about 0.1 mg/kg, about 0.2 mg/kg, about 0.3 mg/kg, about 0.4 mg/kg, about 0.5 mg/kg, about 0.6 mg/kg, about 0.7 mg/kg, about 0.8 mg/kg, about 0.9 mg/kg, about 1 mg/kg, about 1.5 mg/kg, about 2 mg/kg, about 2.5 mg/kg, about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, or about 10 mg/kg, for example 0.01 mg/kg to about 1 mg/kg body weight, about 0.05 mg/kg to about 5 mg/kg body weight, about 0.2 mg/kg to about 2 mg/kg body weight, or about 1.0 mg/kg to about 10 mg/kg body weight.

[0412] In some embodiments, the dosage a set amount of a disclosed PreF antigen, or a nucleic acid or a viral vector encoding, expressing or including a PreF antigen includes for children, adults, elderly, etc., such as from about 1-300 µg, for example, a dosage of about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, or about 300 µg of the PreF antigens, or a nucleic acid or a viral vector encoding, expressing or including a PreF antigen. The dosage and number of doses will depend on the setting, for example, in an adult or anyone primed by prior RSV infection or immunization, a single dose may be a sufficient booster. In naive infants, in some examples, at least two doses would be given, for example, at least three doses. In some embodiments, an annual boost is given to elderly subjects (e.g., humans over age 60) once per year, for example, along with an annual influenza vaccination. Methods for preparing administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as Remingtons Pharmaceutical Sciences, 19th Ed., Mack Publishing Company, Easton, Pennsylvania, 1995.

[0413] Dosage can be varied by the attending clinician to maintain a desired concentration at a target site (for example, systemic circulation). Higher or lower concentrations can be selected based on the mode of delivery, for example, trans-epidermal, rectal, oral, pulmonary, or intranasal delivery versus intravenous or subcutaneous delivery. Dosage can also be adjusted based on the release rate of the administered formulation, for example, of an intrapulmonary spray versus powder, sustained release oral versus injected particulate or transdermal delivery formulations, and so forth. To achieve the same serum concentration level, for example, slow-release particles with a release rate of 5 nanomolar (under standard conditions) would be administered at about twice the dosage of particles with a release rate of 10 nanomolar.

[0414] Upon administration of an immunogenic composition of this disclosure, the immune system of the subject typically responds to the immunogenic composition by producing antibodies specific for the prefusion conformation of the RSV F protein. Such a response signifies that an effective dose of the immunogenic composition was delivered.

[0415] In several embodiments, it may be advantageous to administer the immunogenic compositions disclosed herein with other agents such as proteins, peptides, antibodies, and other antiviral agents, such as anti-RSV agents. Non-limiting examples of anti-RSV agents include the monoclonal antibody palivizumab (SYNAGIS®; Medimmune, Inc.) and the small molecule anti-viral drug ribavirin (manufactured by many sources, e.g., Warrick Pharmaceuticals, Inc.). In certain embodiments, immunogenic compositions are administered concurrently with other anti-RSV agents. In certain embodiments, the immunogenic compositions are administered sequentially with other anti-RSV therapeutic agents, such as before or after the other agent. One of ordinary skill in the art would know that sequential administration can mean immediately following or after an appropriate period of time, such as hours, days, weeks, months, or even years later.

[0416] In additional embodiments, a therapeutically effective amount of a pharmaceutical composition including a nucleic acid encoding a disclosed PreF antigen is administered to a subject in order to generate an immune response. In one specific, non-limiting example, a therapeutically effective amount of a nucleic acid encoding a disclosed antigen is administered to a subject to treat or prevent or inhibit RSV infection.

[0417] One approach to administration of nucleic acids is direct immunization with plasmid DNA, such as with a mammalian expression plasmid. As described above, the nucleotide sequence encoding a disclosed antigen can be placed under the control of a promoter to increase expression of the molecule. Another approach would use RNA (such as Nonviral delivery of self-amplifying RNA vaccines, see e.g., Geall et al., Proc Natl Acad Sci USA, 109:14604-9, 2012.

[0418] Immunization by nucleic acid constructs is well known in the art and taught, for example, in U.S. Patent No. 5,643,578 (which describes methods of immunizing vertebrates by introducing DNA encoding a desired antigen to elicit a cell-mediated or a humoral response), and U.S. Patent No. 5,593,972 and U.S. Patent No. 5,817,637 (which describe operably linking a nucleic acid sequence encoding an antigen to regulatory sequences enabling expression). U.S. Patent No. 5,880,103 describes several methods of delivery of nucleic acids encoding immunogenic peptides or other antigens to an organism. The methods include liposomal delivery of the nucleic acids (or of the synthetic peptides themselves), and immune-stimulating constructs, or ISCOMS™, negatively charged cage-like structures of 30-40 nm in size formed spontaneously on mixing cholesterol and Quil A™ (saponin). Protective immunity has been generated in a variety of experimental models of infection, including toxoplasmosis and Epstein-Barr virus-induced tumors, using ISCOMS™ as the delivery vehicle for antigens (Mowat and Donachie, Immunol. Today 12:383, 1991). Doses of antigen as low as 1 µg encapsulated in ISCOMS™ have been found to produce Class I mediated CTL responses (Takahashi et al., Nature 344:873, 1990).

[0419] In another approach to using nucleic acids for immunization, a disclosed antigen can also be expressed by attenuated viral hosts or vectors or bacterial vectors. Recombinant vaccinia virus, adenovirus, adeno-associated virus (AAV), herpes virus, retrovirus, cytomegalovirus or other viral vectors can be used to express the peptide or protein, thereby eliciting a CTL response. For example, vaccinia vectors and methods useful in immunization protocols are described in U.S. Patent No. 4,722,848. BCG (Bacillus Calmette Guerin) provides another vector for expression of the peptides (see Stover, Nature 351:456-460, 1991).

[0420] In one embodiment, a nucleic acid encoding a disclosed PreF antigen is introduced directly into cells. For example, the nucleic acid can be loaded onto gold microspheres by standard methods and introduced into the skin by a device such as Bio-Rad's HELIOS™ Gene Gun. The nucleic acids can be "naked," consisting of plasmids under control of a strong promoter. Typically, the DNA is injected into muscle, although it can also be injected directly into other sites, including tissues in proximity to metastases. Dosages for injection are usually around 0.5 µg/kg to about 50 mg/kg, and typically are about 0.005 mg/kg to about 5 mg/kg (see, *e.g.,* U.S. Patent No. 5,589,466).

[0421] In addition to the therapeutic methods provided above, any of the disclosed PreF antigens can be utilized to produce antigen specific immunodiagnostic reagents, for example, for serosurveillance. Immunodiagnostic reagents can be designed from any of the antigens described herein. For example, in the case of the disclosed antigens, the presence of serum antibodies to RSV is monitored using the isolated antigens disclosed herein, such as to detect an RSV infection and/or the presence of antibodies that specifically bind to the prefusion conformation of RSV F protein.

[0422] Generally, the method includes contacting a sample from a subject, such as, but not limited to a blood, serum, plasma, urine or sputum sample from the subject with one or more of the RSV F protein antigen stabilized in a prefusion conformation disclosed herein and detecting binding of antibodies in the sample to the disclosed immunogens. The binding can be detected by any means known to one of skill in the art, including the use of labeled secondary antibodies that specifically bind the antibodies from the sample. Labels include radiolabels, enzymatic labels, and fluorescent labels.

[0423] In addition, the detection of the prefusion RSV F binding antibody also allows the response of the subject to immunization with the disclosed antigen to be monitored. In still other embodiments, the titer of the prefusion RSV F antibody binding antibodies is determined. The binding can be detected by any means known to one of skill in the art, including the use of labeled secondary antibodies that specifically bind the antibodies from the sample. Labels include radiolabels, enzymatic labels, and fluorescent labels. In other embodiments, a disclosed immunogen is used to isolate antibodies present in a subject or biological sample obtained from a subject.

G. Kits

[0424] Kits are also provided. For example, kits for treating or preventing an RSV infection in a subject, or for detecting the presence of RSV F protein prefusion specific antibodies in the sera of a subject. The kits will typically include one or more of the PreF antigens, or a nucleic acid or a viral vector encoding, expressing or including the antigen.

[0425] The kit can include a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container typically holds a composition including one or more of the disclosed PreF antigens, or a nucleic acid or a viral vector encoding, expressing or including the antigen, which is effective for treating or preventing RSV infection. In several embodiments the container may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The label or package insert indicates that the composition is used for treating the particular condition.

[0426] The label or package insert typically will further include instructions for use of a PreF antigen, or a nucleic acid or a viral vector encoding, expressing or including the antigen, for example, in a method of treating or preventing a RSV infection. The package insert typically includes instructions customarily included in commercial packages of therapeutic products that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products. The instructional materials may be written, in an electronic form (such as a computer diskette or compact disk) or may be visual (such as video files). The kits may also include additional components to facilitate the particular application for which the kit is designed. The kits may additionally include buffers and other reagents routinely used for the practice of a particular method. Such kits and appropriate contents are well known to those of skill in the art.

[0427] As used herein, reference to:

"any one of SEQ ID NOs: 1-184" refers to "SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 105, SEQ ID NO: 106, SEQ ID NO: 107, SEQ

ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 121, SEQ ID NO: 122, SEQ ID NO: 123, SEQ ID NO: 124, SEQ ID NO: 125, SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, SEQ ID NO: 130, SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137, SEQ ID NO: 138, SEQ ID NO: 139, SEQ ID NO: 140, SEQ ID NO: 141, SEQ ID NO: 142, SEQ ID NO: 143, SEQ ID NO: 144, SEQ ID NO: 145, SEQ ID NO: 146, SEQ ID NO: 147, SEQ ID NO: 148, SEQ ID NO: 149, SEQ ID NO: 150, SEQ ID NO: 151, SEQ ID NO: 152, SEQ ID NO: 153, SEQ ID NO: 154, SEQ ID NO: 155, SEQ ID NO: 156, SEQ ID NO: 157, SEQ ID NO: 158, SEQ ID NO: 159, SEQ ID NO: 160, SEQ ID NO: 161, SEQ ID NO: 162, SEQ ID NO: 163, SEQ ID NO: 164, SEQ ID NO: 165, SEQ ID NO: 166, SEQ ID NO: 167, SEQ ID NO: 168, SEQ ID NO: 169, SEQ ID NO: 170, SEQ ID NO: 171, SEQ ID NO: 172, SEQ ID NO: 173, SEQ ID NO: 174, SEQ ID NO: 175, SEQ ID NO: 176, SEQ ID NO: 177, SEQ ID NO: 178, SEQ ID NO: 179, SEQ ID NO: 180, SEQ ID NO: 181, SEQ ID NO: 182, SEQ ID NO: 183, or SEQ ID NO: 184"

"SEQ ID NOs: 698-828" refers to "any one of "SEQ ID NO: 699, SEQ ID NO: 700, SEQ ID NO: 701, SEQ ID NO: 702, SEQ ID NO: 703, SEQ ID NO: 704, SEQ ID NO: 705, SEQ ID NO: 706, SEQ ID NO: 707, SEQ ID NO: 708, SEQ ID NO: 709, SEQ ID NO: 710, SEQ ID NO: 711, SEQ ID NO: 712, SEQ ID NO: 713, SEQ ID NO: 714, SEQ ID NO: 715, SEQ ID NO: 716, SEQ ID NO: 717, SEQ ID NO: 718, SEQ ID NO: 719, SEQ ID NO: 720, SEQ ID NO: 721, SEQ ID NO: 722, SEQ ID NO: 723, SEQ ID NO: 724, SEQ ID NO: 725, SEQ ID NO: 726, SEQ ID NO: 727, SEQ ID NO: 728, SEQ ID NO: 729, SEQ ID NO: 730, SEQ ID NO: 731, SEQ ID NO: 732, SEQ ID NO: 733, SEQ ID NO: 734, SEQ ID NO: 735, SEQ ID NO: 736, SEQ ID NO: 737, SEQ ID NO: 738, SEQ ID NO: 739, SEQ ID NO: 740, SEQ ID NO: 741, SEQ ID NO: 742, SEQ ID NO: 743, SEQ ID NO: 744, SEQ ID NO: 745, SEQ ID NO: 746, SEQ ID NO: 747, SEQ ID NO: 748, SEQ ID NO: 749, SEQ ID NO: 750, SEQ ID NO: 751, SEQ ID NO: 752, SEQ ID NO: 753, SEQ ID NO: 754, SEQ ID NO: 755, SEQ ID NO: 756, SEQ ID NO: 757, SEQ ID NO: 758, SEQ ID NO: 759, SEQ ID NO: 760, SEQ ID NO: 761, SEQ ID NO: 762, SEQ ID NO: 763, SEQ ID NO: 764, SEQ ID NO: 765, SEQ ID NO: 766, SEQ ID NO: 767, SEQ ID NO: 768, SEQ ID NO: 769, SEQ ID NO: 770, SEQ ID NO: 771, SEQ ID NO: 772, SEQ ID NO: 773, SEQ ID NO: 774, SEQ ID NO: 775, SEQ ID NO: 776, SEQ ID NO: 777, SEQ ID NO: 778, SEQ ID NO: 779, SEQ ID NO: 780, SEQ ID NO: 781, SEQ ID NO: 782, SEQ ID NO: 783, SEQ ID NO: 784, SEQ ID NO: 785, SEQ ID NO: 786, SEQ ID NO: 787, SEQ ID NO: 788, SEQ ID NO: 789, SEQ ID NO: 790, SEQ ID NO: 791, SEQ ID NO: 792, SEQ ID NO: 793, SEQ ID NO: 794, SEQ ID NO: 795, SEQ ID NO: 796, SEQ ID NO: 797, SEQ ID NO: 798, SEQ ID NO: 799, SEQ ID NO: 800, SEQ ID NO: 801, SEQ ID NO: 802, SEQ ID NO: 803, SEQ ID NO: 804, SEQ ID NO: 805, SEQ ID NO: 806, SEQ ID NO: 807, SEQ ID NO: 808, SEQ ID NO: 809, SEQ ID NO: 810, SEQ ID NO: 811, SEQ ID NO: 812, SEQ ID NO: 813, SEQ ID NO: 814, SEQ ID NO: 815, SEQ ID NO: 816, SEQ ID NO: 817, SEQ ID NO: 818, SEQ ID NO: 819, SEQ ID NO: 820, SEQ ID NO: 821, SEQ ID NO: 822, SEQ ID NO: 823, SEQ ID NO: 824, SEQ ID NO: 825, SEQ ID NO: 826, SEQ ID NO: 827, or SEQ ID NO: 828."

"SEQ ID NOs: 1474-1478" refers to "any one of SEQ ID NO: 1474, SEQ ID NO: 1475, SEQ ID NO: 1476, SEQ ID NO: 1477, or SEQ ID NO: 1478."

"SEQ ID NOs: 829-1025" refers to "any one of SEQ ID NO: 829, SEQ ID NO: 830, SEQ ID NO: 831, SEQ ID NO: 832, SEQ ID NO: 833, SEQ ID NO: 834, SEQ ID NO: 835, SEQ ID NO: 836, SEQ ID NO: 837, SEQ ID NO: 838, SEQ ID NO: 839, SEQ ID NO: 840, SEQ ID NO: 841, SEQ ID NO: 842, SEQ ID NO: 843, SEQ ID NO: 844, SEQ ID NO: 845, SEQ ID NO: 846, SEQ ID NO: 847, SEQ ID NO: 848, SEQ ID NO: 849, SEQ ID NO: 850, SEQ ID NO: 851, SEQ ID NO: 852, SEQ ID NO: 853, SEQ ID NO: 854, SEQ ID NO: 855, SEQ ID NO: 856, SEQ ID NO: 857, SEQ ID NO: 858, SEQ ID NO: 859, SEQ ID NO: 860, SEQ ID NO: 861, SEQ ID NO: 862, SEQ ID NO: 863, SEQ ID NO: 864, SEQ ID NO: 865, SEQ ID NO: 866, SEQ ID NO: 867, SEQ ID NO: 868, SEQ ID NO: 869, SEQ ID NO: 870, SEQ ID NO: 871, SEQ ID NO: 872, SEQ ID NO: 873, SEQ ID NO: 874, SEQ ID NO: 875, SEQ ID NO: 876, SEQ ID NO: 877, SEQ ID NO: 878, SEQ ID NO: 879, SEQ ID NO: 880, SEQ ID NO: 881, SEQ ID NO: 882, SEQ ID NO: 883, SEQ ID NO: 884, SEQ ID NO: 885, SEQ ID NO: 886, SEQ ID NO: 887, SEQ ID NO: 888, SEQ ID NO: 889, SEQ ID NO: 890, SEQ ID NO: 891, SEQ ID NO: 892, SEQ ID NO: 893, SEQ ID NO: 894, SEQ ID NO: 895, SEQ ID NO: 896, SEQ ID NO: 897, SEQ ID NO: 898, SEQ ID NO: 899, SEQ ID NO: 900, SEQ ID NO: 901, SEQ ID NO: 902, SEQ ID NO: 903, SEQ ID NO: 904, SEQ ID NO: 905, SEQ ID NO: 906, SEQ ID NO: 907, SEQ ID NO: 908, SEQ ID NO: 909, SEQ ID NO: 910, SEQ ID NO: 911, SEQ ID NO: 912, SEQ ID NO: 913, SEQ ID NO: 914, SEQ ID NO: 915, SEQ ID NO: 916, SEQ ID NO: 917, SEQ ID NO: 918, SEQ ID NO: 919, SEQ ID NO: 920, SEQ ID NO: 921, SEQ ID NO: 922, SEQ ID NO: 923, SEQ ID NO: 924, SEQ ID NO: 925, SEQ ID NO: 926, SEQ ID NO: 927, SEQ ID NO: 928, SEQ ID NO: 929, SEQ ID NO: 930, SEQ ID NO: 931, SEQ ID NO: 932, SEQ ID NO: 933, SEQ ID NO: 934, SEQ ID NO: 935, SEQ ID NO: 936, SEQ ID NO: 937, SEQ ID NO: 938, SEQ ID NO: 939, SEQ ID NO: 940, SEQ ID NO: 941, SEQ ID NO: 942, SEQ ID NO: 943, SEQ ID NO: 944, SEQ ID NO: 945, SEQ ID NO: 946, SEQ ID NO: 947, SEQ ID NO: 948, SEQ ID NO: 949, SEQ ID NO: 950, SEQ ID NO: 951, SEQ ID NO: 952, SEQ ID NO: 953, SEQ ID NO: 954, SEQ ID NO: 955, SEQ ID NO: 956, SEQ ID NO: 957, SEQ ID NO: 958, SEQ ID NO: 959, SEQ ID NO: 960, SEQ ID NO: 961, SEQ ID NO: 962, SEQ ID NO: 963, SEQ ID NO: 964, SEQ ID NO: 965, SEQ ID NO: 966, SEQ ID NO: 967, SEQ ID NO: 968, SEQ ID NO: 969, SEQ ID NO: 970, SEQ ID NO: 971, SEQ ID NO: 972, SEQ ID NO: 973, SEQ ID NO: 974, SEQ ID NO: 975, SEQ ID NO: 976, SEQ ID NO: 977, SEQ ID NO: 978, SEQ ID NO: 979, SEQ ID NO: 980, SEQ ID NO: 981, SEQ ID NO: 982, SEQ ID NO: 983, SEQ ID NO: 984, SEQ ID NO: 985, SEQ ID NO: 986, SEQ ID NO: 987, SEQ ID NO: 988, SEQ ID NO: 989, SEQ ID NO: 990, SEQ ID NO: 991, SEQ ID NO: 992, SEQ ID NO: 993, SEQ ID NO: 994, SEQ ID NO: 995, SEQ ID NO: 996, SEQ ID NO: 997, SEQ ID NO: 998, SEQ ID NO: 999, SEQ ID NO: 1000, SEQ ID NO: 1001, SEQ ID NO: 1002, SEQ ID NO: 1003, SEQ ID NO: 1004, SEQ ID NO: 1005, SEQ ID NO: 1006, SEQ ID NO: 1007, SEQ ID NO: 1008, SEQ ID NO: 1009, SEQ ID NO: 1010, SEQ ID NO: 1011, SEQ ID NO: 1012, SEQ ID NO: 1013, SEQ ID NO: 1014, SEQ ID NO: 1015, SEQ ID NO: 1016, SEQ ID NO: 1017, SEQ ID NO: 1018, SEQ ID NO: 1019, SEQ ID NO: 1020, SEQ ID NO: 1021, SEQ ID NO: 1022, SEQ ID NO: 1023, SEQ ID NO: 1024, or SEQ ID NO: 1025."

"SEQ ID NOs: 969-1025" refers to "any one of SEQ ID NO: 969, SEQ ID NO: 970, SEQ ID NO: 971, SEQ ID NO: 972, SEQ ID NO: 973, SEQ ID NO: 974, SEQ ID NO: 975, SEQ ID NO: 976, SEQ ID NO: 977, SEQ ID NO: 978, SEQ ID NO: 979, SEQ ID NO: 980, SEQ ID NO: 981, SEQ ID NO: 982, SEQ ID NO: 983, SEQ ID NO: 984, SEQ ID NO: 985, SEQ ID NO: 986, SEQ ID NO: 987, SEQ ID NO: 988, SEQ ID NO: 989, SEQ ID NO: 990, SEQ ID NO: 991, SEQ ID NO: 992, SEQ ID NO: 993, SEQ ID NO: 994, SEQ ID NO: 995, SEQ ID NO: 996, SEQ ID NO: 997, SEQ ID NO: 998, SEQ ID NO: 999, SEQ ID NO: 1000, SEQ ID NO: 1001, SEQ ID NO: 1002, SEQ ID NO: 1003, SEQ ID NO: 1004, SEQ ID NO: 1005, SEQ ID NO: 1006, SEQ ID NO: 1007, SEQ ID NO: 1008, SEQ ID NO: 1009, SEQ ID NO: 1010, SEQ ID NO: 1011, SEQ ID NO: 1012, SEQ ID NO: 1013, SEQ ID NO: 1014, SEQ ID NO: 1015, SEQ ID NO: 1016, SEQ ID NO: 1017, SEQ ID NO: 1018, SEQ ID NO: 1019, SEQ ID NO: 1020, SEQ ID NO: 1021, SEQ ID NO: 1022, SEQ ID NO: 1023, SEQ ID NO: 1024, or SEQ ID NO: 1025."

"SEQ ID NOs: 901-968" refers to "any one of SEQ ID NO: 901, SEQ ID NO: 902, SEQ ID NO: 903, SEQ ID NO: 904, SEQ ID NO: 905, SEQ ID NO: 906, SEQ ID NO: 907, SEQ ID NO: 908, SEQ ID NO: 909, SEQ ID NO: 910, SEQ ID NO: 911, SEQ ID NO: 912, SEQ ID NO: 913, SEQ ID NO: 914, SEQ ID NO: 915, SEQ ID NO: 916, SEQ ID NO: 917, SEQ ID NO: 918, SEQ ID NO: 919, SEQ ID NO: 920, SEQ ID NO: 921, SEQ ID NO: 922, SEQ ID NO: 923, SEQ ID NO: 924, SEQ ID NO: 925, SEQ ID NO: 926, SEQ ID NO: 927, SEQ ID NO: 928, SEQ ID NO: 929, SEQ ID NO: 930, SEQ ID NO: 931, SEQ ID NO: 932, SEQ ID NO: 933, SEQ ID NO: 934, SEQ ID NO: 935, SEQ ID NO: 936, SEQ ID NO: 937, SEQ ID NO: 938, SEQ ID NO: 939, SEQ ID NO: 940, SEQ ID NO: 941, SEQ ID NO: 942, SEQ ID NO: 943, SEQ ID NO: 944, SEQ ID NO: 945, SEQ ID NO: 946, SEQ ID NO: 947, SEQ ID NO: 948, SEQ ID NO: 949, SEQ ID NO: 950, SEQ ID NO: 951, SEQ ID NO: 952, SEQ ID NO: 953, SEQ ID NO: 954, SEQ ID NO: 955, SEQ ID NO: 956, SEQ ID NO: 957, SEQ ID NO: 958, SEQ ID NO: 959, SEQ ID NO: 960, SEQ ID NO: 961, SEQ ID NO: 962, SEQ ID NO: 963, SEQ ID NO: 964, SEQ ID NO: 965, SEQ ID NO: 966, SEQ ID NO: 967, or SEQ ID NO: 968."

"SEQ ID NOs: 1027-1218" refers to "any one of SEQ ID NO: 1027, SEQ ID NO: 1028, SEQ ID NO: 1029, SEQ ID NO: 1030, SEQ ID NO: 1031, SEQ ID NO: 1032, SEQ ID NO: 1033, SEQ ID NO: 1034, SEQ ID NO: 1035, SEQ ID NO: 1036, SEQ ID NO: 1037, SEQ ID NO: 1038, SEQ ID NO: 1039, SEQ ID NO: 1040, SEQ ID NO: 1041, SEQ ID NO: 1042, SEQ ID NO: 1043, SEQ ID NO: 1044, SEQ ID NO: 1045, SEQ ID NO: 1046, SEQ ID NO: 1047, SEQ ID NO: 1048, SEQ ID NO: 1049, SEQ ID NO: 1050, SEQ ID NO: 1051, SEQ ID NO: 1052, SEQ ID NO: 1053, SEQ ID NO: 1054, SEQ ID NO: 1055, SEQ ID NO: 1056, SEQ ID NO: 1057, SEQ ID NO: 1058, SEQ ID NO: 1059, SEQ ID NO: 1060, SEQ ID NO: 1061, SEQ ID NO: 1062, SEQ ID NO: 1063, SEQ ID NO: 1064, SEQ ID NO: 1065, SEQ ID NO: 1066, SEQ ID NO: 1067, SEQ ID NO: 1068, SEQ ID NO: 1069, SEQ ID NO: 1070, SEQ ID NO: 1071, SEQ ID NO: 1072, SEQ ID NO: 1073, SEQ ID NO: 1074, SEQ ID NO: 1075, SEQ ID NO: 1076, SEQ ID NO: 1077, SEQ ID NO: 1078, SEQ ID NO: 1079, SEQ ID NO: 1080, SEQ ID NO: 1081, SEQ ID NO: 1082, SEQ ID NO: 1083, SEQ ID NO: 1084, SEQ ID NO: 1085, SEQ ID NO: 1086, SEQ ID NO: 1087, SEQ ID NO: 1088, SEQ ID NO: 1099, SEQ ID NO: 1100, SEQ ID NO: 1101, SEQ ID NO: 1102, SEQ ID NO: 1103, SEQ ID NO: 1104, SEQ ID NO: 1105, SEQ ID NO: 1106, SEQ ID NO: 1107, SEQ ID NO: 1108, SEQ ID NO: 1109, SEQ ID NO: 1110, SEQ ID NO: 1111, SEQ ID NO: 1112, SEQ ID NO: 1113, SEQ ID NO: 1114, SEQ ID NO: 1115, SEQ ID NO: 1116, SEQ ID NO: 1117, SEQ ID NO: 1118, SEQ ID NO: 1119, SEQ ID NO: 1120, SEQ ID NO: 1121, SEQ ID NO: 1122, SEQ ID NO: 1123, SEQ ID NO: 1124, SEQ ID NO: 1125, SEQ ID NO: 1126, SEQ ID NO: 1127, SEQ ID NO: 1128,

SEQ ID NO: 1129, SEQ ID NO: 1130, SEQ ID NO: 1131, SEQ ID NO: 1132, SEQ ID NO: 1133, SEQ ID NO: 1134, SEQ ID NO: 1135, SEQ ID NO: 1136, SEQ ID NO: 1137, SEQ ID NO: 1138, SEQ ID NO: 1139, SEQ ID NO: 1140, SEQ ID NO: 1141, SEQ ID NO: 1142, SEQ ID NO: 1143, SEQ ID NO: 1144, SEQ ID NO: 1145, SEQ ID NO: 1146, SEQ ID NO: 1147, SEQ ID NO: 1148, SEQ ID NO: 1149, SEQ ID NO: 1150, SEQ ID NO: 1151, SEQ ID NO: 1152, SEQ ID NO: 1153, SEQ ID NO: 1154, SEQ ID NO: 1155, SEQ ID NO: 1156, SEQ ID NO: 1157, SEQ ID NO: 1158, SEQ ID NO: 1159, SEQ ID NO: 1160, SEQ ID NO: 1161, SEQ ID NO: 1162, SEQ ID NO: 1163, SEQ ID NO: 1164, SEQ ID NO: 1165, SEQ ID NO: 1166, SEQ ID NO: 1167, SEQ ID NO: 1168, SEQ ID NO: 1169, SEQ ID NO: 1170, SEQ ID NO: 1171, SEQ ID NO: 1172, SEQ ID NO: 1173, SEQ ID NO: 1174, SEQ ID NO: 1175, SEQ ID NO: 1176, SEQ ID NO: 1177, SEQ ID NO: 1178, SEQ ID NO: 1179, SEQ ID NO: 1180, SEQ ID NO: 1181, SEQ ID NO: 1182, SEQ ID NO: 1183, SEQ ID NO: 1184, SEQ ID NO: 1185, SEQ ID NO: 1186, SEQ ID NO: 1187, SEQ ID NO: 1188, SEQ ID NO: 1189, SEQ ID NO: 1190, SEQ ID NO: 1191, SEQ ID NO: 1192, SEQ ID NO: 1193, SEQ ID NO: 1194, SEQ ID NO: 1195, SEQ ID NO: 1196, SEQ ID NO: 1197, SEQ ID NO: 1198, SEQ ID NO: 1199, SEQ ID NO: 1200, SEQ ID NO: 1201, SEQ ID NO: 1202, SEQ ID NO: 1203, SEQ ID NO: 1204, SEQ ID NO: 1205, SEQ ID NO: 1206, SEQ ID NO: 1207, SEQ ID NO: 1208, SEQ ID NO: 1209, SEQ ID NO: 1210, SEQ ID NO: 1211, SEQ ID NO: 1212, SEQ ID NO: 1213, SEQ ID NO: 1214, SEQ ID NO: 1215, SEQ ID NO: 1216, SEQ ID NO: 1217, or SEQ ID NO: 1218."

"SEQ ID NOs: 1429-1442" refers to "any one of SEQ ID NO: 1429, SEQ ID NO: 1430, SEQ ID NO: 1431, SEQ ID NO: 1432, SEQ ID NO: 1433, SEQ ID NO: 1434, SEQ ID NO: 1435, SEQ ID NO: 1436, SEQ ID NO: 1437, SEQ ID NO: 1438, SEQ ID NO: 1439, SEQ ID NO: 1440, SEQ ID NO: 1441, or SEQ ID NO: 1442."

"SEQ ID NOs: 1219-1428" refers to any one of SEQ ID NO: 1219, SEQ ID NO: 1220, SEQ ID NO: 1221, SEQ ID NO: 1222, SEQ ID NO: 1223, SEQ ID NO: 1224, SEQ ID NO: 1225, SEQ ID NO: 1226, SEQ ID NO: 1227, SEQ ID NO: 1228, SEQ ID NO: 1229, SEQ ID NO: 1230, SEQ ID NO: 1231, SEQ ID NO: 1232, SEQ ID NO: 1233, SEQ ID NO: 1234, SEQ ID NO: 1235, SEQ ID NO: 1236, SEQ ID NO: 1237, SEQ ID NO: 1238, SEQ ID NO: 1239, SEQ ID NO: 1240, SEQ ID NO: 1241, SEQ ID NO: 1242, SEQ ID NO: 1243, SEQ ID NO: 1244, SEQ ID NO: 1245, SEQ ID NO: 1246, SEQ ID NO: 1247, SEQ ID NO: 1248, SEQ ID NO: 1249, SEQ ID NO: 1250, SEQ ID NO: 1251, SEQ ID NO: 1252, SEQ ID NO: 1253, SEQ ID NO: 1254, SEQ ID NO: 1255, SEQ ID NO: 1256, SEQ ID NO: 1257, SEQ ID NO: 1258, SEQ ID NO: 1259, SEQ ID NO: 1260, SEQ ID NO: 1261, SEQ ID NO: 1262, SEQ ID NO: 1263, SEQ ID NO: 1264, SEQ ID NO: 1265, SEQ ID NO: 1266, SEQ ID NO: 1267, SEQ ID NO: 1268, SEQ ID NO: 1269, SEQ ID NO: 1270, SEQ ID NO: 1271, SEQ ID NO: 1272, SEQ ID NO: 1273, SEQ ID NO: 1274, SEQ ID NO: 1275, SEQ ID NO: 1276, SEQ ID NO: 1277, SEQ ID NO: 1278, SEQ ID NO: 1279, SEQ ID NO: 1280, SEQ ID NO: 1281, SEQ ID NO: 1282, SEQ ID NO: 1283, SEQ ID NO: 1284, SEQ ID NO: 1285, SEQ ID NO: 1286, SEQ ID NO: 1287, SEQ ID NO: 1288, SEQ ID NO: 1289, SEQ ID NO: 1290, SEQ ID NO: 1291, SEQ ID NO: 1292, SEQ ID NO: 1293, SEQ ID NO: 1294, SEQ ID NO: 1295, SEQ ID NO: 1296, SEQ ID NO: 1297, SEQ ID NO: 1298, SEQ ID NO: 1299, SEQ ID NO: 1300, SEQ ID NO: 1301, SEQ ID NO: 1302, SEQ ID NO: 1303, SEQ ID NO: 1304, SEQ ID NO: 1305, SEQ ID NO: 1306, SEQ ID NO: 1307, SEQ ID NO: 1308, SEQ ID NO: 1309, SEQ ID NO: 1310, SEQ ID NO: 1311, SEQ ID NO: 1312, SEQ ID NO: 1313, SEQ ID NO: 1314, SEQ ID NO: 1315, SEQ ID NO: 1316, SEQ ID NO: 1317, SEQ ID NO: 1318, SEQ ID NO: 1319, SEQ ID NO: 1320, SEQ ID NO: 1321, SEQ ID NO: 1322, SEQ ID NO: 1323, SEQ ID NO: 1324, SEQ ID NO: 1325, SEQ ID NO: 1326, SEQ ID NO: 1327, SEQ ID NO: 1328, SEQ ID NO: 1329, SEQ ID NO: 1330, SEQ ID NO: 1331, SEQ ID NO: 1332, SEQ ID NO: 1333, SEQ ID NO: 1334, SEQ ID NO: 1335, SEQ ID NO: 1336, SEQ ID NO: 1337, SEQ ID NO: 1338, SEQ ID NO: 1339, SEQ ID NO: 1340, SEQ ID NO: 1341, SEQ ID NO: 1342, SEQ ID NO: 1343, SEQ ID NO: 1344, SEQ ID NO: 1345, SEQ ID NO: 1346, SEQ ID NO: 1347, SEQ ID NO: 1348, SEQ ID NO: 1349, SEQ ID NO: 1350, SEQ ID NO: 1351, SEQ ID NO: 1352, SEQ ID NO: 1353, SEQ ID NO: 1354, SEQ ID NO: 1355, SEQ ID NO: 1356, SEQ ID NO: 1357, SEQ ID NO: 1358, SEQ ID NO: 1359, SEQ ID NO: 1360, SEQ ID NO: 1361, SEQ ID NO: 1362, SEQ ID NO: 1363, SEQ ID NO: 1364, SEQ ID NO: 1365, SEQ ID NO: 1366, SEQ ID NO: 1367, SEQ ID NO: 1368, SEQ ID NO: 1369, SEQ ID NO: 1370, SEQ ID NO: 1371, SEQ ID NO: 1372, SEQ ID NO: 1373, SEQ ID NO: 1374, SEQ ID NO: 1375, SEQ ID NO: 1376, SEQ ID NO: 1377, SEQ ID NO: 1378, SEQ ID NO: 1379, SEQ ID NO: 1380, SEQ ID NO: 1381, SEQ ID NO: 1382, SEQ ID NO: 1383, SEQ ID NO: 1384, SEQ ID NO: 1385, SEQ ID NO: 1386, SEQ ID NO: 1387, SEQ ID NO: 1388, SEQ ID NO: 1389, SEQ ID NO: 1390, SEQ ID NO: 1391, SEQ ID NO: 1392, SEQ ID NO: 1393, SEQ ID NO: 1394, SEQ ID NO: 1395, SEQ ID NO: 1396, SEQ ID NO: 1397, SEQ ID NO: 1398, SEQ ID NO: 1399, SEQ ID NO: 1400, SEQ ID NO: 1401, SEQ ID NO: 1402, SEQ ID NO: 1403, SEQ ID NO: 1404, SEQ ID NO: 1405, SEQ ID NO: 1406, SEQ ID NO: 1407, SEQ ID NO: 1408, SEQ ID NO: 1409, SEQ ID NO: 1410, SEQ ID NO: 1411, SEQ ID NO: 1412, SEQ ID NO: 1413, SEQ ID NO: 1414, SEQ ID NO: 1415, SEQ ID NO: 1416, SEQ ID NO: 1417, SEQ ID NO: 1418, SEQ ID NO: 1419, SEQ ID NO: 1420, SEQ ID NO: 1421, SEQ ID NO: 1422, SEQ ID NO: 1423, SEQ ID NO: 1424, SEQ ID NO: 1425, SEQ ID NO: 1426, SEQ ID NO: 1427, or SEQ ID NO: 1428."

[0428] In some aspects of the disclosure, a disclosed recombinant RSV F protein can include an amino acid sequence at least 80% (such as at least 90%, at least 95%, or at least 98%, or 100%) identical to any one of SEQ ID NO: 185, SEQ ID NO: 186, SEQ ID NO: 187, SEQ ID NO: 188, SEQ ID NO: 189, SEQ ID NO: 190, SEQ ID NO: 191, SEQ ID NO: 192, SEQ ID NO: 193, SEQ ID NO: 194, SEQ ID NO: 195, SEQ ID NO: 196, SEQ ID NO: 197, SEQ ID NO: 198, SEQ ID NO: 199, SEQ ID NO: 200, SEQ ID NO: 201, SEQ ID NO: 202, SEQ ID NO: 203, SEQ ID NO: 204, SEQ ID NO: 205, SEQ ID NO: 206, SEQ ID NO: 207, SEQ ID NO: 208, SEQ ID NO: 209, SEQ ID NO: 210, SEQ ID NO: 211, SEQ ID NO: 212, SEQ ID NO: 213, SEQ ID NO: 214, SEQ ID NO: 215, SEQ ID NO: 216, SEQ ID NO: 217, SEQ ID NO: 218, SEQ ID NO: 219, SEQ ID NO: 220, SEQ ID NO: 221, SEQ ID NO: 222, SEQ ID NO: 223, SEQ ID NO: 224, SEQ ID NO: 225, SEQ ID NO: 226, SEQ ID NO: 227, SEQ ID NO: 228, SEQ ID NO: 229, SEQ ID NO: 230, SEQ ID NO: 231, SEQ ID NO: 232, SEQ ID NO: 233, 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NO: 705, SEQ ID NO: 706, SEQ ID NO: 707, SEQ ID NO: 708, SEQ ID NO: 709, SEQ ID NO: 710, SEQ ID NO: 711, SEQ ID NO: 712, SEQ ID NO: 713, SEQ ID NO: 714, SEQ ID NO: 715, SEQ ID NO: 716, SEQ ID NO: 717, SEQ ID NO: 718, SEQ ID NO: 719, SEQ ID NO: 720, SEQ ID NO: 721, SEQ ID NO: 722, SEQ ID NO: 723, SEQ ID NO: 724, SEQ ID NO: 725, SEQ ID NO: 726, SEQ ID NO: 727, SEQ ID NO: 728, SEQ ID NO: 729, SEQ ID NO: 730, SEQ ID NO: 731, SEQ ID NO: 732, SEQ ID NO: 733, SEQ ID NO: 734, SEQ ID NO: 735, SEQ ID NO: 736, SEQ ID NO: 737, SEQ ID NO: 738, SEQ ID NO: 739, SEQ ID NO: 740, SEQ ID NO: 741, SEQ ID NO: 742, SEQ ID NO: 743, SEQ ID NO: 744, SEQ ID NO: 745, SEQ ID NO: 746, SEQ ID NO: 747, SEQ ID NO: 748, SEQ ID NO: 749, SEQ ID NO: 750, SEQ ID NO: 751, SEQ ID NO: 752, SEQ ID NO: 753, SEQ ID NO: 754, SEQ ID NO: 755, SEQ ID NO: 756, SEQ ID NO: 757, SEQ ID NO: 758, SEQ ID NO: 759, SEQ ID NO: 760, SEQ ID NO: 761, SEQ ID NO: 762, SEQ ID NO: 763, SEQ ID NO: 764, SEQ ID NO: 765, SEQ ID NO: 766, SEQ ID NO: 767, 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In several embodiments the immunogen specifically binds to the antibody or includes the antigenic site after incubation in PBS at pH 7.4 at 20°C for 24 hours. In some embodiments, the recombinant RSV F polypeptide includes one of the sequences from Sequence set F, and further includes up to 20 (such as up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19) amino acid substitutions (such as conservative amino acid substitutions, wherein the recombinant RSV F polypeptide specifically binds to a RSV F prefusion specific antibody (such as D25) and/or includes a RSV F prefusion specific antigenic site (such as antigenic site Ø). The person of ordinary skill in the art will appreciate that the sequences listed above may include leader sequences, purification tags, protease cleavage sites to remove purification tags trimerization domains, protein nanoparticle subunit domains or other sequences that are unrelated to the recombinant RSV F protein. In several embodiments, an immunogen provided herein includes a recombinant RSV F protein of one of the above sequences but does not include the leader sequences, purification tags, protease cleavage sites to remove purification tags trimerization domains, protein nanoparticle subunit domains or other sequences that are unrelated to the recombinant RSV F protein. Nucleic acid molecules encoding these protein sequences are also provides, as are methods of using the recombinant RSV F proteins to generate an immune response to RSV in a subject, or to prevent or treat RSV infection in a subject.

III. EXAMPLES

[0429] The following examples are provided to illustrate particular features of certain embodiments, but the scope of the claims should not be limited to those features exemplified.

Example 1

Structure of Respiratory Syncytial Virus Prefusion F Trimer Bound to a Human Antibody

[0430] The prefusion conformation of the respiratory syncytial virus (RSV) fusion (F) glycoprotein is the target of most RSV-neutralizing antibodies in human sera, but its metastability has hindered characterization. To overcome this obstacle, antibodies that do not bind the postfusion conformation of F and are >10-fold more potent than the prophylactic antibody palivizumab (Synagis®), were identified. The co-crystal structure for one of these antibodies, D25, in complex with the F glycoprotein reveals that D25 locks F in its prefusion state. Comparisons of prefusion and postfusion F conformations define the rearrangements required to mediate RSV entry. The D25-F glycoprotein structure reveals a new site-of-vulnerability, antigenic site Qj, at the top of the F glycoprotein that is prefusion-specific and quaternary in character. The prefusion RSV F trimer structure, along with definition of antigenic site Ø, should enable the design of improved vaccine antigens and guide new approaches for passive prevention of RSV-induced disease.

[0431] Respiratory syncytial virus (RSV) is ubiquitous, infecting nearly all children by 3 years of age (Glezen et al., Am. J. Dis. Child., 140, 543 (1986)). In the US, RSV bronchiolitis is the leading cause of hospitalization in infants and a major cause of asthma and wheezing throughout childhood (Shay et al., JAMA, 282, 1440 (1999); Hall et al., N. Engl. J. Med., 360, 588 (2009)). Globally, RSV is responsible for 66,000-199,000 deaths each year for children younger than five years of age (Nair et al., Lancet, 375, 1545 (2010)), and accounts for 7% of deaths among infants 1 month to 1 year old-more than any other single pathogen except malaria (Lozano et al., Lancet, 380, 2095 (2013)). The only available intervention is passive administration of the licensed monoclonal antibody palivizumab (Synagis®), which recognizes the RSV fusion (F) glycoprotein (Johnson et al., J. Infect. Dis., 176, 1215 (1997); Beeler and van Wyke Coelingh, J. Virol., 63, 2941 (1989)) and reduces incidence of severe disease (The IMpact-RSV Study Group, Pediatrics, 102, 531 (1998)). Clinical evidence that RSV F-specific antibodies can protect against disease has prompted a search for better antibodies (Collarini et al., J. Immunol., 183, 6338 (2009); Wu et al., J. Mol. Biol., 368, 652 (2007); Kwakkenbos et al., Nat. Med., 16, 123 (2010)) and a concerted effort to develop an effective vaccine (Graham, Immunol. Rev., 239, 149 (2011)).

[0432] The RSV F glycoprotein facilitates fusion of viral and cellular membranes (Walsh and Hruska, J. Virol., 47, 171 (1983)); it is a type I fusion protein, with a metastable prefusion conformation that stores folding energy, released during a structural rearrangement to a highly stable postfusion conformation. Three antigenic sites (I, II, and IV) have been found to elicit neutralizing activity (Arbiza et al., J. Gen. Virol., 73, 2225 (1992); Lopez et al., J. Virol., 72, 6922 (1998); Lopez et al., J.

Virol., 64, 927 (1990)), and all exist on the postfusion form of F as determined by structural and biophysical studies (McLellan et al., J. Virol., 85, 7788 (2011); Swanson et al., Proc. Natl. Acad. Sci. U.S.A., 108, 9619 (2011)). Absorption of human sera with postfusion F, however, fails to remove the majority of F-specific neutralizing activity, suggesting that the prefusion form may harbor novel neutralizing antigenic sites (Magro et al., Proc. Natl. Acad. Sci. U.S.A., 109, 3089 (2012)). Despite extensive effort, a homogeneous preparation of soluble prefusion RSV F has not been obtained. Thus, determination of the prefusion F structure and identification of novel F-specific antigenic sites have become converging priorities for development of new prophylactic and therapeutic antibodies and vaccines. In line with these objectives, F-specific antibodies that could neutralize RSV, but not bind to postfusion F were identified, and structure of RSV F recognized by these antibodies was defined. The results reveal the prefusion conformation of RSV F, the mechanism of neutralization for a category of remarkably potent antibodies, and atomic-level details for a prefusion-specific antigenic site that should serve as a target of improved antibody-based therapies and provide a basis for the development of effective vaccine antigens.

[0433] Two human antibodies-D25 and AM22-were determined to be ~ 50-fold more potent than palivizumab (FIG. 1A) for neutralizing RSV F, and which also do not bind to a soluble form of RSV F stabilized in the postfusion conformation (McLellan et al., J. Virol., 85, 7788 (2011)) (FIG. 1B). D25 and AM22 were previously disclosed (Kwakkenbos et al., Nat. Med., 16, 123 (2010); U.S. Pat. Pub. 2010/0239593; U.S. Pat. Pub. 2012/0070446). The lack of D25 and AM22 binding to the postfusion form of RSV F suggested these antibodies might recognize the metastable prefusion conformation.

[0434] Structural efforts were focused on the human antibodies, AM22 and D25. A 96-well microtiter plate expression format (Pancera et al., PLoS One. 2013;8(2):e55701, 2013) was used to screen binding of these antibodies to a panel of RSV F glycoprotein variants that were captured from cell supernatants on Ni^{2+}-NTA ELISA plates. Antibody binding to an F glycoprotein construct (RSV F(+) Fd), comprising RSV F residues 1-513 fused to a C-terminal fibritin trimerization domain was tested (Frank et al., J. Mol. Biol., 308, 1081 (2001)). However, complexes were not formed by mixing purified RSV F(+) Fd with purified D25 or AM22 antibody. It was determined that purification of the soluble F glycoprotein triggered the metastable prefusion state (Chaiwatpongsakorn et al., J. Virol., 85, 3968 (2011)); to overcome this instability, cells expressing RSV F(+) Fd were incubated with antigen-binding fragments (Fabs) or immunoglobulins (the latter with an HRV3C protease-cleavage site in the hinge region (McLellan et al., Nature 480, 336, (2011)) in order to trap F in the prefusion state. Alternatively, cells expressing RSV F(+) Fd were cotransfected with separate DNA-expression cassettes encoding antibody-heavy and -light chains (FIG. 5). Optimal expression of a D25-F glycoprotein complex was obtained from cotransfection of DNA encoding D25 Fab with DNA encoding RSV F(+) Fd; reasonable complex yields were also observed from the addition of soluble Fab.

[0435] Crystallizations were screened for Fab D25 and AM22, alone or in complex with RSV F(+) Fd. X-ray diffraction data to 1.6 Å resolution were obtained on hexagonal crystals of Fab D25 by itself, and the structure was solved by molecular replacement and refined to R_{cryst}/R_{free} of 24.5/25.7 % (Table 9). Data to 3.6 Å resolution were obtained on cubic crystals of Fab D25 in complex with RSV F (+) Fd, and this structure was solved by molecular replacement using the unbound D25 structure and portions of the previously determined postfusion RSV F structure (McLellan et al., J. Virol., 85, 7788 (2011); Swanson et al., Proc. Natl. Acad. Sci. U.S.A., 108, 9619 (2011)) as search models, along with clues from a gold derivative. The structure of the complex was refined to R_{cryst}/R_{free} of 21.3/26.7% (FIG. 1C) (Table 9).

[0436] A complex of one D25 Fab bound to one molecule of the RSV F glycoprotein was present in the asymmetric unit of the cubic lattice. Three-fold lattice symmetry positioned two other D25-RSV F complexes to generate an extensive RSV F trimeric interface of 2,098 $Å^2$. Continuous electron density was observed for residues 26 to 513, except for residues 98-136 that included the 27 amino-acid fragment removed by proteolytic cleavage of the F_0 precursor to form the F_2 and F_1 subunits (corresponding to N- and C-terminal fragments, respectively) of the mature F glycoprotein. Three sites of N-linked glycosylation were detected in the electron density at asparagine residues 27, 70 and 500 (FIG. 2A).

[0437] Overall, the D25-bound RSV F structure consists of two lobes packed at either end of a 7-stranded antiparallel open-ended barrel, two strands of which (β2 and β7) extend between the two lobes, hydrogen-bonding for over 70 Å and forming integral portions of both lobes and of the central barrel. The membrane-proximal lobe, which contains the F_2 N-terminus and F_1 C-terminus, consists of a triple layered β-sandwich and three helices (α8, α9 and α10). Helix α10 forms part of a helix that appeared to extend into the viral membrane and to which the fibrin trimerization domain was appended. The membrane-distal lobe, approximately 90 Å from the viral membrane, consists of seven helices, packed around a three-stranded antiparallel sheet and a β-hairpin (β3+β4). Extensive inter-protomer contacts appeared to stabilize the trimeric structure, particularly the hydrophobic N-terminus of the F_1 subunit (also known as the fusion peptide), which was cradled by the triple β-sandwich from the membrane-proximal lobe of a neighboring protomer. The fusion peptide, contained within the otherwise hollow cavity of the trimer, is connected to the surface-exposed α2 and α3 helices through a cylindrical opening between the protomers that is roughly 10 Å in diameter; this opening may be used as an exit path for the fusion peptide during triggering.

[0438] The structure of the D25-bound F glycoprotein resembled the prefusion structure of the related parainfluenza virus 5 (PIV5) F glycoprotein (Welch et al., Proc. Natl. Acad. Sci. U.S.A., 109, 16672 (2012); Yin et al., Nature, 439, 38 (2006)) (FIGs. 6 and 7). The D25-bound form of RSV F thus appeared to be in the prefusion conformation (FIG. 2). To define the structural rearrangements between pre- and post-fusion F, D25-bound form of RSV F was compared with its postfusion conformation, which was recently determined (McLellan et al., J. Virol., 85, 7788 (2011); Swanson et al., Proc. Natl. Acad. Sci. U.S.A., 108, 9619 (2011).

[0439] Pre- and post-fusion conformations of RSV F revealed dramatic changes in overall shape, from a relatively compact oval-shaped structure with a height of 110 Å to an extended cone approximately 50% longer (170 Å) (FIG. 2A). Despite this remarkable change in conformation, the majority of the F glycoprotein secondary and tertiary structure was preserved in both pre- and post-fusion states, with 215 residues showing less than 2 Å Cα deviation between the two structures (FIGs. 2A,B). Two regions of striking conformational change occur. In the membrane-distal lobe, the fusion peptide and five secondary structure elements (α2, α3, β3, β4, and α4) join with the α5-helix to form a single extended postfusion helix ($α5_{post}$) of over 100 Å in length, which is capped at its N-terminus by the fusion peptide (to aid in clarity, secondary structure elements of the postfusion structure are labeled with "post" subscript). In the membrane-proximal lobe, the sole parallel strand (β22) of the triple β-sandwich-which in the prefusion structure hydrogen bonds to β1-unravels, allowing the prefusion α10-helix to join with the $α5_{post}$-helix. Together, the $α5_{post}$ and $α10_{post}$ helices juxtapose F_1 N- and C-termini to form the coiled-coil structure characteristic of type I fusion proteins in their postfusion conformation (Colman and Lawrence, Nat. Rev. Mol. Cell Biol., 4, 309 (2003)). Overall, portions of the α10 helix move more than 170 Å between pre- and post-fusion conformations.

[0440] In comparison to the previously reported protease-cleaved, prefusion type I structures of influenza hemagglutinin (Wilson et al., Nature, 289, 366 (1981)), Ebola GP (Lee et al., Nature, 454, 177 (2008)) and PIV5 F(Welch et al., Proc. Natl. Acad. Sci. U.S.A., 109, 16672 (2012)), the location of the RSV fusion peptide is most similar to that of hemagglutinin (FIG. 7), which is surprising given that PIV5 and RSV are both paramyxoviruses. The RSV F fusion peptide is buried in the center of the hollow trimer cavity, and is located more than 40 Å away from the last visible F_2 residue. This suggests that a substantial structural rearrangement of the fusion peptide occurs after the F_0 precursor is cleaved by the furin-like host protease to produce F_1/F_2. In addition, dramatic structural rearrangements occur between pre- and post-fusion conformations in both the membrane-proximal and membrane-distal lobes, providing insight into the difficulty of stabilizing the prefusion conformation of RSV F. Unlike PIV5 F and human metapneumovirus F, which can be stabilized in the prefusion state solely by appending a GCN4-trimerization motif to the C-terminus (Yin et al., Nature, 439, 38 (2006); Wen et al., Nat. Struct. Mol. Biol., 19, 461 (2012)), the prefusion RSV F conformation requires stabilization of both the membrane-proximal lobe (accomplished by appending a fibritin trimerization domain (Frank et al., J. Mol. Biol., 308, 1081 (2001)) and the membrane-distal lobe (which occurs through binding of the D25 antibody).

[0441] The D25 antibody recognizes the membrane-distal apex of the RSV F glycoprotein (FIG. 1C). It binds to a quaternary epitope, with the D25-heavy chain interacting with one protomer (involving 638 $Å^2$ of buried interactive-surface area on RSV) and the D25-light chain binding to both the same protomer (373 $Å^2$) and a neighboring protomer (112 $Å^2$) (FIG. 3A). RSV F contacts are made by 5 of the 6 complementarity-determining loops of D25, with the heavy chain 3^{rd} CDR (CDR H3) interacting with the α4-helix (F_1 residues 196-210) and forming intermolecular hydrogen bonds with F_2 residues 63, 65, 66 and 68 in the loop between strand β2 and helix α1. While the secondary structural elements of the D25 epitope remain mostly unchanged, their relative orientation changes substantially, with α4-helix pivoting ~180° relative to strand β2 in pre- and post-fusion conformations (FIG. 3B). This structural rearrangement explains the failure of D25 to bind postfusion F molecules and suggests D25 inhibits membrane fusion by stabilizing the prefusion conformation of the trimeric F glycoprotein complex. Although F proteins from human RSV A and B subtypes are highly related in sequence (447/472 or 94.7% of the amino acids comprising the mature F_2/F_1 ectodomain are identical between known subtypes), six naturally observed positions of RSV-sequence variation (residues 67 and 74 in F_2, and residues 200, 201, 209, and 213 in F_1) are located in the region bound by D25 (FIG. 3C). Similarly, of the 56 amino acids in bovine RSV F that are not identical to the mature ectodomain of human RSV F subtype A, 13 are found in this same region (FIG. 3C). Thus, the D25 epitope, at the apex of the prefusion RSV F structure, may be under immune pressure and serve as a determinant of subtype-specific immunity (Chambers et al., J. Gen. Virol., 73, 1717 (1992)). For example, based on sequence analysis, a loop region in F glycoproteins was hypothesized to exist within the Paramyxoviridae family that might be under immune pressure (Chambers et al., J. Gen. Virol., 73, 1717 (1992)). It has been demonstrated that binding of RSV sub-group specific monoclonal antibodies can be affected by site-directed mutations between F_1 residues 200 and 216 (Connor et al., J. Med. Virol., 63, 168 (2001)), and that a peptide comprising F_1 residues 205-225 could elicit neutralizing activity in rabbits, although a specific epitope was not defined (Corvaisier et al., Arch. Virol., 142, 1073 (1997)).

[0442] To understand the relationship of the D25 epitope relative to epitopes recognized by other RSV-neutralizing antibodies, competition for D25 binding to RSV-infected cells was tested (FIG. 4A). Notably, AM22 competed with D25 for RSV F binding, suggesting that they recognized the same antigenic site. To further define the site recognized by these antibodies, negative stain EM on Fab-RSV F complexes was performed. EM images of Fab D25-RSV F complexes resembled the crystal structure of Fab D25-RSV F, and also EM images of Fab AM22-RSV F (FIG. 4B). Together, these results suggested antibodies D25 and AM22 recognize the same or a highly related antigenic site, which was named "antigenic site Ø".

[0443] To characterize antibodies that recognize antigenic site Ø, their functional properties were examined. In addition to their extraordinary potency and prefusion-specificity (FIG. 1A), all three antibodies strongly inhibited fusion when added post-attachment (FIG. 4C), and all three were unable to block cell-surface attachment (FIG. 4D), suggesting that the RSV F receptor binds to a region on F not blocked by these three antibodies. The receptor-binding domain on the related human metapneumovirus F protein is an RGD motif (Cseke et al., Proc. Natl. Acad. Sci. U.S.A., 106, 1566 (2009)) that corresponds to RSV F residues 361-363, which reside at the tip of a loop of the central barrel, on the side of the prefusion RSV F trimer not blocked by D25-binding. Although these antibodies do not prevent attachment, the regions of both F_2 and F_1 comprising antigenic site Ø are known to contribute to heparin binding (Feldman et al., J. Virol., 74, 6442 (2000); Crim et al., J. Virol., 81, 261 (2007)), and it is possible that this region may contribute to non-specific attachment to heparin sulfate moieties on glycosaminoglycans in concert with the G glycoprotein and other regions of F. Lastly, AM22 and D25 antibodies neutralized similarly in both Fab and immunoglobulin contexts (FIG. 8), indicating that avidity did not play a dominant role as it does for some influenza-virus antibodies (Ekiert et al., Nature, 489, 526 (2012)). Overall, the shared binding-specificity and neutralization phenotypes of D25 and AM22 and suggest that these properties may be characteristic of antibodies that recognize antigenic site QJ. By contrast, none of the antibodies that recognize other antigenic sites on RSV F associated with neutralizing activity (sites I, II, and IV) share similar properties of neutralizing potency and prefusion F specificity (FIGs. 9A-9B).

[0444] Despite antigenic site Ø being partially shielded from immune recognition by multiple mechanisms including conformational masking (it is only present in the metastable prefusion state), quaternary assembly (the site is shared by RSV protomers), antigenic variation (it is one of the most variable portions of RSV F), and glycan shielding (the N-linked glycan attached to Asn70 is at the top of the prefusion F trimer), all three prefusion-specific antibodies appear to target a similar epitope. The location of antigenic site Ø at the apex of the prefusion F trimer should be readily accessible even on the crowded virion surface, which may explain the observation that most neutralizing activity in human sera induced by natural RSV infection is directed against the prefusion form of RSV F (Magro et al., Proc. Natl. Acad. Sci. U.S.A., 109, 3089 (2012), although other prefusion-specific antigenic sites cannot be ruled out. The high potency of antibodies against antigenic site Ø suggests they could be developed for passive prophylaxis of RSV-induced disease in neonates. Also, vaccine-based prefusion-specific antibody elicitation may be assisted by stabilization of the prefusion form of RSV F, perhaps facilitated by linking mobile and immobile portions of the F structure through structure-based design of RSV F variants with disulfide bonds. It is noted that prefusion-stabilized F contains all of the previously characterized neutralizing epitopes as well as antigenic site Ø. Definition of the D25-RSV F structure thus provides the basis for multiple new approaches to prevent RSV-induced disease.

Materials and Methods

[0445] Viruses and cells. Viral stocks were prepared and maintained as previously described (Graham et al., J. Med. Virol., 26, 153 (1988)) RSV-expressing Green Fluorescent Protein (GFP) RSV-GFP was constructed as previously reported (Hallak et al., Virology. 271, 264 (2000)). The titer of the RSV-GFP stocks used for flow cytometry-based neutralization and fusion assays was 2.5×10^7 pfu/ml. The titer of the RSV A2 stock used for attachment assay was 1.02×10^8 pfu/ml. HEp-2 cells were maintained in Eagle's minimal essential medium containing 10% fetal bovine serum (10% EMEM) and were supplemented with glutamine, penicillin and streptomycin.

[0446] Creation of antibody expression plasmids. DNA encoding antibody heavy and light variable regions were codon-optimized for human expression and synthesized. AM22 and D25 heavy and light variable regions were subcloned into pVRC8400 expression plasmids containing in-frame human constant domains (IgG1 for heavy chain and kappa for light chain). Variants of the AM22 and D25 heavy chain expression plasmids were made by inserting either an HRV3C protease site (GLEVLFQGP; SEQ ID NO: 355) or a stop codon into the hinge region.

[0447] Expression and purification of antibodies and Fab fragments. Antibodies were expressed by transient co-transfection of heavy and light chain plasmids into HEK293F cells in suspension at 37°C for 4-5 days. The cell supernatants were passed over Protein A agarose, and bound antibodies were washed with PBS and eluted with IgG elution buffer into 1/10th volume of 1 M Tris-HCl pH 8.0. AM22 and D25 Fabs were created by digesting the IgG with Lys-C. The digestion was inhibited by the addition of Complete protease inhibitor cocktail tablets, and the Fab and Fc mixtures was passed back over Protein A agarose to remove Fc fragments. The Fab that flowed through the column was further purified by size exclusion chromatography.

[0448] RSV neutralization assays. Antibody-mediated neutralization was measured by a flow cytometry neutralization assay (Chen et al., J. Immunol. Methods, 362, 180 (2010). Briefly, HEp-2 cells were infected with RSV-GFP and infection was monitored as a function of GFP expression at 18 hours post-infection by flow cytometry. Data were analyzed by curve fitting and non-linear regression (GraphPad Prism, GraphPad Software Inc., San Diego CA).

[0449] Postfusion RSV F-binding assay. Purified, soluble RSV F protein in the postfusion conformation was prepared as described in (McLellan et al., J. Virol., 85, 7788 (2011). A kinetic ELISA was used to test binding of monoclonal antibodies to postfusion RSV F as described previously (McLellan et al., J. Mol. Biol., 409, 853

(2011). Briefly, 96-well Ni^{2+}-NTA-coated plates (ThermoFisher Scientific) were coated with 100 µl postfusion RSV F (1 µg/ml) for one hour at room temperature. 100 µl of diluted antibody was added to each well and incubated for one hour at room temperature. Bound antibodies were detected by incubating the plates with 100 µl HRP-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) or HRP-conjugated antihuman IgG (Santa Cruz Biolotechnology, Inc, Santa Cruz, CA) for 1 hour at room temperature. Then, 100 µl of Super AquaBlue ELISA substrate (eBioscience, San Diego CA) was added to each well and plates were read immediately using a Dynex Technologies microplate reader at 405nm (Chantilly, VA). Between steps, plates were washed with PBS-T.

[0450] Crystallization and X-ray data collection of unbound D25 Fab. Crystallization conditions were screened using a Cartesian Honeybee crystallization robot, and initial crystals were grown by the vapor diffusion method in sitting drops at 20°C by mixing 0.2 µl of D25 Fab with 0.2 µl of reservoir solution (22% (w/v) PEG 4000, 0.1 M sodium acetate pH 4.6). Crystals were manually reproduced in hanging drops by combining protein and reservoir solution at a 2:1 ratio. Crystals were flash frozen in liquid nitrogen in 27.5% (w/v) PEG 4000, 0.1 M sodium acetate pH 4.5, and 15% (v/v) 2R,3R-butanediol. X-ray diffraction data to 1.6 Å were collected at a wavelength of 1.00 Å at the SER-CAT beamline ID-22 (Advanced Photon Source, Argonne National Laboratory).

[0451] Structure determination and refinement of unbound D25 Fab. X-ray diffraction data were integrated and scaled with the HKL2000 suite (Otwinowski and Minor, in Methods Enzymol. (Academic Press, vol. 276, pp. 307-326, 1997)), and a molecular replacement solution using Ig domains from PDB ID: 3GBM (Ekiert et al., Science, 324, 246 (2009)) and 3IDX (Chen et al., Science, 326, 1123 (2009)) as search models was obtained using PHASER (McCoy et al., J. Appl. Crystallogr., 40, 658 (2007)). Manual model building was carried out using COOT (Emsley et al., Acta Crystallogr D Biol Crystallogr, 66, 486 (2010)), and refinement of individual sites, TLS parameters, and individual B-factors was performed in PHENIX (Adams et al., Acta Crystallogr D Biol Crystallogr, 66, 213 (2010)). The electron density for the D25 variable domains was excellent, but the electron density for the constant domains was poor, possibly a result of flexibility in the elbow angle. Final data collection and refinement statistics are presented in Table 8.

[0452] Expression and purification of RSV F(+) Fd in complex with D25 Fab. The RSV F (+) Fd protein construct was derived from the A2 strain (accession P03420) with three naturally occurring substitutions (P102A, I379V, and M447V) to enhance expression. A mammalian codon-optimized gene encoding RSV F residues 1-513 with a C-terminal T4 fibritin trimerization motif (Frank et al., J. Mol. Biol., 308, 1081 (2001)), thrombin site, 6x His-tag, and StreptagII was synthesized and subcloned into a mammalian expression vector derived from pLEXm (Aricescu et al., Acta Crystallogr D Biol Crystallogr, 62, 1243 (2006)). Plasmids expressing RSV F(+) Fd, the D25 light chain, and the D25 heavy chain (with or without a stop codon in the hinge region) were simultaneously transfected into HEK293 $GnTI^{-/-}$ cells (Reeves et al., Proc. Natl. Acad. Sci. U.S.A., 99, 13419 (2002)) in suspension. Alternatively, just the RSV F(+) Fd plasmid could be transfected, with purified D25 Fab added to the $GnTI^{-/-}$ cells 3 hours post-transfection. After 4-5 days, the cell supernatant was harvested, centrifuged, filtered and concentrated. The complex was initially purified via Ni^{2+}-NTA resin (Qiagen, Valencia, CA) using an elution buffer consisting of 20 mM Tris-HCl pH 7.5, 200 mM NaCl, and 250 mM imidazole pH 8.0. The complex was then concentrated and further purified over StrepTactin resin as per the manufacturer's instructions (Novagen, Darmstadt, Germany). After an overnight incubation with thrombin protease (Novagen) to remove the His and Strep tags, an excess of D25 Fab was added to the complex, which was then purified on a Superose6 gel filtration column (GE Healthcare) with a running buffer of 2 mM Tris-HCl pH 7.5, 350 mM NaCl, and 0.02% NaN_3. The eluted complex was diluted with an equal volume of water and concentrated to ~5 mg/ml. Similar procedures were used to express and purify AM22 Fab complexes.

[0453] Crystallization and X-ray data collection of RSV F(+) Fd in complex with D25 Fab. Initial crystals were grown by the vapor diffusion method in sitting drops at 20°C by mixing 0.1 µl of RSV F(+) Fd bound to D25 Fab with 0.1 µl of reservoir solution (40% (w/v) PEG 400, 5% (w/v) PEG 3350, and 0.1 M sodium acetate pH 5.5) (Majeed et al., Structure, 11, 1061 (2003)). Crystals were manually reproduced in hanging drops, and the crystal that diffracted to 3.6 Å was grown using a reservoir solution containing 30% (w/v) PEG 400, 3.75% (w/v) PEG 3350, 0.1 M HEPES pH 7.5, and 1% (v/v) 1,2-butanediol. The crystal was directly transferred from the drop into the cryostream, and X-ray diffraction data were collected remotely at a wavelength of 1.00 Å at the SER-CAT beamline ID-22.

[0454] Structure determination and refinement of RSV F(+)Fd in complex with D25 Fab. X-ray diffraction data were integrated and scaled with the HKL2000 suite (Otwinowski and Minor, in Methods Enzymol. (Academic Press, vol. 276, pp. 307-326, 1997)), and a molecular replacement solution was obtained by PHASER (McCoy et al., J. Appl. Crystallogr., 40, 658 (2007)) using the unbound D25 Fab structure and residues 29-42, 49-60, 78-98, 219-306, 313-322, 333-343, and 376-459 from the postfusion RSV F structure (PDB ID: 3RRR, McLellan et al., J. Virol., 85, 7788 (2011)) as search models. Six sites from a $NaAuCl_4$ derivative mapped to known reactive side chains (F residues Met97/His159, Met264/Met274, His317, and Met396; D25 heavy chain residues Met19/His82 and His 59). Manual model building was carried out using COOT (Emsley et al., Acta Crystallogr D Biol Crystallogr, 66, 486 (2010)), with secondary structure elements being built first. Refinement of individual sites, TLS parameters, and individual B-factors was performed in PHENIX (Adams et al., Acta Crystallogr D Biol Crystallogr, 66, 213 (2010)), using the unbound D25 Fab structure, and portions of the postfusion RSV F structure as reference models during the refinement. All RSV F residues in the mature protein were built except for those residues in F_2 C-terminal to Met97. Final data collection and refinement statistics are presented in Table 9.

[0455] RSV F competition binding assay. Competition binding of antibodies was performed on RSV infected HEp-2 cells. HEp-2 cells were infected with 3 MOI (multiplicity of infection) of RSV for 18-20 hours. After infection, cells were separated using cell dissociation solution (Cellstripper, Mediatech Inc., Herndon, VA), and washed with PBS. Cells were seeded at 5×10^4 /well in 96-well U-bottom plates in PBS. Monoclonal antibodies AM22, D25, and 101F were diluted starting at a concentration of 100 µg/ml, and added to HEp-2 cells. After 30 minutes 100ul of Alexa 488 conjugated D25 was added at a concentration of 1 µg/ml and incubated at 4°C for one hour. Cells were washed once with PBS, and then fixed with 0.5% paraformaldehyde. The binding of D25-Alexa 488 on cells was measured by flow cytomery (LSR II instrument, Becton Dickinson, San Jose, CA). Data were analyzed by using FlowJo software, version 8.5 (Tree Star, San Carlos, CA).

[0456] Negative staining electron microscopy analysis. Samples were adsorbed to freshly glow-discharged carbon-coated grids, rinsed shortly with water, and stained with freshly made 0.75% uranyl formate. Images were recorded on an FEI T20 microscope with an Eagle CCD camera. Image analysis and 2D averaging was performed with Bsoft (Heymann and Belnap, J. Struct. Biol., 157, 3 (2007) and EMAN (Ludtke et al., J. Struct. Biol., 128, 82 (1999)).

[0457] RSV virus-to-cell fusion inhibition assay. The ability of antibodies to inhibit RSV virus-to-cell fusion was measured as described previously (McLellan et al., J. Virol., 84, 12236 (2010)). Briefly, HEp-2 cells were seeded in 96-well plates, cultured for 24 hours at 37°C, and then chilled at 4°C for one hour prior to assay. RSV-GFP was added to pre-chilled cells at 4°C, and then cells were washed in cold PBS to remove unbound virus. Serially-diluted antibodies were added to chilled cells and incubated for 1 hour at 4°C, before transferring to 37°C for 18 hours. After incubation, cells were trypsinized, fixed in 0.5% paraformaldehyde, and analyzed by flow cytometry to determine the frequency of GFP-expressing cells.

[0458] RSV attachment inhibition assay. The ability of antibodies to inhibit RSV attachment to cells was measured as described previously (McLellan et al., J. Virol., 84, 12236 (2010)). Briefly, HEp-2 cells were dispersed into media, washed with cold PBS, seeded in 96-well v-bottom plates, and chilled for 1 hour at 4°C before use. Antibodies and heparin, a known RSV attachment inhibitor, were distributed in serial dilutions, then mixed with RSV A2 strain virus for one hour at 37°C. Medium from chilled cells was removed after centrifugation and virus or mixtures of virus and reagents were added to chilled cells and incubated for 1 hour at 4°C. After incubation, cells were washed in cold PBS to remove unbound virus, and fixed with 0.5% paraformaldehyde. Viruses bound on cells were detected with FITC-conjugated goat anti-RSV antibody. Cells were washed in cold PBS and evaluated by flow cytometry. Median fluorescence intensities of bound virus were analyzed with FlowJo software, version 8.5 (Tree Star, San Carlos, CA).

Table 9. Crystallographic data collection and refinement statistics.

		D25 Fab	D25 Fab + RSV F
Data collection			
Space group		$P6_122$	$P2_13$
Cell constants			
	a, b, c (Å)	108.7, 108.7, 139.9	152.3, 152.3, 152.390.0,
	α, β, γ (°)	90.0, 90.0, 120.0	90.0, 90.0
Wavelength (Å)		1.00	1.00
Resolution (Å)		50.0-1.6 (1.63-1.60)	50.0-3.6 (3.73-3.60)
R_{merge}		11.2 (68.0)	12.7 (81.4)
$I / \sigma I$		27.3 (2.1)	16.4 (2.0)
Completeness (%)		98.3 (86.1)	99.6 (99.3)
Redundancy		11.0 (5.3)	6.5 (5.2)
Refinement			
Resolution (Å)		35.4-1.6 (1.62-1.60)	42.2-3.6 (3.88-3.60)
Unique reflections		63,360 (2,241)	13,877 (2,742)
R_{work} / R_{free} (%)		24.1/25.5	21.3/26.7
No. atoms			
	Protein	3,305	6,778
	Ligand/ion	0	0
	Water	270	0
B-factors (Å^2)			
	Protein	53.0	128.1
	Ligand/ion	-	-
	Water	44.1	-
R.m.s. deviations			
	Bond lengths (Å)	0.007	0.003
	Bond angles (°)	1.20	0.91
Ramachandran			
	Favored (%)	96.5	92.0
	Allowed (%)	3.0	7.3
	Outliers (%)	0.5	0.7

Example 2

Stabilization of RSV F Proteins

[0459] This example illustrates design of exemplary RSV F proteins stabilized in a prefusion conformation. The crystal structure of the RSV F protein in complex with D25 Fab (*i.e.*, in a prefusion conformation) compared to the structure of the postfusion RSV F protein (disclosed, *e.g.*, in McLellan et al., J. Virol., 85, 7788, 2011, with coordinates deposited as PDB Accession No. 3RRR) shows dramatic structural rearrangements between pre- and post-fusion conformations in both the membrane-proximal and membrane-distal lobes, providing guidance for the stabilization of the prefusion conformation of RSV F. Based on a comparison of the pre- and post-fusion RSV F structures, there are two regions that undergo large conformational changes, located at the N- and C-termini of the F_1 subunit. For example, as illustrated in FIG. 2, the positions 137-216 and 461-513 of the F_1 polypeptide undergo structural rearrangement between the Pre-and Post- F protein conformations, whereas positions 271-460 of the F_1 polypeptide remain relatively unchanged. This example illustrates several strategies of stabilizing the RSV F protein in its prefusion conformation.

[0460] To stabilize the N-terminal region of F_1, which is a component of antigenic site Ø and is involved in binding to antibody D25, various strategies have been designed, including introduction of intra-protomer disulfide bonds, inter-protomer disulfide bonds, cavity filling amino acid substitutions, repacking substitutions, introduction of N-linked glycosylation sites, and combinations thereof.

Intra-protomer disulfide bonds

[0461] Introduction of two cysteine residues that are within a sufficiently close distance to form an intra-protomer disulfide bond in the prefusion, but not postfusion, conformation can lock the F protein in the prefusion conformation. An intra-molecular disulfide bond can be formed within a single F_2/F_1 protomer within the trimer, and thus would not cross-link the three protomers together. Specifically, a disulfide bond formed between a region that changes conformation and a region that does not change conformation in the pre- and post-fusion structures should lock the protein in the prefusion conformation. One example is that of the S155C/S290C mutant, where Ser155 is located in a region that changes conformation, whereas Ser290 is in a region that does not change conformation. Additionally, formation of a disulfide bond between two regions that both change conformation, such as two residues located within F_1 positions 137-216, or two residues located within F_1 positions 461-513, or one residue within F_1 positions 137-216 and the second within F_1 positions 461-513, may also be sufficient to lock the protein in the prefusion conformation.

[0462] Using the methods described above, several pairs of residues of the RSV F protein were determined to be in close enough proximity in the prefusion conformation, but not the postfusion conformation, to form an intra-protomer disulfide bond if cysteines were introduces at the corresponding residue pair positions.

These residue pairs, as well as the corresponding amino acid substitutions to SEQ ID NO: 1 needed to introduce cysteine residues at these positions, are indicated in Table 10. Table 10 also lists a SEQ ID NO containing the indicated substitutions, and corresponding to a precursor F_0 construct including a signal peptide, F_2 polypeptide (positions 26-109), pep27 polypeptide (positions 110-136), F_1 polypeptide (positions 137-513), a trimerization domain (a Foldon domain) and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))).

Table 10. Exemplary Cross-Linked Cysteine Pairs for Intra-Protomer Disulfide Bond Stabilization

F protein Residue Pair(s) for Cysteine Substitution	A.A. substitutions corresponding to SEQ ID NO: 1	SEQ ID NO
F_1 Substitutions		
155 and 290	S155C and S290C	185
151 and 288	G151C and I288C	189
137 and 337	F137C and T337C	213
397 and 487	T397C and E487C	247
138 and 353	L138C and P353C	257
341 and 352	W341C and F352C	267
403 and 420	S403C and T420C	268
319 and 413	S319C and 1413C	269
401 and 417	D401C and Y417C	270
381 and 388	L381C and N388C	271
320 and 415	P320C and S415C	272
319 and 415	S319C and S415C	273
331 and 401	N331C and D401C	274
320 and 335	P320C and T335C	275
406 and 413	V406C and I413C	277
381 and 391	L381C and Y391C	278
357 and 371	T357C and N371C	279
403 and 417	S403C and Y417C	280
321 and 334	L321C and L334C	281
338 and 394	D338C and K394C	282
288 and 300	I288C and V300C	284
F_2 and F_1 Substitutions		
60 and 194	E60C and D194C	190
33 and 469	Y33C and V469C	211
54 and 154	T54C and V154C	212
59 and 192	I59C and V192C	246
46 and 311	S46C and T311C	276
48 and 308	L48C and V308C	283
30 and 410	E30C and L410C	285

<u>**Intermolecular disulfide bonds**</u>

[0463] Introduction of two cysteine residues that are within a sufficiently close distance to form an inter-protomer disulfide bond in the prefusion, but not postfusion, conformation can lock the F protein in the prefusion conformation. An inter-protomer disulfide bond would be formed between adjacent protomers within the trimer, and thus would cross-link the three protomers together. Specifically, a disulfide bond formed between a region that changes conformation and a region that does not change conformation in the pre- and post-fusion structures should lock the protein in the prefusion conformation. One example is that of the A153C/K461C mutant, where Ala153 is located in a region that changes conformation, whereas Lys461 is in a region that does not change conformation. Additionally, formation of a disulfide bond between two regions that both change conformation, such as two residues located within F_1 positions 137-216, or two residues located within F_1 positions 461-513, or one residue within F_1 positions 137-216 and the second within F_1 positions 461-513, may also be sufficient to lock the protein in the prefusion conformation.

[0464] Using the methods described above, several pairs of residues of the RSV F protein were determined to be in close enough proximity in the prefusion conformation, but not the post-fusion conformation, to form an inter-protomer disulfide bond if cysteines were introduced at the corresponding residue pair positions. These residue pairs, as well as the corresponding amino acid substitutions needed to introduce cysteine residues at these positions, are indicated in Table 11. Table 11 also lists a SEQ ID NO containing the indicated substitutions, and corresponding to a precursor F_0 construct also including a signal peptide, F_2 polypeptide (positions 26-109), pep27 polypeptide (positions 110-136), F_1 polypeptide (positions 137-513), a trimerization domain (a Foldon domain) and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))).

Table 11. Exemplary Cross-Linked Cysteine Pairs for Inter-Protomer Disulfide Bond Stabilization

F protein Residue pair(s)	A.A. substitutions corresponding to SEQ ID NO: 1	SEQ ID NO
F_1 Substitutions		
400 and 489	T400C and D489C	201
144 and 406	V144C and V406C	202
153 and 461	A153C and K461C	205
149 and 458	A149C and Y458C	207
143 and 404	G143C and S404S	209

F protein Residue pair(s)	A.A. substitutions corresponding to SEQ ID NO: 1	SEQ ID NO
F_1 Substitutions		
346 and 454	S346C and N454C	244
399 and 494	K399C and Q494C	245
146 and 407	S146C and I407C	264
374 and 454	T374C and N454C	265
369 and 455	T369C and T455C	266
402 and 141	V402C and L141C	302
F_2 and F_1 Substitutions		
74 and 218	A74C and E218C	243

[0465] Additionally, multiple stabilizing mutations described herein can be combined to generate a PreF antigen containing more than one stabilizing mutation. Examples of such constructs containing a first and second residue pair that form an intra- or an inter-protomer disulfide bond are provided in Table 12. Table 12 also lists a SEQ ID NO containing the indicated substitutions, and corresponding to a precursor F0 construct also including a signal peptide, F_2 polypeptide (positions 26-109), pep27 polypeptide (positions 110-136), F_1 polypeptide (positions 137-513), a trimerization domain (a Foldon domain) and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))).

Table 12. Exemplary Cross-Linked Cysteine Pairs for Combinations of Intra- and Inter-Protomer Disulfide Bond Stabilization.

F protein Residue pair(s)	Substitutions corresponding to SEQ ID NO: 1	SEQ ID NO
155 and 290 (Intra); and 402 and 141 (Inter)	S155C and S290C; and V402C and L141C	303
155 and 290(Intra); and 74 and 218	S155C and S290C; and A74C and E218C	263

[0466] Further, amino acids can be inserted (or deleted) from the F protein sequence to adjust the alignment of residues in the F protein structure, such that particular residue pairs are within a sufficiently close distance to form an intra- or inter-protomer disulfide bond in the prefusion, but not postfusion, conformation, which, as discussed above, will stabilize the F protein in the prefusion conformation. Examples of such modification are provided in Table 13. Table 13 also lists a SEQ ID NO containing the indicated substitutions, and corresponding to a precursor F_0 construct also including a signal peptide, F_2 polypeptide (positions 26-109), pep27 polypeptide (positions 110-136), F_1 polypeptide (positions 137-513), a trimerization domain (a Foldon domain) and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))).

Table 13. Using amino acid insertions to orient F proteins to accept inter-intra-protomer disulfide bonds, or combinations thereof.

F protein Residue pair(s)	Substitutions corresponding to SEQ ID NO: 1	SEQ ID NO
155 and 290 (Intra); and 146 and 460 (Inter); G insertion between position 460/461	S155C and S290C; and S146C and N460C; G insertion between position 460/461	258
155 and 290 (Intra); and 345 and 454(Inter); C insertion between positions 453/454	S155C and S290C; and N345C and N454G; C insertion between positions 453/454	259
155 and 290 (Intra); and 374 and 454(Inter); C insertion between positions 453/454	S155C and S290C; and T374C and N454G; C insertion between positions 453/454	260
155 and 290 (Intra); and 239 and 279(Inter); C insertion between positions 238/239	S155C and S290C; and S238G and Q279C; C insertion between positions 238/239	261
155 and 290 (Intra); and 493 paired with C insertion between positions 329/330	S155C and S290C; and S493C paired with a C insertion between positions 329/330	262
183 and 428 (Inter), G insertion between positions 182/183	N183C and N428C; G insertion between positions 182/183	296
183 and 428 (Inter), C insertion between positions 427/428	N183C and N427G; C insertion between positions 427/428	297
155 and 290 (Intra); and 183 and 428(Inter); G insertion between positions 182/183	S155C and S290C; and N183C and N428C; G insertion between positions 182/183	298
155 and 290 (Intra); and 183 and 428(Inter); C insertion between positions 427/428	S155C and S290C; and N183C and N427G; C insertion between positions 427/428	299
145 and 460 (Inter), AA insertion between positions 146/147	S145C and 460C; AA insertion between positions 146/147	338
183 and 423 (Inter), AAA insertion between positions 182/183	N183C and K423C; AAA insertion between positions 182/183	339
330 and 430 (Inter); CAA insertion between positions 329/330	A329C and S430C; and a CAA insertion between positions 329/330	340

Cavity-filling substitutions

[0467] Comparison of the crystal structure of the RSV F protein in complex with D25 Fab (*i.e.*, in a prefusion conformation) compared to the structure of the postfusion RSV F protein (disclosed, *e.g.*, in

[0468] McLellan et al., J. Virol., 85, 7788, 2011; structural coordinates of the RSV F protein in its postfusion conformation are deposited in the Protein Data Bank (PDB) as PDB Accession No. 3RRR) identifies several internal cavities or pockets in the prefusion conformation that must collapse for F to transition to the postfusion conformation. These cavities are listed in Table 14. Accordingly, filling these internal cavities stabilizes F in the prefusion state, by preventing transition to the postfusion conformation. Cavities are filled by substituting amino acids with large side chains for those with small sidechains. The cavities can be intra-protomer cavities, or inter-protomer cavities. One example of a RSV F cavity-filling modification to stabilize the RSV protein in its prefusion conformation is the S190F/V207L mutant.

[0469] Using this strategy, several cavity filling modifications were identified to stabilize the RSV F protein in its prefusion conformation. These modifications, are indicated in Table 14. Table 14 also lists a SEQ ID NO containing the indicated substitutions, and corresponding to a precursor F_0 construct including a signal peptide, F_2 polypeptide (positions 26-109), pep27 polypeptide (positions 110-136), F_1 polypeptide (positions 137-513), a trimerization domain (a Foldon domain) and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))).

Table 14. Exemplarity cavity-filling amino acid substitution

Cavity	A.A. Substitutions	SEQ ID NO:
Ser190 and Val207	190F and 207L	191
Val207	207L and 220L	193
Ser190 and Val296	296F and 190F	196
Ala153 and Val207	220L and 153W	197
Val207	203W	248
Ser190 and Val207	83W and 260W	192
Val296	58W and 298L	195
Val90	87F and 90L	194

[0470] The indicated cavities are referred to by a small residue abutting the cavity that can be mutated to a larger residue to fill the cavity. It will be understood that other residues (besides the one the cavity is named after) could also be mutated to fill the same cavity.

Repacking substitutions

[0471] Additionally, the prefusion conformation of the RSV F protein may be stabilized by increasing the interactions of neighboring residues, such as by enhancing hydrophobic interactions or hydrogen-bond formation. Further, the prefusion conformation of the RSV F protein may be stabilized by reducing unfavorable or repulsive interactions of neighboring residues that lead to metastability of the prefusion conformation. This can be accomplished by eliminating clusters of similarly charged residues. Examples of such modifications are indicated in Table 15. Table 15 also lists a SEQ ID NO containing the indicated substitutions, and corresponding to a precursor F_0 construct including a signal peptide, F_2 polypeptide (positions 26-109), pep27 polypeptide (positions 110-136), F_1 polypeptide (positions 137-513), a trimerization domain (a Foldon domain) and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))).

Table 15. Repacking Amino Acid Substitutions

Substitutions	SEQ ID NO
I64L, I79V, Y86W, L193V, L195F, Y198F, I199F, L203F, V207L, I214L	227
I64L, I79L, Y86W, L193V, L195F, Y198F, I199F, L203F, I214L	228
I64W, I79V, Y86W, L193V, L195F, Y198F, I199F, L203F, V207L, I214L	229
I79V, Y86F, L193V, L195F, Y198F, I199F, L203F, V207L, I214L	230
I64V, I79V, Y86W, L193V, L195F, Y198F, I199Y, L203F, V207L, I214L	231
I64F, I79V, Y86W, L193V, L195F, Y198F, I199F, L203F, V207L, I214L	232
I64L, I79V, Y86W, L193V, L195F, I199F, L203F, V207L, I214L	233
V56I, T58I, V164I, L171I, V179L, L181F, V187I, I291V, V296I, A298I	234
V56I, T58I, V164I, V179L, T189F, I291V, V296I, A298I	235
V56L, T58I, L158W, V164L, I167V, L171I, V179L, L181F, V187I, I291V, V296L	236
V56L, T58I, L158Y, V164L, I167V, V1871, T189F, I291V, V296L	237
V56I, T58W, V1641, I167F, L171I, V179L, L181V, V187I, I291V, V296I	238
V56I, T58I, I64L, I79V, Y86W, V1641, V179L, T189F, L193V, L195F, Y198F, I199F, L203F, V207L, I214L, I291V, V296I, A298I	239
V56I, T58I, I79V, Y86F, V1641, V179L, T189F, L193V, L195F, Y198F, I199F, L203F, V207L, I214L, I291V, V296I, A298I	240
V56I, T58W, I64L, I79V, Y86W, V1641, I167F, L171I, V179L, L181V, V1871, L193V, L195F, Y198F, I199F, L203F, V207L, I214L, I291V, V296I	241
V56I, T58W, I79V, Y86F, V164I, I167F, L171I, V179L, L181V, V1871, L193V, L195F, Y198F, I199F, L203F, V207L, I214L, I291V, V296I	242
D486N, E487Q, D489N, and S491A	249
D486H, E487Q, and D489H	250
T400V, D486L, E487L, and D489L	251
T400V, D486I, E487L, and D489I,	252
T400V, S485I, D486L, E487L, D489L, Q494L, and K498L	253
T400V, S485I, D486I, E487L, D489I, Q494L, and K498L	254
K399I, T400V, S485I, D486L, E487L, D489L, Q494L, E497L, and K498L	255
K399I, T400V, S485I, D486I, E487L, D489I, Q494L, E497L, and K498L	256
L375W, Y391F, and K394M	286
L375W, Y391F, and K394W	287
L375W, Y391F, K394M, D486N, E487Q, D489N, and S491A	288
L375W, Y391F, K394M, D486H, E487Q, and D489H	289
L375W, Y391F, K394W, D486N, E487Q, D489N, and S491A	290
L375W, Y391F, K394W, D486H, E487Q, and D489H	291

Substitutions	SEQ ID NO
L375W, Y391F, K394M, T400V, D486L, E487L, D489L, Q494L, and K498M	292
L375W, Y391F, K394M, T400V, D486I, E487L, D489I, Q494L, and K498M	293
L375W, Y391F, K394W, T400V, D486L, E487L, D489L, Q494L, and K498M	294
L375W, Y391F, K394W, T400V, D486I, E487L, D489I, Q494L, and K498M	295
F137W and R339M	326
F137W and F140W	327
F137W, F140W, and F488W	328
D486N, E487Q, D489N, S491A, and F488W	329
D486H, E487Q, D489H, and F488W	330
T400V, D486L, E487L, D489L, and F488W	331
T400V, D486I, E487L, D489I, and F488W	332
D486N, E487Q, D489N, S491A, F137W, and F140W	333
D486H, E487Q, D489H, F137W, and F140W	334
T400V, D486L, E487L, D489L, F137W, and F140W	335
L375W, Y391F, K394M, F137W, and F140W or	336
L375W, Y391F, K394M, F137W, F140W, and R339M	337

Glycosylation mutations

[0472] Additionally, introduction of N-linked glycosylation sites that would be solvent-accessible in the prefusion RSV F conformation but solvent-inaccessible in the postfusion RSV F conformation may stabilize RSV F in the prefusion state by preventing adoption of the postfusion state. To create an N-linked glycosylation site, the sequence Asn-X-Ser/Thr (where X is any amino acid except Pro) may be introduced. This can be accomplished by substitution of a Ser/Thr amino acid two residues C-terminal to a native Asn residue, or by substitution of an Asn amino acid two residues N-terminal to a native Ser/Thr residue, or by substitution of both an Asn and Ser/Thr residue separated by one non-proline amino acid.

[0473] Using this strategy, several locations for N-linked glycosylation sites that would be solvent-accessible in the prefusion RSV F conformation but solvent-inaccessible in the postfusion RSV F conformation were identified. These modifications are indicated in Table 16. Table 16 also lists the SEQ ID NO containing the indicated substitutions, and corresponding to a precursor F_0 construct including a signal peptide, F_2 polypeptide (positions 26-109), pep27 polypeptide (positions 110-136), F_1 polypeptide (positions 137-513), a trimerization domain (a Foldon domain) and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))).

Table 16. Exemplary N-linked glycosylation

N-linked glycosylation position	Exemplary substitutions	Exemplary SEQ ID NO
506	I506N and K508T	198
175	A177S	199
178	V178N	200
276	V278T	203
476	Y478T	204
185	V185N and V187T	214
160	L160N and G162S	215
503	L503N and a F505S	216
157	V157N	217

Example 3

Stabilizing the membrane proximal lobe of PreF antigens

[0474] As discussed above, the crystal structure of the RSV F protein in complex with D25 Fab (*i.e.*, in a prefusion conformation) compared to the structure of the postfusion RSV F protein ((disclosed, *e.g.*, in McLellan et al., J. Virol., 85, 7788, 2011, with coordinates deposited as PDB Accession No. 3RRR)) shows dramatic structural rearrangements between pre- and post-fusion conformations in the membrane-distal lobe. Based on a comparison of the pre- and post-fusion RSV F structures, there are two regions that undergo large conformational changes, located at the N- and C-termini of the F_1 subunit. For example, as illustrated in FIG. 2, the positions 137-216 and 461-513 of the F_1 polypeptide undergo structural rearrangement between the Pre-and Post- F protein conformations, whereas positions 271-460 of the F_1 polypeptide remain relatively unchanged. This example illustrates several strategies of stabilizing the C-terminal region of F_1, which includes the membrane proximal lobe of the RSV F protein. Various strategies have been identified, including introduction of a trimerization domain (as discussed above), introduction of cysteine pairs that can form a disulfide bond that stabilizes the C-terminal region of F1, and introduction of a transmembrane domain (e.g., for applications including a membrane-bound PreF antigen).

Disulfide bonds

[0475] One strategy for stabilizing the membrane proximal lobe of the F protein is to introduce one or more cysteine substitutions that introduce a disulfide bond that that stabilizes the C-terminal portion of F_1 (for example, for an application including a soluble PreF antigen). Such a strategy can be combined with any of the stabilization modifications provided herein, for example, those described in Example 2, such as a F_1 protein with a S155C/S290C cysteine substitution. One strategy includes introduction of two cysteine residues that are within a sufficiently close distance to form an inter-protomer disulfide bond that links the C-terminal region of the F_1 protein in the prefusion conformation. An inter-protomer disulfide bond would be formed between adjacent protomers within the trimer, and thus would cross-link the three protomers together. Using the methods described above, several pairs of residues of the RSV F protein were determined to be in close enough proximity in the prefusion conformation, to form an inter-protomer disulfide bond if cysteines were introduces at the corresponding residue pair positions.

[0476] Examples of cysteine substitutions that can be introduced to generate a disulfide bond that stabilizes the membrane proximal lobe include cysteine substitutions at residue pairs:

1. (a) 486 and 487
2. (b) 486 and 487; with a P insertion between positions 486/487
3. (c) 512 and 513
4. (d) 493; C insertion between 329/330
5. (e) 493; C insertion between 329/330, and G insertion between 492/493

[0477] Further, the length of the F_1 polypeptide can be varied, depending on the position of the of the C-terminal cysteine pair. For example, the F_1 polypeptide can include positions 137-481, which eliminate the α10 helix from the F_1 polypeptide.

[0478] Examples of constructs containing modifications including cysteines at these residue pairs, as well as additional description are listed in Table 17. Table 17 also lists a SEQ ID NO containing the indicated substitutions, and corresponding to a precursor F_0 construct also including a signal peptide, F_2 polypeptide (positions 26-109), pep27 polypeptide (positions 110-136), F_1 polypeptide (with varying positions).

Table 17. Disulfide bonds to stabilize the membrane proximal lobe of F protein.

Substitutions/insertion	Description	F_1 positions	SEQ ID NO
D486C/E487C; S155C/S290C	The D486C and E487C mutant allows inter-protomer disulfide bond formation while the S155C/S290C mutations stabilize the prefusion format, this construct does not have a Foldon or alpha-10 helix.	137-481	304
S155C/S290C; D486C/E487C; P insertion between positions 486/487	The D486C and E487C mutant should allow inter-protomer disulfide bond formation while the S155C/S290C mutations stabilize the prefusion format, this construct does not have a Foldon or alpha-10 helix.	137-481	305
N183C/N428C; D486C/E487C; G insertion between 182/183	The D486C and E487C mutant should allow inter-protomer disulfide bond formation while the 183C and 428C mutations stabilize the prefusion format. This construct removes the Foldon sequence and the alpha-10 sequence.	137-481	306
N183C/K427G; C insertion between 247/428; D486C/E487C P; insertion between positions 486/487	The D486C and E487C mutant should allow inter-protomer disulfide bond formation while the 183C and 428C mutations stabilize the prefusion format. This construct removes the Foldon sequence and the alpha-10 sequence.	137-481	307
V402C/L141C; L512C/L513C	The 141C and 402C stabilize the prefusion form by locking down the fusion peptide. While the 512C and 513C create an inter-protomer disulfide bond; this construct does not have a foldon domain.	1-513	308
S155C/S290C; V402C/L141C L512C/L513C	The 141C and 402C stabilize the prefusion form by locking down the fusion peptide in conjunction with the S155C/S290C. While the 512C and 513C create an inter-protomer disulfide bond, the foldon sequence is removed.	1-513	309
S155C/S290C; S493C; C insertion between 329/330	Removal of the "foldon" and the facilitation of intermolecular disulfide bond stabilization while the S155C/S290C mutations stabilize the prefusion format	137-491	310
S155C/S290C; S493C; C insertion between 329/330; G insertion between	Removal of the "foldon" and the facilitation of intermolecular disulfide bond stabilization while the S155C/S290C mutations stabilize the prefusion format	137-491	311
492/493			

<u>**Transmembrane domains**</u>

[0479] Another strategy for stabilizing the membrane proximal lobe of the F protein is to include a transmembrane domain on the F_1 protein, for example, for an application including a membrane anchored PreF antigen. For example, the presence of the transmembrane sequences is useful for expression as a transmembrane protein for membrane vesicle preparation. The transmembrane domain can be linked to a F_1 protein containing any of the stabilizing mutations provided herein, for example, those described in Example 2, such as a F_1 protein with a S155C/S290C cysteine substitution. Additionally, the transmembrane domain can be further linked to a RSV F_1 cytosolic tail. Examples of precursor F_0 constructs including a signal peptide, F_2 polypeptide (positions 26-109), pep27 polypeptide (positions 110-136), F_1 polypeptide (positions 137-513), a RSV transmembrane domain are provided as SEQ ID NOs: 323 (without a cytosolic domain) and 324 (with a cytosolic domain).

Example 4

Single chain PreF antigens

[0480] This example illustrates recombinant RSV F proteins that lack the native furin cleavage sites, such that the F protein protomer is formed as a single polypeptide chain, instead of a F_2/F_1 heterodimer.

[0481] Table 18 lists several single chain PreF antigens that include deletion of F positions 98-149, which removes the two furin cleavage sites, the pep27 polypeptide, and the fusion peptide. The remaining portions of the F_1 and F_2 polypeptides are joined by a linker. Additionally, several strategies can be employed to stabilize the single chain constructs in a prefusion conformation, including use of the strategies described in examples 2 and 3, above. Table 18 also lists a SEQ ID

NO containing the indicated substitutions, and corresponding to a precursor F_0 construct also including a signal peptide, F_2 polypeptide (positions 26-109), pep27 polypeptide (positions 110-136), F_1 polypeptide (with varying positions).

Table 18. Single chain PreF antigens

Substitutions	Discussion	F2/F_1 Linker	C-term Stabilization	SEQ ID NO
S155C/S290C L373R Δ98-149	(A) The rationale for this construct is to create a single chain RSV fusion molecule, remove the nucleus localization signal (L373R), and the fusion peptide (, while the S155C/S290C mutations stabilize the prefusion format	GSGNVGLGG (SEQ ID NO: 356)	Foldon	313
S155C/S290C L373R Δ98-149	Same as (A)	GSGNWGLGG (SEQ ID NO: 357)	Foldon	314
S155C/S290C L373R Δ98-149	Same as (A)	GSGNIGLGG (SEQ ID NO: 358)	Foldon	315
S155C/S290C L373R Δ98-149	Same as (A)	GSGGNGIGLGG (SEQ ID NO: 359)	Foldon	316
S155C/S290C L373R Δ98-149	Same as (A)	GSGGSGGSGG (SEQ ID NO: 360)	Foldon	317
S155C/S290C L373R Δ98-149	Same as (A)	GSGNVLGG (SEQ ID NO: 361)	Foldon	318
S155C/S290C L373R Δ98-149	(B) The rationale for this construct is to create a single chain RSV fusion molecule, remove the nucleus localization signal, and the fusion peptide and also the alpha 10 helix and Foldon, while the S155C/S290C mutations stabilize the prefusion format	GSGNVGLGG (SEQ ID NO: 362)	D486C/E487C; P insertion between positions 486/487	319
S155C/S290C/ L373R Δ98-149	Same as (B)	GSGNVGLGG (SEQ ID NO: 363)	L512C/L513C	320
S155C/S290CL 373R Δ98-149	Same as (A)	GSGNIGLGG (SEQ ID NO: 364)	TM	322

Example 5

RSV F protein stabilized with a disulfide bond and a trimerization domain

[0482] This example illustrates production of a RSV F protein stabilized with a disulfide bond and a trimerization domain. As illustrated in FIG. 10, the serine residues at positions 155 and 290 (indicated by arrows and red highlighting in the ribbon diagrams) are adjacent to each other in the prefusion conformation of RSV F protein, but not in the post fusion conformation of the RSV F protein. Further, the side chains of these residues are oriented towards one another. However, the side chains of the residues adjacent to serine 155, valine 154 and lysine 156, are oriented away from the side chain of serine 290. In view of these findings, a recombinant RSV F protein was constructed with S155C and S290C substitutions. It was expected that the cysteine residues in this 155/290 construct would form a disulfide bond that would lock the recombinant RSV F protein in the prefusion conformation, but that incorporation of cysteines at positions 154 or 156 (instead of position 155) would fail to produce a stabilizing disulfide bond.

[0483] A nucleic acid molecule encoding a native RSV F_0 polypeptide was mutated using standard molecular biology techniques to encode the RSV F protein called RSVF(+)FdTHS S155C, S290C, and set forth as SEQ ID NO: 185:

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCNGTDA
KVKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNYTLNNAKKTNVTLSKKRKRRFLGFLLGV
GSAIASGVAVCKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPIVNKQSCSISNI
ETVIEFQQKNNRLLEITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMCI
IKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSN
RVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNRGIIKTFSN
GCDYVSNKGVDTVSVGNTLYYVNKQEGKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSD
ELL**SAIGGYIPEAPRDGQAYVRKDGEWVLLSTFL**GGLVPRGSHHHHHHSAWSHPQFEK (SEQ ID NO: 185).

[0484] RSVF(+)FdTHS S155C, S290C includes a signal peptide (residues 1-25), F_2 polypeptide (residues 26-109), Pep27 polypeptide (residues (110-136), F_1 polypeptide (residues 137-513), Foldon domain (residues 514-544), and a thrombin cleavage site (LVPRGS (positions 547-552 of SEQ ID NO: 185)) and purification tags (his-tag (HHHHHH (positions 553-558 of SEQ ID NO: 185)) and Strep Tag II (SAWSHPQFEK (positions 559-568 of SEQ ID NO: 185))). Control constructs were also generated with V154C or K156C substitutions instead of the S155C substitution.

[0485] When expressed in cells, RSVF(+)FdTHS S155C, S290C was processed and expressed as a stable and soluble RSV F protein; however, the control constructs with 154/290 or 156/290 substitutions failed to express (likely because they failed to fold in a soluble conformation) (see FIG. 10).

[0486] The RSVF(+)FdTHS S155C, S290C construct was purified and tested for antibody binding to the prefusion specific antibodies AM22 and D25, as well as 131-2a antibody (which binds antigenic site I, present on pre- and post-fusion RSV F conformations), motavizumab and palivizumab (which bind antigenic site II, present on pre- and post-fusion RSV F conformations), and 101F antibody (which binds antigenic site IV, present on pre- and post-fusion RSV F conformations). As shown in FIG. 11 (left graph), all of these antibodies specifically bound to the purified RSVF(+)FdTHS S155C, S290C construct, indicating that RSVF(+)FdTHS S155C, S290C maintains a prefusion conformation. The results further indicate that this construct maintains antigenic sites I, II and IV, common to both the pre- and post-fusion conformations of RSV F.

[0487] To demonstrate that purified RSVF(+)FdTHS S155C, S290C is in a trimeric conformation, this construct was passed over a size-exclusion chromatography column. As shown in FIG. 11 (right graphs) a preparation of purified RSVF(+)FdTHS S155C, S290C eluted in a single peak corresponding to the molecular weight of the trimeric F protein. In contrast, a preparation of a control construct lacking the S155C and S290C substitutions, which is not expected to be stabilized in the prefusion conformation, eluted in multiple peaks, indicating the presence of rosettes of triggered F protein and aggregates, indicating that this control construct is not stable in a homogeneous prefusion conformation.

[0488] To further confirm that the RSVF(+)FdTHS S155C, S290C construct is stabilized in a prefusion conformation, electron microscopy studies were performed (FIG. 12) and demonstrate that RSVF(+)FdTHS S155C, S290C form homogeneous population of structures with a shape similar to that of the prefusion conformation of RSV F, and significantly different from that of the postfusion F protein (right image, from Martin et al., J. Gen. Virol., 2006).

[0489] Crystallography studies were performed to demonstrate that purified RSVF(+)FdTHS S155C, S290C is homogeneous in solution. Formation of crystals in aqueous solution is a stringent test for the homogeneity of a protein in solution. FIG. 15 shows pictures of the crystals formed by purified RSVF(+)FdTHS S155C, S290C in aqueous buffer containing 0.2 M lithium sulfate, 1.64 M Na/K tartrate and 0.1 M CHES, at pH 9.5. The formation of RSVF(+)FdTHS S155C, S290C crystals in aqueous buffer demonstrates that this protein is substantially homogeneous in solution.

Example 6

Induction of a neutralizing immune response using a PreF antigen

[0490] This example illustrates use of a PreF antigen to elicit a RSV neutralizing immune response in a subject.

[0491] Eight week old pathogen-free CB6F1/J mice (Jackson Labs) were divided into 5 groups of 10 each, and immunized with the following regimens:

1. 1) live RSV A2 (RSV) at 5×10^6 pfu intranasally;
2. 2) formalin-inactivated alum-precipitated RSV(FI-RSV) intramuscularly (IM);
3. 3) stabilized prefusion RSV F (RSVF(+)FdTHS S155C, S290C; prefusion F) 20 µg in polyI:C 50 µg IM;
4. 4) postfusion RSV F trimer ((postfusion RSV) 20 µg in polyI:C 50 µg IM; and

[0492] Group 1 (live RSV) was infected once at time 0, and all other groups were immunized at 0 and 3 weeks. Serum was obtained at week 5, two weeks after the 2nd IM injection or five weeks post RSV infection. Neutralizing activity was determined by the following method: Sera were distributed as four-fold dilutions from 1:10 to 1:40960, mixed with an equal volume of recombinant mKate-RSV expressing prototypic F genes from either strain A2 (subtype A) or 18537 (subtype B) and the Katushka fluorescent protein, and incubated at 37°C for one hour. Next, 50 µl of each serum dilution/virus mixture was added to HEp-2 cells that had been seeded at a density of 1.5×10^4 in 30 µl MEM (minimal essential medium) in each well of 384-well black optical bottom plates, and incubated for 20-22 hours before spectrophotometric analysis at Ex 588 nm and Em 635 nm (SpectraMax Paradigm, Molecular Devices, Sunnyvale, CA 94089). The IC50 for each sample was calculated by curve fitting and non-linear regression using GraphPad Prism (GraphPad Software Inc., San Diego CA). P values were determined by Student's T-test. The above method for measuring RSV neutralization was performed substantially as described previously (see, e.g., Chen et al. J. Immunol. Methods., 362:180-184, 2010), except that the readout was by a fluorescent plate-reader instead of flow cytometry.

[0493] Using this assay, generally antibody responses above ~100 EC_{50} would be considered to be protective. As shown in FIGs. 13 and 14, mice administered an RSV F protein stabilized in a prefusion conformation (RSV F (RSVF(+)FdTHS S155C, S290C) produced a neutralizing immune response to RSV A ~15-fold greater than that produced by mice administered a RSV F protein in a postfusion conformation, and a response to RSV B ~5-fold greater than that produced by mice administered a RSV F protein in a postfusion conformation. FIG. 13 shows the results after 5 weeks post-initial immunization, and FIG. 14 shows results after 7 weeks post immunization. The mean elicited IC50 values are also shown in FIGs. 13 and 14. The difference in neutralization between RSV A and B subgroups is not surprising as the RSVF(+)FdTHS S155C, S290C construct is derived from a F protein from an RSV A subgroup. It is expected that immunization with a corresponding construct derived from a RSV B strain would generate neutralizing sera more specific for RSV B (see FIG. 41).

[0494] Further, it was shown that the stabilized prefusion F can be formulated in alum as well as polyI:C and retain immunogenicity conferred by antibody responses to antigenic site Ø. BALB/c mice were immunized with 20 µg of the DS S155C/S290C version of stabilized prefusion F derived from subtype A and formulated with alum (aluminum hydroxide gel 10 mg/ml, Brenntag, Frederikssund, Denmark) or polyI:C. Mice were inoculated at 0 and 3 weeks, and at the 5 week time point (2 weeks after the second injection), serum was obtained for neutralization assays (see FIG. 42). The results show that immunization with a RSV F protein stabilized in a prefusion conformation produces a protective immune response to RSV.

Example 7

Treatment of subjects with the disclosed antigens

[0495] This example describes methods that can be used to treat a subject that has or is at risk of having an RSV infection by administration of one or more of the disclosed PreF antigens, or a nucleic acid or a viral vector encoding, expressing or including a PreF antigen. In particular examples, the method includes screening a subject having, thought to have, or at risk of having (for example due to impaired immunity, physiological status, or exposure to RSV) an RSV infection. Subjects of an unknown infection status can be examined to determine if they have an infection, for example using serological tests, physical examination, enzyme-linked immunosorbent assay (ELISA), radiological screening or other diagnostic technique known to those of ordinary skill in the art. In some examples, a subject is selected that has an RSV infection or is at risk of acquiring an RSV infection. Subjects found to (or known to) have an RSV infection and thereby treatable by administration of the disclosed PreF antigens, or a nucleic acid or a viral vector encoding, expressing or including a PreF antigen are selected to receive the PreF antigens, or a nucleic acid or a viral vector encoding, expressing or including a PreF antigen. Subjects may also be selected who are at risk of developing an RSV infection for example, the elderly, the immunocompromised and the very young, such as infants.

[0496] Subjects selected for treatment can be administered a therapeutic amount of disclosed PreF antigens. An immunogenic composition including the PreF antigen can be administered at doses of 0.1 µg/kg body weight to about 1 mg/kg body weight per dose, such as 0.1 µg/kg body weight to about 1 µg/kg body weight, 0.1 µg/kg body weight to about 10 µg/kg body weight per dose, 1 µg/kg body weight -100 µg/kg body weight per dose, 100 µg/kg body weight - 500 µg/kg body weight per dose, or 500 µg/kg body weight - 1000 µg/kg body weight per dose or even greater. In some embodiments, about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, or about 100 µg of the PreF antigen is included in the immunogenic composition that is administered to the subject in a single dose. The immunogenic composition can be administered in several doses, for example continuously, daily, weekly, or monthly. The mode of administration can be any used in the art, such as nasal administration. The amount of agent administered to the subject can be determined by a clinician, and may depend on the particular subject treated. Specific exemplary amounts are provided herein (but the disclosure is not limited to such doses).

Example 8

RSV F protein stabilized with a disulfide bond, cavity filling substitutions, and a trimerization domain

[0497] This example illustrates production of a RSV F protein stabilized with a disulfide bond and a trimerization domain.

[0498] Figure 16 shows the design of a recombinant RSV F protein stabilized by engineered disulfide bond mutations S155C and S290C (termed "DS"), cavity-filling mutations S190F and V207L (termed "Cavl"), and a heterologous trimerization domain appended to the C-terminus of the F1 polypeptide of the F protein. The three-dimensional structure depicted is the D25-bound RSV F structure, and is shown with two of the protomers displayed as a molecular surface colored pink and tan, and the third protomer displayed as ribbons. The N- and C-terminal residues of F_1 that move more than 5Å between the pre and postfusion conformations are shown. Insets show the engineered disulfide bond between residues S155C and S290C, as well as the space-filling cavity mutations S190F and V207L. A model of the T4 phage fibritin trimerization domain is shown at the base of the prefusion trimer.

[0499] A RSV F protein including the S155C, S290C, S190F and V207L (Cav1) substitutions in human RSV subtype A, and the appended C-terminal heterologous foldon domain, was expressed and purified using methods described in Example 1 and 5, and is termed RSV_A F(+)FdTHS DSCav1.

[0500] The antigenic characterization of RSV_A F(+)FdTHS DSCav1 are shown in Figure 17. The association and dissociation rates of soluble D25, AM22, 5C4, 101F, Motavizumab, and Palivizumab Fab with immobilized RSV_A F(+)FdTHS DSCavl were measured using an OctetRED 384™ instrument (ForteBio, Melno Park, CA). Equilibrium dissociation constants for each antibody are provided.

[0501] The purity of RSV_A F(+)FdTHS DSCav1 is illustrated by size exclusion chromatography (FIG. 18). Purified protein, after thrombin cleavage to remove the tags, was passed over a 16/70 Superose 6 size exclusion column. The elution volume is consistent with a glycosylated trimer.

[0502] The antigenic and physical characteristics, including yield from transiently expressed plasmids, antigenicity against various antigenic sites, and the retention of D25-binding (provided as a fractional amount) after 1 hour of incubation at various temperatures (350 mM NaCl pH 7.0, at 50°C, 70°C, or 90°C), pHs (350 mM NaCl pH 3.5 or pH 10, at 25°C), and osmolality (10 mM or 3000 mM osmolarity, pH 7.0, at 25°C), or to 10 cycles of freeze-thaw (in 350 mM NaCl pH 7.0), of RSV_A F(+)FdTHS variants stabilized by DS, Cav1 or DSCav1 mutations are shown in FIG. 19. The DSCav1 variant retains antigenic site Ø recognition, with improved physical stability, as judged by higher retention of D25-reactivity after exposure to extremes of temperature, pH, osmolality and freeze-thaw, then either DS or Cavl variants.

[0503] To investigate the structural properties of the DSCavl mutant, the three dimensional structure of RSV_A F(+)FdTHS DSCavl was determined using X-ray crystallography. FIG. 20 shows a ribbon representation of the 3.1 Å crystal structure of RSV_A F(+)FdTHS DSCav1. Warmer colors and thicker ribbons correspond to increasing B-factors. Despite stabilizing mutations, antigenic site Ø, at the trimer apex, retains significant flexibility. FIG. 21 shows comparison of the structure of RSV_A F(+)FdTHS DSCav1 to the structure of D25-bound RSV F. FIG. 22 highlights the stabilizing mutations in RSV_A F(+)FdTHS DSCavl structure. Observed electron density corresponding to the disulfide bond between cysteine residues 155 and 290 (left), as well as the cavity-filling Phe190 residue (right), indicates that these modifications are present in the crystal.

[0504] To determine the immunogenicity of the RSV_A F(+)FdTHS DSCavl construct, mice and non-human primates were inoculated with this construct and sera obtained from the inoculated animals was tested for neutralization of RSV (FIGs. 23 and 24). Mice were immunized, and the neutralization activity of the resulting sera was tested, as described in Example 6, above. Briefly, ten CB6 mice per group were immunized with 10 µg of the indicated RSV F protein mixed with 50 µg of poly I:C adjuvant. Immunizations occurred at 0 and 3 weeks, and sera from week 5 and week 7 were tested for neutralization of RSV subtype A (RSV_A) and B (RSV_B). Mean values are indicated by horizontal red lines. *Macaca mulatta* animals of Indian origin weighing 8.76-14.68 kg were intramuscularly injected with immunogens at week 0 and week 4. Blood was collected every other week for up to 6 weeks. Four RSV-naïve rhesus macaques per group were immunized intramuscularly with 50 µg of the indicated RSV F protein mixed with 500 µg of poly I:C adjuvant. Immunizations occurred at 0 and 4 weeks, and sera from week 6 were tested for neutralization of RSV subtype A (left) and B (right). Mean values are indicated by horizontal red lines. Taken together, these results show that the RSV_A F(+)FdTHS DSCav1 construct successfully generated a neutralizing response in mice and non-human primates.

Example 9

Structure-Based Design of a Fusion Glycoprotein Vaccine for Respiratory Syncytial Virus

[0505] Abstract. Respiratory syncytial virus (RSV) is the leading cause of hospitalization for children under five years of age. To elicit protective humoral responses against RSV, efforts were focused on antigenic site Ø, a metastable site specific to the prefusion state of the fusion (F) glycoprotein, as this site is the principal target of highly potent RSV-neutralizing antibodies elicited by natural infection. Structure-based design to engineer stabilized versions of F that preserved antigenic site Ø to extremes of pH and temperature was used. Six stabilized-F crystal structures provided atomic-level details for introduced cysteine residues and filled hydrophobic cavities and revealed subtly different "prefusion" F conformations. Immunization with site Ø-stabilized variants of RSV F elicited -in both mice and non-human primates- RSV-specific neutralizing activity 3-15-fold higher than those elicited by RSV F in its postfusion state. Atomic-level design to present a supersite of viral vulnerability can thus have a transformative effect on vaccine development.

[0506] Introduction. Respiratory syncytial virus (RSV) is estimated to be responsible for 6.7% of deaths in children 1mo-1yr of age and causes excess mortality in the elderly at levels comparable to that caused by infection with influenza virus. Although RSV infection does not induce fully protective immunity, antibodies against the RSV fusion (F) glycoprotein can prevent severe disease in humans as demonstrated by passive prophylaxis with the F-directed antibody, palivizumab (Synagis®).

[0507] The proven success of palivizumab has spurred vaccine efforts aimed at eliciting protective RSV F-directed antibodies. These efforts have been complicated by the structural diversity of RSV F, a type I fusion protein that assumes at least two conformations: a metastable prefusion state and a stable postfusion state. Both states share epitopes targeted by neutralizing antibodies, including that of palivizumab, and postfusion RSV F is being developed as a vaccine candidate. As described herein, the dominant target of RSV-neutralizing antibodies elicited by natural infection was found to reside primarily on the prefusion conformation of RSV F, and antibodies such as AM22, and D25 (see, e.g., U.S. 12/600,950, and U.S. 12/898,325) - substantially more potent than palivizumab - target antigenic site Ø, a metastable site specific to prefusion F, which is located at the membrane-distal apex of the prefusion RSV F trimer.

[0508] To enhance elicitation of these potent antibodies, engineered soluble variants of RSV F were designed to stably expose antigenic site Ø. These variants were characterized both antigenically and crystallographically, and tested for immunogenicity in mice and non-human primates. The results provide insight into the interplay between design, antigenicity, structure, and immunogenicity and show how structure-based engineering to preserve and to present an appropriate antigenic target can have a transformative effect on the elicitation of protective humoral responses.

[0509] The structure-based vaccine strategy described herein included a four step strategy: (1) to identify a supersite of viral vulnerability targeted by antibodies with potent neutralizing activity, (2) to determine the structure of the supersite in complex with a representative antibody, (3) to engineer the stable presentation of the supersite in the absence of recognizing antibody, and (4) to elicit high titer protective responses through immunization with engineered antigens that present the supersite (FIG. 26).

Engineering of RSV F Antigens

[0510] Because of its recognition by extraordinarily potent RSV-neutralizing antibodies, antigenic site Ø was chosen as the target supersite; its structure in complex with the D25 antibody is described herein (FIG. 26B). To engineer variants of RSV F that stably presented site Ø, the structure of RSV F bound by D25 was analyzed. Mechanistically, there are a number of ways to stabilize a protein conformation. Mechanisms to stabilize site Ø without compromising its recognition were tested these in combination with a T4-phage fibritin trimerization domain ("foldon") (Efimov et al., J Mol Biol 242, 470 (1994); Boudko et al., European journal of biochemistry / FEBS 269, 833 (2002)) appended to the C-terminus of the RSV F ectodomain (McLellan et al., J. Virol. 85, 7788 (2011)).

[0511] Introducing cysteine pairs predicted to form a disulfide bond in the target conformation, but widely separated in alternative conformations, is one approach to stabilize a select structure. The β-carbons of serine residues 155 and 290 are 4.4 Å apart in the D25-bound RSV F structure (see Example 1) and 124.2 Å apart in the postfusion structure (McLellan et al., J. Virol. 85, 7788 (2011; described above and see FIGs. 27 and 32). A S155C-S290C double mutant, termed named "DS", formed stable RSV F trimers, expressed at 1.4 mg/L, retained antigenic site Ø, and was homogeneous as judged by negative stain EM (described above; see also FIG. 31, FIG. 33). Other cysteine modifications, such as those between regions of RSV F that are compatible with both the pre- and postfusion states (e.g. S403C and T420C), did not stabilize antigenic site Ø (FIG. 31). a number of potential inter-subunit double cysteine modifications was also tested; none of the tested inter-subunit double cysteine substitutions, however, expressed more than 0.1 mg/L.

[0512] Cavity-filling hydrophobic substitutions provide another means to stabilize a select conformation. The D25-bound RSV F structure was analyzed for hydrophobic cavities unique to the D25-bound conformation of RSV F that abutted regions that differed in pre- and postfusion F states. A number of such cavities were identified in the membrane-distal "head" of the prefusion structure, close to the binding site of D25, and modeled hydrophobic alterations to fill these cavities. S190F and V207L alterations adopted prevalent side chain conformations with minimal clashes, while K87F, V90L, V220L and V296F alterations showed less steric compatibility, filling these cavities with pairs of changes was assessed. A S190F-V207L pair, which was named "Cav1" (FIG. 27), formed stable RSV F trimers, expressed at 2.2 mg/L, and retained antigenic site Ø (FIG. 31). Meanwhile, K87F-V90L, S190F-V296F and V207L-V220L variants showed enhanced retention of D25 recognition, but less than 0.1 mg/l yields of RSV F trimer (FIG. 31).

[0513] Other cavities towards the center of prefusion RSV F were close to the fusion peptide, the trimer axis, and an acidic patch comprising residues Asp486, Glu487, and Asp489. A number of cavity-filling alterations were modeled including F137W, F140W, and F488W, and analyzed these alterations in combination with D486H, E487Q, and D489H (FIG. 31). Of the six combinations tested, only two (F488W and D486H-E487Q-F488W-D489H) expressed levels of purified RSV F trimer at greater than 0.1 mg/l and retained D25 recognition. The D486H-E487Q-F488W-D489H variant, designated "TriC", formed stable RSV F trimers, expressed at 0.8 mg/l, and retained antigenic site Ø (FIG. 31, FIG. 27).

[0514] The impact of destabilizing the postfusion conformation on the preservation of antigenic site Ø was also tested. V178N, predicted to introduce an N-linked glycan compatible with the prefusion but not the postfusion conformation of F, did not appear to stabilize antigenic site Ø, nor did V185E or I506K, which would place a glutamic acid or a lysine into the interior of the postfusion six-helix bundle (FIG. 31). These mutations likely result in some intermediate conformation of RSV F that is "triggered", but is unable to adopt the postfusion conformation. In all, over 100 RSV F variants were constructed, expressed in a 96-well transfection format (Pancera et al., PLoS ONE 8, e55701 (2013)), and tested by ELISA for binding to D25 and motavizumab. Fifteen constructs were compatible with D25 binding, six of which retained D25 recognition for at least 7 days at 4°C, and three of these could be purified to homogeneous trimers that retained antigenic site Ø (FIG. 31). Overall, a strong correlation was observed between retention of D25 binding for at least 7 days at 4°C in 96-well supernatants and yield of purified trimers from large scale expression and purification (FIG. 34).

Combinatorial Optimization of Site Ø Stability

[0515] DS, Cavl, and TriC variants displayed a variety of physical and antigenic properties. The DS variant was the least stable to pH and temperature extremes, but more permanently stabilized in the trimeric state, while constant interconversion from trimer to aggregate was observed for Cavl and TriC. To assess whether a more optimal variant of RSV F might be obtained by combining DS, Cav1, and TriC, all combinations were made.

[0516] Combinations generally improved retention of D25 reactivity to physical extremes. Thus, for example, all combinations showed improved stability to incubation at 50°C or pH 10.0. However, the low tolerance to freeze-thaw exhibited by TriC was also observed in both Cav 1- TriC and DS-Cav 1- TriC. Overall, the DS-Cav1 combination appeared optimal in terms of trimer yield and physical stability to extremes of temperature, pH, osmolality, and freeze-thaw (FIG. 31, Fig 35), and was homogeneous as judged by negative stain EM (FIG. 33).

Crystallographic Analysis

[0517] To provide atomic-level feedback, crystal structures of site Ø-stabilized variants of RSV F were determined (FIG. 28). The DS, Cav1, DS-Cav1 and DS-Cav1-TriC variants all crystallized in similar 1.5 M tartrate pH 9.5 conditions, and these cubic crystals diffracted X-rays to resolutions of 3.1 Å, 3.1 Å, 3.8 Å and 2.8 Å resolutions, respectively (FIG. 40). Molecular replacement solutions were obtained by using the D25-bound RSV F structure as a search model, and these revealed a single RSV F protomer in the asymmetric unit, with the trimeric F axis aligned along the crystallographic threefold. Tetragonal crystals of Cav1 and cubic crystals of DS-Cav1 were also obtained from 1.7 M ammonium sulfate pH 5.5 conditions, and these diffracted to resolutions of 2.4 Å and 3.0 Å, respectively (FIG. 40).

Molecular replacement revealed the tetragonal lattice to have a full RSV F trimer in the asymmetric unit, and to be highly related to the tartrate cubic lattices. Overall these structures revealed the engineered RSV F variants to be substantially in the D25-bound conformation (The engineered RSV F variants had CD-root mean square deviations from the D25-bound conformation between 0.68-1.5 Å and from the postfusion conformation of approximately 30 Å).

[0518] Although the structure of the DS variant (FIG. 28, left most column) was stable as a soluble trimer, with the cysteine substituted residues at 155 and 290 indeed forming a disulfide bond that largely prevented triggering to the postfusion state, much of the membrane-distal portion of the RSV F trimer, including antigenic site Ø, was either disordered (residues 63-72 and 169-216) or in a different conformation. Thus, for example, residues 160-168 in the DS structure extend the α2-helix instead of forming a turn and initiating the a3-helix as in the D25-bound F structure (FIG. 28B, left most panel). One non-limiting explanation for the differences between DS structure and the D25-bound RSV F structure is that the crystallized DS is in a conformation that does not bind D25. Overall the DS variant retained many of the features of the prefusion state of RSV F, including the fusion peptide in the interior of the trimeric cavity.

[0519] In comparison to DS, the Cavl structure (FIG. 28, 2nd and 3rd columns) was more ordered in the membrane-distal apex, with the a3-helix, β3/β4 hairpin, and the α4-helix clearly defined. Residues 137-202, which contain the S190F substitution, had a Ca-rmsd of 0.6 Å when compared to the D25-bound F structure. The higher degree of structural order was likely due to the S190F mutation that filled a cavity observed in the D25-bound F structure, and increased van der Waal's contacts with residues Ile57, Lys176, Val192, Glu256, Ser259 and Leu260. The other cavity-filling mutation in Cav1, V207L, was shifted by 5.5Å compared to the D25-bound F structure, with the C-terminal portion of the a4-helix kinking near Pro205 and adopting distinct conformations in the two crystallization conditions (FIG. 28B, 2nd and 3rd panels from left).

[0520] A striking feature of the Cavl structure in the tetragonal crystal lattice is the C-terminus of F_2, which is disordered in the D25-bound F structure, but in Cavl, tunnels into the trimeric cavity alongside the fusion peptide. Interestingly, the C-terminus ends with Ala107, and not Arg109, as expected after cleavage of the furin site (Arg106-Ala107-Arg108-Arg109). In the Cav1 structure, the positive charge of Arg106 is offset by an ordered sulfate ion (FIG. 28C). Biologically, the interior position of the F_2 C-terminus may play a role in triggering of the prefusion F conformation.

[0521] Comparison of the DS-Cavl structures from the two tetragonal crystal forms (FIG. 28, 2nd and 3rd columns from right) to those of Cavl revealed only minor differences (Cα rmsd of 0.86 Å for residues between Cavl and DS-Cavl grown in ammonium sulfate conditions; Cα rmsd of 0.47 Å for 447 residues in the cubic lattice). The largest differences occurred at the RSV F apex, including antigenic site Ø and specifically at residues 64-73 and 203-216. Notably, the atomic mobility (B-factor) was highest in this apex region for all of the site Ø-stabilized variants, perhaps indicative of intrinsic site Ø flexibility. Interestingly, however, site Ø has low atomic mobility when bound by D25, revealing the ability of D25 to stabilize both overall and local RSV F conformations.

[0522] The structure of the DS-Cav 1- TriC triple combination (FIG. 28, far right column) was also highly similar to other Cavl-containing RSV F variant structures. One difference in the electron density, however, corresponded to an expanse of weak density at the membrane-proximal region, which corresponded to the dimensions of the T4-fibritin trimerization domain (Stetefeld et al., Structure 11, 339 (2003)), which is not visible in other crystallized RSV F structures which contained this domain, including the D25-bound structure. Small structural differences in packing likely allow for the partial ordering of this domain (and may also account for its increased diffraction limit of the DS-Cav 1- TriC crystals relative to the other cubic variants), rather than differences in the interaction between the DS-Cav 1- Tri C stabilized RSV F and this trimerization domain.

[0523] In terms of the TriC alterations of residues 486-489, the critical F488W substitution packed directly against the F488W substitutions of neighboring protomers of the RSV F trimer. The indole side chain of Trp488 pointed towards the trimer apex and also formed ring-stacking interactions with the side chain of 140Phe of the fusion peptide (FIG. 28C, far right panel). This fusion peptide interaction, which is not observed in any of the Phe488-containing structures, likely inhibits the extraction of the fusion peptide from the prefusion trimer cavity, providing a structural rationale for the ability of the F488W alteration to stabilize the prefusion state of RSV F (FIG. 31).

Immunogenicity of Antigenic Site Ø-Stabilized RSV F

[0524] To assess the effect of site Ø-stabilization on the elicitation of RSV-protective humoral responses, CB6 mice were immunized with various forms of RSV F, injecting each mouse with 10 µg RSV F combined with 50 µg poly I:C adjuvant at weeks 0 and 3, and measured the ability of week 5 sera to prevent RSV infection of HEp-2 cells. DS, Cavl, and TriC each elicited high titers of neutralizing activity (geometric mean 50% effective concentrations (EC_{50}s) of 1826-2422). This level was ~3-fold higher than elicited by postfusion F (504 EC_{50}), and ~20-fold higher than the protective threshold. By comparison, DS-Cavl elicited neutralizing activity of 3937 EC_{50}, roughly 7-fold higher than postfusion F and 40-fold higher than the protective threshold (FIG. 29A). (When palivizumab (Synagis®) is dosed at a concentration of 15 mg/kg, serum levels at trough are ~40 µg/ml, which provides protection in infants from severe disease. In the neutralization assay, 40 µg/ml of palivizumab in serum yields an EC50 of 100. This titer is also associated with complete protection from lower respiratory tract infection in mice and cotton rats challenged with RSV.)

[0525] To quantify the elicitation of antibodies between different sites on prefusion RSV F, antigenic site Ø-occluded forms of RSV F were utilized. CB6 mice immunized with 20 µg RSV F bound by antigenic site Ø-directed antibodies (comprising ~10 µg of RSV F and ~10 µg of the antigen-binding fragment of antibody) developed week 5 geometric mean neutralizing titers of 911 and 1274 EC_{50} for AM22 and D25 complexes, respectively, roughly double that of postfusion at 10 µg/ml and comparable to those elicited by postfusion at 20 µg/ml (FIG. 29A). These findings suggest that the very high titers elicited by immunization with RSV F variants stabilized in the prefusion state - especially DS-Cav1 - were due to antibodies targeting antigenic site Ø.

[0526] To examine the generality of site Ø elicitation, rhesus macaques were immunized with DS-Cavl, DS and postfusion forms of RSV F, injecting each macaque with 50 µg RSV F mixed with 500 µg poly I:C adjuvant at weeks 0 and 4 and measuring the ability of week 6 sera to inhibit RSV infection. Formulated proteins retained expected antigenic profiles as measured by D25 binding (FIG. 38). DS and DS-Cav1 elicited geometric mean titers of 1222 and 2578 EC_{50}, respectively, roughly 5- and 10-fold higher than postfusion F (287 EC_{50}) (FIG. 29B), thereby demonstrating a conservation of the relative immunogenicity for the different forms of RSV F immunogen between mice and primates, and the ability of DS-Cavl to generate high RSV-protective titers in a primate immune system.

Optimization of RSV F protective responses

[0527] The matrix of information (FIG. 26C) generated by the interplay between design, physical and antigenic properties, atomic-level structure, and immunogenicity provides a basis for further optimization (Nabel, Science 326, 53 (2009)). For example, to obtain insight into the relationship between various antigenic and physical properties of engineered RSV Fs and the elicitation of RSV-protective responses, one can correlate properties (FIG. 31) with immunogenicity (FIG. 29). Such correlations indicate that increasing site Ø stability to physical extremes (but not trimer yield nor D25 affinity) should increase protective titers elicited upon immunization (FIG. 30A), thereby providing design insight into further optimization. Similarly, correlations between various conformational states or regions of

RSV F (FIG. 28) and immunogenicity (FIG. 29) provide design insight into the conformation of RSV F that provides the most protective responses. In this case, the results indicate that enhancing structural mimicry of antigenic site QJ in its D25-bound conformation should lead to improved protective titers (FIG. 30B).

[0528] In addition to providing direction for improvement, the matrix of information can also provide an estimate for the degree that such improvement can occur. That is, once a correlation has been established say between physical stability or structural mimicry and protective responses, one can maximize physical stability (e.g. to 100% retention of D25 binding) or structural mimicry (e.g. to exact mimicry of the D25-bound conformation) to gain an idea of the maximal improvement of the elicited protective response relative to that particular parameter. These results (FIG. 30A,B) suggest that additional structural mimicry would likely not have much effect on immunogenicity, but additional physical stabilization of antigenic site Ø might substantially improve the antigenic quality of the protective titers. Independent parameters such as adjuvant, multimerization, or immunization regimes are likely to allow improvement of the elicited response, and such parameters can be independently analyzed and optimized (Flexibility of an antigenic site may increase its immunogenicity by allow the site to conform to a wider diversity of antibodies. We note in this context that the atomic-mobility factors of antigenic site Ø were among the highest in the RSV F ectodomain).

[0529] Experimentally mixing parameters can also provide insight. For example, to determine the focus of RSV F-elicited sera, immunogenicity can be interrogated antigenically (FIG. 30C). To measure the antigenicity of sera elicited by different forms of RSV F, the different forms of RSV F were coupled to an Octet biosensor tip, and measured the reactivity of elicited sera as well as "preabsorbed" sera, to which different forms of RSV Fs had been added (FIG. 30C). With DS-Cav1 on the sensor, biosensor responses to postfusion F-, DS-, and DS-Cav1-immunized macaques showed increasing responses (FIG. 30C, left panel); with postfusion F on the sensor, biosensor responses to the same sera showed decreasing responses (FIG. 30C, right panel); and with sera that had been preabsorbed with postfusion F and with DS-Cav1 on the sensor, responses from postfusion F-, DS- and DS-Cav1-immunized macaques trending with elicited titers of protection (FIG. 30C left panel). Overall, elicited EC_{50} titers did not trend with antigenic responses measured against either prefusion or postfusion forms of RSV F, but did correlate with the level of prefusion-specific responses, either measured as a difference or as a ratio (p=0.005) between prefusion and postfusion RSV F-directed responses (FIG. 30D) (For the "prefusion" form of RSV F, the DS-Cav1 stabilized variant of RSV F was used). These results suggest that the quality of the immune response is substantially better for RSV F immunogens in the prefusion versus the postfusion conformation, a finding that may relate to the superior neutralization potency observed for prefusion-specific antibodies that target antigenic site Ø (it should be possible to deconvolute the elicited response, by using structurally defined probes, as shown with D25 and motavizumab-bond RSV F in FIG. 39).

[0530] Without being bound by theory, antigenic sites that contain multiple epitopes targeted by antibodies that derive from multiple germline genes may be ideal vaccine targets since these "supersites" have a high probability of eliciting multiple lineages of neutralizing antibodies. Antigenic site Ø on RSV F is an example of an antigenic supersite that is also a site of viral vulnerability. Many of the lessons learned from the efforts with RSV described herein, such as the importance of examining the natural human immune response and of selecting the appropriate target site, are likely to be generally applicable. Overall, by focusing structure-based design on supersites of vulnerability, structural vaccinology may be on the brink of achieving a paradigm-altering shift in the development of vaccines against viral pathogens.

Materials and Methods

[0531] Viruses and cells. Viral stocks were prepared and maintained as previously described (Graham et al., . J. Med. Virol. 26, 153 (1988)). RSV-expressing Green Fluorescent Protein (GFP) RSV-GFP was constructed and provided as previously reported (Hallak et al., Virology 271, 264 (2000)). The titer of the RSV-GFP stocks used for flow cytometry-based neutralization and fusion assays was 2.5×10^7 pfu/ml. The titer of the RSV A2 stock used for attachment assay was 1.02×10^8 pfu/ml. HEp-2 cells were maintained in Eagle's minimal essential medium containing 10% fetal bovine serum (10% EMEM) and were supplemented with glutamine, penicillin and streptomycin.

[0532] Expression and purification of antibodies and Fab fragments. Antibodies were expressed by transient co-transfection of heavy and light chain plasmids into HEK293F cells in suspension at 37°C for 4-5 days (see above, and also McLellan et al., Nat Struct Mol Biol 17, 248 (2010); McLellan et al., J Virol 84, 12236 (2010)). The cell supernatants were passed over Protein A agarose, and bound antibodies were washed with PBS and eluted with IgG elution buffer into 1/10th volume of 1 M Tris-HCl pH 8.0. Fabs were created by digesting the IgG with Lys-C or HRV3C protease (McLellan et al., Nature 480, 336 (2011)), and the Fab and Fc mixtures was passed back over Protein A agarose to remove Fc fragments. The Fabs that flowed through the column was further purified by size exclusion chromatography.

[0533] Screening of prefusion-stabilized RSV F constructs. Prefusion RSV F variants were derived from the RSV F (+) Fd construct (see Example 1), which consists of RSV F residues 1-513 with a C-terminal T4 fibritin trimerization motif (McLellan et al., Nature 480, 336 (2011)), thrombin site, 6x His-tag, and StreptagII. A 96-well microplate-formatted transient gene expression approach was used to achieve high-throughput expression of various RSV F proteins as described previously (Pancera et al., PLoS ONE 8, e55701 (2013)). Briefly, 24 hours prior to transfection HEK 293T cells were seeded in each well of a 96-well microplate at a density of 2.5×10^5 cells/ml in expression medium (high glucose DMEM supplemented with 10% ultra-low IgG fetal bovine serum and 1x-non-essential amino acids), and incubated at 37°C, 5% CO_2 for 20 h. Plasmid DNA and TrueFect-Max (United BioSystems, MD) were mixed and added to the growing cells, and the 96-well plate was incubated at 37°C, 5% CO_2. One day post transfection, enriched medium (high glucose DMEM plus 25% ultra-low IgG fetal bovine serum, 2x non-essential amino acids, 1x glutamine) was added to each well, and returned to incubator for continuous culture. On day five post transfection, the expressed RSV F protein in the supernatant was harvested and tested by ELISA for binding to D25 and motavizumab antibodies using Ni^{2+}-NTA microplates. After incubating the harvested supernatants at 4° C for one week, the ELISAs were repeated.

[0534] Large-scale expression and purification of RSV F constructs. Soluble postfusion RSV F was expressed and purified as described previously (McLellan, J Virol 85, 7788 (2011)). Prefusion variants were expressed by transient transfection in Expi293F cells using TrueFect-Max (United BioSystems, MD). The culture supernatants were harvested 5 days post transfection and centrifuged at 10,000 g to remove cell debris. The culture supernatants were sterile filtered prior to buffer exchange and concentrated using tangential flow filtration (van Reis, J Membrane Sci 159, 133 (1999)). RSV F glycoproteins were purified by immobilized nickel- and streptactin-affinity chromatography, and relevant fractions containing the RSV F variants were pooled, concentrated and subjected to size-exclusion chromatography (see Example 1). Affinity tags were removed by digestion with thrombin followed by size exclusion chromatography. Glycoproteins used in the non-human primate immunizations were tested for endotoxins using the limulus amebocyte lysate assay and if necessary, proteins were passed over an EndoTrap Red (BioVendor) column to remove endotoxins prior to immunizations. Endotoxin level was < 5 EU/kg body weight/hr, as measured by the Endpoint Chromogenic Limulus Amebocyte Lysate (LAL) test kit (Lonza, Basel, Switzerland).

[0535] Stabilized RSV F antigenic characterization. A fortéBio Octet Red384 instrument was used to measure binding kinetics of RSV F to antibodies that target antigenic site Ø (D25, AM22), site I (131-2a), site II (pavlizumab, motavizumab) and site IV (101F). All assays were performed with agitation set to 1,000 rpm in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) in order to minimize nonspecific interactions. The final volume for all solutions was 100 µl/well. Assays were performed at 30°C in solid black 96-well plates (Geiger Bio-One). *Strep*MAB-Immo (35 µg/ml) in PBS buffer was used to load anti-mouse Fc probes for 300 s, which were then used to capture relevant RSV F variant proteins that contained a C-terminal *Strep*-tag. Typical capture levels for each loading step were between 0.7 and 1 nm, and variability within a row of eight tips did not exceed 0.1 nm for each of these steps. Biosensor tips were then equilibrated for 300 s in PBS + 1% BSA prior to measuring association with antigen binding fragments (Fabs) in solution (0.002 μM to 1 μM) for 300 s; Fabs were then allowed to dissociate for 400 s-1200 s depending on the observed dissociation rate. Dissociation wells were used only once to prevent contamination. Parallel correction to subtract systematic baseline drift was carried out by subtracting the measurements recorded for a loaded sensor incubated in PBS + 1% BSA. To remove nonspecific binding responses, a HIV-1 gp120 molecule with a C-terminal *Strep*-tag was loaded onto the anti-mouse Fc probes and incubated with RSV Fabs, and the nonspecific responses were subtracted from RSV F variant response data. Data analysis and curve fitting were carried out using Octet software, version 7.0. Experimental data were fitted with the binding equations describing a 1:1 interaction. Global analyses of the complete data sets assuming reversible binding (full dissociation) were carried out using nonlinear least-squares fitting allowing a single set of binding parameters to be obtained simultaneously for all concentrations used in each experiment.

[0536] Physical stability of RSV F variants. To assess the physical stability of designed RSV F proteins under various stress conditions, the proteins were treated with a variety of pharmaceutically relevant stresses such as extreme pH, high temperature, low and high osmolality as well as repeated freeze/thaw cycles. The physical stability of treated RSV F proteins was evaluated by their degree of preservation of antigenic site Ø after treatment, a critical parameter assessed by binding of the site Ø-specific antibody D25.

[0537] In the pH treatment, RSV F protein was diluted to an initial concentration of 50 μg/ml, adjusted to pH 3.5 and pH 10 with appropriate buffers and incubated at room temperature for 60 minutes before neutralized back to pH7.5 and adjusted to 40 μg/ml. In the temperature treatment, RSV protein at 40 μg/ml was incubated at 50°C, 70°C and 90°C for 60 minutes in PCR cyclers with heated lids to prevent evaporation. In the osmolality treatment, 100 μl of RSV F protein solutions (40 μg/ml) originally containing 350 mM NaCl were either diluted with 2.5 mM Tris buffer (pH 7.5) to an osmolality of 10 mM NaCl or adjusted with 4.5 M MgCl2 to a final concentration of 3.0 M. The protein solutions were incubated for 60 minutes at room temperature and then brought back to 350 mM NaCl by adding 5M NaCl or dilution with 2.5 mM Tris buffer, respectively, before concentration down to 100 μl. The freeze/thaw treatment was carried out 10 times by repeated liquid nitrogen freezing and thawing at 37°C. Binding of antibody D25 to the treated RSV F proteins were measured with an Octet instrument with protocols described above. The degrees of physical stability were shown as the ratio of steady state D25-binding level before and after stress treatment.

[0538] Crystallization and X-ray data collection of prefusion-stabilized RSV F proteins. Crystals of RSV F DS, CavI, DSCavI, and DSCavITriC were grown by the vapor diffusion method in hanging drops at 20°C by mixing 1 μl of RSV F with 1 μl of reservoir solution (1.4 M K/Na tartrate, 0.1M CHES pH 9.5, 0.2 M $LiSO_4$). Crystals were directly frozen in liquid nitrogen. Crystals of RSV F Cav1 and DSCav1 were also grown by the vapor diffusion method in hanging drops at 20°C by mixing 1 μl of RSV F with 0.5 μl of reservoir solution (1.7 M ammonium sulfate, 0.1 M citrate pH 5.5). Crystals were transferred to a solution of 3.2 M ammonium sulfate, 0.1 M citrate pH 5.5, and flash frozen in liquid nitrogen. All X-ray diffraction data were collected at a wavelength of 1.00 Å at the SER-CAT beamline ID-22.

Structure determination, refinement and analysis of prefusion-stabilized RSV F.

[0539] X-ray diffraction data were integrated and scaled with the HKL2000 suite (Otwinowski and Minor, in Methods Enzymol. (Academic Press, 1997), vol. 276, pp. 307-326)), and molecular replacement solutions were obtained by PHASER (McCoy et al., Phaser crystallographic software. J. Appl. Crystallogr. 40, 658 (2007)) using the D25-bound RSV F structure (PDB ID: 4JHW, (see example 1)) as a search model. Manual model building was carried out using COOT (Emsley et al., Acta Crystallogr D Biol Crystallogr 66, 486 (2010)), and refinement was performed in PHENIX (Adams et al., Acta Crystallogr D Biol Crystallogr 66, 213 (2010)). Final data collection and refinement statistics are presented in FIG. 40. Superimpositions of RSV F structures were performed using residues 225-455 which showed high levels of structural similarity. Antigenic site Ø rmsd calculations were based on residues 61-71 and 194-219 which were within 10 Å of the D25 antibody in the RSV F-D25 complex structure.

[0540] Negative staining electron microscopy analysis. Samples were adsorbed to freshly glow-discharged carbon-film grids, rinsed twice with buffer, and stained with freshly made 0.75% uranyl formate. Images were recorded on an FEI T20 microscope with a 2k × 2k Eagle CCD camera at a pixel size of 1.5 Å. Image analysis and 2D averaging was performed with Bsoft (Heymann, J. Struct. Biol. 157, 3 (2007)) and EMAN (Ludtke et al., J. Struct. Biol. 128, 82 (1999)).

[0541] NHP immunizations. All animal experiments were reviewed and approved by the Animal Care and Use Committee of the Vaccine Research Center, NIAID, NIH, and all animals were housed and cared for in accordance with local, state, federal, and institute policies in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited facility at the NIH. *Macaca mulatta* animals of Indian origin weighing 8.76-14.68 kg were intramuscularly injected with immunogens at week 0 and week 4. Blood was collected every other week for up to 6 weeks.

[0542] RSV neutralization assays. Sera were distributed as four-fold dilutions from 1:10 to 1:40960, mixed with an equal volume of recombinant mKate-RSV expressing prototypic F genes from strain A2 and the Katushka fluorescent protein, and incubated at 37°C for one hour. Next, 50 μl of each serum dilution/virus mixture was added to HEp-2 cells that had been seeded at a density of 1.5×10^4 in 30 μl MEM (minimal essential medium) in each well of 384-well black optical bottom plates, and incubated for 20-22 hours before spectrophotometric analysis at Ex 588 nm and Em 635 nm (SpectraMax Paradigm, Molecular Devices, Sunnyvale, CA 94089). The IC_{50} for each sample was calculated by curve fitting and non-linear regression using GraphPad Prism (GraphPad Software Inc., San Diego CA). P values were determined by Student's T-test.

[0543] Sera antigenicity analysis. A fortéBio Octet Red384 instrument was used to measure sera reactivity to RSV F variant proteins with agitation, temperature, 96-well plates, buffer and volumes identical to those used for kinetic measurements. RSV F DSCav1 and postfusion F were immobilized to amine coupling probes via probe activation in a EDC/NHS activation mixture for 300 s in 10 mM acetate pH 5. The probe reactivity was quenched using 10 mM ethanolamine pH 8.5. Typical capture levels were between 0.7 and 1 nm, and variability within a row of eight tips did not exceed 0.1 nm for each of these steps. Biosensor tips were then equilibrated for 300 s in PBS + 1% BSA buffer prior to binding measurements. Sera were diluted to a 1/50 and 1/100 dilution in PBS + 1% BSA and binding was assessed for 300s. Sera depletion was carried out by using 1 μg of DSCav1 or postfusion F proteins per 1 μl of animal sera. Parallel correction to subtract non-specific sera binding was carried out by subtracting binding levels of an unloaded probe incubated with the sera. Site-specific antigenicity was assessed by incubating the RSV F variant-loaded probes with 1 or 2 μM D25 Fab for site Ø assessment and motavizumab Fab for site II assessment or both antibodies to assess the remaining non-site Ø/II reactivity.

Example 10

Single Chain RSV F proteins stabilized in a prefusion conformation

[0544] This example illustrates additional recombinant RSV F proteins that lack the native furin cleavage sites, such that the F protein protomer is formed as a single polypeptide chain, instead of a F_2/F_1 heterodimer. Schematic diagrams illustrating design of the additional prefusion-stabilized single-chain RSV F proteins are provided in FIGs. 43 and 44.

[0545] FIGs. 43-45 illustrate the design of a series of single chain constructs, including single-chain RSV F construct no. 9 (scF no. 9; BZGJ9 DSCavl; SEQ ID NO: 669). Variables for the single chain constructs include the linker size, the F1 and F2 end points and the mechanism used to induce trimerization of the single chain construct. Additionally, several strategies can be employed to stabilize the single chain constructs in a prefusion conformation, including use of the strategies described herein. The indicated single chain constructs were expressed in cells and characterized by size exclusion chromatography (FIG. 46) and binding to RSV F specific antibodies (FIG. 47).

[0546] To further characterize the RSV F construct no. 9 (scF no. 9; BZGJ9 DSCavl; SEQ ID NO: 669), the three dimensional structure of this protein was solved by X-ray crystallography (see FIGs. 48-51). Cubic crystals were grown using the vapor diffusion method in a reservoir solution of 1.19 M Li_2SO_4, 3.33% PEG 400, 0.12 M $MgSO_4$, 0.1 M NaOAc pH 5.5. Crystals grew to ~120 µm before they were flash-frozen in a reservoir solution containing 2 M lithium sulfate. The diffraction data was collected to a resolution of 3.2 Å with an intensity over error of 2.84. The crystal structure illustrated the location of the GS linker in construct No. 9 (FIGs. 49 and 50), was used to predict the location of other linker sizes (FIG. 51), and as the basis of the design of additional single chain constructs BZGJ9-1 through 9-10 (see FIG. 55). Single-chain construct codon-optimized genes with a C-terminal T4 fibritin trimerization motif, thrombin site, 6x His-tag, and StreptagII was synthesized and subcloned into a mammalian expression vector derived from pLEXm. Plasmids expressing RSV F(+) Fd, were transfected into HEK293 GnTI-/- Cells in suspension. After 4-5 days, the cell supernatant was harvested, centrifuged, filtered and concentrated. The protein was initially purified via Ni2+-NTA resin (Qiagen, Valencia, CA) using an elution buffer consisting of 20 mM Tris-HCl pH 7.5, 200 mM NaCl, and 250 mM imidazole pH 8.0. The complex was then concentrated and further purified over StrepTactin resin as per the manufacturer's instructions (Novagen, Darmstadt, Germany). After an overnight incubation with thrombin protease (Novagen) to remove the His and Strep tags, an excess of D25 Fab was added to the complex, which was then purified on a Superdex-200 gel filtration column (GE Healthcare) with a running buffer of 2 mM Tris-HCl pH 7.5, 350 mM NaCl, and 0.02% NaN3 or phosphate buffered saline (PBS) pH7.4. Single-chain-Ferritin single gene products were expressed and purified in a similar manner.

[0547] Several single chain constructs were selected for immunogenicity testing in animal models (FIG. 53). BZGJ9 DS-Cav1, BZGJ9, BZGJ11 DS-Cav1 (monomer), BZGJ10 (monomer and trimer fractions), BZGJ8 (monomer), BZGJ4 DS-Cav1 and BZGJ11 DS-Cav1-Lumazine synthase (60mer oligomer) were all tested for immunogenicity in groups of 10 CB6F1/J mice by injecting 10 ug of protein in the presence of 50 ug Poly I:C at week 0 and week 3. Sera from week 5 was tested for immunogenicity. Control groups of RSV F subtype A DS-Cav1 and Postfusion protein were also tested and immunized in a similar manner.

[0548] To assess neutralization against RSV subtype A and Subtype B, sera from immunized animals were distributed as four-fold dilutions from 1:10 to 1:40960, mixed with an equal volume of recombinant mKate-RSV expressing prototypic F genes from subtype A (strain A2) or subtype B (strain 18537) and the Katushka fluorescent protein, and incubated at 37 °C for 1 h. Next, 50 µl of each serum dilution/virus mixture was added to HEp-2 cells that had been seeded at a density of 1.5x104 in 30 µl MEM (minimal essential medium) in each well of 384-well black optical bottom plates, and incubated for 20-22 h before spectrophotometric analysis at 588 nm excitation and 635 nm emission (SpectraMax Paradigm, Molecular Devices, CA). The IC50 for each sample was calculated by curve fitting and non-linear regression using GraphPad Prism (GraphPad Software Inc., CA). P-values were determined by Student's t-test.

[0549] The neutralization results show that all tested single chain constructs are immunogenic.

[0550] The single chain constructs were linked to ferritin to produce ferritin nanoparticles including the scF antigens (FIG. 56). Briefly, the C-terminus of the F1 polypeptide included in the scF protein was linked to ferritin, and the recombinant protein was expressed in cells to produce scF-ferritin nanoparticles. One example is the "BZGJ9-DS-Cav1-LongLink-Ferritin" protein (SEQ ID NO: 1429), which includes a recombinant RSV F single chain protein including a GS linker between RSV F positions 105 and 145 and a ferritin subunit linked to position 513 of the RSV F protein by a heterologous peptide linker generated by linking the C-terminus of the F1 polypeptide in scF no.9 to a ferritin subunit. The scF-ferritin nanoparticles were expressed, purified, and characterized for temperature, pH, and osmolarity stability (FIG. 57). Additionally, the ferritin nanoparticles were administered to animals to demonstrate that they are immunogenic (FIG. 58). The three constructs tested were RSV F DSCav1 (SEQ ID NO: 371), BZGJ9-DS-Cav1-LongLink-Ferritin (SEQ ID NO: 1429), and scF no. 9 (also termed BZGJ9 DS-Cavl, SEQ ID NO: 669). These are the same immunogenicity/neutralization as described above.

[0551] Several single chains sequences are provided in the SEQ ID NOs listed in Table 19, as well as an indication of design approach.

Table 19. Exemplary Single Chain RSV F proteins

Name	Concept	Background		Mutations	SEQ ID NO
Non-cleavable Foldon					
BZGJ9-1	SC BZGJ9-DS-Cav1 w/IL			G linker between residue 105 to 145	698
BZGJ9-2	SC BZGJ9-DS-Cav1 w/IL			GG linker between residue 105 to 145	699
BZGJ9-3	SC BZGJ9-DS-Cav1 w/IL			GQG linker between residue 105 to 145	700
BZGJ9-4	SC BZGJ9-DS-Cav1 w/IL			GGSG (Seq_1443) linker between residue 105 to 145	701
BZGJ9-5	SC BZGJ9-DS-Cav1 w/IL			GGSG (Seq_1443) linker between residue 105 to 145	702
BZGJ9-6	SC BZGJ9-DS-Cav1 w/IL			GGSG (Seq_1443) linker between residue 105 to 145	703
BZGJ9-7	SC BZGJ9-DS-Cav1 w/IL			GGSGGS (Seq_1444) linker between residue 105 to 145	704
BZGJ9-8	SC BZGJ9-DS-Cav1 w/IL			GGSGGSG (Seq_1445) linker between residue 105 to 145	705
BZGJ9-9	SC BZGJ9-DS-Cav1 w/IL			Fusion of residue 103 to 145	706
BZGJ9-10	SC BZGJ9-DS-Cav1 w/IL			GS linker between residue 103 to 145	707
Cleavable Foldon					
scRSVF9aCCextxFd	Inter-DS	BZGJ9a (SeqID 669 w/o DSCavl mut.)	xFd	512CChnvnagkstt	708
scRSVF9aC485C494xFd	Inter-DS	BZGJ9a	xFd	485C, 494C	709

Cleavable Foldon					
scRSVF9aC519C520extx Fd	Inter-DS	BZGJ9a	xFd	512LLhnvnaCCstt	710
scRSVF9aC99C362xFd	Inter-DS	BZGJ9a	xFd	99C, 362C	711
scRSVF9aC99C361xFd	Inter-DS	BZGJ9a	xFd	99C, 361C	712
scRSVF9aC153C461xFd	Inter-DS	BZGJ9a	xFd	153C, 461C	713
scRSVF9aC102C359xFd	Inter-DS	BZGJ9a	xFd	102C, 359C	714
scRSV F 9axFd	xFd	BZGJ9a	xFd	512LLSAI	715
scRSV F 9aextxFd	xFd	BZGJ9a		|xFd|512LLhnvnagkstt	716
Ferritin Particles - No Foldon					
OpFer1	ES of RSV F	BZGJ9a	Fer	S190V	717
OpFer2	ES of RSV F	BZGJ9a	Fer	K226L	718
OpFer3	ES of RSV F	BZGJ9a	Fer	T58I, A298M	719
OpFer4	ES of RSV F	BZGJ9a	Fer	S190V, K226L	720
OpFer5	ES of RSV F	BZGJ9a	Fer	S190V, T58I, A298M	721
OpFer6	ES of RSV F	BZGJ9a	Fer	K226L, T58I, A298M	722
OpFer7	ES of RSV F	BZGJ9a	Fer	T58I, A298M, S190V, K226L	723
OpFer8	ES of RSV F	BZGJ9a	Fer	CavI	724
OpFer9	ES of RSV F	BZGJ9a	Fer	NoMutationsSC9a	725
OpFer10	ES of RSV F	BZGJ9a	Fer	S190V with optimized coil coil - 8aa linker	726
OpFer11	ES of RSV F	BZGJ9a	Fer	S190V With CC and optimized coiled coil - 8aa linker	727
OpFer12	ES of RSV F	BZGJ9a	Fer	S190V FIRKSDELLSAIGGYIPS APSGSG-Fer	728
OpFer13	ES of RSV F	BZGJ9a	Fer	S190V SC-Foldon-8aa-Fer	729
OpFer14	ES of RSV F	BZGJ9a	Fer	S190V optimized leader	730
Non-cleavable Foldon					
OpFd1	ES of RSV F	BZGJ9a	Fd	S190V	731
OpFd2	ES of RSV F	BZGJ9a	Fd	K226L	732
OpFd3	ES of RSV F	BZGJ9a	Fd	T58I, A298M	733
OpFd4	ES of RSV F	BZGJ9a	Fd	S190V, K226L	734
OpFd5	ES of RSV F	BZGJ9a	Fd	S190V, T58I, A298M	735
OpFd6	ES of RSV F	BZGJ9a	Fd	K226L, T58I, A298M	736
OpFd7	ES of RSV F	BZGJ9a	Fd	T58I, A298M, S190V, K226L	737
OpFd8	ES of RSV F	BZGJ9a	Fd	S190F, V207L	738
OpFd9	ES of RSV F	BZGJ9a	Fd	NoMutationsSC9a	739
OpFd10	ES of RSV F	BZGJ9a	Fd	S190V with optimized coil coil	740
OpFd11	ES of RSV F	BZGJ9a	Fd	S190V With CC and optimized coiled coil	741
OpFd14	ES of RSV F	BZGJ9a	Fd	S190V optimized leader	742
OpFd14	ES of RSV F	BZGJ9a	Fd	S190V optimized leader	743
Cleavable Foldon					
scRSVF9a 74C218C xFd	Inter-DS	BZGJ9a	xFd	74C, 218C	744
scRSVF9a 146C460C xFd	Inter-DS	BZGJ9a	xFd	146C, 460C	745
scRSVF9a 149C458C xFd	Inter-DS	BZGJ9a	xFd	149C, 458C	746
scRSVF9a 374C454C xFd	Inter-DS	BZGJ9a	xFd	374C, 454C	747
scRSVF 74C218C xFd	Inter-DS	SEQ_669 (BZGJ9 DSCavI)	xFd	74C, 218C	748
scRSVF 146C460C xFd	Inter-DS	BZGJ9 DSCavI	xFd	146C, 460C	749
scRSVF 149C458C xFd	Inter-DS	BZGJ9 DSCavI	xFd	149C, 458C	750
scRSVF 374C454C xFd	Inter-DS	BZGJ9 DSCavI	xFd	374C, 454C	751
scRSVF9 C485C494xFd	Inter-DS	BZGJ9 DSCavI	xFd	485C, 494C	752
scRSVF9 C519C520extxFd	Inter-DS	BZGJ9 DSCavI	xFd	519C, 520C	753
scRSVF9 C99C362xFd	Inter-DS	BZGJ9 DSCavI	xFd	99C, 362C	754
scRSVF9 C99C361xFd	Inter-DS	BZGJ9 DSCavI	xFd	99C, 361C	755
scRSVF9 C153C461xFd	Inter-DS	BZGJ9 DSCavI	xFd	153C, 461C	756
scRSVF9 C102C359xFd	Inter-DS	BZGJ9 DSCavI	xFd	102C, 359C	757
Non-Cleavable Foldon					
BZGJ9pi I217W	Inter-DS	BZGJ9 DSCavI	Fd	1217W	758
BZGJ9pi 1221W	Inter-DS	BZGJ9 DSCavI	Fd	1221W	759
BZGJ9pi D486F	Inter-DS	BZGJ9 DSCavI	Fd	H486F	760
BZGJ9pi T400F	Inter-DS	BZGJ9 DSCavI	Fd	T400F	761
BZGJ9pi V278F	Inter-DS	BZGJ9 DSCavI	Fd	V278F	762
BZGJ9pi Q224D,L78K	Inter-DS	BZGJ9 DSCavI	Fd	Q224D, L78K	763
BZGJ9pi 1217W, 1221W	Inter-DS	BZGJ9 DSCavI	Fd	1217W, 1221W	764

Non-Cleavable Foldon					
BZGJ9pi I217W, 1221W, L78F	Inter-DS	BZGJ9 DSCavl	Fd	1217W, 1221W, L78F	765
GSJscINT_1	SC RSV F	DSCAV1	Fd	F2 linked to full fusion peptide by (Gly)n linker	766
GSJscINT_2	SC RSV F	DSCAV2	Fd	F2 linked to full fusion peptide by (Gly)n linker	767
GSJscINT_3	SC RSV F	DSCAV3	Fd	F2 linked to full fusion peptide by (Gly)n linker	768
GSJscINT_2 F488W	SC RSV F	DSCAV4	Fd	F2 linked to full fusion peptide by (Gly)n linker	769
GSJscINT_1 Q354A	SC RSV F	DSCAVS	Fd	F2 linked to full fusion peptide by (Gly)n linker	770
GSJscINT_MBE	SC RSV F	DSCAV6	Fd	F2 linked to full fusion peptide by (Gly)n linker	771
GSJscINT_2 F488Wsh	SC RSV F	DSCAV7	Fd	F2 linked to full fusion peptide by (Gly)n linker	772
GSJscINT_1 F488Wsh	SC RSV F	DSCAV8	Fd	F2 linked to full fusion peptide by (Gly)n linker	773
BZGJ9-11	SC BZGJ9-DS-Cav1 w/IL		Fd	GS linker between 102 to 145	774
BZGJ9-12	SC BZGJ9-DS-Cav1 wilL		Fd	GS linker between 101 to 145	775
BZGJ9-13	SC BZGJ9-DS-Cav1 wilL		Fd	GS linker between 100 to 145	776
BZGJ9-14	SC BZGJ9-DS-Cav1 wilL		Fd	GS linker between 99 to 145	777
BZGJ9-15	SC BZGJ9-DS-Cav1 wilL		Fd	GS linker between 98 to 145	778
BZGJ9-16	SC BZGJ9-DS-Cav1 wilL		Fd	GS linker between 97 to 145	779
BZGJ9-17	SC BZGJ9-DS-Cav1 w/IL		Fd	GGSG linker between 103 to 145	780
BZGJ9-18	SC BZGJ9-DS-Cav1 wilL		Fd	GGSGG linker between 103 to 145	781
BZGJ9-19	SC BZGJ9-DS-Cav1 w/IL		Fd	GGSGGSG linker between 103 to 145	782
BZGJ9-20	SC BZGJ9-DS-Cav1 wilL		Fd	GGSGN linker between 103 to 145	783
BZGJ9-21	SC BZGJ9-DS-Cav1 wilL		Fd	N linker between 102 to 145	784
BZGJ9-22	SC BZGJ9-DS-Cav1 w/IL		Fd	ISSTSATGS linker between 96 to 145	785
BZGJ9-23	SC BZGJ9-DS-Cav1 w/IL		Fd	VTSTSATGS linker between 96 to 145	786
BZGJ9-24	SC BZGJ9-DS-Cav1 w/IL		Fd	NSALSATGS linker between 96 to 145	787
BZGJ9-25	SC BZGJ9-DS-Cav1 w/IL		Fd	ISSTTSTGS linker between 96 to 145	788
BZGJ9-26	SC BZGJ9-DS-Cav1 w/IL		Fd	VTSTTSTGS linker between 96 to 145	789
BZGJ9-27	SC BZGJ9-DS-Cav1 w/IL		Fd	NSALSSTGS linker between 96 to 145	790
BZGJ9-28	SC BZGJ9-DS-Cav1 w/IL		Fd	ISSTSATVGGS linker between 96 to 145	791
BZGJ9-29	SC BZGJ9-DS-Cav1 w/IL		Fd	VTSTSATTGGS linker between 96 to 145	792
BZGJ9-30	SC BZGJ9-DS-Cav1 w/IL		Fd	NSALSATGGS linker between 96 to 145	793
BZGJ9-31	SC BZGJ9-DS-Cav1 w/IL		Fd	LISSTTSTVGGS linke r between 96 to 145	794
BZGJ9-32	SC BZGJ9-DS-Cav1 w/IL		Fd	VTSTTSTTGGS linker between 96 to 145	795
BZGJ9-33	SC BZGJ9-DS-Cav1 w/IL		Fd	NSALSSTGGS linker between 96 to 145	796
Lumazine Synthase Particles					
BZGJ10-DSCav1-LS	Monomer RSV F SC on LS		LS	SEKS Furin site I and SEKSGS Furin site II	797
BZGJ10-DSCav1 DEF-LS	Monomer RSV F SC on LS		LS	SEKS Furin site I and SEKSGS Furin site II	798
BZGJ11-DSCav1-LS	Monomer RSV F SC on LS		LS	SEKS Furin site I and SEKSGS Furin site II	799
BZGJ11-DSCav1-SS-LS	Monomer RSV F SC on LS		LS	SEKS Furin site I and SEKSGS Furin site II	800
Non-cleavable Foldon, with Pep27					
A2-PP1	SC w/o furin sites to maintain pep27		Fd	SEKS Furin site I and SEKSGS Furin site II	801
B18537-PP1	SC w/o furin		Fd	SEKS Furin site I and SEKSGS Furin site II	802

Non-cleavable Foldon, with Pep27					
	sites to maintain pep27				
A2-DS-Cav1-PP1	SC w/o furin sites to maintain pep27		Fd	SEKS Furin site I and SEKSGS Furin site II	803
B18537 DS-Cav1-PP1	SC w/o furin sites to maintain pep27		Fd	SEKS Furin site I and SEKSGS Furin site II	804
A2-DS-Cav1-PP1-dFold	SC w/o furin		Fd	SEKS Furin site I	805
	sites to maintain pep27			and SEKSGS Furin site II	
B18537 DS-Cav1-PP1-dFold	SC w/o furin sites to maintain pep27		Fd	SEKS Furin site I and SEKSGS Furin site II	806
A2-DS-Cav1-PP1-GCN4	SC w/o furin sites to maintain pep27		Fd	SEKS Furin site I and SEKSGS Furin site II	807
B18537 DS-Cav1-PP1-GCN4	SC w/o furin sites to maintain pep27		Fd	SEKS Furin site I and SEKSGS Furin site II	808
BZGJ9-dscav1 N155Q	SC BZGJ9-DS-Cav1 w/IL		Fd	Removal of introduced glycan site on BZGJ9-DS-Cav1 (SEQ ID:669 located in the linker region on Asn 105	809
BZGJ9-DS-Cav1-FerritinHis	Add Histidines to Ferr to improve expression and purification		Fd	Introduction of six His residues to the Ferritin molecule to enable purification of RSV F molecules in the Ferritin context without using His-tag or Strep-Tag sequences.	810
Additional constructs					
B18537-BZGJ9-9	B18537 strain		Fd	Single chain RSV F subtype B (strain B18537)with direct fusion of residue 103 to 145	811
B18537-BZGJ9-10	B18537 strain		Fd	Single chain RSV F subtype B (strain B18537)with GS linker between residue 103 to 145	812
B1-BZGJ9-9	B1 strain		Fd	Single chain RSV F subtype B (strain B1) with direct fusion of residue 103 to 145	813
B1-BZGJ9-10	B1 strain		Fd	Single chain RSV F subtype B (strain B)with GS linker between residue 103 to 145	814
BZGJ9ext-9			xFd	Single chain RSV F DS-CavI (BZGJ9 #669)with direct fusion of residue 103 to 145 and an elongation of the C-terminus adding residues hnvnagkstt after L513 and prior to the Thrombin-His-Strep tags.	815
BZGJ9ext-10			xFd	Single chain RSV F	816
				DS-Cav1 (BZGJ9 #669)with GS linker between residue 103 to 145 and an elongation of the C-terminus adding residues hnvnagkstt after L513 and prior to the Thrombin-His-Strep tags.	
B18537-BZGJ9ext-9	B18537 strain		xFd	Single chain RSV F DS-Cav1 (subtype B (strain B18537) with direct fusion of residue 103 to 145 and an elongation of the C-terminus adding residues hnvnagkstt after L513 and prior to the Thrombin-His-Strep tags.	817
B18537-BZGJ9ext-10	B18537 strain		xFd	Single chain RSV F DS-Cav1 (subtype B (strain B18537) with GS linker between residue 103 to 145 and an elongation of the C-terminus adding residues hnvnagkstt after L513 and prior to the Thrombin-His-Strep tags.	818
B1-BZGJ9ext-9	B1 strain		xFd	Single chain RSV F DS-Cav1 (subtype B (strain B1) with direct fusion of residue 103 to 145 and an elongation of the C-terminus adding residues hnvnagkstt after L513 and prior to the Thrombin-His-Strep tags.	819
B1-BZGJ9ext-10	B1 strain		xFd	Single chain RSV F DS-Cav1 (subtype B (strain B1) with GS linker between residue 103 to 145 and an elongation of the C-terminus adding residues hnvnagkstt after L513 and prior to the Thrombin-His-Strep tags.	820
BZGJ9extxFd-9				Single chain RSV F DS-Cav1 (BZGJ9 #669)with direct fusion of residue	821
				103 to 145 and an elongation of the C-terminus adding residues hnvnagkstt after L513 and prior to the cleavable Foldon and Thrombin-His-Strep tags.	
BZGJ9extxFd-10				Single chain RSV F DS-Cav1 (BZGJ9 #669)with GS linker between residue 103 to 145 and an elongation of the C-terminus adding residues hnvnagkstt after L513 and prior to the cleavable	822

Additional constructs					
				Foldon and Thrombin-His-Strep tags.	
B18537-BZGJ9extxFd-9	B18537 strain			Single chain RSV F DS-Cav1 (subtype B (strain B18537) with direct fusion of residue 103 to 145 and an elongation of the C-terminus adding residues hnvnagkstt after L513 and prior to the cleavable Foldon and Thrombin-His-Strep tags.	823
B18537-BZGJ9extxFd-10	B18537 strain			Single chain RSV F DS-Cav1 (subtype B (strain B18537) with GS linker between residue 103 to 145 and an elongation of the C-terminus adding residues hnvnagkstt after L513 and prior to the cleavable Foldon and Thrombin-His-Strep tags.	824
B1-BZGJ9extxFd-9	B1 strain			Single chain RSV F DS-Cav1 (subtype B (strain B1) with direct fusion of residue 103 to 145 and an elongation of the C-terminus adding residues hnvnagkstt after	825
				L513 and prior to the cleavable Foldon and Thrombin-His-Strep tags.	
B1-BZGJ9extxFd-10	B1 strain			Single chain RSV F DS-Cavl (subtype B (strain B1) with GS linker between residue 103 to 145 and an elongation of the C-terminus adding residues hnvnagkstt after L513 and prior to the Thrombin-His-Strep tags.	826
BZGJ9-CS1				Single chain RSV F with linker MTSVLHRFDTDAF between 96 and 150	1474
BZGJ9-CS2				Single chain RSV F with linker MTSVLWFGDTDAFA between 96 and 150	1475
BZGJ9-10-GSJCCtail5xFd			xFd	Single chain RSV F based on sequence #707 with C-terminalsequence CChnvnagksttnGGLVP RGS encoding disulphide bonds and cleavable foldon	1476
BZGJ9-10-GSJCCtail6xFd			xFd	Single chain RSV F based on sequence #707 with C-terminalsequence LLhnvnaCCsttnGGLVP RGS encoding disulphide bonds and cleavable foldon	1477
BZGJ9-10-GSJCCtail9xFd			xFd	Single chain RSV F based on sequence #707 with C-terminal sequence CChnvnaCCsttnGGLVP RGS encoding disulphide bonds and cleavable foldon	1478
BZGJ9-9-DS-Cav1-Ferritin				RSV F single chain BZGJ9-9 (#706) with Fusion of residue 103 to 145in the Ferritin context	827
BZGJ9-10-DS-Cav1-Ferritin				RSV F single chain BZGJ9-10 (#707) GS linker between residue 103 to 145	828
BZGJ9-DS-Cav1-LongLink-Ferritin				RSV F single chain BZGJ9 (#669) in the Ferritin context with a long linker from the RSV F C-terminus to the Ferritin N-terminus	1429
BZGJ9-9-DS-Cav1-LongLink-Ferritin				RSV F single chain BZGJ9-9 (#706) with Fusion of residue 103 to 145 in the Ferritin context with a long linker from RSV C-terminus to Ferritin N-terminus	1430
BZGJ9-10-DS-Cav1-LongLink-Ferritin				RSV F single chain BZGJ9-10 (#707) GS linker between residue 103 to 145 in the Ferritin context with a long linker from RSV C-terminus to Ferritin N-terminus	1431
BZGJ9-DS-Cav1-LongLinkFerritinHis	Add Histidines to Ferr to improve expression and purification			RSV F single chain BZGJ9 (#669) in the Ferritin context with a long linker from the RSV F C-terminus to the Ferritin N-terminus with added histidines to the Ferritin molecule to facilitate purification of Ferritin nanoparticles without the use of His or Strep-tags.	1432
BZGJ9-IG1	SC BZGJ9-DS-Cav1 w/IL			ARLLGSGSG linker from 96 to 147	1433
BZGJ9-IG2	SC BZGJ9-DS-Cav1 w/IL			ARLLGGSG linker from 96 to 147	1434
BZGJ9-IG3	SC BZGJ9-DS-Cav1 w/IL			ARLLGGSG linker from 96 to 148	1435
BZGJ9-IG4	SC BZGJ9-DS-Cav1 w/IL			LARLLGSG linker from 96 to 147	1436
BZGJ9-IG5	SC BZGJ9-DS-Cav1 w/IL			mqstGGSG linker from 96 to 147	1437
BZGJ9-IG6	SC BZGJ9-DS-Cav1 w/IL			aqstGGSG linker from 96 to 147	1438
				RSV F single chain BZGJ9-9 (#811) with Fusion	

Additional constructs					
B18537-BZGJ9-9-LongLink-Ferritin	B18537 strain			of	1439
				residue 103 to 145 in the Ferritin context with a long linker from RSV C-terminus to Ferritin N-terminus	
B18537-BZGJ9-10-LongLink-Ferritin	B18537 strain			RSV F single chain BZGJ9-10 (#812) GS linker between residue 103 to 145 in the Ferritin context with a long linker from RSV C-terminus to Ferritin N-terminus	1440
B1-BZGJ9-9-LongLink-Ferritin	B1 strain			RSV F single chain BZGJ9-9 (#813) with Fusion of residue 103 to 145 in the Ferritin context with a long linker from RSV C-terminus to Ferritin N-terminus	1441
B1-BZGJ9-10-LongLink-Ferritin	B1 strain			RSV F single chain BZGJ9-10 (#814) GS linker between residue 103 to 145 in the Ferritin context with a long linker from RSV C-terminus to Ferritin N-terminus	1442

The yield of protein was calculated for several of the recombinant F proteins, and is shown below in Table 27.

Table 27. Yield of recombinant RSV F protein expression

Construct Name	Yield (mg/L)	SEQ ID NO
scRSVF9aCCextxFd	12.7	708
scRSVF9aC485C494xFd	4.1	709
scRSVF9aC419C420ext xFd	11.4	710
scRSVF9aC99C362xFd	2.2	711
scRSV F 9axFd	15.7	715
scRSV F 9aextxFd	29.6	716
GSJscINT 1	0.84	766
GSJscINT 3	0.9	768

Example 11

The structure of an RSV F protein from the B18537 strain with the DSCav1 mutations

[0552] This examples illustrated the similarity of the RSV protein with the stabilizing DSCavl substitutions across RSV subtypes. The DSCavl substitutions were introduced into the RSV F protein from the B 18537 strain. And the three dimensional structure of the resulting recombinant protein, including a C-terminal Foldon domain, was solved using methods similar to those described above. As shown in FIGs. 59-62, the DSCavl substitutions could be successfully introduced into a RSV F glycoprotein B subtype to stabilize antigenic site Ø to generate a DSCavl mutant on the subtype B background that specifically binds to prefusion specific antibodies. Table 25, below, provides a summary of the crystallographic data for DSCavl on RSV F subtype B.

Table 25. Crystallographic data concerning DSCavl subtype B

		RSV B18537 F
PDB accession code		
Data collection		
Space group		$P4_132$
Cell constants		
	a, b, c (Å)	167.9, 167.9, 167.9
	a, b, g (°)	90, 90, 90
Wavelength (Å)		1.00
Resolution (Å)		50.0-1.94 (2.01-1.94)
R_{merge}		10 (78.8)
I / s*I*		11.96 (1.12)
Completeness (%)		95.9 (79.7)
Redundancy		4.0 (2.4)
Refinement		
Resolution (Å)		1.94
Unique reflections		57,616
R_{work} / R_{free} (%)		18.71/21.52
No. atoms		
	Protein	3552
	Ligand/ion	5
	Water	401
B-factors (Å^2)		
	Protein	46.2
	Ligand/ion	78.3
	Water	53.2

Refinement		
R.m.s. deviations		
	Bond lengths (Å)	0.009
	Bond angles (°)	1.17
Ramachandran		
	Favored regions (%)	96.18
	Allowed regions (%)	3.82
	Disallowed regions (%)	0

Example 12

Design and production of recombinant RSV F proteins without a trimerization domain.

[0553] This example illustrated the design and production of recombinant RSV F proteins that are stabilized in a prefusion conformation but which do not include a C-terminal trimerization domain to maintain stability of the membrane proximal lobe of the RSV F protein.

[0554] Briefly, in place of the C-terminal trimerization domain, a ring of disulfide bonds is introduced into the C-terminus of the FI polypeptide by substituting cysteine residues for amino acids of the α10 helix. The three α10 helixes of the RSV F Ectodomain for a coil-coil that stabilized the membrane proximal portion of the protein. When expressed in cells, inter-protomer disulfide bonds form between the cysteines introduced into the al0 helix, thereby "locking" the three α10 helix's in close proximity and preventing movement of the membrane proximal domain from the pre-to the post-fusion conformation. The α10 helix of the RSV F protein includes residues 492 to the transmembrane domain (residue 529).

[0555] In this example, the recombinant RSV F protein with the stabilizing cysteine ring is initially expressed as a recombinant protein that includes a trimerization domain. The trimerization domain can be proteolytically removed following initial expression. The cleavage can be performed before, after, or during purification of the RSV F protein. Currently we purify the RSV F protein using tendem Ni2+ IMAC and Streptactin immobilization steps via the C-terminal His6 and StrepII tag, followed by thrombin digestion at room temperature for 12 hours then separation of the foldon from the RSV F protein by size-exclusion chromatography. It would be possible to also purify the cleaved RSV F protein by ion exchange.

[0556] FIGs. 63-68 show gel filtration results and coommassie blue staining of reduced and non-reduced PAGE analysis of several of the recombinant F proteins without trimerization domain as designed listed below. Table 22 provides antigenic and physical characteristics of the indicated constructs, which include the DSCavl substitutions, and cysteine substitutions in the al0 helix at positions 525 and 526 (CCTail4xFd), 512 and 513 (CCTail5xFd), 519 and 520 (CCTail6xFd), and 512 and 512 (CCLongxFd). The corresponding SEQ ID NO for each construct is shown in FIG. 66.

Table 22. Antigenic and physical characteristics of engineered RSV F glycoprotein variants.

	Physical characterization (Fractional D25 reactivity)						
	1 hour incubation Temp (°)		**pH**		**Osmolality (mM)**		**10 times Freeze-thaw**
Construct	**50**	**70**	**3.5**	**10**	**10**	**3000**	
GSJ CCtail 4x Fd Uncleaved	0.9	0.5	0.9	0.9	1.0	.08	0.3
GSJ CC tail 4x Fd Cleaved	0.9	0.4	0.9	1.0	1.0	0.7	0.4
GSJ CC tail 5x Fd Uncleaved	0.9	0.6	0.9	1.0	1.0	0.7	0.2
GSJ CC tail 5x Fd Cleaved	0.9	0.2	0.9	1.0	1.0	1.0	0.2
GSJ CCtail 6x Fd Uncleaved	0.9	0.4	0.9	0.9	1.0	0.7	0.2
GSJ CC tail6 xFd Cleaved	1.0	0.3	0.9	0.9	1.0	0.7	0.3
GSJ CC tail Long xFd Unleaved	0.9	0.1	0.7	0.8	0.8	0.6	0.1
GSJ CC tail Long xFd Cleaved	1.0	0.3	0.9	1.0	1.0	0.8	0.2

[0557] Several RSV F protein sequences without trimerization domains, or with a cleavable trimerization domain are provided in the SEQ ID NOs listed in Table 23, as well as an indication of design approach. The name, α10 cysteine ring, presence or absence of C-terminal Foldon or cleavable Foldon, background sequence (e.g., "DSCAV1" indicates that the construct includes the DSCavl substitutions), the design concept, and corresponding SEQ ID NO are indicated. In Table 23, the following acronyms are used: DSCAV1: S155C, S290C, S190F, V207L substitutions; Op - Optimized coil coil; OpCC - Optimized Coil Coil with disulfides; InterC - Interprotomer disulfide at C-terminal helix; Multi-InterC - Multiple interprotomer disulfide stabilization; ECC: Enhanced coil-coil stability; FP-CC: Fusion peptide Cys bridge; 190P: 190 pocket alternative amino acid; Fd (non-cleavable Foldon), xFd (cleavable foldon), N (no Foldon); CFM: Cavity Filling mutation; ICFM: Interface cavity filling mutations

[0558] The recombinant RSV F proteins with no or with a cleavable trimerization domain listed in Table 23 were expressed in cells under conditions where the proteins are secreted from the cells in the cell media as described above in Example 9. Each construct contains a leader sequence that causes the protein to enter the secretory system and be secreted. The medium was then centrifuged and the supernatant used for antigenicity testing for binding to the Site Ø specific antibody D25 and the Site II specific antibody Motavizumab ("Mota", FIGs. 69A-69E). The conditions tested include D25 and Mota binding on day 0 (conditions 1 and 2), D25 and Mota binding on day 0 after incubation at 70°C for one hour (conditions 3 and 4), and D25 and Mota binding after 1 week at 4°C (conditions 5 and 6). The control is the DSCavl construct with a foldon domain. Specific antigenicity data for each construct is provided in FIGs. 69A-69E (the conditions tested are noted in the header rows).

Table 23. Recombinant RSV F proteins that lack a trimerization domain, or that have a protease cleavable trimerization domain.

Construct Name	Motif	C-Term	Background	Design concept	SEQ ID NO
CS/GSJ ext2Opti1	512LLhnvnagLstVnimLttVl	N	DSCAV1	Op	829
CS/GSJ ext2Opti2	512LLhnvnagLstVnKmLttVl	N	DSCAV1	Op	830
CS/GSJ ext2Opti3	512LLhnvnKkLstVnKmLttVl	N	DSCAV1	Op	831

Construct Name	Motif	C-Term	Background	Design concept	SEQ ID NO
CS/GSJ ext2OpCC1	512CChnvnagLstVnKmLttVl	N	DSCAV1	OpCC	832
CS/GSJ ext2OpCC2	512LLhnvnaCCstVnKmLttVl	N	DSCAV1	OpCC	833
CS/GSJ ext2OpCC3	512LLhnvnagLstVnKCCttVl	N	DSCAV1	OpCC	834
CS/GSJ ext2OpCC4	512FQNAVESTINTLQTTLEAV AQAl	N	DSCAV1	Op	835
CS/GSJ GCN4ccl	512IEDKIEEILSKQYHIENEIA RCC	N	DSCAV1	OpCC	836
CS/GSJ GCN4cc2	512CCDKIEEILSKQYHIENEIA RIK	N	DSCAV1	OpCC	837
CS/GSJ CartOp	512LLhnvnagLstVnKmLttVlKcc	N	DSCAV1	OpCC	838
GSJ CClongxFd	512CCHNVNAGKSGG	xFd	DSCAV1	InterC	839
GSJ CCtaillxFd	512CChnvnagksttnimitt	xFd	DSCAV1	InterC	840
GSJ CCtail2xFd	512LLhnvnaCCsttnimitt	xFd	DSCAV1	InterC	841
GSJ CCtail3xFd	512LLhnvnagksttniCCtt	xFd	DSCAV1	InterC	842
GSJ CCtail4xFd	512LLhnvnagksttnCCitt	xFd	DSCAV1	InterC	843
GSJ CCtail5xFd	512CChnvnagksttn	xFd	DSCAV1	InterC	844
GSJ CCtail6xFd	512LLhnvnaCCsttn	xFd	DSCAV1	InterC	845
GSJ CCtail7xFd	5 PLLhnvnagksttn	xFd	DSCAV1	Extended C-terminal helix	846
GSJ CCtail8xFd	512LLhnvnagksttnimitt	xFd	DSCAV1	Extended C-terminal helix	847
GSJ CCtail9.1xFd	5 PCChnvnaCCsttnimitt	xFd	DSCAV1	Multi-InterC	848
GSJ CCtail9xFd	512CChnvnaCCsttn	xFd	DSCAV1	Multi-InterC	849
GSJ CCtail10xFd	485C494C 512Cchnvnagksttn	xFd	DSCAV1	Multi-InterC	850
GSJ CCtailllxFd	485C494C 512CchnvnaCCsttn	xFd	DSCAV1	Multi-InterC	851
Tail11 s_ds_F505W_oxFd	SeqID 566 + 485C494C 512CchnvnaCCsttn	xFd	SeqID 566 (Based on DSCav1)	Multi-InterC	852
GSJ CCtaill2xFd	512CChnvnaCCsttniCCtt	xFd	DSCAV1	Multi-InterC	853
GSJ CCtaill3xFd	485C494C 512CchnvnaCCsttniCCtt	xFd	DSCAV1	Multi-InterC	854
GSJ CCtaill4xFd	SeqID 566 + 485C494C 512CchnvnaCCsttniCCtt	xFd	SeqID 566 (Based on DSCav1)	Multi-InterC	855
GSJ CCtaill5xFd	SeqID 566 + 485C494C 512CchnvnaCCsttn	xFd	SeqID 566 (Based on DSCav1)	Multi-InterC	856
GSJ CCtaill6xFd	SeqID 566 + 485C494C 512CchnvnaGKsttniCCtt	xFd	SeqID 566 (Based on DSCav1)	Multi-InterC	857
GSJT11	DSCav1-S509W, L512C, L513C	N	DSCAV1	ECC	858
GSJT12	DSCavl-S509F, L512C, L513C	N	DSCAV1	ECC	859
GSJT13	DSCav1-L512F, L513C, 514E, 515C	N	DSCAV1	ECC	860
GSJT14	DSCav1-S509W, L513C, 514E, 515C	N	DSCAV1	ECC	861
GSJT15	DSCav1-S509F, L513C, 514E, 515C	N	DSCAV1	ECC	862
GSJT16	DSCav1-S509W, L512F, L513C, 514E, 515C	N	DSCAV1	ECC	863
GSJT17	DSCav1-L512F, L513C, 514E, 515E, 516C	N	DSCAV1	ECC	864
GSJT18	DSCav1-S509W, L513C, 514E, 515E, 516C	N	DSCAV1	ECC	865
GSJT19	DSCav1-S509F, L513C, 514E, 515E, 516C	N	DSCAV1	ECC	866
GSJT20	DSCav1-S509W, L512F, L513C, 514E, 515E, 516C	N	DSCAV1	ECC	867
GSJT21	DSCav1- L512C, L513E, 514C	N	DSCAV1	ECC	868
GSJT22	DSCav1- L512C, L513E, 514E, 516C	N	DSCAV1	ECC	869
GSJT23	DSCav1- A515C, I516C	N	DSCAV1	ECC	870
GSJT24	DSCav1- L512T, L513E	N	DSCAV1	ECC	871
GSJT25	DSCav1-L512T, L513E, A515C, I516C	N	DSCAV1	ECC	872
GSJT26	DSCav1- L512S, L513E	N	DSCAV1	ECC	873
GSJT27	DSCav1-L512S, L513E, A515C, I516C	N	DSCAV1	ECC	874
GSJT28	DSCav1- L512S, L513D	N	DSCAV1	ECC	875
GSJT29	DSCav1-L512S, L513D, A515C, I516C	N	DSCAV1	ECC	876
GSJT30	DSCav1-L512F, A515C, I516C	N	DSCAV1	ECC	877
GSJT31	DSCav1-L513F, A515C, I516C	N	DSCAV1	ECC	878
GSJT32	DSCav1-L512F, L513F, A515C, I516C	N	DSCAV1	ECC	879
GSJT33	DSCav1-L512Y, L513Y, A515C, I516C	N	DSCAV1	ECC	880
GSJT34	DSCav1-L512F, L513Y, A515C, I516C	N	DSCAV1	ECC	881
GSJT35	DSCav1-L512W, L513W, A515C, I516C	N	DSCAV1	ECC	882
GSJT36	DSCav1- L5132W, L513Y, A515C, I516C	N	DSCAV1	ECC	883
GSJT37	DSCav1-S509W, A515C, I516C	N	DSCAV1	ECC	884
GSJT38	DSCav1-S509F, A515C, I516C	N	DSCAV1	ECC	885
GSJT39	DSCav1-S509W, L512F, A515C, I516C	N	DSCAV1	ECC	886
GSJT40	DSCav1-S509W, L512F, L513F, A515C,	N	DSCAV1	ECC	887

Construct Name	Motif	C-Term	Background	Design concept	SEQ ID NO
	I516C				
GSJT41	DSCav1-S509F, L512A, L513A, A515C, I516C	N	DSCAV1	ECC	888
GSJT42	DSCav1-S509W, L512A, L513A, A515C, I516C	N	DSCAV1	ECC	889
GSJT43	DSCav1- F505W, I506W, S509F, L512C, L513C	N	DSCAV1	ECC	890
GSJT44	DSCav1- F505W, I506W, S509F, A515C, I516C	N	DSCAV1	ECC	891
GSJT45	DSCav1- F505W, I506W, S509F, L512S, L513E, A515C, I516C	N	DSCAV1	ECC	892
GSJT46	DSCav1- F505W, I506W, S509F, L512A, L513A, A515C, I516C	N	DSCAV1	ECC	893
GSJT47	DSCavl- F505K, I506D, S509F, L512C, L513C	N	DSCAV1	ECC	894
GSJT48	DSCav1- F505K, I506D, S509F, A515C, I516C	N	DSCAV1	ECC	895
GSJT49	DSCav1- F505K, I506D, L512C, L513C	N	DSCAV1	ECC	896
GSJT50	DSCav1- F505K, I506D, A515C, I516C	N	DSCAV1	ECC	897
GSJT51	DSCav1- F505K, I506D, L512A, L513A, A515C, I516C	N	DSCAV1	ECC	898
GSJT52	DSCav1- F505K, I506D, L512S, L513E, L512C, L513C	N	DSCAV1	ECC	899
GSJT53	DSCav1- F505K, I506D, L512S, L513D, A515C, I516C	N	DSCAV1	ECC	900
GSJ-FP1	DSCav1-F137C, R339C	Fd	DSCAV1	FP-CC	969
GSJ-FP2	DSCavl- F137C, T337C	Fd	DSCAV1	FP-CC	970
GSJ-FP3	DSCav1-G139C, Q354C	Fd	DSCAV1	FP-CC	971
GSJ-FP4	F137C, R339C	Fd	DSCAV1	FP-CC	972
GSJ-FP5	F137C, T337C	Fd	DSCAV1	FP-CC	973
GSJ-FP6	G139C, Q354C	Fd	DSCAV1	FP-CC	974
GSJ-190P1	L260F	Fd	DSCAV1	190P	975
GSJ-190P2	L260W	Fd	DSCAV1	190P	976
GSJ-190P3	L260Y	Fd	DSCAV1	190P	977
GSJ-190P4	L260R	Fd	DSCAV1	190P	978
GSJ-190P5	L188F	Fd	DSCAV1	190P	979
GSJ-190P6	L188W	Fd	DSCAV1	190P	980
GSJ-190P7	L188Y	Fd	DSCAV1	190P	981
GSJ-190P8	L188R	Fd	DSCAV1	190P	982
GSJ-190P9	I57F	Fd	DSCAV1	190P	983
GSJ-190P10	I57W	Fd	DSCAV1	190P	984
GSJ-190P11	I57R	Fd	DSCAV1	190P	985
GSJ-190P12	L252F	Fd	DSCAV1	190P	986
GSJ-190P13	L252W	Fd	DSCAV1	190P	987
GSJ-190P14	L252R	Fd	DSCAV1	190P	988
GSJ-190P15	V192F	Fd	DSCAV1	190P	989
GSJ-190P16	V192W	Fd	DSCAV1	190P	990
GSJ-190P17	V192R	Fd	DSCAV1	190P	991
GSJ-DS1	S150C, Y458C	Fd	DSCAV1	Stabilizing Disulfides	992
GSJ-DS2	A149C, N460C	Fd	DSCAV1	Stabilizing Disulfides	993
GSJ-DS3	S146C, N460C	Fd	DSCAV1	Stabilizing Disulfides	994
GSJ-DS4	A149C, Y458C	Fd	DSCAV1	Stabilizing Disulfides	995
GJ-3-1	V220F	Fd	DSCAV1	CFM	996
GJ-3-2	V220W	Fd	DSCAV1	CFM	997
GJ-3-3	V220M	Fd	DSCAV1	CFM	998
GJ-3-4	T219F	Fd	DSCAV1	CFM	999
GJ-3-5	T219M	Fd	DSCAV1	CFM	1000
GJ-3-6	T219W	Fd	DSCAV1	CFM	1001
GJ-3-7	T219R	Fd	DSCAV1	CFM	1002
GSJ-Int-FdF-1	I22IF	Fd	DSCAV1	ICFM	1003
GSJ-Int-FdF-2	I221Y	Fd	DSCAV1	ICFM	1004
GSJ-Int-FdF-3	I21W	Fd	DSCAV1	ICFM	1005
GSJ-Int-FdF-4	Q224D, L78K	Fd	DSCAV1	ICFM	1006

Construct Name	Motif	C-Term	Background	Design concept	SEQ ID NO
GSJ-Int-FdF-5	V278F	Fd	DSCAV1	ICFM	1007
GSJ-Int-FdF-6	Q279F	Fd	DSCAV1	ICFM	1008
GSJ-Int-FdF-7	N277D, S99K	Fd	DSCAV1	ICFM	1009
GSJ-Int-FdF-8	Q361F	Fd	DSCAV1	ICFM	1010
GSJ-Int-FdF-9	V402F	Fd	DSCAV1	ICFM	1011
GSJ-Int-FdF-10	T400F	Fd	DSCAV1	ICFM	1012
GSJ-Int-FdF-11	T400W	Fd	DSCAV1	ICFM	1013
GSJ-Int-FdF-12	H486F	Fd	DSCAV1	ICFM	1014
GSJ-Int-FdF-13	H486W	Fd	DSCAV1	ICFM	1015
GSJ-Int-FdF-14	I217F	Fd	DSCAV1	ICFM	1016
GSJ-Int-FdF-15	I217Y	Fd	DSCAV1	ICFM	1017
GSJ-Int-FdF-16	I217W	Fd	DSCAV1	ICFM	1018
DSCav1OpFd1	F190V	Fd	DSCAV1	Enhanced stability of DSCav1	1019
DSCav1OpFd2	K226L	Fd	DSCAV1	Enhanced stability of DSCav1	1020
DSCav1OpFd3	T58I, A298M	Fd	DSCAV1	Enhanced stability of DSCav1	1021
DSCavlOpFd4	F190V, K226L	Fd	DSCAV1	Enhanced stability of DSCav1	1022
DSCav1OpFd5	F190V, T58I, A298M	Fd	DSCAV1	Enhanced stability of DSCav1	1023
DSCav1OpFd6	K226L, T58I, A298M	Fd	DSCAV1	Enhanced stability of DSCav1	1024
DSCav1OpFd7	T58I, A298M, F190V, K226L	Fd	DSCAV1	Enhanced stability of DSCav1	1025
CSGSJ1		xFd	DSCAV1	Engineered alpha 10 coil coil with GCN4 internal motifs	1456
CSGSJ2		xFd	DSCAV1	Engineered alpha 10 coil coil with GCN4 internal motifs	1457
CSGSJ3		xFd	DSCAV1	Engineered alpha 10 coil coil with GCN4 internal motifs	1458
CSGSJ4		xFd	DSCAV1	Engineered alpha 10 coil coil with GCN4 internal	1459
				motifs	
CSGSJ5		xFd	DSCAV1	Engineered alpha 10 coil coil with GCN4 internal motifs	1460
CSGSJ6		xFd	DSCAV1	Engineered alpha 10 coil coil with GCN4 internal motifs	1461
CSGSJ7		xFd	DSCAV1	Engineered alpha 10 coil coil with GCN4 internal motifs	1462
BZGJ9-10-TMCC1		xFd	DSCAV1	single chain F, alpha10 disulfide, Furin site, Foldon, TM region	1463
BZGJ9-10-TMCC2		xFd	DSCAV1	single chain F, alpha10 disulfide, Furin site, Foldon, TM region	1464
GSJCCTail9-TMCC1		xFd	DSCAV1	F, alpha10 disulfide, Furin site, Foldon, TM region	1465
GSJCCTail9-TMCC2		xFd	DSCAV1	F, alpha10 disulfide, Furin site, Foldon, TM region	1466
GSJCCTail9-TMCC3		xFd	DSCAV1	F, alpha10 disulfide, Furin site, TM region	1467
GSJCCTail9-TMCC4		xFd	DSCAV1	F, alpha10 disulfide, Furin site, TM region	1468

The yield of protein was calculated for several of the recombinant F proteins, and is shown below in Table 26.

Table 26. Yield of recombinant RSV F protein expression

Design Name	Yield mg/L	SEQ ID NO
CS/GSJ ext2OpCC1	0.39	832
CS/GSJ ext2OpCC2	0.33	833
CS/GSJ ext2OpCC3	0.72	834
CS/GSJ ext2OpCC4	0.48	835
CS/GSJ GCN4ccl	2.85	836
CS/GSJ GCN4cc2	0.75	837
CS/GSJ CartOp	0.36	838
GSJ CClongxFd	6.3	839
GSJ CCtail1xFd	1.05	840
GSJ CCtail2xFd	0.33	841
GSJ CCtail3xFd	0.99	842
GSJ CCtail4xFd	1.53	843
GSJ CCtail5xFd	2.13	844
GSJ CCtail6xFd	1.65	845

Design Name	Yield mg/L	SEQ ID NO
CSGSJ7	0.66	1462
GSJ 1Cav1 13	8	913
JCB GSJ 4	8	942
JCB GSJ 5	8	943

Example 13

Additional mutations to stabilize the membrane distal portion of the RSV F Ectodomain.

[0559] This example illustrates additional mutations that were made to RSV F to stabilize the protein in its prefusion conformation.

[0560] Several RSV F protein sequences without trimerization domains were designed and are provided in the SEQ ID NOs listed in Table 24, as well as an indication of design approach. The name, mutation relative to SEQ ID NO: 1026, presence or absence of C-terminal Foldon domain, background sequence (e.g., "WT" indicates wild-type RSV F), the design concept, and corresponding SEQ ID NO are indicated.

[0561] The recombinant RSV F proteins with a C-terminal trimerization domain listed in Table 24 were expressed in cells under conditions where the proteins are secreted from the cells in the cell media. Each construct contains a leader sequence that causes the protein to enter the secretory system and be secreted as described above in Example 9. The medium was then centrifuged and the supernatant used for antigenicity testing for binding to the Site Ø specific antibody D25 and the Site II specific antibody Motavizumab ("Mota", FIGs. 69A-69E). The conditions tested include D25 and Mota binding on day 0 (conditions 1 and 2), D25 and Mota binding on day 0 after incubation at 70°C for one hour (conditions 3 and 4), and D25 and Mota binding after 1 week at 4°C (conditions 5 and 6). The control is the DSCavl construct with a foldon domain. Specific antigenicity data for each construct is provided in FIGs. 69A-69E (the conditions tested are noted in the header rows).

Table 24. New stabilization with Foldon domain

Construct Name	Mutation(s) relative to SEQ ID NO: 1026	C-Term	Background	Design concept	SEQ ID NO
GSJ 1Cav1 1	S190W	Fd	WT	S190 AA Scan	901
GSJ 1Cav1 2	S190L	Fd	WT	S190 AA Scan	902
GSJ 1Cav1 3	S190R	Fd	WT	S190 AA Scan	903
GSJ 1Cav1 4	S190E	Fd	WT	S190 AA Scan	904
GSJ 1Cav1 5	S190A	Fd	WT	S190 AA Scan	905
GSJ 1Cav1 6	S190Q	Fd	WT	S190 AA Scan	906
GSJ 1Cav1 7	S190Y	Fd	WT	S190 AA Scan	907
GSJ 1Cav1 8	S190G	Fd	WT	S190 AA Scan	908
GSJ 1Cav1 9	S190P	Fd	WT	S190 AA Scan	909
GSJ 1Cav1 10	S190I	Fd	WT	S190 AA Scan	910
GSJ 1Cav1 11	S190T	Fd	WT	S190 AA Scan	911
GSJ 1Cav1 12	S190C	Fd	WT	S190 AA Scan	912
GSJ 1Cav1 13	S190V	Fd	WT	S190 AA Scan	913
GSJ 1Cav1 14	S190D	Fd	WT	S190 AA Scan	914
GSJ 1Cav1 15	S190N	Fd	WT	S190 AA Scan	915
GSJ 1Cav1 16	S190H	Fd	WT	S190 AA Scan	916
GSJ 1Cav1 17	S190K	Fd	WT	S190 AA Scan	917
GSJ 1Cav1 18	DSV207L	Fd	WT	S190 AA Scan	918
GSJ 1Cav1 19	DS S190F	Fd	WT	S190 AA Scan	919
GSJ 1Cav1 20	V207G	Fd	WT	S190 AA Scan	920
GSJ 1Cav1 21	V207A	Fd	WT	S190 AA Scan	921
GSJ 1Cav1 22	V207S	Fd	WT	S190 AA Scan	922
GSJ 1Cav1 23	V207T	Fd	WT	S190 AA Scan	923
GSJ 1Cav1 24	V207C	Fd	WT	S190 AA Scan	924
GSJ 1Cav1 25	V207L	Fd	WT	S190 AA Scan	925
GSJ 1Cav1 26	V207I	Fd	WT	S190 AA Scan	926
GSJ 1Cav1 27	V207M	Fd	WT	S190 AA Scan	927
GSJ 1Cav1 28	V207P	Fd	WT	S190 AA Scan	928
GSJ 1Cav1 29	V207F	Fd	WT	S190 AA Scan	929
GSJ 1Cav1 30	V207Y	Fd	WT	S190 AA Scan	930
GSJ 1Cav1 31	V207W	Fd	WT	S190 AA Scan	931
GSJ 1Cav1 32	V207D	Fd	WT	S190 AA Scan	932
GSJ 1Cav1 33	V207E	Fd	WT	S190 AA Scan	933
GSJ 1Cav1 34	V207N	Fd	WT	S190 AA Scan	934
GSJ 1Cav1 35	V207Q	Fd	WT	S190 AA Scan	935
GSJ 1Cav1 36	V207H	Fd	WT	S190 AA Scan	936
GSJ 1Cav1 37	V207K	Fd	WT	S190 AA Scan	937

Construct Name	Mutation(s) relative to SEQ ID NO: 1026	C-Term	Background	Design concept	SEQ ID NO
GSJ 1Cav1 38	V207R	Fd	WT	S190 AA Scan	938
JCB GSJ 1	Y198F	Fd	WT	Probing JCB16/18/24 residues	939
JCB GSJ 2	T219L	Fd	WT	Probing JCB16/18/24 residues	940
JCB GSJ 3	V296I	Fd	WT	Probing JCB16/18/24 residues	941
JCB GSJ 4	K226M	Fd	WT	Probing JCB16/18/24 residues	942
JCB GSJ 5	K226L	Fd	WT	Probing JCB16/18/24 residues	943
IG1-V192M	V192M	Fd	WT		944
IG2-A298M RSVF(+)FdTHS-paH	A298M	Fd	WT		945
IG2-T58I_A298M	T58I_A298M	Fd	WT		946
IG2-T58I_V192F_A298I_RSVF(+)FdTHS-paH	T58I, V192F, A298I	Fd	WT		947
IG2-T58I_V192M_A298I_RSVF(+)FdTHS-paH	T58I, V192M, A298I	Fd	WT		948
il67m-a298m	I167M, A298M	Fd	WT		949
i167m-l181m	I167M, L181M	Fd	WT		950
il99f	I199F	Fd	WT		951
i57c-s190c	I57C, S190C	Fd	WT		952
ig2-t58l-a298m	T58L, A298M	Fd	WT		953
ig2-t58m	T58M	Fd	WT		954
ig2-t58m-a298i	T58M, A298I	Fd	WT		955
ig2-t58m-a298L	T58M, A298L	Fd	WT		956
ig2-v192c-ins192-193-g-e256c	V192C, G insertion 192/193, E256C	Fd	WT		957
rsv f ths_s_f505w_o_s509f	ths_s_F505W_o_S50 9F	N	WT		958
rsv f ths _ s _f505w _ s509f	ths_s_F505W_S509F	N	WT		959
t58i-a298i	T58I, A298I	Fd	WT		960
t58m-a298m	T58M, A298M	Fd	WT		961
v179l-t189f	V179L, T189F	Fd	WT		962
v192f	V192F	Fd	WT		963
v192f-l252a	V192F, L252A	Fd	WT		964
v56m-i167m-l181m	V56M, I167M, L181M	Fd	WT		965
v56m-i167m-v296m	V56M, I167M, V296M	Fd	WT		966
v56m-l181f	V56M, L181F	Fd	WT		967
w52c-s150c	W52C, S150C	Fd	WT		968

Example 14

Minimal Site Ø immunogens

[0562] The site Ø epitope of RSV F is located on the apex of the trimer spike and includes the region recognized by the three neutralizing antibodies D25, AM22 and 5C4. More specifically, as delineated by the crystal structure of the RSV F/D25 complex, this epitope comprises the outer surface of helix a4 (residues 196-210) and the adjacent loop (residues 63-68) between β2 and α1. This example illustrates the design and characterization of antigens that present site Ø alone with minimal adjoining residues, and which can be used to elicit a site Ø immune response and can be more cost effective to produce than full length pre-fusion stabilized RSV F trimer.

General concepts for the design of minimal site Ø RSV F immunogens

[0563] The minimal site Ø immunogens were designed utilizing four primary design concepts: circular permutation, scaffolded circular permutation, domain III immunogens, and multimerization.

[0564] Circular permutations involve altering the native connections within a protein structure while keeping the spatial orientation(s) of the component parts. The minimal site Ø epitope components α4 and the β2-α1 loop are each part of two separate loop segments within RSV FI. To create stable site Ø folds, the two loop segments were connected (C-terminal to N-terminal) with short flexible amino acid linkers in the two different possible orders, thereby creating two separate folds, each of which preserve the site Ø epitope (FIG. 70A).

[0565] To create scaffolded circular permutations, the short flexible linkers of circularly permutated site Ø proteins were replaced by small rigid segments from other proteins that potentially provide greater stability than simple amino acid linkers (FIG. 70B).

[0566] Domain III (residues 50-306) is a larger domain of approximately 250 amino acids of the RSV F protein that contains the site Ø epitope (see FIG. 70D). The domain III residues surrounding site Ø provides further structural stability to site Ø while not adding significant additional distracting surface epitopes to the immunogen. Domain III contains a natural furin cleavage site between residues 136 and 137 which exposes the fusion peptide. Domain III can be further stabilized by replacing the cleavage site with an amino acid linker or by performing a circular permutation to link the original N- and C-termini or domain III and create a new N- and C-termini at the cleavage site. Both of these methods were utilized to stabilize various domain III immunogens.

[0567] Lastly, site Ø immunogens were multimerized to enhance immunogenicity (FIG. 70D and 70E). Trimerization was utilized to mimic the native trimer observed in the pre-fusion RSV F viral spike and larger defined oligomers such as 24mers and 60mers were utilized to specifically enhance immunogenicity. The was accomplished by introducing disulfide bonds between constructs, or by covalently linking constructs together as dimers or trimers using amino acid linkers or by linking constructs to multimerization domains using amino acid linkers. Some constructs utilized a combination of these strategies. The smallest multimerization domains used were trimers (e.g. GCN4) and the largest were 60mers (e.g. lumazine synthase). We also used pentamers, 12mers and 24mers.

[0568] In addition to the major design concepts delineated above, the immunogens were stabilized by using several other methods including addition of disulfide bonds, cavity filling mutations, reduction of surface hydrophobicity, addition of charged surface residues, and addition of N-linked glycans and truncation of potentially flexible regions. A listing of several minimal site Ø immunogens is provided in Tables 20 (site Ø non-particle immunogens) and 21 (site Ø immunogens on a protein nanoparticle), as well as an indication of design approach. The name, concept, residues of RSV F protein, scaffold or other added protein, and corresponding SEQ ID NO are indicated. In Tables 20 and 21, the following acronyms are used: SØ: minimal Site Ø; CP: circular permutation; DS: Disulfide; CAV: cavity filling; Charge: Adding charged residues; SC: single chain; TD3: tandem domain III domain; D3: domain III; RH: Reduce Hydrophobicity; Fd: T4 Fd trimerization domain; CCMPTD: chicken cartilage matrix protein trimerization domain; MTQ-CC: MTQ coiled coil trimerization motif; CXVIII: Collagen XVIII trimerization domain; 2M0E: Miz-1 zinc finger 6 (2M0E) scaffold; ATCase: aspartate carbamoyltransferase (ATCase) trimerization domain (1GQ3); GCN4: GCN4 trimerization domain; Fer: Ferritin; Dps: Microbacterium Arborescens Dps; LS: A. aeolicus Lumazine Synthase; Thr: thrombin; EH: exposed hydrophobic; HCP1: P. aeruginosa hcpl (1y12).

[0569] The minimal site Ø immunogens were expressed in cells using a system that results in secretion of the minimal site QS immunogens into the tissue culture medium as described above in Example 9. The medium was then centrifuged and the supernatant used for antigenicity testing for binding to the Site Ø specific antibodies D25, AN22 and 5C4 by ELISA (FIGs. 72A-72F). The conditions tested include D25 binding after 0 and 1 week at 4°C (conditions 1 and 2), D25 binding after 1 hr. at 60°C (condition 3), 70°C (condition 4), 80°C (condition 5), 90°C (condition 6), or 100°C (condition 7), AM22 binding after two weeks at 4°C (condition 8), 5C4 binding at week 0 4°C(condition 9). The average of D25, AM22, and D25 binding after 1 hour at 70°C is also shown (condition 10). A summary of the antigenicity data is provided in FIG. 71, which shows the number of site Ø immunogens that fall within each design category and which produced an ELISA result of at least 1.5. Specific antigenicity data for each construct is provided in FIGs. 72A-72F (the conditions tested are noted in the header rows). The results indicate that the minimal site Ø immunogens specifically bind to prefusion specific antibodies; and thus, are useful for inducing an immune response in a subject to antigenic site Ø. Additionally, the results indicate that the minimal site Ø constructs can be used as probes for isolating and detecting RSV F prefusion specific antibodies from a sample.

[0570] Based on the antigenicity data, 14 of the initial constructs were selected as representative for evaluation in animal models for producing an immune response, and for additional physical and structural characterization. The metric for choosing the 14 included selecting constructs showing average of ELISAs for D25 (weekl), AM22 (week 2) and D25 after 1 hour at 70 degrees. To prevent several very similar constructs being chosen for each category, each of the categories was subdivided into further categories (SEQ ID NO in parentheses):

<u>Category 1: Monomers:</u>

site Ø circular permutation: TZ-13 (354567-108) Avg: 3.18 (SEQ ID NO: 1040)

site Ø circular permutation with scaffold: JG 2KN0 (354567-417) Avg: 3.00 (SEQ ID NO: 1053)

domain III: E-CP_RBD51-307_14mutDS-Cav1_THS (354567-273) Avg: 3.17 (SEQ ID NO: 1156)

domain III dimer: GSJnh4-TWIN (354567-693) Avg: 3.06 (SEQ ID NO: 1194)

<u>Category 2: Trimers:</u>

site Ø circular permutations: TZ-19 (354567-126) Avg: 3.08 (SEQ ID NO: 1106)

domain III (two are tied): RSVF(+)THS_s_to_hp2_foldon (354567-210) Avg: 3.08 (SEQ ID NO: 1170), and MS_08 (354567-447) Avg: 3.08 (SEQ ID NO: 1188)

domain III dimer: GSJnh4Fd-TWIN (354567-705) Avg: 3.01 (SEQ ID NO: 1212)

<u>Category 3: multivalent monomers:</u>

site Ø circular permutation on ferritin: 2m0e-resurfl-Ferritin (354567-621) Avg: 2.81 (SEQ ID NO: 1276)

domain III on ferritin: GSJnh2F (354567-471) Avg: 3.10 (SEQ ID NO: 1220)

monomer on a non-ferritin oligomer: LS1-E-CP_RBD51-307_11mutDS-Cav1_THS (354567-315) Avg: 2.72 (SEQ ID NO: 1281)

Additional: MP11 (354567-642) Avg: 3.05 (SEQ ID NO: 1263)

<u>Category 4: multivalent trimers:</u>
domain III on nanoparticles (2): GSJnh2Fd-F (354567-483) Avg: 2.57 (SEQ ID NO: 1266), and GSJnh4Fd-F (354567-489) Avg: 2.02 (SEQ ID NO: 1268)

Table 20. Minimal Site Ø immunogens (not on a protein nanoparticle)

Name	Concept	Region of RSVF (residue #s)	scaffold or other added protein	SEQ ID NO
Circular permutation of site Ø (26)				
JCB 01	CP-SØ+CAV	60-94, 192-232	APGG linker (Seq 1454)	1027
JCB 02	CP-SØ+CAV	60-94, 192-232	APGG (Seq_1454)linker, DS	1028
JCB 03	CP-SØ+CAV	60-94, 192-232	APGG(Seq_1454) linker, DS	1029
JCB 04	CP-SØ+CAV	60-94, 192-232	AGSG(Seq_1455) linker	1030
JCB 05	CP-SØ+CAV	60-94, 192-232	AGSG (Seq_1455)linker, DS	1031
JCB 06	CP-SØ+CAV	60-94, 192-232	AGSG (Seq_1455)linker, DS	1032
JCB 07	CP-SØ+CAV	60-94, 192-229	GSG linker	1033

Name	Concept	Region of RSVF (residue #s)	scaffold or other added protein	SEQ ID NO
Circular permutation of site Ø (26)				
JCB 08	CP-SØ+CAV	60-94, 192-229	GSG linker, DS	1034
JCB 09	CP-SØ+CAV	60-94, 192-229	GSG linker, DS	1035
TZ-09	CP-SØ+DS+CAV+glycan	192-242, 60-97	GGSGSGG (Seq_1446)linker	1036
TZ-10	CP-SØ+DS+CAV+charge	192-242, 60-97	GGSGSGG(Seq_1446)linker	1037
TZ-11	shorter CP-SØ+DS+CAV+charge	192-242, 60-97	GGSGSGG(Seq_1446) linker	1038
TZ-12	CP-SØ+DS+CAV+charge	192-242, 60-97	GGSGSGG(Seq_1446) linker	1039
TZ-13	CP-SØ+DS+CAV+glycan	192-242, 60-97	GGSGSGG(Seq_1446) linker	1040
TZ-14	CP-SØ+DS+CAV+glycan	192-242, 60-97	GGSGSGG(Seq_1446) linker	1041
RSVF(+)THS_m e	CP-SØ	62-69 - ggsggggsggsg(S eq_1447) - 196-212	Ggsggggsggsg (Seq 1447) linker	1042
RSVF(+)THS_m e hp1	CP-SØ	62-69 - ggsggggsggsg(S eq_1447) - 196-212	ggsggggsggsg(Seq_1447) linker	1043
RSVF(+)THS_m e_ds	CP-SØ	62-69 - ggsggggsggsg(S eq_1447) - 196-212	ggsggggsggsg (Seq 1447) linker	1044
RSVF(+)THS_m e_hp1_ds	CP-SØ	62-69 - ggsggggsggsg(S eq_1447) - 196-212	ggsggggsggsg (Seq 1447) linker	1045
JG_circ1	CP-SØ	60-94, 193-237	GGSGG (Seq_1448) linker	1046
JG_circ1_ds	CP-SØ+DS	60-94, 193-237	GGSGG(Seq_1448) linker	1047
JG_circ1_del K	CP-SØ+deletion	60-94, 193-237	GGSGG(Seq_1448) linker	1048
JG_circ1_sol ds	CP-SØ+DS	60-94, 193-237	GGSGG(Seq_1448) linker	1049
JG_circ1_sol	CP-SØ	60-94, 193-237	GGSGG(Seq_1448) linker	1050
JG_Circ2	CP-SØ	60-75, 193-218	GGSGG(Seq_1448) linker	1051
JG_Circ2_sol	CP-SØ	60-75, 193-218	GGSGG(Seq_1448) linker	1052
Circular permutation with scaffold connection (19)				
JG_2KN0	CP-SØ+section of TENC1 (2KNO) scaffold	60-75, 193-218	GGSGGSG (Seq_1445) linker and TENC1 (2KNO) scaffold	1053
Site_0_2a90_ 1 GYC	CP-SØ+CAV+RH+section of 2A90 scaffold	61-96, 192-235	WWE domain fragment (2A90)	1054
Site_0_2a90_ 2 GYC	CP-SØ+CAV+RH+DS+section of 2A90 scaffold	61-96, 192-235	WWE domain fragment (2A90)	1055
Site_0_2a90_ 3 GYC	CP-SØ+CAV+RH+DS+section of 2A90 scaffold	61-96, 192-235	WWE domain fragment (2A90)	1056
Site_0_2w59_ 1 GYC	CP-SØ+CAV+RH+section of 2W59 scaffold	60-96, 193-238	IgY fragment (2W59)	1057
Site_0_2w59_ 2 GYC	CP-SØ+CAV+RH+DS+section of 2W59 scaffold	60-96, 193-238	IgY fragment (2W59)	1058
Site_0_2w59_ 3 GYC	CP-SØ+CAV+RH+DS+section of 2W59 scaffold	60-96, 193-238	IgY fragment (2W59)	1059
Site_0_3u2e_ 1 GYC	CP-SØ+CAV+RH+section of 3U2E scaffold	61-96, 192-238	EAL domain fragment (3U2E)	1060
Site_0_3u2e_ 2 GYC	CP-SØ+CAV+RH+DS+section of 3U2E scaffold	61-96, 192-238	EAL domain fragment (3U2E)	1061
Site_0_3u2e_ 3 GYC	CP-SØ+CAV+RH+DS+section of 3U2E scaffold	61-96, 192-238	EAL domain fragment (3U2E)	1062
Site_0_2vj1_ 1 GYC	CP-SØ+CAV+RH+section of 2VJ1 scaffold	61-96, 192-240	SARS proteinase fragment (2VJ1)	1063
Site_0_2vj1_ 2 GYC	CP-SØ+CAV+RH+DS+section of 2VJ1 scaffold	61-96, 192-240	SARS proteinase fragment (2VJ1)	1064
Site_0_2vj1_ 3 GYC	CP-SØ+CAV+RH+DS+section of 2VJ1 scaffold	61-96, 192-240	SARS proteinase fragment (2VJ1)	1065
Site_0_1chd_ 1 GYC	CP-S0+CAV+RH+section of 1CHD scaffold	60-95, 192-240	CheB methylesterase fragment (1CHD)	1066
Site_0_1chd_ 2_GYC	CP-SØ+CAV+RH+DS+section of 1CHD scaffold	60-95, 192-240	CheB methylesterase fragment (1CHD)	1067
Site_0_1chd_ 3_GYC	CP-SØ+CAV+RH+DS+section of 1CHD scaffold	60-95, 192-240	CheB methylesterase fragment (1CHD)	1068
Site_0_1pqz_ 1 GYC	CP-S0+CAV+RH+section of 1PQZ scaffold	60-96, 192-239	Immunomodulatory protein M144 fragment (1PQZ)	1069
Site_0_1pqz_ 2 GYC	CP-SØ+CAV+RH+DS+section of 1PQZ scaffold	60-96, 192-239	Immunomodulatory protein M144 fragment (1PQZ)	1070
Site_0_1pqz_ 3 GYC	CP-SØ+CAV+RH+DS+section of 1PQZ scaffold	60-96, 192-239	Immunomodulatory protein M144 fragment (1PQZ)	1071
Circular permutation of site Ø with trimer (39)				
JCB 10	CP-SØ+CAV+GCN4	60-94, 192-232	APGG linker, GCN4	1072
JCB 11	CP-SØ+CAV+DS+GCN4	60-94, 192-232	APGG linker, GCN4	1073
JCB 12	CP-SØ+CAV+DS+GCN4	60-94, 192-232	APGG linker, GCN4	1074
JCB 13	CP-SØ+CAV+DS+GCN4	60-94, 192-232	APGG linker, GCN4	1075

Circular permutation of site Ø with trimer (39)				
JCB 14	CP-SØ+CAV+DS+GCN4	60-94, 192-232	APGG linker, GCN4	1076
JCB 15	CP-SØ+CAV+DS+GCN4	60-94, 192-232	APGG linker, GCN4	1077
JCB 16	CP-SØ+CAV+GCN4	60-94, 192-229	GSG linker, GCN4	1078
JCB 17	CP-SØ+CAV+DS+GCN4	60-94, 192-229	GSG linker, DS, GCN4	1079
JCB 18	CP-SØ+CAV+DS+GCN4	60-94, 192-229	GSG linker, DS, GCN4	1080
JCB 19	CP-SØ+CAV+DS+GCN4	60-94, 192-229	GSG linker, GCN4	1081
JCB 20	CP-SØ+CAV+DS+GCN4	60-94, 192-229	GSG linker, DS, GCN4	1082
JCB 21	CP-SØ+CAV+DS+GCN4	60-94, 192-229	GSG linker, DS, GCN4	1083
TZ-01	CP-SØ+interchain DS	192-242, 60-97	GGSGSGG(Seq_1446) linker	1084
TZ-02	CP-SØ+interchain DS+CAV	192-242, 60-97	GGSGSGG(Seq_1446) linker	1085
TZ-03	CP-SØ+interchain DS+CAV	192-242, 60-97	GGSGSGG(Seq_1446) linker	1086
TZ-04	CP-SØ+interchain DS+CAV	192-242, 60-97	GGSGSGG(Seq_1446) linker	1087
TZ-05	CP-SØ+interchain DS+CAV+charge	192-242, 60-97	GGSGSGG(Seq_1446) linker	1088
TZ-06	CP-SØ+interchain DS+CAV+charge	192-242, 60-97	GGSGSGG(Seq_1446) linker	1099
TZ-07	CP-SØ+interchain DS+CAV+glycan	192-242, 60-97	GGSGSGG(Seq_1446) linker	1100
TZ-08	CP-SØ+interchain DS+CAV+glycan	192-242, 60-97	GGSGSGG(Seq_1446) linker	1101
TZ-15	CP-SØ+DS+CXVIII	58-97, 192-242	GGSGSGSG(Seq_1449) linker , CXVIII	1102
TZ-16	CP-SØ+DS+CAV+CXVIII	58-97, 192-242	GGSGSGSG(Seq_1449) linker, CXVIII	1103
TZ-17	CP-SØ+DS+CAV+CXVIII	58-97, 192-242	GGSGSGSG(Seq_1449) linker, CXVIII	1104
TZ-18	CP-SØ+DS+CAV+CXVIII	58-97, 192-242	GGSGSGSG(Seq_1449) linker, CXVIII	1105
TZ-19	CP-SØ+DS+CAV+charge+glycan+CXVIII	58-97, 192-242	GGSGSGSG(Seq_1449) linker, CXVIII	1106
TZ-20	CP-SØ+DS+CAV+charge+glycan+CXVIII	58-97, 192-242	GGSGSGSG(Seq_1449) linker, CXVIII	1107
AO 1	sc CP-SØ trimers	60-97, 194-239	multiple glycine linkers	1108
AO 2	sc CP-SØ trimers	60-97, 194-239	multiple glycine linkers	1109
AO 3	sc CP-SØ trimers	60-97, 194-239	multiple glycine linkers	1110
AO_4	sc CP-SØ trimers	60-97, 194-239	multiple glycine linkers	1111
AO 5	sc CP-SØ trimers	60-97, 194-239	multiple glycine linkers	1112
AO 6	sc CP-SØ trimers	60-97, 194-239	multiple glycine linkers	1113
AO 7	CP-SØ+N-terminal Fd	60-97, 194-239	Glycine linkers and Fd	1114
AO 8	CP-SØ+C-terminal Fd	60-97, 194-239	Glycine linkers and Fd	1115
AO 9	CP-SØ+C-terminal ATCase	60-97, 194-239	E coli ATCase trimerization domain	1116
MP5	CP-SØ+Fd	56-97 GG - 189-211	GG linker and Fd	1117
MP6	CP-SØ+Fd	56-97 G 189-211	GG linker and Fd	1118
MP7	CP-SØ+GCN4ization domain	56-97 GG 189-211	GG linker and GCN4ization domain	1119
MP8	CP-SØ+ C-terminal ATCase	56-97 G-ATCAse-189-211	ATCase	1120
Site Ø minimal epitope on a scaffold (6)				
2m0e-resurf1	Minimal SØ on 2M0E		2M0E	1121
2m0e-resurf2	Minimal SØ on 2M0E		2M0E	1122
2m0e-resurf3	Minimal SØ on 2M0E		2M0E	1123
2M0E_r04	Minimal SØ on 2M0E	196-212	2M0E	1124
2M0E_r05	Minimal SØ on 2M0E	196-212	2M0E	1125
2MOE_r06	Minimal SØ on 2M0E	196-212	2M0E	1126
Domain III (42)				
RBD51-307 11mut DS-Cav1	D3+DS+RH	51-307		1127
RBD51-307 11mut DS-Cav1 2sug	D3+DS+RH, add glycans	51-307		1128
RBD51-304 11mut DS-Cav1 3sug	D3+DS+RH, add glycans	51-304		1129
RBD51-307 10mut DS-Cav1	D3+reduce hydrophobicity	51-307		1130
RBD51-307 10mut DS-Cav1 2sug	D3+RH, add glycans	51-307		1131
RBD51-304 10mut DS-Cav1 3sug	D3+RH, add glycans	51-304		1132
CP RBD51-307 11mut DS-Cav1	CP-D3+DS, RH	51-307		1133
CP RBD51-307 11mut DS-Cav1 2sug	CP-D3+DS, RH	51-307		1134

Domain III (42)				
CP RBD51-304 11mut DS-Cav1 3sug	CP-D3+DS, RH	51-304		1135
CP RBD51-307 10mut DS-Cav1	CP-D3, RH	51-307		1136
CP RBD51-307 10mut DS-Cav1 2sug	CP-D3, RH, add glycans	51-307		1137
CP RBD51-304 10mut DS-Cav1 3sug	CP-D3, RH, add glycans	51-304		1138
JCB 28	D3+CAV	50-96, 149-306	GSGGGSG(Seq_1450) linker	1139
JCB 29	D3+CAV	50-96, 149-306	GSGGGSG(Seq_1450) linker	1140
RSVF(+)THS_s _to	CP-D3+DS-Cav1	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1141
RSVF(+)THS_s _to_hp2	CP-D3, RH + DS-Cavl	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1142
RSVF(+)THS_s _to_hp12	CP-D3, RH + DS-Cavl	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1143
RSVF(+)THS_s _to_hp2_I221 F	CP-D3, RH + DS-Cavl	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1144
RSVF(+)THS_s _to_hp2_ds	CP-D3, RH + DS-Cavl	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1145
RSVF(+)THS_s _to_hp23	CP-D3, RH + DS-Cavl	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1146
RSVF(+)THS_s _to_hp123	CP-D3, RH + DS-Cavl	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1147
RSVF(+)THS_s _to A102C-A241C	CP-D3+DS-Cav1	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1148
RSVF(+)THS_s _to_hp2 A102C-A241C	CP-D3, RH + DS-Cavl	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1149
RSVF(+)THS_s _to_hp12 A102C-A241C	CP-D3, RH + DS-Cavl	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1150
RSVF(+)THS_s _to_hp2_I221 F A102C-A241C	CP-D3, RH + DS-Cavl	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1151
RSVF(+)THS_s _to_hp2_ds A102C-A241C	CP-D3, RH + DS-Cavl	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1152
RSVF(+)THS_s _to_hp23 A102C-A241C	CP-D3, RH + DS-Cavl	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1153
RSVF(+)THS_s _to_hp123 A102C-A241C	CP-D3, RH + DS-Cavl	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1154
RSVF(+)THS_s _to_hp1234 A102C-A241C	CP-D3, RH + DS-Cavl	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1155
E-CP_RBD51-307 14mutDS-Cav1 THS	CP-D3, RH + DS-Cavl		GG linker	1156
E-RBD51-307 14mut DS -Cav1 THS	CP-D3, RH + DS-Cavl			1157
RSVF(+)THS_ s _to_hp1234 A102C-A241C K196C-E60C	CP-D3, RH + DS-Cavl	146-306 - ggsgg(Seq_1448)-50-105	GGSGG(Seq_1448) linker	1158
E-CP_RBD51-307 14mutDS-Cav1 THS K196C-E60C	CP-D3, RH + DS-Cavl		GG linker	1159
E-RBD51-307 14mut DS -Cav1_THS K196C-E60C	CP-D3, RH + DS-Cavl			1160
E-CP_RBD51-307 11mutDS-Cav1 THS	CP-D3, RH + DS-Cavl		GG linker	1161
E-RBD51-307 11mut DS -Cav1 THS	CP-D3, RH + DS-Cavl			1162
E-CP_RBD51-307 11mut-K196C-E60C-DS-Cavl THS	CP-D3, RH + DS-Cavl		GG linker	1163
E-RBD51-307 11mut-K196C-E60C-DS-Cav1 THS	CP-D3, RH + DS-Cavl			1164
GSJnh1	Truncated D3	46-310	GG linker	1165
GSJnh2	Truncated D3	46-310	GG linker	1166
GSJnh3	Truncated D3	51-305	GG linker	1167
GSJnh4	Truncated D3	51-305	GSG linker	1168

Domain III (42)				
Domain III with trimer (22)				
RSVF(+)THS_s	CP-D3+DS-Cav1+Fd	1146-306 -	Glycine linkers and Fd	11169
_to_foldon		ggsgg(Seq_1448)- 50-105 - ggsggsg (Seq_1445) - Fd		
RSVF(+)THS_s _to_hp2_fold on	CP-D3+ RH+DS-Cav1+C-terminal Fd	146-306 - ggsgg(Seq_1448)- 50-105 - ggsggsg (Seq_1445) - Fd	Glycine linkers and Fd	1170
RSVF(+)THS_s _to_hp12_fol don	CP-D3+ RH+DS-Cav1+C-terminal Fd	146-306 - ggsgg(Seq_1448)- 50-105 - ggsggsg (Seq_1445) - Fd	Glycine linkers and Fd	1171
RSVF(+)THS_s _to_hp2_fold on_I221F	CP-D3+ RH+DS-Cav1+C-terminal Fd	146-306 - ggsgg(Seq_1448)- 50-105 " - ggsggsg (Seq_1445) - Fd	Glycine linkers and Fd	1172
RSVF(+)THS_s _to_hp2_fold on ds	CP-D3+ RH+DS-Cav1+C-terminal Fd	146-306 - ggsgg(Seq_1448)- 50-105 - ggsggsg (Seq_1445) - Fd	Glycine linkers and Fd	1173
RSVF(+)THS_s _to_foldon A102C-A241C	CP-D3+DS-Cav1+Fd	146-306 - ggsgg(Seq_1448)- 50-105 " - ggsggsg (Seq_1445) - Fd	Glycine linkers and Fd	1174
RSVF(+)THS_s _to_hp2_fold on A102C-A241C	CP-D3+ RH+DS-Cav1+C-terminal Fd	146-306 - ggsgg(Seq_1448)- 50-105 - ggsggsg (Seq_1445) - Fd	Glycine linkers and Fd	1175
RSVF(+)THS_s _to_hp12_fol don A102C-A241C	CP-D3+ RH+DS-Cav1+C-terminal Fd	146-306 - ggsgg(Seq_1448)- 50-105 " - ggsggsg (Seq_1445) - Fd	Glycine linkers and Fd	1176
RSVF(+)THS_s _to_hp2_fold on_I221F A102C-A241C	CP-D3+ RH+DS-Cav1+C-terminal Fd	146-306 - ggsgg(Seq_1448)- 50-105 - ggsggsg (Seq_1445) - Fd	Glycine linkers and Fd	1177
RSVF(+)THS_s _to_hp2_fold on ds A102C-A241C	CP-D3+ RH+DS-Cav1+C-terminal Fd	146-306 - ggsgg(Seq_1448)- 50-105 " - ggsggsg (Seq_1445) - Fd	Glycine linkers and Fd	1178
GSJnhFd1	Truncated D3+Fd		Fd	1179
GSJnhFd2	Truncated D3+Fd		Fd	1180
MS_01	D3+C-terminal CCMPTD	51-103, 146-307	GGPGG(Seq_1451) linker turn, C-terminal CCMPTD	1181
MS_02	D3+C-terminal CCMPTD	51-103, 146-307	GGPGG(Seq_1451) turn, longer linker, C-terminal CCMPTD	1182
MS_03	D3+N-terminal CCMPTD	51-103, 139-307	GGPGG(Seq_1451) turn, C-terminal CCMPTD	1183
MS_04	D3+N-terminal CCMPTD	51-103, 137-307	GGPGG(Seq_1451) turn plus fusion peptide, C-terminal CCMPTD	1184
MS_05	D3+N-terminal CCMPTD	51-103, 146-307	GGPGG(Seq_1451) turn, C-terminal CCMPTD	1185
MS_06	CP-D3, C-terminal MTQ-CC	51-103, 146-307	GGPGG(Seq_1451) turn, C-terminal MTQ-CC	1186
MS_07	CP-D3, C-terminal MTQ-CC	51-103, 146-307	GGPGG(Seq_1451) turn, longer linker, C-terminal MTQ-CC	1187
MS_08	CP-D3, N-terminal MTQ-CC	51-103, 146-307	GGPGG(Seq_1451) turn, C-terminal MTQ-CC	1188
MS_09	CP-D3, N-terminal MTQ-CC	51-103, 139-307	GGPGG(Seq_1451) turn plus fusion peptide, C-terminal MTQ-CC	1189
MS_10	CP-D3, N-terminal MTQ-CC	51-103, 139-307	GGPGG(Seq_1451) turn plus fusion peptide, C-terminal MTQ-CC	1190
Tandem domain III (18)				
GSJnh1-TWIN	TD3	(47-307, 103GG147)GSG(4 7-307, 103GG147)	Glycine linkers	1191
GSJnh2-TWIN	TD3	(47-307, 104GSG146)GSG(47-307, 104GSG146)	Glycine linkers	1192
GSJnh3-TWIN	TD3	(51-305, 103GG147)GSG(5 1-305, 103GG147)	Glycine linkers	1193
GSJnh4-TWIN	TD3	(51-305, 104GSG146)GSG(51-305, 104GSG146)	Glycine linkers	1194
GSJnh1-TWINLg	TD3	(47-307, 103GG147)GGGSG GGG(47-307, 103GG147)	Glycine linkers	1195
GSJnh2-TWINLg	TD3	(47-307, 104GSG146)GGGS GGGG(47-307, 104GSG146)	Glycine linkers	1196
GSJnh3-TWINLg	TD3	(51-305, 103GG147)GGGSG GGG(51-305, 103GG147)	Glycine linkers	1197
GSJnh4-TWINLg	TD3	(51-305, 104GSG146)GGGS GGGG(51-305, 104GSG146)	Glycine linkers	1198
LC-DH01	CP-TD3+long linker	145-306, 52-96	GGGSGGGSGGGSGGG(Seq_1452	1199

Tandem domain III (18)				
			)linker	
LC-DH02	CP-TD3+long linker+DS	145-306, 52-96	GGGSGGGSGGGSGGG(Seq_1452) linker	1200
LC-DH03	CP-TD3+short linker	145-306, 52-96	GGGSGGGSGGG (Seq 1453)linker	1201
LC-DH04	CP-TD3+short linker	145-306, 52-96	GGGSGGGSGGG(Seq_1453) linker	1202
LC-DH05	LM leader+CP-TD3+short linker	145-306, 52-96	GGGSGGGSGGG(Seq_1453) linker	1203
LC-DH06	LM leader+CP-TD3+short linker+DS	145-306, 52-96	GGGSGGGSGGG(Seq 1453) linker	1204
LC-DH07	LM leader+CP-TD3+long linker	145-306, 52-96	GGGSGGGSGGGSGGG(Seq 1452) linker	1205
LC-DH08	LM leader+CP-TD3+long linker+DS	145-306, 52-96	GGGSGGGSGGGSGGG(Seq_1452) linker	1206
LC-DH09	LM leader+CP-TD3+long linker+Arg	145-306, 52-96	GGGSGGGSGGG(Seq_1453) linker	1207
LC-DH10	LM leader+CP-TD3+long linker+Arg+DS	145-306, 52-96	GGGSGGGSGGG(Seq_1453) linker	1208
Tandem domain III with a trimer (10)				
GSJnh1Fd-TWIN	TD3+Fd	(47-307, 103GG147) GG-Fd-GG(47-307, 103GG147)	Glycine linkers	1209
GSJnh2Fd-TWIN	TD3+Fd	(47-307, 104GSG146) GG-Fd-GG(47-307, 104GSG146)	Glycine linkers	1210
GSJnh3Fd-TWIN	TD3+Fd	(51-305, 103GG147)GG-Fd-GG(51-305, 103GG147)	Glycine linkers	1211
GSJnh4Fd-TWIN	TD3+Fd	(51-305, 104GSG146) GG-Fd-GG(51-305, 104GSG146)	Glycine linkers	1212
GSJnhFd3a TWIN	TD3+Fd	(F1/GSG/F2/Fd/ F1/GSG/F2/Thbn/H/S)	Glycine linkers	1213
GSJnhFd3b TWIN	TD3+Fd	(H/S/Thbn/F1/GSG/F2/Fd/ F1/GSG/F2)	Glycine linkers	1214
GSJnh1-TWINGFd	TD3+Fd	(47-307, 103Fd147)GSGGSG(47-307, 103GG147)	Glycine linkers	1215
GSJnh2-TWINGFd	TD3+Fd	(47-307, 104Fd146)GSGGSG(47-307, 104GSG146)	Glycine linkers	1216
GSJnh1-TWINFdG	TD3+Fd	(47-307, 103Fd147)GSGGSG(47-307, 103GG147)	Glycine linkers	1217
GSJnh2-TWINFdG	TD3+Fd	(47-307, 104Fd146)GSGGSG(47-307, 104GSG146)	Glycine linkers	1218

Table 21. Minimal site Ø immunogens on a protein nanoparticle.

	Concept					
Construct name		**Part icle**	**RSV F Region (residue #s)**	**scaffold or other added protein**	**His tag (N, I or C)**	**SEQ ID NO**
Domain III on ferritin (45)						
GSJnh1F	TD3+Fer	Fer	46-103 GG 147-310	GG linker, C-terminal Fer	N-H8-Strep-GG-Thr-GGS	1219
GSJnh2F	TD3+Fer	Fer	46-104 GSG 146-310	GSG linker, C-term. Fer	N-H8-Strep-GG-Thr-GGS	1220
GSJnh3F	TD3+Fer	Fer	51-103 GG 147-305	GG linker, C-term. Fer	N-H8-Strep-GG-Thr-GGS	1221
GSJnh4F	TD3+Fer	Fer	51-104 GSG 146-305	GSG linker, C-term. Fer	N-H8-Strep-GG-Thr-GGS	1222
TK_01	CP-SØ+DS+DPS	Dps	59-97,194-240	Dps	N-H6-Thr	1223
TK_02	CP-SØ+DS+DPS	Dps	59-97,194-240	Dps	N-H6-Thr	1224
TK_03	CP-SØ+DS+DPS	Dps	59-97,194-240	Dps	N-H6-Thr	1225
TK_04	CP-SØ+DS+DPS	Dps	59-97,194-240	Dps	N-H6-Thr	1226
TK_05	CP-SØ+CAV+DPS	Dps	59-97,194-240	Dps	N-H6-Thr	1227
TK 06	CP-SØ+CAV+DPS	Dps	59-97,194-240	Dps	N-H6-Thr	1228
TK_07	CP-SØ+CAV+DPS	Dps	59-97,194-240	Dps	N-H6-Thr	1229
TK 08	CP-SØ+CAV+DPS	Dps	59-97,194-240	Dps	N-H6-Thr	1230
TK 09	CP-SØ +CAV+DS+DPS	Dps	59-97,194-240	Dps	N-H6-Thr	1231
TK_10	CP-SØ +CAV+DS+DPS	Dps	59-97,194-240	Dps	N-H6-Thr	1232

Construct name	Concept	Particle	RSV F Region (residue #s)	scaffold or other added protein	His tag (N, I or C)	SEQ ID NO
Domain III on ferritin (45)						
TK_11	CP-SØ +CAV+DS+DPS	Dps	59-97,194-240	Dps	N-H6-Thr	1233
TK_12	CP-SØ +CAV+DS+DPS	Dps	59-97,194-240	Dps	N-H6-Thr	1234
TK_13	D3+DS+CAV+Fer	Fer	53-97,148-305	Fer	N-H6-Thr	1235
TK_14	D3+DS+CAV+Fer	Fer	53-97,148-306	Fer	N-H6-Thr	1236
TK_15	D3+DS+CAV+Fer	Fer	53-97,148-307	Fer	N-H6-Thr	1237
TK_16	D3+DS+CAV+Fer	Fer	53-97,148-308	Fer	N-H6-Thr	1238
TK_17	D3+DS+CAV+Fer	Fer	53-97,148-309	Fer	N-H6-Thr	1239
TK_18	D3+DS+CAV+Fer	Fer	53-97,148-310	Fer	N-H6-Thr	1240
TK_19	D3+DS+CAV+Fer	Fer	53-97,148-311	Fer	N-H6-Thr	1241
TK_20	D3+DS+CAV+Fer	Fer	53-97,148-312	Fer	N-H6-Thr	1242
TK_21	D3+DS+CAV+Fer	Fer	53-97,148-313	Fer	N-H6-Thr	1243
TK_22	D3+DS+CAV+Fer	Fer	53-97,148-314	Fer	N-H6-Thr	1244
TK_23	D3+DS+CAV+Fer	Fer	53-97,148-315	Fer	N-H6-Thr	1245
TK 24	D3+DS+CAV+Fer	Fer	53-97,148-316	Fer	N-H6-Thr	1246
TK 25	D3+DS+CAV+Fer	Fer	53-97,148-317	Fer	N-H6-Thr	1247
TK_26	D3+DS+CAV+Fer	Fer	53-97,148-318	Fer	N-H6-Thr	1248
TK 27	D3+DS+CAV+Fer	Fer	53-97,148-319	Fer	N-H6-Thr	1249
TK_28	D3+DS+CAV+Fer	Fer	53-97,148-320	Fer	N-H6-Thr	1250
TK_29	D3+DS+RH+Fer	Fer	53-104,145-307	Fer	N-H6-Thr	1251
TK 30	D3+DS+RH+Fer	Fer	53-104,145-307	Fer	N-H6-Thr	1252
RSVF(+)THS_s_to+Fer_ 3ln	CP-D3, RH + DS-Cav1+Fer	Fer	146-306 - ggsgg(Seq_1448)- 50-105 - sgg- Fer	Glycine linkers, Fer	N-Strep-H8-HRV3C	1253
RSVF(+)THS_s_to_hp2+ Fer_3ln	CP-D3, RH + DS-Cav1+Fer	Fer	146-306 - ggsgg(Seq_1448)- 50-105 - sgg- Fer	Glycine linkers, Fer	N-Strep-H8-HRV3C	1254
RSVF(+)THS_s_to+Fer_ 5ln	CP-D3, RH + DS-Cav1+Fer	Fer	146-306 - ggsgg(Seq_1448)- 50-105 - ggsgg(Seq_1448)- Fer	Glycine linkers, Fer	N-Strep-H8-HRV3C	1255
RSVF(+)THS_s_to_hp2+ Fer_5ln	CP-D3, RH + DS-Cav1+Fer	Fer	146-306 - ggsgg(Seq_1448)- 50-105 - ggsgg(Seq_1448)- Fer	Glycine linkers, Fer	N-Strep-H8-HRV3C	1256
RSVF(+)THS_s_to_hp12 +Fer_5ln	CP-D3, RH + DS-Cav1+Fer	Fer	146-306 - ggsgg(Seq_1448)- 50-105 - ggsgg(Seq_1448)- Fer	Glycine linkers, Fer	N-Strep-H8-HRV3C	1257
RSVF(+)THS_s_to_hp2+ Fer_3ln_I221F	CP-D3, RH + DS-Cav1+Fer	Fer	146-306 - ggsgg(Seq_1448)- 50-105 - sgg- Fer	Glycine linkers, Fer	N-Strep-H8-HRV3C	1258
RSVF(+)THS_s_to_hp2+ Fer 5ln 1221F	CP-D3, RH + DS-Cav1+Fer	Fer	146-306 - ggsgg(Seq_1448	Glycine linkers, Fer	N-Strep-H8-HRV3C	1259
			)- 50-105 - ggsgg(Seq_1448)- Fer			
MP1	D3+Cav+Fer	Fer	50-306 GSG	Glycine linker, Fer	N-Strep-H8-Thr	1260
MP2	D3+Cav+Fer	Fer	50-306 GGSGG(Seq _1448 -	Glycine linker, Fer	N-Strep-H8-Thr	1261
MP10	D3+Fer	Fer	SC-GGSGG (Seq_1448	Glycine linker, Fer	N-Strep-H8-Thr	1262
MP11	D3+Fer	Fer	SC-GGSGG(Seq_1448)	Glycine linker, Fer	N-Strep-H8-Thr	1263
Minimal epitope with trimer on ferritin (1)						
MP9	Minimal CP-SØ +ATCase trimerization domain+Fer	Fer	56-76 G-ATCase-G-189-211GGSGG (Sea 1448)	Fer	N-Strep-H8-Thr	1264
Domain III wi th t rimer on ferr itin (4)						
GSJnh1Fd-F	Truncated sc D3+Fd+Fer	Fer	46-310	103GG147, C-term. Fd-Fer	N-H8StrepGG-Thr-GGS	1265
GSJnh2Fd-F	Truncated sc D3+Fd+Fer	Fer	46-310	104GSG146, C-term. Fd-Fer	N-H8StrepGG-	1266

Domain III wi th t rimer on ferr itin (4)						
					Thr-GGS	
GSJnh3Fd-F	Truncated sc D3+Fd+Fer	Fer	51-305	103GG147, C-term. Fd-Fer	C-term. H8StrepGG-Thr-GGS	1267
GSJnh4Fd-F	Truncated sc D3+Fd+Fer	Fer	51-305	104GSG146, C-term. Fd-Fer	C-term. H8StrepGG-Thr-GGS	1268
Minimal epitope on ferritin (10)						
JCB 22	CP-SØ+Fer	Fer	60-94, 192-229	PGG linker, Fer	N-H6-HRV3C	1269
JCB 23	CP-SØ+Fer	Fer	60-94, 192-229	PGG linker, Fer	N-H6-HRV3C	1270
JCB 24	CP-SØ+Fer	Fer	60-94, 192-229	PGG linker, Fer	N-H6-HRV3C	1271
JCB 25	CP-SØ+Fer	Fer	60-94, 192-229	GSG linker, Fer	N-H6-HRV3C	1272
JCB 26	CP-SØ+Fer	Fer	60-94, 192-229	GSG linker, Fer	N-H6-HRV3C	1273
JCB 27	CP-SØ+Fer	Fer	60-94, 192-229	GSG linker, Fer	N-H6-HRV3C	1274
2m0e-resurf1-Fer	Minimal SØ on a 2M0E+Fer	Fer		Miz-1 zinc finger 6 (2M0E) fragment+Fer	N-Strep, H6, Thr	1275
2m0e-resurf1-Fer	Minimal SØ on a 2M0E+Fer	Fer		Miz-1 zinc finger 6 (2M0E) fragment+Fer	N-Strep, H6, Thr	1276
MP3	CP-SØ+Fer	Fer	56-97 GG 189-240 GSG	GG linker+Fer	N-Strep-H8-Thr	1277
MP4	CP-SØ+Fer	Fer	same as MP3 with GGSGG(Seq_14 48)	GGSGG (Seq_1448)li nker+Fer	N-Strep-H8-Thr	1278
Minimal epitope on LS (2)						
2m0e-resurf1-LS	Minimal SØ on a 2M0E+LS	LS		LS	C-term. Thr H6-strep	1279
2m0e-resurf1-1y12	Minimal SØ on a 2M0E+hcp1	HCP 1		HCP1	C-term. Thr H6-strep	1280
Domain III on LS (2)						
LS1-E-CP_RBD51-307_11mutDS-Cav1_THS	CP-D3, RH + DS-Cav1+LS	LS		LS	N-Strep, H6, Thr	1281
LS2-E-CP_RBD51-307_11mutDS-Cav1_THS	CP-D3, RH + DS-Cav1+LS	LS		LS	N-Strep, H6, Thr	1282
Domain III on hcpl (4)						
1y12- E-CP_RBD51-307_11mutDS-Cav1_THS	CP-D3, RH + DS-Cav1+hcp1	HCP 1		HCP1	C-term. Thr H6-strep	1283
1y12- E-RBD51-307_11mut_DS-Cav1_THS	CP-D3, RH + DS-Cav1+hcp1	HCP 1		HCP1	C-term. Thr H6-strep	1284
1y12- E-CP_RBD51-307_14mutDS-Cav1_THS	CP-D3, RH + DS-Cav1+hcp1	HCP 1		HCP1	C-term. Thr H6-strep	1285
1y12- E-RBD51-307_14mut_DS-Cav1_THS	CP-D3, RH + DS-Cav1+hcp1	HCP 1		HCP1	C-term. Thr H6-strep	1286
Monomers on ferritin (40)						
JCB_1_GSGGSG_ferr	CP-SØ+CAV+Fer	Fer	60-94, 192-232	APGG linker+Fer	N-Strep-H8-Thr	1287
JCB_2_GSGGSG_ferr	CP-SØ+CAV+Fer	Fer	60-94, 192-232	APGG linker, DS+Fer	N-Strep-H8-Thr	1288
JCB_5_GSGGSG_ferr	CP-SØ+CAV+Fer	Fer	60-94, 192-232	AGSG linker, DS+Fer	N-Strep-H8-Thr	1289
JCB_7_GSGGSG_ferr	CP-SØ+CAV+Fer	Fer	60-94, 192-229	GSG linker+Fer	N-Strep-H8-Thr	1290
JCB_8_GSGGSG_ferr	CP-SØ+CAV+Fer	Fer	60-94, 192-229	GSG linker, DS+Fer	N-Strep-H8-Thr	1291
JCB_28_GSGGGSG_ferr	D3+CAV+Fer	Fer	53-96, 149-304	Glycine linkers, Fer	N-Strep-H8-Thr	1292
TZ_09r_GGSG_ferr	CP-SØ+DS+CAV+gly can+Fer	Fer	192-242, 60-97	Glycine linkers, Fer	N-Strep-H8-Thr	1293
TZ_12r_GGSG_Ferr	CP-SØ +DS+CAV+cha rge+Fer	Fer	192-242, 60-97	Glycine linkers, Fer	N-Strep-H8-Thr	1294
TZ_13r_GGSG_Ferr	CP-SØ+DS+CAV+gly can+Fer	Fer	192-242, 60-97	Glycine linkers, Fer	N-Strep-H8-Thr	1295
TZ_14r_GGG_Ferr	CP-SØ+DS+CAV+gly	Fer	192-242, 60-97	Glycine linkers,	N-Strep-H8-	1296

Monomers on ferritin (40)						
	can+Fer			Fer	Thr	
Site_0_1chd_3_GYC_GG SGGSGGSGGSGGG_ferr	CP-SØ +CAV+RH+DS+ section of 1CHD scaffold+Fer	Fer	60-95, 192-240	CheB methylestera se fragment (1CHD)+Fer	N-Strep-H8-Thr	1297
JG_circ1_sol_ds_ferr	CP-SØ+DS+Fer	Fer	60-94, 193-237	Glycine linkers, Fer	N-Strep-H8-Thr	1298
JG_2KN0_ferr	CP-SØ+section of TENC1 (2KNO) scaffold+Fer	Fer	60-75, 193-218	Glycine linkers+TENC 1 (2KNO) scaffold+Fer	N-Strep-H8-Thr	1299
JG_Circ2_ferr	CP-SØ+Fer	Fer	60-75, 193-218	Glycine linkers, Fer	N-Strep-H8-Thr	1300
JG_Circ2_sol_ Ferr	CP-SØ+Fer	Fer	60-75, 193-218	Glycine linkers, Fer	N-Strep-H8-Thr	1301
GSJnh2-Fer	Truncated sc D3	Fer	46-310	Glycine linkers, Fer	N- H8-Strep-Thr	1302
GSJnh3-Fer	Truncated sc D3	Fer	51-305	Glycine linkers, Fer	N- H8-Strep-Thr	1303
GSJnh4-Fer	Truncated sc D3	Fer	51-305	Glycine linkers, Fer	N- H8-Strep-Thr	1304
GSJnh2-TWIN-Fer	TD3	Fer	47-307, 47-307	Glycine linkers, Fer	N- H8-Strep-Thr	1305
GSJnh3-TWIN-Fer	TD3	Fer	51-305, 51-305	Glycine linkers, Fer	N- H8-Strep-Thr	1306
GSJnh4-TWIN-Fer	TD3	Fer	51-305, 51-305	Glycine linkers, Fer	N- H8-Strep-Thr	1307
GSJnh2Fd-TWIN-Fer	TD3 with Fd	Fer	47-307, 47-307	Glycine linkers+T4 Fd+Fer	N- H8-Strep-Thr	1308
GSJnh4Fd-TWIN-Fer	TD3 with Fd	Fer	51-305, 51-305	Glycine linkers+T4 Fd+Fer	N- H8-Strep-Thr	1309
GSJnhFd2-Fer	TD3 with Fd	Fer		Glycine linkers+T4 Fd+Fer	N- H8-Strep-Thr	1310
GSJnh2-TWINLg-Fer	TD3	Fer	47-307, 47-307	Glycine linkers, Fer	N- H8-Strep-Thr	1311
GSJnh3-TWINLg-Fer	TD3	Fer	51-305, 51-305	Glycine linkers, Fer	N- H8-Strep-Thr	1312
GSJnh4-TWINLg-Fer	TD3	Fer	51-305, 51-305	Glycine linkers, Fer	N- H8-Strep-Thr	1313
RSVF(+)THS_s_to A102C-A241C_sgg_ferr	CP D3 + DS-Cav1	Fer	146-306 - ggsgg (Seq_14 48)- 50-105	Glycine linkers, Fer	N-Strep-H8-HRV3C	1314
RSVF(+)THS_s_to_fold on A102C-A241C_ggsggggsgg_fer r	CP D3 + DS-Cav1 + Fd	Fer	146-306 - ggsgg (Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers, Fer	N-Strep-H8-HRV3C	1315
RSVF(+)THS_s_to_hp12 A102C-A241C_ggsgg_ferr	CP D3 + EH + DS-Cav1	Fer	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, Fer	N-Strep-H8-HRV3C	1316
RSVF(+)THS_s_to_hp12 3 A102C-A241C_ggsgg_ferr	CP D3 + EH + DS-CavI	Fer	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, Fer	N-Strep-H8-HRV3C	1317
RSVF(+)THS_s_to_hp12 34 A102C-A241C_ggsgg_ferr	CP D3 + EH + DS-CavI	Fer	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, Fer	N-Strep-H8-HRV3C	1318
RSVF(+)THS_s_to_hp12 3_ggsgg_ferr	CP D3 + EH + DS-Cav1	Fer	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, Fer	N-Strep-H8-HRV3C	1319
RSVF(+)THS_s_to_hp2 A102C-A241C_sgg_ferr	CP D3 + EH + DS-Cav1	Fer	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, Fer	N-Strep-H8-HRV3C	1320
RSVF(+)THS_s_to_hp23 A102C-A241C_sgg_ferr	CP D3 + EH + DS-Cav1	Fer	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, Fer	N-Strep-H8-HRV3C	1321
RSVF(+)THS_s_to_hp23 _ggsgg_ferr	CP D3 + EH + DS-Cav1	Fer	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, Fer	N-Strep-H8-HRV3C	1322
RSVF(+)THS_s_to_hp2_ ds A102C-A241C_sgg_ferr	CP D3 + EH + DS-Cav1	Fer	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, Fer	N-Strep-H8-HRV3C	1323
RSVF(+)THS_s_to_hp2_ ds_ggsgg_ferr	CP D3 + EH + DS-Cav1	Fer	146-306 - ggsgg- 50-105	Glycine linkers, Fer	N-Strep-H8-HRV3C	1324

Monomers on ferritin (40)						
RSVF(+)THS_s_to_hp2_ 1221F A102C-A241C_sgg_ferr	CP D3 + EH + DS-Cav1	Fer	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, Fer	N-Strep-H8-HRV3C	1325
C-Trimer Fer: leader-Strep-HISx6-Thr-L1H1-K1-H2L2H3-GGSG	CP-SØ+Fer	Fer		Glycine linker+Fer	N- Leader-Strep-HISx6-Thr	1326
Monomers on LS (44)						
JCB_1_GSGGSG_LS	CP-SØ+CAV+LS	LS	60-94, 192-232	APGG linker+LS	C-term. Thr, Strep, and H8	1327
JCB_2_GSGGSG_LS	CP-SØ+CAV+LS	LS	60-94, 192-232	APGG linker, DS+LS	C-term. Thr, Strep, and H8	1328
JCB_5_GSGGSG_LS	CP-SØ+CAV+LS	LS	60-94, 192-232	AGSG linker, DS+LS	C-term. Thr, Strep, and H8	1329
JCB_7_GSGGSG_LS	CP-SØ+CAV+LS	LS	60-94, 192-229	GSG linker+LS	C-term. Thr, Strep, and H8	1330
JCB_8_GSGGSG_LS	CP-SØ+CAV+LS	LS	60-94, 192-229	GSG linker, DS+LS	C-term. Thr, Strep, and H8	1331
JCB_28_GSGGSG_LS	D3+CAV+LS	LS	53-96, 149-304	Glycine linkers+LS	C-term. Thr, Strep, and H8	1332
TZ_12r_GGSGG_LS	CP-SØ +DS+CAV+cha rge+LS	LS	192-242, 60-97	Glycine linkers+LS	N-Strep-H8-Thr	1333
TZ_13r_GGSGG_LS	CP-SØ+DS+CAV+gly can+LS	LS	192-242, 60-97	Glycine linkers+LS	N-Strep-H8-Thr	1334
TZ_14r_GGSGSG_LS	CP-SØ+DS+CAV+gly can+LS	LS	192-242, 60-97	Glycine linkers+LS	N-Strep-H8-Thr	1335
Site_0_lchd_3_GYC_GG SGGSGGSGGSGGG_LS	CP-SØ +CAV+RH+DS+ section of 1CHD scaffold+LS	LS	60-95, 192-240	CheB methylesteras e fragment (1CHD)+LS	C-term. Thr-H6Strep	1336
JG_circ1_sol_ds_LS	CP-SØ+DS+LS	LS	60-94, 193-237	Glycine linkers+LS	C-Thr-His-Strep	1337
JG_2KN0_LS	CP-SØ+section of TENC1 (2KNO) scaffold+LS	LS	60-75, 193-218	Glycine linkers+TENC1 (2KNO) scaffold+Fer	C-Thr-His-Strep	1338
JG Circ2 LS	CP-SØ+LS	LS	60-75, 193-218	Glycine linkers+LS	C-Thr-His-Strep	1339
JG_Circ2_sol_LS	CP-SØ+LS	LS	60-75, 193-218	Glycine linkers+LS	C-Thr-His-Strep	1340
GSJnh2-LS	Truncated sc D3	LS	46-310	Glycine linkers, LS	N- H8-Strep-Thr	1341
GSJnh3-LS	Truncated sc D3	LS	51-305	Glycine linkers, LS	N- H8-Strep-Thr	1342
GSJnh4-LS	Truncated sc D3	LS	51-305	Glycine linkers, LS	N- H8-Strep-Thr	1343
GSJnh2-TWIN-LS	TD3	LS	47-307, 47-307	Glycine linkers, LS	N- H8-Strep-Thr	1344
GSJnh3-TWIN-LS	TD3	LS	51-305, 51-305	Glycine linkers, LS	N- H8-Strep-Thr	1345
GSJnh4-TWIN-LS	TD3	LS	51-305, 51-305	Glycine linkers, LS	N- H8-Strep-Thr	1346
GSJnh2Fd-TWIN-LS	TD3 with Fd	LS	47-307, 47-307	Glycine linkers+T4 Fd+LS	N- H8-Strep-Thr	1347
GSJnh4Fd-TWIN-LS	TD3 with Fd	LS	51-305, 51-305	Glycine linkers+T4 Fd+LS	N- H8-Strep-Thr	1348
GSJnhFd2-LS	TD3 with Fd	LS		Glycine linkers+T4 Fd+LS	N- H8-Strep-Thr	1349
GSJnh2-TWINLg-LS	TD3	LS	47-307, 47-307	Glycine linkers, LS	N- H8-Strep-Thr	1350
GSJnh3-TWINLg-LS	TD3	LS	51-305, 51-305	Glycine linkers, LS	N- H8-Strep-Thr	1351
GSJnh4-TWINLg-LS	TD3	LS	51-305, 51-305	Glycine linkers, LS	N- H8-Strep-Thr	1352
RSVF(+)THS_s_to_gagg gsggsggsggg_ls	CP D3 + DS-Cav1	LS	146-306 -	Glycine linkers,	N-Strep-H8-	1353

Monomers on LS (44)						
			ggsgg(Seq_14 48)- 50-105	LS	HRV3C	
RSVF(+)THS_s_to_hp2_ gagggsggsggsggg_ls	CP D3 + EH + DS-Cav1	LS	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, LS	N-Strep-H8-HRV3C	1354
RSVF(+)THS_s_to_hp12 _gagggsggsggsggg_ls	CP D3 + EH + DS-Cav1	LS	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, LS	N-Strep-H8-HRV3C	1355
RSVF(+)THS_s_to_hp2_ I221F_gagggsggsggsgg g ls	CP D3 + EH + DS-Cav1	LS	146-306 - ggsgg(Seq 14 48)- 50-105	Glycine linkers, LS	N-Strep-H8-HRV3C	1356
RSVF(+)THS_s_to A102C-A241C_gagggsggsggsgg g_ls	CP D3 + DS-Cav1	LS	146-306 - ggsgg(Seq 14 48)- 50-105	Glycine linkers, LS	N-Strep-H8-HRV3C	1357
RSVF(+)THS_s_to_hp12 A102C-A241C_gagggsggsggsgg g_ls	CP D3 + DS-Cav1 + Fd	LS	146-306 - ggsgg(Seq 14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+LS	N-Strep-H8-HRV3C	1358
RSVF(+)THS_s_to_hp12 3_gagggsggsggsggg_ls	CP D3 + DS-Cav1 + Fd	LS	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+LS	N-Strep-H8-HRV3C	1359
RSVF(+)THS_s_to_hp12 3 A102C-A241C_gagggsggsggsgg g ls	CP D3 + EH + DS-Cav1	LS	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, LS	N-Strep-H8-HRV3C	1360
RSVF(+)THS_s_to_hp12 34 A102C-A241C_gagggsggsggsgg g_ls	CP D3 + EH + DS-Cav1	LS	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, LS	N-Strep-H8-HRV3C	1361
RSVF(+)THS_s_to_hp2 A102C-A241C_gagggsggsggsgg g ls	CP D3 + EH + DS-Cav1	LS	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, LS	N-Strep-H8-HRV3C	1362
RSVF(+)THS_s_to_hp2_ ds_gagggsggsggsggg_l s	CP D3 + EH + DS-Cav1	LS	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, LS	N-Strep-H8-HRV3C	1363
RSVF(+)THS_s_to_hp2_ ds A102C-A241C _gagggsggsggsgg g_ls	CP D3 + EH + DS-CavI + Fd	LS	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+LS	N-Strep-H8-HRV3C	1364
RSVF(+)THS_s_to_hp2_ 1221F A102C-A241C _gagggsggsggsgg g_ls	CP D3 + EH + DS-CavI + Fd	LS	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+LS	N-Strep-H8-HRV3C	1365
RSVF(+)THS_s_to_hp23 _gagggsggsggsggg_ls	CP D3 + EH + DS-Cav1	LS	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, LS	N-Strep-H8-HRV3C	1366
RSVF(+)THS_s_to_hp23 A102C-A241C_gagggsggsggsgg g_ls	CP D3 + EH + DS-Cav1	LS	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, LS	N-Strep-H8-HRV3C	1367
C-Trimer LS: leader-Strep-HISx6-Thr-L1H1-K1-H2L2H3-GGSGGGSG	CP-SØ+LS	LS		Glycine linker+LS	N- Leader-Strep-HISx6-Thr	1368
C-Trimer LS: leader-L1H1-K1-H2L2H3-GGSGGGSG-LS-Thr-HISx6-Strep:	CP-SØ+LS	LS		Glycine linker+LS	C-Leader-Thr-H6-Strep	1369
Trimers on ferritin (30)						
JCB_13_GSGGGSG_ferr	CP-SØ +CAV+DS+GCN 4+Fer	Fer	60-94, 192-232	APGG linker, GCN4, inter-DS+Fer	N-Strep-H8-Thr	1370
JCB_19_GSGGSG_ferr	CP-SØ +CAV+DS+GCN 4+Fer	Fer	60-94, 192-229	GSG linker, GCN4, inter-DS+Fer	N-Strep-H8-Thr	1371
TZ_05_GGSG_ferr	CP-SØ+interchain DS+CAV+charge +Fer	Fer	192-242, 60-97	Glycine linkers, Fer	N-Strep-H8-Thr	1372
TZ_08r_GGSG_ferr	CP-SØ+interchain DS+CAV+glycan +Fer	Fer	192-242, 60-97	Glycine linkers, Fer	N-Strep-H8-Thr	1373
TZ_15_GGSG_3Hferr	CP-SØ+interchain DS+CXVIII+Fer	Fer	58-97, 192-242	Glycine linkers+CXVII I+Fer	N-Strep-H8-Thr	1374
TZ_16_GGSG_3Hferr	CP-SØ+interchain DS+CAV+CXVIII +Fer	Fer	58-97, 192-242	Glycine linkers+CXVII I+Fer	N-Strep-H8-Thr	1375
TZ_17_GGSGSG_3Hferr	CP-SØ+interchain DS+CAV+CXVIII +Fer	Fer	58-97, 192-242	Glycine linkers+CXVII I+Fer	N-Strep-H8-Thr	1376
TZ_19_GGSGSGG_3Hferr	CP-SØ+interchain DS+CAV+charge +glycan+CXVII I+Fer	Fer	58-97, 192-242	Glycine linkers+CXVII I+Fer	N-Strep-H8-Thr	1377

Trimers on ferritin (30)						
TZ_19_GGSGG_ferr	CP-S∅+interchain DS+CAV+charge +glycan+Fer	Fer	58-97, 192-242	Glycine linkers, Fer	N-Strep-H8-Thr	1378
TZ_20_GGSGSGG_3Hferr	CP-S∅ +DS+CAV+cha rge+glycan+CX VIII+Fer	Fer	58-97, 192-242	Glycine linkers+CXVII I+Fer	N-Strep-H8-Thr	1379
MS_03_GGGSSGSGGGSSGG GSSGGGS_Ferr	D3+N-term. CCMPTD+Fer	Fer	51-103, 139-307	Glycine linkers, Fer	N-Strep-H8-Thr	1380
MS_05_GGGSSGSGGGSSGG GSSGGGS_Ferr	D3+N-term. CCMPTD+Fer	Fer	51-103, 146-307	Glycine linkers, Fer	N-Strep-H8-Thr	1381
MS_07_ GSGGGSSGSGGGSSGGGSSG GGS_Ferr	CP-D3, C-term. MTQ-CC+Fer	Fer	51-103, 146-307	Glycine linkers, Fer	N-Strep-H8-Thr	1382
MS_08_GGGSSGSGGGSSGGGSSGGG S Ferr	CP-D3, C-term. MTQ-CC+Fer	Fer	51-103, 146-307	Glycine linkers, Fer	N-Strep-H8-Thr	1383
MS_09_GGGSSGSGGGSSGG GSSGGGS_Ferr	CP-D3, C-term. MTQ-CC+Fer	Fer	51-103, 139-307	Glycine linkers, Fer	N-Strep-H8-Thr	1384
GSJnh2-Fer A74C E218C	Truncated sc D3, interchain DS	Fer	46-310	Glycine linkers, Fer	N- H8-Strep-Thr	1385
GSJnh3-Fer A74C E218C	Truncated sc D3, interchain DS	Fer	51-305	Glycine linkers, Fer	N- H8-Strep-Thr	1386
GSJnh4-Fer A74C E218C	Truncated sc D3, interchain DS	Fer	51-305	Glycine linkers, Fer	N- H8-Strep-Thr	1387
GSJnh4-TWIN-Fer A74C E218C	TD3, interchain DS	Fer	51-305, 51-305	Glycine linkers, Fer	N- H8-Strep-Thr	1388
RSVF(+)THS_s_to_fold on_ggsggggsgg_ferr	CP D3 + DS-Cav1 + Fd	Fer	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers, Fer	N-Strep-H8-HRV3C	1389
RSVF(+)THS_s_to_hp12 _foldon A102C-A241C_ _ggsggggsgg_fer r	CP D3 + EH + DS-CavI + Fd	Fer	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+Fer	N-Strep-H8-HRV3C	1390
RSVF(+)THS_s_to_hp12 _foldon_ggsggggsgg_f err	CP D3 + EH + DS-CavI + Fd	Fer	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+Fer	N-Strep-H8-HRV3C	1391
RSVF(+)THS_s_to_hp2_ foldon A102C-A241C_ggsggggsgg_fer r	CP D3 + EH + DS-CavI + Fd	Fer	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+Fer	N-Strep-H8-HRV3C	1392
RSVF(+)THS_s_to_hp2_ foldon_ds A102C-A241C_ggsggggsgg_fer r	CP D3 + EH + DS-Cav1	Fer	146-306 - ggsgg(Seq_14 48)- 50-105	Glycine linkers, Fer	N-Strep-H8-HRV3C	1393
RSVF(+)THS_s_to_hp2_ foldon_ds_ggsggggsgg ferr -	CP D3 + EH + DS-CavI + Fd	Fer	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+Fer	N-Strep-H8-HRV3C	1394
RSVF(+)THS_s_to_hp2 foldon_ggsggggsgg_fe rr	CP D3 + EH + DS-CavI + Fd	Fer	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+Fer	N-Strep-H8-HRV3C	1395
RSVF(+)THS_s_to_hp2_ foldon_I221F A102C-A241C_ggsggggsgg_fer r	CP D3 + EH + DS-CavI + Fd	Fer	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+Fer	N-Strep-H8-HRV3C	1396
RSVF(+)THS_s_to_hp2_ foldon_I221F_ggsgggg	CP D3 + EH + DS-CavI + Fd	Fer	146-306 - ggsgg(Seq 14	Glycine linkers+T4	N-Strep-H8-HRV3C	1397
sgg ferr			48)- 50-105 -ggsggsg (Seq_1445) - Fd	Fd+Fer		
C-Trimer 1GQ3-Fer: leader-Strep-HISx6-Thr-L1H1-K1-H2L2H3-GGSGGGSG-1GQ3-GGSGGGSGGGSGGGSG-Fer	CP-S∅+C-term. ATCase+Fer	Fer		Glycine linkers+ATCas e (1GQ3)+Fer	N- Leader-Strep-HISx6-Thr	1398
C-Trimer 1GQ3-Fer: leader-Strep-HISx6-Thr-L1H1-K1-H2L2H3-GGSGGGSG-1GQ3-GGSGGGSGGGSGGGSGGGSG -Fer	CP-S∅+C-term. ATCase+Fer	Fer		Glycine linkers+ATCas e (1GQ3)+Fer	N- Leader-Strep-HISx6-Thr	1399
Trimers on LS (30)						
JCB_13_GSGGGSG_LS	CP-S∅ +CAV+DS+GCN 4+LS	LS	60-94, 192-232	APGG linker, GCN4, inter-DS+LS	C-term. Thr, Strep, and H8	1400
JCB_19_GSGGSG_LS	CP-S∅ +CAV+DS+GCN 4+LS	LS	60-94, 192-229	GSG linker, GCN4, inter-DS+LS	C-term. Thr, Strep, and H8	1401
TZ_05_GGSGGG_LS	CP-S∅ +DS+CAV+cha	LS	192-242, 60-97	Glycine linkers+LS	N-Strep-H8-Thr	1402

Trimers on LS (30)						
	rge+LS					
TZ_08r_GGSGGG_LS	CP-S∅+DS+CAV+gly can+LS	LS	192-242, 60-97	Glycine linkers+LS	N-Strep-H8-Thr	1403
TZ_09r_GGSGGG_LS	CP-S∅+DS+CAV+gly can+LS	LS	192-242, 60-97	Glycine linkers+LS	N-Strep-H8-Thr	1404
TZ_15_GGG-LS	CP-S∅+DS+LS	LS	58-97, 192-242	Glycine linkers+LS	N-Strep-H8-Thr	1405
TZ_16_GGG_LS	CP-S∅+DS+CAV+LS	LS	58-97, 192-242	Glycine linkers+LS	N-Strep-H8-Thr	1406
TZ_17_GGG_LS	CP-S∅+DS+CAV+LS	LS	58-97, 192-242	Glycine linkers+LS	N-Strep-H8-Thr	1407
TZ_19_GGG_LS	CP-S∅ +DS+CAV+cha rge+glycan+LS	LS	58-97, 192-242	Glycine linkers+LS	N-Strep-H8-Thr	1408
TZ_20_GGG_LS	CP-S∅ +DS+CAV+cha rge+glycan+LS	LS	58-97, 192-242	Glycine linkers+LS	N-Strep-H8-Thr	1409
MS_03_GGGSSGSGGGSSGG GSSGGGS_LS	D3+N-term. CCMPTD+LS	LS	51-103, 139-307	Glycine linkers+CCMPT D+LS	N-Strep-H8-Thr	1410
MS_05_GGGSSGSGGGSSGG GSSGGGS_LS	D3+N-term. CCMPTD+LS	LS	51-103, 146-307	Glycine linkers+CCMPT D+LS	N-Strep-H8-Thr	1411
MS_07_ GSGGGSSGSGGGSSGGGSSG GGS_LS	CP-D3, C-term. MTQ-CC+LS	LS	51-103, 146-307	Glycine linkers+CCMPT D+LS	N-Strep-H8-Thr	1412
MS_08_ GGGSSGSGGGSSGGGSSGGG S_LS	CP-D3, C-term. MTQ-CC+LS	LS	51-103, 146-307	Glycine linkers+CCMPT D+LS	N-Strep-H8-Thr	1413
MS_09_GGGSSGSGGGSSGG GSSGGGS_LS	CP-D3, C-term. MTQ-CC+LS	LS	51-103, 139-307	Glycine linkers+CCMPT D+LS	N-Strep-H8-Thrombin	1414
RSVF(+)THS_s_to_fold on_ gagggsggsggsggg_ls	CP D3 + EH + DS-Cav1	LS	146-306 - ggsgg(Seq_14 48)- 50-105+Fd	Glycine linkers, LS	N-Strep-H8-HRV3C	1415
RSVF(+)THS_s_to_fold on A102C-A241C_gagggsggsggsgg g_ls	CP D3 + EH + DS-Cav1	LS	146-306 - ggsgg(Seq_14 48)- 50-105+Fd	Glycine linkers, LS	N-Strep-H8-HRV3C	1416
RSVF(+)THS_s_to_hp12 _foldon_gagggsggsggs ggg_ls	CP D3 + EH + DS-Cav1	LS	146-306 - ggsgg(Seq_14 48)- 50-105+Fd	Glycine linkers, LS	N-Strep-H8-HRV3C	1417
RSVF(+)THS_s_to_hp12 _foldon A102C-A241C _gagggsggsggsgg g_ls	CP D3 + EH + DS-Cavl + Fd	LS	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+LS	N-Strep-H8-HRV3C	1418
RSVF(+)THS_s_to_hp2_ foldon_gagggsggsggsg gg_ls	CP D3 + EH + DS-Cav1	LS	146-306 - ggsgg(Seq_14 48)- 50-105+Fd	Glycine linkers, LS	N-Strep-H8-HRV3C	1419
RSVF(+)THS_s_to_hp2_ foldon A102C-A241C_gagggsggsggsgg g_ls	CP D3 + EH + DS-Cavl + Fd	LS	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+LS	N-Strep-H8-HRV3C	1420
RSVF(+)THS_s_to_hp2_ foldon_ds_gagggsggsg gsggg_ls	CP D3 + EH + DS-Cavl + Fd	LS	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+LS	N-Strep-H8-HRV3C	1421
RSVF(+)THS_s_to_hp2_ foldon_ds A102C-A241C _gagggsggsggsgg g_ls	CP D3 + EH + DS-Cavl + Fd	LS	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+LS	N-Strep-H8-HRV3C	1422
RAVF(+)THS_s_to_hp2_ foldon_1221F_gagggsg gsggsggg_ls	CP D3 + EH + DS-Cavl + Fd	LS	146-306 - ggsgg(Seq_14 48)- 50-105 -ggsggsg (Seq_1445) - Fd	Glycine linkers+T4 Fd+LS	N-Strep-H8-HRV3C	1423
RSVF(+)THS_s_to_hp2_ foldon_1221F A102C-A241C_gagggsggsggsgg g ls	CP D3 + EH + DS-Cav1+Fd	LS	146-306 - ggsgg(Seq_14 48)- 50-105+Fd	Glycine linkers, LS	N-Strep-H8-HRV3C	1424
C-Trimer 1GQ3-LS_60mer: leader-Strep-HISx6-Thr-L1H1-K1-H2L2H3-GGSGGGSG-1GQ3-GGSGGGSGGGSGGGSG-LS	CP-S∅+C-term. ATCase +LS	LS		Glycine linkers+E coli ATCase (1GQ3)+LS	N- Leader-Strep-HISx6-Thr	1425
C-Trimer 1GQ3-LS_60mer: leader-Strep-HISx6-Thr-L1H1-K1-H2L2H3-GGSGGGSG-1GQ3-GGSGGGSGGGSGGGSGGGSG -LS	CP-S∅+C-term. ATCase +LS	LS		Glycine linkers+E coli ATCase (1GQ3)+LS	N- Leader-Strep-HISx6-Thr	1426

Trimers on LS (30)						
C-Trimer 1GQ3-LS_60mer: leader-L1H1-K1-H2L2H3-GGSGGGSG-1GQ3-GGSGGGSGGGSGGGSG-LS-Thr-HISx6-Strep	CP-SØ+C-term. ATCase +LS	LS		Glycine linkers+E coli ATCase (1GQ3)+LS	C-Leader-Thr-H6-Strep	1427
C-Trimer 1GQ3-LS_60mer: leader-L1H1-K1-H2L2H3-GGSGGGSG-1GQ3-GGSGGGSGGGSGGGSGGGSG -LS-Thr-HISx6-Strep	CP-SØ+C-term. ATCase +LS	LS		Glycine linkers+E coli ATCase (1GQ3)+LS	C-Leader-Thr-H6-Strep	1428

Example 15

Immunogenicity of prefusion stabilized F protein

[0571] A series of assays (in addition to those provided above) were performed to illustrate the immunogenicity of the recombinant RSV F proteins provided herein that are stabilized in a prefusion conformation. The results show that the provided recombinant RSV F proteins stabilized in a prefusion conformation can be used to induce an immune response in multiple animal models, and further that induction of this immune response protects against future viral challenge.

[0572] Unless indicated otherwise, in FIGs. 73-84, and in this example, reference is made to the following recombinant RSV F proteins:

DS (Subtype A) = RSV A2 F(+)FdTHS S155C, S290C (SEQ ID NO: 185)

DS (Subtype B) = RSV B18537 F(+)FdTHS S155C, S290C (SEQ ID NO: 1479)

DS-CavI (Subtype A) = RSV A2 F(+)FdTHS S155C, S290C, S190F, V207L (SEQ ID NO: 371)

DS-CavI (Subtype B) = RSV B18537 F(+)FdTHS S155C, S290C, S190F, V207L (SEQ ID NO: 372)

Postfusion F (Subtype A) = RSV A2 F(+) dFPTHS

[0573] FIG. 73 illustrates that, using Ribi as adjuvant, a single chain version of DS-CavI presented in the context of a ferritin nanoparticle given IM elicits a small but detectable neutralizing antibody response after 2 weeks in rhesus macaques after a single dose. Based on these small but detectable responses after one dose it is expected that after boosting with a second dose a significant neutralizing antibody response will be induced. This would be consistent with the immunogenicity of 2 mcg of cleaved DS stabilized prefusion F trimer presented on a ferritin nanoparticle formulated with Ribi after 2 doses in mice, discussed below.

[0574] As illustrated in FIG. 74, mice (CB6F1/J) an immune response to the DS version of stabilized prefusion is induced in mice immunized with 20 mcg of DS F in 50 mcg of poly ICLC on weeks 0 and 3. Neutralizing activity was maintained at a high level in DS immunized mice for more than 12 weeks.

[0575] As illustrated in FIG. 75, immunization with DS (Subtype A) = RSV A2 F(+)FdTHS S155C, S290C (SEQ ID NO: 185) can prevent RSV infection in an animal model. Mice were immunized IM with the DS version of the stabilized F protein (SEQ ID NO: 185) at week 0 and week 3. Mice were challenged intranasally with 10e7 pfu of homologous RSV A2 virus on week 19, four months after the last vaccination. On day 5 lungs and noses were removed to measure virus load in tissue. The results show that mice immunized with the DS version of prefusion F had no detectable virus in lung or nose.

[0576] Further, the mice administered DS (Subtype A) = RSV A2 F(+)FdTHS S155C, S290C (SEQ ID NO: 185) did not undergo a Type 2 cytokine response to the immunogen (FIG. 76). Cytokine content was measured in lung and nose supernatants on day 5 following initial immunization with control (PBS), wild-type RSV (RSV), formalin inactivated RSV (FIRSV), DS (SEQ ID NO: 185; "pre-fusion F), or a stabilized post fusion F construct (post-fusion F). Mice undergoing primary infection had significant levels of IFN-gamma and MIP-lalpha as expected. FI-RSV immunized mice had significant levels of type 2 cytokines (IL-4, IL-5, and IL-13) and cytokines associated with epithelial damage (IL-6) typical of responses associated with vaccine-enhanced disease. Mice immunized with prefusion F (DS) had a modest level of IFN-gamma and IL-10 associated with an effective and regulated response and no illness or weight loss.

[0577] The neutralization activity of serum from non-human primate models immunized with the recombinant RSV F DSCavI protein (SEQ ID NO: 371) was assayed over the course of a three-dose immunization (FIG. 77). Rhesus macaques, 4 per group, were immunized twice at 0 and 4 weeks with 50 mcg IM with either DS-CavI prefusion F (SEQ ID NO: 371) or postfusion F based on subtype A sequence and formulated with poly ICLC. On week 26, both groups were boosted with 50 mcg IM of DS-CavI prefusion F formulated with poly ICLC. After 2 doses of DS-CavI, significant neutralizing activity is induced and sustained above the protective threshold for more than 5 months. Postfusion F was immunogenic and induced detectable neutralizing activity after 2 doses, but was only transiently above the protective threshold. Boosting the postfusion F group with a 3rd dose of DS-CavI stabilized prefusion F resulted in a rise in neutralizing activity above that achieved after the 2nd dose. After the 3rd dose neutralizing activity against the homologous subtype A was stably maintained for over 10 weeks as highlighted in the red boxed areas.

[0578] To demonstrate that the DSCavI construct can be formulated with Alum, purified DSCavI (SEQ ID NO: 371) was mixed with Alum hydroxide gel or Alum phosphate gel at various ratios. BALB/c mice were immunized IM with 10 mcg of DS-CavI version of stabilize prefusion F formulated with alum (either aluminum hydroxide gel or aluminum phosphate gel) at 0 and 3 weeks. The protein:alum wt:wt ratios were varied between 1:1 and 1:10. All formulations were immunogenic (FIG. 78). In addition, use of Alum as an adjuvant for DSCavI immunization was demonstrated in a non-human primate model (FIG. 79). Rhesus macaques were immunized at week 0, 4, and 26 with purified DS protein (SEQ ID NO: 185). The week 0 and 4 injections were comprised of the DS version of stabilized RSV prefusion F (50 mcg) formulated in poly ICLC. The week 26 boost was 50 mcg of the DS prefusion stabilized F formulated in aluminum phosphate gel. Therefore alum is an effective adjuvant for the stabilized prefusion F in NHP.

[0579] To show that another immunization protocol is effective for inducing an effective immune response with DSCavI, mice were immunized with a gene-based vector expressing DSCavI, and the resulting immune response to RSV F was evaluated (FIG. 80). CB6F1/J mice were immunized with a recombinant adenovirus serotype 5 vector expressing the wild-type version of F at 0 and 3 weeks or were immunized at week 0 with rAd5 expressing the DS-CavI version of preF membrane anchored (non-secreted) and boosted with 10 mcg of DS-CavI formulated in alum at week 3. rAd5-preF primed mice boosted with DS-CavI in alum produced as much neutralizing antibody as mice given two doses of protein only indicating that prefusion F delivered by a gene-based vector is immunogenic and can prime for a subsequent protein boost..

[0580] Additionally, the DSCavl protein was effective for boosting an immune response to wild-type (WT) RSV F (FIG. 81). Non-human primates primed with recombinant adenovirus vectors expressing WT versions of RSV F (subtype A) more than 2 years before boost, were boosted with a single 50 mcg dose of DS-Cavl subtype A or subtype B formulated in alum. Two weeks after boosting neutralizing activity was significantly increased by both subtype A and B DS-Cavl proteins (FIGs. 81-82).

[0581] To demonstrate the cross-subtype effectiveness of the DS (S155C, S290C) version of stabilized F, CB6F1/J mice were immunized IM with 10 mcg of DS formulated in Ribi at week 0 and week 3 (FIG. 83). Neutralizing antibody was induced by both A (SEQ ID NO: 185) and B (SEQ ID NO: 1479) subtype proteins against both subtype A and B viruses. The group receiving both A and B received a total of 20 mcg of protein.
treated with glycosidases or mutant versions were made to remove glycosylation sites at N27 and N70. The F protein could not be produced if the N500 glycosylation was mutated suggesting that glycosylation at that site is required for expression. Neutralizing activity was detected at week 5 (solid bars) and week 7 (hatched bars) in mice immunized by any of the glycosylation variants of F. However, altering glycosylation appeared to reduce immunogenicity compared to the original DS version of stabilized prefusion F. **** = P<0.0001.

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Patentkrav

1. Isoleret immunogen, der omfatter:
et rekombinant RSV-F-protein eller fragment deraf, der omfatter aminosyresubstitutioner sammenlignet med et nativt RSV-F-protein som angivet som én af SEQ ID NOs: 1-184, der stabiliserer det rekombinante RSV-F-protein i en præfusionskonformation, som specifikt binder til et præfusionsspecifikt RSV-F-antistof, hvor:
det rekombinante RSV-F-protein er stabiliseret i RSV-F-protein-præfusionskonformationen med S155C-, S290C-, S190F- og V207L-substitutioner,
præfusionskonformationen af det rekombinante RSV-F-protein eller fragmentet deraf omfatter et antigen-site Ø, der omfatter rest 62-69 og 196-209 af en nativ RSV-F-protein-sekvens angivet som én af SEQ ID NOs: 1-184,
immunogenet specifikt binder til det præfusionsspecifikke antistof efter inkubation ved 20 °C i phosphatbufret saltopløsning ved fysiologisk pH-værdi i mindst 24 timer i fravær af det præfusionsspecifikke antistof, og
det præfusionsspecifikke RSV-F-antistof ikke binder til et RSV-F-protein i en postfusionskonformation; og
det præfusionsspecifikke antistof er et D25-antistof, der omfatter VH- og VL-domæner som angivet i henholdsvis SEQ ID NOs 368 og 369.

2. Immunogen ifølge krav 1, hvor det rekombinante RSV-F-protein omfatter eller består af en aminosyresekvens, der omfatter mindst 80 % identitet med henholdsvis rest 26-109 og 137-513 eller henholdsvis rest 26-103 og 145-513 eller henholdsvis rest 26-105 og 145-513 af én af SEQ ID NOs: 371 (DS-Cav1, subtype A), 372 (DSCav1, subtype B) eller 373 (DSCav1, bovin).

3. Immunogen ifølge krav 2, hvor det rekombinante RSV-F-protein omfatter eller består af en aminosyresekvens, der omfatter mindst 80 % identitet med henholdsvis rest 26-109 og 137-513 eller henholdsvis rest 26-103 og 145-513 eller henholdsvis rest 26-105 og 145-513 af SEQ ID NO: 371 (DS-Cav1, subtype A).

4. Immunogen ifølge krav 2, hvor det rekombinante RSV-F-protein omfatter eller

består af en aminosyresekvens, der omfatter mindst 80 % identitet med henholdsvis rest 26-109 og 137-513 eller henholdsvis rest 26-103 og 145-513 eller henholdsvis rest 26-105 og 145-513 af SEQ ID NO: 372 (DS-Cav1, subtype B).

5. Immunogen ifølge krav 2, hvor det rekombinante RSV-F-protein omfatter henholdsvis rest 26-109 og 137-513 eller henholdsvis rest 26-103 og 145-513 eller henholdsvis rest 26-105 og 145-513 af SEQ ID NO: 371 (DS-Cav1, subtype A).

6. Immunogen ifølge krav 2, hvor det rekombinante RSV-F-protein omfatter henholdsvis rest 26-109 og 137-513 eller henholdsvis rest 26-103 og 145-513 eller henholdsvis rest 26-105 og 145-513 af SEQ ID NO: 372 (DSCav1, subtype B).

7. Immunogen ifølge et hvilket som helst af de foregående krav, der omfatter en multimer af det rekombinante RSV-F-protein eller fragmentet deraf ifølge krav 1-7.

8. Immunogen ifølge et hvilket som helst af de foregående krav, hvor det rekombinante RSV-F-protein eller fragmentet deraf ifølge krav 1-7 er bundet til et trimeriseringsdomæne.

9. Immunogen ifølge krav 8, hvor den C-terminale ende af F1-polypeptidet af det rekombinante RSV-F-protein er bundet til trimeriseringsdomænet.

10. Immunogen ifølge krav 8 eller krav 9, hvor trimeriseringsdomænet er et foldon-domæne.

11. Immunogen ifølge krav 8, hvor det rekombinante RSV-F-protein, der er bundet til trimeriseringsdomænet, omfatter eller består af en aminosyresekvens, der omfatter mindst 80 % identitet med rest 26-109 og 137-544 eller 26-103 og 145-544 af én af SEQ ID NOs: 371 (DS-Cav1, subtype A), 372 (DSCav1, subtype B) eller 373 (DSCav1, bovin).

12. Immunogen ifølge krav 11, hvor det rekombinante RSV-F-protein, der er bundet til trimeriseringsdomænet, omfatter eller består af en aminosyresekvens, der omfatter mindst 80 % identitet med rest 26-109 og 137-544 eller 26-103 og 145-544 af SEQ ID

NO: 371 (DS-Cav1, subtype A).

13. Immunogen ifølge krav 11, hvor det rekombinante RSV-F-protein, der er bundet til trimeriseringsdomænet, omfatter eller består af en aminosyresekvens, der omfatter mindst 80 % identitet med rest 26-109 og 137-544 eller 26-103 og 145-544 af SEQ ID NO: 372 (DS-Cav1, subtype B).

14. Immunogen ifølge krav 11, hvor det rekombinante RSV-F-protein, der er bundet til trimeriseringsdomænet, omfatter rest 26-109 og 137-544 eller 26-103 og 145-544 af SEQ ID NO: 371 (DS-Cav1, subtype A).

15. Immunogen ifølge krav 11, hvor det rekombinante RSV-F-protein, der er bundet til trimeriseringsdomænet, omfatter rest 26-109 og 137-544 eller 26-103 og 145-544 af SEQ ID NO: 372 (DS-Cav1, subtype B).

16. Viruslignende partikel, der omfatter immunogenet ifølge et hvilket som helst af kravene 1-15.

17. Proteinnanopartikel, der omfatter immunogenet ifølge et hvilket som helst af kravene 1-15.

18. Nukleinsyremolekyle, der koder for immunogenet eller proteinnanopartiklen ifølge et hvilket som helst af kravene 1-17.

19. Nukleinsyremolekyle ifølge krav 18, der er operabelt forbundet med en promotor.

20. Vektor, der omfatter nukleinsyremolekylet ifølge krav 19.

21. Vektor ifølge krav 20, hvor vektoren er en virusvektor.

22. Nukleinsyremolekyle eller vektor ifølge et hvilket som helst af kravene 19-21, der omfatter nukleotidsekvensen angivet som SEQ ID NO: 383, SEQ ID NO: 384, SEQ ID NO: 385 eller SEQ ID NO: 386.

23. Immunogen sammensætning, der omfatter en effektiv mængde af immunogenet, den viruslignende partikel, proteinnanopartiklen, nukleinsyremolekylet eller vektoren ifølge et hvilket som helst af kravene 1-22; og en farmaceutisk acceptabel bærer.

24. Immunogen sammensætning ifølge krav 23 til anvendelse i en fremgangsmåde til generering af en immunrespons på RSV-F hos et individ, som har behov derfor, hvilken fremgangsmåde omfatter administration til individet af en effektiv mængde af den immunogene sammensætning med henblik på at generere immunresponsen.

25. Immunogen sammensætning til anvendelse ifølge krav 24, hvor fremgangsmåden omfatter en prime-boost-administration af den immunogene sammensætning.

26. Immunogen sammensætning til anvendelse ifølge krav 24 eller 25, hvor individet er et menneske eller et dyreindivid.

27. Immunogen sammensætning til anvendelse ifølge et hvilket som helst af kravene 25-26, hvor individet er mindre end ét år gammelt, mere end 65 år gammelt eller gravid.

28. Kit, der omfatter immunogenet, den viruslignende partikel, proteinnanopartiklen, nukleinsyremolekylet, vektoren, værtscellen eller den immunogene sammensætning ifølge et hvilket som helst af kravene 1-27; og instruktioner til anvendelse af kittet.

29. Immunogen ifølge et hvilket som helst af kravene 1-15 til anvendelse til inhibering af RSV-infektion hos et individ.

30. Immunogen ifølge et hvilket som helst af kravene 1-15 til anvendelse i en fremgangsmåde til induktion af en immunrespons på RSV-F-protein hos et individ, som har behov derfor.

DRAWINGS

Drawing

FIG. 1A

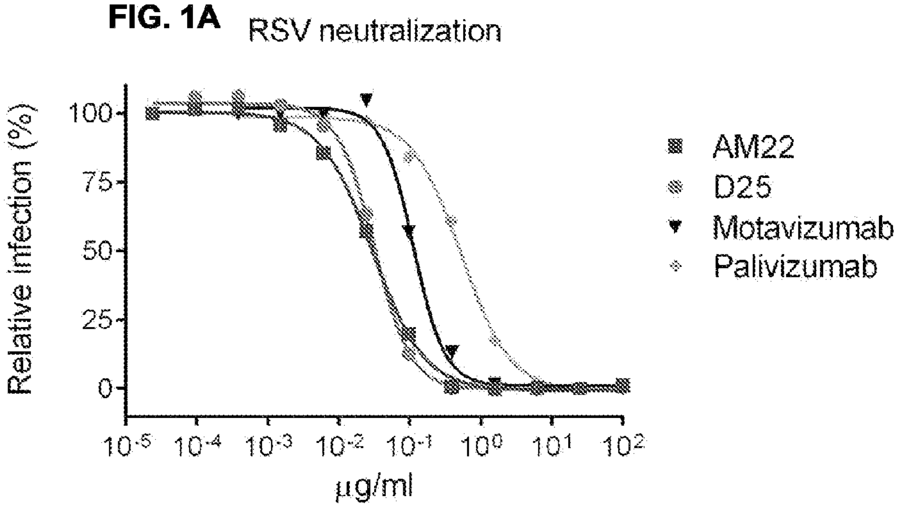


FIG. 1B

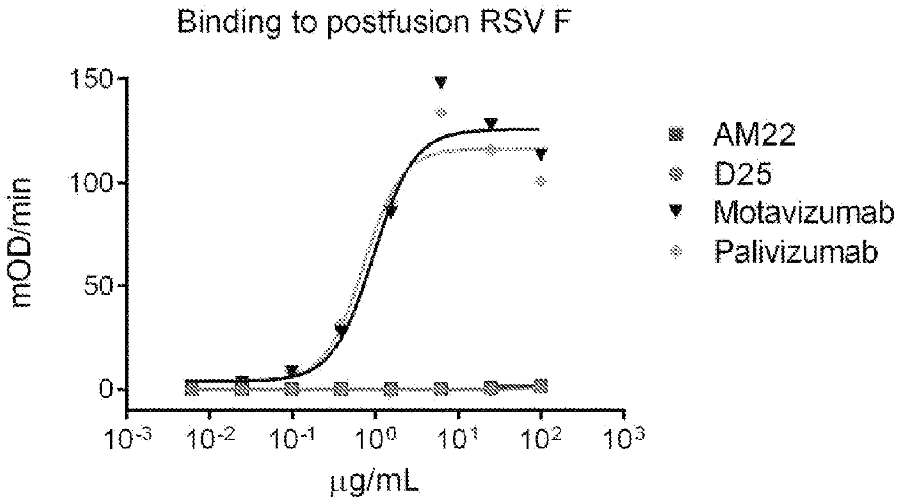


FIG. 1C

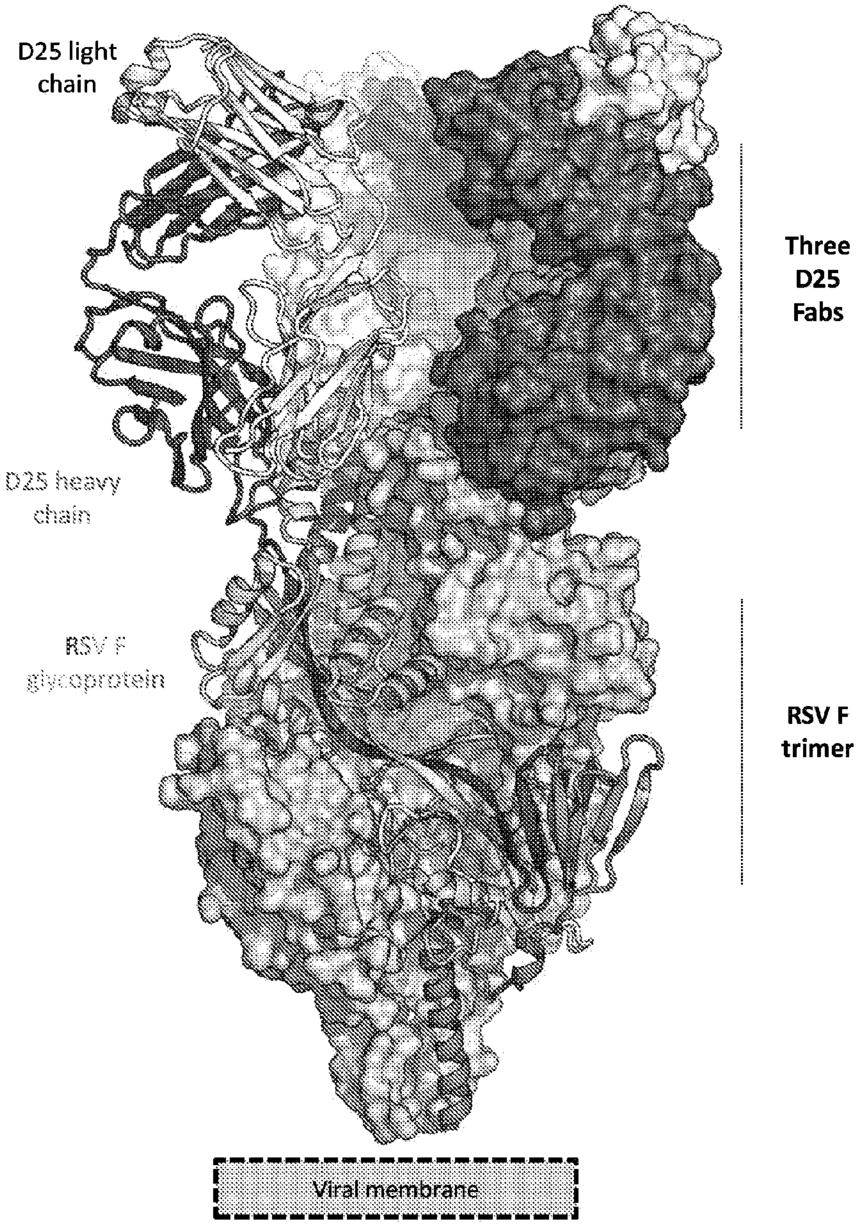
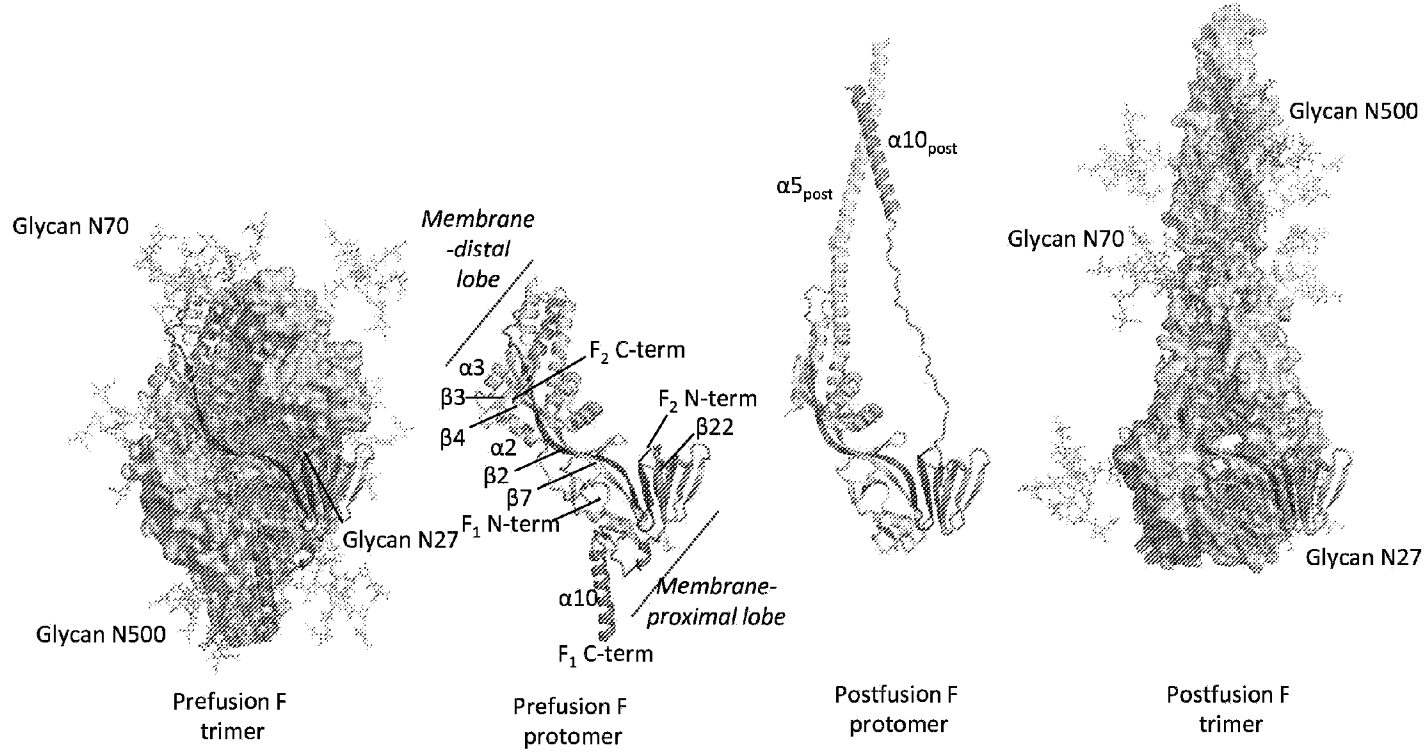

D25 light chain
D25 heavy chain
RSV F glycoprotein
Three D25 Fabs
RSV F trimer
Viral membrane

FIG. 2A



Glycan N70
Glycan N500
Glycan N27
Prefusion F trimer
Membrane -distal lobe
α3
β3
β4
α2
β2
β7
F1 N-term
F2 C-term
F2 N-term
β22
α10
F1 C-term
Membrane- proximal lobe
Prefusion F protomer
α5post
α10post
Postfusion F protomer
Glycan N500
Glycan N70
Glycan N27
Postfusion F trimer

FIG. 2B

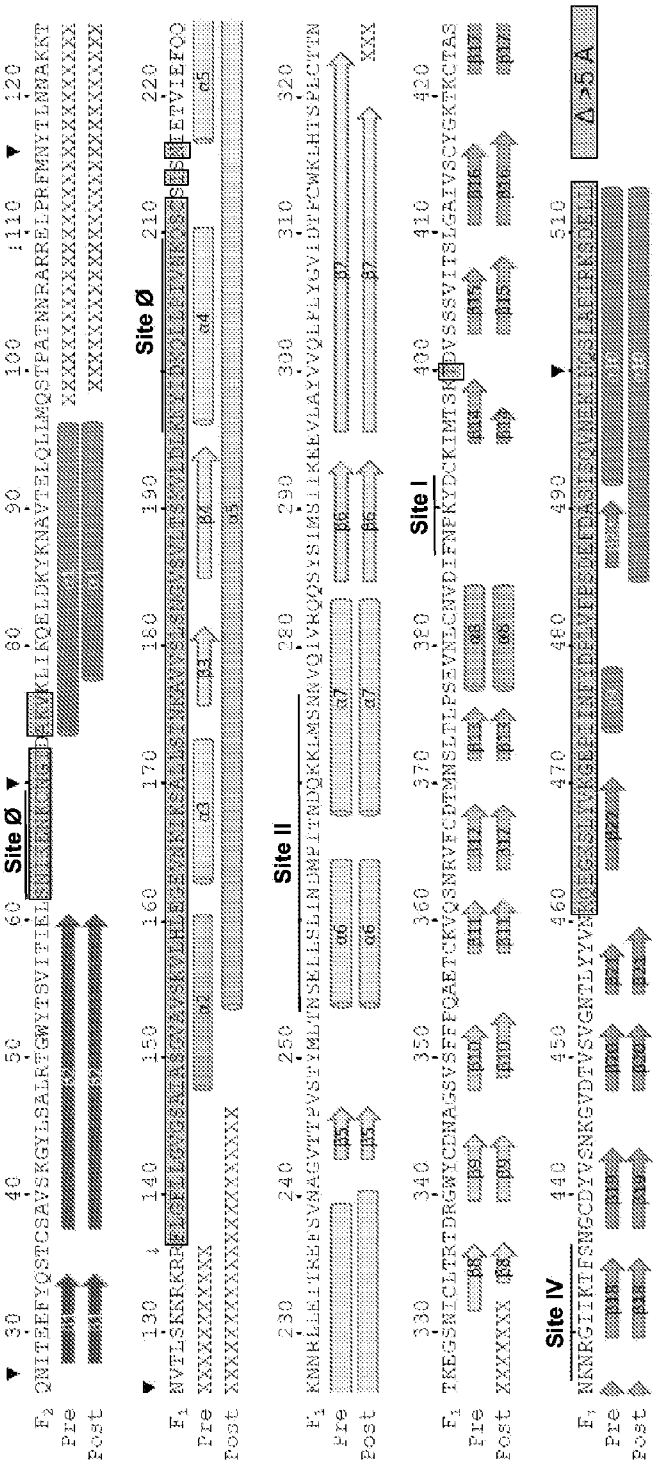


FIG. 3A

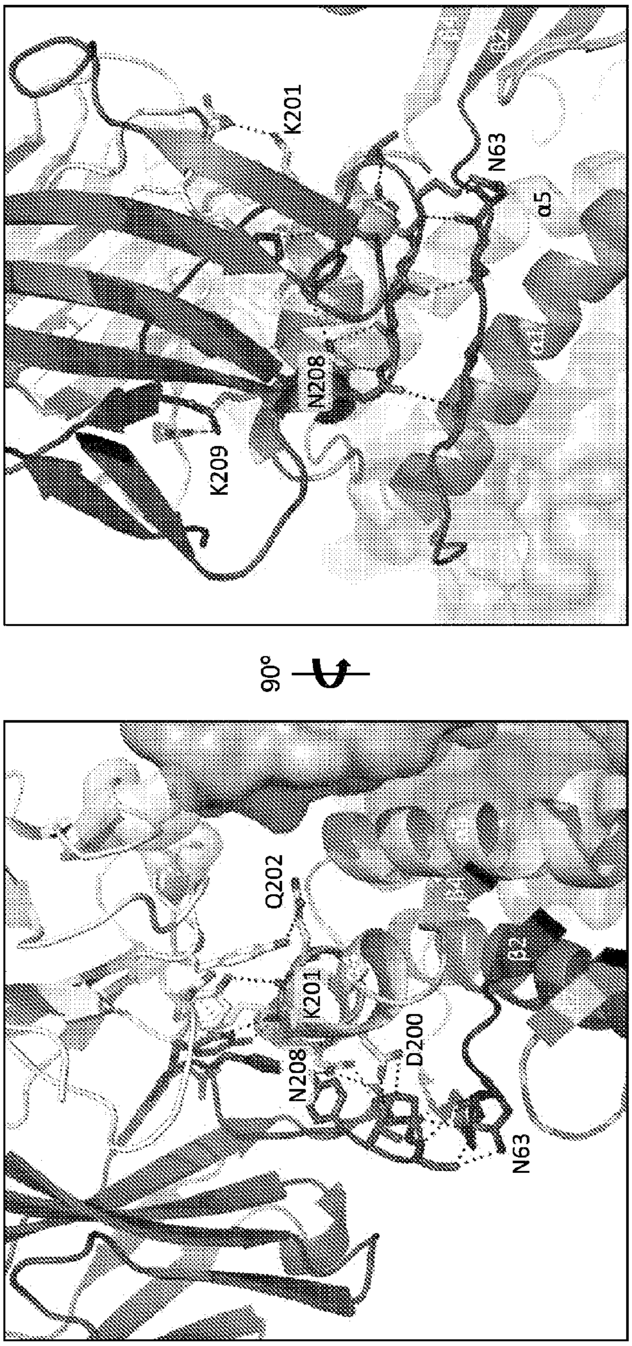


FIG. 3B

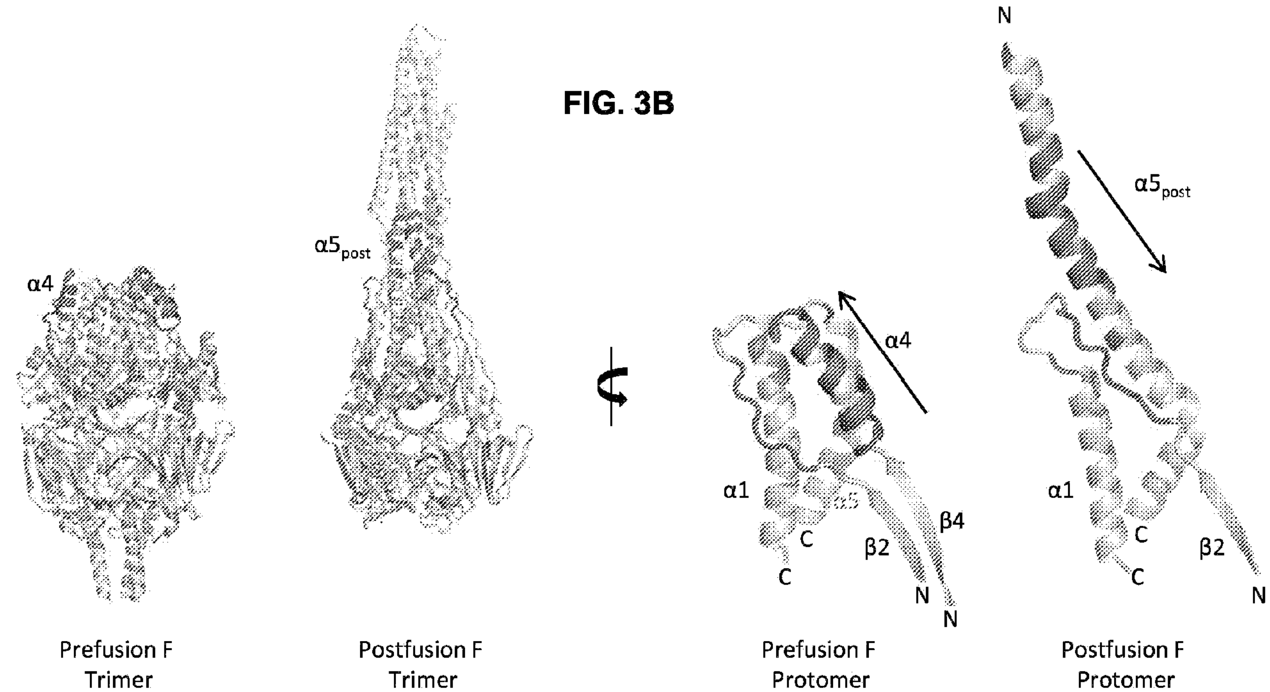


FIG. 3C

	F2	F1	Identity with hRSV/A ectodomain: epitope region	Identity with hRSV/A ectodomain: non-epitope	Changes within epitope
hRSV/A	63-NIKENKCNGTDA-74	200-DKQLLPIVNKQSCS-213			
hRSV/B	63-NIKETKCNGTDT-74	200-NNQLLPIVNQQSCR-213	30/36 (77%)	427/446 (95.7%)	6/35 (34%)
bRSV	63-EIQKNVCKSTDS-74	200-DKELLPKVNNHDCR-213	13/36 (50%)	403/446 (90.4%)	13/36 (33%)

FIG. 4A

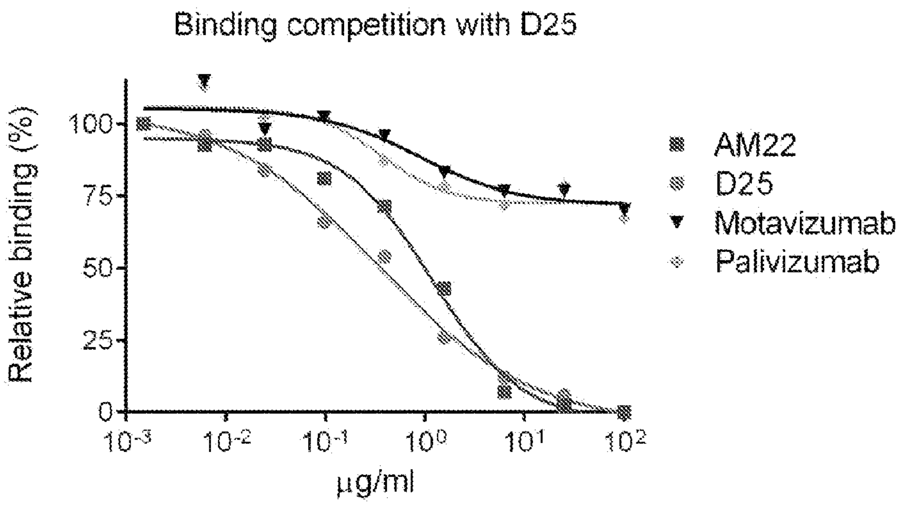

Binding competition with D25
Relative binding (%)
100
75
50
25
0
10-3
10-2
10-1
10^0
10^1
10^2
μg/ml
AM22
D25
Motavizumab
Palivizumab

FIG. 4B

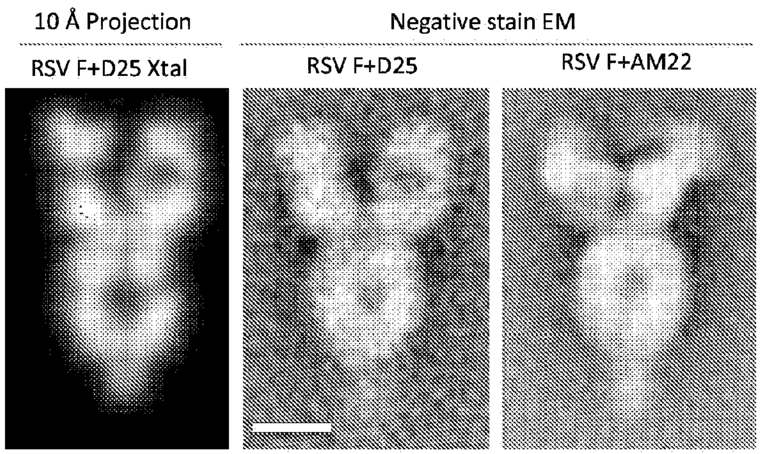

10 Å Projection
Negative stain EM
RSV F+D25 Xtal
RSV F+D25
RSV F+AM22

FIG. 4C

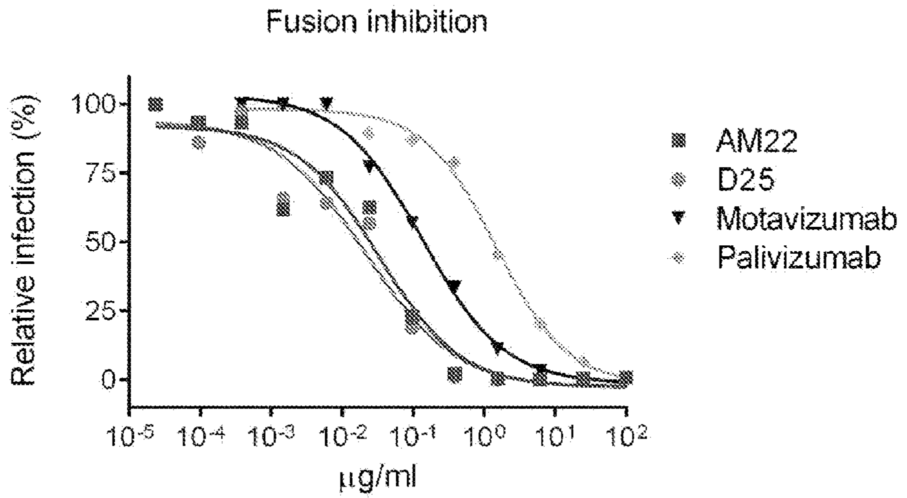

Fusion inhibition
Relative infection (%)
100
75
50
25
0
10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2}
μg/ml
AM22
D25
Motavizumab
Palivizumab

FIG. 4D

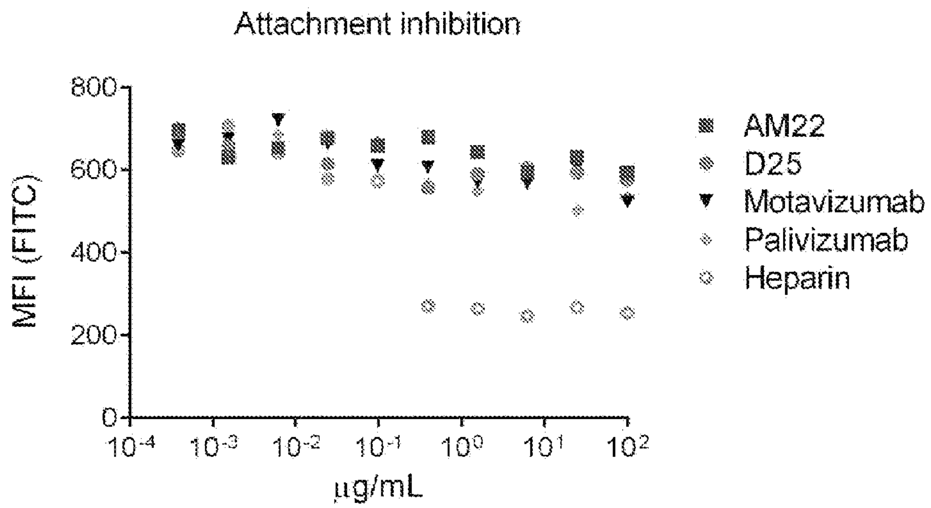

Attachment inhibition
MFI (FITC)
800
600
400
200
0
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2}
μg/mL
AM22
D25
Motavizumab
Palivizumab
Heparin

FIG. 5

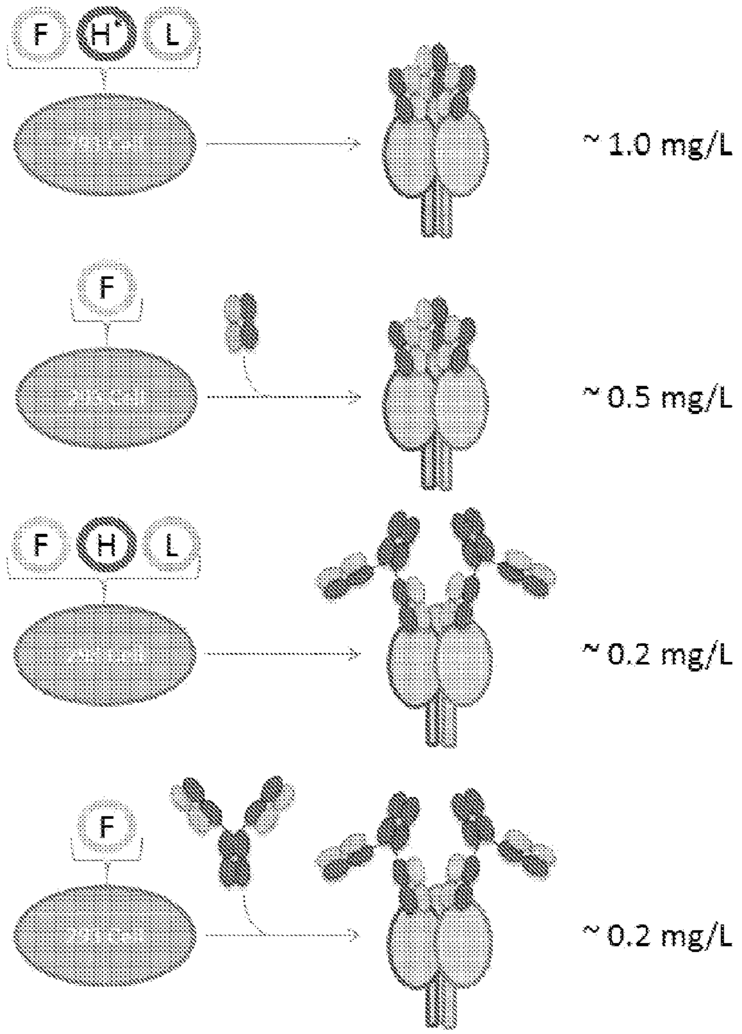


FIG. 6

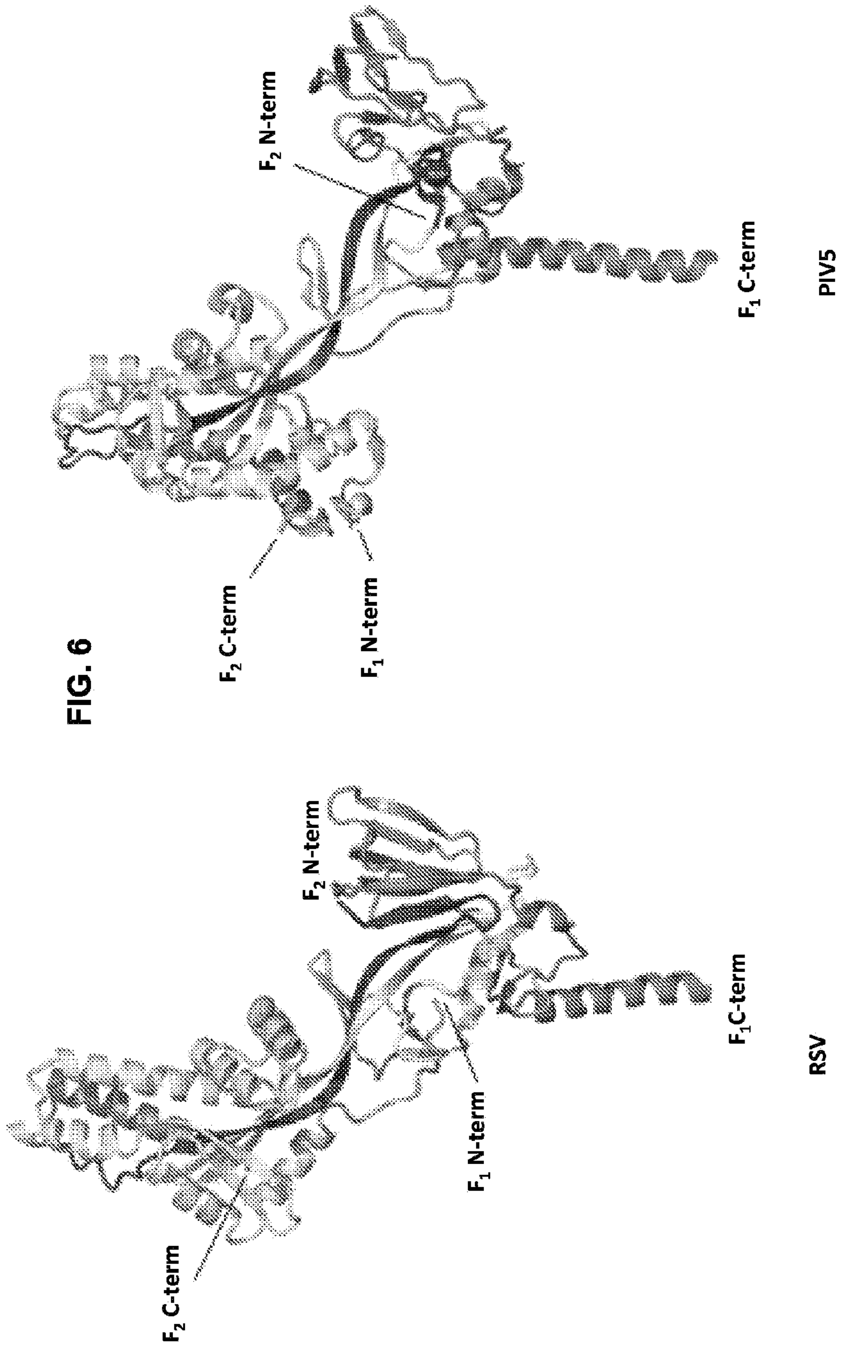

F2 N-term
F1 C-term
PIV5
F2 C-term
F1 N-term
F2 N-term
F1C-term
RSV
F1 N-term
F2 C-term

FIG. 7

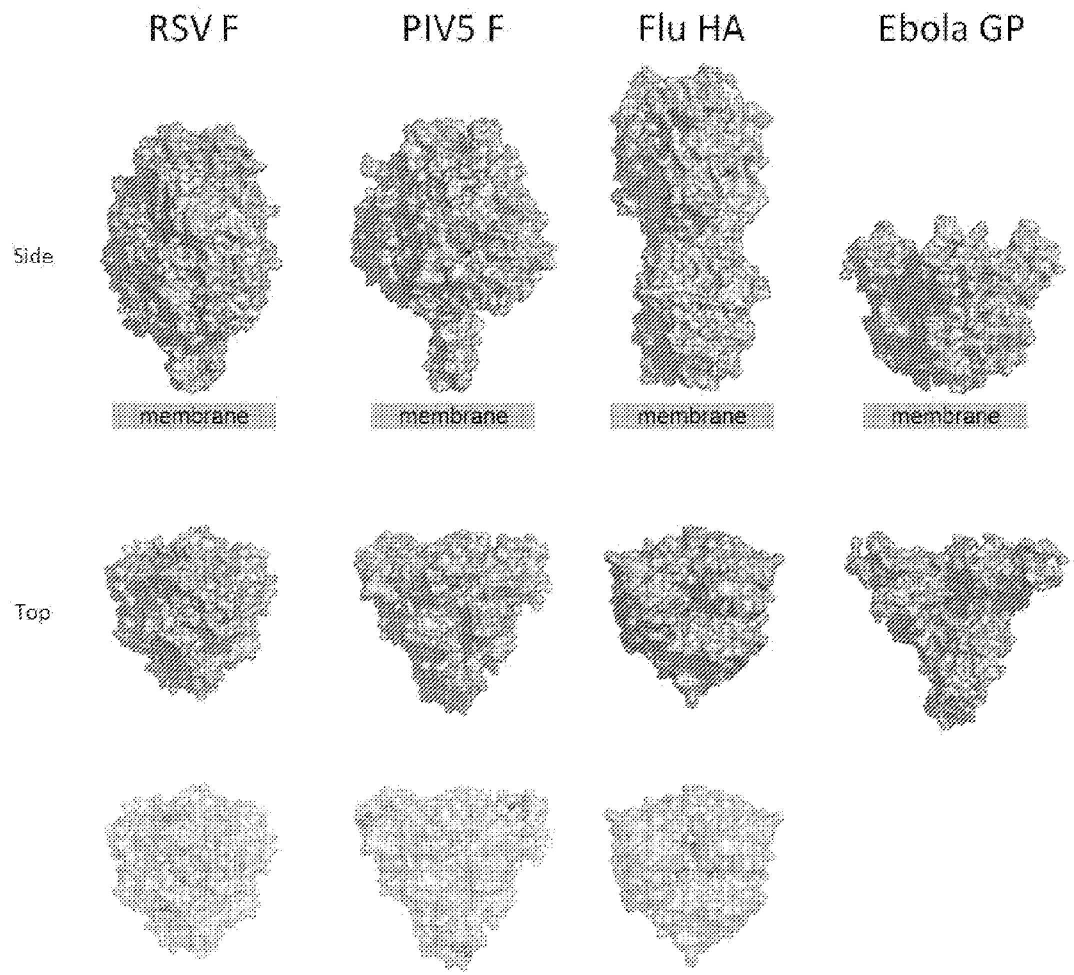

RSV F
PIV5 F
Flu HA
Ebola GP
Side
Top
membrane
membrane
membrane
membrane

FIG. 8

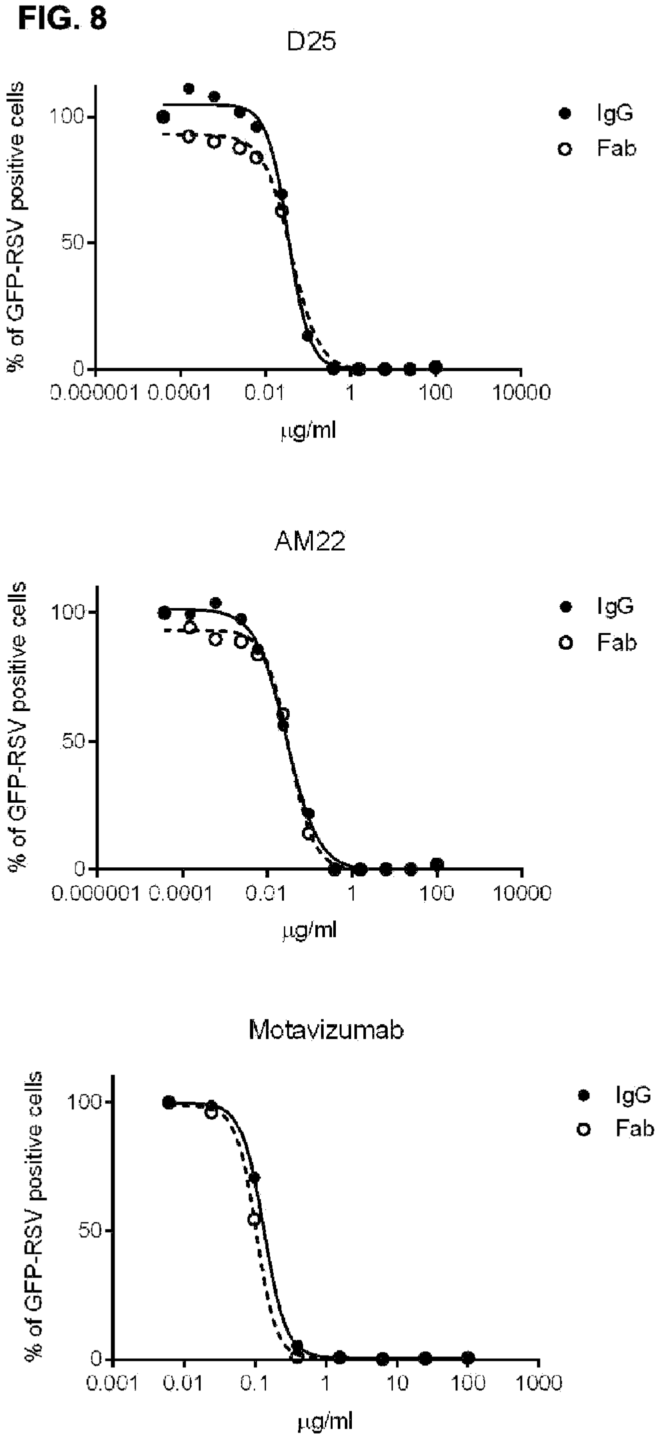

D25
% of GFP-RSV positive cells
100
50
0
0.000001
0.0001
0.01
1
100
10000
μg/ml
IgG
Fab
AM22
% of GFP-RSV positive cells
100
50
0
0.000001
0.0001
0.01
1
100
10000
μg/ml
IgG
Fab
Motavizumab
% of GFP-RSV positive cells
100
50
0
0.001
0.01
0.1
1
10
100
1000
μg/ml
IgG
Fab

FIG. 9A

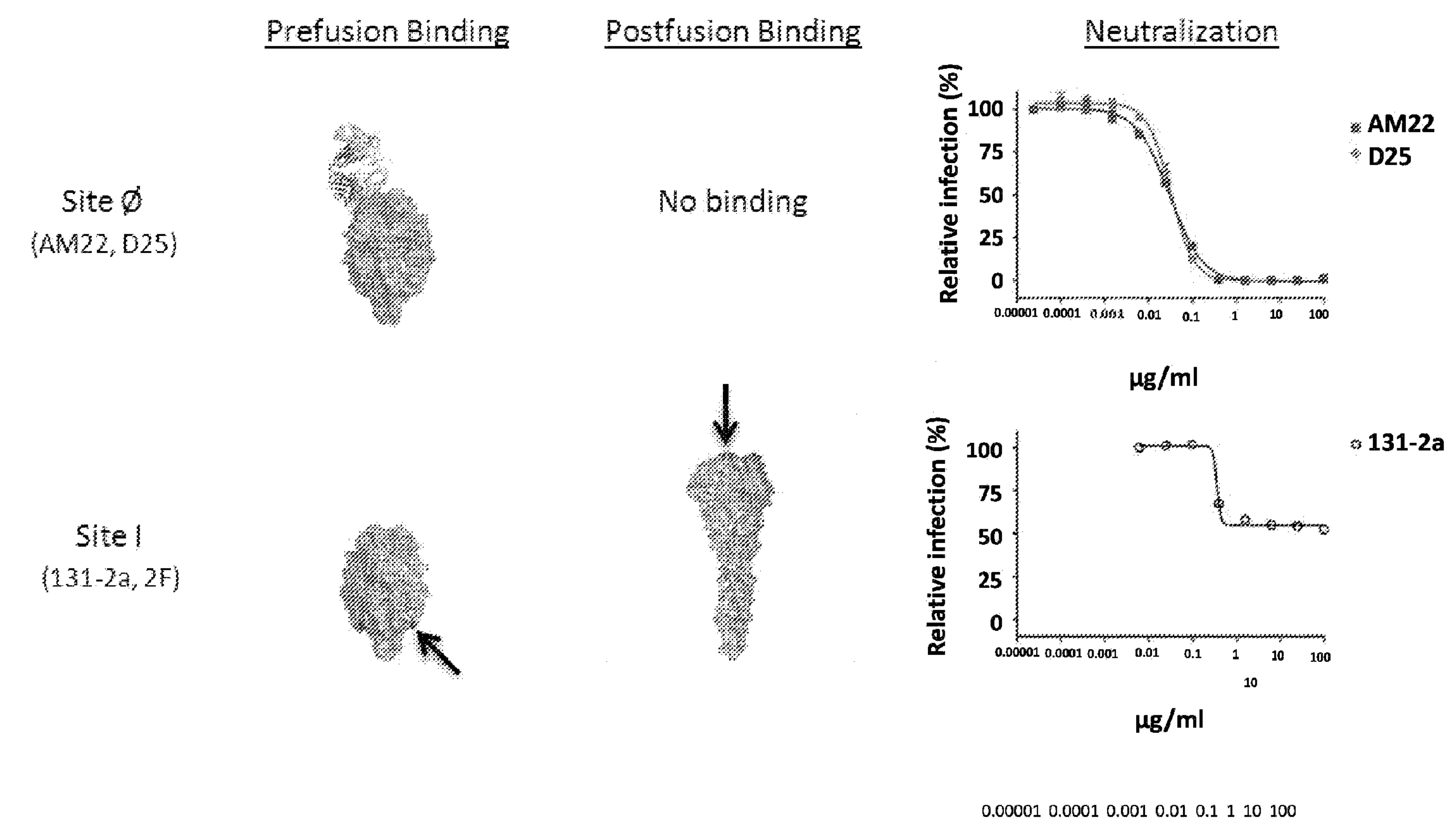

Prefusion Binding
Postfusion Binding
Neutralization
Site Ø
(AM22, D25)
No binding
Site I
(131-2a, 2F)
Relative infection (%)
100
75
50
25
0
0.00001 0.0001 0.001 0.01 0.1 1 10 100
μg/ml
AM22
D25
131-2a
10
0.00001 0.0001 0.001 0.01 0.1 1 10 100

FIG. 9B

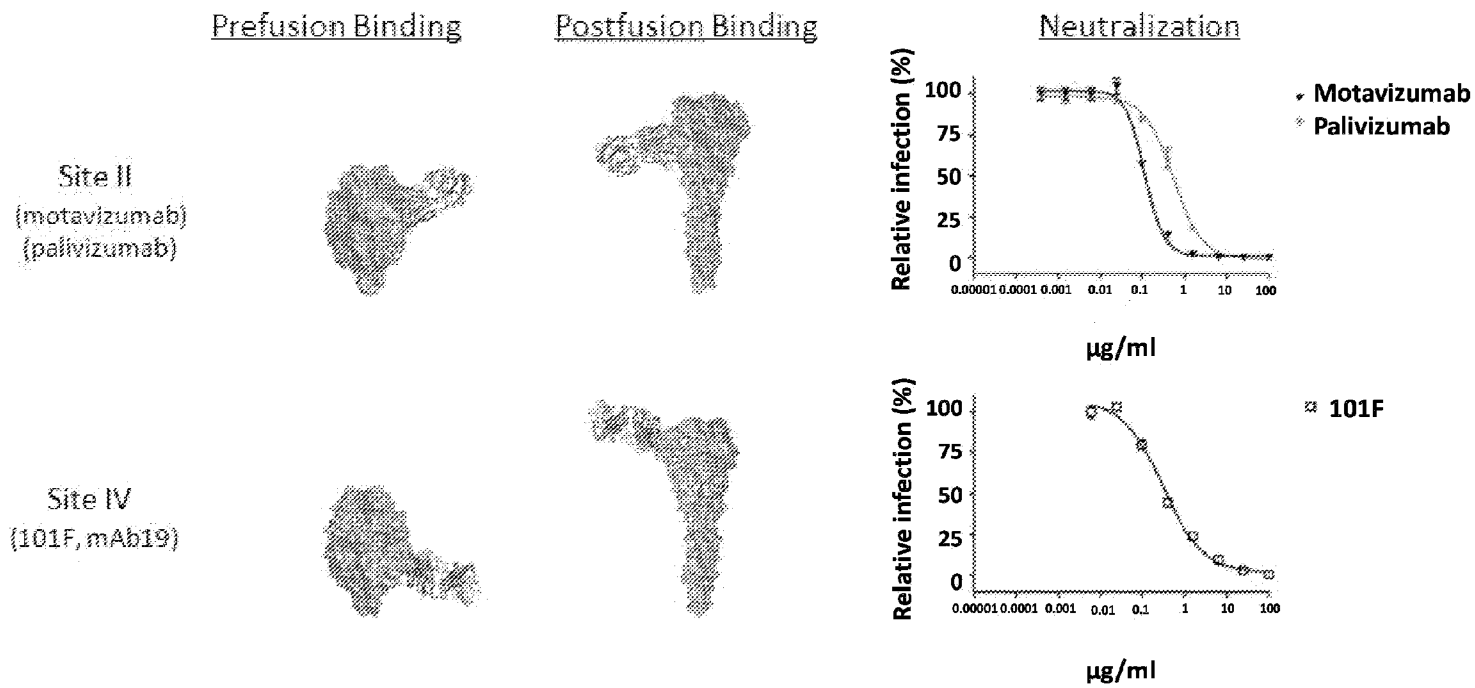

Prefusion Binding
Postfusion Binding
Neutralization
Site II
(motavizumab)
(palivizumab)
Site IV
(101F, mAb19)
Relative infection (%)
100
75
50
25
0
0.00001 0.0001 0.001 0.01 0.1 1 10 100
μg/ml
Motavizumab
Palivizumab
101F

FIG. 10

Mutation Ser155Cys/Ser290Cys Can Form a Disulfide Bond Only in Prefusion State

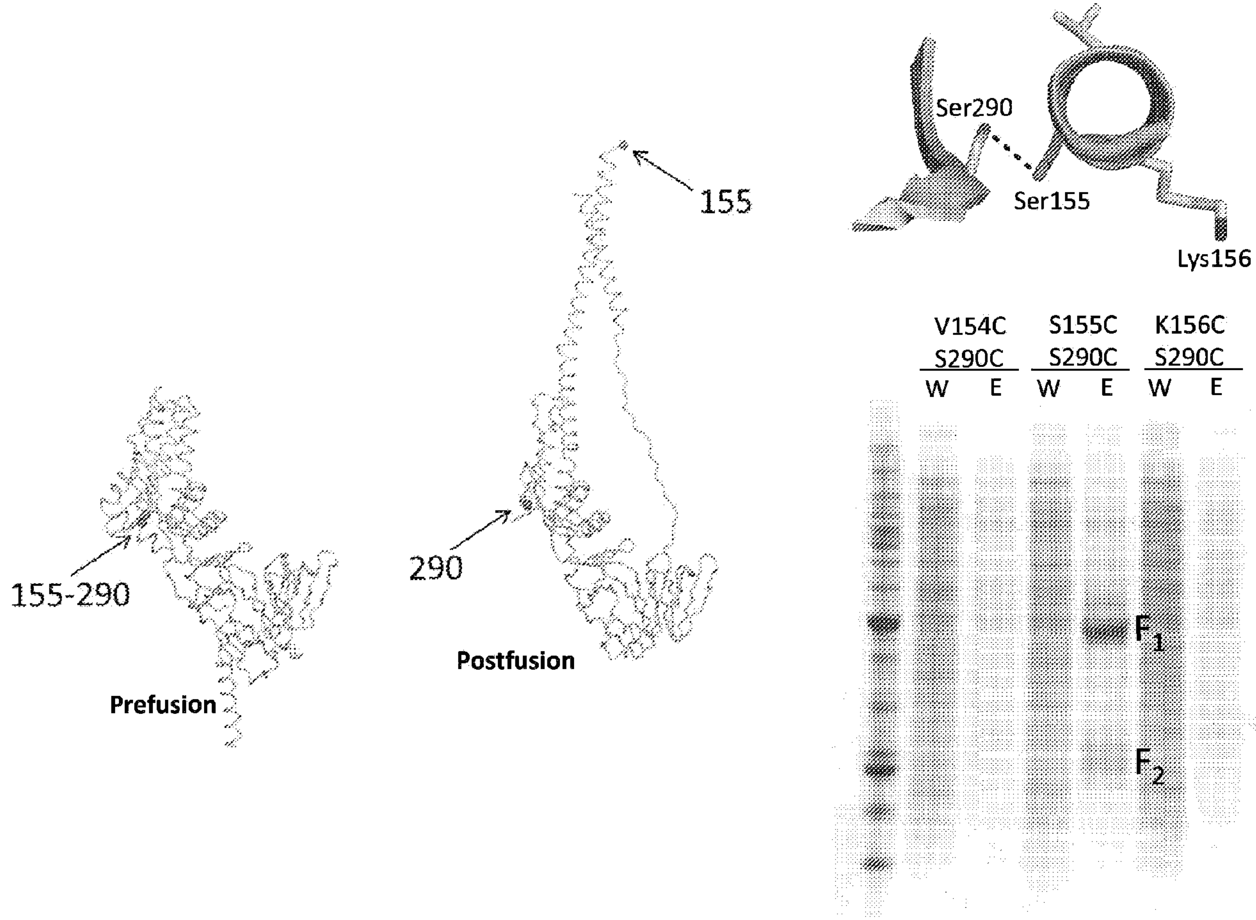


FIG. 11

Ser155Cys/Ser290Cys Stabilizes Prefusion Conformation of RSV F

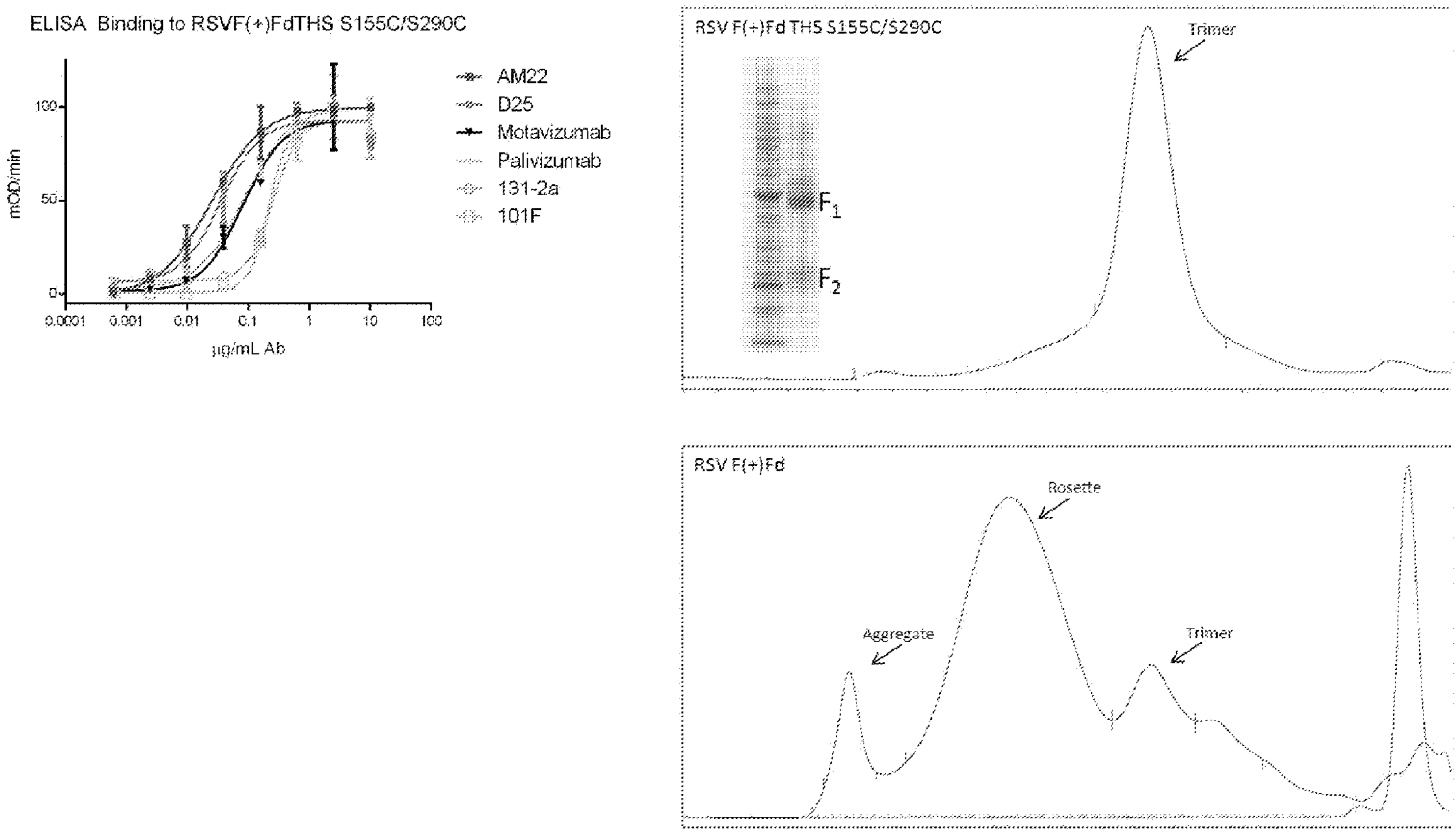


FIG. 12

Ser155Cys/Ser290Cys Results in Homogeneous Prefusion-Stabilized RSV F

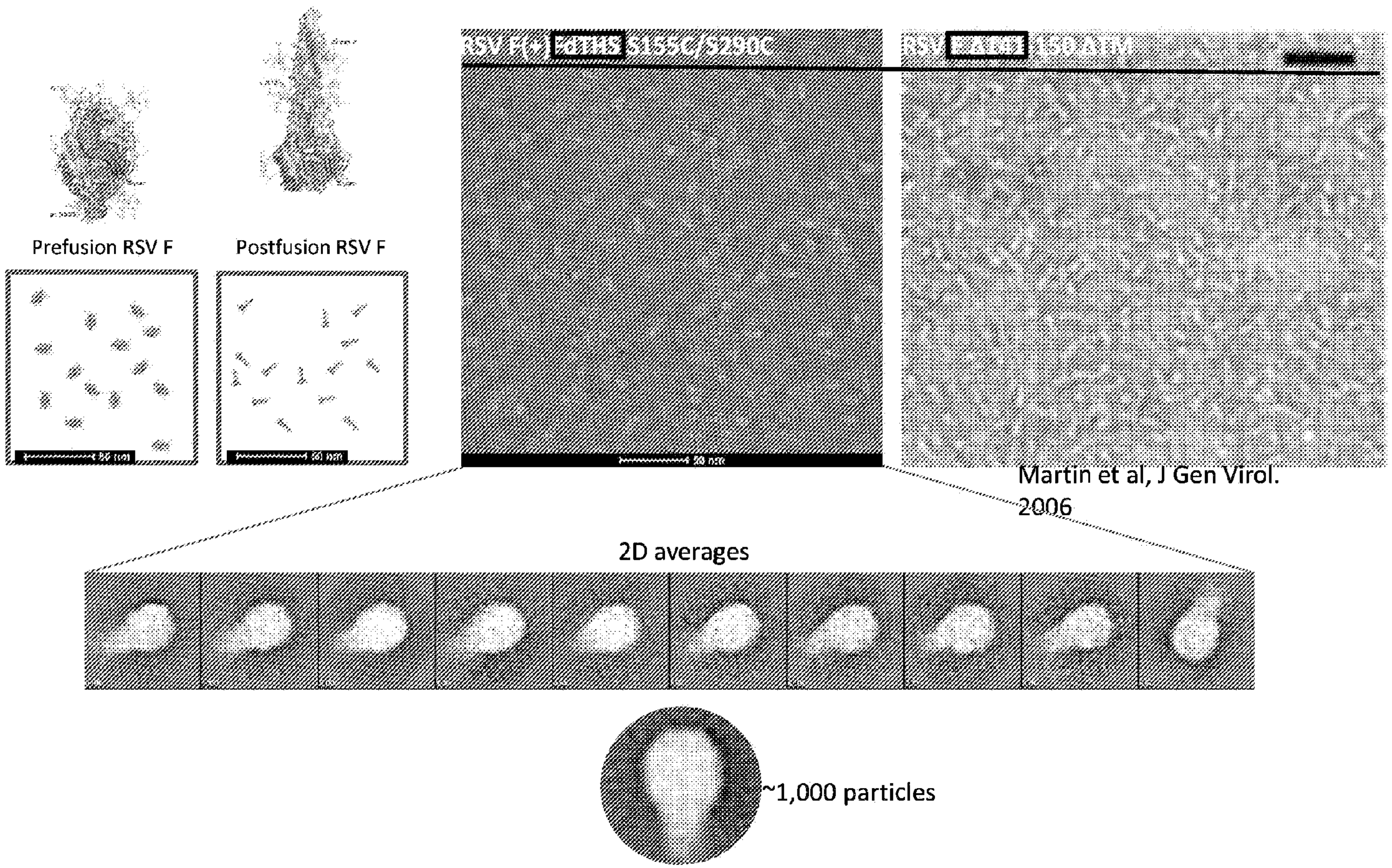
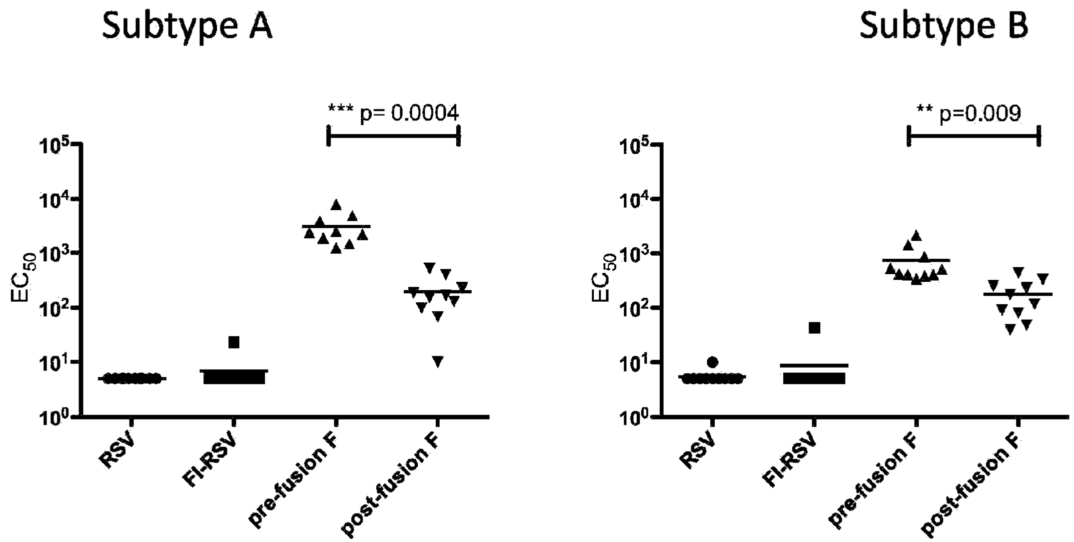


FIG. 13

Neutralization of RSV subtypes A and B
Week 5



RSV Line 19 (Subtype A)

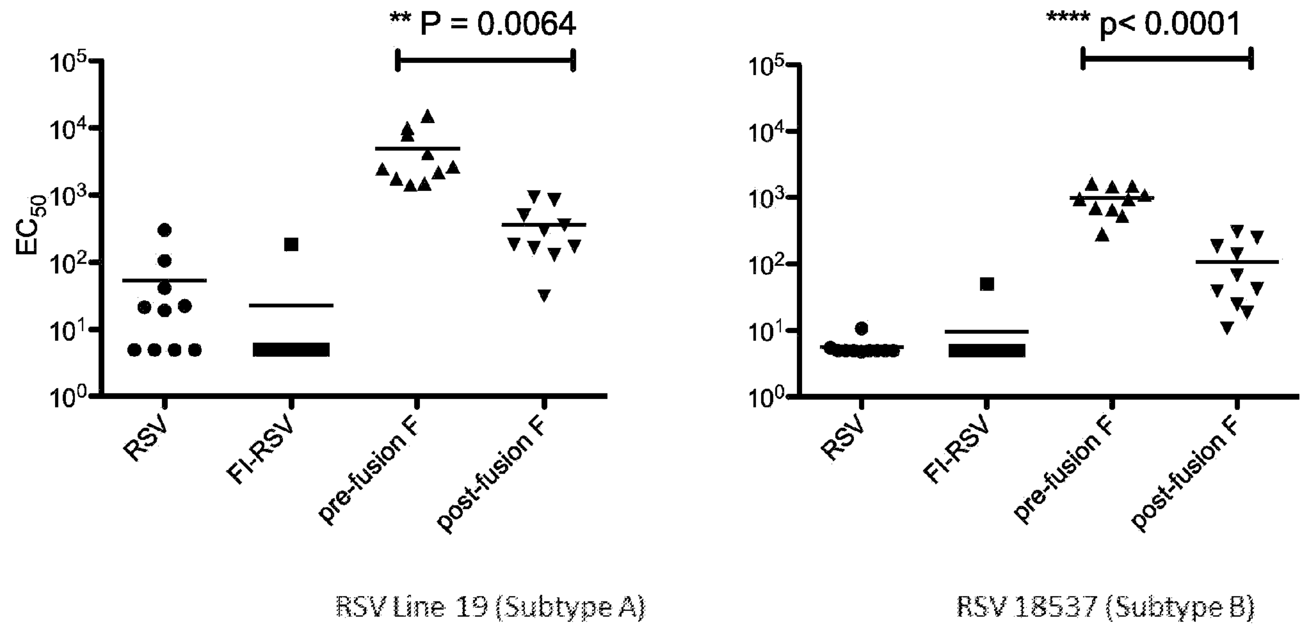
	RSV	FI-RSV	pre-fusion F	post-fusion F
Mean	0	7	3143	198

RSV 18537 (Subtype B)

RSV	FI-RSV	pre-fusion F	post-fusion F
0	0	1068	230

FIG. 14

Neutralization of RSV subtypes A and B
week 7



	RSV	FI-RSV	pre-fusion F	post-fusion F
Mean	51	18	4930	360

RSV	FI-RSV	pre-fusion F	post-fusion F
2	5	971	108

FIG. 15

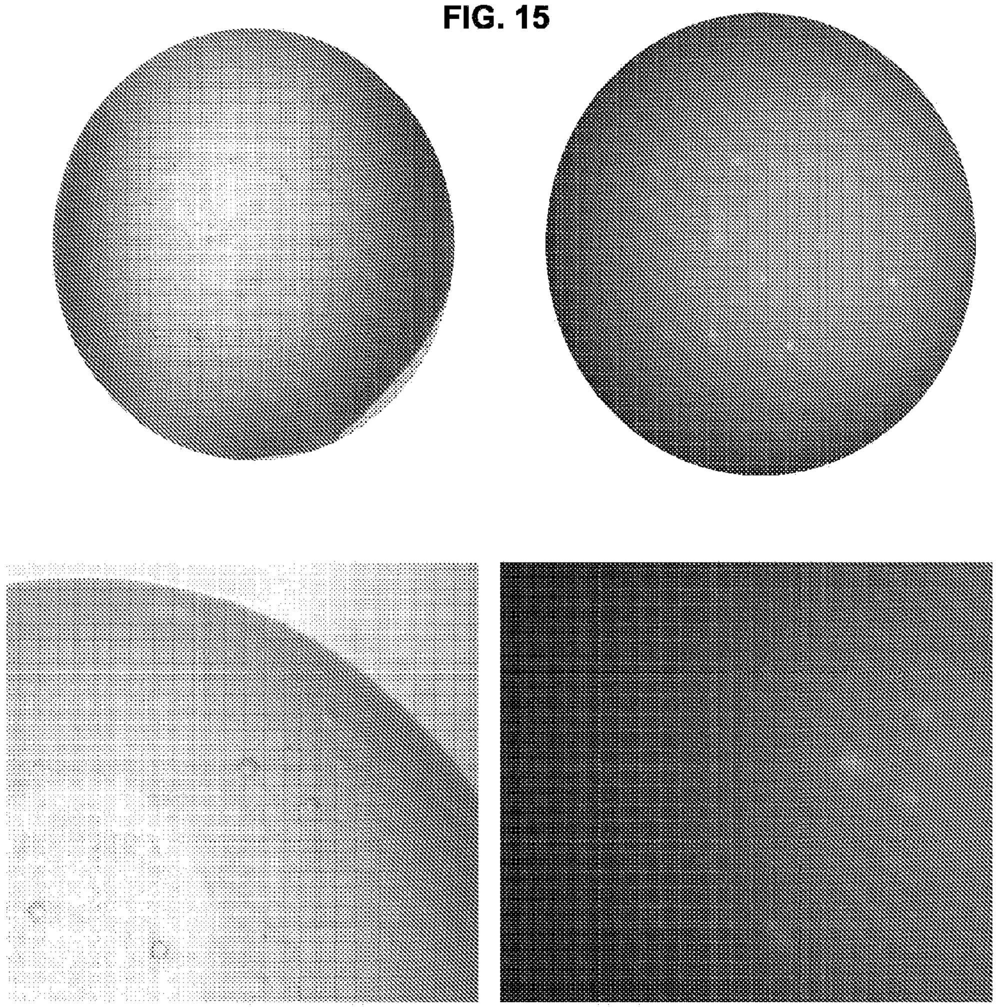


FIG. 16

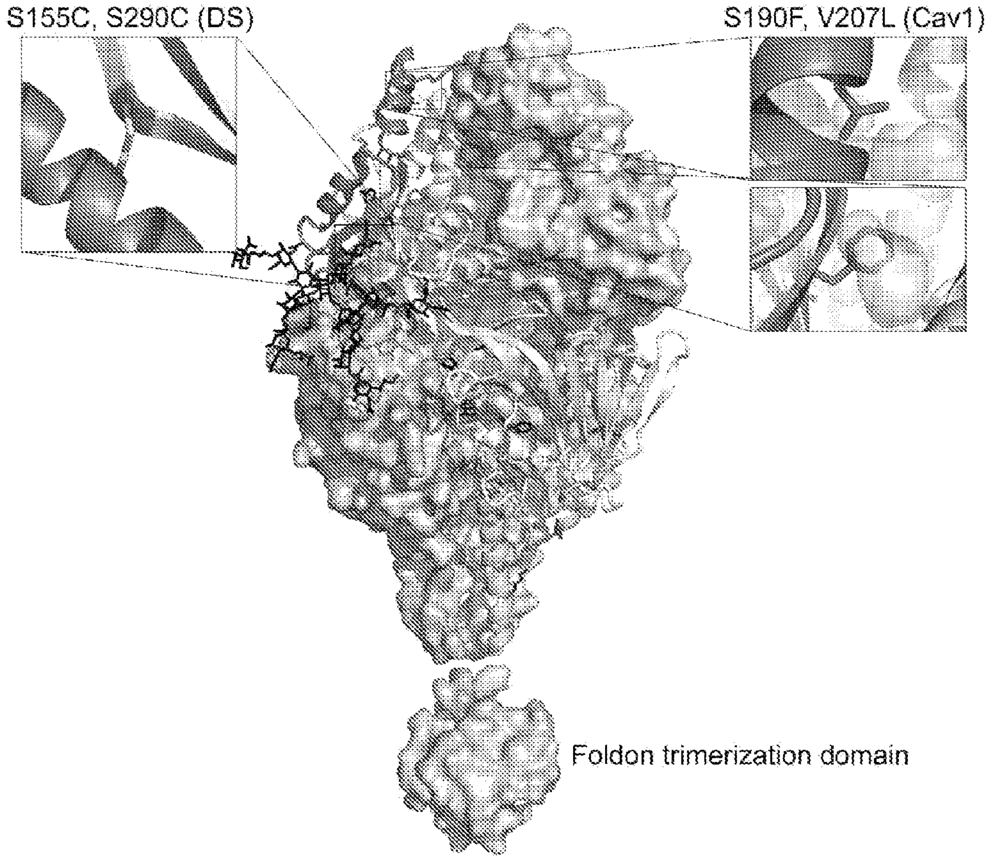
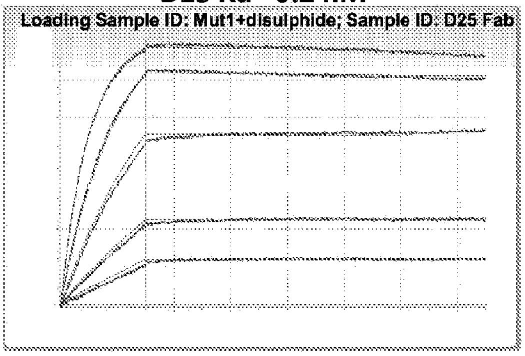
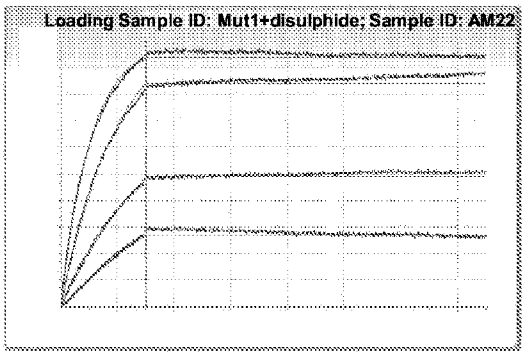

S155C, S290C (DS)
S190F, V207L (Cav1)
Foldon trimerization domain

FIG. 17

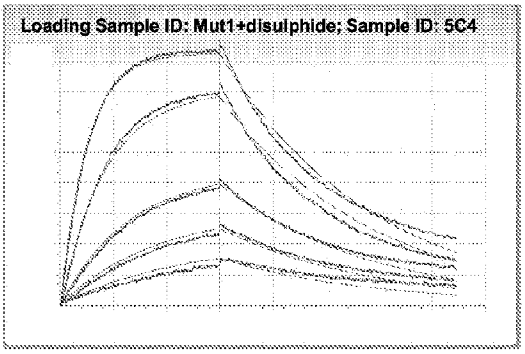
D25 Kd= 0.2 nM



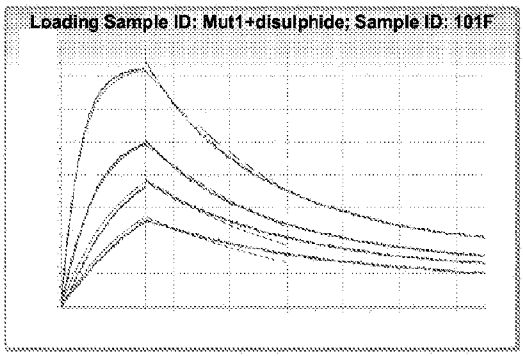
AM22 Kd< 0.01 nM



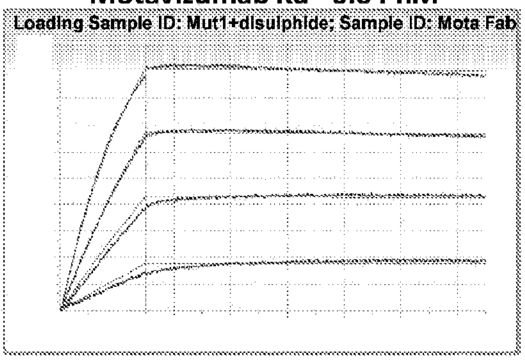
5C4 Kd= 13.2 nM



101F Kd= 3.2 nM



Motavizumab Kd= 0.04 nM



Palivizumab Kd= 23.4 nM

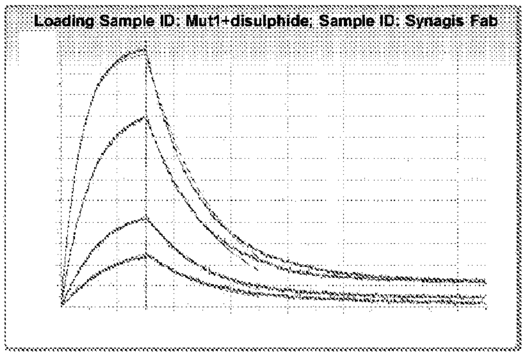


FIG. 18

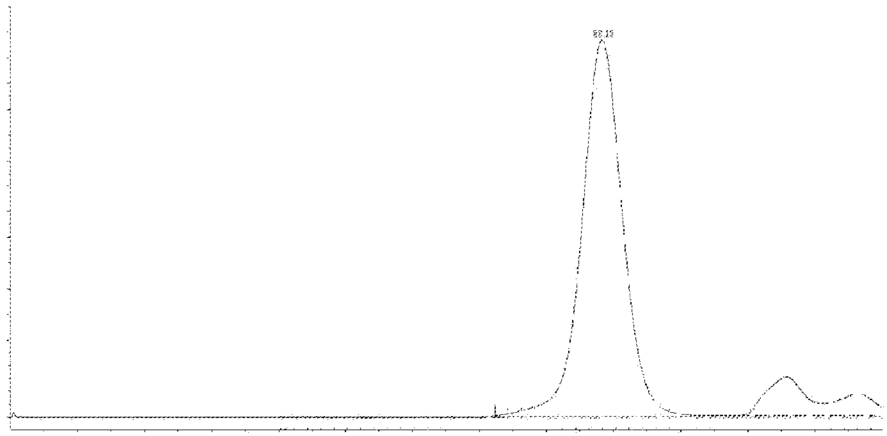


FIG. 19

RSV F variant	Yield (mg/L)*	Antibody K_D value (nM)							Physical characterization (fractional D25 reactivity)							
		Site Ø			Site I	Site II		Site IV	Temp (°)			pH		Osmolality (mM)		Freeze-Thaw
		D25	AM22	5C4	131-2a	Paliv	Mota	101F	50	70	90	3.5	10.0	10	3000	
S155C, S290C (DS)	1.4	0.3	<0.01	35.4	3.4	2.8	0.04	2.2	0.3	0	0	0.1	0.8	1.3	0.8	0.3
S190F, V207L (Cav1)	2.2	0.23	<0.10	9.31	>1000	42.90	<0.01	2.94	0.8	0.1	0.1	0.7	0.8	1.0	0.7	0.6
DSCav1	1.9	0.2	<0.01	13.2	>1000	23.4	0.04	3.2	0.9	0	0	0.8	0.9	1.0	0.8	0.7

*Yield is shown for trimeric fraction
>1000 nM = no binding at 1 μM Fab concentration

FIG. 20

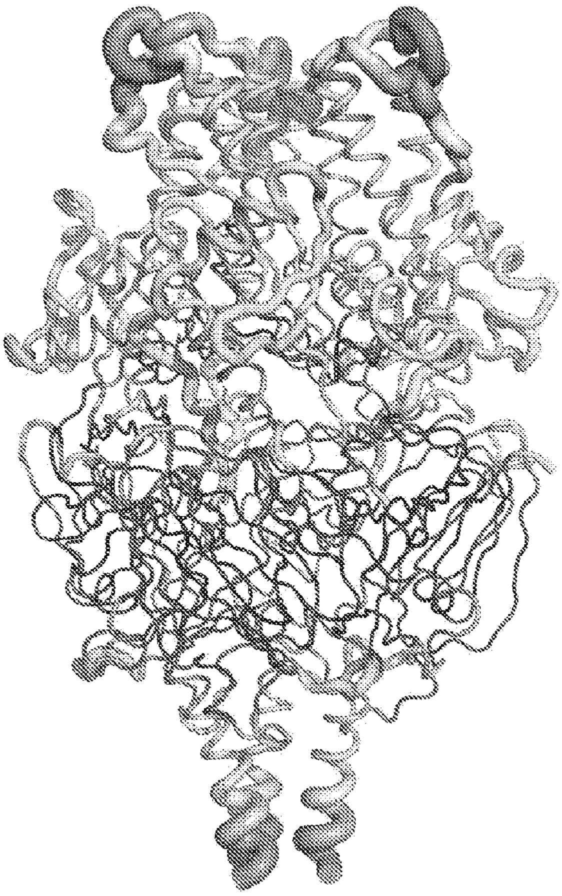


FIG. 21

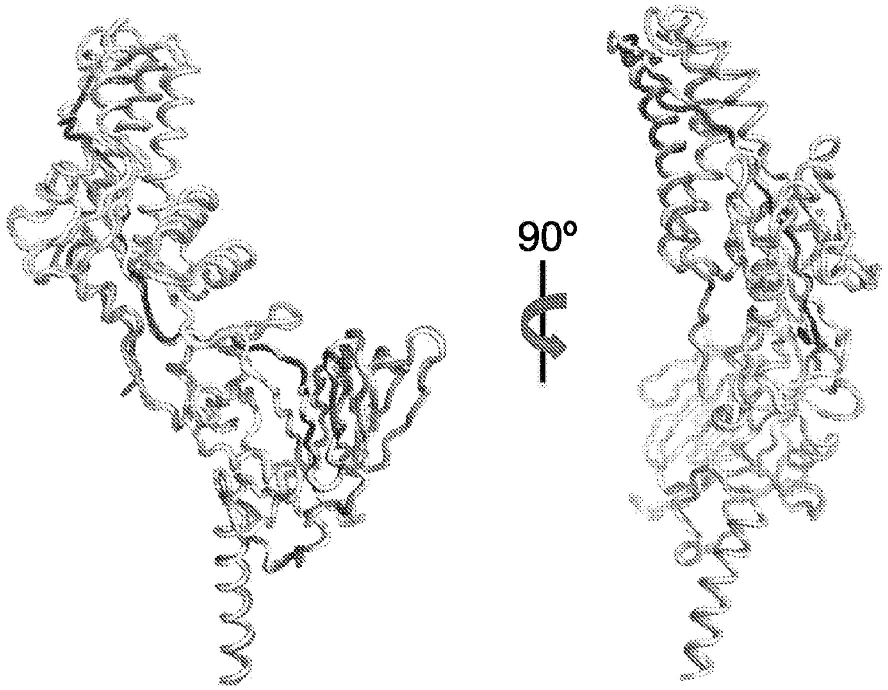


FIG. 22

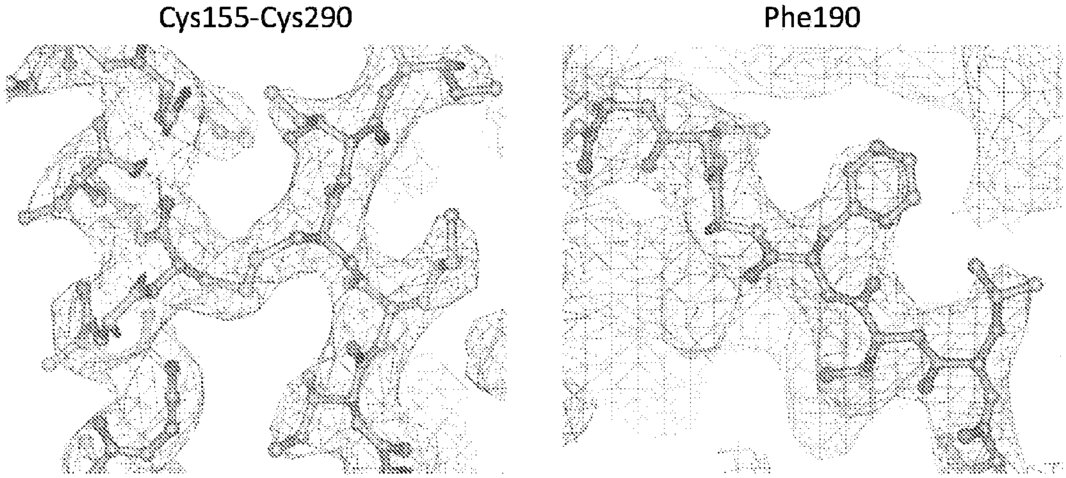


FIG. 23

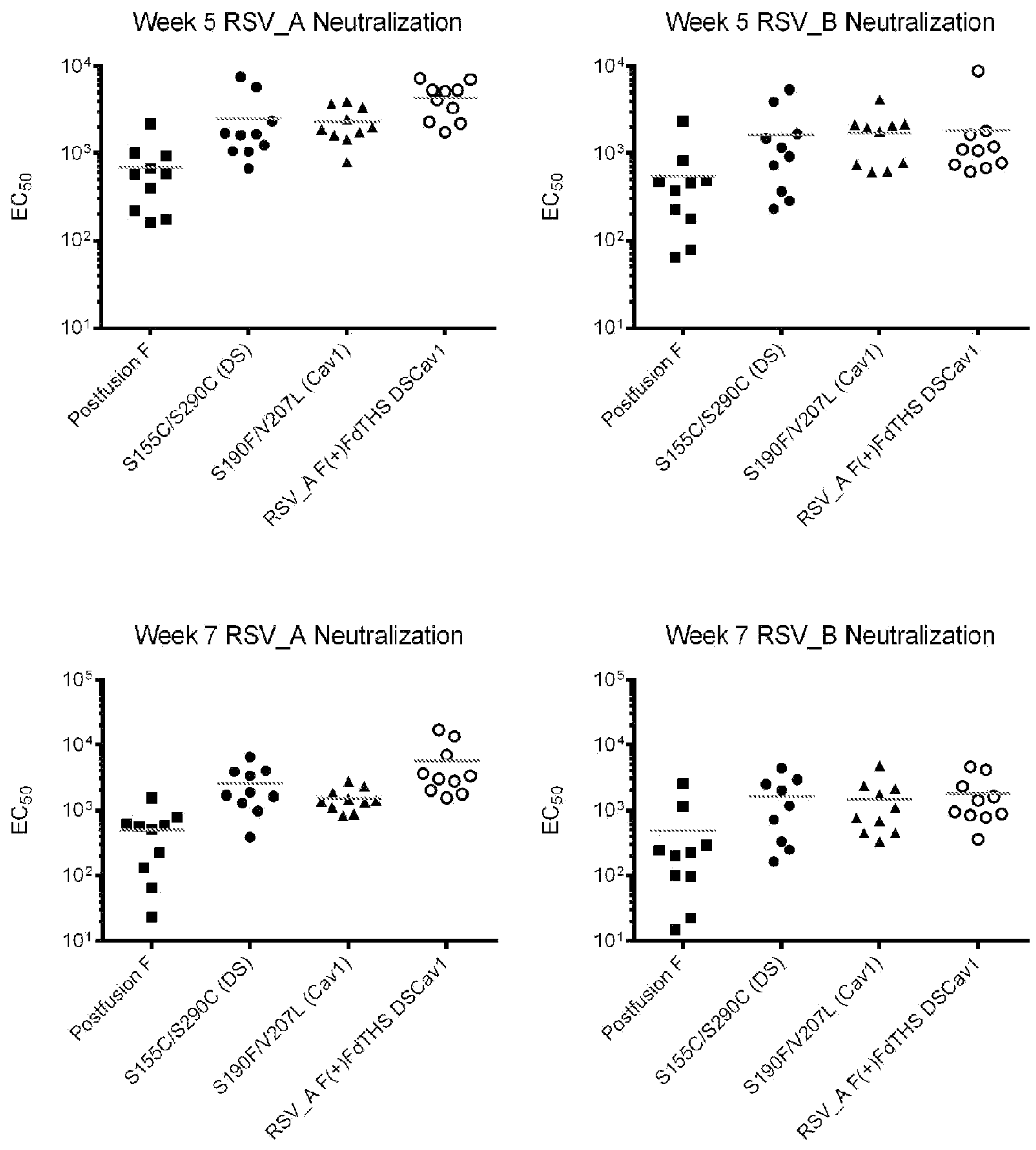

Week 5 RSV_A Neutralization
Week 5 RSV_B Neutralization
Week 7 RSV_A Neutralization
Week 7 RSV_B Neutralization
EC50
10^1
10^2
10^3
10^4
10^5
Postfusion F
S155C/S290C (DS)
S190F/V207L (Cav1)
RSV_A F(+)FdTHS DSCav1

FIG. 24

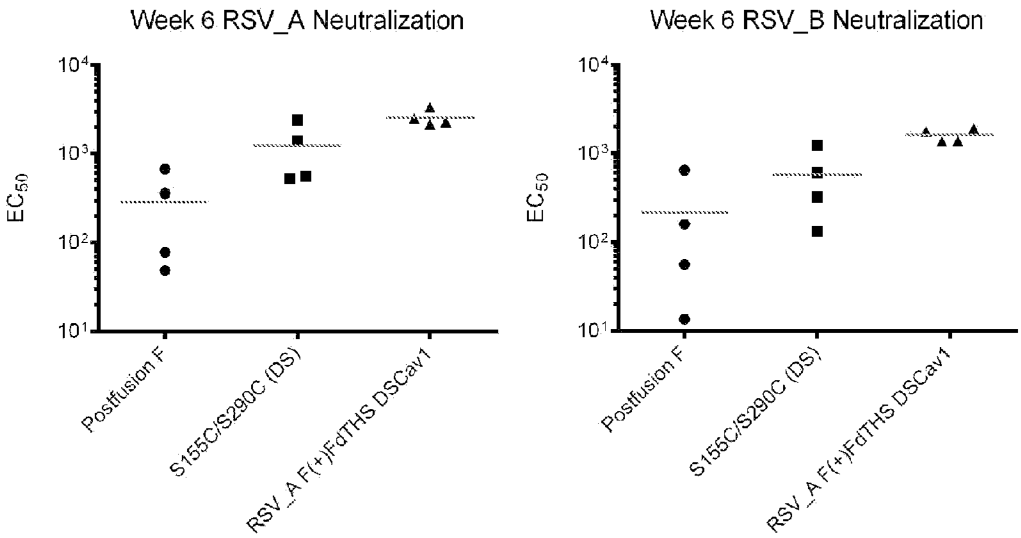


FIG. 25A

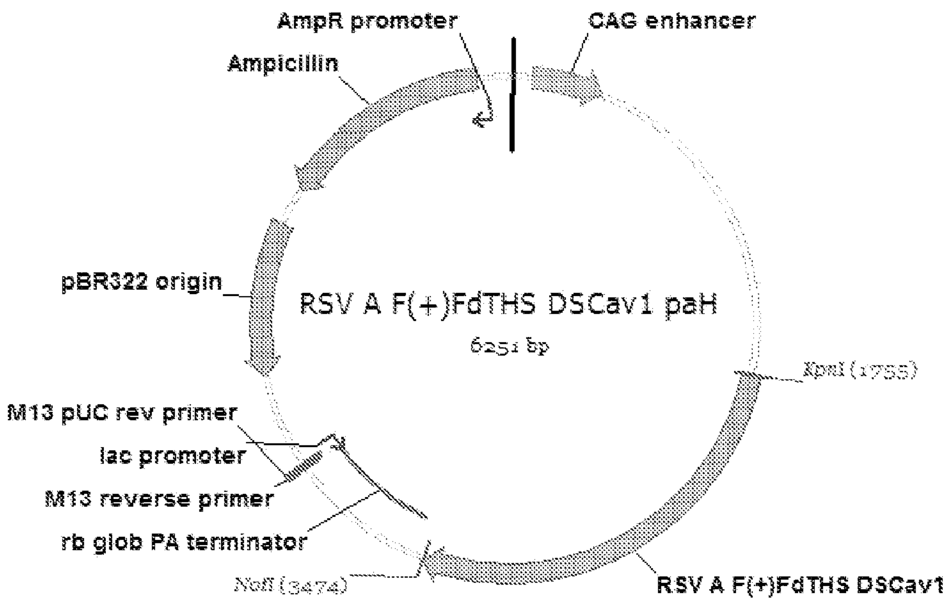


FIG. 25B

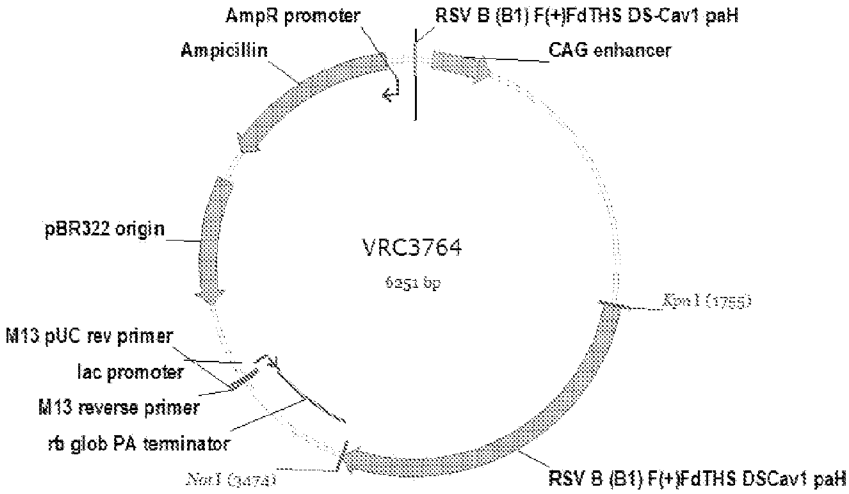


FIG. 25C

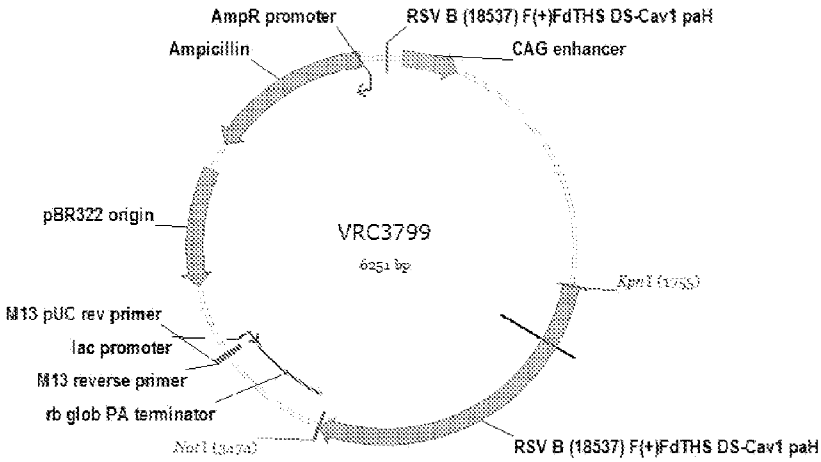


FIG. 26A

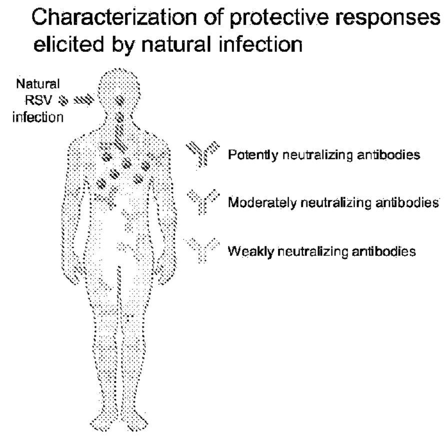
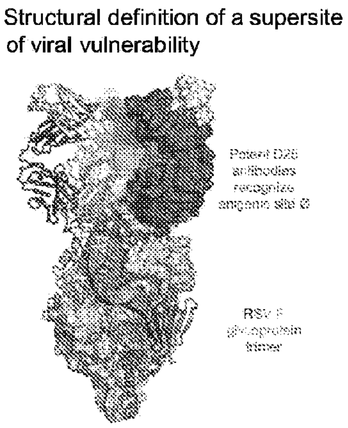


FIG. 26B



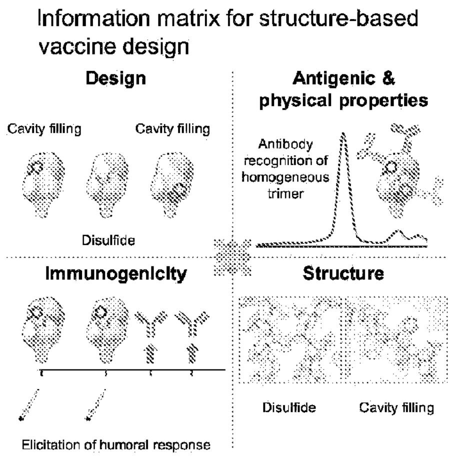


FIG. 26C

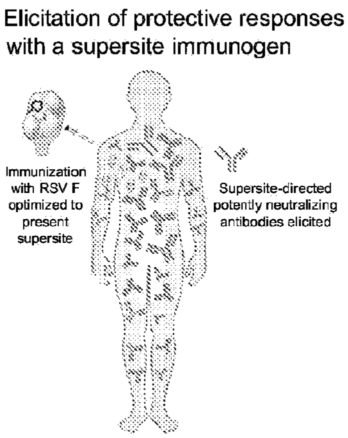


FIG. 26D

FIG. 27

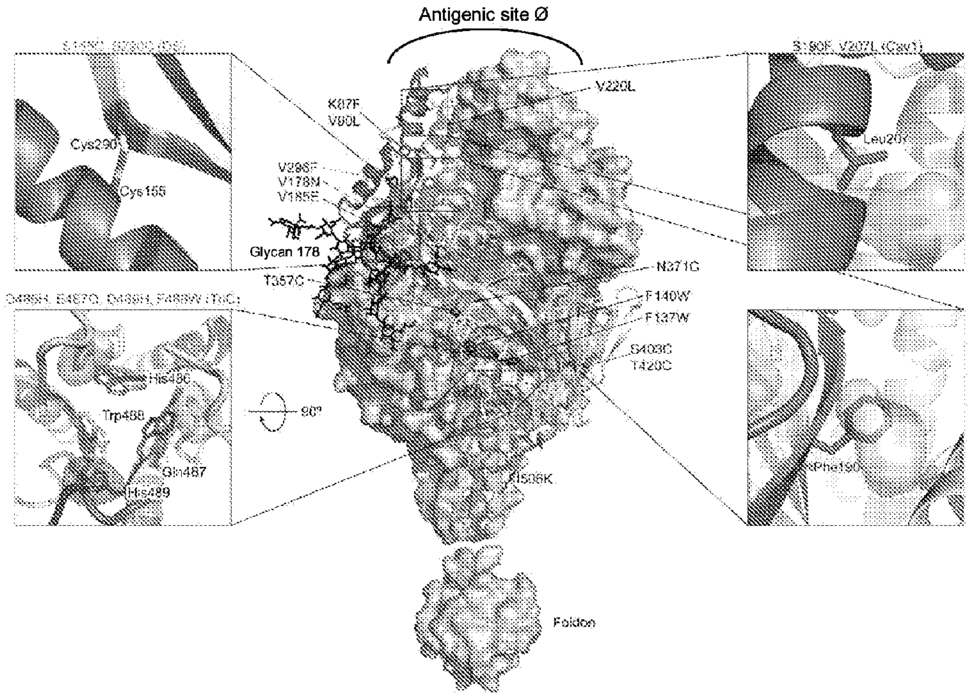

Antigenic site Ø
S190F, V207L (Cav1)
Cys290
Cys155
Leu207
K87F
V90L
V220L
V296F
V178N
V185E
Glycan 178
T357C
N371C
F140W
F137W
S403C
T420C
His486
Trp488
Gln487
His489
90°
Phe190
I506K
Foldon

FIG. 28A

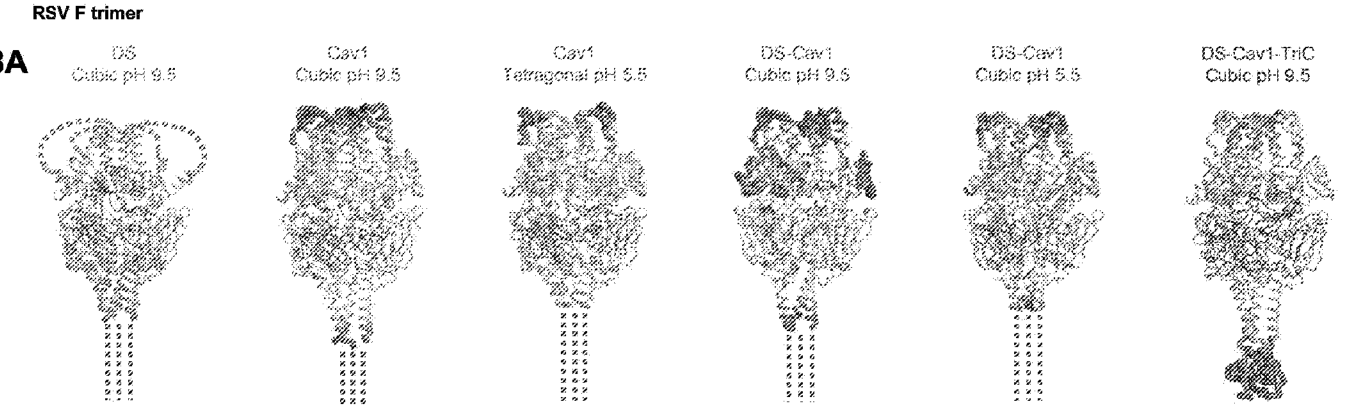


FIG. 28B

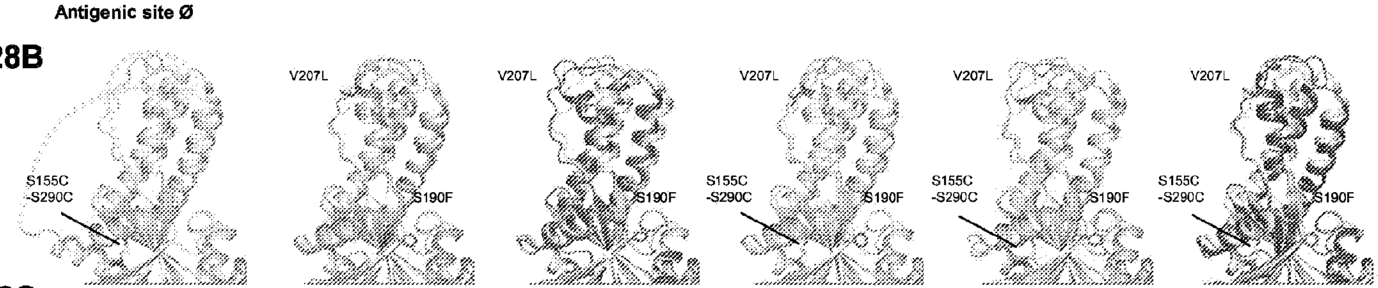


FIG. 28C

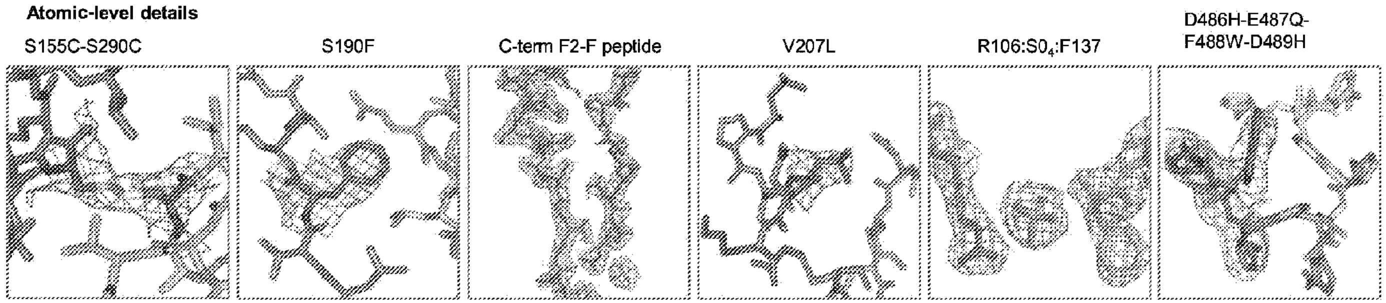


FIG. 29A

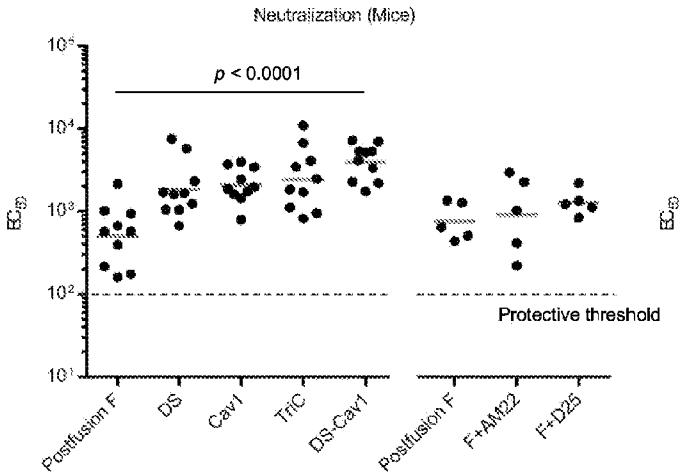


FIG. 29B

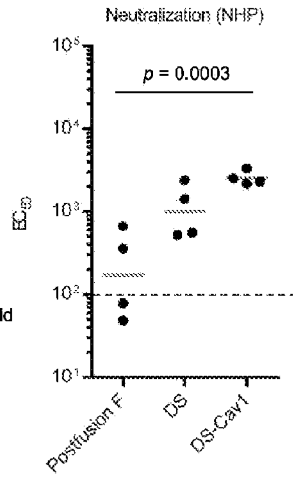


FIG. 30A

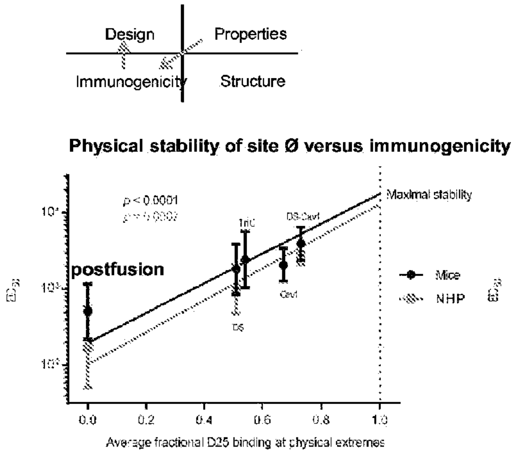
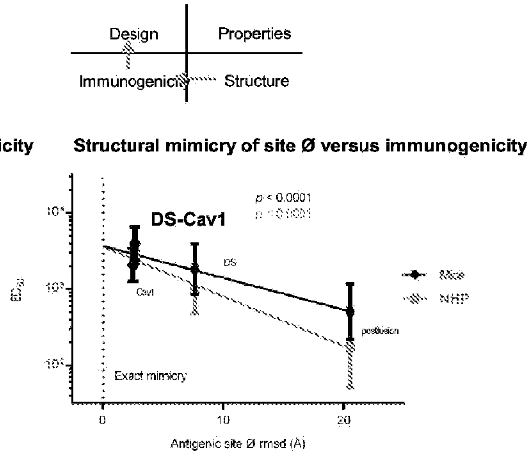


FIG. 30B



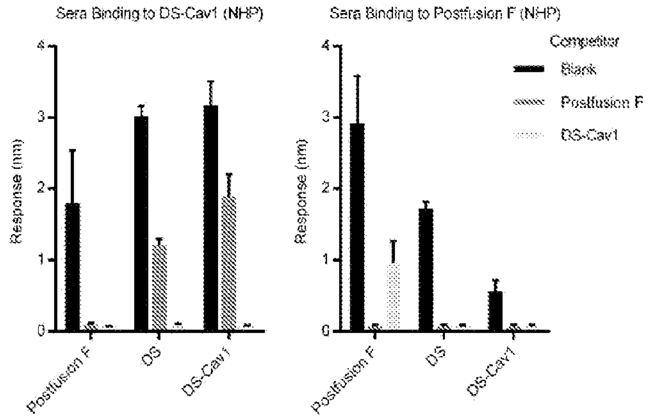


FIG. 30C

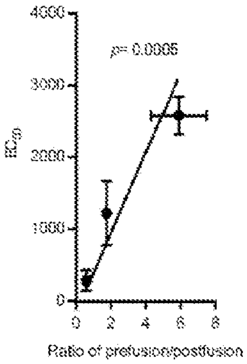


FIG. 30D

FIG. 31A

RSV F variant	Oligomeric state#	Yield (mg/L)*	Antibody K_D value (nM)						
			Site Ø			Site I	Site II		Site IV
			D25	AM22	5C4	131-2a	Paliv	Mota	101 F
K87F, V90L	Aggregate	0.3	>1000	>1000	>1000	7.6	1.68	0.17	1.57
F137W, F140W	N.D.	<0.1	N/A	N/A	N/A	N/A	N/A	N/A	N/A
F137W, F140W, F488W	N.D.	<0.1	N/A	N/A	N/A	N/A	N/A	N/A	N/A
S155C, S290C (DS)	Trimer	1.4	0.3	<0.01	35.4	3.4	2.8	0.04	2.2
V178N	Aggregate	<0.1	>1000	>1000	>1000	7.18	3.13	0.11	1.64
V185E	Aggregate	<0.1	>1000	>1000	>1000	3.53	1.70	0.11	1.64
S190F, V207L (Cav1)	Trimer	2.2	0.23	<0.10	9.31	>1000	42.90	<0.01	2.94
S190F, V296F (Cav2)	Aggregate	0.4	>1000	>1000	>1000	4.17	1.67	<0.01	1.59
V207L, V220L (Cav3)	Aggregate	0.4	>1000	>1000	>1000	2.79	0.99	0.01	0.69
T357C, N371C	N.D.	<0.1	N/A	N/A	N/A	N/A	N/A	N/A	N/A
S403C, T420C	Aggregate	0.3	>1000	>1000	>1000	3.05	3.31	0.05	1.85
D486H, E487Q, D489H	Aggregate	0.1	>1000	>1000	>1000	>1000	9.5	0.57	12.7
D486H, E487Q, D489H, F488W (TriC)	Trimer	0.8	0.01	1.0	33.6	>1000	30.7	0.5	4.0
F488W	Trimer	1.7	0.09	0.25	26.89	>1000	31.6	0.1	4.74
I506K	Aggregate	<0.1	>1000	>1000	>1000	4.55	1.71	0.05	1.39
Doubles									
DS, Cav1	Trimer	1.9	0.2	<0.01	13.2	>1000	23.4	0.04	3.2
DS, TriC	Trimer	0.6	0.17	<0.01	33		4.8	0.05	3.1
Cav1, TriC	Trimer	0.2	0.99	0.086	5.1	>1000	32.28	0.17	3.09
Triple									
DS, Cav1, TriC	Trimer	1.3	0.17	0.02	17.8	>1000	19.72	0.10	3.17

FIG. 31B

RSV F variant	Physical characterization (fractional D25 reactivity)							
	Temp (°)			pH		Osmolality (mM)		Freeze-Thaw
	50	**70**	**90**	**3.5**	**10.0**	**10**	**3000**	**10x**
K87F, V90L	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
F137W, F140W	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
F137W, F140W, F488W	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
S155C, S290C (DS)	0.3	0	0	0.1	0.8	1.3	0.8	0.3
V178N	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
V185E	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
S190F, V207L (Cav1)	0.8	0.1	0.1	0.7	0.8	1.0	0.7	0.6
S190F, V296F (Cav2)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
V207L, V220L (Cav3)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
T357C, N371C	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
S403C, T420C	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
D486H, E487Q, D489H	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
D486H, E487Q, D489H, F488W (TriC)	0.8	0.1	0.1	0.1	0.8	1.3	0.6	0.1
F488W	0.9	0.1	0	0.1	0.7	1.1	0.5	0
I506K	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Doubles								
DS, Cav1	0.9	0	0	0.8	0.9	1.0	0.8	0.7
DS, TriC	0.9	0	0	0.3	0.9	0.5	0.9	0.5
Cav1, TriC	0.9	0.1	0.1	0.3	0.8	0.6	0.5	0
Triple								
DS, Cav1, TriC	0.9	0.1	0.1	0.6	0.9	0.6	0.6	0

FIG. 32

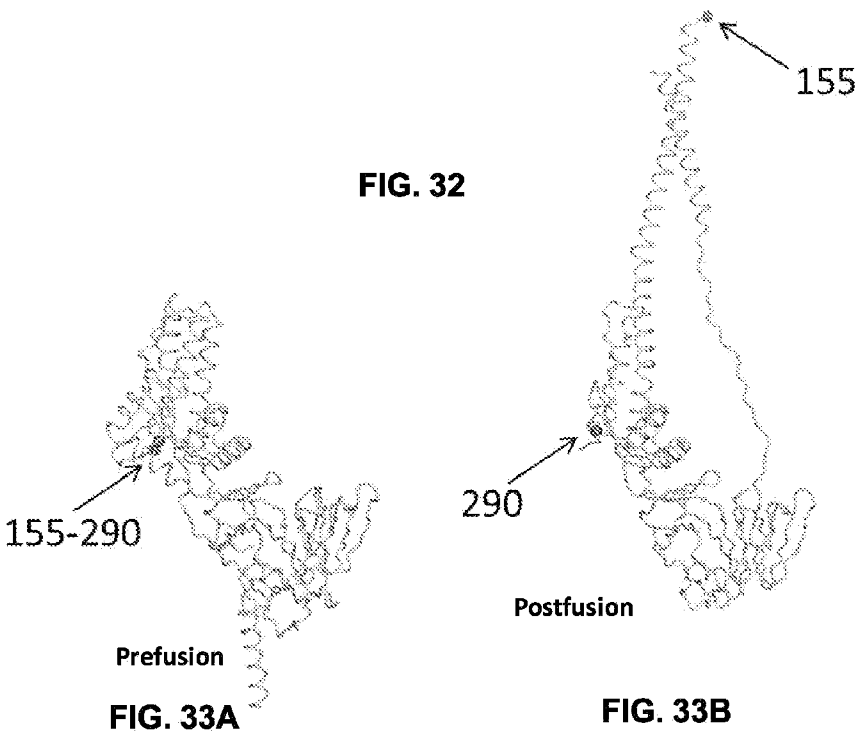


FIG. 33A

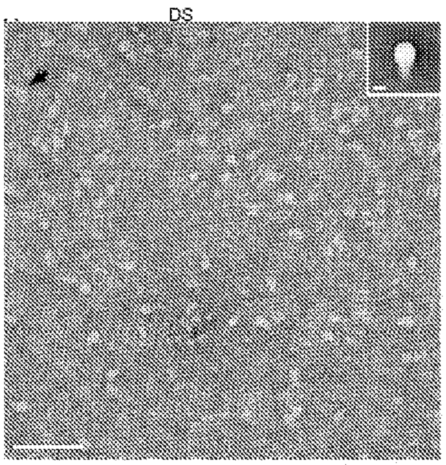


FIG. 33B

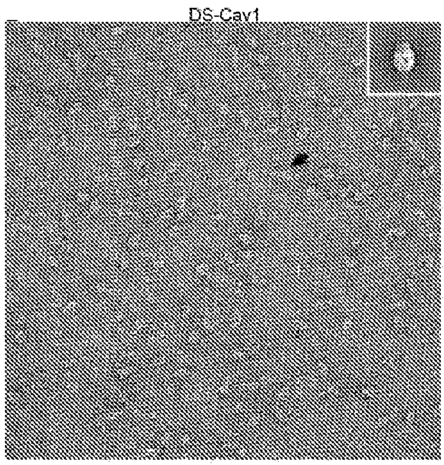


FIG. 33C

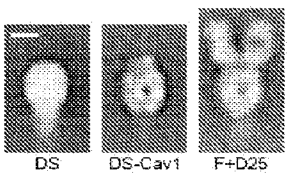


FIG. 34

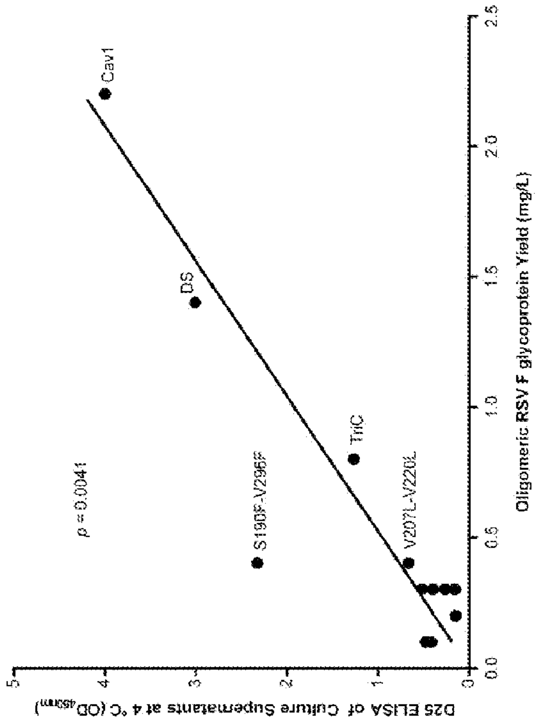
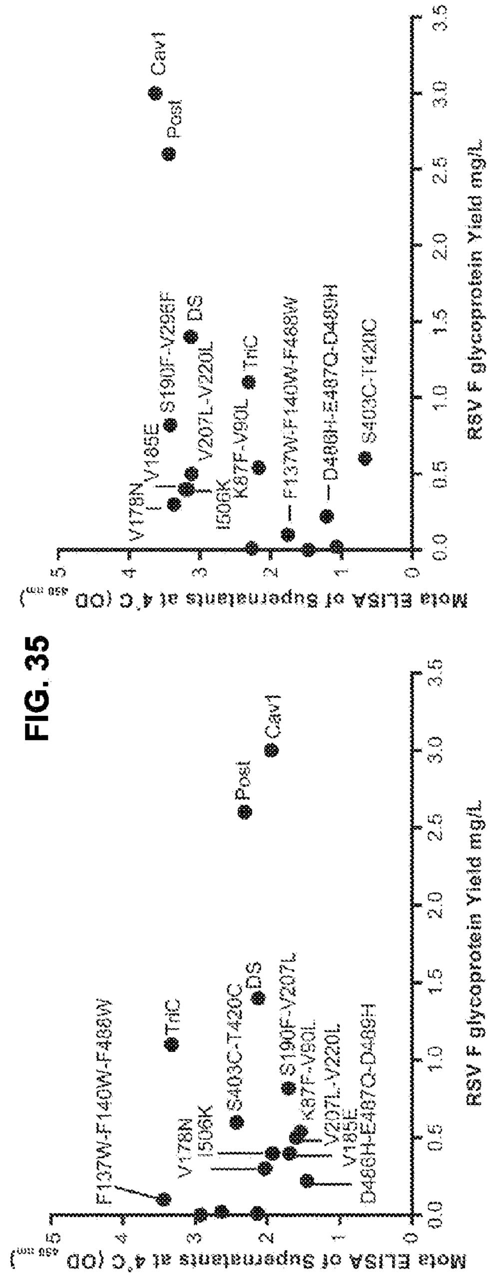

D25 ELISA of Culture Supernatants at 4 °C (OD450nm)
Oligomeric RSV F glycoprotein Yield (mg/L)
p = 0.0041
Cav1
DS
TriC
S190F-V296F
V207L-V220L

FIG. 35


Mota ELISA of Supernatants at 4°C (OD 450 nm)
RSV F glycoprotein Yield mg/L
Cav1
Post
DS
TriC
S190F-V296F
V207L-V220L
V178N
V185E
I506K
K87F-V90L
F137W-F140W-F488W
D486H-E487Q-D489H
S403C-T420C
S190F-V207L

a = Cav1
b = Cav1-TriC
c = DS-Cav1-TriC
d = F488W
e = DS-Cav1
f = TriC
g = DS-TriC
h = DS
i = S190F-V296F
j = K87F-V90L
k = V207L-V220L
l = V178N
m = S403C-T420C
n = I506K
o = V185E
p = F137W-F140W- F488W
q = D486H-E487Q-D489H

FIG. 36

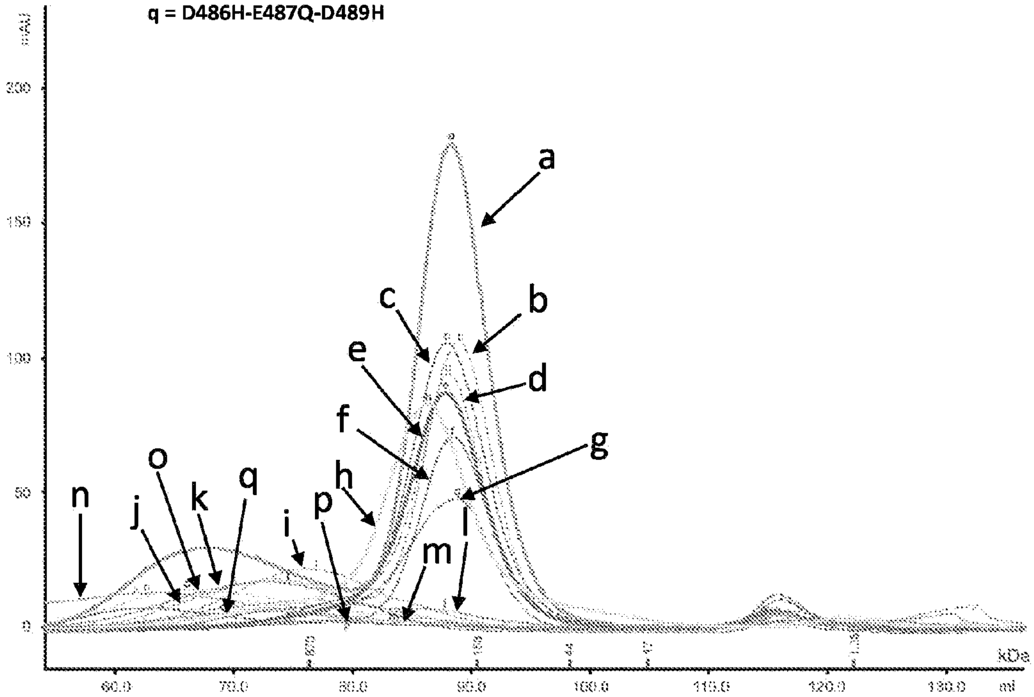


FIG. 37

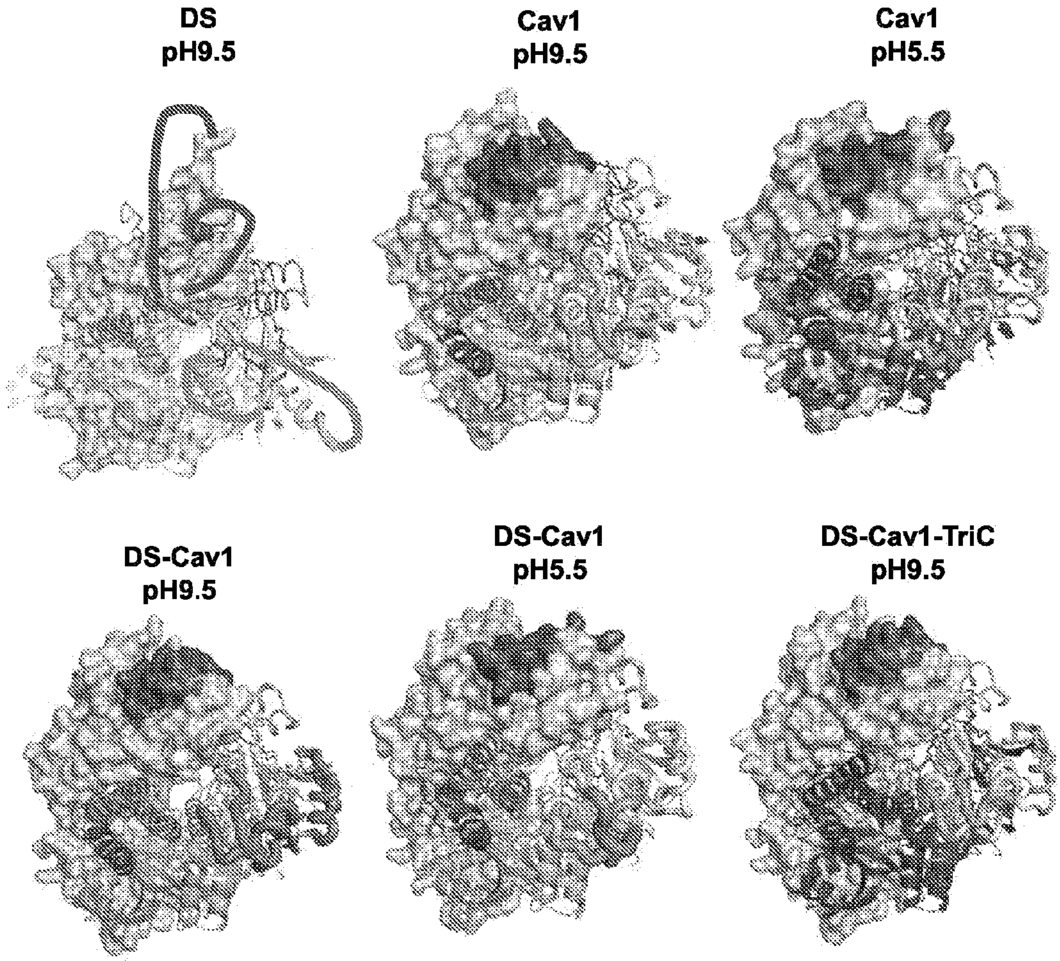

DS
pH9.5
Cav1
pH9.5
Cav1
pH5.5
DS-Cav1
pH9.5
DS-Cav1
pH5.5
DS-Cav1-TriC
pH9.5

FIG. 38A

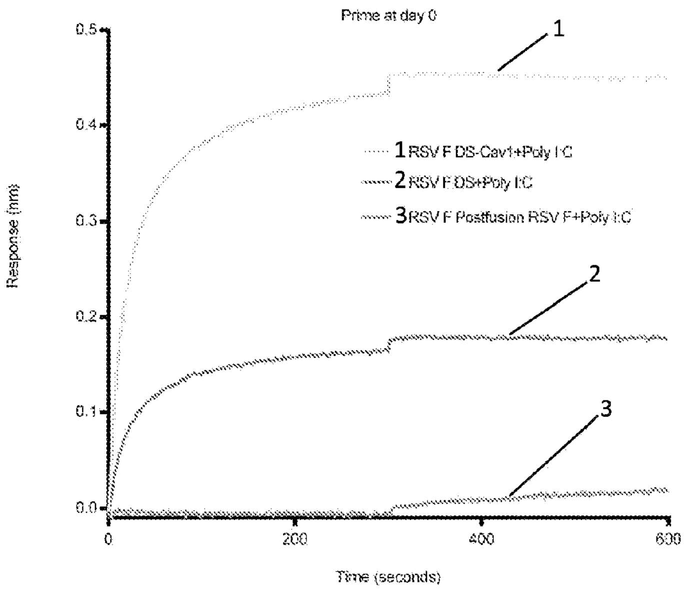


FIG. 38B

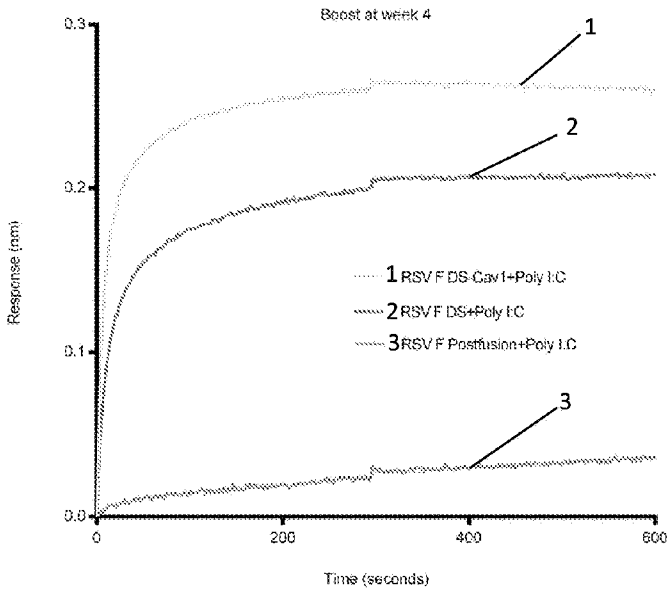
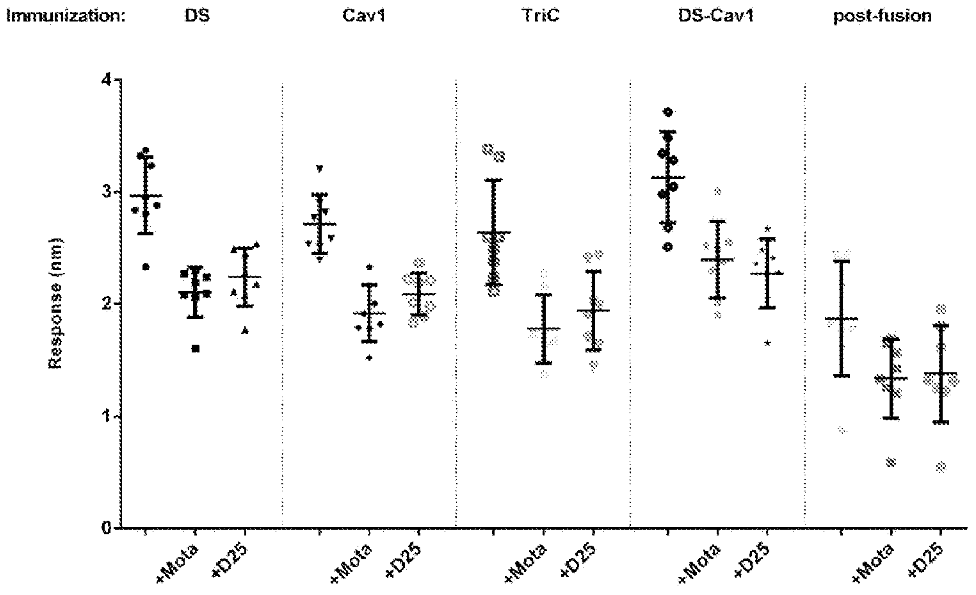


FIG. 39A



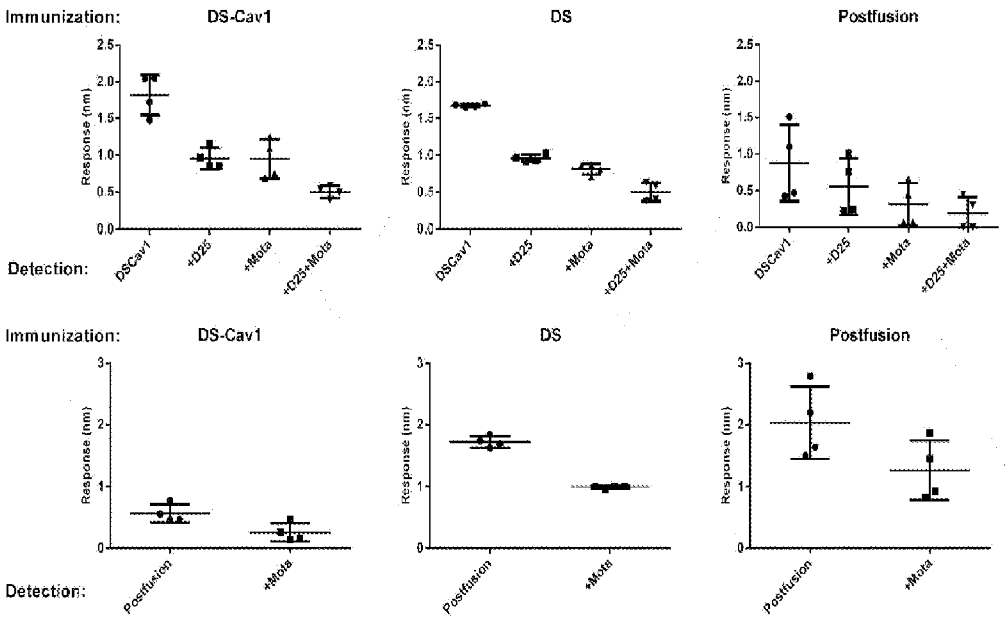


FIG. 39B

Table S1. Crystallographic data collection and refinement statistics

	DS (pH9.5)	Cav1 (pH9.5)	Cav1 (pH5.5)	DS-Cav1 (pH9.5)	DS-Cav1 (pH5.5)	DS-Cav1-TriC (pH9.5)
Data collection						
Space group	$P4_132$	$P4_132$	$P4_12_12$	$P4_132$	$P4_132$	$P4_132$
Cell constants						
a, b, c (Å)	168.4, 168.4, 168.4	170.9, 170.9, 170.9	170.7, 170.7, 163.9	168.6, 168.6, 168.6	168.6, 168.6, 168.6	170.4, 170.4, 170.4
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Wavelength (Å)	1.00	1.00	1.00	1.00	1.00	1.00
Resolution (Å)	50-3.25 (3.31-3.25)	50-3.1 (3.21-3.1)	50-2.40 (2.49-2.40)	50-3.85 (3.99-3.85)	50-3.0 (3.11-3.0)	50-2.8 (2.90-2.80)
R_{merge}	14.8 (64.3)	20.5 (70.7)	11.1 (59.8)	21.4 (95.3)	13.0 (79.3)	13.0 (92.6)
$I/\sigma I$	14.8 (2.2)	11.8 (1.5)	12.6 (2.1)	9.1 (1.2)	22.4 (2.2)	42.9 (1.8)
Completeness (%)	99.6 (96.4)	99.1 (91.7)	95.6 (97.7)	98.5 (89.9)	99.8 (98.1)	99.9 (99.4)
Redundancy	8.3 (4.1)	11.7 (3.8)	4.4 (4.0)	8.1 (4.3)	16.3 (7.5)	12.6 (6.2)
Refinement						
Resolution (Å)	3.25	3.10	2.40	3.85	3.00	2.80
Unique reflections	13,345	15,925	90,779	8,287	16,999	21,005
R_{work}, R_{free} (%)	23.7, 27.4	20.6, 23.7	18.7, 21.4	24.7, 28.6	18.6, 23.9	22.5, 25.9
No. atoms						
Protein	3033	3546	10,421	3523	3526	3771
Ligand/ion	30	-	70	-	156	-
Water	0	39	522	-	108	69
B-factors (Å^2)						
Protein	75.1	114.9	56.4	198.7	76.3	106.9
Ligand/ion	136.4	-	101.5	-	155.8	-
Water	-	87.6	48.5	-	63.4	80.0
R.m.s. deviations						
Bond lengths (Å)	0.008	0.012	0.003	0.011	0.010	0.005
Bond angles (°)	1.35	1.24	0.84	1.67	1.42	0.93
Ramachandran						
Favored regions (%)	95.8	95.3	95.1	93.4	93.3	96.2
Allowed regions (%)	3.9	4.3	4.5	5.3	6.0	3.3
Disallowed regions (%)	0.3	0.4	0.4	1.3	0.7	0.5

Values in parentheses are for highest-resolution shell

FIG. 40

FIG. 41

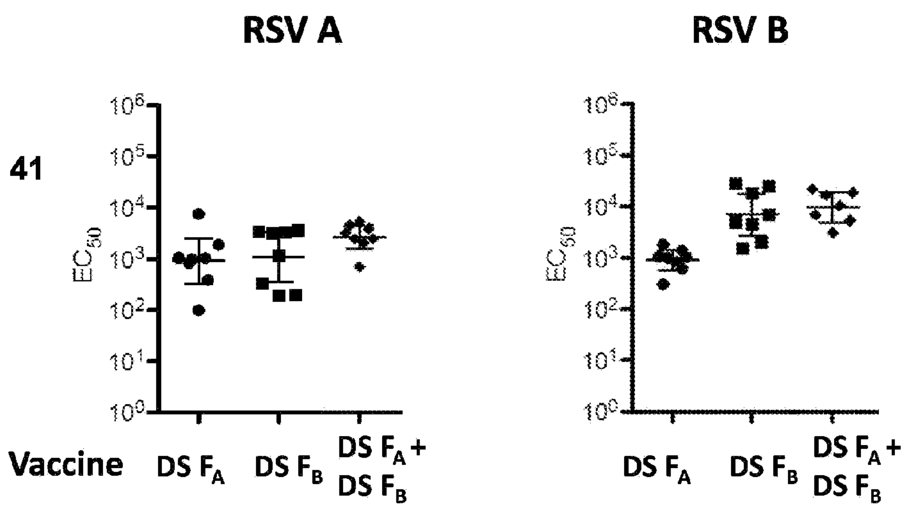

RSV A
RSV B
10^6
10^5
10^4
10^3
10^2
10^1
10^0
EC_{50}
Vaccine
DS F_A
DS F_B
DS F_A + DS F_B

FIG. 42

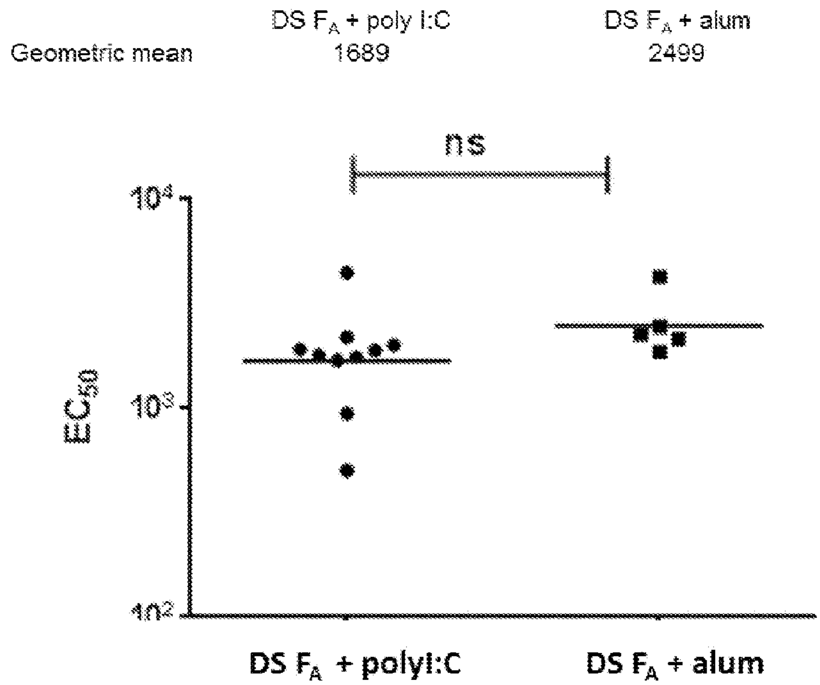

DS F_A + poly I:C
DS F_A + alum
Geometric mean
1689
2499
ns
10^4
10^3
10^2
EC_{50}
DS F_A + polyI:C
DS F_A + alum

FIG. 43

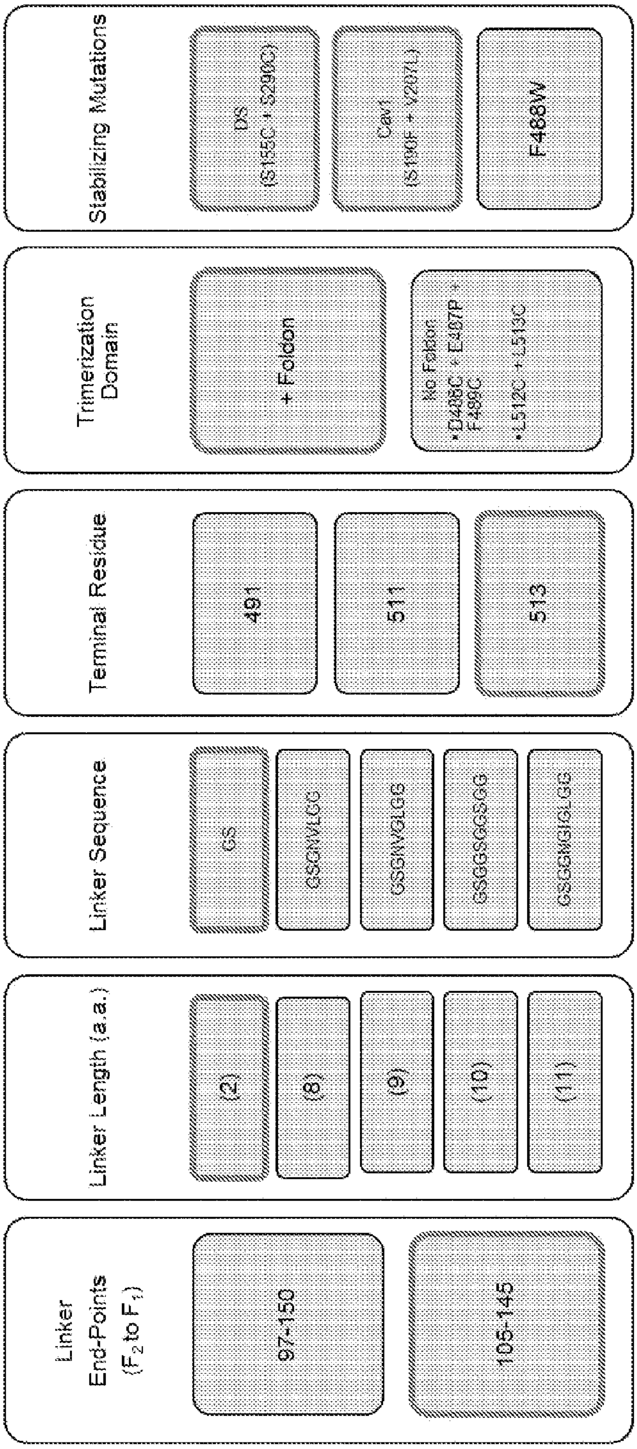
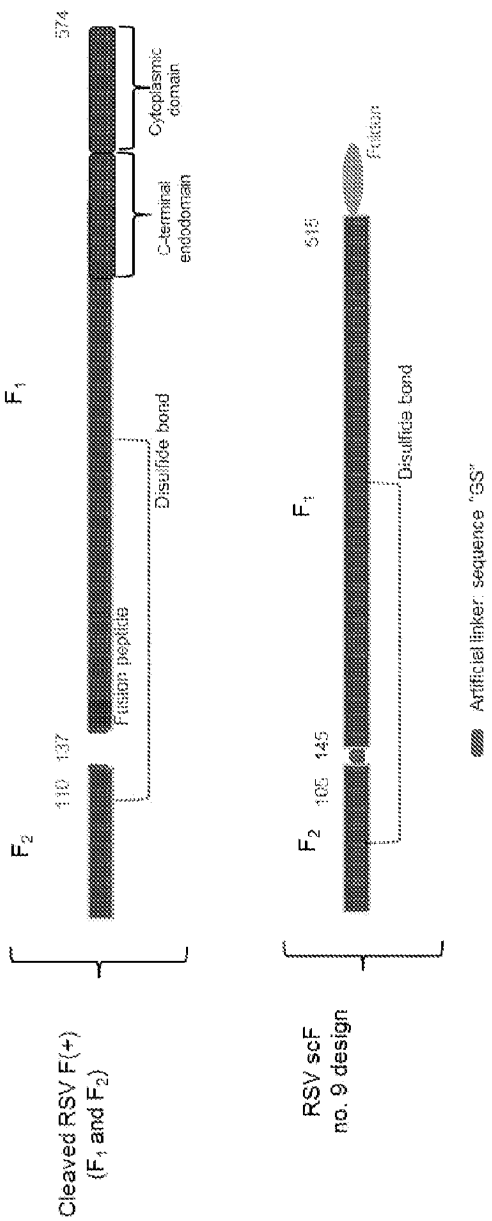

Linker End-Points (F_2 to F_1)
97-150
105-145
Linker Length (a.a.)
(2)
(8)
(9)
(10)
(11)
Linker Sequence
GS
GSGNVLGG
GSGNVGLGG
GSGGSGGSGG
GSGGNGIGLGG
Terminal Residue
491
511
513
Trimerization Domain
+ Foldon
No Foldon
• D486C + E487P + F489C
• L512C + L513C
Stabilizing Mutations
DS (S155C + S290C)
Cav1 (S190F + V207L)
F488W

FIG. 44A



Cleaved RSV F(+)
(F_1 and F_2)
F_2
110
137
F_1
Fusion peptide
Disulfide bond
C-terminal endodomain
Cytoplasmic domain
574
RSV scF
no. 9 design
F_2
105
145
F_1
Disulfide bond
515
Foldon
Artificial linker: sequence "GS"

FIG. 44B

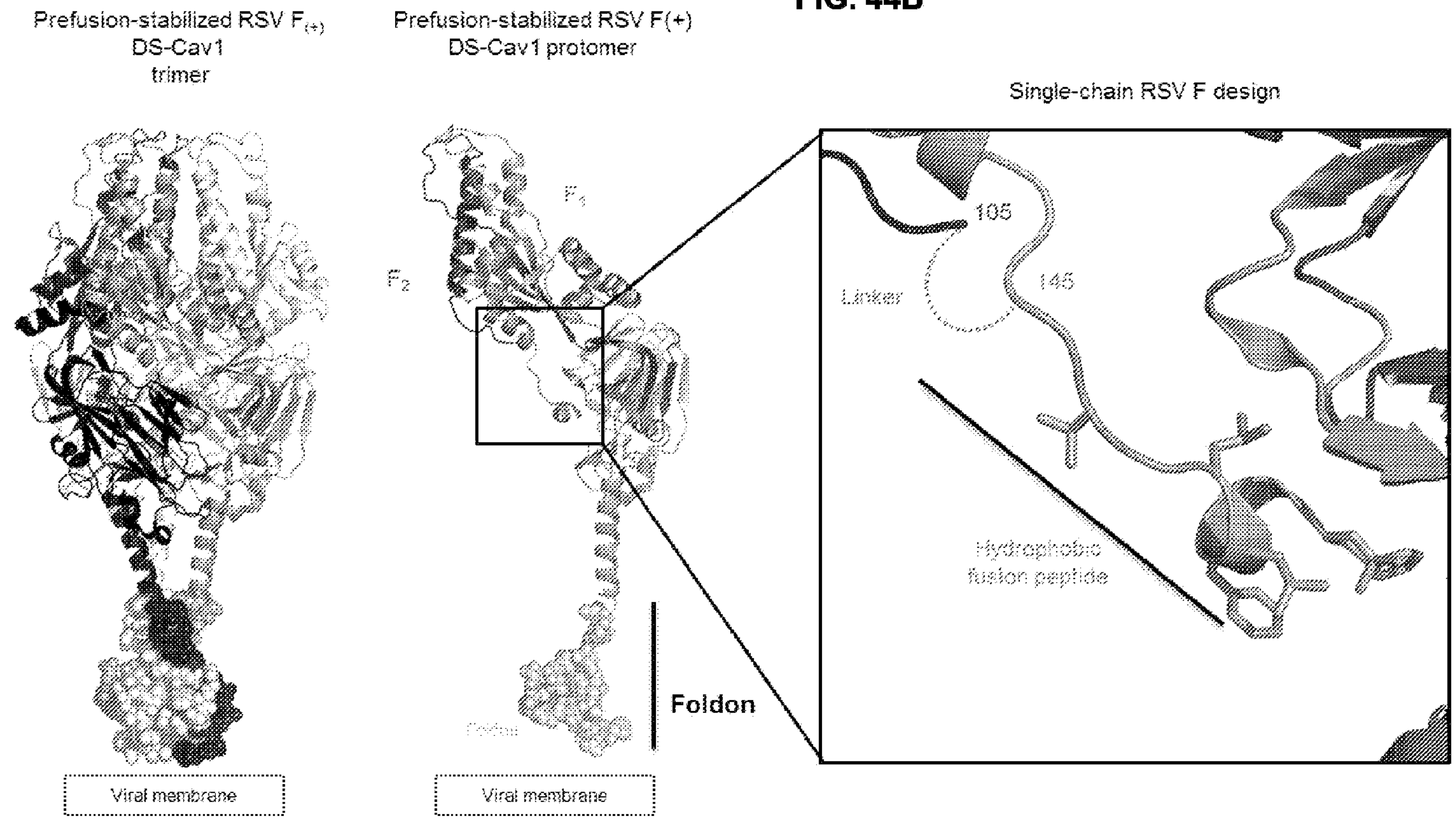

Prefusion-stabilized RSV $F_{(+)}$
DS-Cav1
trimer
Prefusion-stabilized RSV F(+)
DS-Cav1 protomer
Single-chain RSV F design
F_1
F_2
105
145
Linker
Hydrophobic
fusion peptide
Foldon
Viral membrane
Viral membrane

FIG. 45

	Prefusion Stabilization		Linker length				Production	
Construct	Variant	Trimerization domain	C-terminal residue	Linker end points	Linker length	Linker sequence	Oligomeric state	Yield (mg/L)
3	DS-Cav1	Foldon	513	97-150	9	GSGNIGLGG	Trimer	2.8
4	DS		513	97-150	11	GSGGNGIGLGG	N.D.	N.D.
	DS-Cav1		513	97-150	11	GSGGNGIGLGG	Trimer	3.4
	a		513	97-150	11	GSGGNGIGLGG	N.D.	N.D.
6	DS		513	97-150	8	GSGNVLGG	N.D.	N.D.
	DS-Cav1		513	97-150	8	GSGNVLGG	Trimer	1.0
7	DS	CPC	491	97-150	9	GSGNVGLGG	N.D.	N.D>
	Cav1	CPC	491	97-150	9	GSGNVGLGG	Aggregate	0.45
	DS-Cav1	CPC	491	97-150	9	GSGNVGLGG	Aggregate	0.3
8	DS	CC	511	97-150	9	GSGNVGLGG	Monomer	0.6
					9		Trimer	0.5
	DS-Cav1	CC	511	97-150	9	GSGNVGLGG	Monomer	0.8
					2		Trimer	1.0
	DS-Cav1	None	511	97-150	2	GSGNVGLGG	Monomer	0.5
					2		Trimer	0.8
9	DS-Cav1	Foldon	513	105-145	2	GS	Trimer	3.1
	a	Foldon	513	105-145	2	GS	Trimer	4.8
	DS	Foldon	513	105-145	2	GS	Trimer	4.3
	Cav1	Foldon	513	105-145	2	GS	Trimer	5.8
10	DS	CPC	491	105-145	2	GS	Monomer	0.3
							Trimer	0.4
	DS-Cav1	CPC	491	105-145	2	GS	Monomer	0.5
							Trimer	0.5
11	DS	CC	513	105-145	2	GS	Monomer	0.8
	Cav1	CC	513	105-145	2	GS	Monomer	1.0

FIG. 46A

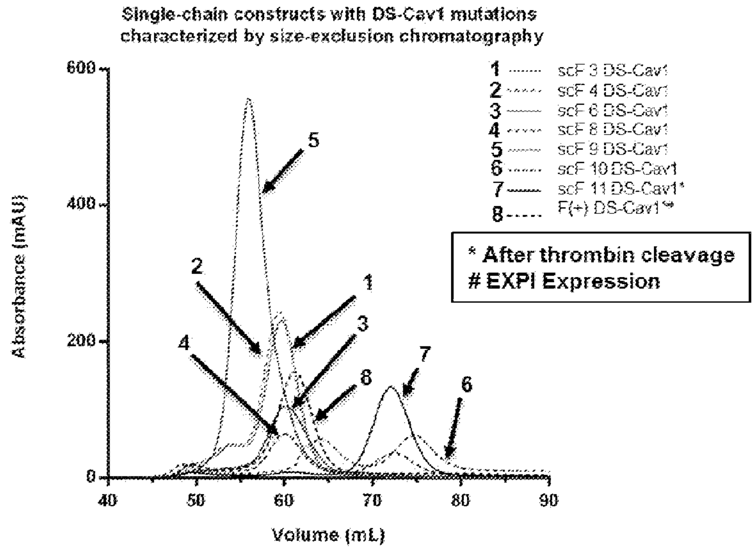


FIG. 46B

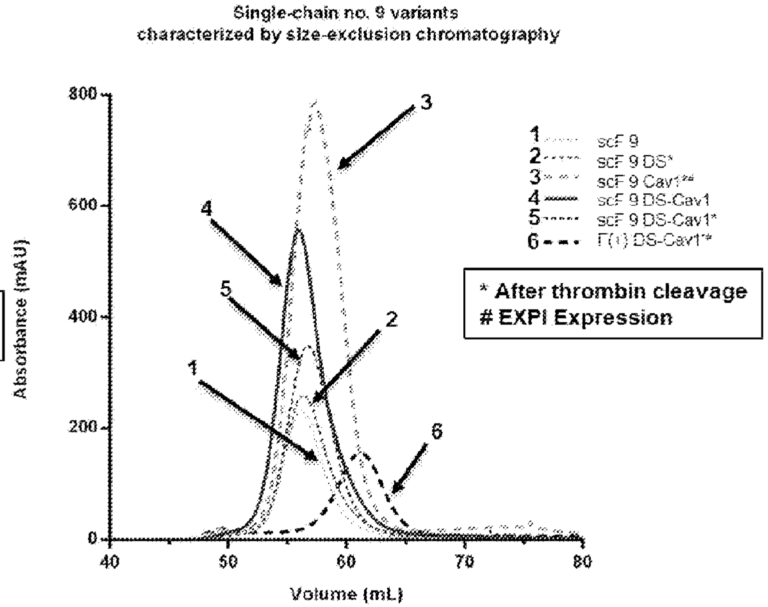


FIG. 47

		Antibody K_D value (nM)					
		Site Ø			Site II[$]		Site IV
Construct	Variant	D25	AM22	5C4	Paliv	Mota	101F
F(+)	DS-Cav1	0.15	<0.01	13	23	0.04	3.2
scF no. 9	DS-Cav1	0.06	0.1	6.0	4.0	8.9	2.7

$ Palivizumab (Paliv); Motavizumab (Mota).

FIG. 48

Data collection and refinement	Single-chain RSV F no. 9 DS-Cav1
Space group	P 4_1 3 2
Resolution (Å)	50-3.21 (3.36-3.21)
I / σI	2.84
No. reflections	521334
Unique reflections	76368
Completeness (%)	98
Average redundancy	4.3
R_{work} / R_{free} (%)	19.3 / 23.9

R = Σhkl||Fobs| − |Fcalc||/Σhkl|Fobs|.
R_{free} is calculated from 5% of the reflections excluded from refinement.

FIG. 49A


Antigenic site Ø
"GS" linker
Ser 106
Gly 145
Asn460
Gln462
Lys461
Adjacent protomer
Viral membrane

FIG. 49B

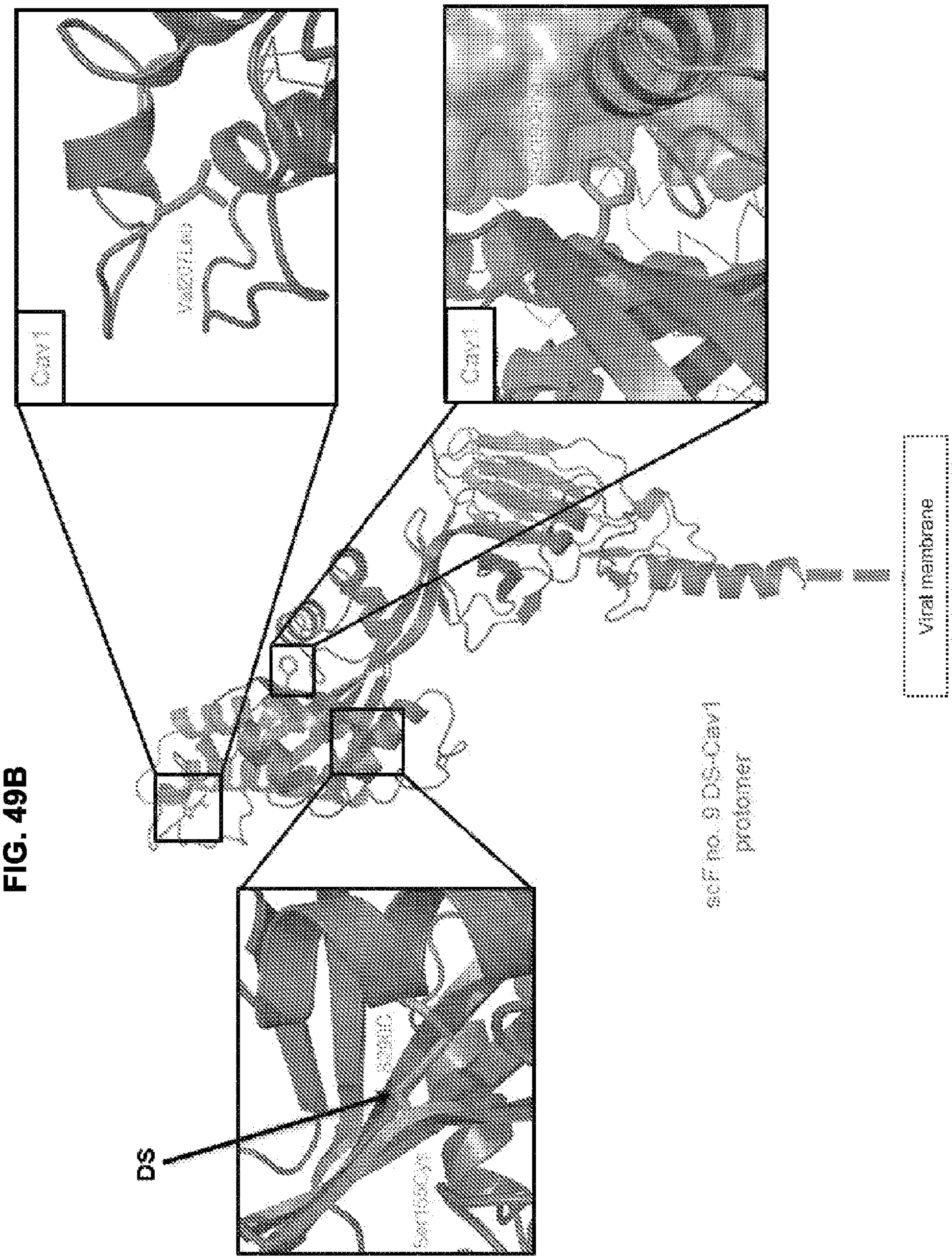


FIG. 50

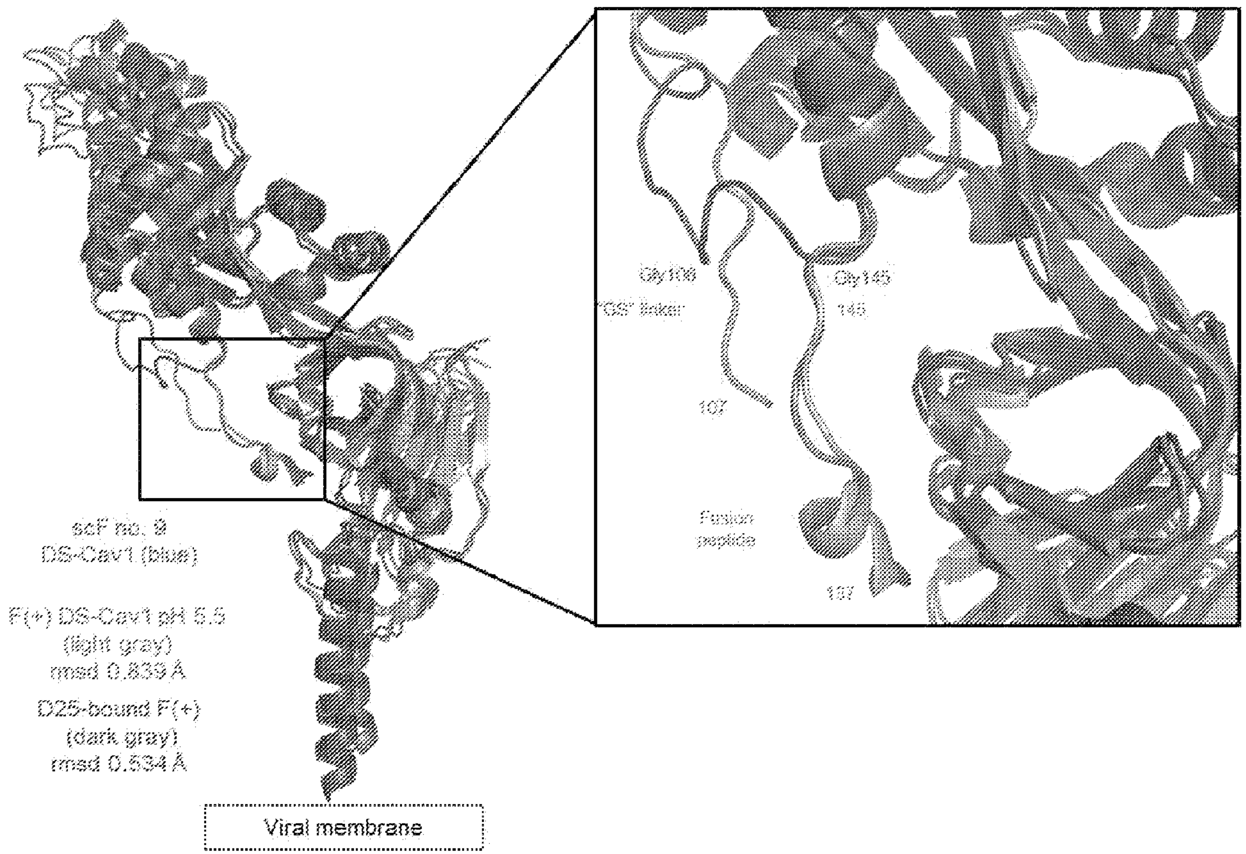

Gly106
"GS" linker
107
Gly145
145
Fusion
peptide
137
scF no. 9
DS-Cav1 (blue)
F(+) DS-Cav1 pH 5.5
(light gray)
rmsd 0.839 Å
D25-bound F(+)
(dark gray)
rmsd 0.534 Å
Viral membrane

FIG. 51

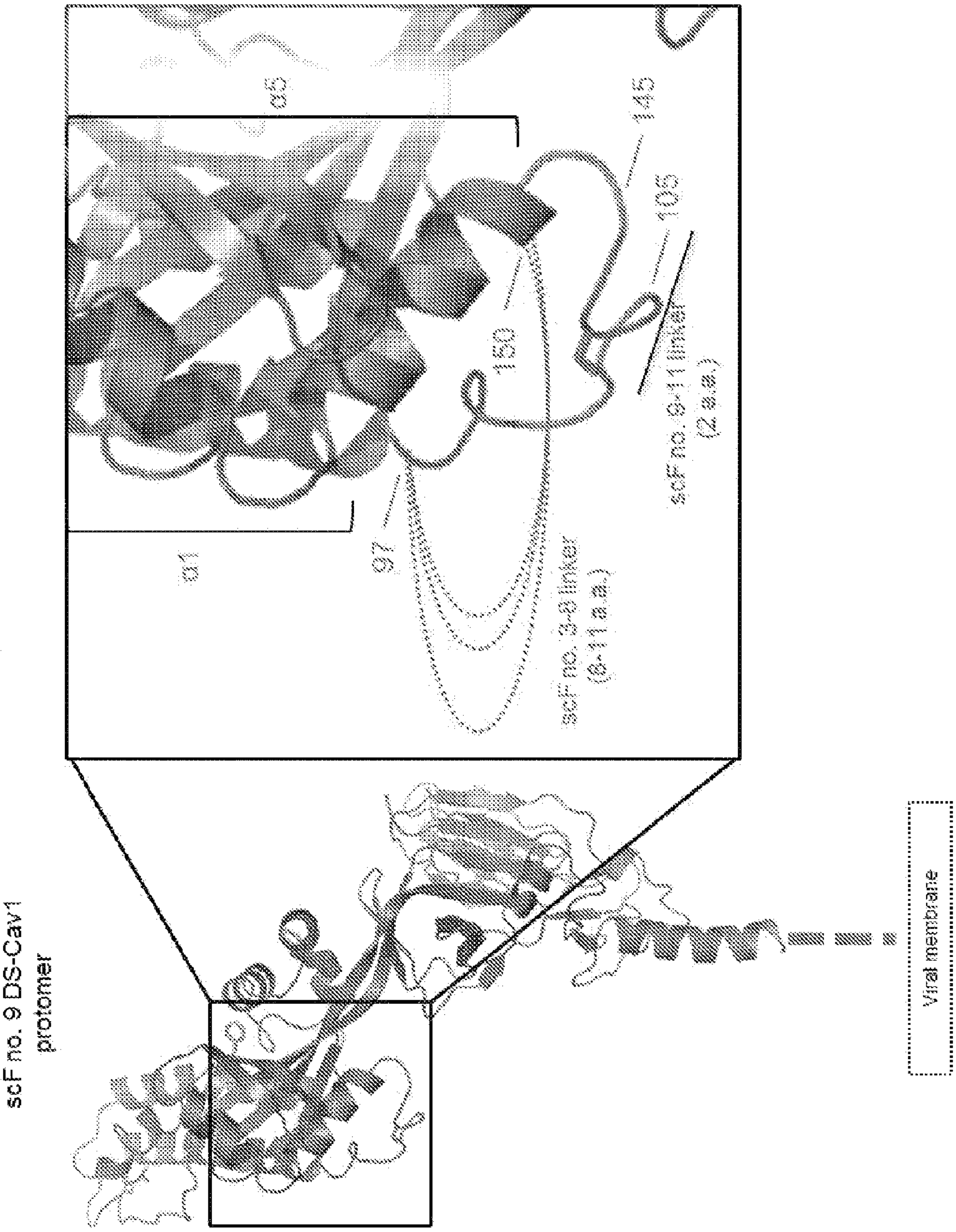

scF no. 9 DS-Cav1
protomer
d1
d5
97
150
145
105
scF no. 3-9 linker
(6-11 a.a.)
scF no. 9-11 linker
(2 a.a.)
Viral membrane

FIG. 52

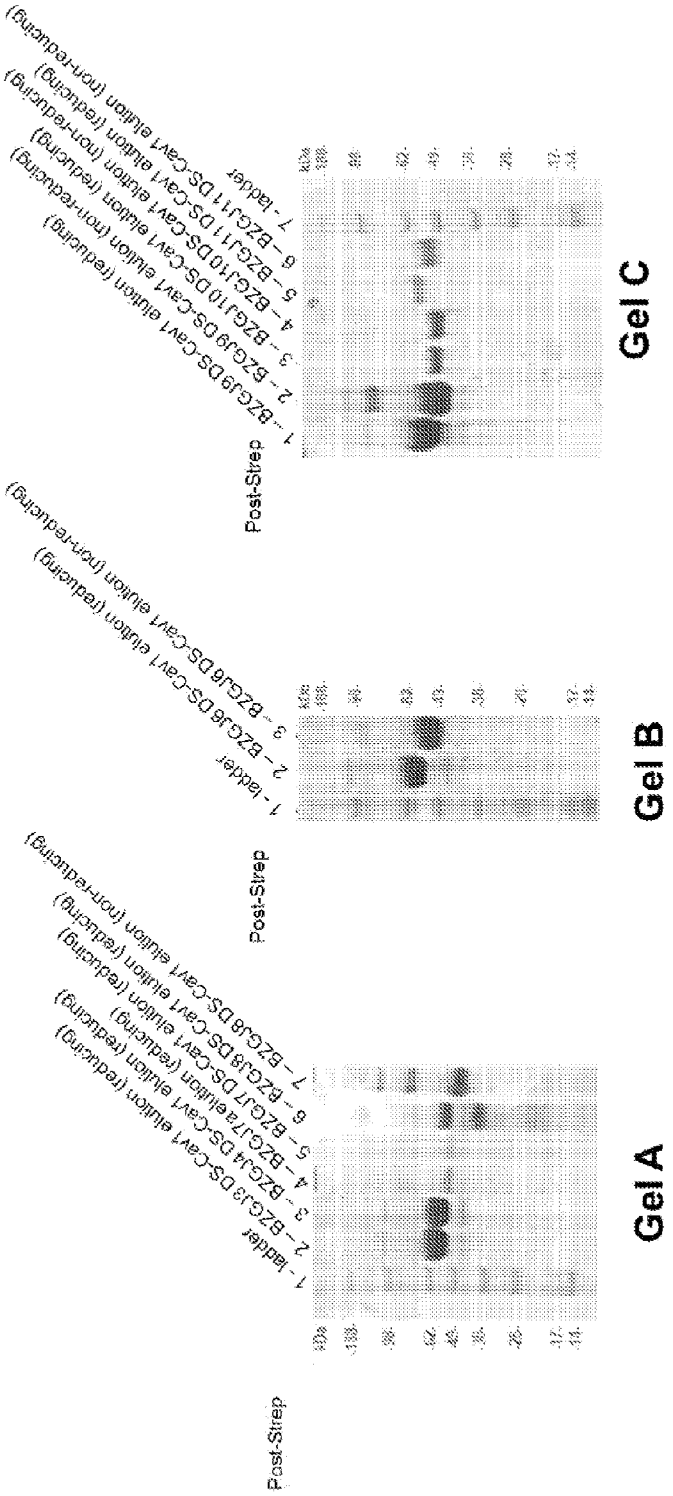
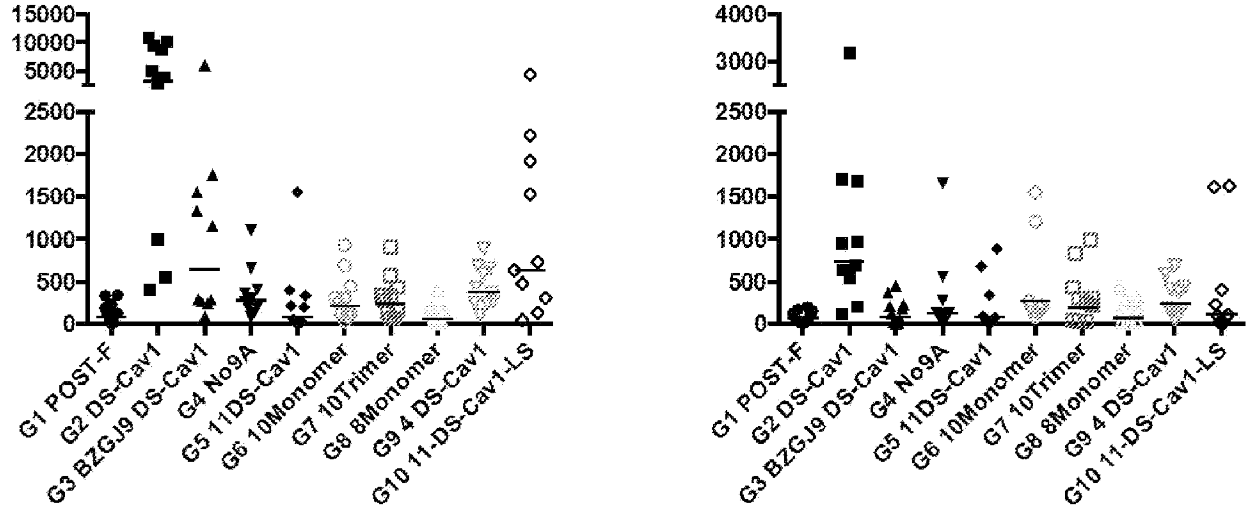

Post-Strep
1 - ladder
2 - BZGJ3 DS-Cav1 elution (reducing)
3 - BZGJ4 DS-Cav1 elution (reducing)
4 - BZGJ7a elution (reducing)
5 - BZGJ7 DS-Cav1 elution (reducing)
6 - BZGJ8 DS-Cav1 elution (reducing)
7 - BZGJ8 DS-Cav1 elution (non-reducing)
Gel A
Post-Strep
1 - ladder
2 - BZGJ6 DS-Cav1 elution (reducing)
3 - BZGJ6 DS-Cav1 elution (non-reducing)
Gel B
Post-Strep
1 - BZGJ9 DS-Cav1 elution (reducing)
2 - BZGJ9 DS-Cav1 elution (non-reducing)
3 - BZGJ10 DS-Cav1 elution (reducing)
4 - BZGJ10 DS-Cav1 elution (non-reducing)
5 - BZGJ11 DS-Cav1 elution (reducing)
6 - BZGJ11 DS-Cav1 elution (non-reducing)
7 - ladder
Gel C

FIG. 53 Week 5 neutralization data 10 animals/group

Immunizations at Week 0 and Week 3 with 10 ug protein + 50 ug Poly I:C per animal

Subtype A **Subtype B**



SUBTYPE A	POST-F	DS-Cav1	BZGJ9 DS-Cav1	BZGJ9A	BZGJ11 DS-Cav1	BZGJ10 Monomer	BZGJ10 Trimer	BZGJ8 Monomer	BZGJ4 DS-Cav1	BZGJ11-DS-Cav1-LS
Mean	151.1	5260	1308	388.5	281.9	329.3	329.5	118.2	449.4	1238
Geometric mean	82.98	3190	849.8	279.6	79.18	220	240	80.88	378.9	537.7

SUBTYPE B	POST-F	DS-Cav1	BZGJ9 DS-Cav1	BZGJ9A	BZGJ11 DS-Cav1	BZGJ10 Monomer	BZGJ10 Trimer	BZGJ8 Monomer	BZGJ4 DS-Cav1	BZGJ11-DS-Cav1-LS
Mean	94.51	1070	166.5	305.2	223.8	446.3	344.4	166.8	318.1	420.6
Geometric mean	61.97	737.9	78.17	130.5	76.07	258.9	193	68.73	243.8	112.9

FIG. 54

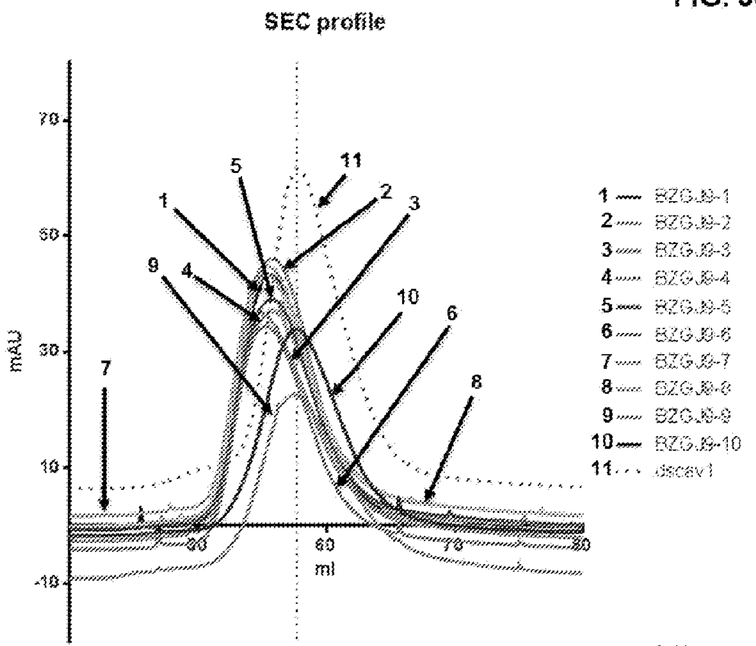
Single chain linker region may be improved by removing Proline 101 or shortening/mutating the linker residues and adjacent residues

FIG. 55

Further improvement of Single Chain (SC) #669 and assessment by gel-filtration

Superose 6 (120 ml column)

FIG. 55



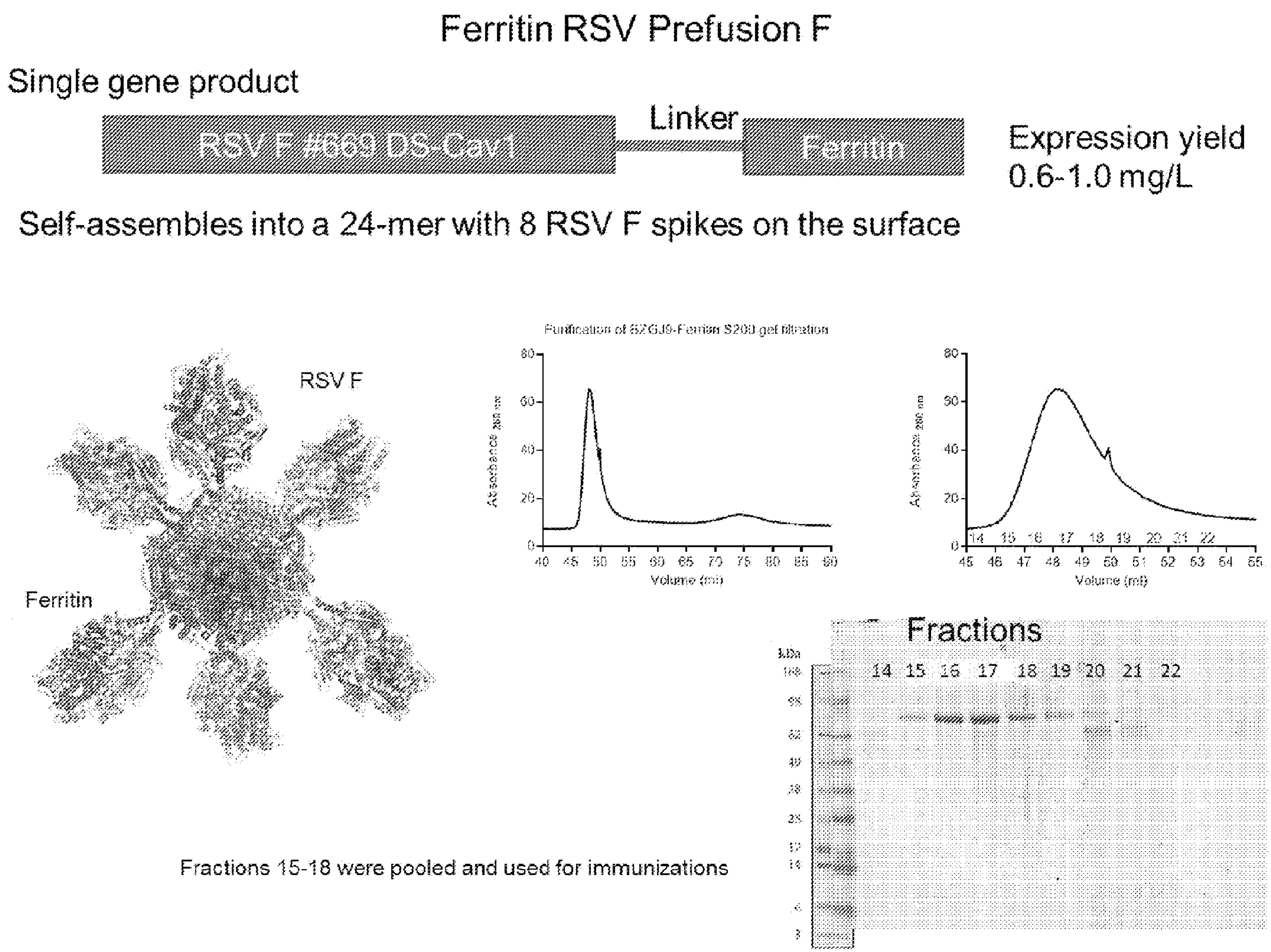
Linker sequence

1. MQSTPATNN GSAIASGVAVCKVLH
2. MQSTPATNN GSAIASGVAVCKVLH
3. MQSTPATNN GSAIASGVAVCKVLH
4. MQSTPATNN GSAIASGVAVCKVLH
5. MQSTPATNN GSAIASGVAVCKVLH
6. MQSTPATNN GSAIASGVAVCKVLH
7. MQSTPATNN GSAIASGVAVCKVLH
8. MQSTPATNN GSAIASGVAVCKVLH
9. MQSTPAT GSAIASGVAVCKVLH
10. MQSTPAT GSAIASGVAVCKVLH

All protein yields ~ 12 mg/L Expi cells

Removing residues 104NN105 results in improved trimer species

FIG. 56


Ferritin RSV Prefusion F
Single gene product
RSV F #669 DS-Cav1
Linker
Ferritin
Expression yield
0.6-1.0 mg/L
Self-assembles into a 24-mer with 8 RSV F spikes on the surface
RSV F
Ferritin
Absorbance 280 nm
Volume (ml)
Fractions
14 15 16 17 18 19 20 21 22
Fractions 15-18 were pooled and used for immunizations

Physical stability of #669 linked to Ferritin compared to RSV F DS-Cav1

FIG. 57

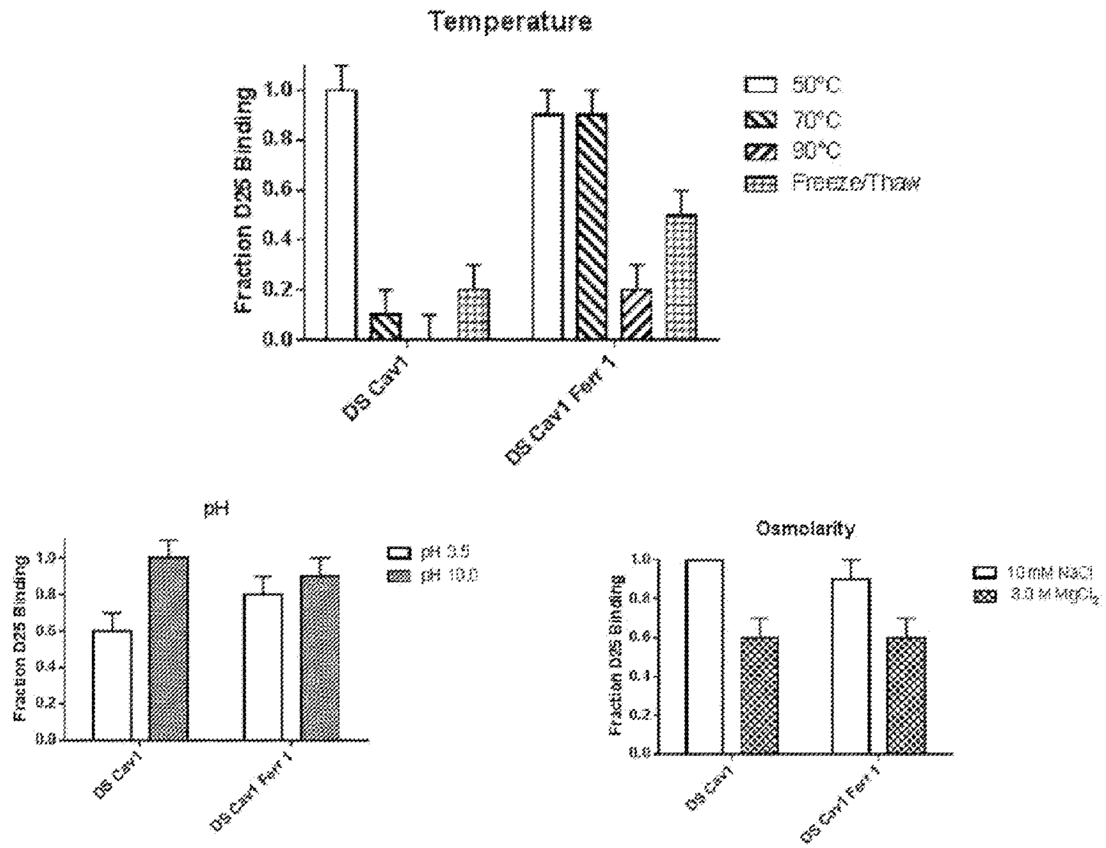


FIG. 58A

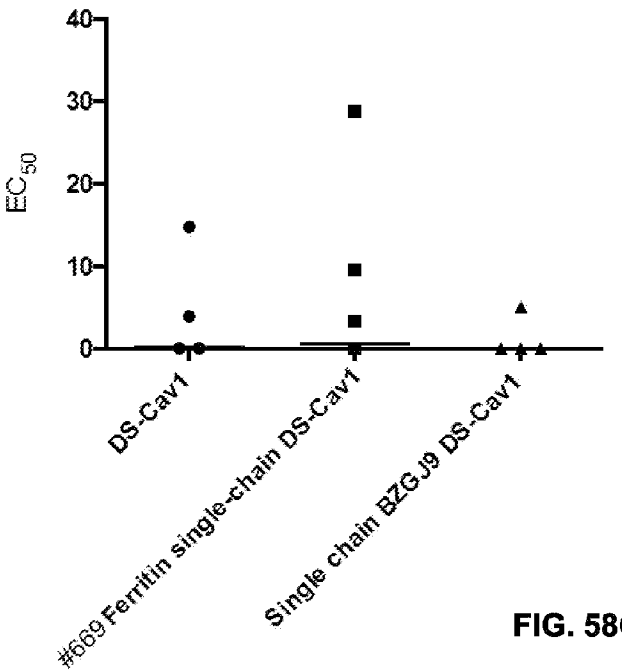


FIG. 58B

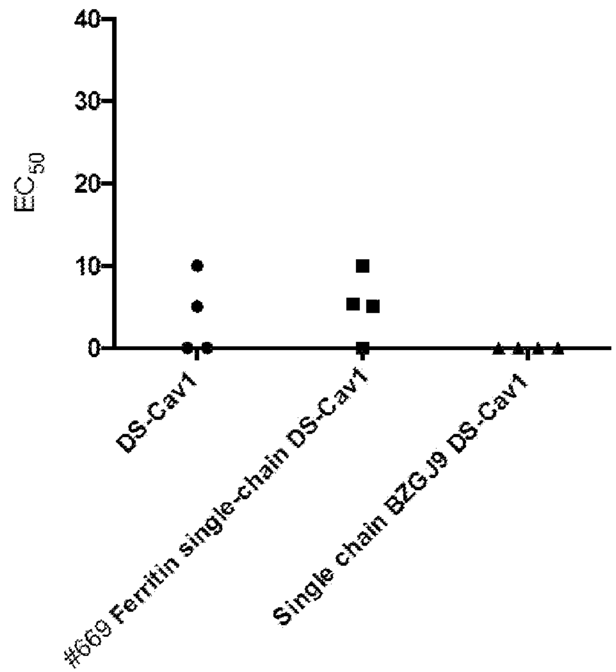


FIG. 58C

	DS-Cav1	Ferritin single-chain DS-Cav1	Single chain BZGJ9 DS-Cav1
Number of values	4	4	4
Mean	4.666	10.41	1.25
Std. Deviation	6.974	12.86	2.5
Std. Error of Mean	3.487	6.431	1.25
Geometric mean	0.2038	0.5074	0.0002659

FIG. 59A

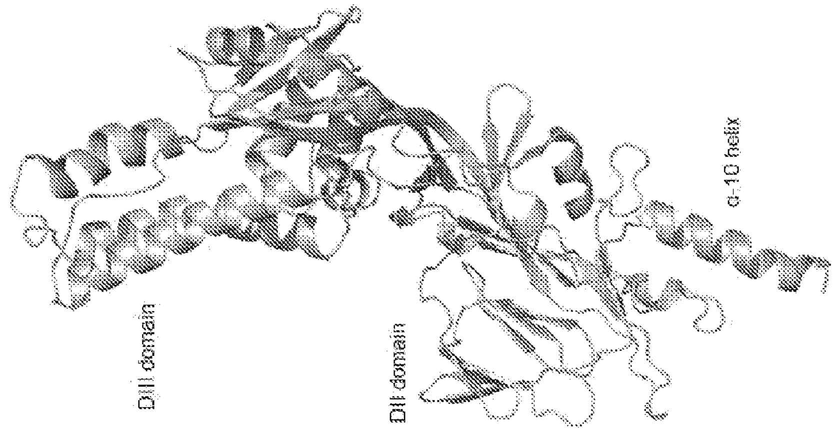
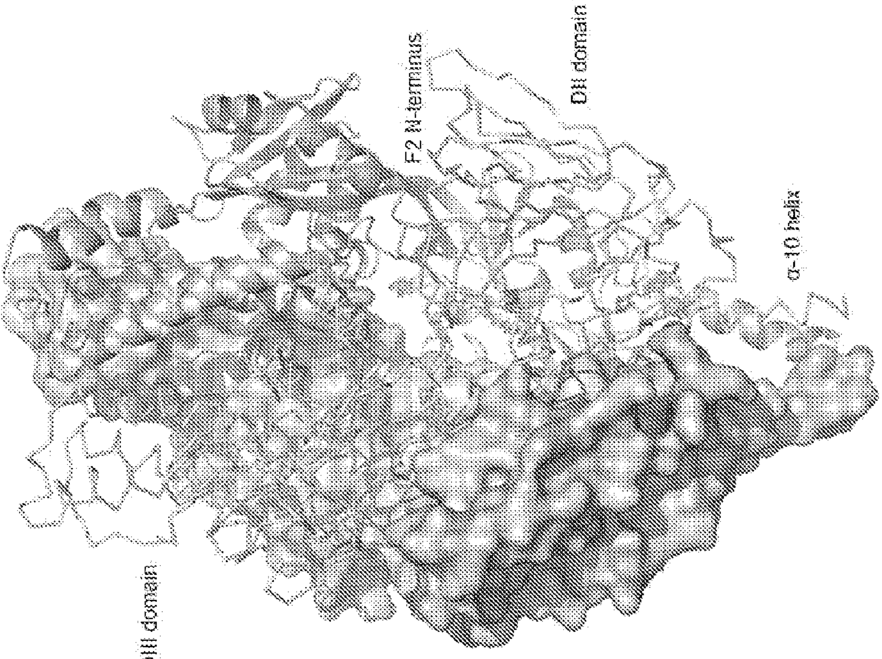


FIG. 59B



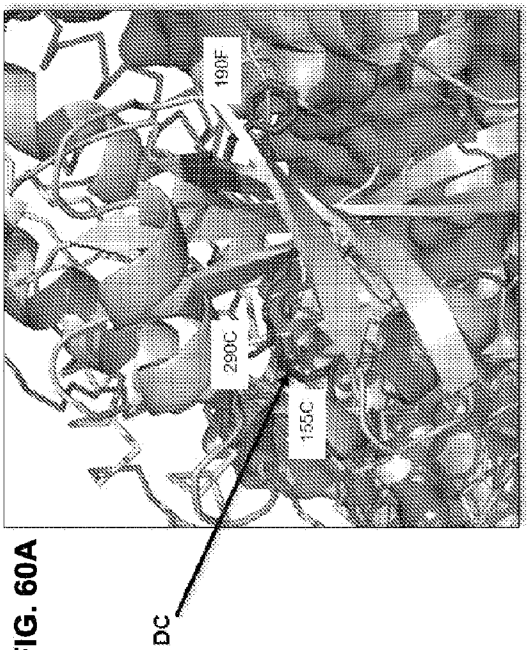


FIG. 60A

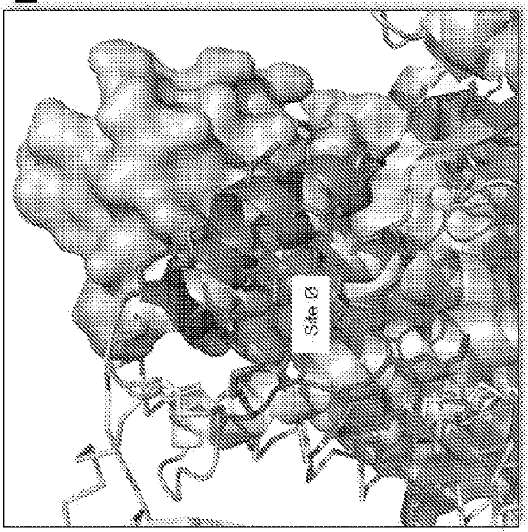


FIG. 60B

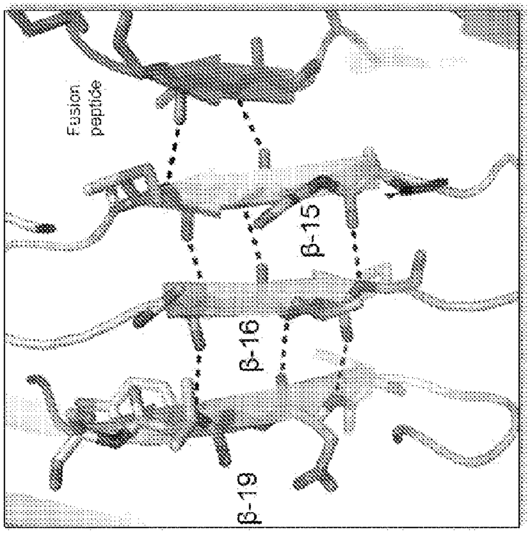


FIG. 60C

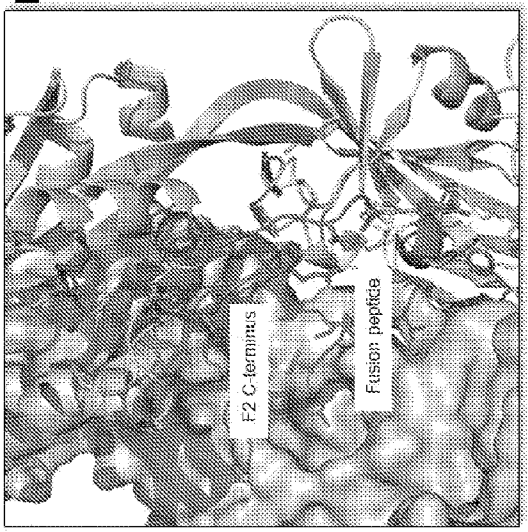
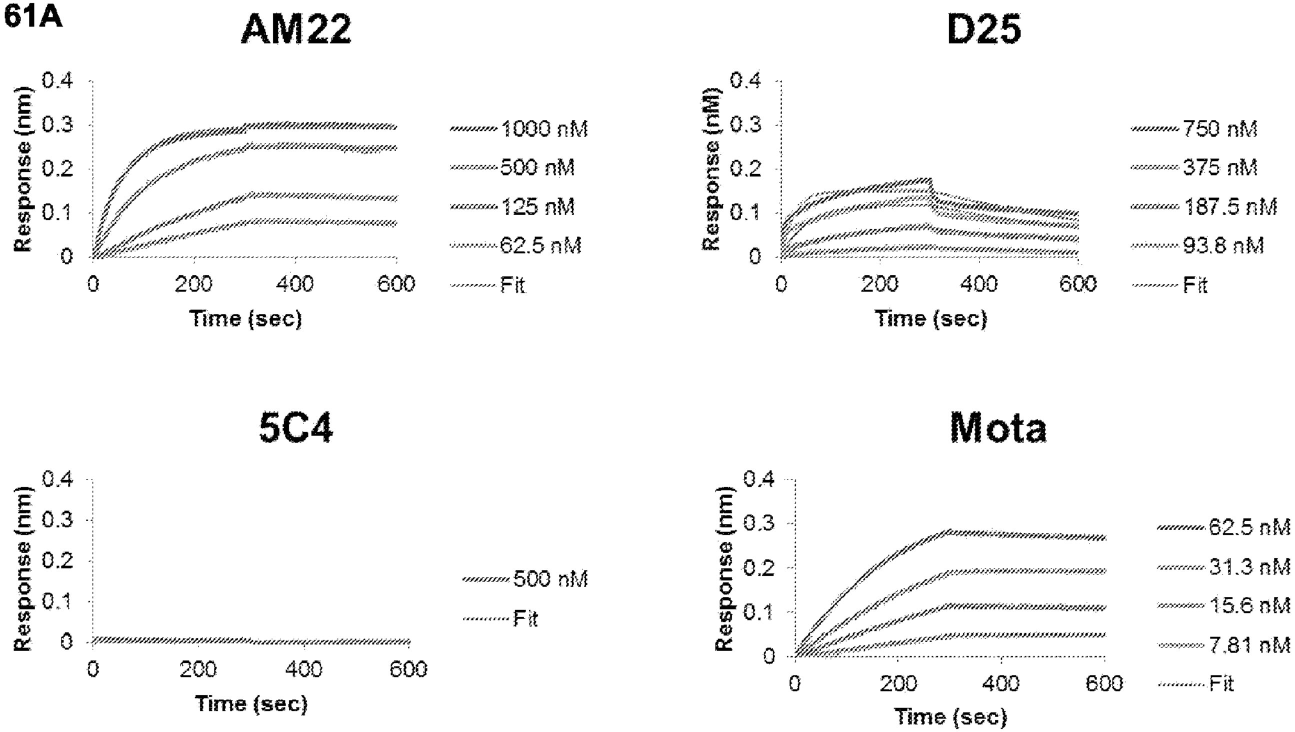


FIG. 60D

FIG. 61A



Antibody	KD (nM)	KD Error (nM)
AM22 Fab	<0.001	0.312
D25 Fab	**37.1**	**0.776**
Mota Fab	0.862	0.049
5C4 Fab	0	0

FIG. 61B

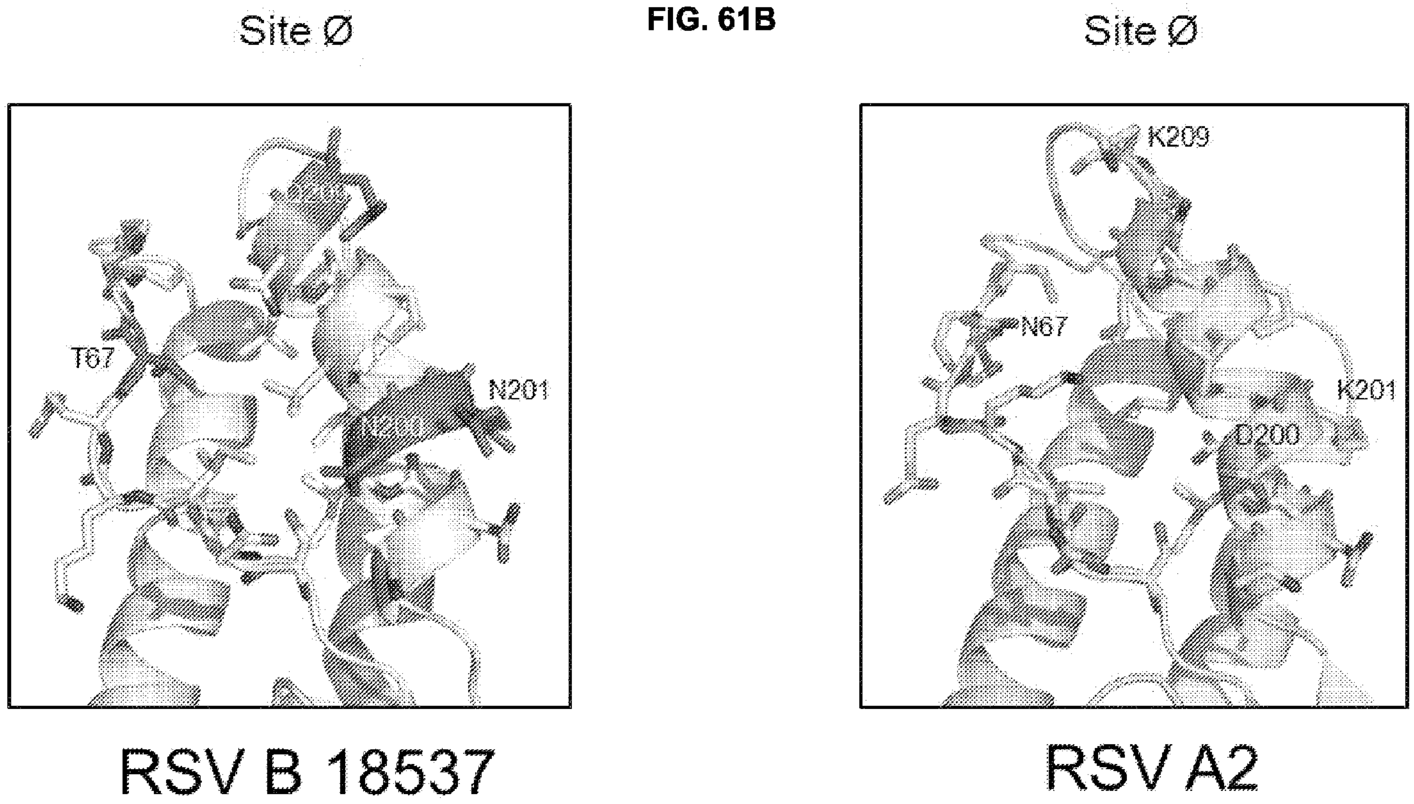


FIG. 62A

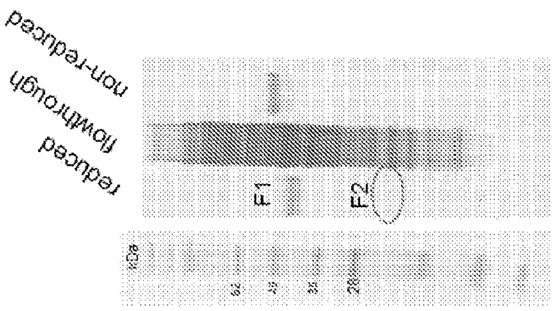


FIG. 62B

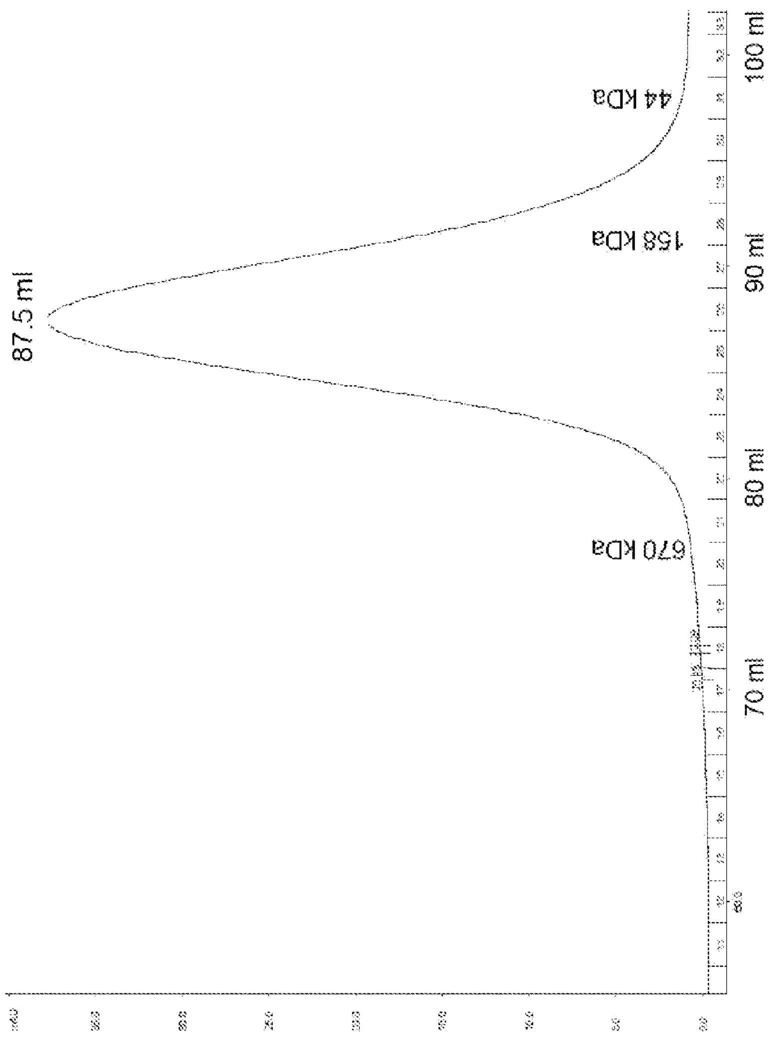


FIG. 63

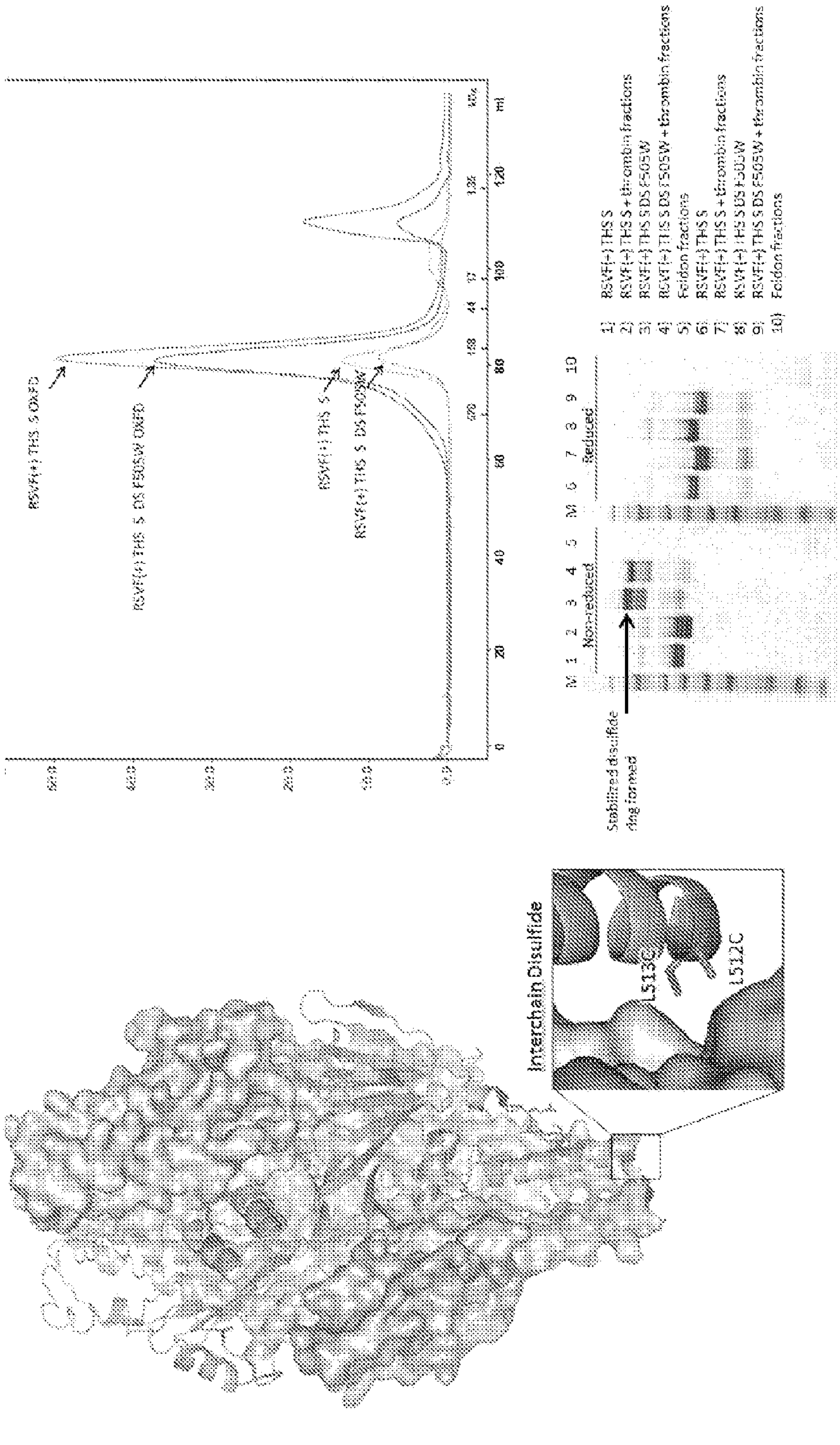
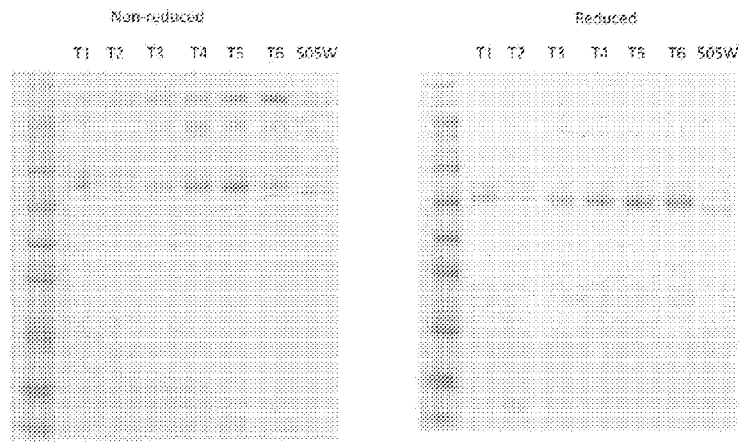

Interchain Disulfide
L513C
L512C
RSVF(+) THS S OxFD
RSVF(+) THS S DS F505W OxFD
RSVF(+) THS S
RSVF(+) THS S DS F505W
ml
Stabilized disulfide ring formed
M 1 2 3 4 5 M 6 7 8 9 10
Non-reduced
Reduced
1) RSVF(+) THS S
2) RSVF(+) THS S + thrombin fractions
3) RSVF(+) THS S DS F505W
4) RSVF(+) THS S DS F505W + thrombin fractions
5) Foldon fractions
6) RSVF(+) THS S
7) RSVF(+) THS S + thrombin fractions
8) RSVF(+) THS S DS F505W
9) RSVF(+) THS S DS F505W + thrombin fractions
10) Foldon fractions

FIG. 64

GSJCCTail1-6xFd and DS F505W post-streptactin



Construct	mg	mg/L	Motif between 511 and cleavable foldon	SEQ ID NO:
GSJCCtail1xFd	1.05	1.05	CChnvnagksttnimitt	840
GSJCCtail2xFd	0.33	0.33	LLhnvnaCCsttnimitt	841
GSJCCtail3xFd	0.99	0.99	LLhnvnagksttniCCtt	842
GSJCCtail4xFd	1.53	1.53	LLhnvnagksttnCCitt	843
GSJCCtail5xFd	2.13	2.13	CChnvnagksttn	844
GSJCCtail6xFd	1.65	1.65	LLhnvnaCCsttn	845
RSVF + THS S DS F505W OXFD	0.81	0.81	CC	

FIG. 65

Gel filtration profiles of RSV F proteins

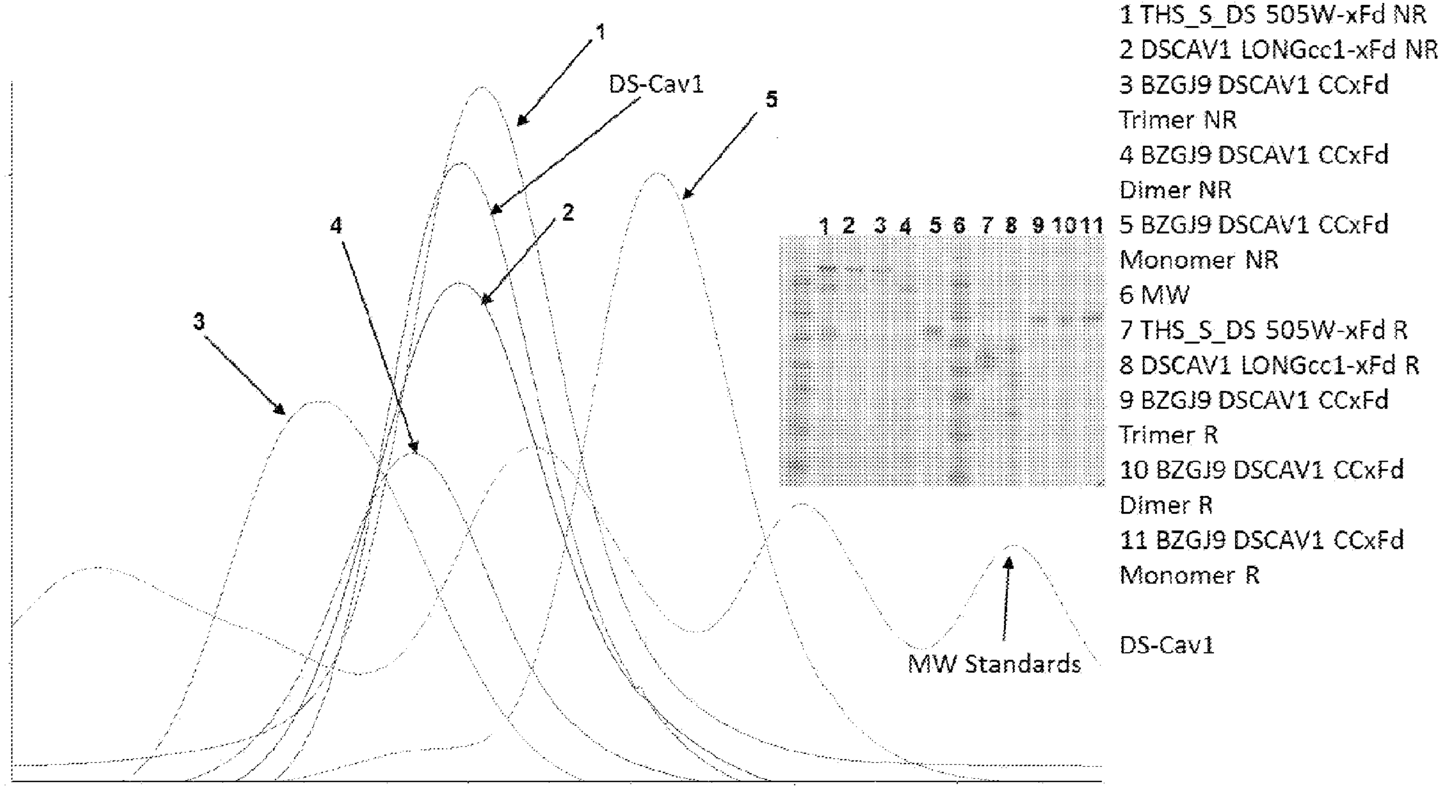
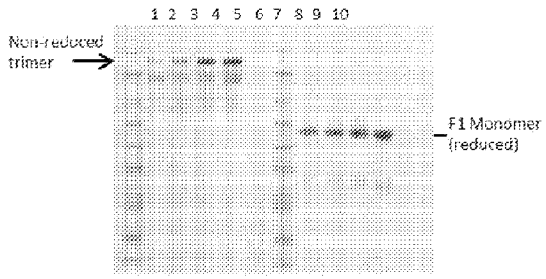


FIG. 66

Interprotomer disulfides stabilizing F protein trimer after removal of the foldon

SEQ ID No:			
840	GSJCCtail1xFd		512CCHNVNAGKSTTNIMITT
841	GSJCCtail2xFd		512LLHNVNACCSTTNIMITT
842	GSJCCtail3xFd		512LLHNVNAGKSTTNICCTT
843	GSJCCtail4xFd		512LLHNVNAGKSTTNCCITT
844	GSJCCtail5xFd		512CCHNVNAGKSTTN
445	GSJCCtail6xFd		512LLHNVNACCSTTN
839	GSJCCLongxFd		512CCHNVNAGKS
847	GSJCCtail8xFd		512LLHNVNAGKSTTNIMITT
849	GSJCCtail9xFd		512CCHNVNACCSTTN
850	GSJCCtail10xFd	485C494C	512CCHNVNAGKSTTN
851	GSJCCtail11xFd	495C494C	512CCHNVNACCSTTN
852	Tail11 s_ds_F505W_cxFd	SEQID 566 + 485C494C	512CCHNVNACCSTTN
853	GSJCCtail12xFd		512CCHNVNACCSTTNICCTT
854	GSJCCtail13xFd	485C494C	512CCHNVNACCSTTNICCTT
855	GSJCCtail14xFd	SEQID 566 + 485C494C	512CCHNVNACCSTTNICCTT

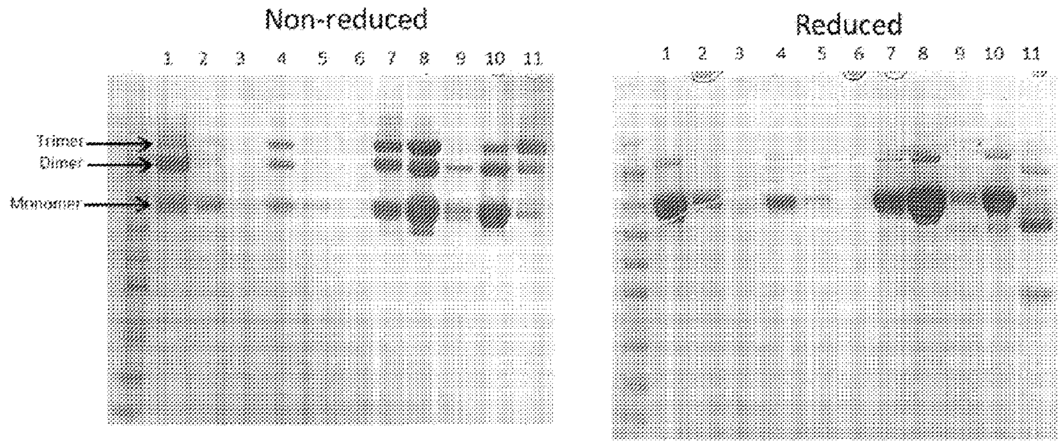


1 GSJCCtail4xFd N/R
2 GSJCCtail5xFd N/R
3 GSJCCtail6xFd N/R
4 DSCAV1 LONGcc1-xFd N/R

6 MW
7 GSJCCtail4xFd Reduced
8 GSJCCtail5xFd Reduced
9 GSJCCtail6xFd Reduced
10 DSCAV1 LONGcc1-xFd Reduced

FIG. 67

Disulfide screening of protomer interface in single chain and DS Cav1 format



	SEQ ID No:
1 scBZGJ9 DSCav1 74C, 219C xFd	744
2 scBZGJ9 DSCav1 149C, 458C xFd	746
3 scBZGJ9 DSCav1 374C, 454C xFd	747
4 scBZGJ9 DSCav1 99C, 362C xFd	754
5 scBZGJ9 DSCav1 146C, 460C xFd	745
6 scBZGJ9 DSCav1 153C, 461C xFd	756
7 scBZGJ9 DSCav1 485C, 494C xFd	752
8 scBZGJ9 DSCav1 519C, 520C xFd	753
9 scBZGJ9 DSCav1 99C, 361C xFd	755
10 scBZGJ9 DSCav1 102C, 359C xFd	757
11 GSJCCTail9xFd	849

FIG. 68 Comparison of CSGSJ (no foldon) proteins with DS Cav1 showing monomer and trimer peak formation

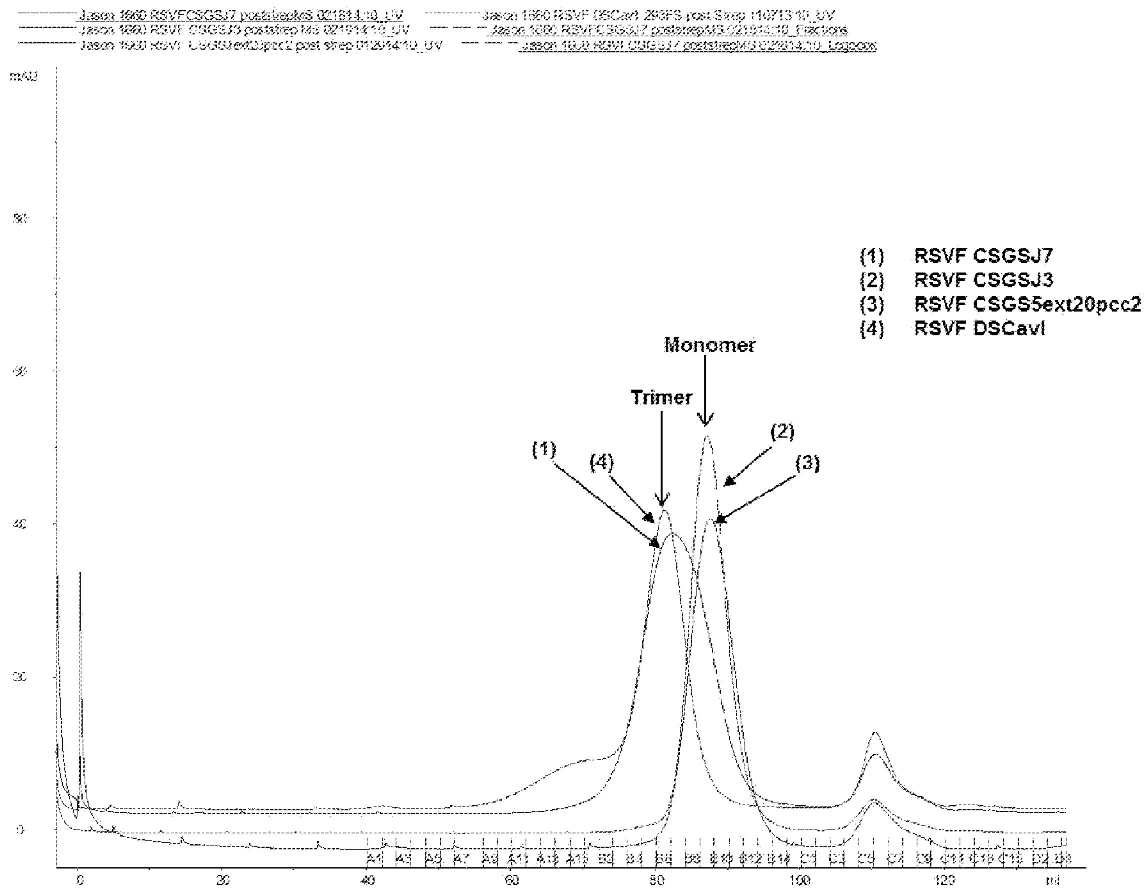


FIG. 69A

		Condition					
	SEQ ID NO	(1)	(2)	(3)	(4)	(5)	(6)
GS-Cav1 control		2.9722	2.629	0.079	2.456	2.6019	3.0760
GSJ 1Cav1 1	901	2.9394	2.9999	0.0598	2.494	1.9186	3.0448
GSJ 1Cav1 2	902	2.8747	2.8486	0.0934	2.721	2.9776	2.8101
GSJ 1Cav1 3	903	2.6728	2.9517	0.0604	2.267	3.0476	3.0533
GSJ 1Cav1 4	904	2.7302	2.9117	0.0881	1.532	0.0919	2.529
GSJ 1Cav1 5	905	2.984	2.9964	0.0619	2.331	2.0366	3.0638
GSJ 1Cav1 6	906	3.0393	3.0238	0.0601	2.28	0.3496	2.8567
GSJ 1Cav1 7	907	2.9335	3.049	0.0896	2.557	3.0614	3.1202
GSJ 1Cav1 8	908	1.5173	2.151	0.0533	0.622	0.1222	1.2086
GSJ 1Cav1 9	909	0.0664	0.1252	1.4955	0.071	0.0666	0.2809
GSJ 1Cav1 10	910	2.926	3.0756	0.075	2.455	3.0581	3.0857
GSJ 1Cav1 11	911	2.984	3.049	0.0642	2.473	1.6633	3.0621
GSJ 1Cav1 12	912	2.728	2.9347	0.081	1.685	1.9661	2.9011
GSJ 1Cav1 13	913	2.9293	3.093	0.0718	2.491	3.0264	3.0535
GSJ 1Cav1 14	914	0.074	0.1171	0.0865	0.11	0.058	1.9173
GSJ 1Cav1 15	915	2.8859	2.9177	0.0566	2.248	0.1957	2.8954
GSJ 1Cav1 16	916	2.8717	3.0962	0.0931	2.524	0.699	2.9927
GSJ 1Cav1 17	917	2.8605	3.1047	0.0891	2.147	0.6287	2.9251
GSJ 1Cav1 18	918	2.981	2.9848	0.1134	2.34	1.3975	3.0765
GSJ 1Cav1 20	920	2.7496	2.9874	0.0728	2.379	0.1579	2.5803
GSJ 1Cav1 21	921	2.6721	2.9259	0.0535	2.28	0.1022	2.6841
GSJ 1Cav1 22	922	2.9434	2.8613	0.072	2.168	0.1202	2.8014
GSJ 1Cav1 23	923	2.8094	3.0166	0.0689	2.378	0.1273	2.7775
GSJ 1Cav1 24	924	2.6291	2.7995	2.1119	2.754	0.2752	2.3836
GSJ 1Cav1 25	925	3.1039	3.1097	0.1162	2.192	0.369	3.1036
GSJ 1Cav1 26	926	3.0783	3.0849	0.1055	2.268	0.4974	3.0849
GSJ 1Cav1 27	927	3.0306	3.0256	0.0626	2.322	0.3391	2.6006
GSJ 1Cav1 28	928	0.8729	2.3566	0.0761	1.891	0.082	2.2764
GSJ 1Cav1 29	929	2.7403	3.1072	0.0572	2.309	1.8155	2.86
GSJ 1Cav1 30	930	2.7515	2.928	0.0764	2.2	0.1129	2.7988
GSJ 1Cav1 31	931	2.8769	2.997	0.0716	2.22	0.2543	2.8772
GSJ 1Cav1 32	932	2.7309	2.9971	0.1275	2.826	0.097	2.9804
GSJ 1Cav1 33	933	2.6077	2.7011	0.0517	2.664	0.0884	2.6221
GSJ 1Cav1 34	934	2.6916	2.9784	0.0619	2.387	0.1185	2.8935
GSJ 1Cav1 35	935	2.6504	2.8824	0.0603	2.253	0.0877	2.7279
GSJ 1Cav1 36	936	2.8615	2.9404	0.0976	2.19	0.1221	2.8286
GSJ 1Cav1 37	937	2.9057	2.9104	0.08	2.092	0.0919	2.75
GSJ 1Cav1 38	938	2.7711	2.858	0.0559	1.923	0.0572	2.481
JCB GSJ 1	939	2.9562	2.8762	0.0932	2.551	0.8961	2.9355

FIG. 69B

	SEQ ID NO	(1)	(2)	(3)	(4)	(5)	(6)
JCB GSJ 2	940	2.5514	2.8462	0.0989	2.103	0.1986	2.7591
JCB GSJ 3	941	3.0163	2.9573	0.1007	2.717	1.9165	2.9945
JCB GSJ 4	942	2.9577	2.9605	0.0842	2.239	2.061	3.0062
JCB GSJ 5	943	2.8765	2.9654	0.0783	2.059	3.0692	2.9273
IG1-V192M	944	0.0622	0.1164	0.053	0.146	0.0632	0.3497
IG2-A298M_RSVF(+)FdTHS-paH	945	3.053	3.0139	0.1081	2.686	0.3677	2.8677
IG2-T58I_A298M	946	3.0122	2.9796	0.0866	2.645	2.7714	2.9833
IG2-T58I_V192F_A298I_RSVF(+)FdTHS-paH	947	0.0912	0.0934	0.0882	0.127	0.0734	0.1541
IG2-T58I_V192M_A298I_RSVF(+)FdTHS-paH	948	0.0908	0.1119	0.0825	0.11	0.0832	0.7898
i167m-a298m	949	0.0995	0.3355	0.0492	0.345	0.0628	0.7184
i167m-l181m	950	0.0579	0.2538	0.052	0.367	0.0772	0.6506
i199f	951	2.5468	2.7464	0.4405	2.429	0.2993	2.7677
i57c-s190c	952	0.2652	0.5291	0.0661	0.094	0.0725	0.461
ig2-t58i-a298m	953	1.0703	1.5305	0.0717	0.552	0.1018	1.2218
ig2-t58m	954	3.0989	2.9985	0.1685	2.792	0.797	2.8626
ig2-t58m-a298i	955	2.8471	2.8545	0.056	2.765	0.8859	2.8106
ig2-t58m-a298L	956	3.038	2.9793	0.0879	2.795	2.3546	2.8514
ig2-v192c-ins192-193-g-e256c	957	0.0696	0.1069	0.0556	0.11	0.0852	0.3724
rsv f ths_s_f505w_c_s509f	958	2.1277	2.6243	0.1037	1.738	2.3049	2.6123
rsv f ths_s_f505w_s509f	959	1.3529	1.6774	0.1193	0.159	1.4003	2.3193
t58i-a298i	960	2.6436	2.6535	0.0875	2.243	0.2619	2.5531
t58m-a298m	961	2.8397	2.715	0.1073	2.712	2.0852	2.9196
v179l-t189f	962	0.856	1.2011	0.0499	0.824	0.0621	0.9012
v192f	963	0.053	0.178	0.0816	0.186	0.0609	0.318
v192f-l252a	964	0.0515	0.0803	0.1396	0.074	0.0663	0.3239
v56m-i167m-l181m	965	0.0464	0.0855	0.062	0.071	0.0549	0.4488
v56m-i167m-v296m	966	0.0521	0.1116	0.0754	0.111	0.0518	0.4902
v56m-l181f	967	0.0511	0.1818	0.1008	0.475	0.0612	0.4797
w52c-s150c	968	0.1112	0.2253	0.0782	0.103	0.08	0.3329
GSJT12	859	1.8695	1.9726	0.0574	0.166	1.6895	2.3914
GSJT13	860	1.4253	1.7736	0.0572	0.144	1.6425	2.2703
GSJT14	861	2.2902	2.3344	0.0737	0.422	1.6947	2.4307
GSJT15	862	1.5401	1.7335	0.0752	0.114	1.744	2.2699
GSJT16	863	1.3708	1.6618	0.062	0.093	1.6552	2.3271
GSJT17	864	1.2053	1.5253	0.0702	0.103	1.5696	2.2797
GSJT18	865	1.181	1.5089	0.1298	0.272	1.1712	2.1823

FIG. 69C

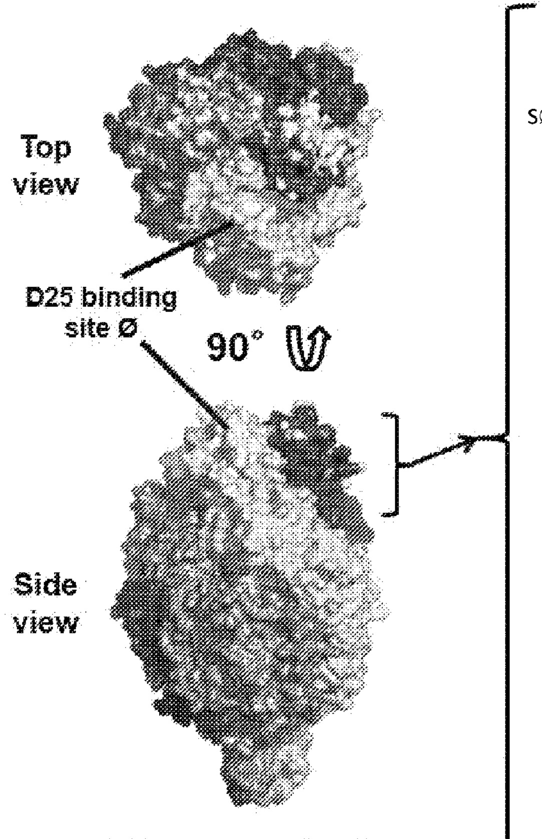
	SEQ ID NO	(1)	(2)	(3)	(4)	(5)	(6)
GSJT19	866	0.9276	1.2456	0.0969	0.161	0.9697	1.3834
GSJT20	867	0.934	1.3047	0.0527	0.154	0.9109	2.38
GSJT21	868	1.1944	1.4919	0.1448	0.159	1.3432	2.125
GSJT22	869	2.449	2.556	0.0687	0.594	2.2867	2.62
GSJT23	870	1.0262	1.3655	0.0679	0.257	1.1962	2.1363
GSJT24	871	1.9053	2.1459	0.0885	0.255	1.5913	2.2505
GSJT25	872	0.8893	1.43	0.0981	0.361	0.8439	1.9153
GSJT26	873	1.4387	2.0503	0.0532	0.421	1.6024	2.2934
GSJT27	874	1.0373	1.4579	0.0577	0.419	0.8234	1.9723
GSJT28	875	1.92	2.0962	0.0566	0.346	1.5545	2.2083
GSJT29	876	1.1084	1.5343	0.0723	0.408	0.8422	1.942
GSJT30	877	1.5348	1.9793	0.1053	0.354	1.6307	2.4172
GSJT31	878	1.7044	2.1351	0.0569	0.284	1.7527	2.3803
GSJT32	879	1.5763	1.9587	0.0518	0.296	1.6372	2.2152
GSJT33	880	1.677	1.9659	0.0586	0.228	1.5347	2.1997
GSJT34	881	1.8105	1.9678	0.0968	0.388	1.7569	2.372
GSJT35	882	1.91	2.1785	0.0773	0.34	1.7139	2.2363
GSJT36	883	1.6632	1.9904	0.0934	0.296	1.6547	2.1367
GSJT37	884	1.6236	1.9141	0.0612	0.342	1.7493	2.4074
GSJT38	885	1.5627	2.0942	0.0588	0.13	1.7619	2.4227
GSJT39	886	1.6508	2.07	0.0611	0.113	1.6541	2.425
GSJT40	887	1.576	1.8917	0.0645	0.177	1.6654	2.3938
GSJT41	888	1.5413	1.824	0.081	0.151	1.6104	2.3619
GSJT42	889	2.1906	2.252	0.068	0.352	1.9041	2.3642
GSJT43	890	1.6024	2.0164	0.0559	0.378	1.5314	2.4296
GSJT44	891	1.3943	1.6933	0.0751	0.141	1.3954	2.2111
GSJT45	892	1.5485	1.9654	0.0623	0.144	1.5211	2.4807
GSJT46	893	1.7633	2.0335	0.0673	0.231	1.39	2.2563
GSJT47	894	2.1206	2.2091	0.0566	0.318	1.9313	2.4835
GSJT48	895	1.5802	2.1433	0.0875	0.682	1.6569	2.3015
GSJT49	896	1.8774	2.1642	0.0609	0.437	1.6532	2.3664
GSJT50	897	1.6151	1.998	0.0735	0.508	1.6905	2.2291
GSJT51	898	2.0185	2.2042	0.056	0.668	1.5997	2.2547
GSJT52	899	1.7521	2.2572	0.0635	0.791	1.4597	2.4122
GSJT53	900	1.625	1.899	0.0666	0.482	1.3792	2.3026
GSJ-FP1	969	1.7611	2.1208	0.2051	0.399	1.9283	2.4804
GSJ-FP2	970	2.365	2.4107	0.1963	0.515	2.2619	2.8949
GSJ-FP3	971	0.126	0.234	0.0566	0.09	0.34	1.2832
GSJ-FP4	972	0.1049	0.1701	0.0785	0.084	0.1021	0.6274
GSJ-FP5	973	0.0691	0.178	0.0531	0.119	0.1454	0.5375

FIG. 69D

	SEQ ID NO	(1)	(2)	(3)	(4)	(5)	(6)
GSJ-FP6	974	0.0634	0.0904	0.0525	0.066	0.0822	0.5962
GSJ-190P1	975	0.8047	1.1312	0.0688	0.182	0.1205	0.6337
GSJ-190P2	976	0.0573	0.0813	0.0523	0.065	0.0735	0.6404
GSJ-190P3	977	0.0739	0.1148	0.0516	0.07	0.0653	0.3209
GSJ-190P4	978	0.0924	0.093	0.0536	0.062	0.0807	0.2631
GSJ-190P5	979	2.7932	2.8717	0.0679	2.014	0.0989	2.5646
GSJ-190P6	980	2.8847	2.9283	0.0697	2.401	0.4255	2.9114
GSJ-190P7	981	2.8983	2.9739	0.0745	2.392	0.5633	2.9715
GSJ-190P8	982	2.1604	2.6693	0.1063	2.27	0.175	2.6687
GSJ-190P9	983	0.0745	0.2046	0.0878	0.394	0.0654	0.7136
GSJ-190P10	984	0.0601	0.2007	0.061	0.069	0.0564	0.4225
GSJ-190P11	985	0.0699	0.0992	0.0595	0.107	0.0723	0.3176
GSJ-190P12	986	0.0709	0.1044	0.0528	0.061	0.0895	2.1549
GSJ-190P13	987	0.0592	0.1057	0.0475	0.083	0.0985	0.2632
GSJ-190P14	988	0.0583	0.0955	0.0468	0.058	0.0895	0.31
GSJ-190P15	989	0.0539	0.1363	0.0486	0.233	0.0695	0.3087
GSJ-190P16	990	0.0656	0.1091	0.1299	0.173	0.0791	0.492
GSJ-190P17	991	0.0583	0.1179	0.0502	0.092	0.0981	0.292
GSJ-DS1	992	0.0598	0.1296	0.0476	0.087	0.0823	0.3217
GSJ-DS2	993	0.0517	0.0942	0.0487	0.068	0.0698	0.2403
GSJ-DS3	994	0.0514	0.104	0.0714	0.094	0.0673	0.2739
GSJ-DS4	995	0.0562	0.1153	0.0542	0.08	0.0685	0.2281
GJ-3-1	996	2.6144	2.91	0.0528	2.225	0.2481	2.5224
GJ-3-2	997	2.4331	2.6384	0.0597	1.348	0.0674	1.9871
GJ-3-3	998	1.6954	2.1696	0.0777	1.264	0.102	2.4298
GJ-3-4	999	0.5157	1.3561	0.0551	1.246	0.168	1.527
GJ-3-5	1000	2.5823	2.8132	0.0606	1.904	0.2602	2.5274
GJ-3-6	1001	0.0791	0.772	0.046	1.231	0.0677	1.7194
GJ-3-7	1002	0.6065	1.8109	0.0927	1.621	0.0625	1.5747
GSJ-Int-FdF-1	1003	1.9736	2.1439	0.0467	0.295	1.6327	1.5662
GSJ-Int-FdF-2	1004	1.8225	2.0812	0.0824	0.195	1.7008	2.3449
GSJ-Int-FdF-3	1005	1.7866	1.8727	0.0537	0.222	1.7458	2.2908
GSJ-Int-FdF-4	1006	0.7215	1.0353	0.0522	0.095	0.6795	1.4721
GSJ-Int-FdF-5	1007	1.3574	1.6888	0.0555	0.118	1.1065	1.8092
GSJ-Int-FdF-6	1008	0.7496	1.0469	0.052	0.091	0.6161	1.285
GSJ-Int-FdF-7	1009	0.6324	0.7985	0.1179	0.15	0.5634	1.3309
GSJ-Int-FdF-8	1010	1.4654	1.8779	0.0997	1.153	1.352	2.1494
GSJ-Int-FdF-9	1011	1.4669	1.8242	0.147	0.158	1.4619	2.4151
GSJ-Int-FdF-10	1012	1.836	2.0029	0.0532	0.624	1.5126	2.1783
GSJ-Int-FdF-11	1013	1.7458	1.8869	0.0512	0.173	1.3535	2.0495

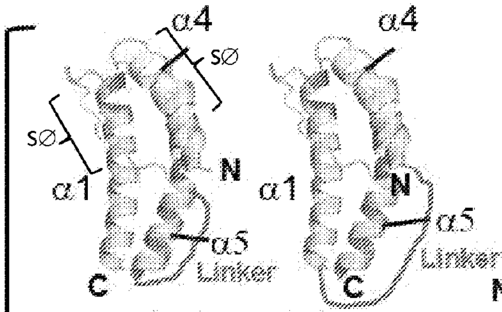
FIG. 69E

	SEQ ID NO	(1)	(2)	(3)	(4)	(5)	(6)
GSJ-Int-FdF-12	1014	1.663	2.0524	0.0676	0.714	1.6219	2.2935
GSJ-Int-FdF-13	1015	1.8807	2.052	0.0534	0.894	1.4138	2.2218
GSJ-Int-FdF-14	1016	1.2368	1.6639	0.0777	0.209	1.2947	2.1821
GSJ-Int-FdF-15	1017	1.9938	2.0424	0.0531	0.18	1.8005	2.2882
GSJ-Int-FdF-16	1018	2.0878	2.1147	0.0637	0.177	1.8094	2.3236
IG1-V192M	944	0.0622	0.1164	0.053	0.146	0.0632	0.3497
IG2-A298M_RSVF(+)FdTHS-paH	945	3.053	3.0139	0.1081	2.686	0.3677	2.8677
IG2-T58I_A298M	946	3.1122	2.9796	0.0866	2.645	2.7714	2.9533
IG2-T58I_V192F_A298I_RSVF(+)FdTHS-paH	947	0.0912	0.0934	0.0882	0.127	0.0734	0.1541
IG2-T58I_V192M_A298I_RSVF(+)FdTHS-paH	948	0.0908	0.1119	0.0825	0.11	0.0832	0.7888
i167m-a298m	949	0.0995	0.3355	0.0492	0.345	0.0628	0.7184
i167m-l181m	950	0.0579	0.2538	0.052	0.367	0.0772	0.6506
i199f	951	2.6468	2.7464	0.4405	2.429	0.2993	2.7677
i57c-s190c	952	0.2652	0.5291	0.0661	0.094	0.0725	0.461
ig2-t58i-a298m	953	1.0703	1.5365	0.0717	0.552	0.1018	1.2218
ig2-t58m	954	3.0496	2.9985	0.1685	2.792	0.797	2.8626
ig2-t58m-a298i	955	2.6471	2.8545	0.056	2.765	0.8859	2.8106
ig2-t58m-a298L	956	3.039	2.9793	0.0879	2.795	2.1593	2.3514
ig2-v192c-ins192-193-g-e256c	957	0.0696	0.1069	0.0556	0.11	0.0852	0.3724
rsv f ths_s_f505w_o_s509f	958	2.1277	2.6243	0.1037	1.738	2.3049	2.6125
rsv f ths_s_f505w_s509f	959	1.3529	1.6774	0.1193	0.158	1.4003	2.3193
t58i-a298i	960	2.6436	2.6535	0.0875	2.243	0.2619	2.5531
t58m-a298m	961	2.8387	2.715	0.1073	2.712	2.0658	2.9156
v179l-t189f	962	0.856	1.2011	0.0499	0.824	0.0621	0.9012
v192f	963	0.053	0.178	0.0816	0.186	0.0609	0.318
v192f-l252a	964	0.0515	0.0803	0.1396	0.074	0.0663	0.3239
v56m-i167m-l181m	965	0.0464	0.0855	0.062	0.071	0.0549	0.4488
v56m-i167m-v296m	966	0.0521	0.1116	0.0754	0.111	0.0518	0.4902
v56m-l181f	967	0.0511	0.1818	0.1008	0.475	0.0612	0.4797
w52c-s150c	968	0.1112	0.2253	0.0782	0.103	0.08	0.3329



RSV F pre-fusion trimer

FIG. 70A



Site Ø circular permutations

FIG. 70B

Site Ø scaffolds

FIG. 70C

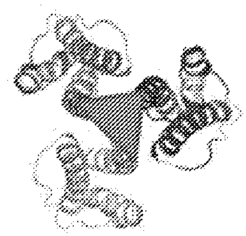
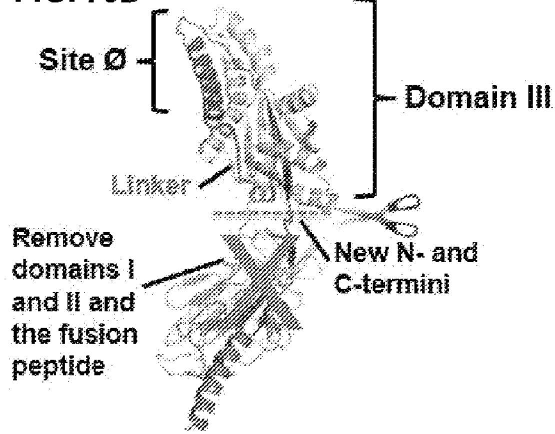

Site Ø trimers

FIG. 70D



RSV F domain III only

FIG. 70E

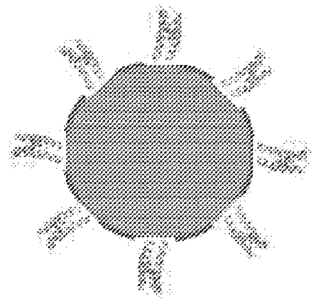

Site Ø Nanoparticles

FIG. 71

RSV F Antigenic Site Ø Immunogens

- 249 immunogens expressed and evaluated antigenically:

  26 Circularly permutated site Ø monomers
  25 Circularly permutated scaffolded site Ø monomers
  39 Trimerized circularly permutated site Ø
  11 Circularly permutated site Ø monomer nanoparticles
  1 Circularly permutated site Ø trimer on ferritin nanoparticles
  52 Domain III constructs
  12 Trimerized domain III constructs
  18 Circularly permutated domain III dimers
  10 Circularly permutated trimerized domain III dimers
  51 Domain III on nanoparticles
  4 Trimerized domain III nanoparticles

- Antigenic readout

  D25 binding at 0 and 1 week at 4°C
  D25 binding after 1 hr. at 60°C, 70°C, 80°C, 90°C and 100°C
  AM22 and 5C4 binding

- Summary of antigenicity results (ELISA ≥ 1.5):

Immunogens designed	D25 wk 0 4C	D25 wk 1 4C	D25 1hr 60C	D25 1hr 70C	D25 1 hr 80C	D25 1hr 90C	D25 1 hr 100C	AM22 wk2 4C	5C4 wk 0 4C
Monomers (121)	54	49	45	49	21	9	10	54	44
Trimers (61)	36	31	40	28	13	4	6	42	42
Monomers on nanoparticles (63)	20	12	20	12	0	0	0	28	23
Trimers on nanoparticles (4)	0	2	1	2	0	0	0	4	4

FIG. 72A

SEQ ID NO	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Con	1.7749	2.4042	1.3312	0.063	0.0546	0.0611	ND	2.7959	2.6353	1.754
Con	1.928	0.1536	0.1846	0.051	0.0596	0.0907	ND	0.915	ND	0.373
Circular permutation of site 0 (26)										
1027	0.994	0.7916	0.4133	2.798	1.2895	0.7806	ND	0.3243	0.1245	1.305
1028	0.2347	0.1646	0.0961	1.508	2.7552	0.7053	ND	0.4055	0.0814	0.693
1029	0.0597	0.0619	0.0531	0.055	0.0652	0.0583	ND	0.1138	0.0892	0.077
1030	1.0318	0.6279	0.4113	0.959	1.8999	0.4427	ND	1.1626	0.0856	0.916
1031	0.3199	0.3181	0.1695	2.678	2.5887	1.4001	1.9429	0.8191	0.0751	1.272
1032	0.0661	0.0616	0.0562	0.067	0.0791	0.065	ND	0.1363	0.0932	0.088
1033	1.3594	0.849	0.4444	2.711	2.1708	2.3641	1.8722	0.2756	0.0867	1.279
1034	0.5424	0.6069	0.1407	2.364	2.4148	1.5628	2.7861	0.1339	0.0842	1.035
1035	0.071	0.0661	0.0564	0.076	0.084	0.1459	ND	0.0943	0.1136	0.079
1036	2.3735	2.1015	1.5353	2.232	2.2364	0.1381	ND	3.0121	0.075	2.448
1037	2.5576	1.4036	1.4876	0.434	0.3958	0.4036	ND	2.0542	0.1077	1.297
1038	2.7399	1.6952	1.376	0.629	0.0524	0.333	ND	2.1779	0.0858	1.501
1039	3.0073	2.9769	2.9504	2.7	0.2338	0.2418	ND	3.1817	0.1113	2.953
1040	3.1199	3.2391	3.1501	3.045	0.0559	2.5247	2.9345	3.2581	0.0604	3.181
1041	3.021	2.901	2.9543	2.749	0.4102	0.8312	1.6808	3.1678	0.0613	2.946
1042	0.0777	0.071	0.085	0.052	0.0859	0.0496	ND	0.2679	0.0933	0.130
1043	0.0564	0.0648	0.0617	0.051	0.0819	0.0485	ND	0.1483	0.187	0.088
1044	0.053	0.0523	0.0574	0.049	0.0847	0.0498	ND	0.0832	0.1699	0.061
1045	0.0491	0.0571	0.0561	0.046	0.074	0.0468	ND	0.1178	0.2204	0.074
1046	0.0719	0.1451	0.218	0.145	2.3869	2.579	ND	2.3759	2.9192	0.889
1047	0.0786	0.0507	0.2224	0.051	0.1093	0.4426	ND	1.1171	0.9184	0.406
1048	0.0855	0.2682	0.0826	0.268	1.6515	2.2777	ND	2.15	0.1436	0.895
1049	0.856	2.8029	0.2993	2.803	2.9675	2.6686	3.2757	0.3899	0.1003	1.999
1050	0.0607	0.4221	0.1522	0.422	0.6564	2.8585	ND	0.2904	0.1102	0.378
1051	0.1055	1.9886	0.1805	1.989	2.6382	2.9702	3.2262	0.4197	0.1127	1.466
1052	2.578	2.7939	2.9126	2.794	2.0478	2.7466	2.4877	2.7874	3.1874	2.792
Circular permutation with scaffold connection (19)										
1053	2.6567	3.1872	2.6488	3.187	3.1226	2.9381	3.2123	2.6379	3.0295	3.004
1054	0.0728	0.0849	0.0929	0.063	0.0677	0.0465	ND	0.3494	0.0902	0.166
1055	0.5403	0.3211	0.3096	0.249	0.1933	0.0526	ND	1.0104	0.1344	0.527
1056	2.1931	1.5791	1.9433	0.304	0.1792	0.0586	ND	2.122	0.0816	1.335
1057	0.7436	0.3687	0.4128	0.083	0.085	0.0479	ND	0.4357	0.0715	0.296
1058	0.0633	0.0816	0.06	0.058	0.085	0.0521	ND	0.1304	0.0744	0.090
1059	0.0641	0.065	0.0717	0.054	0.0648	0.0546	ND	0.2744	0.1045	0.131
1060	2.114	1.8611	2.0589	1.006	0.0925	0.0498	ND	3.0045	2.5961	1.957
1061	0.0754	0.0712	0.0716	0.058	0.0682	0.0505	ND	0.2135	0.1304	0.114
1062	0.4145	0.1479	0.1602	0.133	0.1876	0.0542	ND	0.5636	0.1938	0.281
1063	0.085	0.0982	0.0661	0.069	0.0828	0.0496	ND	0.343	0.0841	0.170
1064	0.0541	0.0569	0.0632	0.052	0.1114	0.0567	ND	0.105	0.0799	0.071
1065	0.0562	0.214	0.0571	0.06	0.0848	0.0641	ND	0.1566	0.0759	0.144
1066	0.0676	0.0694	0.0521	0.061	0.1022	0.0507	ND	0.09	0.0813	0.073
1067	0.0492	0.0514	0.0566	0.049	0.0677	0.0552	ND	0.1413	0.0937	0.080
1068	2.3614	2.9114	2.9171	2.57	0.2665	0.082	ND	3.0351	2.9698	2.839
1069	0.07	0.0696	0.0656	0.054	0.0758	0.0499	ND	0.2169	0.1646	0.113
1070	0.0523	0.0527	0.0546	0.05	0.079	0.0473	ND	0.1313	0.1131	0.078
1071	0.0448	0.0484	0.0913	0.046	0.0703	0.049	ND	0.1503	0.0733	0.082

FIG. 72B

SEQ ID NO	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Circular permutation of site 0 with trimer (39)										
1072	0.3581	0.4078	0.7423	0.481	1.1851	1.6631	ND	0.3298	0.0868	0.406
1073	1.9816	1.8505	2.1982	1.327	2.7423	1.9532	3.2163	1.1248	0.0861	1.434
1074	0.1814	0.2503	0.3292	0.114	0.2837	0.1077	ND	0.2319	0.0806	0.199
1075	2.8516	2.6792	2.9809	2.791	3.2015	2.8639	3.2468	0.4305	0.0997	1.967
1076	1.902	1.3076	2.0732	1.305	2.4359	1.4035	1.8783	0.1298	0.0862	0.914
1077	1.472	0.825	1.9265	1.15	1.732	0.5228	ND	0.2167	0.0854	0.731
1078	0.8158	0.2163	0.8631	0.306	1.9185	1.2613	ND	0.099	0.0767	0.207
1079	1.7333	1.5426	2.3438	1.409	2.2722	1.8603	2.7799	1.4647	0.107	1.472
1080	0.4502	0.2953	0.4333	0.187	0.3934	0.1679	ND	0.218	0.0819	0.233
1081	2.8737	2.477	2.903	2.634	3.1623	2.9098	3.0618	0.3779	0.1206	1.830
1082	0.3473	0.3027	0.5607	0.371	0.8177	0.4034	ND	0.0757	0.0815	0.250
1083	0.4663	0.2718	0.8992	0.42	0.9627	0.2882	ND	0.0854	0.079	0.259
1084	0.3509	0.363	0.2706	0.519	0.4042	0.0582	ND	1.3032	0.0717	0.728
1085	0.1433	0.1191	0.2362	0.365	0.3129	0.0557	ND	0.9482	0.1128	0.477
1086	0.3671	0.2472	0.5002	0.551	0.4468	0.0653	ND	1.64	0.0853	0.813
1087	0.0741	0.0731	0.0846	0.11	0.1371	0.0512	ND	0.263	0.1618	0.149
1088	2.4789	1.9043	2.1233	2.153	2.5609	0.2414	ND	2.8283	0.0775	2.295
1099	1.6689	0.8883	1.2592	1.073	1.3876	0.1011	ND	1.5505	0.078	1.171
1100	1.1362	0.5942	0.7754	0.682	0.9898	0.0765	ND	2.0515	0.0844	1.109
1101	2.0625	1.5285	1.6158	1.916	2.2367	0.1205	ND	2.8305	0.0928	2.092
1102	3.0513	2.9073	2.8972	1.923	2.3869	0.0829	ND	3.1173	0.0746	2.649
1103	2.8736	2.9112	2.7143	1.609	0.1093	0.0562	ND	3.0903	0.0686	2.537
1104	2.9502	3.0009	2.6871	2.854	1.6515	0.0737	ND	3.0888	0.0638	2.961
1105	2.1426	1.7548	2.2434	0.969	0.0737	0.0519	ND	2.6865	0.1038	1.804
1106	3.0297	3.0861	3.0603	2.995	1.4528	0.0601	ND	3.1395	0.0849	3.060
1107	3.0488	3.0707	3.0183	2.941	0.5496	0.0541	ND	2.7789	0.1179	2.930
1108	0.0592	0.057	0.0537	0.057	0.0462	0.1491	ND	0.6247	0.1318	0.246
1109	0.0814	0.0709	0.0718	0.071	0.1027	0.5605	ND	0.464	0.1334	0.202
1110	0.0477	0.0615	0.0575	0.062	0.1888	0.1249	ND	0.5142	0.1537	0.212
1111	0.051	0.0559	0.0564	0.056	0.0935	0.2652	ND	0.6155	0.1604	0.242
1112	0.0533	0.0513	0.0547	0.051	0.0655	0.1411	ND	0.5763	0.1488	0.226
1113	0.0713	0.0623	0.0659	0.062	0.1656	0.2525	ND	0.5906	0.1695	0.238
1114	0.0546	0.0528	0.1484	0.053	0.0823	0.7941	ND	0.7087	0.1844	0.271
1115	0.1118	0.0548	0.1034	0.055	0.0533	1.0703	ND	1.9905	0.1999	0.700
1116	3.1519	2.903	2.7587	2.906	0.5106	0.0828	ND	2.8276	3.2044	2.861
1117	0.0632	0.0606	0.0677	0.061	0.0582	0.2678	ND	0.523	0.1354	0.215
1118	0.0821	0.1203	0.11	0.12	0.0819	0.091	ND	0.4687	0.109	0.236
1119	0.0581	0.0587	0.0563	0.059	0.0614	0.0524	ND	0.2851	0.1118	0.134
1120	0.0555	0.0518	0.0592	0.052	0.0595	0.0498	ND	0.2154	0.1156	0.106
Site 0 minimal epitope on a scaffold (6)										
1121	0.0588	0.0565	0.0558	0.057	0.1035	0.0593	ND	0.2015	0.0825	0.105
1122	0.0567	0.0579	0.0597	0.058	0.0997	0.0629	ND	0.1782	0.1011	0.098
1123	0.0535	0.0527	0.1198	0.053	0.1266	0.0767	ND	0.1795	0.1359	0.095
1124	0.0547	0.1717	0.6951	0.172	0.0968	0.054	ND	1.0895	1.644	0.478
1125	0.0498	0.0523	0.0699	0.048	0.0803	0.0618	ND	0.623	0.1274	0.241
1126	0.0467	0.0511	0.0507	0.055	0.0846	0.0602	ND	0.1205	0.1162	0.075
Domain III (42)										
1127	2.3497	2.1859	ND	1.798	ND	ND	ND	ND	ND	ND

FIG. 72C

SEQ ID NO	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
1128	1.8913	1.3526	ND	0.182	ND	ND	ND	ND	ND	ND
1129	0.1181	0.1009	ND	0.067	ND	ND	ND	ND	ND	ND
1130	2.2318	1.9028	ND	0.568	ND	ND	ND	ND	ND	ND
1131	1.4159	0.9357	ND	0.208	ND	ND	ND	ND	ND	ND
1132	0.1072	0.1636	ND	0.075	ND	ND	ND	ND	ND	ND
1133	2.8957	2.8256	ND	2.963	ND	ND	ND	ND	ND	ND
1134	1.6943	1.3663	ND	0.25	ND	ND	ND	ND	ND	ND
1135	0.2983	0.2993	ND	0.11	ND	ND	ND	ND	ND	ND
1136	2.9005	2.8267	ND	2.192	ND	ND	ND	ND	ND	ND
1137	0.9978	1.1904	ND	0.28	ND	ND	ND	ND	ND	ND
1138	0.5312	0.7704	ND	0.305	ND	ND	ND	ND	ND	ND
1139	2.0258	2.061	2.0601	1.869	0.1128	0.0592	ND	2.1613	2.8734	2.031
1140	0.1182	0.2949	0.1563	0.094	0.0826	0.0517	ND	1.2182	1.5279	0.536
1141	2.5559	2.541	2.333	2.562	0.2226	0.0622	ND	3.1111	2.6692	2.738
1142	2.6619	2.8985	2.5202	2.636	0.2683	0.0536	ND	3.0737	2.7379	2.869
1143	2.7804	2.8597	2.5537	2.192	0.8527	0.224	ND	3.1262	2.4711	2.726
1144	2.8499	3.0864	3.0749	2.745	0.3977	0.1391	ND	3.11	3.0637	2.981
1145	2.7083	2.6366	2.7459	2.572	1.7732	0.0521	ND	3.1076	3.0516	2.772
1146	3.1125	3.1886	2.954	1.907	1.0453	0.1286	ND	3.2298	3.0826	2.775
1147	2.9579	3.2076	2.9367	2.183	2.7782	1.13	2.1693	3.2534	2.9833	2.881
1148	2.8409	2.8971	2.8098	2.579	0.7371	0.3829	ND	1.4542	2.8212	2.310
1149	2.7887	2.9015	2.6135	2.551	0.5153	0.2845	ND	3.1136	2.7058	2.855
1150	2.7865	2.8584	2.5003	1.859	0.4836	0.075	ND	3.0944	2.6091	2.604
1151	2.8132	3.0253	3.0237	2.762	0.9838	0.0662	ND	3.1032	3.09	2.970
1152	2.7828	2.5105	2.7218	2.334	1.4915	0.0614	ND	3.0998	3.0544	2.648
1153	3.1438	3.1697	3.0513	2.713	0.8313	0.2322	ND	3.1979	3.1925	3.027
1154	3.0847	3.1601	3.0265	2.585	1.2956	0.224	ND	2.48	3.1684	2.742
1155	2.8643	3.0609	3.0251	2.738	1.5185	0.2005	ND	3.1526	3.0995	2.984
1156	3.0902	3.1988	3.0863	3.061	3.0275	0.7406	ND	3.2332	3.1654	3.171
1157	3.03	3.2543	3.1566	2.806	1.0701	0.1974	ND	3.2473	3.0829	3.103
1158	1.81	1.0186	0.9501	0.208	0.3739	0.0864	ND	2.806	1.871	1.344
1159	2.626	2.8175	2.61	2.869	1.6898	0.1447	ND	3.321	2.9622	3.063
1160	2.0768	2.839	2.1299	0.076	1.5314	0.1097	ND	2.6298	3.054	1.848
1161	2.8665	3.2047	3.1815	3.205	2.8295	0.8036	ND	2.8899	3.2611	3.100
1162	2.7123	3.1616	2.9949	3.162	2.8515	0.1987	ND	2.8523	3.2335	3.059
1163	2.323	3.1018	2.8147	3.102	1.3027	0.2541	ND	2.8146	3.2003	3.006
1164	0.5076	2.8125	2.1352	2.813	1.778	0.1256	ND	2.8194	3.0376	2.815
1165	2.5424	1.285	2.0781	1.285	0.0445	0.0903	ND	2.8442	3.057	1.805
1166	2.4769	3.0629	3.023	3.063	0.1207	0.0893	ND	2.9292	3.1297	3.018
1167	2.3672	1.7585	2.8717	1.759	0.0664	0.0703	ND	2.8358	3.209	2.118
1168	2.5499	2.9419	3.0947	2.942	0.1674	0.0705	ND	2.8555	3.1965	2.913
Domain III with trimer (22)										
1169	2.9087	3.1685	3.0345	3.03	1.0033	0.1147	ND	2.3982	2.8127	2.865
1170	2.8831	3.1103	2.9567	2.955	1.3358	0.4711	ND	3.1749	2.8526	3.080
1171	2.9096	3.0151	2.7308	2.766	2.2196	1.3086	2.2228	3.1102	2.9257	2.964
1172	2.8697	3.0289	2.7027	2.948	0.7138	0.1834	ND	3.1221	2.6216	3.033
1173	2.9601	3.0572	3.0328	2.947	1.2007	0.1174	ND	3.0647	3.0356	3.023
1174	2.9753	3.1399	3.0062	2.911	0.4101	0.0565	ND	0.8609	3.0055	2.304
1175	2.8916	3.0532	2.8612	2.755	0.3803	0.0639	ND	1.2822	2.9703	2.363
1176	2.8284	3.0866	2.7402	2.078	0.4938	0.1352	ND	3.1426	2.9844	2.769

FIG. 72D

SEQ ID NO	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
1177	2.9084	2.9269	2.696	2.592	0.2782	0.0624	ND	3.1318	2.6926	2.883
1178	2.9339	3.0762	3.0639	2.945	1.7652	0.0711	ND	3.1215	3.2297	3.048
1179	2.8755	0.0982	3.1576	0.098	0.0688	0.0638	ND	2.9749	3.2621	1.057
1180	2.8435	2.7472	3.1025	2.747	0.1038	0.0698	ND	3.0339	3.3278	2.843
1181	2.6187	1.392	2.9138	1.392	0.2403	0.1386	ND	2.7881	3.2213	1.857
1182	2.5762	1.3382	2.8281	1.338	0.2278	0.1296	ND	2.7143	3.2032	1.797
1183	2.0026	2.1708	2.5307	2.171	0.0668	0.0524	ND	2.7349	3.0949	2.359
1184	0.0549	0.0533	0.0805	0.053	0.0519	0.0585	ND	0.7682	0.3922	0.292
1185	2.1649	3.0485	2.8212	3.049	0.1002	0.0551	ND	2.7643	3.1423	2.954
1186	2.4898	1.4644	3.0314	1.464	0.2314	0.1102	ND	2.7345	3.2544	1.888
1187	2.6496	1.588	3.0289	1.588	0.2269	0.1206	ND	2.7482	3.2885	1.975
1188	2.7763	3.2268	3.006	3.227	0.1278	0.0592	ND	2.788	3.2753	3.081
1189	2.4998	2.7034	2.7576	2.703	0.1162	0.0614	ND	2.7434	3.2517	2.717
1190	0.4011	0.1884	0.7964	0.188	0.0616	0.0521	ND	2.375	2.4944	0.917
Tandem domain III (18)										
1191	1.8368	0.8202	2.2007	0.82	0.0574	0.0618	ND	2.8501	3.1536	1.497
1192	2.5446	3.1211	2.7713	3.121	0.0691	0.0734	ND	2.8646	3.2782	3.036
1193	2.6237	2.5047	2.643	2.505	0.0583	0.0726	ND	2.9049	3.2067	2.638
1194	2.748	3.1383	2.5969	3.138	0.1009	0.066	ND	2.9005	3.1654	3.059
1195	1.5986	1.0687	1.9038	1.069	0.0794	0.0514	ND	2.6989	3.0144	1.612
1196	2.7203	3.0435	2.7253	3.044	0.0762	0.0593	ND	2.7855	3.174	2.959
1197	2.5384	2.7383	2.451	2.738	0.0576	0.0473	ND	2.7646	3.1412	2.747
1198	2.291	3.0732	2.4519	3.073	0.1422	0.0503	ND	2.7156	3.1633	2.954
1199	0.0522	0.0518	0.0592	0.052	0.053	0.0455	ND	0.4433	0.1375	0.182
1200	2.5794	0.5879	2.4456	0.588	0.2308	0.0942	ND	2.6989	3.1526	1.292
1201	0.0699	0.0527	0.0626	0.053	0.0475	0.0547	ND	0.2363	0.1469	0.114
1202	0.5592	0.1981	1.4615	0.198	0.0562	0.051	ND	2.5259	2.8012	0.974
1203	0.0815	0.0506	0.0973	0.051	0.04	0.0528	ND	0.301	0.2133	0.134
1204	0.1143	0.7387	0.3154	0.739	0.3958	0.0577	ND	2.1174	1.2797	1.198
1205	0.0526	0.049	0.1158	0.049	0.0524	0.0464	ND	0.1603	0.1179	0.086
1206	0.0978	0.6093	0.1909	0.609	0.2338	0.0493	ND	1.5509	0.9296	0.923
1207	0.0591	0.052	0.0893	0.052	0.0559	0.0465	ND	0.3483	0.1432	0.151
1208	0.1188	0.5431	0.2391	0.543	0.4102	0.0467	ND	1.9059	1.5241	0.997
Tandem domain III with a trimer (10)										
1209	0.6093	0.0806	0.8155	0.081	0.0438	0.0938	ND	2.7016	2.5006	0.954
1210	1.3484	2.4624	2.2383	2.462	0.0624	0.0769	ND	2.9038	3.0104	2.610
1211	1.2145	0.7313	2.302	0.731	0.0648	0.0658	ND	2.9505	3.0608	1.471
1212	2.494	3.0396	2.9677	3.04	0.0758	0.065	ND	2.9579	3.1809	3.012
1213	2.3155	0.8101	2.5948	0.81	0.0712	0.0731	ND	2.9564	3.2253	1.526
1214	2.6101	1.4694	2.7332	0.056	0.0612	0.049	ND	2.7111	3.1491	1.412
1215	0.2802	0.0612	0.346	0.061	0.0687	0.046	ND	2.414	2.3453	0.845
1216	0.4552	0.1039	0.9587	0.104	0.0634	0.0464	ND	2.503	2.7455	0.904
1217	0.264	0.065	1.9952	0.065	0.0577	0.0622	ND	2.2431	2.3756	0.791
1218	0.4957	0.1884	0.8309	0.188	0.0491	0.0521	ND	2.4909	2.6829	0.956

FIG. 72E

SEQ ID NO	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
	1.7749	2.4042	1.331	0.C628	0.0546	0.0611	ND	2.7959	2.6353	1.754
	1.928	0.1536	0.185	0.051	0.0596	0.0907	ND	0.915	ND	0.373
Domain III on ferritin (45)										
1219	2.6369	2.5119	2.725	2.5119	0.065	0.0473	ND	2.7715	3.2062	2.5984
1220	2.7353	3.2496	2.909	3.2496	0.3082	0.0755	ND	2.9063	3.2815	3.1018
1221	2.6214	2.8132	2.767	2.8132	0.1323	0.0531	ND	2.7568	3.2087	2.7944
1222	2.6679	2.8848	2.541	2.8848	0.2636	0.0577	ND	2.7639	3.1706	2.8445
1223	0.0612	0.0736	0.061	0.C736	0.0735	0.0813	ND	0.7882	0.1386	0.3118
1224	0.0565	0.0708	0.052	0.C708	0.0824	0.1425	ND	0.6484	0.1213	0.2633
1225	0.0694	0.0861	0.064	0.C861	0.0815	0.0829	ND	0.7931	0.1601	0.3218
1226	0.0584	0.0614	0.061	0.C614	0.0698	0.108	ND	0.8759	0.1194	0.3329
1227	0.055	0.0566	0.057	0.C566	0.0655	0.0654	ND	0.6022	0.1469	0.2385
1228	0.0546	0.0591	0.052	0.C591	0.062	0.1387	ND	0.3552	0.1366	0.1578
1229	0.1014	0.1232	0.144	0.1232	0.0653	0.0543	ND	1.557	0.5376	0.6011
1230	0.1051	0.1351	0.105	0.1351	0.059	0.0605	ND	1.1219	0.2913	0.4640
1231	0.0566	0.057	0.058	0.057	0.0745	0.1529	ND	0.6168	0.1501	0.2436
1232	0.056	0.061	0.066	0.061	0.076	0.1061	ND	0.5295	0.1523	0.2172
1233	0.0722	0.0668	0.063	0.C668	0.0734	0.0919	ND	0.8361	0.2903	0.3232
1234	0.0856	0.0682	0.086	0.C682	0.0703	0.0575	ND	1.164	0.3927	0.4335
1235	0.0565	0.0688	0.058	0.C688	0.0713	0.0547	ND	0.648	0.2045	0.2619
1236	0.0905	0.0981	0.1	0.C981	0.0712	0.1001	ND	1.4816	0.4691	0.5593
1237	0.1075	0.1501	0.14	0.1501	0.0741	0.0547	ND	1.5077	0.5662	0.6026
1238	0.1118	0.1694	0.13	0.1694	0.0762	0.0534	ND	1.5024	0.5431	0.6137
1239	0.0961	0.0877	0.115	0.C877	0.0755	0.051	ND	1.4138	0.5617	0.5297
1240	0.074	0.0465	0.048	0.C465	0.0672	0.0468	ND	0.7411	0.2309	0.2780
1241	0.0637	0.0613	0.054	0.C613	0.0706	0.0534	ND	0.5896	0.2442	0.2374
1242	0.0801	0.0551	0.066	0.C551	0.0687	0.0501	ND	1.185	0.3432	0.4317
1243	0.0489	0.0743	0.052	0.C743	0.0702	0.0585	ND	0.4026	0.1753	0.1837
1244	0.0658	0.0898	0.088	0.C898	0.0849	0.0886	ND	1.1825	0.3517	0.4540
1245	0.0437	0.0525	0.05	0.C525	0.0899	0.0621	ND	0.354	0.198	0.1530
1246	0.0915	0.1202	0.121	0.1202	0.0835	0.0683	ND	1.4024	0.5728	0.5476
1247	0.0519	0.054	0.053	0.054	0.0869	0.056	ND	0.3511	0.2163	0.1530
1248	0.0538	0.0529	0.05	0.C529	0.085	0.0533	ND	0.6254	0.2197	0.2437
1249	0.054	0.0604	0.071	0.C604	0.0727	0.0638	ND	0.2686	0.1958	0.1298
1250	0.0498	0.052	0.05	0.052	0.0783	0.061	ND	0.4175	0.2043	0.1738
1251	0.7164	0.0893	1.281	0.C893	0.1008	0.0816	ND	2.6399	2.2949	0.9395
1252	0.4948	0.0911	1.178	0.C911	0.328	0.1121	ND	2.6141	2.3564	0.9321
1253	2.9674	2.0803	2.457	2.C803	0.0885	0.1008	ND	2.9133	3.1299	2.3580
1254	2.538	2.2394	3.09	2.2394	0.0897	0.0923	ND	2.835	3.1713	2.4379
1255	2.8665	2.7791	3.148	2.7791	0.0871	0.0778	ND	2.9032	3.227	2.8205
1256	2.8432	1.2219	3.19	1.2219	0.0949	0.0832	ND	2.8499	3.2365	1.7646
1257	3.0299	0.5965	3.112	0.5965	0.1684	0.1345	ND	2.8652	3.2205	1.3527
1258	3.1963	0.7303	3.162	0.7303	0.0687	0.0793	ND	2.8689	3.2227	1.4432
1259	3.0929	0.9592	2.982	0.9592	0.0654	0.0825	ND	2.8042	3.2544	1.5742
1260	0.0859	0.0482	0.068	0.C482	0.0762	0.1101	ND	1.5692	0.4392	0.5552
1261	0.0694	0.0545	0.075	0.C545	0.0549	0.0839	ND	1.8636	0.4449	0.6575
1262	2.5715	3.0539	2.394	3.C539	0.6392	0.084	ND	2.8932	3.1093	3.0003
1263	2.9839	3.0974	3.084	3.C974	0.845	0.1029	ND	2.9696	3.2732	3.0548

FIG. 72F

Minimal epitope with trimer on ferritin (1)										
SEQ ID NO	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
1264	0.0742	0.0546	0.069	0.0546	0.0842	0.2906	ND	0.5426	0.161	0.2173
Domain III with trimer on ferritin (4)										
1265	0.5925	0.1983	0.864	0.1983	0.047	0.0535	ND	2.436	2.5114	0.9442
1266	1.6402	2.5253	2.263	2.5253	0.097	0.0591	ND	2.671	3.0447	2.5739
1267	0.8118	0.6634	1.349	0.6634	0.0617	0.0524	ND	2.4671	2.7241	1.2646
1268	0.9489	1.784	1.533	1.784	0.0972	0.0509	ND	2.5067	2.8226	2.0249
Minimal epitope on LS (2)										
1269	0.0636	0.0609	0.087	0.0609	0.0805	0.1106	ND	0.5099	0.1762	0.2106
1270	0.0575	0.0568	0.078	0.0568	0.2386	0.1018	ND	0.7801	0.1876	0.2979
1271	0.0578	0.1292	0.051	0.1292	0.0464	0.0994	ND	0.916	0.1568	0.3915
1272	0.0673	0.0555	0.06	0.0555	0.051	0.1013	ND	0.6001	0.1717	0.2370
1273	0.0645	0.0667	0.063	0.0667	0.0972	0.1059	ND	0.3102	0.152	0.1479
1274	0.0583	0.0493	0.057	0.0493	0.0716	0.1201	ND	0.4213	0.1671	0.1733
1275	1.4152	1.3084	1.816	1.3084	0.0853	0.0692	ND	2.5677	2.8052	1.7282
1276	3.0125	2.8056	2.934	2.8056	0.2304	0.1167	ND	2.8184	3.222	2.8099
1277	0.0467	0.0755	0.05	0.0755	0.0683	0.0682	ND	0.9456	0.1632	0.3655
1278	0.0511	0.0563	0.052	0.0563	0.0586	0.0705	ND	0.6354	0.1483	0.2493
Minimal epitope on ferritin (10)										
1279	0.4585	0.3412	0.516	0.3412	0.0572	0.0542	ND	2.0056	1.4897	0.8960
1280	0.0868	0.0545	0.074	0.0545	0.0569	0.0485	ND	0.5017	0.1722	0.2036
Domain III on LS (2)										
1281	3.005	3.0975	3.039	2.2394	1.0938	0.2008	ND	2.8081	3.2029	2.7150
1282	2.6493	2.162	2.756	2.7791	0.1031	0.0552	ND	2.7199	3.101	2.5537
Domain III on hcp1 (4)										
1283	2.6893	1.3892	2.374	1.2219	0.3614	0.1108	ND	2.7309	3.2143	1.7973
1284	1.9742	1.3049	1.746	0.5965	0.124	0.0564	ND	2.7153	3.2268	1.5389
1285	2.0274	0.5993	2.258	0.7303	0.212	0.0755	ND	2.7217	3.2638	1.3504
1286	2.1559	0.6031	1.812	0.9592	0.0736	0.0595	ND	2.7465	3.2466	1.4363

FIG. 73

Single chain DS-Cav1 or Cleaved Stabilized Prefusion F on ferritin is Immunogenic

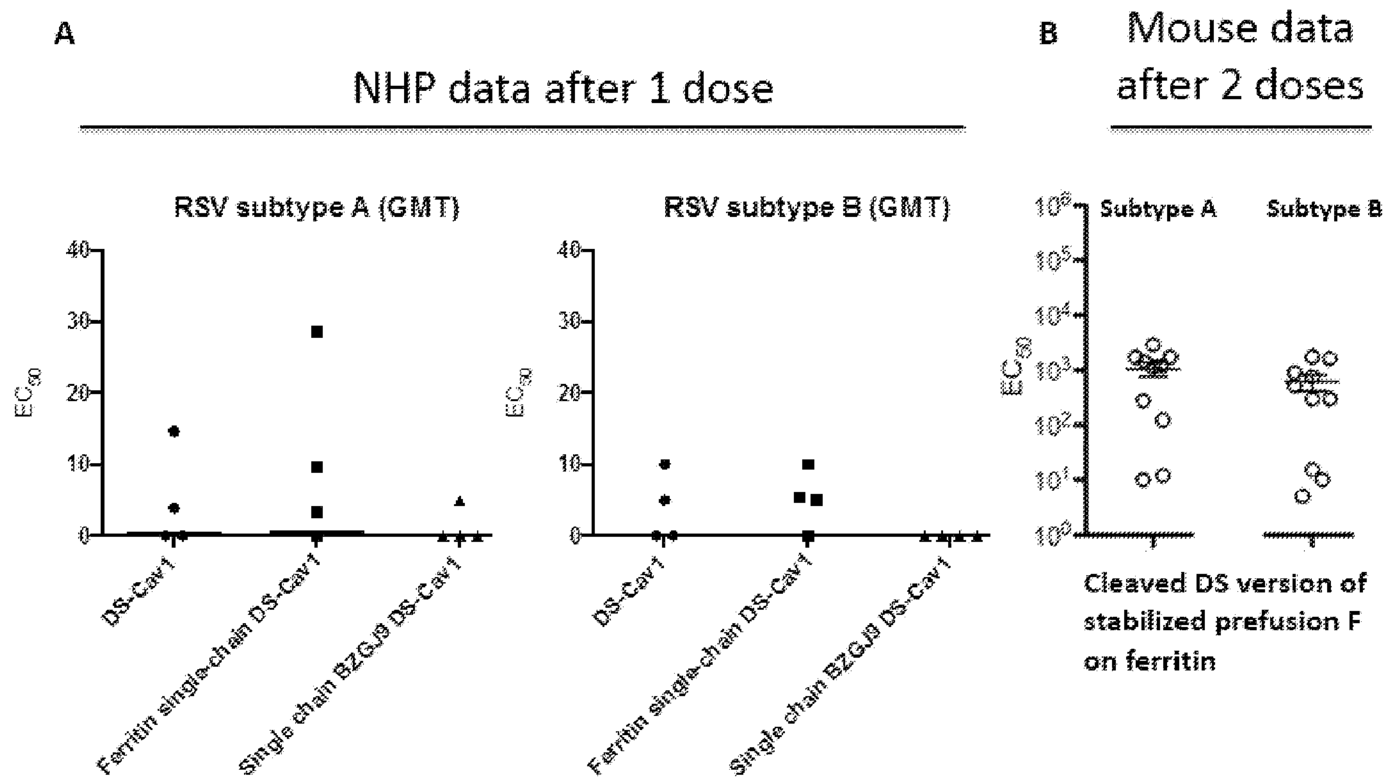


FIG. 74

Antibody response is durable in mice after 2 doses

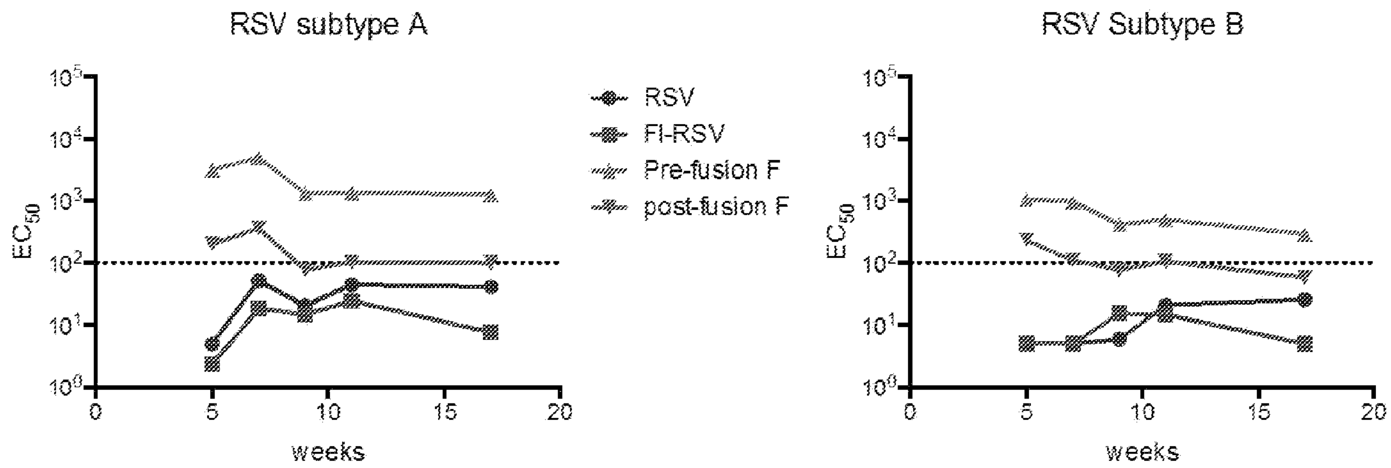


FIG. 75

DS immunization can prevent RSV infection in mice

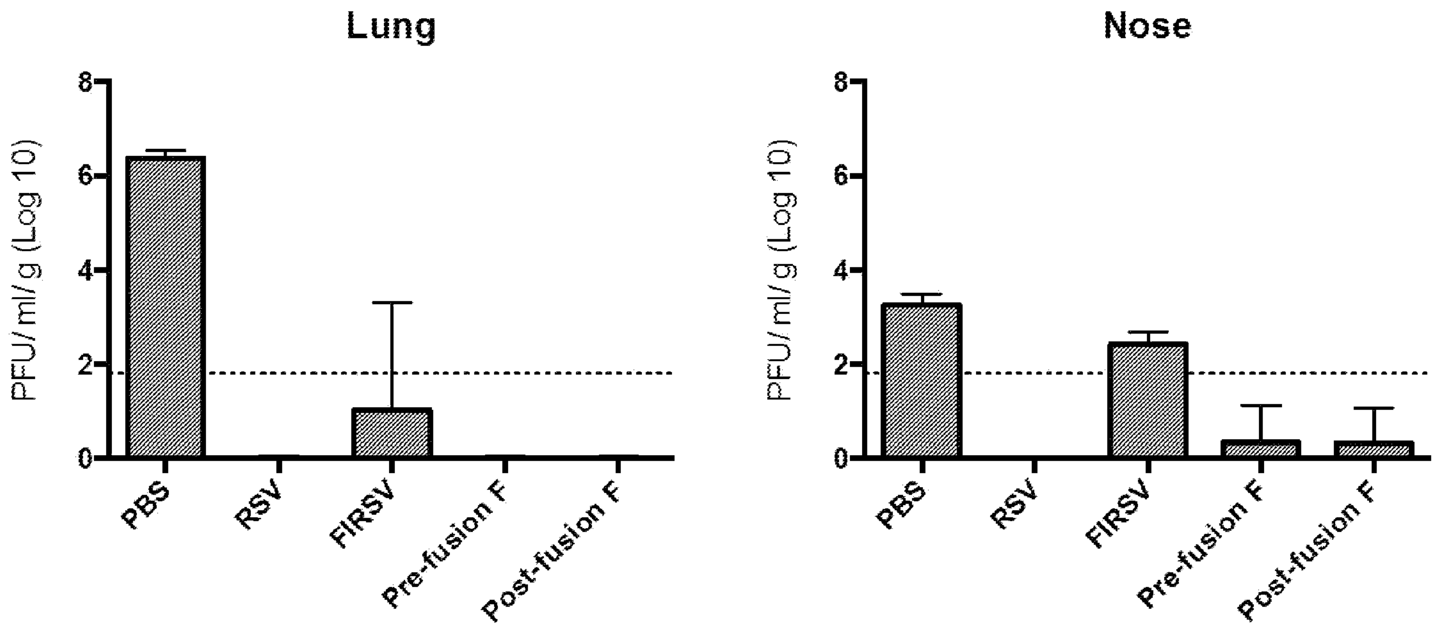


FIG. 76

DS immunization does not induce Type 2 cytokine responses in mice post-challenge

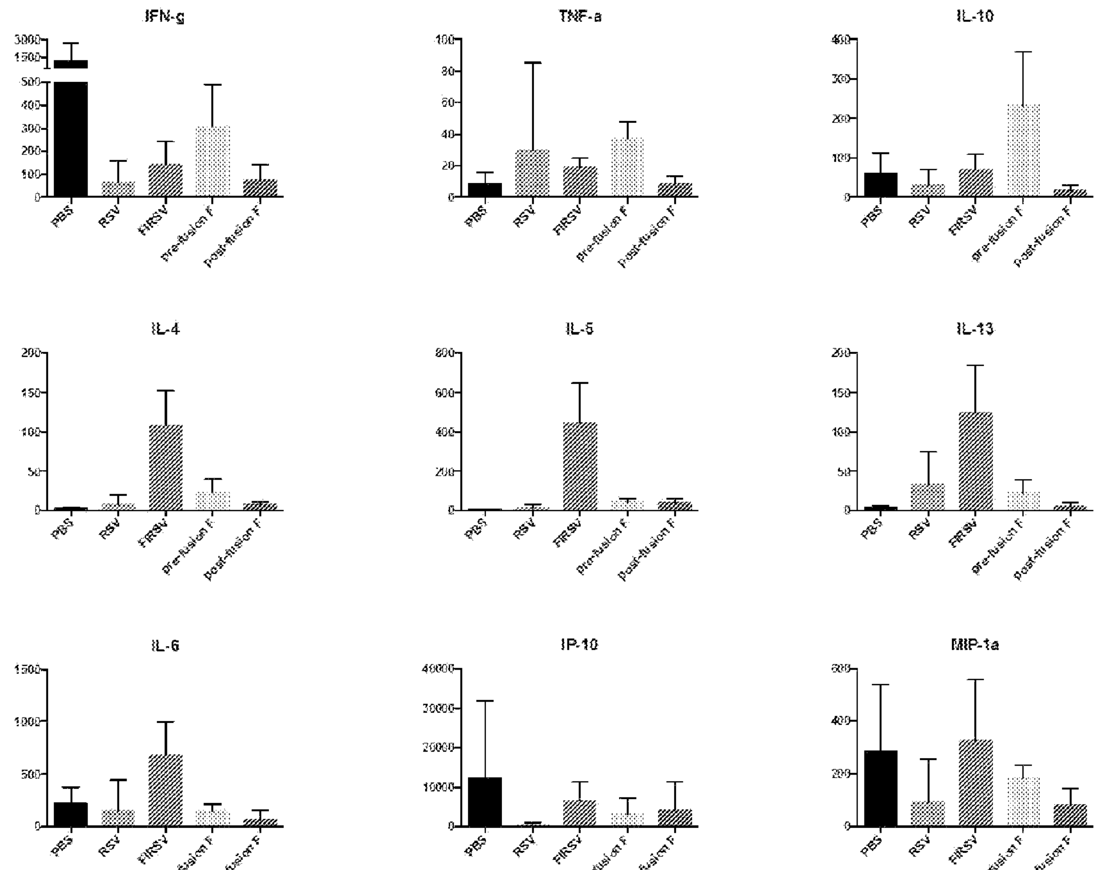


FIG. 77

Neutralizing activity is boosted and sustained after 3rd dose in NHP

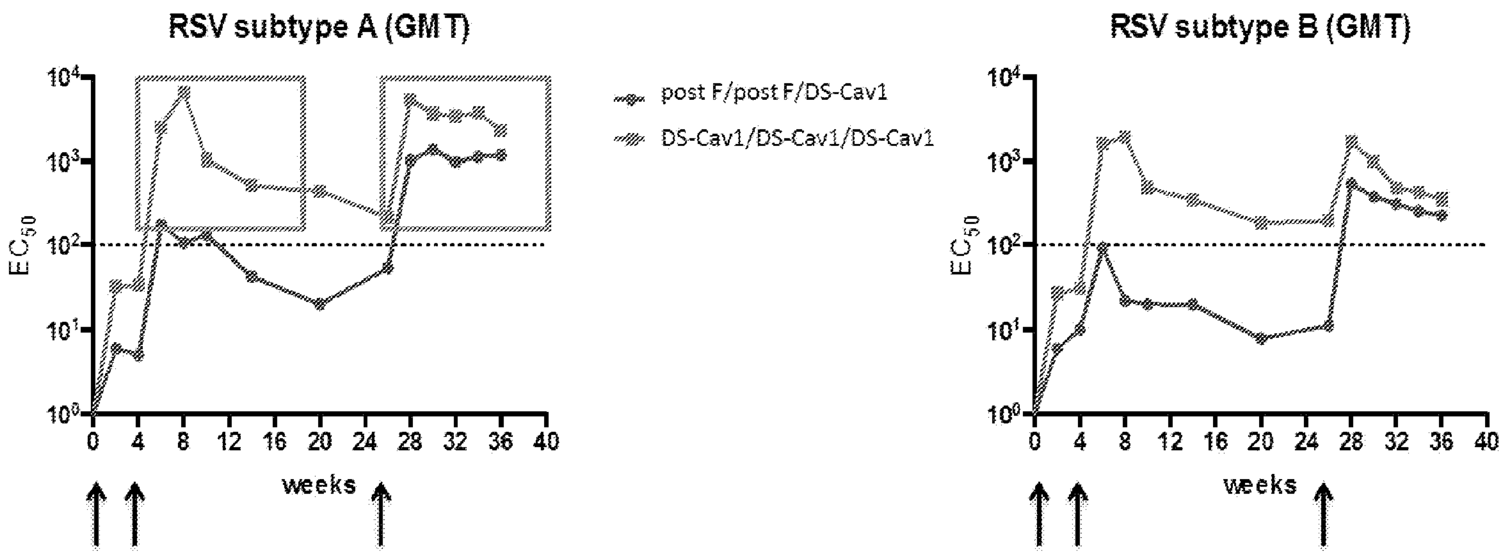


FIG. 78

DS-CAV1 can be effectively formulated in alum and retain immunogenicity

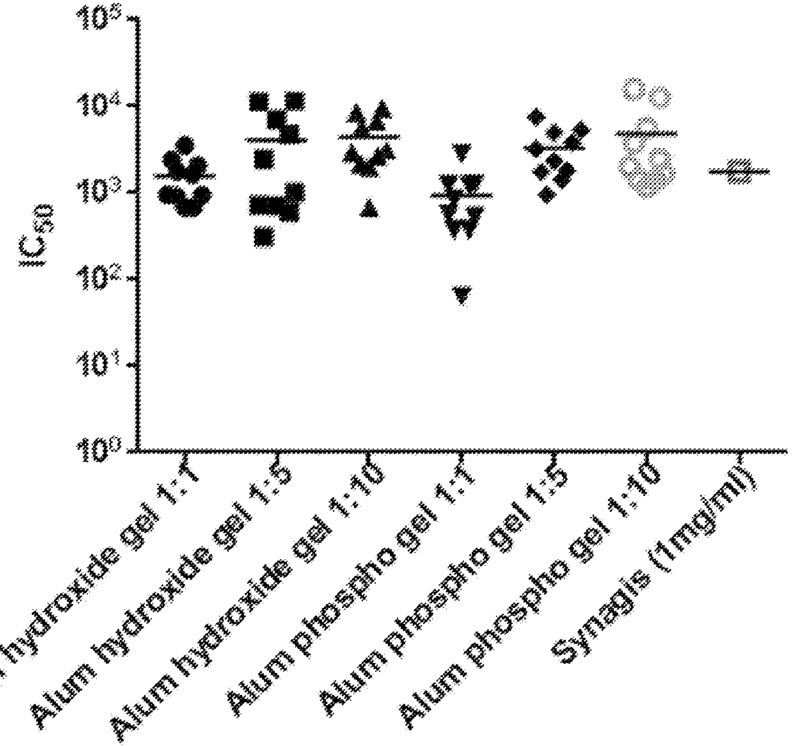
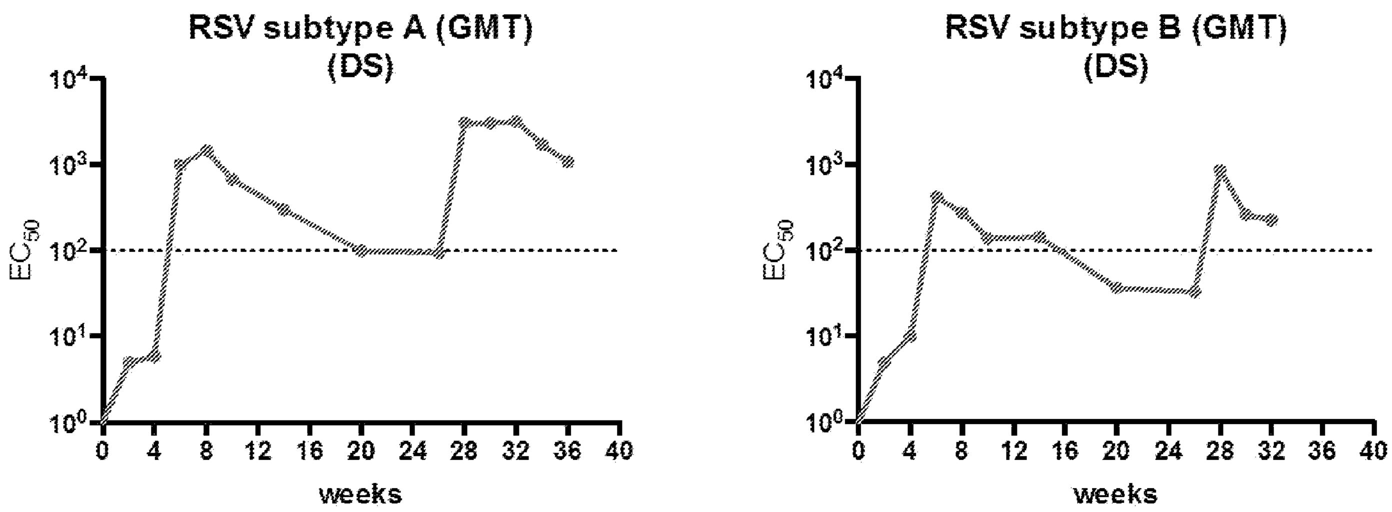


FIG. 79

Alum is an effective adjuvant in NHP



Immunization: w0, w4, w26
DS+polyI:C/DS+poly I:C/DS+Alum

FIG. 80

DS-CAV1 is immunogenic when expressed from a gene-based vector either alone or as priming for a protein boost

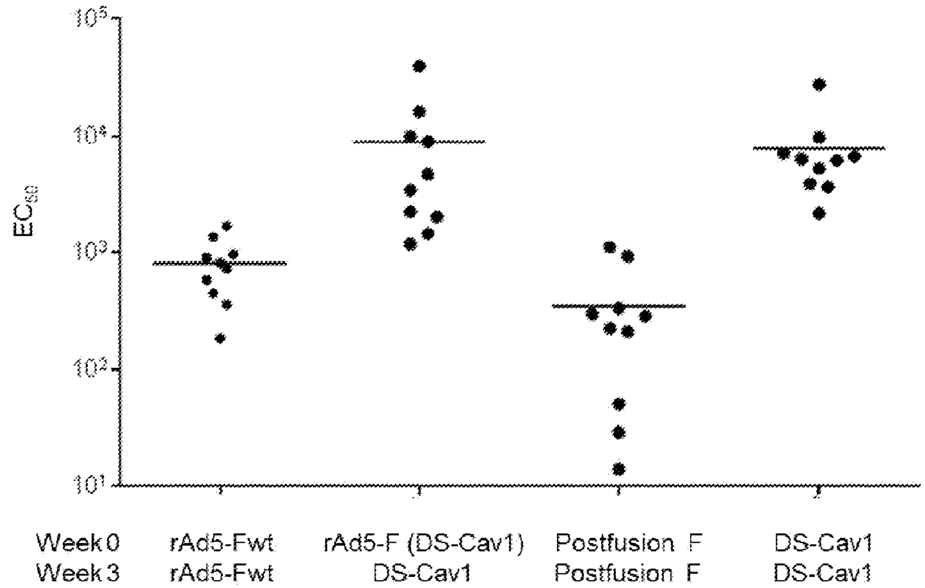
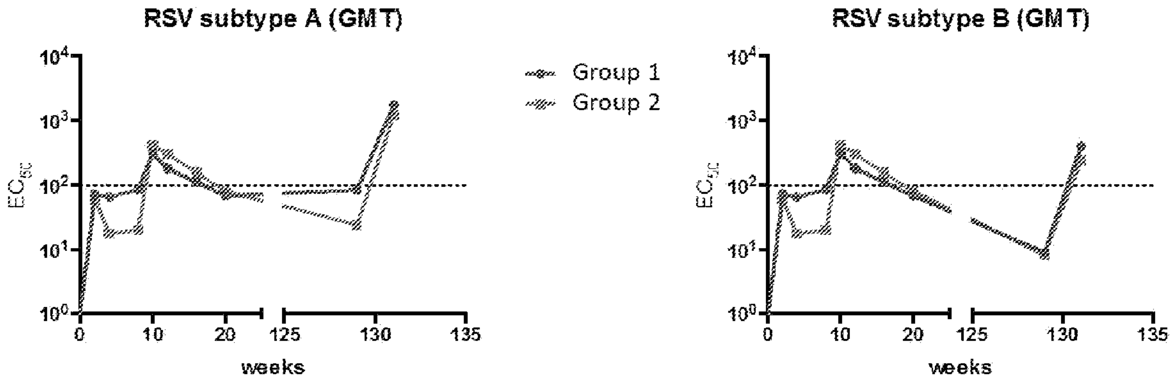


FIG. 81

DS-Cav1 RSV F Subtype A or B Boosts rAd-F(A)WT-Primed NHP



Group	N	Week 0	Week 8	Week 129
1	6	rAd5-F	rAd5-F	DS-Cav1-A in alum
				DS-Cav1-B in alum
2	6	sAd7-F	rAd5-F	DS-Cav1-A in alum
				DS-Cav1-B in alum

FIG. 82

DS-Cav1 RSV F Subtype A or B Boosts rAd-F(A)WT-Primed NHP

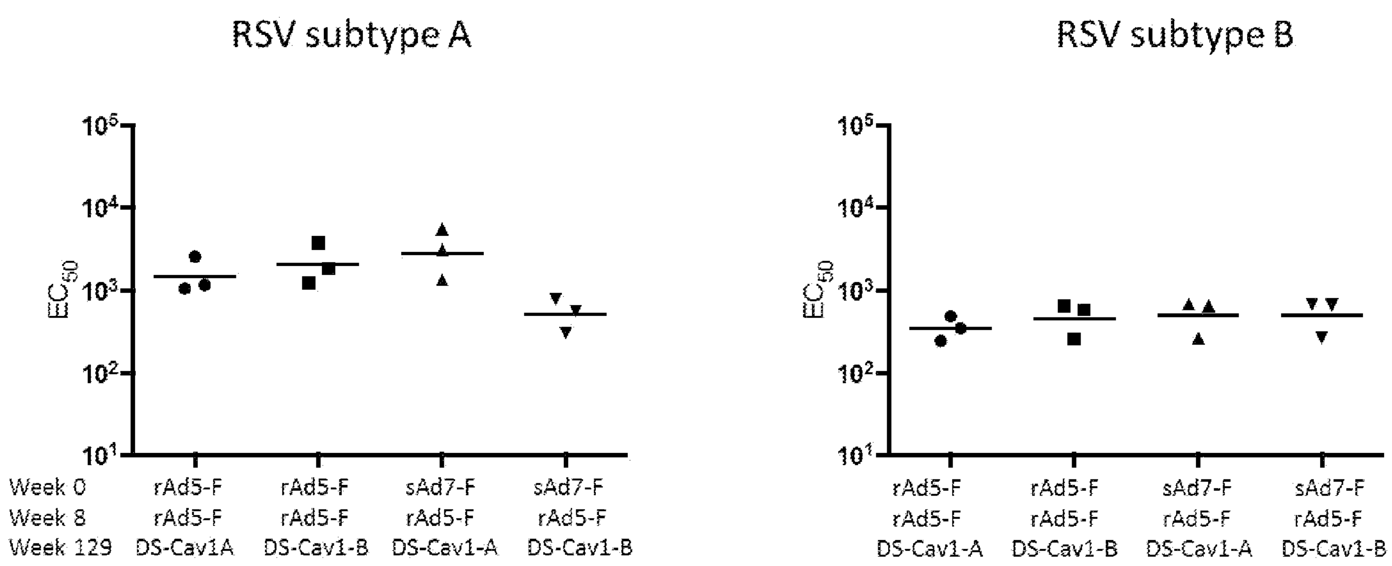


FIG. 83

Immunization with DS version of stabilized prefusion F subtype A or B or both induces neutralizing activity against both subtypes

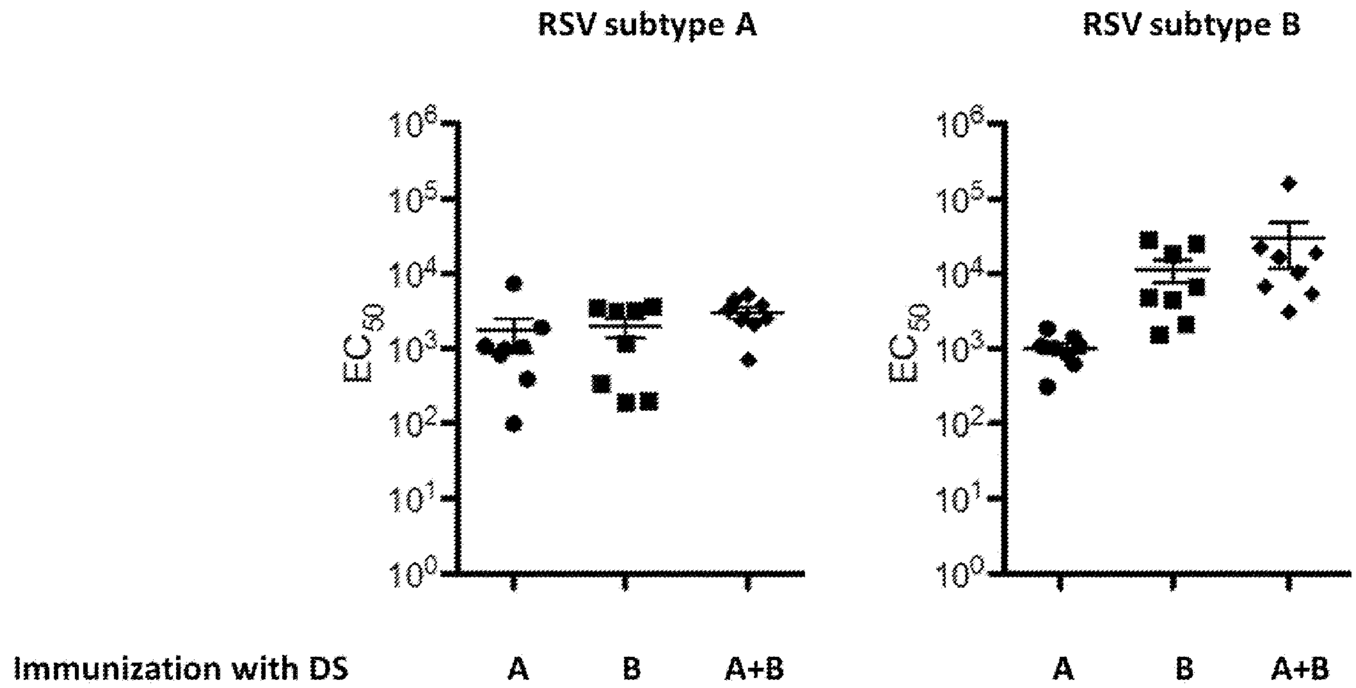
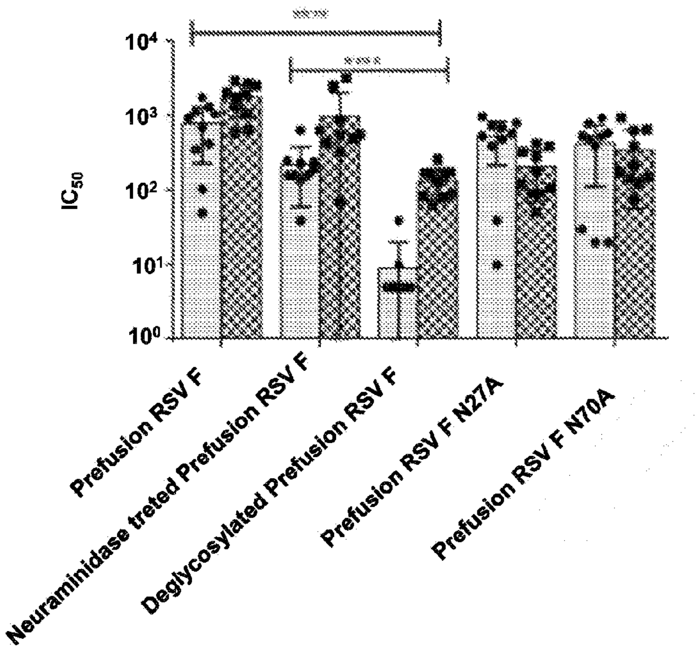


FIG. 84

Altering glycosylation reduces immunogenicity of stabilized prefusion F



SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

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