RECOMBINANT NANOPARTICLE RSV F VACCINE FOR RESPIRATORY SYNCYTIAL VIRUS

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims priority to U.S. Provisional Application Serial No. 61/542,040, filed September 30, 2011, U.S. Provisional Application Serial No. 61/542,721 filed October 3, 2011, U.S. Provisional Application Serial No. 61/611,834 filed March 16, 2012, and to 61/614,286 filed March 22, 2012, the disclosures of which are each incorporated by reference in their entirety for all purposes.

[002] The contents of the text file submitted electronically are incorporated herein by reference in their entirety: A computer readable format copy of the Sequence Listing (filename: NOVV_048_04WO_SeqList.txt, date recorded: September 27, 2012; file size: 74 kilobytes).

TECHNICAL FIELD

[003] The present invention is generally related to modified or mutated respiratory syncytial virus fusion (F) proteins and methods for making and using them, including immunogenic compositions such as vaccines for the treatment and/or prevention of RSV infection.

BACKGROUND OF THE INVENTION

[004] Respiratory syncytial virus (RSV) is a member of the genus *Pneumovirus* of the family *Paramyxoviridae*. Human RSV (HRSV) is the leading cause of severe lower respiratory tract disease in young children and is responsible for considerable morbidity and mortality in humans. RSV is also recognized as an important agent of disease in immunocompromised adults and in the elderly. Due to incomplete resistance to RSV in the infected host after a natural infection, RSV may infect multiple times during childhood and adult life.

[005] This virus has a genome comprised of a single strand negative-sense RNA, which is tightly associated with viral protein to form the nucleocapsid. The viral envelope is composed of a plasma membrane derived lipid bilayer that contains virally encoded structural proteins. A viral polymerase is packaged with the virion and transcribes genomic RNA into mRNA. The RSV genome encodes three transmembrane structural proteins, F, G, and SH,

two matrix proteins, M and M2, three nucleocapsid proteins N, P, and L, and two nonstructural proteins, NS1 and NS2.

[006] Fusion of HRSV and cell membranes is thought to occur at the cell surface and is a necessary step for the transfer of viral ribonucleoprotein into the cell cytoplasm during the early stages of infection. This process is mediated by the fusion (F) protein, which also promotes fusion of the membrane of infected cells with that of adjacent cells to form a characteristic syncytia, which is both a prominent cytopathic effect and an additional mechanism of viral spread. Accordingly, neutralization of fusion activity is important in host immunity. Indeed, monoclonal antibodies developed against the F protein have been shown to neutralize virus infectivity and inhibit membrane fusion (Calder *et al.*, 2000, *Virology* 271: 122-131).

[007] The F protein of RSV shares structural features and limited, but significant amino acid sequence identity with F glycoproteins of other paramyxoviruses. It is synthesized as an inactive precursor of 574 amino acids (F0) that is cotranslationally glycosylated on asparagines in the endoplasmic reticulum, where it assembles into homo-oligomers. Before reaching the cell surface, the F0 precursor is cleaved by a protease into F2 from the N terminus and F1 from the C terminus. The F2 and F1 chains remain covalently linked by one or more disulfide bonds.

[008] Immunoaffinity purified full-length F proteins have been found to accumulate in the form of micelles (also characterized as rosettes), similar to those observed with other full-length virus membrane glycoproteins (Wrigley *et al.*, 1986, in *Electron Microscopy of Proteins*, Vol 5, p. 103-163, Academic Press, London). Under electron microscopy, the molecules in the rosettes appear either as inverted cone-shaped rods (~70%) or lollipop-shaped (~30%) structures with their wider ends projecting away from the centers of the rosettes. The rod conformational state is associated with an F glycoprotein in the pre-fusion inactivate state while the lollipop conformational state is associated with an F glycoprotein in the post-fusion, active state.

[009] Electron micrography can be used to distinguish between the prefusion and postfusion (alternatively designated prefusogenic and fusogenic) conformations, as demonstrated by Calder *et al.*, 2000, *Virology* 271:122-131. The prefusion conformation can also be distinguished from the fusogenic (postfusion) conformation by liposome association assays. Additionally, prefusion and fusogenic conformations can be distinguished using antibodies (e.g., monoclonal antibodies) that specifically recognize conformation epitopes present on one or the other of the prefusion or fusogenic form of the RSV F protein, but not on the other

form. Such conformation epitopes can be due to preferential exposure of an antigenic determinant on the surface of the molecule. Alternatively, conformational epitopes can arise from the juxtaposition of amino acids that are non-contiguous in the linear polypeptide.

[010] It has been shown previously that the F precursor is cleaved at two sites (site I, after residue 109 and site II, after residue 136), both preceded by motifs recognized by furin-like proteases. Site II is adjacent to a fusion peptide, and cleavage of the F protein at both sites is needed for membrane fusion (Gonzalez-Reyes *et al.*, 2001, *PNAS* 98(17): 9859-9864). When cleavage is completed at both sites, it is believed that there is a transition from cone-shaped to lollipop-shaped rods.

SUMMARY OF THE INVENTION

[011] As described herein, the present inventors have found that surprisingly high levels of expression of the fusion (F) protein can be achieved when certain modifications are made to the structure of the RSV F protein. Such modifications also unexpectedly reduce the cellular toxicity of the RSV F protein in a host cell. In addition, the modified F proteins of the present invention demonstrate an improved ability to exhibit the post-fusion "lollipop" morphology as opposed to the pre-fusion "rod" morphology. Thus, in one aspect, the modified F proteins of the present invention can also exhibit improved immunogenicity as compared to wild-type F proteins. These modifications have significant applications to the development of vaccines and methods of using said vaccines for the treatment and/or prevention of RSV. The present invention provides recombinant RSV F proteins that demonstrate increased expression, reduced cellular toxicity, and/or enhanced immunogenic properties as compared to wild-type RSV F proteins.

[012] In one aspect, the invention provides recombinant RSV F proteins comprising modified or mutated amino acid sequences as compared to wild-type RSV F proteins. In general, these modifications or mutations increase the expression, reduce the cellular toxicity, and/or enhance the immunogenic properties of the RSV F proteins as compared to wild-type RSV F proteins. In certain exemplary embodiments, the RSV F proteins are human RSV F proteins.

[013] The RSV F protein preferably comprises a modified or mutated amino acid sequence as compared to the wild-type RSV F protein (*e.g.* as exemplified in SEQ ID NO: 2). In one embodiment, the RSV F protein contains a modification or mutation at the amino acid corresponding to position P102 of the wild-type RSV F protein (SEQ ID NO: 2). In another

embodiment, the RSV F protein contains a modification or mutation at the amino acid corresponding to position I379 of the wild-type RSV F protein (SEQ ID NO: 2). In another embodiment, the RSV F protein contains a modification or mutation at the amino acid corresponding to position M447 of the wild-type RSV F protein (SEQ ID NO: 2).

[014] In one embodiment, the RSV F protein contains two or more modifications or mutations at the amino acids corresponding to the positions described above. In another embodiment, the RSV F protein contains three modifications or mutations at the amino acids corresponding to the positions described above.

[015] In one specific embodiment, the invention is directed to RSV F proteins wherein the proline at position 102 is replaced with alanine. In another specific embodiment, the invention is directed to RSV F proteins wherein the isoleucine at position 379 is replaced with valine. In yet another specific embodiment, the invention is directed to RSV F proteins wherein the methionine at position 447 is replaced with valine. In certain embodiments, the RSV F protein contains two or more modifications or mutations at the amino acids corresponding to the positions described in these specific embodiments. In certain other embodiments, the RSV F protein contains three modifications or mutations at the amino acids corresponding to the positions described in these specific embodiments. In an exemplary embodiment, the RSV protein has the amino acid sequence described in SEQ ID NO: 4.

[016] In one embodiment, the coding sequence of the RSV F protein is further optimized to enhance its expression in a suitable host cell. In one embodiment, the host cell is an insect cell. In an exemplary embodiment, the insect cell is an Sf9 cell.

[017] In one embodiment, the coding sequence of the codon optimized RSV F gene is SEQ ID NO: 3. In another embodiment, the codon optimized RSV F protein has the amino acid sequence described in SEQ ID NO: 4.

[018] In one embodiment, the RSV F protein further comprises at least one modification in the cryptic poly(A) site of F2. In another embodiment, the RSV F protein further comprises one or more amino acid mutations at the primary cleavage site (CS). In one embodiment, the RSV F protein contains a modification or mutation at the amino acid corresponding to position R133 of the wild-type RSV F protein (SEQ ID NO: 2) or the codon optimized RSV F protein (SEQ ID NO: 4). In another embodiment, the RSV F protein contains a modification or mutation at the amino acid corresponding to position R135 of the wild-type RSV F protein (SEQ ID NO: 2) or the codon optimized RSV F protein (SEQ ID NO: 4). In yet another embodiment, the RSV F protein contains a modification or mutation at the amino

acid corresponding to position R136 of the wild-type RSV F protein (SEQ ID NO: 2) or the codon optimized RSV F protein (SEQ ID NO: 4).

[019] In one specific embodiment, the invention is directed to RSV F proteins wherein the arginine at position 133 is replaced with glutamine. In another specific embodiment, the invention is directed to RSV F proteins wherein the arginine at position 135 is replaced with glutamine. In yet another specific embodiment, the invention is directed to RSV F proteins wherein arginine at position 136 is replaced with glutamine. In certain embodiments, the RSV F protein contains two or more modifications or mutations at the amino acids corresponding to the positions described in these specific embodiments. In certain other embodiments, the RSV F protein contains three modifications or mutations at the amino acids corresponding to the positions described in these specific embodiments. In an exemplary embodiment, the RSV protein has the amino acid sequence described in SEQ ID NO: 6.

[020] In another embodiment, the RSV F protein further comprises a deletion in the N-terminal half of the fusion domain corresponding to amino acids 137-146 of SEQ ID NO: 2, SEQ ID NO: 4, and SEQ ID NO: 6. In an exemplary embodiment, the RSV F protein has the amino acid sequence described in SEQ ID NO: 8. In an alternative embodiment, the RSV F protein has the amino acid sequence described in SEQ ID NO: 10.

[021] Further included within the scope of the invention are RSV F proteins, other than human RSV F protein (SEQ ID NO: 2), which contain alterations corresponding to those set out above. Such RSV F proteins may include, but are not limited to, the RSV F proteins from A strains of human RSV, B strains of human RSV, strains of bovine RSV, and strains of avian RSV.

[022] In some embodiments, the invention is directed to modified or mutated RSV F proteins that demonstrate increased expression in a host cell as compared to wild-type RSV F proteins, such as the one shown by SEQ ID NO: 2. In other embodiments, the invention is directed to modified or mutated RSV F proteins that demonstrate reduced cellular toxicity in a host cell as compared to wild-type RSV F proteins, such as the one shown by SEQ ID NO: 2. In yet other embodiments, the invention is directed to modified or mutated RSV F proteins that demonstrate enhanced immunogenic properties as compared to wild-type RSV F proteins, such as the one shown by SEQ ID NO: 2.

[023] In additional aspects, the invention provides immunogenic compositions comprising one or more modified or mutated RSV F proteins as described herein. In one embodiment, the invention provides a micelle comprised of one or more modified or mutated RSV F proteins (*e.g.* an RSV F micelle).

[024] In another embodiment, the present invention provides a virus-like particle (VLP) comprising a modified or mutated RSV F protein. In some embodiments, the VLP further comprises one or more additional proteins.

[025] In one embodiment, the VLP further comprises a matrix (M) protein. In one embodiment, the M protein is derived from a human strain of RSV. In another embodiment, the M protein is derived from a bovine strain of RSV. In other embodiments, the matrix protein may be an M1 protein from an influenza virus strain. In one embodiment, the influenza virus strain is an avian influenza virus strain. In other embodiments, the M protein may be derived from a Newcastle Disease Virus (NDV) strain.

[026] In additional embodiments, the VLP further comprises the RSV glycoprotein G. In another embodiment, the VLP further comprises the RSV glycoprotein SH. In yet another embodiment, the VLP further comprises the RSV nucleocapsid N protein.

[027] The modified or mutated RSV F proteins may be used for the prevention and/or treatment of RSV infection. Thus, in another aspect, the invention provides a method for eliciting an immune response against RSV. The method involves administering an immunologically effective amount of a composition containing a modified or mutated RSV F protein to a subject, such as a human or animal subject.

[028] In another aspect, the present invention provides pharmaceutically acceptable vaccine compositions comprising a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein.

[029] In one embodiment, the invention comprises an immunogenic formulation comprising at least one effective dose of a modified or mutated RSV F protein. In another embodiment, the invention comprises an immunogenic formulation comprising at least one effective dose of an RSV F micelle comprising a modified or mutated RSV F protein. In yet another embodiment, the invention comprises an immunogenic formulation comprising at least one effective dose of a VLP comprising a modified or mutated RSV F protein.

[030] In another embodiment, the invention provides for a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the vaccine formulations of the invention.

[031] In another embodiment, the invention provides a method of formulating a vaccine or antigenic composition that induces immunity to an infection or at least one disease symptom thereof to a mammal, comprising adding to the formulation an effective dose of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein,

or a VLP comprising a modified or mutated RSV F protein. In a preferred embodiment, the infection is an RSV infection.

[032] The modified or mutated RSV F proteins of the invention are useful for preparing compositions that stimulate an immune response that confers immunity or substantial immunity to infectious agents. Thus, in one embodiment, the invention provides a method of inducing immunity to infections or at least one disease symptom thereof in a subject, comprising administering at least one effective dose of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein.

[033] In yet another aspect, the invention provides a method of inducing substantial immunity to RSV virus infection or at least one disease symptom in a subject, comprising administering at least one effective dose of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein.

[034] Compositions of the invention can induce substantial immunity in a vertebrate (*e.g.* a human) when administered to the vertebrate. Thus, in one embodiment, the invention provides a method of inducing substantial immunity to RSV virus infection or at least one disease symptom in a subject, comprising administering at least one effective dose of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein. In another embodiment, the invention provides a method of vaccinating a mammal against RSV comprising administering to the mammal a protection-inducing amount of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein.

[035] In another embodiment, the invention comprises a method of inducing a protective antibody response to an infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein.

[036] In another embodiment, the invention comprises a method of inducing a protective cellular response to RSV infection or at least one disease symptom in a subject, comprising administering at least one effective dose of a modified or mutated RSV F protein. In another embodiment, the invention comprises a method of inducing a protective cellular response to RSV infection or at least one disease symptom in a subject, comprising administering at least

one effective dose of an RSV F micelle comprising a modified or mutated RSV F protein. In yet another embodiment, the invention comprises a method of inducing a protective cellular response to RSV infection or at least one disease symptom in a subject, comprising administering at least one effective dose of a VLP, wherein the VLP comprises a modified or mutated RSV F protein.

[037] In yet another aspect, the invention provides an isolated nucleic acid encoding a modified or mutated RSV F protein of the invention. In an exemplary embodiment, the isolated nucleic acid encoding a modified or mutated RSV F protein is selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, or SEQ ID NO: 9.

[038] In yet another aspect, the invention provides an isolated cell comprising a nucleic acid encoding a modified or mutated RSV F protein of the invention. In an exemplary embodiment, the isolated nucleic acid encoding a modified or mutated RSV F protein is selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, or SEQ ID NO: 9.

[039] In yet another aspect, the invention provides a vector comprising a nucleic acid encoding a modified or mutated RSV F protein of the invention. In an exemplary embodiment, the isolated nucleic acid encoding a modified or mutated RSV F protein is selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, or SEQ ID NO: 9. In one embodiment, the vector is a baculovirus vector.

[040] In yet another aspect, the invention provides a method of making a RSV F protein, comprising (a) transforming a host cell to express a nucleic acid encoding a modified or mutated RSV F protein of the invention; and (b) culturing said host cell under conditions conducive to the production of said RSV F protein. In one embodiment, the nucleic acid encoding a modified or mutated RSV F protein is selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, or SEQ ID NO: 9. In another embodiment, the host cell is an insect cell. In a further embodiment, the host cell is an is an insect cell transfected with a baculovirus vector comprising a modified or mutated RSV F protein of the invention.

[041] In yet another aspect, the invention provides a method of making a RSV F protein micelle, comprising (a) transforming a host cell to express a nucleic acid encoding a modified or mutated RSV F protein of the invention; and (b) culturing said host cell under conditions conducive to the production of said RSV F protein micelle. In one embodiment, the nucleic acid encoding a modified or mutated RSV F protein is selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, or SEQ ID NO: 9. In one embodiment, the

host cell is an insect cell. In an exemplary embodiment, the host cell is an is an insect cell transfected with a baculovirus vector comprising a modified or mutated RSV F protein of the invention.

[042] In one aspect, the present invention is directed to an RSV fusion surface glycoprotein (F) nanoparticle vaccine. In one embodiment, the vaccine comprises full length F protein. In a further embodiment, the full length F protein is cleaved into disulfide linked F1 and F2 trimers. The F1 and F2 trimers, in one embodiment, are present in micelles having a diameter of about 20 nm to about 40 nm.

[043] In another aspect, an antibody generated by the vaccine of the invention is provided.

[044] In yet another aspect, a method for vaccinating a subject in need thereof is provided. In one embodiment, the method comprises administering to the subject a recombinant RSV fusion glycoprotein (F) nanoparticle vaccine. In a further embodiment, the nanoparticle vaccine comprises full length F protein. In even a further embodiment, the full length F protein is cleaved into disulfide linked F1 and F2 trimers. The F1 and F2 trimers, in one embodiment, are present in micelles having a diameter of about 20 nm to about 40 nm.

[045] In one embodiment, the vaccine of the invention is administered at a dose is selected from the group consisting of 5 μg, 15 μg, 30 μg and 60 μg.

[046] In another aspect, a method for vaccinating a subject in need thereof is provided. In one embodiment, the method comprises administering to the subject a recombinant RSV fusion glycoprotein (F) nanoparticle vaccine comprising full length F protein and an adjuvant. In a further embodiment, the adjuvant is alum.

BRIEF DESCRIPTION OF THE FIGURES

[047] **Figure 1** depicts the structure of wild type HRSV F_0 protein and the primary (SEQ ID NO: 32) and secondary (SEQ ID NO: 33) cleavage sites.

[048] **Figure 2** depicts structures of modified RSV F_0 proteins with cleavage site mutations as described in Example 3, corresponding to SEQ ID NOs: 28 (KKQKQQ), 29 (GRRQQR), 30 (RAQQ), and 31 (KKQKRQ).

[049] **Figure 3** depicts conservative substitutions (R133Q, R135Q and R136Q) in the primary cleavage site of modified HRSV F protein BV #541 (SEQ ID NO: 6).

[050] **Figure 4** depicts sequence and structure of modified HRSV F protein BV #541 (SEQ ID NO: 6).

[051] **Figure 5** depicts sequence and structure of modified HRSV F protein BV #622 (SEQ ID NO: 10).

[052] **Figure 6** depicts SDS-PAGE coomassie-stained gel of purified recombinant HRSV F protein BV #622 with or without the presence of βME.

[053] **Figure 7A** depicts a Western blot analysis of the RSV F fusion domain mutants. **Figure 7B** depicts cell surface RSV F protein immunostaining of the RSV F fusion domain mutants. **Figure 7C** depicts the structure of modified HRSV F protein BV #683 (SEQ ID NO: 8). **Figure 7D** depicts the parent clone BV#541 (Δ0) and mutants with Δ2 Δ4, Δ6, Δ8, Δ10 (BV#683), Δ12, Δ14, Δ16, and Δ18 deletions in the fusion domain. BV#541 comprises a protein in which the amino acid sequence of the fusion domain comprises position 137 to 154 of SEQ ID NO: 6). The amino acid sequences of the fusion domain portions of the deletion mutants comprise position 139 to 154 of SEQ ID NO: 6 (Δ2), position 141 to 154 of SEQ ID NO: 6 (Δ4), position 143 to 154 of SEQ ID NO: 6 (Δ6), position 145 to 154 of SEQ ID NO: 6 (Δ8), position 147 to 154 of SEQ ID NO: 6 (Δ10; BV#683), position 149 to 154 of SEQ ID NO: 6 (Δ12), position 151 to 154 of SEQ ID NO: 6(Δ14), or position 153 to 154 of SEQ ID NO: 6 (Δ16). The entire fusion domain corresponding to position 137-154 in SEQ ID NO: 6 is deleted in the mutant with a Δ18 deletion.

[054] **Figure 8** depicts SDS-PAGE coomassie-stained gels of purified recombinant HRSV F proteins BV #622 and BV #683 with or without the presence of βME (on the left), and their structures.

[055] **Figure 9** depicts SDS-PAGE coomassie-stained gel (on the left) and Western Blot (on the right) analysis of purified recombinant HRSV F protein BV #683 with or without the presence of βME.

[056] **Figure 10** depicts SDS-PAGE coomassie-stained gel used in purity analysis by scanning densitometry (on the left) and Western Blot (on the right) of purified recombinant HRSV F protein BV #683.

[057] **Figure 11** depicts images of purified recombinant HRSV F protein BV #683 micelles (rosettes) taken in negative stain electron microscopy.

[058] **Figure 12A** depicts reverse phase HPLC analysis of HRSV F protein BV #683. **Figure 12B** depicts size exclusion HPLC analysis of HRSV F protein BV #683. **Figure 12C** depicts particle size analysis of HRSV F protein BV #683 micelles.

[059] **Figure 13** depicts SDS-PAGE coomassie-stained gel (on the left) and Western Blot (on the right) analysis of modified HRSV F proteins BV #622 and BV #623 (SEQ ID NO: 21) with or without co-expression with HRSV N and BRSV M proteins in the crude cell

culture harvests (intracellular) or pelleted samples by 30% sucrose gradient separation, and structures of BV #622 and BV #623.

[060] **Figure 14** depicts SDS-PAGE coomassie-stained gel (on the left) and Western Blot (on the right) analysis of modified HRSV F protein BV #622, double tandem chimeric BV #636 (BV #541 + BRSV M), BV #683, BV #684 (BV #541 with YIAL L-domain), and BV #685 (BV #541 with YKKL L-domain) with or without co-expression with HRSV N and BRSV M proteins in the crude cell culture harvests (intracellular) samples, and structure of each analyzed modified HRSV F protein.

[061] **Figure 15** depicts SDS-PAGE coomassie-stained gel (on the left) and Western Blot (on the right) analysis of modified RSV F protein BV #622 (SEQ ID NO: 10), double tandem chimeric BV #636 (BV #541 +BRSV M), BV #683 (SEQ ID NO: 8), BV #684 (BV #541 with YIAL L-domain), and BV #685 (BV #541 with YKKL L-domain) with or without co-expression with HRSV N and BRSV M proteins in the pelleted samples by 30% sucrose gradient separation, and structure of each analyzed modified HRSV F protein.

[062] **Figure 16 A-D** depict structure, clone name, description, Western Blot and SDS-PAGE coomassie results, and conclusion for each modified RSV F protein as described in Example 9.

[063] **Figure 17** depicts experimental procedures of the RSV challenge study as described in Example 10.

[064] **Figure 18** depicts results of RSV neutralization assay at day 31 and day 46 of mice immunized with PBS, live RSV, FI-RSV, 1 μg PFP, 1 μg PFP + Alum, 10 μg PFP, 10 μg PFP + Alum, 30 μg PFP, and positive control (anti-F sheep).

[065] **Figure 19** depicts RSV titers in lung tissues of mice immunized with PBS, live RSV, FI-RSV, 1 μg PFP, 1 μg PFP + Alum, 10 μg PFP, 10 μgPFP + Alum, and 30 μg PFP, 4 days after challenge of infectious RSV.

[066] **Figure 20** depicts SDS-PAGE gel stained with coomassie of purified recombinant RSV F protein BV #683 stored at 2 – 8°C for 0, 1, 2, 4, and 5 weeks.

[067] **Figure 21** depicts RSV A and RSV B neutralizing antibody responses following immunization with live RSV (RSV), formalin inactivated RSV (FI-RSV), RSV-F protein BV #683 with and without aluminum (PFP and PFP + Aluminum Adjuvant), and PBS controls.

[068] **Figure 22** depicts lung pathology following challenge with RSV in rats immunized with live RSV, formalin inactivated RSV (FI-RSV), RSV-F protein BV #683 with and without aluminum (F-micelle (30 μg) and F-micelle (30 μg) + Aluminum Adjuvant), and PBS controls.

[069] **Figure 23** is a graph showing the neutralizing antibody responses against RSV A in cotton rat (y-axis, expressed as Log2 titers) vs. various vaccination treatment groups (x-axis). The line for each group is the geometric mean of end point titer that neutralized 100%.

[070] **Figure 24** is a graph showing the neutralizing antibody responses against RSV A in cotton rat (y-axis, expressed as Log2 titers) vs. various vaccination treatment groups (x-axis). The line for each group is the geometric mean of end point titer that neutralized 100%.

[071] **Figure 25** is a graph showing the lung viral titers of cotton rats (expressed as log10 pfu/gram of tissue) vs. various vaccination treatment groups (x-axis). The viral titers are shown ±SEM.

[072] **Figure 26A** is a graph showing ELISA units vs. vaccination group, and provides a measure for antibody production in animals treated with the RSV F vaccine, FI-RSV, live RSV, or PBS. **Figure 26 B** is a graph showing antibody production in each vaccine group as measured by RSV-F IgG titer. **Figure 26C** is a graph depicting serum neutralizing antibody titers against RSV in each vaccination group. **Figure 26D** is a graph showing palivizumab competitive IgG titers from pooled sera from each vaccination group.

[073] **Figure 27** are representative micrographs of lung tissue harvested from rats after treatment with the nanoparticle vaccine of the invention and a subsequent challenge with RSV.

[074] **Figure 28** is a graph showing the binding competition between the palivizumab epitope (SEQ ID NO: 35) and antibodies produced by the vaccine of the present invention.

[075] **Figure 29A** is a graph showing the binding of various concentrations of Synagis® mAb to palivizumab epitope peptide. **Figure 29B** is a graph showing the binding of various concentrations of Synagis® to recombinant RSV F micelles.

[076] **Figure 30** provides schemes of various assays carried out to test the immunogenicity of the nanoparticle vaccines of the invention.

[077] **Figure 31** is a graph showing the results of an ELISA study using human sera from subjects treated with the vaccine of the present invention.

[078] **Figure 32** is a graph showing anti-RSV F (A) and anti-RSV G (B) IgG detected in human sera from subjects treated with the vaccine of the present invention.

[079] **Figure 33** is a graph showing the geometric mean fold rise in anti-RSV F IgG levels for the alum treatment groups.

[080] **Figure 34** is a graph showing the plague reduction neutralization titers for subjects at various timepoints, before or after treatment with the nanoparticle vaccine of the invention.

[081] **Figure 35** shows the reverse cumulative distribution for Day 0, Day 30 and Day 60 PRNTs in the placebo and 30 μg + Alum groups.

[082] **Figure 36A** shows the positive assay controls for the BIAcore SPR-based antigen binding assay utilized to assess the avidity of antibodies in the human sera for RSV F. **Figure 36 B** shows a sensorgram for sera from Day 0 and placebo controls, in comparison to the positive control palivizumab.

[083] **Figure 37** shows the binding curve for palivizumab and a representative sample from the vaccine group, as measured using the BIAcore SPR-based antigen binding assay.

[084] **Figure 38** is a graph showing the geometric mean rise in antibody titer levels for both (1) anti-F IgG (left bar) and (2) MN (right bar) for the various treatment groups.

[085] **Figure 39** is a graph showing antibody geometric mean titers (GMT) in patients administered RSV nanoparticle vaccine at various dosages. The antibody response is to the antigenic site II peptide 254-278.

[086] **Figure 40A** is a graph showing antibodies generated by the RSV F protein nanoparticle vaccine are competitive with Palivizumab. **Figure 40B** is a graph showing palivizumab competitive antibodies post dose 1 and post dose 2 in all vaccine groups.

[087] **Figure 41** is a graph showing the results of a Palivizumab competition assay. The results show that RSV F nanoparticle vaccine induced antibodies correlate with antibodies that compete for the Palivizumab binding site.

[088] **Figure 42** shows the RSV F binding titers of the indicated monoclonal antibodies and the antibody recognition sites for each monoclonal antibody on the full length RSV F antigen.

[089] **Figure 43** is a graph showing the anti-RSV F IgG antibody titer in sera from cotton rats immunized with FI-RSV, RSV-F nanoparticle vaccine with or without adjuvant, or live RSV at Day 0, 28, and 49 post-immunization.

[090] **Figure 44** is a graph showing neutralizing antibody responses at Day 0, 28, and 49 after immunization of cotton rats with FI-RSV, RSV-F nanoparticle vaccine with or without adjuvant, or live RSV.

[091] **Figure 45** is a graph showing the fusion inhibition titers in sera from cotton rats immunized with FI-RSV, RSV-F nanoparticle vaccine with or without adjuvant, or live RSV.

[092] **Figure 46** is a graph showing competitive ELISA titers in sera from cotton rats immunized with FI-RSV, RSV-F nanoparticle vaccine with or without adjuvant, or live RSV.

[093] **Figure 47** shows the titers of vaccine-induced antibodies competitive with the indicated neutralizing RSV F-specific monoclonal antibody in sera from cotton rats immunized with FI-RSV, RSV-F nanoparticle vaccine with or without adjuvant, or live RSV.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[094] As used herein, the term "adjuvant" refers to a compound that, when used in combination with a specific immunogen (*e.g.* a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein) in a formulation, will augment or otherwise alter or modify the resultant immune response. Modification of the immune response includes intensification or broadening the specificity of either or both antibody and cellular immune responses. Modification of the immune response can also mean decreasing or suppressing certain antigen-specific immune responses.

[095] As used herein, the term "antigenic formulation" or "antigenic composition" refers to a preparation which, when administered to a vertebrate, especially a bird or a mammal, will induce an immune response.

[096] As used herein, the term "avian influenza virus" refers to influenza viruses found chiefly in birds but that can also infect humans or other animals. In some instances, avian influenza viruses may be transmitted or spread from one human to another. An avian influenza virus that infects humans has the potential to cause an influenza pandemic, i.e., morbidity and/or mortality in humans. A pandemic occurs when a new strain of influenza virus (a virus against which humans have no natural immunity) emerges, spreading beyond individual localities, possibly around the globe, and infecting many humans at once.

[097] As used herein, an "effective dose" generally refers to that amount of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein of the invention sufficient to induce immunity, to prevent and/or ameliorate an infection or to reduce at least one symptom of an infection or disease, and/or to enhance the efficacy of another dose of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein. An effective dose may refer to the amount of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein sufficient to delay or minimize the onset of an infection or disease. An effective dose may also refer to the amount of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or

mutated RSV F protein that provides a therapeutic benefit in the treatment or management of an infection or disease. Further, an effective dose is the amount with respect to a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein of the invention alone, or in combination with other therapies, that provides a therapeutic benefit in the treatment or management of an infection or disease. An effective dose may also be the amount sufficient to enhance a subject's (*e.g.*, a human's) own immune response against a subsequent exposure to an infectious agent or disease. Levels of immunity can be monitored, *e.g.*, by measuring amounts of neutralizing secretory and/or serum antibodies, *e.g.*, by plaque neutralization, complement fixation, enzyme-linked immunosorbent, or microneutralization assay, or by measuring cellular responses, such as, but not limited to cytotoxic T cells, antigen presenting cells, helper T cells, dendritic cells and/or other cellular responses. T cell responses can be monitored, *e.g.*, by measuring, for example, the amount of $CD4^+$ and $CD8^+$ cells present using specific markers by fluorescent flow cytometry or T cell assays, such as but not limited to T-cell proliferation assay, T-cell cytotoxic assay, TETRAMER assay, and/or ELISPOT assay. In the case of a vaccine, an "effective dose" is one that prevents disease and/or reduces the severity of symptoms.

[098] As used herein, the term "effective amount" refers to an amount of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein necessary or sufficient to realize a desired biologic effect. An effective amount of the composition would be the amount that achieves a selected result, and such an amount could be determined as a matter of routine experimentation by a person skilled in the art. For example, an effective amount for preventing, treating and/or ameliorating an infection could be that amount necessary to cause activation of the immune system, resulting in the development of an antigen specific immune response upon exposure to a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein of the invention. The term is also synonymous with "sufficient amount."

[099] As used herein, the term "expression" refers to the process by which polynucleic acids are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleic acid is derived from genomic DNA, expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA. In the context of the present

invention, the term also encompasses the yield of RSV F gene mRNA and RSV F proteins achieved following expression thereof.

[0100] As used herein, the term "F protein" or "Fusion protein" or "F protein polypeptide" or "Fusion protein polypeptide" refers to a polypeptide or protein having all or part of an amino acid sequence of an RSV Fusion protein polypeptide. Similarly, the term "G protein" or "G protein polypeptide" refers to a polypeptide or protein having all or part of an amino acid sequence of an RSV Attachment protein polypeptide. Numerous RSV Fusion and Attachment proteins have been described and are known to those of skill in the art. WO/2008/114149, which is herein incorporated by reference in its entirety, sets out exemplary F and G protein variants (for example, naturally occurring variants).

[0101] As used herein, the terms "immunogens" or "antigens" refer to substances such as proteins, peptides, and nucleic acids that are capable of eliciting an immune response. Both terms also encompass epitopes, and are used interchangeably.

[0102] As used herein the term "immune stimulator" refers to a compound that enhances an immune response *via* the body's own chemical messengers (cytokines). These molecules comprise various cytokines, lymphokines and chemokines with immunostimulatory, immunopotentiating, and pro-inflammatory activities, such as interferons (IFN-γ), interleukins (*e.g.*, IL-1, IL-2, IL-3, IL-4, IL-12, IL-13); growth factors (*e.g.*, granulocyte-macrophage (GM)-colony stimulating factor (CSF)); and other immunostimulatory molecules, such as macrophage inflammatory factor, Flt3 ligand, B7.1; B7.2, etc. The immune stimulator molecules can be administered in the same formulation as VLPs of the invention, or can be administered separately. Either the protein or an expression vector encoding the protein can be administered to produce an immunostimulatory effect.

[0103] As used herein, the term "immunogenic formulation" refers to a preparation which, when administered to a vertebrate, *e.g.* a mammal, will induce an immune response.

[0104] As used herein, the term "infectious agent" refers to microorganisms that cause an infection in a vertebrate. Usually, the organisms are viruses, bacteria, parasites, protozoa and/or fungi.

[0105] As used herein, the terms "mutated," "modified," "mutation," or "modification" indicate any modification of a nucleic acid and/or polypeptide which results in an altered nucleic acid or polypeptide. Mutations include, for example, point mutations, deletions, or insertions of single or multiple residues in a polynucleotide, which includes alterations arising within a protein-encoding region of a gene as well as alterations in regions outside of a protein-encoding sequence, such as, but not limited to, regulatory or promoter sequences.

A genetic alteration may be a mutation of any type. For instance, the mutation may constitute a point mutation, a frame-shift mutation, an insertion, or a deletion of part or all of a gene. In some embodiments, the mutations are naturally-occurring. In other embodiments, the mutations are the results of artificial mutation pressure. In still other embodiments, the mutations in the RSV F proteins are the result of genetic engineering.

[0106] As used herein, the term "multivalent" refers to compositions which have one or more antigenic proteins/peptides or immunogens against multiple types or strains of infectious agents or diseases.

[0107] As used herein, the term "pharmaceutically acceptable vaccine" refers to a formulation which contains a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein of the present invention, which is in a form that is capable of being administered to a vertebrate and which induces a protective immune response sufficient to induce immunity to prevent and/or ameliorate an infection or disease, and/or to reduce at least one symptom of an infection or disease, and/or to enhance the efficacy of another dose of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein. Typically, the vaccine comprises a conventional saline or buffered aqueous solution medium in which the composition of the present invention is suspended or dissolved. In this form, the composition of the present invention can be used conveniently to prevent, ameliorate, or otherwise treat an infection. Upon introduction into a host, the vaccine is able to provoke an immune response including, but not limited to, the production of antibodies and/or cytokines and/or the activation of cytotoxic T cells, antigen presenting cells, helper T cells, dendritic cells and/or other cellular responses.

[0108] As used herein, the phrase "protective immune response" or "protective response" refers to an immune response mediated by antibodies against an infectious agent or disease, which is exhibited by a vertebrate (*e.g.*, a human), that prevents or ameliorates an infection or reduces at least one disease symptom thereof. Modified or mutated RSV F proteins, RSV F micelles comprising a modified or mutated RSV F protein, or VLPs comprising a modified or mutated RSV F protein of the invention can stimulate the production of antibodies that, for example, neutralize infectious agents, blocks infectious agents from entering cells, blocks replication of the infectious agents, and/or protect host cells from infection and destruction. The term can also refer to an immune response that is mediated by T-lymphocytes and/or other white blood cells against an infectious agent or disease, exhibited by a vertebrate (*e.g.*,

a human), that prevents or ameliorates infection or disease, or reduces at least one symptom thereof.

[0109] As used herein, the term "vertebrate" or "subject" or "patient" refers to any member of the subphylum cordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species. Farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats (including cotton rats) and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like are also non-limiting examples . The terms "mammals" and "animals" are included in this definition. Both adult and newborn individuals are intended to be covered. In particular, infants and young children are appropriate subjects or patients for a RSV vaccine.

[0110] As used herein, the term "virus-like particle" (VLP) refers to a structure that in at least one attribute resembles a virus but which has not been demonstrated to be infectious. Virus-like particles in accordance with the invention do not carry genetic information encoding for the proteins of the virus-like particles. In general, virus-like particles lack a viral genome and, therefore, are noninfectious. In addition, virus-like particles can often be produced in large quantities by heterologous expression and can be easily purified.

[0111] As used herein, the term "chimeric VLP" refers to VLPs that contain proteins, or portions thereof, from at least two different infectious agents (heterologous proteins). Usually, one of the proteins is derived from a virus that can drive the formation of VLPs from host cells. Examples, for illustrative purposes, are the BRSV M protein and/or the HRSV G or F proteins. The terms RSV VLPs and chimeric VLPs can be used interchangeably where appropriate.

[0112] As used herein, the term "vaccine" refers to a preparation of dead or weakened pathogens, or of derived antigenic determinants that is used to induce formation of antibodies or immunity against the pathogen. A vaccine is given to provide immunity to the disease, for example, influenza, which is caused by influenza viruses. In addition, the term "vaccine" also refers to a suspension or solution of an immunogen (*e.g.* a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein) that is administered to a vertebrate to produce protective immunity, *i.e.*, immunity that prevents or reduces the severity of disease associated with infection. The present invention provides for vaccine compositions that are immunogenic and may provide protection against a disease associated with infection.

RSV F Proteins

[0113] RSV-F proteins and methods are described in U.S. Patent Application Serial No. 12/633,995, filed December 9, 2009 (published September 23, 2010 as U.S. Publication No. 2010/0239617), U.S. Provisional Application Serial No. 61/121,126, filed December 9, 2008, and U.S. Provisional Application Serial No. 61/169,077, filed April 14, 2009, and U.S. Provisional Application Serial No. 61/224,787, filed July 10, 2009, the disclosures of which are each incorporated by reference in their entirety for all purposes.

[0114] Two structural membrane proteins, F and G proteins, are expressed on the surface of RSV, and have been shown to be targets of neutralizing antibodies (Sullender, W., 2000, *Clinical Microbiology Review* 13, 1-15). These two proteins are also primarily responsible for viral recognition and entry into target cells; G protein binds to a specific cellular receptor and the F protein promotes fusion of the virus with the cell. The F protein is also expressed on the surface of infected cells and is responsible for subsequent fusion with other cells leading to syncytia formation. Thus, antibodies to the F protein can neutralize virus or block entry of the virus into the cell or prevent syncytia formation. Although antigenic and structural differences between A and B subtypes have been described for both the G and F proteins, the more significant antigenic differences reside on the G protein, where amino acid sequences are only 53% homologous and antigenic relatedness is 5% (Walsh *et al.* (1987) J. Infect. Dis. 155, 1198-1204; and Johnson *et al.* (1987) Proc. Natl. Acad. Sci. USA 84,5625-5629). Conversely, antibodies raised to the F protein show a high degree of cross-reactivity among subtype A and B viruses.

[0115] The RSV F protein directs penetration of RSV by fusion between the virion's envelope protein and the host cell plasma membrane. Later in infection, the F protein expressed on the cell surface can mediate fusion with neighboring cells to form syncytia. The F protein is a type I transmembrane surface protein that has a N-terminal cleaved signal peptide and a membrane anchor near the C-terminus. RSV F is synthesized as an inactive F_0 precursor that assembles into a homotrimer and is activated by cleavage in the trans-Golgi complex by a cellular endoprotease to yield two disulfide-linked subunits, F_1 and F_2 subunits. The N-terminus of the F_1 subunit that is created by cleavage contains a hydrophobic domain (the fusion peptide) that inserts directly into the target membrane to initiate fusion. The F_1 subunit also contains heptad repeats that associate during fusion, driving a conformational shift that brings the viral and cellular membranes into close proximity (Collins and Crowe,

2007, Fields Virology, 5th ed., D.M Kipe *et al.*, Lipincott, Williams and Wilkons, p. 1604). SEQ ID NO: 2 (GenBank Accession No. AAB59858) depicts a representative RSV F protein, which is encoded by the gene shown in SEQ ID NO: 1 (GenBank Accession No. M11486).

[0116] In nature, the RSV F protein is expressed as a single polypeptide precursor, 574 amino acids in length, designated FO. In vivo, FO oligomerizes in the endoplasmic reticulum and is proteolytically processed by a furin protease at two conserved furin consensus sequences (furin cleavage sites), RARR (SEQ ID NO: 23) (secondary) and KKRKRR (SEQ ID NO: 24) (primary) to generate an oligomer consisting of two disulfide-linked fragments. The smaller of these fragments is termed F2 and originates from the N-terminal portion of the FO precursor. It will be recognized by those of skill in the art that the abbreviations FO, Fl and F2 are commonly designated F_0, F_1 and F_2 in the scientific literature. The larger, C-terminal Fl fragment anchors the F protein in the membrane via a sequence of hydrophobic amino acids, which are adjacent to a 24 amino acid cytoplasmic tail. Three F2-F1 dimers associate to form a mature F protein, which adopts a metastable prefusogenic ("prefusion") conformation that is triggered to undergo a conformational change upon contact with a target cell membrane. This conformational change exposes a hydrophobic sequence, known as the fusion peptide, 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.

[0117] The F1 fragment contains at least two heptad repeat domains, designated HRA and HRB, and is situated in proximity to the fusion peptide and transmembrane anchor domains, respectively. In the prefusion conformation, the F2-F1 dimer forms a globular head and stalk structure, in which the HRA domains are in a segmented (extended) conformation in the globular head. In contrast, the HRB domains form a three-stranded coiled coil stalk extending from the head region. During transition from the prefusion to the postfusion conformations, the HRA domains collapse and are brought into proximity to the HRB domains to form an anti-parallel six helix bundle. In the postfusion state the fusion peptide and transmembrane domains are juxtaposed to facilitate membrane fusion.

[0118] Although the conformational description provided above is based on molecular modeling of crystallographic data, the structural distinctions between the prefusion and postfusion conformations can be monitored without resort to crystallography. For example, electron micrography can be used to distinguish between the prefusion and postfusion (alternatively designated prefusogenic and fusogenic) conformations, as demonstrated by Calder *et al.*, *Virology*, 271:122-131 (2000) and Morton *et al.*, *Virology*, 311: 275-288, which are incorporated herein by reference for the purpose of their technological teachings. The

prefusion conformation can also be distinguished from the fusogenic (post-fusion) conformation by liposome association assays as described by Connolly *et al*, *Proc. Natl. Acad. Sci. USA*, 103:17903-17908 (2006), which is also incorporated herein by reference for the purpose of its technological teachings. Additionally, prefusion and fusogenic conformations can be distinguished using antibodies (*e.g.*, monoclonal antibodies) that specifically recognize conformation epitopes present on one or the other of the prefusion or fusogenic form of the RSV F protein, but not on the other form. Such conformation epitopes can be due to preferential exposure of an antigenic determinant on the surface of the molecule. Alternatively, conformational epitopes can arise from the juxtaposition of amino acids that are non-contiguous in the linear polypeptide.

Modified or Mutated RSV F Proteins

[0119] The present inventors have found that surprisingly high levels of expression of the fusion (F) protein can be achieved when specific modifications are made to the structure of the RSV F protein. Such modifications also unexpectedly reduce the cellular toxicity of the RSV F protein in a host cell. In addition, the modified F proteins of the present invention demonstrate an improved ability to exhibit the post-fusion "lollipop" morphology as opposed to the pre-fusion "rod" morphology. Thus, in one aspect, the modified F proteins of the present invention can also exhibit improved (*e.g.* enhanced) immunogenicity as compared to wild-type F proteins (*e.g.* exemplified by SEQ ID NO: 2, which corresponds to GenBank Accession No. AAB59858). These modifications have significant applications to the development of vaccines and methods of using said vaccines for the treatment and/or prevention of RSV.

[0120] In accordance with the invention, any number of mutations can be made to native or wild-type RSV F proteins, and in a preferred aspect, multiple mutations can be made to result in improved expression and/or immunogenic properties as compared to native or wild-type RSV F proteins. Such mutations include point mutations, frame shift mutations, deletions, and insertions, with one or more (e.g., one, two, three, or four, etc.) mutations preferred.

[0121] The native F protein polypeptide can be selected from any F protein of an RSV A strain, RSV B strain, HRSV A strain, HRSV B strain, BRSV strain, or avian RSV strain, or from variants thereof (as defined above). In certain exemplary embodiments, the native F protein polypeptide is the F protein represented by SEQ ID NO: 2 (GenBank Accession No AAB59858). To facilitate understanding of this disclosure, all amino acid residue positions, regardless of strain, are given with respect to (that is, the amino acid residue position

corresponds to) the amino acid position of the exemplary F protein. Comparable amino acid positions of the F protein from other RSV strains can be determined easily by those of ordinary skill in the art by aligning the amino acid sequences of the selected RSV strain with that of the exemplary sequence using readily available and well-known alignment algorithms (such as BLAST, e.g., using default parameters). Numerous additional examples of F protein polypeptides from different RSV strains are disclosed in WO/2008/114149 (which is incorporated herein by reference in its entirety). Additional variants can arise through genetic drift, or can be produced artificially using site directed or random mutagenesis, or by recombination of two or more preexisting variants. Such additional variants are also suitable in the context of the modified or mutated RSV F proteins disclosed herein.

[0122] Mutations may be introduced into the RSV F proteins of the present invention using any methodology known to those skilled in the art. Mutations may be introduced randomly by, for example, conducting a PCR reaction in the presence of manganese as a divalent metal ion cofactor. Alternatively, oligonucleotide directed mutagenesis may be used to create the mutant or modified RSV F proteins which allows for all possible classes of base pair changes at any determined site along the encoding DNA molecule. In general, this technique involves annealing an oligonucleotide complementary (except for one or more mismatches) to a single stranded nucleotide sequence coding for the RSV F protein of interest. The mismatched oligonucleotide is then extended by DNA polymerase, generating a double-stranded DNA molecule which contains the desired change in sequence in one strand. The changes in sequence can, for example, result in the deletion, substitution, or insertion of an amino acid. The double-stranded polynucleotide can then be inserted into an appropriate expression vector, and a mutant or modified polypeptide can thus be produced. The above-described oligonucleotide directed mutagenesis can, for example, be carried out via PCR.

Additional RSV Proteins

[0123] The invention also encompasses RSV virus-like particles (VLPs) comprising a modified or mutated RSV F protein that can be formulated into vaccines or antigenic formulations for protecting vertebrates (*e.g.* humans) against RSV infection or at least one disease symptom thereof. In some embodiments, the VLP comprising a modified or mutated RSV F protein further comprises additional RSV proteins, such as M, N, G, and SH. In other embodiments, the VLP comprising a modified or mutated RSV F protein further comprises proteins from heterologous strains of virus, such as influenza virus proteins HA, NA, and M1.

In one embodiment, the influenza virus protein M1 is derived from an avian influenza virus strain.

[0124] RSV N protein binds tightly to both genomic RNA and the replicative intermediate anti-genomic RNA to form RNAse resistant nucleocapsid. SEQ ID NOs: 16 (wild-type) and 18 (codon-optimized) depict representative amino acid sequences of the RSV N protein and SEQ ID NOs: 15 (wild-type) and 17 (codon-optimized) depict representative nucleic acid sequences encoding the RSV N protein. Encompassed in this invention are RSV N proteins that are at least about 20%, about 30%, about 40%, about 50%, about 60%, about 70% or about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98% or about 99% identical to SEQ ID NO: 18, and all fragments and variants (including chimeric proteins) thereof.

[0125] RSV M protein is a non-glycosylated internal virion protein that accumulates in the plasma membrane that interacts with RSV F protein and other factors during virus morphogenesis. In certain preferred embodiments, the RSV M protein is a bovine RSV (BRSV) M protein. SEQ ID NOs: 12 (wild-type) and 14 (codon-optimized) depict representative amino acid sequences of the BRSV M protein and SEQ ID NOs: 11 (wild-type) and 13 (codon-optimized) depict representative nucleic acid sequences encoding the BRSV M protein. Encompassed in this invention are RSV (including, but not limited to, BRSV) M proteins that are at least about 20%, about 30%, about 40%, about 50%, about 60%, about 70% or about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98% or about 99% identical to SEQ ID NOs: 12 and 14, and all fragments and variants (including chimeric proteins) thereof.

[0126] RSV G protein is a type II transmembrane glycoprotein with a single hydrophobic region near the N-terminal end that serves as both an uncleaved signal peptide and a membrane anchor, leaving the C-terminal two-thirds of the molecule oriented externally. RSV G is also expressed as a secreted protein that arises from translational initiation at the second AUG in the ORF (at about amino acid 48), which lies within the signal/anchor. Most of the ectodomain of RSV G is highly divergent between RSV strains (*Id.*, p. 1607). SEQ ID NO: 26 depicts a representative RSV G protein, which is encoded by the gene sequence shown in SEQ ID NO: 25. Encompassed in this invention are RSV G proteins that are at least about 20%, about 30%, about 40%, about 50%, about 60%, about 70% or about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98% or about 99% identical to SEQ ID NO: 26, and all fragments and variants (including chimeric proteins) thereof.

[0127] The SH protein of RSV is a type II transmembrane protein that contains 64 (RSV subgroup A) or 65 amino acid residues (RSV subgroup B). Some studies have suggested that the RSV SH protein may have a role in viral fusion or in changing membrane permeability. However, RSV lacking the SH gene are viable, cause syncytia formation and grow as well as the wild-type virus, indicating that the SH protein is not necessary for virus entry into host cells or syncytia formation. The SH protein of RSV has shown the ability of inhibit TNF-α signaling. SEQ ID NO: 27 depicts a representative amino acid sequence of the RSV SH protein. Encompassed in this invention are RSV SH proteins that are at least about 20%, about 30%, about 40%, about 50%, about 60%, about 70% or about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98% or about 99% identical to SEQ ID NO: 27, and all fragments and variants (including chimeric proteins) thereof.

RSV Vaccines

[0128] Currently, the only approved approach to prophylaxis of RSV disease is passive immunization. Initial evidence suggesting a protective role for IgG was obtained from observations involving maternal antibody in ferrets (Prince, G. A., Ph.D. diss., University of California, Los Angeles, 1975) and humans (Lambrecht *et al.*, (1976) J. Infect. Dis. 134, 211-217; and Glezen *et al.* (1981) J. Pediatr. 98,708-715). Hemming *et al.* (Morell *et al.*, eds., 1986, Clinical Use of Intravenous Immunoglobulins, Academic Press, London at pages 285-294) recognized the possible utility of RSV antibody in treatment or prevention of RSV infection during studies involving the pharmacokinetics of an intravenous immunoglobulin (IVIG) in newborns suspected of having neonatal sepsis. They noted that one infant, whose respiratory secretions yielded RSV, recovered rapidly after IVIG infusion. Subsequent analysis of the IVIG lot revealed an unusually high titer of RSV neutralizing antibody. This same group of investigators then examined the ability of hyper-immune serum or immunoglobulin, enriched for RSV neutralizing antibody, to protect cotton rats and primates against RSV infection (Prince *et al.* (1985) Virus Res. 3, 193-206; Prince *et al.* (1990) J. Virol. 64, 3091-3092. Results of these studies suggested that RSV neutralizing antibody given prophylactically inhibited respiratory tract replication of RSV in cotton rats. When given therapeutically, RSV antibody reduced pulmonary viral replication both in cotton rats and in a nonhuman primate model. Furthermore, passive infusion of immune serum or immune globulin did not produce enhanced pulmonary pathology in cotton rats subsequently challenged with RSV.

[0129] Since RSV infection can be prevented by providing neutralizing antibodies to a vertebrate, a vaccine comprising a modified or mutated RSV F protein may induce, when administered to a vertebrate, neutralizing antibodies *in vivo*. The modified or mutated RSV F proteins are favorably used for the prevention and/or treatment of RSV infection. Thus, another aspect of this disclosure concerns a method for eliciting an immune response against RSV. The method involves administering an immunologically effective amount of a composition containing a modified or mutated RSV F protein to a subject (such as a human or animal subject). Administration of an immunologically effective amount of the composition elicits an immune response specific for epitopes present on the modified or mutated RSV F protein. Such an immune response can include B cell responses (e.g., the production of neutralizing antibodies) and/or T cell responses (e.g., the production of cytokines). Preferably, the immune response elicited by the modified or mutated RSV F protein includes elements that are specific for at least one conformational epitope present on the modified or mutated RSV F protein. In one embodiment, the immune response is specific for an epitope present on an RSV F protein found in the "lollipop" post-fusion active state. The RSV F proteins and compositions can be administered to a subject without enhancing viral disease following contact with RSV. Preferably, the modified or mutated RSV F proteins disclosed herein and suitably formulated immunogenic compositions elicit a Th1 biased immune response that reduces or prevents infection with a RSV and/or reduces or prevents a pathological response following infection with a RSV.

[0130] In one embodiment, the RSV F proteins of the present invention are found in the form of micelles (*e.g.* rosettes). The micelles obtainable in accordance with the invention consist of aggregates of the immunogenically active F spike proteins having a rosette-like structure. The rosettes are visible in the electron microscope (Calder *et al.*, 2000, *Virology* 271: 122-131). Preferably, the micelles of the present invention comprising modified or mutated RSV F proteins exhibit the "lollipop" morphology indicative of the post-fusion active state. In one embodiment, the micelles are purified following expression in a host cell. When administered to a subject, the micelles of the present invention preferably induce neutralizing antibodies. In some embodiments, the micelles may be administered with an adjuvant. In other embodiments, the micelles may be administered without an adjuvant.

[0131] In another embodiment, the invention encompasses RSV virus-like particles (VLPs) comprising a modified or mutated RSV F protein that can be formulated into vaccines or antigenic formulations for protecting vertebrates (*e.g.* humans) against RSV infection or at least one disease symptom thereof. The present invention also relates to RSV VLPs and

vectors comprising wild-type and mutated RSV genes or a combination thereof derived from different strains of RSV virus, which when transfected into host cells, will produce virus like particles (VLPs) comprising RSV proteins.

[0132] In some embodiments, RSV virus-like particles may further comprise at least one viral matrix protein (*e.g.* an RSV M protein). In one embodiment, the M protein is derived from a human strain of RSV. In another embodiment, the M protein is derived from a bovine strain of RSV. In other embodiments, the matrix protein may be an M1 protein from a strain of influenza virus. In one embodiment, the strain of influenza virus is an avian influenza strain. In a preferred embodiment, the avian influenza strain is the H5N1 strain A/Indonesia/5/05. In other embodiments, the matrix protein may be from Newcastle Disease Virus (NDV).

[0133] In some embodiments, the VLPs may further comprise an RSV G protein. In one embodiment, the G protein may be from HRSV group A. In another embodiment, the G protein may be from HRSV group B. In yet another embodiment, the RSV G may be derived from HRSV group A and/or group B.

[0134] In some embodiments, the VLPs may further comprise an RSV SH protein. In one embodiment, the SH protein may be from HRSV group A. In another embodiment, the SH protein may be from HRSV group B. In yet another embodiment, the RSV SH may be derived from HRSV group A and/or group B.

[0135] In some embodiments, VLPs may further comprise an RSV N protein. In one embodiment, the N protein may be from HRSV group A. In another embodiment, the N protein may be from HRSV group B. In yet another embodiment, the RSV N may be derived from HRSV group A and/or group B.

[0136] In further embodiments, VLPs of the invention may comprise one or more heterologous immunogens, such as influenza hemagglutinin (HA) and/or neuraminidase (NA).

[0137] In some embodiments, the invention also comprises combinations of different RSV M, F, N, SH, and/or G proteins from the same and/or different strains in one or more VLPs. In addition, the VLPs can include one or more additional molecules for the enhancement of an immune response.

[0138] In another embodiment of the invention, the RSV VLPs can carry agents such as nucleic acids, siRNA, microRNA, chemotherapeutic agents, imaging agents, and/or other agents that need to be delivered to a patient.

[0139] VLPs of the invention are useful for preparing vaccines and immunogenic compositions. One important feature of VLPs is the ability to express surface proteins of interest so that the immune system of a vertebrate induces an immune response against the protein of interest. However, not all proteins can be expressed on the surface of VLPs. There may be many reasons why certain proteins are not expressed, or are poorly expressed, on the surface of VLPs. One reason is that the protein is not directed to the membrane of a host cell or that the protein does not have a transmembrane domain. As an example, sequences near the carboxyl terminus of influenza hemagglutinin may be important for incorporation of HA into the lipid bilayer of the mature influenza enveloped nucleocapsids and for the assembly of HA trimer interaction with the influenza matrix protein M1 (Ali, *et al.*, (2000) J. Virol. 74, 8709-19).

[0140] Thus, one embodiment of the invention comprises chimeric VLPs comprising a modified or mutated F protein from RSV and at least one immunogen which is not normally efficiently expressed on the cell surface or is not a normal RSV protein. In one embodiment, the modified or mutated RSV F protein may be fused with an immunogen of interest. In another embodiment, the modified or mutated RSV F protein associates with the immunogen via the transmembrane domain and cytoplasmic tail of a heterologous viral surface membrane protein, *e.g.*, MMTV envelope protein.

[0141] Other chimeric VLPs of the invention comprise VLPs comprising a modified or mutated RSV F protein and at least one protein from a heterologous infectious agent. Examples of heterologous infectious agents include but are not limited to a virus, a bacterium, a protozoan, a fungus and/or a parasite. In one embodiment, the immunogen from another infectious agent is a heterologous viral protein. In another embodiment, the protein from a heterologous infectious agent is an envelope-associated protein. In another embodiment, the protein from another heterologous infectious agent is expressed on the surface of VLPs. In another embodiment, the protein from an infectious agent comprises an epitope that will generate a protective immune response in a vertebrate. In one embodiment, the protein from another infectious agent is co-expressed with a modified or mutated RSV F protein. In another embodiment, the protein from another infectious agent is fused to a modified or mutated RSV F protein. In another embodiment, only a portion of a protein from another infectious agent is fused to a modified or mutated RSV F protein. In another embodiment, only a portion of a protein from another infectious agent is fused to a portion of a modified or mutated RSV F protein. In another embodiment, the portion of the protein

from another infectious agent fused to modified or mutated RSV F protein is expressed on the surface of VLPs.

[0142] The invention also encompasses variants of the proteins expressed on or in the VLPs of the invention. The variants may contain alterations in the amino acid sequences of the constituent proteins. The term "variant" with respect to a protein refers to an amino acid sequence that is altered by one or more amino acids with respect to a reference sequence. The variant can have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, *e.g.*, replacement of leucine with isoleucine. Alternatively, a variant can have "nonconservative" changes, *e.g.*, replacement of a glycine with a tryptophan. Analogous minor variations can also include amino acid deletion or insertion, or both. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without eliminating biological or immunological activity can be found using computer programs well known in the art, for example, DNASTAR software.

[0143] Natural variants can occur due to mutations in the proteins. These mutations may lead to antigenic variability within individual groups of infectious agents, for example influenza. Thus, a person infected with, for example, an influenza strain develops antibody against that virus, as newer virus strains appear, the antibodies against the older strains no longer recognize the newer virus and re-infection can occur. The invention encompasses all antigenic and genetic variability of proteins from infectious agents for making VLPs.

[0144] General texts which describe molecular biological techniques, which are applicable to the present invention, such as cloning, mutation, cell culture and the like, include Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology volume 152 Academic Press, Inc., San Diego, Calif. (Berger); Sambrook *et al.*, Molecular Cloning--A Laboratory Manual (3rd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 2000 ("Sambrook") and Current Protocols in Molecular Biology, F. M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., ("Ausubel"). These texts describe mutagenesis, the use of vectors, promoters and many other relevant topics related to, *e.g.*, the cloning and mutating F and/or G molecules of RSV, etc. Thus, the invention also encompasses using known methods of protein engineering and recombinant DNA technology to improve or alter the characteristics of the proteins expressed on or in the VLPs of the invention. Various types of mutagenesis can be used to produce and/or isolate variant nucleic acids that encode for protein molecules and/or to further modify/mutate the proteins in or on the VLPs of the invention. They include but are not limited to site-directed, random point mutagenesis, homologous recombination

(DNA shuffling), mutagenesis using uracil containing templates, oligonucleotide-directed mutagenesis, phosphorothioate-modified DNA mutagenesis, mutagenesis using gapped duplex DNA or the like. Additional suitable methods include point mismatch repair, mutagenesis using repair-deficient host strains, restriction-selection and restriction-purification, deletion mutagenesis, mutagenesis by total gene synthesis, double-strand break repair, and the like. Mutagenesis, *e.g.*, involving chimeric constructs, is also included in the present invention. In one embodiment, mutagenesis can be guided by known information of the naturally occurring molecule or altered or mutated naturally occurring molecule, *e.g.*, sequence, sequence comparisons, physical properties, crystal structure or the like.

[0145] The invention further comprises protein variants which show substantial biological activity, *e.g.*, able to elicit an effective antibody response when expressed on or in VLPs of the invention. Such variants include deletions, insertions, inversions, repeats, and substitutions selected according to general rules known in the art so as have little effect on activity.

[0146] Methods of cloning the proteins are known in the art. For example, the gene encoding a specific RSV protein can be isolated by RT-PCR from polyadenylated mRNA extracted from cells which had been infected with a RSV virus. The resulting product gene can be cloned as a DNA insert into a vector. The term "vector" refers to the means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include plasmids, viruses, bacteriophages, pro-viruses, phagemids, transposons, artificial chromosomes, and the like, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that is not autonomously replicating. In many, but not all, common embodiments, the vectors of the present invention are plasmids or bacmids.

[0147] Thus, the invention comprises nucleotides that encode proteins, including chimeric molecules, cloned into an expression vector that can be expressed in a cell that induces the formation of VLPs of the invention. An "expression vector" is a vector, such as a plasmid that is capable of promoting expression, as well as replication of a nucleic acid incorporated therein. Typically, the nucleic acid to be expressed is "operably linked" to a promoter and/or enhancer, and is subject to transcription regulatory control by the promoter and/or enhancer. In one embodiment, the nucleotides encode for a modified or mutated RSV F protein (as

discussed above). In another embodiment, the vector further comprises nucleotides that encode the M and/or G RSV proteins. In another embodiment, the vector further comprises nucleotides that encode the M and/or N RSV proteins. In another embodiment, the vector further comprises nucleotides that encode the M, G and/or N RSV proteins. In another embodiment, the vector further comprises nucleotides that encode a BRSV M protein and/or N RSV proteins. In another embodiment, the vector further comprises nucleotides that encode a BRSV M and/or G protein, or influenza HA and/or NA protein. In another embodiment, the nucleotides encode a modified or mutated RSV F and/or RSV G protein with an influenza HA and/or NA protein. In another embodiment, the expression vector is a baculovirus vector.

[0148] In some embodiments of the invention, proteins may comprise mutations containing alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded protein or how the proteins are made. Nucleotide variants can be produced for a variety of reasons, *e.g.*, to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by insect cells such as Sf9 cells. *See* U.S. Patent Publication 2005/0118191, herein incorporated by reference in its entirety for all purposes.

[0149] In addition, the nucleotides can be sequenced to ensure that the correct coding regions were cloned and do not contain any unwanted mutations. The nucleotides can be subcloned into an expression vector (*e.g.* baculovirus) for expression in any cell. The above is only one example of how the RSV viral proteins can be cloned. A person with skill in the art understands that additional methods are available and are possible.

[0150] The invention also provides for constructs and/or vectors that comprise RSV nucleotides that encode for RSV structural genes, including F, M, G, N, SH, or portions thereof, and/or any chimeric molecule described above. The vector may be, for example, a phage, plasmid, viral, or retroviral vector. The constructs and/or vectors that comprise RSV structural genes, including F, M, G, N, SH, or portions thereof, and/or any chimeric molecule described above, should be operatively linked to an appropriate promoter, such as the AcMNPV polyhedrin promoter (or other baculovirus), phage lambda PL promoter, the *E. coli* lac, phoA and tac promoters, the SV40 early and late promoters, and promoters of retroviral LTRs are non-limiting examples. Other suitable promoters will be known to the skilled artisan depending on the host cell and/or the rate of expression desired. The expression constructs will further contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome-binding site for translation. The coding portion of the

transcripts expressed by the constructs will preferably include a translation initiating codon at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

[0151] Expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Among vectors preferred are virus vectors, such as baculovirus, poxvirus (*e.g.*, vaccinia virus, avipox virus, canarypox virus, fowlpox virus, raccoonpox virus, swinepox virus, etc.), adenovirus (*e.g.*, canine adenovirus), herpesvirus, and retrovirus. Other vectors that can be used with the invention comprise vectors for use in bacteria, which comprise pQE70, pQE60 and pQE-9, pBluescript vectors, Phagescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5. Among preferred eukaryotic vectors are pFastBac1 pWINEO, pSV2CAT, pOG44, pXT1 and pSG, pSVK3, pBPV, pMSG, and pSVL. Other suitable vectors will be readily apparent to the skilled artisan. In one embodiment, the vector that comprises nucleotides encoding for RSV genes, including modified or mutated RSV F genes, as well as genes for M, G, N, SH or portions thereof, and/or any chimeric molecule described above, is pFastBac.

[0152] The recombinant constructs mentioned above could be used to transfect, infect, or transform and can express RSV proteins, including a modified or mutated RSV F protein and at least one immunogen. In one embodiment, the recombinant construct comprises a modified or mutated RSV F, M, G, N, SH, or portions thereof, and/or any molecule described above, into eukaryotic cells and/or prokaryotic cells. Thus, the invention provides for host cells which comprise a vector (or vectors) that contain nucleic acids which code for RSV structural genes, including a modified or mutated RSV F; and at least one immunogen such as but not limited to RSV G, N, and SH, or portions thereof, and/or any molecule described above, and permit the expression of genes, including RSV F, G, N, M, or SH or portions thereof, and/or any molecule described above in the host cell under conditions which allow the formation of VLPs.

[0153] Among eukaryotic host cells are yeast, insect, avian, plant, *C. elegans* (or nematode) and mammalian host cells. Non limiting examples of insect cells are, *Spodoptera frugiperda* (Sf) cells, *e.g.* Sf9, Sf21, *Trichoplusia ni* cells, *e.g.* High Five cells, and *Drosophila* S2 cells. Examples of fungi (including yeast) host cells are *S. cerevisiae*, *Kluyveromyces lactis* (*K. lactis*), species of Candida including *C. albicans* and *C. glabrata*, *Aspergillus nidulans*, *Schizosaccharomyces pombe* (S. pombe), *Pichia pastoris*, and *Yarrowia lipolytica*. Examples

of mammalian cells are COS cells, baby hamster kidney cells, mouse L cells, LNCaP cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, and African green monkey cells, CV1 cells, HeLa cells, MDCK cells, Vero and Hep-2 cells. Xenopus laevis oocytes, or other cells of amphibian origin, may also be used. Examples of prokaryotic host cells include bacterial cells, for example, *E. coli, B. subtilis, Salmonella typhi* and mycobacteria.

[0154] Vectors, *e.g.*, vectors comprising polynucleotides of a modified or mutated RSV F protein; and at least one immunogen including but not limited to RSV G, N, or SH or portions thereof, and/or any chimeric molecule described above, can be transfected into host cells according to methods well known in the art. For example, introducing nucleic acids into eukaryotic cells can be by calcium phosphate co-precipitation, electroporation, microinjection, lipofection, and transfection employing polyamine transfection reagents. In one embodiment, the vector is a recombinant baculovirus. In another embodiment, the recombinant baculovirus is transfected into a eukaryotic cell. In a preferred embodiment, the cell is an insect cell. In another embodiment, the insect cell is a Sf9 cell.

[0155] This invention also provides for constructs and methods that will increase the efficiency of VLP production. For example, the addition of leader sequences to the RSV F, M, G, N, SH, or portions thereof, and/or any chimeric or heterologous molecules described above, can improve the efficiency of protein transporting within the cell. For example, a heterologous signal sequence can be fused to the F, M, G, N, SH, or portions thereof, and/or any chimeric or heterologous molecule described above. In one embodiment, the signal sequence can be derived from the gene of an insect cell and fused to M, F, G, N, SH, or portions thereof, and/or any chimeric or heterologous molecules described above. In another embodiment, the signal peptide is the chitinase signal sequence, which works efficiently in baculovirus expression systems.

[0156] Another method to increase efficiency of VLP production is to codon optimize the nucleotides that encode RSV including a modified or mutated RSV F protein, M, G, N, SH or portions thereof, and/or any chimeric or heterologous molecules described above for a specific cell type. For examples of codon optimizing nucleic acids for expression in Sf9 cell see SEQ ID Nos: 3, 5, 7, 9, 13, 17, 19, and 25.

[0157] The invention also provides for methods of producing VLPs, the methods comprising expressing RSV genes including a modified or mutated RSV F protein, and at least one additional protein, including but not limited to RSV M, G, N, SH, or portions thereof, and/or any chimeric or heterologous molecules described above under conditions that allow VLP

formation. Depending on the expression system and host cell selected, the VLPs are produced by growing host cells transformed by an expression vector under conditions whereby the recombinant proteins are expressed and VLPs are formed. In one embodiment, the invention comprises a method of producing a VLP, comprising transfecting vectors encoding at least one modified or mutated RSV F protein into a suitable host cell and expressing the modified or mutated RSV F protein under conditions that allow VLP formation. In another embodiment, the eukaryotic cell is selected from the group consisting of, yeast, insect, amphibian, avian or mammalian cells. The selection of the appropriate growth conditions is within the skill or a person with skill of one of ordinary skill in the art.

[0158] Methods to grow cells engineered to produce VLPs of the invention include, but are not limited to, batch, batch-fed, continuous and perfusion cell culture techniques. Cell culture means the growth and propagation of cells in a bioreactor (a fermentation chamber) where cells propagate and express protein (*e.g.* recombinant proteins) for purification and isolation. Typically, cell culture is performed under sterile, controlled temperature and atmospheric conditions in a bioreactor. A bioreactor is a chamber used to culture cells in which environmental conditions such as temperature, atmosphere, agitation and/or pH can be monitored. In one embodiment, the bioreactor is a stainless steel chamber. In another embodiment, the bioreactor is a pre-sterilized plastic bag (*e.g.* Cellbag®, Wave Biotech, Bridgewater, NJ). In other embodiment, the pre-sterilized plastic bags are about 50 L to 1000 L bags.

[0159] The VLPs are then isolated using methods that preserve the integrity thereof, such as by gradient centrifugation, *e.g.*, cesium chloride, sucrose and iodixanol, as well as standard purification techniques including, *e.g.*, ion exchange and gel filtration chromatography.

[0160] The following is an example of how VLPs of the invention can be made, isolated and purified. Usually VLPs are produced from recombinant cell lines engineered to create VLPs when the cells are grown in cell culture (see above). A person of skill in the art would understand that there are additional methods that can be utilized to make and purify VLPs of the invention, thus the invention is not limited to the method described.

[0161] Production of VLPs of the invention can start by seeding Sf9 cells (non-infected) into shaker flasks, allowing the cells to expand and scaling up as the cells grow and multiply (for example from a 125-ml flask to a 50 L Wave bag). The medium used to grow the cell is formulated for the appropriate cell line (preferably serum free media, *e.g.* insect medium ExCell-420, JRH). Next, the cells are infected with recombinant baculovirus at the most efficient multiplicity of infection (*e.g.* from about 1 to about 3 plaque forming units per cell).

Once infection has occurred, the modified or mutated RSV F protein, M, G, N, SH, or portions thereof, and/or any chimeric or heterologous molecule described above, are expressed from the virus genome, self assemble into VLPs and are secreted from the cells approximately 24 to 72 hours post infection. Usually, infection is most efficient when the cells are in mid-log phase of growth (4-8 × 10^6 cells/ml) and are at least about 90% viable.

[0162] VLPs of the invention can be harvested approximately 48 to 96 hours post infection, when the levels of VLPs in the cell culture medium are near the maximum but before extensive cell lysis. The Sf9 cell density and viability at the time of harvest can be about 0.5× 10^6 cells/ml to about 1.5 × 10^6 cells/ml with at least 20% viability, as shown by dye exclusion assay. Next, the medium is removed and clarified. NaCl can be added to the medium to a concentration of about 0.4 to about 1.0 M, preferably to about 0.5 M, to avoid VLP aggregation. The removal of cell and cellular debris from the cell culture medium containing VLPs of the invention can be accomplished by tangential flow filtration (TFF) with a single use, pre-sterilized hollow fiber 0.5 or 1.00 μm filter cartridge or a similar device.

[0163] Next, VLPs in the clarified culture medium can be concentrated by ultra-filtration using a disposable, pre-sterilized 500,000 molecular weight cut off hollow fiber cartridge. The concentrated VLPs can be diafiltrated against 10 volumes pH 7.0 to 8.0 phosphate-buffered saline (PBS) containing 0.5 M NaCl to remove residual medium components.

[0164] The concentrated, diafiltered VLPs can be furthered purified on a 20% to 60% discontinuous sucrose gradient in pH 7.2 PBS buffer with 0.5 M NaCl by centrifugation at 6,500 × g for 18 hours at about 4° C to about 10° C. Usually VLPs will form a distinctive visible band between about 30% to about 40% sucrose or at the interface (in a 20% and 60% step gradient) that can be collected from the gradient and stored. This product can be diluted to comprise 200 mM of NaCl in preparation for the next step in the purification process. This product contains VLPs and may contain intact baculovirus particles.

[0165] Further purification of VLPs can be achieved by anion exchange chromatography, or 44% isopycnic sucrose cushion centrifugation. In anion exchange chromatography, the sample from the sucrose gradient (see above) is loaded into column containing a medium with an anion (*e.g.* Matrix Fractogel EMD TMAE) and eluded via a salt gradient (from about 0.2 M to about 1.0 M of NaCl) that can separate the VLP from other contaminates (*e.g.* baculovirus and DNA/RNA). In the sucrose cushion method, the sample comprising the VLPs is added to a 44% sucrose cushion and centrifuged for about 18 hours at 30,000 g. VLPs form a band at the top of 44% sucrose, while baculovirus precipitates at the bottom and

other contaminating proteins stay in the 0% sucrose layer at the top. The VLP peak or band is collected.

[0166] The intact baculovirus can be inactivated, if desired. Inactivation can be accomplished by chemical methods, for example, formalin or β-propiolactone (BPL). Removal and/or inactivation of intact baculovirus can also be largely accomplished by using selective precipitation and chromatographic methods known in the art, as exemplified above. Methods of inactivation comprise incubating the sample containing the VLPs in 0.2% of BPL for 3 hours at about 25°C to about 27°C. The baculovirus can also be inactivated by incubating the sample containing the VLPs at 0.05% BPL at 4°C for 3 days, then at 37 °C for one hour.

[0167] After the inactivation/removal step, the product comprising VLPs can be run through another diafiltration step to remove any reagent from the inactivation step and/or any residual sucrose, and to place the VLPs into the desired buffer (*e.g.* PBS). The solution comprising VLPs can be sterilized by methods known in the art (*e.g.* sterile filtration) and stored in the refrigerator or freezer.

[0168] The above techniques can be practiced across a variety of scales. For example, T-flasks, shake-flasks, spinner bottles, up to industrial sized bioreactors. The bioreactors can comprise either a stainless steel tank or a pre-sterilized plastic bag (for example, the system sold by Wave Biotech, Bridgewater, NJ). A person with skill in the art will know what is most desirable for their purposes.

[0169] Expansion and production of baculovirus expression vectors and infection of cells with recombinant baculovirus to produce recombinant RSV VLPs can be accomplished in insect cells, for example Sf9 insect cells as previously described. In one embodiment, the cells are SF9 infected with recombinant baculovirus engineered to produce RSV VLPs.

Pharmaceutical or Vaccine Formulations and Administration

[0170] The pharmaceutical compositions useful herein contain a pharmaceutically acceptable carrier, including any suitable diluent or excipient, which includes any pharmaceutical agent that does not itself induce the production of an immune response harmful to the vertebrate receiving the composition and which may be administered without undue toxicity, and a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein of the invention. As used herein, the term "pharmaceutically acceptable" means being approved by a

regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopia, European Pharmacopia or other generally recognized pharmacopia for use in mammals, and more particularly in humans. These compositions can be useful as a vaccine and/or antigenic compositions for inducing a protective immune response in a vertebrate.

[0171] The invention encompasses a pharmaceutically acceptable vaccine composition comprising VLPs comprising at least one modified or mutated RSV F protein, and at least one additional protein, including but not limited to RSV M, G, N, SH, or portions thereof, and/or any chimeric or heterologous molecules described above. In one embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs comprising at least one modified or mutated RSV F protein and at least one additional immunogen. In another embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs comprising at least one modified or mutated RSV F protein and at least one RSV M protein. In another embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs comprising at least one modified or mutated RSV F protein and at least one BRSV M protein. In another embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs comprising at least one modified or mutated RSV F protein and at least one influenza M1 protein. In another embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs comprising at least one modified or mutated RSV F protein and at least one avian influenza M1 protein.

[0172] In another embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs further comprising an RSV G protein, including but not limited to a HRSV, BRSV or avian RSV G protein. In another embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs further comprising RSV N protein, including but not limited to a HRSV, BRSV or avian RSV N protein. In another embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs further comprising RSV SH protein, including but not limited to a HRSV, BRSV or avian RSV SH protein.

[0173] In another embodiment, the invention encompasses a pharmaceutically acceptable vaccine composition comprising chimeric VLPs such as VLPs comprising BRSV M and a modified or mutated RSV F protein and/or G, H, or SH protein from a RSV and optionally HA or NA protein derived from an influenza virus, wherein the HA or NA protein is a fused to the transmembrane domain and cytoplasmic tail of RSV F and/or G protein.

[0174] The invention also encompasses a pharmaceutically acceptable vaccine composition comprising modified or mutated RSV F protein, an RSV F micelle comprising a modified or

mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein as described above.

[0175] In one embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs comprising a modified or mutated RSV F protein and at least one additional protein. In another embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs further comprising RSV M protein, such as but not limited to a BRSV M protein. In another embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs further comprising RSV G protein, including but not limited to a HRSV G protein. In another embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs further comprising RSV N protein, including but not limited to a HRSV, BRSV or avian RSV N protein. In another embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs further comprising RSV SH protein, including but not limited to a HRSV, BRSV or avian RSV SH protein. In another embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs comprising BRSV M protein and F and/or G protein from HRSV group A. In another embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs comprising BRSV M protein and F and/or G protein from HRSV group B. In another embodiment, the invention encompasses a pharmaceutically acceptable vaccine composition comprising chimeric VLPs such as VLPs comprising chimeric M protein from a BRSV and optionally HA protein derived from an influenza virus, wherein the M protein is fused to the influenza HA protein. In another embodiment, the invention encompasses a pharmaceutically acceptable vaccine composition comprising chimeric VLPs such as VLPs comprising BRSV M, and a chimeric F and/or G protein from a RSV and optionally HA protein derived from an influenza virus, wherein the chimeric influenza HA protein is fused to the transmembrane domain and cytoplasmic tail of RSV F and/or G protein. In another embodiment, the invention encompasses a pharmaceutically acceptable vaccine composition comprising chimeric VLPs such as VLPs comprising BRSV M and a chimeric F and/or G protein from a RSV and optionally HA or NA protein derived from an influenza virus, wherein the HA or NA protein is a fused to the transmembrane domain and cytoplasmic tail of RSV F and/or G protein.

[0176] The invention also encompasses a pharmaceutically acceptable vaccine composition comprising a chimeric VLP that comprises at least one RSV protein. In one embodiment, the pharmaceutically acceptable vaccine composition comprises VLPs comprising a modified or mutated RSV F protein and at least one immunogen from a heterologous infectious agent or diseased cell. In another embodiment, the immunogen from a heterologous infectious agent

is a viral protein. In another embodiment, the viral protein from a heterologous infectious agent is an envelope associated protein. In another embodiment, the viral protein from a heterologous infectious agent is expressed on the surface of VLPs. In another embodiment, the protein from an infectious agent comprises an epitope that will generate a protective immune response in a vertebrate.

[0177] The invention also encompasses a kit for immunizing a vertebrate, such as a human subject, comprising VLPs that comprise at least one RSV protein. In one embodiment, the kit comprises VLPs comprising a modified or mutated RSV F protein. In one embodiment, the kit further comprises a RSV M protein such as a BRSV M protein. In another embodiment, the kit further comprises a RSV G protein. In another embodiment, the invention encompasses a kit comprising VLPs which comprises a chimeric M protein from a BRSV and optionally HA protein derived from an influenza virus, wherein the M protein is fused to the BRSV M. In another embodiment, the invention encompasses a kit comprising VLPs which comprises a chimeric M protein from a BRSV, a RSV F and/or G protein and an immunogen from a heterologous infectious agent. In another embodiment, the invention encompasses a kit comprising VLPs which comprises a M protein from a BRSV, a chimeric RSV F and/or G protein and optionally HA protein derived from an influenza virus, wherein the HA protein is fused to the transmembrane domain and cytoplasmic tail of RSV F or G protein. In another embodiment, the invention encompasses a kit comprising VLPs which comprises M protein from a BRSV, a chimeric RSV F and/or G protein and optionally HA or NA protein derived from an influenza virus, wherein the HA protein is fused to the transmembrane domain and cytoplasmic tail of RSV F and/or G protein.

[0178] In one embodiment, the invention comprises an immunogenic formulation comprising at least one effective dose of a modified or mutated RSV F protein. In another embodiment, the invention comprises an immunogenic formulation comprising at least one effective dose of an RSV F micelle comprising a modified or mutated RSV F protein. In yet another embodiment, the invention comprises an immunogenic formulation comprising at least one effective dose of a VLP comprising a modified or mutated RSV F protein as described above.

[0179] The immunogenic formulation of the invention comprises a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein, and a pharmaceutically acceptable carrier or excipient. Pharmaceutically acceptable carriers include but are not limited to saline, buffered saline, dextrose, water, glycerol, sterile isotonic aqueous buffer, and combinations thereof. A thorough discussion of pharmaceutically acceptable carriers, diluents, and other

excipients is presented in Remington's Pharmaceutical Sciences (Mack Pub. Co. N.J. current edition). The formulation should suit the mode of administration. In a preferred embodiment, the formulation is suitable for administration to humans, preferably is sterile, non-particulate and/or non-pyrogenic.

[0180] The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a solid form, such as a lyophilized powder suitable for reconstitution, a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, *etc*.

[0181] The invention also provides for a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the vaccine formulations of the invention. In a preferred embodiment, the kit comprises two containers, one containing a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein, and the other containing an adjuvant. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[0182] The invention also provides that the formulation be packaged in a hermetically sealed container such as an ampoule or sachette indicating the quantity of composition. In one embodiment, the composition is supplied as a liquid, in another embodiment, as a dry sterilized lyophilized powder or water free concentrate in a hermetically sealed container and can be reconstituted, *e.g.*, with water or saline to the appropriate concentration for administration to a subject.

[0183] In an alternative embodiment, the composition is supplied in liquid form in a hermetically sealed container indicating the quantity and concentration of the composition. Preferably, the liquid form of the composition is supplied in a hermetically sealed container at least about 50 μg/ml, more preferably at least about 100 μg/ml, at least about 200 μg/ml, at least 500 μg/ml, or at least 1 mg/ml.

[0184] As an example, chimeric RSV VLPs comprising a modified or mutated RSV F protein of the invention are administered in an effective amount or quantity (as defined above) sufficient to stimulate an immune response, each a response against one or more strains of RSV. Administration of the modified or mutated RSV F protein, an RSV F micelle

comprising a modified or mutated RSV F protein, or VLP of the invention elicits immunity against RSV. Typically, the dose can be adjusted within this range based on, *e.g.*, age, physical condition, body weight, sex, diet, time of administration, and other clinical factors. The prophylactic vaccine formulation is systemically administered, *e.g.*, by subcutaneous or intramuscular injection using a needle and syringe, or a needle-less injection device. Alternatively, the vaccine formulation is administered intranasally, either by drops, large particle aerosol (greater than about 10 microns), or spray into the upper respiratory tract. While any of the above routes of delivery results in an immune response, intranasal administration confers the added benefit of eliciting mucosal immunity at the site of entry of many viruses, including RSV and influenza.

[0185] Thus, the invention also comprises a method of formulating a vaccine or antigenic composition that induces immunity to an infection or at least one disease symptom thereof to a mammal, comprising adding to the formulation an effective dose of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein. In one embodiment, the infection is an RSV infection.

[0186] While stimulation of immunity with a single dose is possible, additional dosages can be administered, by the same or different route, to achieve the desired effect. In neonates and infants, for example, multiple administrations may be required to elicit sufficient levels of immunity. Administration can continue at intervals throughout childhood, as necessary to maintain sufficient levels of protection against infections, *e.g.* RSV infection. Similarly, adults who are particularly susceptible to repeated or serious infections, such as, for example, health care workers, day care workers, family members of young children, the elderly, and individuals with compromised cardiopulmonary function may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored, for example, by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to elicit and maintain desired levels of protection.

[0187] Methods of administering a composition comprising a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein (*e.g.* vaccine and/or antigenic formulations) include, but are not limited to, parenteral administration (*e.g.*, intradermal, intramuscular, intravenous and subcutaneous), epidural, and mucosal (*e.g.*, intranasal and oral or pulmonary routes or by suppositories). In a specific embodiment, compositions of the present invention

are administered intramuscularly, intravenously, subcutaneously, transdermally or intradermally. The compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucous, colon, conjunctiva, nasopharynx, oropharynx, vagina, urethra, urinary bladder and intestinal mucosa, etc.) and may be administered together with other biologically active agents. In some embodiments, intranasal or other mucosal routes of administration of a composition of the invention may induce an antibody or other immune response that is substantially higher than other routes of administration. In another embodiment, intranasal or other mucosal routes of administration of a composition of the invention may induce an antibody or other immune response that will induce cross protection against other strains of RSV. Administration can be systemic or local.

[0188] In yet another embodiment, the vaccine and/or immunogenic formulation is administered in such a manner as to target mucosal tissues in order to elicit an immune response at the site of immunization. For example, mucosal tissues such as gut associated lymphoid tissue (GALT) can be targeted for immunization by using oral administration of compositions which contain adjuvants with particular mucosal targeting properties. Additional mucosal tissues can also be targeted, such as nasopharyngeal lymphoid tissue (NALT) and bronchial-associated lymphoid tissue (BALT).

[0189] Vaccines and/or immunogenic formulations of the invention may also be administered on a dosage schedule, for example, an initial administration of the vaccine composition with subsequent booster administrations. In particular embodiments, a second dose of the composition is administered anywhere from two weeks to one year, preferably from about 1, about 2, about 3, about 4, about 5 to about 6 months, after the initial administration. Additionally, a third dose may be administered after the second dose and from about three months to about two years, or even longer, preferably about 4, about 5, or about 6 months, or about 7 months to about one year after the initial administration. The third dose may be optionally administered when no or low levels of specific immunoglobulins are detected in the serum and/or urine or mucosal secretions of the subject after the second dose. In a preferred embodiment, a second dose is administered about one month after the first administration and a third dose is administered about six months after the first administration. In another embodiment, the second dose is administered about six months after the first administration. In another embodiment, the compositions of the invention can be administered as part of a combination therapy. For example, compositions of the invention can be formulated with other immunogenic compositions, antivirals and/or antibiotics.

[0190] The dosage of the pharmaceutical composition can be determined readily by the skilled artisan, for example, by first identifying doses effective to elicit a prophylactic or therapeutic immune response, *e.g.*, by measuring the serum titer of virus specific immunoglobulins or by measuring the inhibitory ratio of antibodies in serum samples, or urine samples, or mucosal secretions. The dosages can be determined from animal studies. A non-limiting list of animals used to study the efficacy of vaccines include the guinea pig, hamster, ferrets, chinchilla, mouse and cotton rat. Most animals are not natural hosts to infectious agents but can still serve in studies of various aspects of the disease. For example, any of the above animals can be dosed with a vaccine candidate, *e.g.* modified or mutated RSV F proteins, an RSV F micelle comprising a modified or mutated RSV F protein, or VLPs of the invention, to partially characterize the immune response induced, and/or to determine if any neutralizing antibodies have been produced. For example, many studies have been conducted in the mouse model because mice are small size and their low cost allows researchers to conduct studies on a larger scale.

[0191] In addition, human clinical studies can be performed to determine the preferred effective dose for humans by a skilled artisan. Such clinical studies are routine and well known in the art. The precise dose to be employed will also depend on the route of administration. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal test systems.

[0192] As also well known in the art, the immunogenicity of a particular composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Adjuvants have been used experimentally to promote a generalized increase in immunity against unknown antigens (*e.g.*, U.S. Pat. No. 4,877,611). Immunization protocols have used adjuvants to stimulate responses for many years, and as such, adjuvants are well known to one of ordinary skill in the art. Some adjuvants affect the way in which antigens are presented. For example, the immune response is increased when protein antigens are precipitated by alum. Emulsification of antigens also prolongs the duration of antigen presentation. The inclusion of any adjuvant described in Vogel *et al.*, "A Compendium of Vaccine Adjuvants and Excipients (2nd Edition)," herein incorporated by reference in its entirety for all purposes, is envisioned within the scope of this invention.

[0193] Exemplary, adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed Mycobacterium tuberculosis), incomplete Freund's adjuvants and aluminum hydroxide adjuvant. Other adjuvants comprise GMCSP, BCG, aluminum hydroxide, MDP compounds, such as thur-MDP and nor-MDP, CGP (MTP-PE),

lipid A, and monophosphoryl lipid A (MPL). RIBI, which contains three components extracted from bacteria, MPL, trehalose dimycolate (TDM) and cell wall skeleton (CWS) in a 2% squalene/Tween 80 emulsion also is contemplated. MF-59, Novasomes®, MHC antigens may also be used.

[0194] In one embodiment of the invention the adjuvant is a paucilamellar lipid vesicle having about two to ten bilayers arranged in the form of substantially spherical shells separated by aqueous layers surrounding a large amorphous central cavity free of lipid bilayers. Paucilamellar lipid vesicles may act to stimulate the immune response several ways, as non-specific stimulators, as carriers for the antigen, as carriers of additional adjuvants, and combinations thereof. Paucilamellar lipid vesicles act as non-specific immune stimulators when, for example, a vaccine is prepared by intermixing the antigen with the preformed vesicles such that the antigen remains extracellular to the vesicles. By encapsulating an antigen within the central cavity of the vesicle, the vesicle acts both as an immune stimulator and a carrier for the antigen. In another embodiment, the vesicles are primarily made of nonphospholipid vesicles. In other embodiments, the vesicles are Novasomes®. Novasomes® are paucilamellar nonphospholipid vesicles ranging from about 100 nm to about 500 nm. They comprise Brij 72, cholesterol, oleic acid and squalene. Novasomes have been shown to be an effective adjuvant for influenza antigens (*see*, U.S. Patents 5,629,021, 6,387,373, and 4,911,928, herein incorporated by reference in their entireties for all purposes).

[0195] The compositions of the invention can also be formulated with "immune stimulators." These are the body's own chemical messengers (cytokines) to increase the immune system's response. Immune stimulators include, but are not limited to, various cytokines, lymphokines and chemokines with immunostimulatory, immunopotentiating, and pro-inflammatory activities, such as interleukins (*e.g.*, IL-1, IL-2, IL-3, IL-4, IL-12, IL-13); growth factors (*e.g.*, granulocyte-macrophage (GM)-colony stimulating factor (CSF)); and other immunostimulatory molecules, such as macrophage inflammatory factor, Flt3 ligand, B7.1; B7.2, etc. The immunostimulatory molecules can be administered in the same formulation as the compositions of the invention, or can be administered separately. Either the protein or an expression vector encoding the protein can be administered to produce an immunostimulatory effect. Thus in one embodiment, the invention comprises antigentic and vaccine formulations comprising an adjuvant and/or an immune stimulator.

Methods of Stimulating an Immune Response

[0196] The modified or mutated RSV F proteins, the RSV F micelles comprising a modified or mutated RSV F protein, or the VLPs of the invention are useful for preparing compositions that stimulate an immune response that confers immunity or substantial immunity to infectious agents. Both mucosal and cellular immunity may contribute to immunity to infectious agents and disease. Antibodies secreted locally in the upper respiratory tract are a major factor in resistance to natural infection. Secretory immunoglobulin A (sIgA) is involved in the protection of the upper respiratory tract and serum IgG in protection of the lower respiratory tract. The immune response induced by an infection protects against reinfection with the same virus or an antigenically similar viral strain. For example, RSV undergoes frequent and unpredictable changes; therefore, after natural infection, the effective period of protection provided by the host's immunity may only be effective for a few years against the new strains of virus circulating in the community.

[0197] Thus, the invention encompasses a method of inducing immunity to infections or at least one disease symptom thereof in a subject, comprising administering at least one effective dose of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein. In one embodiment, the method comprises administering VLPs comprising a modified or mutated RSV F protein and at least one additional protein. In another embodiment, the method comprises administering VLPs further comprising an RSV M protein, for example, a BRSV M protein. In another embodiment, the method comprises administering VLPs further comprising a RSV N protein. In another embodiment, the method comprises administering VLPs further comprising a RSV G protein. In another embodiment, the method comprises administering VLPs further comprising a RSV SH protein. In another embodiment, the method comprises administering VLPs further comprising F and/or G protein from HRSV group A and/or group B. In another embodiment, the method comprises administering VLPs comprising M protein from BRSV and a chimeric RSV F and/or G protein or MMTV envelope protein, for example, HA or NA protein derived from an influenza virus, wherein the HA and/or NA protein is fused to the transmembrane domain and cytoplasmic tail of the RSV F and/or G protein or MMTV envelope protein. In another embodiment, the method comprises administering VLPs comprising M protein from BRSV and a chimeric RSV F and/or G protein and optionally HA or NA protein derived from an influenza virus, wherein the HA or NA protein is fused to the transmembrane domain and cytoplasmic tail of RSV F and/or G protein. In another embodiment, the subject is a

mammal. In another embodiment, the mammal is a human. In another embodiment, RSV VLPs are formulated with an adjuvant or immune stimulator.

[0198] In one embodiment, the invention comprises a method to induce immunity to RSV infection or at least one disease symptom thereof in a subject, comprising administering at least one effective dose of a modified or mutated RSV F protein. In another embodiment, the invention comprises a method to induce immunity to RSV infection or at least one disease symptom thereof in a subject, comprising administering at least one effective dose of an RSV F micelle comprising a modified or mutated RSV F protein. In yet another embodiment, the invention comprises a method to induce immunity to RSV infection or at least one disease symptom thereof in a subject, comprising administering at least one effective dose of RSV VLPs, wherein the VLPs comprise a modified or mutated RSV F protein, M, G, SH, and/or N proteins. In another embodiment, a method of inducing immunity to RSV infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of a RSV VLPs, wherein the VLPs consists essentially of BRSV M (including chimeric M), and RSV F, G, and/or N proteins. The VLPs may comprise additional RSV proteins and/or protein contaminates in negligible concentrations. In another embodiment, a method of inducing immunity to RSV infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of a RSV VLPs, wherein the VLPs consists of BRSV M (including chimeric M), RSV G and/or F. In another embodiment, a method of inducing immunity to RSV infection or at least one disease symptom in a subject, comprises administering at least one effective dose of a RSV VLPs comprising RSV proteins, wherein the RSV proteins consist of BRSV M (including chimeric M), F, G, and/or N proteins, including chimeric F, G, and/or N proteins. These VLPs contain BRSV M (including chimeric M), RSV F, G, and/or N proteins and may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc., but do not contain additional RSV proteins (other than fragments of BRSV M (including chimeric M), BRSV/RSV F, G, and/or N proteins. In another embodiment, the subject is a vertebrate. In one embodiment the vertebrate is a mammal. In another embodiment, the mammal is a human. In another embodiment, the method comprises inducing immunity to RSV infection or at least one disease symptom by administering the formulation in one dose. In another embodiment, the method comprises inducing immunity to RSV infection or at least one disease symptom by administering the formulation in multiple doses.

[0199] The composition may be administered in a suitable protein dosage range. In some aspects, the protein dosage range has an upper limit of about 100 μg, about 80 μg, about 60 μg, about 30 μg, about 15 μg, about 10 μg, or about 5 μg. In other aspects, the dosage range has a lower limit of about 30 μg, about 15 μg, about 5 μg, or about 1 μg. Thus, suitable ranges include, for example, about 1 μg to about 100 μg, about 5 μg to about 80 μg, about 5 μg to about 60 μg, about 15 μg to about 60 μg, about 30 μg to about 60 μg, and about 15 μg to about 30 μg of protein.

[0200] As used throughout the disclosure, the term "about" means ± 10% of the indicated value.

[0201] The invention also encompasses inducing immunity to an infection, or at least one symptom thereof, in a subject caused by an infectious agent, comprising administering at least one effective dose of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein. In one embodiment, the method comprises administering VLPs comprising a modified or mutated RSV F protein and at least one protein from a heterologous infectious agent. In one embodiment, the method comprises administering VLPs comprising a modified or mutated RSV F protein and at least one protein from the same or a heterologous infectious agent. In another embodiment, the protein from the heterologous infectious agent is a viral protein. In another embodiment, the protein from the infectious agent is an envelope associated protein. In another embodiment, the protein from the infectious agent is expressed on the surface of VLPs. In another embodiment, the protein from the infectious agent comprises an epitope that will generate a protective immune response in a vertebrate. In another embodiment, the protein from the infectious agent can associate with RSV M protein such as BRSV M protein, RSV F, G and/or N protein. In another embodiment, the protein from the infectious agent is fused to a RSV protein such as a BRSV M protein, RSV F, G and/or N protein. In another embodiment, only a portion of a protein from the infectious agent is fused to a RSV protein such as a BRSV M protein, RSV F, G and/or N protein. In another embodiment, only a portion of a protein from the infectious agent is fused to a portion of a RSV protein such as a BRSV M protein, RSV F, G and/or N protein. In another embodiment, the portion of the protein from the infectious agent fused to the RSV protein is expressed on the surface of VLPs. In other embodiment, the RSV protein, or portion thereof, fused to the protein from the infectious agent associates with the RSV M

protein. In other embodiment, the RSV protein, or portion thereof, is derived from RSV F, G, N and/or P. In another embodiment, the chimeric VLPs further comprise N and/or P protein from RSV. In another embodiment, the chimeric VLPs comprise more than one protein from the same and/or a heterologous infectious agent. In another embodiment, the chimeric VLPs comprise more than one infectious agent protein, thus creating a multivalent VLP.

[0202] Compositions of the invention can induce substantial immunity in a vertebrate (*e.g.* a human) when administered to the vertebrate. The substantial immunity results from an immune response against compositions of the invention that protects or ameliorates infection or at least reduces a symptom of infection in the vertebrate. In some instances, if the vertebrate is infected, the infection will be asymptomatic. The response may not be a fully protective response. In this case, if the vertebrate is infected with an infectious agent, the vertebrate will experience reduced symptoms or a shorter duration of symptoms compared to a non-immunized vertebrate.

[0203] In one embodiment, the invention comprises a method of inducing substantial immunity to RSV virus infection or at least one disease symptom in a subject, comprising administering at least one effective dose of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein. In another embodiment, the invention comprises a method of vaccinating a mammal against RSV comprising administering to the mammal a protection-inducing amount of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein. In one embodiment, the method comprises administering VLPs further comprising an RSV M protein, such as BRSV M protein. In another embodiment, the method further comprises administering VLPs comprising RSV G protein, for example a HRSV G protein. In another embodiment, the method further comprises administering VLPs comprising the N protein from HRSV group A. In another embodiment, the method further comprises administering VLPs comprising the N protein from HRSV group B. In another embodiment, the method comprises administering VLPs comprising chimeric M protein from BRSV and F and/or G protein derived from RSV wherein the F and/or G protein is fused to the transmembrane and cytoplasmic tail of the M protein. In another embodiment, the method comprises administering VLPs comprising M protein from BRSV and chimeric RSV F and/or G protein wherein the F and/or G protein is a fused to the transmembrane domain and cytoplasmic tail of influenza HA and/or NA protein. In another embodiment, the method comprises administering VLPs comprising M protein from BRSV and chimeric RSV F and/or

G protein and optionally an influenza HA and/or NA protein wherein the F and/or G protein is a fused to the transmembrane domain and cytoplasmic tail of the HA protein. In another embodiment, the method comprises administering VLPs comprising M protein from BRSV and chimeric RSV F and/or G protein, and optionally an influenza HA and/or NA protein wherein the HA and/or NA protein is fused to the transmembrane domain and cytoplasmic tail of RSV F and/or G protein.

[0204] The invention also encompasses a method of inducing substantial immunity to an infection, or at least one disease symptom in a subject caused by an infectious agent, comprising administering at least one effective dose of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein. In one embodiment, the method comprises administering VLPs further comprising a RSV M protein, such as BRSV M protein, and at least one protein from another infectious agent. In one embodiment, the method comprises administering VLPs further comprising a BRSV M protein and at least one protein from the same or a heterologous infectious agent. In another embodiment, the protein from the infectious agent is a viral protein. In another embodiment, the protein from the infectious agent is an envelope associated protein. In another embodiment, the protein from the infectious agent is expressed on the surface of VLPs. In another embodiment, the protein from the infectious agent comprises an epitope that will generate a protective immune response in a vertebrate. In another embodiment, the protein from the infectious agent can associate with RSV M protein. In another embodiment, the protein from the infectious agent can associate with BRSV M protein. In another embodiment, the protein from the infectious agent is fused to a RSV protein. In another embodiment, only a portion of a protein from the infectious agent is fused to a RSV protein. In another embodiment, only a portion of a protein from the infectious agent is fused to a portion of a RSV protein. In another embodiment, the portion of the protein from the infectious agent fused to the RSV protein is expressed on the surface of VLPs. In other embodiment, the RSV protein, or portion thereof, fused to the protein from the infectious agent associates with the RSV M protein. In other embodiment, the RSV protein, or portion thereof, fused to the protein from the infectious agent associates with the BRSV M protein. In other embodiment, the RSV protein, or portion thereof, is derived from RSV F, G, N and/or P. In another embodiment, the VLPs further comprise N and/or P protein from RSV. In another embodiment, the VLPs comprise more than one protein from the infectious agent. In another embodiment, the VLPs comprise more than one infectious agent protein, thus creating a multivalent VLP.

[0205] In another embodiment, the invention comprises a method of inducing a protective antibody response to an infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of a modified or mutated RSV F protein, an RSV F micelle comprising a modified or mutated RSV F protein, or a VLP comprising a modified or mutated RSV F protein as described above.

[0206] As used herein, an "antibody" is a protein comprising one or more polypeptides substantially or partially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. A typical immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. Antibodies exist as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases.

[0207] In one embodiment, the invention comprises a method of inducing a protective cellular response to RSV infection or at least one disease symptom in a subject, comprising administering at least one effective dose of a modified or mutated RSV F protein. In another embodiment, the invention comprises a method of inducing a protective cellular response to RSV infection or at least one disease symptom in a subject, comprising administering at least one effective dose an RSV F micelle comprising a modified or mutated RSV F protein. In yet another embodiment, the invention comprises a method of inducing a protective cellular response to RSV infection or at least one disease symptom in a subject, comprising administering at least one effective dose a VLP, wherein the VLP comprises a modified or mutated RSV F protein as described above. Cell-mediated immunity also plays a role in recovery from RSV infection and may prevent RSV-associated complications. RSV-specific cellular lymphocytes have been detected in the blood and the lower respiratory tract secretions of infected subjects. Cytolysis of RSV-infected cells is mediated by CTLs in concert with RSV-specific antibodies and complement. The primary cytotoxic response is detectable in blood after 6–14 days and disappears by day 21 in infected or vaccinated individuals (Ennis *et al.,* 1981). Cell-mediated immunity may also play a role in recovery

from RSV infection and may prevent RSV-associated complications. RSV-specific cellular lymphocytes have been detected in the blood and the lower respiratory tract secretions of infected subjects.

[0208] As mentioned above, the immunogenic compositions of the invention prevent or reduce at least one symptom of RSV infection in a subject. Symptoms of RSV are well known in the art. They include rhinorrhea, sore throat, headache, hoarseness, cough, sputum, fever, rales, wheezing, and dyspnea. Thus, the method of the invention comprises the prevention or reduction of at least one symptom associated with RSV infection. A reduction in a symptom may be determined subjectively or objectively, *e.g.*, self assessment by a subject, by a clinician's assessment or by conducting an appropriate assay or measurement (*e.g.* body temperature), including, *e.g.*, a quality of life assessment, a slowed progression of a RSV infection or additional symptoms, a reduced severity of a RSV symptoms or a suitable assays (*e.g.* antibody titer and/or T-cell activation assay). The objective assessment comprises both animal and human assessments.

[0209] This invention is further illustrated by the following examples that should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and the Sequence Listing, are incorporated herein by reference for all purposes.

EXAMPLES

Example 1

Generating recombinant bacmids, transfection of insect cells to make recombinant virus stocks, plaque purification, and infecting insect cells with primary virus stock.

[0210] To construct recombinant virus, the viral genes of interest were codon optimized for Sf9 insect cells expression and cloned into pFastBac™ vectors.

[0211] Once the desired constructs were identified and purified, one vial of MAX Efficiency® DH10Bac™ competent cells for each construct was thawed on ice. Approximately 1 ng (5 µl) of the desired pFastBac™ construct plasmid DNA was added to the cells and mixed gently. The cells were incubated on ice for 30 minutes. This was

followed by heat-shock of the cells for 45 seconds at 42° C without shaking. Next, the tubes were transferred to ice and chilled for 2 minutes. Subsequently 900 µl of room temperature S.O.C. Medium was added to each tube. The tubes were put on a shaker at 37°C at 225 rpm for 4 hours. For each pFastBac™ transformation, 10-fold serial dilutions of the cells (10-1, 10-2 and 10-3) were prepared using S.O.C. medium. Next, 100 µl of each dilution was plated on an LB agar plate containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal, and 40 µg/ml IPTG . The plates were incubated for 48 hours at 37° C. White colonies were picked for analysis.

[0212] Different bacmid DNAs from above were made for each construct and were isolated. These DNAs were precipitated and added to Sf9 cells for 5 hours.

[0213] Next, 30 ml of Sf9 insect cells (2 x 10^6 cells/ml) were infected with baculovirus expressing viral proteins of interest with 0.3 ml of plaque eluate and incubated 48-72 hrs. Approximately 1 ml of crude culture (cells + medium) and clarified culture harvests were saved for expression analysis and the rest were saved for purification purposes.

Example 2

Expression, purification, and analysis of modified HRSV F proteins

[0214] Genes encoding modified HRSV F proteins of interest were synthesized in vitro as overlapping oligonucleotides, cloned and expressed in host cells. Cloning and expression of the modified RSV F genes were achieved following the methods known in the art.

[0215] Recombinant plaques containing viral proteins of interest were picked and confirmed. The recombinant virus was then amplified by infection of Sf9 insect cells. In some cases, Sf9 insect cells were co-infected by a recombinant virus expressing modified F protein and another recombinant virus expressing other viral proteins (e.g., BRSV M protein and/or HRSV N protein). A culture of insect cells was infected at ~3 MOI (Multiplicity of infection = virus ffu or pfu/cell) with baculovirus carrying the various constructs. The culture and supernatant were harvested 48-72 hours post-infection. The crude harvest, approximately 30 mL, was clarified by centrifugation for 15 minutes at approximately 800 x g. The resulting crude cell harvests containing modified HRSV F protein were purified as described below.

[0216] Modified HRSV F proteins of interest were purified from the infected Sf9 insect cell culture harvests. Non-ionic surfactant Tergitol® NP-9 (Nonylphenol Ethoxylate) was used in a membrane protein extraction protocol. Crude extraction was further purified by passing

through anion exchange chromatography, lentil lectin affinity/HIC, and cation exchange chromatography.

[0217] Protein expression was analyzed by SDS-PAGE and stained for total proteins by coomassie stain. Equal volumes of cell samples from crude harvest and 2x sample buffer containing βME (beta-mercaptoehtanol) were loaded, approximately 15 to 20 µl (about to 7.5 to 10 µl of the culture)/lane, onto an SDS Laemmli gel.

[0218] In some cases, instead of chromatography, modified HRSV F proteins in the crude cell harvests were concentrated by 30% sucrose gradient separation method, and then were analyzed by SDS-PAGE stained with coomassie, or Western Blot using anti-RSV F monoclonal antibody.

[0219] Crude cell harvest containing modified recombinant F proteins, purified recombinant F proteins, or recombinant F proteins concentrated by sucrose gradient can be further analyzed by Western Blot using anti-RSV F monoclonal antibody and/or anti-RSV F polyclonal antibody.

Example 3

Modified HRSV F gene encoding F protein BV # 541

[0220] Initial attempts to express the full length HRSV F protein proved unsuccessful in achieving high levels of expression. The F gene sequence used in the expression was SEQ ID NO: 1 (wild type HRSV F gene, GenBank Accession No. M11486). It encodes an inactive precursor (F_0) of 574 aa. This precursor is cleaved twice by furin-like proteases during maturation to yield two disulfide-linked polypeptides, subunit F_2 from the N terminus and F_1 from the C terminus (Figure 1). The two cleavages sites are at residues 109 and 136, which are preceded by furin-recognition motifs (RARR, aa 106-109 (SEQ ID NO: 23) and KKRKRR, aa 131-136 (SEQ ID NO: 24)). The F gene sequence of SEQ ID NO: 1 contains suboptimal codon usage for expression in Sf9 insect cells and harbors 3 errors, producing a protein that can exhibit less than optimal folding (SEQ ID NO: 2, GenBank Accession No. AAB59858). In addition, a possible Poly (A) adenylation site (ATAAAA) was identified at the region encoding the F_2 subunit. Moreover, the wild type F gene sequence is approximately 65% AT rich, while desired GC-AT ratio of a gene sequence in Sf9 insect cell expression system is approximately 1:1.

[0221] In attempt to overcome poor expression levels of HRSV F protein, a new F gene sequence was designed so that:

(a) the three GenBank sequencing errors were corrected;

(b) the cryptic poly (A) site at the region encoding F_2 subunit was modified;

(c) F gene codons were optimized; and

(d) the F gene encodes a modified F protein with inactivated primary cleavage site.

[0222] The three corrected amino acids errors were P102A, I379V, and M447V. The cryptic poly (A) site in the HRSV F gene was corrected without changing the amino acid sequence.

[0223] The codon optimization scheme was based on the following criteria: (1) abundance of aminoacyl-tRNAs for a particular codon in Lepidopteran species of insect cells for a given amino acid as described by Levin, D.B. et al. (Journal of General Virology, 2000, vol. 81, pp. 2313-2325), (2) maintenance of GC-AT ratio in gene sequences at approximately 1:1, (3) minimal introduction of palindromic or stem-loop DNA structures, and (4) minimal introduction of transcription and post-transcription repressor element sequences. An example of optimized F gene sequence was shown as SEQ ID NO: 19 (RSV-F BV #368).

[0224] To inactivate the primary cleavage site (1° CS, KKRKRR, aa 131-136) of HRSV F protein, the furin recognition site was mutated to either KKQKQQ (SEQ ID NO: 28) or GRRQQR (SEQ ID NO: 29). Several modified F proteins with such cleavage site mutations were evaluated to determine the efficiency of cleavage prevention. Figure 2 shows several of the modified F proteins that were evaluated. The results indicate that the primary cleavage site of HRSV F protein can be inactivated by three conservative amino acid changes R133Q, R135Q, and R136Q. These conservative amino acid changes from Arginine (R) which is a polar-charged molecule, to Glutamine (Q) which is a polar-neutral molecule, altered the charge status at these sites and prevented cleavage by furin-like proteases (see Figure 3), while still preserving the F protein 3D structure. Prevention of cleavage at 1° CS resulted in reduced membrane fusion activity of the F protein.

[0225] A non-limiting exemplary modified HRSV F gene sequence designed to have all modifications mentioned above is shown in Figure 4. This modified F gene (SEQ ID NO: 5, RSV-F BV #541) encodes a modified F protein of SEQ ID NO: 6. The gene sequence was synthesized in vitro as overlapping oligonucleotides, cloned and expressed in host cells. Modified HRSV F protein BV #541 was purified from the infected Sf9 insect cell culture harvests, and was analyzed by SDS-PAGE stained by coomassie. The method of purification and SDS-PAGE analysis is described in Example 2. The expression level of the F protein

RSV-F BV #541 (*e.g.* F protein 541) was improved as compared to the wild type F_0 protein in Sf9 insect cells.

Example 4

Modified HRSV F protein with F_1 subunit fusion domain partial deletion

[0226] To further improve expression of the RSV F protein, additional modified HRSV F genes were designed that comprised the following modifications:

(a) the three GenBank sequencing errors were corrected;

(b) the cryptic poly (A) site at the region encoding F2 subunit was modified;

(c) F gene codons were optimized; and

(d) the nucleotide sequences encoding the F_1 subunit fusion domain was partially deleted. In one experiment, the nucleotide sequence encoding the first 10 amino acids of the F_1 subunit fusion domain was deleted (corresponding to amino acids 137-146 of SEQ ID NO: 2).

[0227] A non-limiting exemplary modified RSV F gene comprising said modifications is shown in Figure 5, designated as SEQ ID NO: 9 (RSV-F BV #622, *e.g.* F protein 622), encoding a modified F protein of SEQ ID NO: 10. The modified HRSV F protein BV #622 was purified from the infected Sf9 insect cell culture harvests, and was analyzed by SDS-PAGE stained with coomassie. The method of purification and SDS-PAGE analysis is described in Example 2. High expression levels of HRSV F protein BV #622 were observed, as displayed in the SDS-PAGE in Figure 6.

Example 5

Modified HRSV F protein with both inactivated primary cleavage site and F_1 fusion domain partial deletion

[0228] To determine if the combination of inactivated primary cleavage site and F_1 fusion domain partial deletion can further promote expression of the RSV F protein, particularly in the Sf9 insect cells, another modified RSV F gene was designed comprising following modifications:

(a) the three GenBank sequencing errors were corrected;

(b) the cryptic poly (A) site at the region encoding F2 subunit was modified;

(c) F gene codons were optimized;

(d) the primary cleavage site was inactivated; and

(e) the nucleotide sequence encoding the F1 subunit fusion domain was partially deleted.

In one experiment, the nucleotide sequence encoding the first 10 amino acids of the F_1 subunit fusion domain was deleted (corresponding to amino acids 137-146 of SEQ ID NO: 2).

[0229] A non-limiting exemplary modified RSV F gene in which the first 10 amino acids of the F_1 subunit fusion domain were deleted (corresponding to amino acids 137-146 of SEQ ID NO: 2) is shown in Figure 7A, designated as SEQ ID NO: 7 (RSV-F BV #683, *e.g.* F protein 683), encoding the modified F protein of SEQ ID NO: 8. The modified RSV F protein BV #683 (*e.g.* F protein 683) was purified from the infected Sf9 insect cell culture harvests and analyzed by SDS-PAGE stained with coomassie. The method of purification and SDS-PAGE analysis is described in Example 2. Further enhancements in the of expression levels were achieved, as displayed in the SDS-PAGE in Figure 8.

[0230] An non-limiting exemplary modified RSV F gene comprising said in which the first 10 amino acids of the F_1 subunit fusion domain were deleted (corresponding to amino acids 137-146 of SEQ ID NO: 2) is shown in Figure 7A, designated as SEQ ID NO: 7 (RSV-F BV #683, *e.g.* F protein 683), encoding the modified F protein of SEQ ID NO: 8. The modified RSV F protein BV #683 (*e.g.* F protein 683) was purified from the infected Sf9 insect cell culture harvests and analyzed by SDS-PAGE stained with coomassie. The method of purification and SDS-PAGE analysis is described in Example 2. Further enhancements in the of expression levels were achieved, as displayed in the SDS-PAGE in Figure 8.

Alternate Example 5

Modified HRSV F protein fusion domain deletions

[0231] Deletions in the F1 fusion domain of Δ2, Δ4, Δ6, Δ8, Δ10, Δ12, Δ14, Δ16 or Δ18 amino acids from Phe137 - Val154 were introduced into clone #541 (Figure 7A). Sf9 cells infected with baculovirus expressing these deletion mutants were analyzed for RSV F protein extracted from infected cells with a non-ionic detergent (Figure 7B) and by FACS analysis of cells stained for RSV F (Figure 7C). Deletions of up to 10 amino acids Phe137 – Ser146 (Figure 7A Δ2, Δ4, Δ6, Δ8 and Δ10) increased the level of soluble F1 extracted from the cells

relative to the parent clone BV#541 (Figure 7B). A dramatic loss in soluble RSV F was observed with increasing deletions of the fusion domain greater than 10 amino acids, likely due to mis-folding of the molecule. Consistent with these results, constructs with Δ2, Δ6 and Δ10 amino acid deletions of the F1 fusion peptide displayed the highest cell surface expression of RSV F (Figure 7C).

Example 6

Expression and Purification of modified HRSV F protein BV #683

[0232] Modified HRSV F protein BV #683 (*e.g.* F protein 683, SEQ ID NO: 8) was expressed in baculovirus expression system as described in Example 1, and recombinant plaques expressing HRSV F protein BV #683 were picked and confirmed. The recombinant virus was then amplified by infection of Sf9 insect cells. A culture of insect cells was infected at ~3 MOI (Multiplicity of infection = virus ffu or pfu/cell) with baculovirus. The culture and supernatant were harvested 48-72 hrs post-infection. The crude harvest, approximately 30 mL, was clarified by centrifugation for 15 minutes at approximately 800 x g. The resulting crude cell harvests containing HRSV F protein BV #683 were purified as described below.

[0233] HRSV F protein BV #683 was purified from the infected Sf9 insect cell culture harvests. Non-ionic surfactant Tergitol® NP-9 (Nonylphenol Ethoxylate) was used to in a membrane protein extraction protocol. Crude extraction was further purified by passing through anion exchange chromatography, lentil lectin affinity/HIC and cation exchange chromatography.

[0234] Purified HRSV F protein BV #683 was analyzed by SDS-PAGE stained with coomassie, and Western Blot using anti-RSV F monoclonal antibody as described in Example 2. The results were shown in Figure 9. Excellent expression levels of the HRSV F protein BV #683 (*e.g.* F protein 683, SEQ ID NO: 8) were achieved. It was estimated that the expression level was above 10 mg/L in crude cell culture, and recovered F protein BV #683 was about 3.5 mg/L cell culture. In some cases expression levels above 20 mg/L were achieved and about 5mg/L modified F protein BV #683 was recovered (see Figure 10). Purity of the recovered F protein BV #683 reached above 98% as determined by scanning densitometry (see Figure 10).

Example 7

Purified HRSV F protein BV #683 micelles (rosettes)

[0235] Purified HRSV F protein BV #683 was analyzed by negative stain electron microscopy (see Figure 11). F proteins aggregated in the form of micelles (rosettes), similar to those observed for wild type HRSV F protein (Calder *et al.*, 2000, *Virology* 271, pp. 122-131), and other full-length virus membrane glycoproteins (Wrigley et al., Academic Press, London, 1986, vol. 5, pp. 103-163). Under electron microscopy, the F spikes exhibited lollipop-shaped rod morphology with their wider ends projecting away from the centers of the rosettes (Figure 11).

Alternate Example 7

HPLC analysis of of purified HRSV F protein BV #683 micelles (rosettes)

[0236] Purified HRSV F protein BV #683 was analyzed by HPLC. Reverse phase high pressure liquid chromatography (RP-HPLC) analysis showed purified RSV F micelles consisting of 90.1% F1+F2 at retention time of 11.195 min and 9.9% F1 polypeptide at retention time of 6.256 min (Figure 12A). The F1+F2 peak at 11.195 min showed a double peak, suggesting different glycosylation species. The identity of F1+F2 and F1 were confirmed by SDS-PAGE and Western blot analysis of isolated HPLC peak fractions. Intact mass determination by mass spectrometry showed that F1 and F1+F2 had molecular weights of 50KDa and 61Kda, respectively, similar to the predicted molecular weights.

[0237] Purified RSV F nanoparticles were further analyzed using HPLC size exclusion chromatography (SEC). The RSV F nanoparticles consisted primarily of covalently linked F1 and F2 (Figure 12B; F1+F2) with a low level of free F1 subunits. F1+F2 was 95.8% of the total peak area, and F1 was 3.8% of the total peak area. The purity of RSV F particles was estimated to be $\geq$ 98%. The F1+F2 peak was eluted in the void volume of this SEC column and the F1 peak had a mass of about 180Kda, as expected for F1 trimers. An analytical ultracentrifugation (AUC) study showed that the majority of species in RSV F nanoparticles had a molecular weight between about 1million Da to about 8 million Da.

[0238] The length of the single trimer was about 20nm, and the micelle particle diameter was about 40nm (see Figure 12C). These results indicated that HRSV F protein BV #683 has the correct 3D structure for a native, active protein.

[0239] In summary, a modified recombinant HRSV F protein (*e.g.*, BV #683) has been designed, expressed, and purified. This modified full-length F is glycosylated. Modifications of the primary cleavage site and the fusion domain together greatly enhanced expression level

of F protein. In addition, this modified F protein can be cleaved to F1 and F2 subunits, which are disulfide-linked. Trimers of the F1 and F2 subunits form lollipop-shaped spikes of 19.6 nm and particles of 40.2 nm. Moreover, this modified F protein is highly expressed in Sf9 insect cells. Purity of micelles > 98% is achieved after purification. The fact that the spikes of this modified protein have a lollipop morphology, which can further form micelles particles of 40 nm, indicates that modified F protein BV #683 has correct 3D structure of a native protein.

Example 8

Co-expression of modified HRSV F protein with BRSV M and/or HRSV N in VLP production

[0240] The present invention also provides VLPs comprising a modified or mutated RSV F protein. Such VLPs are useful to induce neutralizing antibodies to viral protein antigens and thus can be administered to establish immunity against RSV. For example, such VLPs may comprise a modified RSV F protein, and a BRSV M and/or HRSV N proteins. Codons of genes encoding BRSV M (SEQ ID NO: 14) or HRSV N (SEQ ID NO: 18) proteins can be optimized for expression in insect cells. For example, an optimized BRSV M gene sequence is shown in SEQ ID NO: 13 and an optimized RSV N gene sequence is shown in SEQ ID NO: 17.

[0241] In one experiment, a modified F protein BV #622 and another modified F protein BV #623 (SEQ ID NO: 21, modified such that both cleavage sites are inactivated) were either expressed alone, or co-expressed with HRSV N protein and BRSV M protein. Both crude cell harvests containing VLPs (intracellular) and VLPs pellets collected from 30% sucrose gradient separation were analyzed by SDS-PAGE stained with coomassie, and Western Blot using anti-RSV F monoclonal antibody. Figure 13 shows the structure of the modified F proteins BV #622 and BV #623, and results of SDS-PAGE and Western Blot analysis. BV #622 was highly expressed by itself or co-expressed with HRSV N protein and BRSV M protein, while BV #623 had very poor expression, indicating that inactivation of both cleavage sites inhibits F protein expression.

[0242] In another experiment, modified F protein BV #622, double tandem gene BV #636 (BV #541 + BRSV M), BV #683, BV #684 (BV #541 with YIAL L-domain introduced at the C terminus), and BV #685 (BV #541 with YKKL L-domain introduced at the C terminus) were either expressed alone, or co-expressed with HRSV N protein and BRSV M protein. L-

domain (Late domain) is a conserved sequence in retroviruses, and presents within Gag acting in conjunction with cellular proteins to efficiently release virions from the surface of the cell (Ott *et al.*, 2005, *Journal of Virology* 79: 9038-9045). The structure of each modified F protein is shown in Figure 14. Both crude cell harvests containing VLPs (intracellular) and VLPs pellets collected from 30% sucrose gradient separation were analyzed by SDS-PAGE stained with coomassie, and Western Blot using anti-RSV F monoclonal antibody. Figure 14 shows the results of SDS-PAGE and Western Blot analysis of crude cell harvests containing VLPs (intracellular), and Figure 15 shows results of SDS-PAGE and Western Blot analysis of VLPs pellets collected from 30% sucrose gradient separation. BV #622 and BV #683 were highly expressed by themselves or co-expressed with HRSV N protein and BRSV M protein, while BV #636, BV #684, and BV #685 had poor expression.

Example 9

Screening of chimeric HRSV F proteins with high expression

[0243] Efforts were made to screen for additional RSV F proteins that could be highly expressed in soluble form in insect cells and could form VLPs with better yield. Various F genes were designed, expressed, and analyzed. Both Western Blot and SDS-PAGE were used to evaluate the expression.

[0244] Figure 16A to Figure 16D summarize the structure, clone name, description, Western Blot/coomassie analysis results, and conclusion for each chimeric HRSV F clone.

[0245] As the results indicated, wild type full length F protein was poorly expressed; chimeric HRSV F proteins that contain F_1 but not F_2 subunit could be expressed well, but the products were either insoluble, which might be due to misfolding, or could not assemble with other viral proteins to form VLPs with good yield after co-infections. Inactivation of the primary cleavage site alone did not result in substantial increases in expression, but better expression was achieved when inactivation of the primary cleavage site was combined with other modifications such as deletion of cryptic poly (A) site and correction of GenBank aa errors (*e.g.*, BV #541). Introduction of the YKKL L-domain into the C terminus of BV #541 enhanced the secretion of VLPs containing modified F protein for about 2-3 folds in co-expression with BRSV M and HRSV N proteins. The results further showed that a double tandem chimeric gene consisting of BV #541 and BRSV M genes displayed both improved intracellular expression and improved VLP yield compared to co-infection of BV #541 and BRSV M proteins, indicating that BRSV M protein can facilitate production of VLPs

containing a modified HRSV F protein in insect cells when tandemly expressed. A triple tandem chimeric gene consisting of BV #541, BRSV M, and HRSV N had even higher intracellular expression and much better VLP yield compared to above mentioned double tandem chimeric gene or co-infection of BV #541, BRSV M, and HRSV N proteins. Furthermore, the results suggested that chimeric HRSV F protein BV#683 (*e.g.* F protein 683, SEQ ID NO: 8) had the best intracellular expression. Expression of a double tandem chimeric gene consisting of BV#683 and BRSV M genes, or a triple tandem chimeric gene consisting of BV#683, BRSV M, and HRSV N genes is also embodied herein. These double and triple tandem chimeric gene should further improve VLP production compared to co-infection.

Example 10

RSV neutralization assay and RSV challenge studies in mice

[0246] To test the efficiency of a vaccine comprising modified HRSV F protein BV #683 in prohibiting RSV infection, neutralization assays and RSV challenge studies were conducted in mice. The experimental procedures are shown in Figure 17.

[0247] Groups of mice (n=10) were injected intramuscularly (except for live RSV) with placebo (PBS solution), live RSV (given intranasally), formalin inactivated RSV vaccine (FI-RSV), 1 μg purified F particles (PFP, modified F protein BV #683), 1 μg purified F particles with Alum (PFP + Alum), 10 μg purified F particles, 10 μg purified F particles with Alum (PFP + Alum), or 30 μg purified F particles on day 0 and day 21. Each immunized group was challenged by live RSV on day 42 (21 days after the second immunization). Mouse serum from each group was harvested on day 0, day 31 (10 days after the second immunization), and day 46 (4 days following challenge with live RSV).

[0248] Mouse serum from each treatment group was assayed for the presence of anti-RSV neutralization antibodies. Dilutions of serum from immunized mice were incubated with infectious RSV in 96-well microtiter plates. Serum was diluted from 1:20 to 1:2560. 50 μl diluted serum was mixed with 50 μl live RSV virus (400 pfu) in each well. The virus/serum mixture was incubated first for 60 minutes at room temperature, and then mixed with 100 μl HEp-2 cells and incubated for 4 days. The number of infectious virus plaques were then counted after staining with crystal violet. The neutralization titer for each serum sample, defined as the inverse of the highest dilution of serum that produced 100% RSV neutralization (e.g., no plaques), was determined for each animal. The geometric mean serum

neutralizing antibody titer at day 31 (10 days after the boost) and day 46 (4 days following challenge with live RSV) were graphed for each vaccine group. Figure 18 shows the results of the neutralization assays. The results indicated that 10 μg or 30 μg purified F protein produced much higher neutralization titer as compared to live RSV. In addition, neutralization titers of PFP were enhanced with co-administration of Alum adjuvant.

[0249] RSV challenge studies were carried out to determine if immunization could prevent and/or inhibit RSV replication in the lungs of the immunized animals. The amount of RSV in the lungs of immunized mice was determined by plaque assay using HEp-2 cells. Immunized groups of mice mentioned above were infected with 1 x 10^6 pfu of infectious RSV long strain intranasally on day 42 (11 days after the second immunization). On day 46 (4 days after RSV infection), lungs of mice were removed, weighed, and homogenized. Homogenized lung tissue was clarified. Supernatant of clarified solution was diluted and subjected to plaque assay using HEp-2 cells to determine RSV titer in lung tissue (calculated as pfu/g lung tissue). Results are shown in Figure 19, indicating that all mice immunized with recombinant RSV F protein BV #683 had undetectable RSV in the lungs, and even 1 μg purified recombinant HRSV F protein BV #683 without adjuvant exhibited excellent efficiency in inhibiting RSV replication (reduced more then 1000 times compared to placebo).

[0250] To determine the stability of the RSV PFP vaccine used above, the vaccine was stored at 2-8 °C for 0, 1, 2, 4, and 5 weeks, and then analyzed by SDS-PAGE stained with coomassie (Figure 20). The results show that the RSV PFP vaccine is stable at 2-8 °C, and there is no detectable degradation.

Example 11

Recombinant RSV F Micelle Activity in Cotton Rats

[0251] In this example, groups included cotton rats immunized at days 0 and 21 with live RSV (RSV), formalin inactivated RSV (FI-RSV), RSV-F protein BV #683 with and without aluminum (PFP and PFP + Aluminum Adjuvant), and PBS controls.

[0252] As shown in Figure 21, immunization with 30 μg of the F-micelle vaccine (RSV-F protein BV #683, *i.e.* F protein 683, SEQ ID NO: 8), with and without aluminum, produced robust neutralizing antibody responses following exposure to both RSV A and RSV B. In addition, it was observed that aluminum significantly enhanced the antibody response. Moreover, neutralizing antibodies were increased following a boost at day 46 or day 49 in RSV A and RSV B, respectively.

[0253] While significant lung pathology was observed in rats immunized with formalin inactivated RSV (FI-RSV), no disease enhancement was seen with the F-micelle vaccine (Figure 22). The use of the F-micelle vaccine and the F-micelle vaccine with adjuvant produced lower inflammation scores (4.0 and 2.8, respectively) than the primary RSV infection (PBS + RSV challenge) control group (5.8). As noted above, the FI-RSV treated group had a higher inflammation score than the primary RSV infection (PBS + RSV challenge) control group (9.0 versus 5.8). Moreover, the FI-RSV treated group had a significantly higher mean inflammation score (9.0) than the unchallenged placebo controls, live RSV + RSV challenge, F-micelle + RSV challenge, and F-micelle + aluminum + RSV challenge.

Example 12

Preclinical Efficacy of the RSV F Nanoparticle Vaccine in Rats

[0254] Efficacy of the RSV F Nanoparticle vaccine was tested in cotton rats.

[0255] Neutralizing antibody responses against RSV-A were assessed in cotton rats immunized with RSV F vaccine ± alum.

[0256] In one study, cotton rats were immunized with one of the following treatment groups:

(1) PBS;

(2) RSV;

(3) formalin inactivated-RSV (FI-RSV);

(4) RSV F nanoparticle vaccine (1 μg + alum)

(5) RSV F nanoparticle vaccine (6 μg + alum)

(6) RSV F nanoparticle vaccine (30 μg + alum)

[0257] Rats were then bled on day 21 and day 49. Serum from day 49 was tested against RSV-A in neutralizing assays by using a plaque-reduction neutralization test. Results of this experiment are provided in Figure 23. Figure 23 is a graph showing neutralizing titers vs. each respective treatment group. The line for each treatment group indicates the geometric mean of end point titer that neutralized the RSV-A virus 100%. The results indicated that the vaccine of the invention neutralized RSV to a greater degree than RSV and FI-RSV.

[0258] In another study, cotton rats were immunized with one of the following treatment groups.

(1) PBS;

(2) RSV;

(3) FI-RSV;

(4) RSV F nanoparticle vaccine (1 μg)

(5) RSV F nanoparticle vaccine (1 μg + alum)

(6) RSV F nanoparticle vaccine (10 μg)

(7) RSV F nanoparticle vaccine (10 μg + alum)

(8) RSV F nanoparticle vaccine (30 μg)

[0259] Cotton rats were immunized on day 0 and day 21 at 1 with one of the above treatment groups. Rats were subsequently bled on day 31. Sera from all groups were tested against RSV-A in a CPE assay. Figure 24 shows the neutralizing antibody responses against RSV-A in cotton rats, expressed as Log2 titers, vs. the respective vaccination group (x-axis). The vaccine of the invention neutralized RSV to a greater degree than RSV and FI-RSV. Additionally, nanoparticle vaccines administered with alum produced a greater number of neutralizing titers than nanoparticle vaccines without alum.

[0260] In another experiment, cotton rats were immunized with one of the following treatment groups:

(1) PBS;

(2) RSV;

(3) FI-RSV;

(4) RSV F nanoparticle vaccine (1 μg)

(5) RSV F nanoparticle vaccine (1 μg + alum)

(6) RSV F nanoparticle vaccine (6 μg)

(7) RSV F nanoparticle vaccine (6 μg + alum)

(8) RSV F nanoparticle vaccine (30 μg)

(9) RSV F nanoparticle vaccine (30 μg + alum)

[0261] Cotton Rats were immunized with one of vaccine treatment groups (1)-(9) on day 0 and day 21, and subsequently challenged with RSV A strain virus on day 49.

[0262] Lung tissues were harvested on day 54 (n=8/group). Tissue was then homogenized and evaluated for the presence of RSV virus using a Hep-2 monolayer plaque assay to detect infectious virus. Figure 25 shows that neutralizing antibody elicited by RSV F nanoparticles was effective in preventing RSV virus replication in the lungs of challenged animals. In Figure 25, RSV titers are expressed as log10 pfu/per gram of lung tissue.

[0263] An ELISA assay was also carried out on rat sera, treated with the above nine vaccination groups, to determine the presence or absence of anti-RSV antibodies. Sera was pooled for animals in each group and subject to the ELISA assay. Results are provided in Figures 26A and 26B as measured by ELISA units (corresponding to the 50% titer on a 4

parameter fit dilution curve; Figure 26A) or RSV-F IgG titer (Figure 26B). Animals treated with the vaccine of the present invention produced a greater number of detectable anti-RSV antibodies than animals treated with RSV or FI-RSV. Additionally, alum increased the antibody response.

[0264] Functional activity of anti-RSV F antibody was evaluated in a RSV neutralization assay by determining the dilution of antisera capable of inhibiting 100% RSV cytopathic activity on HEp-2 cell monolayers. Immunization with all doses of unadjuvanted RSV F nanoparticles resulted in the development of RSV neutralizing antibody (Figure 26C). Consistent with results for the anti-RSV F IgG, co-administration of aluminum phosphate adjuvant increased RSV neutralizing antibody titers 3.5 – 18 fold. Neutralizing antibody titers induced with live RSV were comparable to the low dose RSF F adjuvanted group and likely a component was directed against the RSV G protein. Sera from cotton rats immunized with formalin inactivated RSV (FI-RSV) did not exhibit neutralizing activity (Figure 26C; FI-RSV).

[0265] Antigenic site II on the RSV F protein has been shown to be the target of palivizumab, a humanized RSV neutralizing monoclonal antibody used in prophylaxis for the prevention of RSV disease. To determine whether antibodies directed against antigenic site II were induced by immunization with RSV F nanoparticles, a palivizumab competitive ELISA was performed using pooled sera from the individual animals within each group. Sera obtained from either live RSV treated animals, FI-RSV immunized animals, or PBS control animals did not inhibit palivizumab binding (Figure 26D). In contrast, serum pools obtained from animals immunized with RSV F nanoparticles had high levels of antibody that inhibited the binding of Palivizumab to RSV F (Figure 26D). This result was achieved for pools from all doses, with or without aluminum phosphate adjuvant. These data demonstrate that the RSV F protein stimulates antibodies with the same specificity as palivizumab.

Histopathology

[0266] The histopathology of the cotton rat challenged with RSV was also studied. Cotton rats were immunized with one of the following vaccination groups on day 0 and day 21:

(1) RSV F nanoparticle vaccine (1 μg , 6 μg, or 30 μg; +/- alum)

(2) FI-RSV

(3) RSV

(4) PBS

(5) PBS

Rats from groups (1)-(4) were subsequently challenged with RSV A strain virus on day forty-nine. Rats from group (5) were not challenged with the virus. Lung tissue was isolated five days after challenge. Tissue were frozen, sectioned and stained with hematoxylin and eosin. Representative micrographs are provided to indicate peribronchiolitis condition in the control animals and 30 μg + alum vaccine groups (Figure 27).

[0267] Slides were evaluated in a blinded fashion using a score of 0 to 4 (0=no; 1=minimal; 2=mild; 3=moderate; 4=maximum inflammation) in order of increasing severity for each of the following 5 parameters: a) bronchiolitis; b) vasculitis; c) bronchitis; d) alveolitis and e) interstitial pneumonitis (as described in Prince GA, et al. (1986) *J Virol* 57: 721-728). The summary value for each of the five parameters was added together to arrive at a single summary score for each animal. Summary scores for each group were used to arrive at the total average score/group expressed as the arithmetic mean ± the SEM.

[0268] Analysis of the histopathology scores for the various experimental groups (Table 1) demonstrated that FI-RSV exacerbated inflammation upon subsequent challenge with live virus. The total histopathology score for FI-RSV was significantly higher than that observed for non-immune animals challenged with RSV (mean 5.63; $p<0.0001$, t-test). No significant increase in pathology was observed in any RSV F immunized group either in the presence or absence of alum relative to the control groups. The cotton rats infected with RSV and challenged with RSV had a mean score of 0.75. The next lowest histopathology scores of 1.13 and 1.43 were in the adjuvanted vaccine groups immunized respectively with 6 μg or 30 μg RSV F nanoparticles (Table 1). These data demonstrate that the adjuvanted RSV F nanoparticle vaccine inhibits lung inflammation after RSV challenge.

Table 1. Pulmonary histopathological parameters in immunized cotton rats

Total Score for Each Histological Parameter

Treatment	Bronchiolitis	Vasculitis	Bronchitis	Alveolitis	Pneumonitis	Mean, all measures
PBS alone	0	0	1	0	0	0.13
PBS+Chal	5	0	10	5	1	2.63
RSV+Chal	1	1	2	2	0	0.75
FI-RSV+Chal	15	0	19	9	2	5.63
1μg RSV-F + Chal	6	0	9	2	0	2.13
1μg RSV-F with alum + Chal	7	5	7	5	0	3.43
6μg RSV-F + Chal	5	0	11	3	0	2.71
6μg RSV-F with alum + Chal	0	0	7	2	0	1.13
30μg RSV-F + Chal	6	2	13	6	1	3.50
30μg RSV-F with alum + Chal	1	0	6	3	0	1.43

Example 13

Preclinical efficacy of RSV F Nanoparticle Vaccine in Mice

[0269] ELISA plates were coated with RSV F micelle at 2 μg/mL. Pre immune and a pool of day 28 serum from mice immunized on day 0 with 30 μg RSV F with alum were mixed with 50 ng/mL biotin-Palivizumab epitope peptide. These samples were then serially diluted and incubated with purified RSV F coated ELISA plate. Streptavidin was used to determine Palivizumab bound to the plate.

[0270] An unweighted four parameter logistic regression curve is presented in Figure 28. The results indicated that antibodies produced by the nanoparticle vaccine of the invention competed with Palivizumab peptide for binding the target RSV.

Example 14

RSV Nanoparticle Vaccine-Palivizumab Assay

[0271] Binding of the RSV nanoparticle vaccine of the invention to Synagis was assayed.

[0272] Binding of Synagis mAb to Palivizumab Epitope Peptide. ELISA plates were coated with streptavidin at 5 μg/mL. Palivizumab peptide at 1 μg/mL was bound to the plate to the streptavidin via a biotin linker. Synagis® at 10 μg/mL was serially diluted four fold and incubated to the peptide on the plate. Synagis® binding was detected using anti human HRP reaction. The results are provided in Figure 29A (left graph).

[0273] Binding of Synagis® to Recombinant RSV F micelles. ELISA plates were coated with 2μg/ml RSV F micelle antigen. Synagis® at 10 μg/mL concentration serially diluted four fold and reacted to RSV F on the plate. Anti human HRP reaction was used to determine

the Synagis binding to RSV F micelles. An unweighted four parameter logistic regression curve is presented (Figure 29B, right graph). The results indicated that Synagis® recognized and bound the vaccine of the invention.

Example 15

Clinical Safety of the RSV F Nanoparticle Vaccine

[0274] A Phase 1 randomized, observer blinded placebo controlled trial was carried out to evaluate the safety and immunogenicity of the RSV nanoparticle vaccine in healthy adults in a dose-escalating fashion.

[0275] 150 healthy adults were administered two immunizations at day 0 and at day 30. The treatment groups are provided in Table 2, below. Demographic and subject dispositions for the subjects are provided in Tables 3 and 4, respectively.

Table 2.

Cohort	Group Designation	Number of Subjects	RSV nanoparticle dose	Alum dose
Cohort 1 (N=25)	A	20	5 µg	1.2 mg
	E	5	0 µg	0 mg
Cohort 2 (N=25)	B	20	15 µg	1.2 mg
	E	5	0 µg	0 mg
Cohort 3 (N=25)	C	20	30 µg	1.2 mg
	E	5	0 µg	0 mg
Cohort 4 (N=25)	D	20	30 µg	0 mg
	E	5	0 µg	0 mg
Cohort 5 (N=25)	F	20	60 µg	1.2 mg
	E	5	0 µg	0 mg
Cohort 6 (N=25)	G	20	60 µg	0 mg
	E	5	0 µg	0 mg

Table 3.

Vaccine Group	Placebo (group 1)	5 µg + Alum (group 2)	15 µg + Alum (group 3)	30 µg + Alum (group 4)	30 µg (group 5)	60 µg + Alum (group 6)	60 µg (group 7)
# of subjects (n)	30	20	20	20	20	20	20
Age (years)							
Mean (SD)	29.0 (6.92)	35.6 (7.70)	34.9 (9.20)	29.7 (7.41)	31.5 (8.41)	26.4 (5.59)	33.5 (8.55)
Min, Max	19, 47	20, 49	20, 49	20, 46	18, 48	19, 43	20, 49

Age group (%) 18-30	66.7%	20.0%	30.0%	60.0%	50.0%	85.0%	45.0%
Age group (%) 31-40	30.0%	65.0%	45.0%	30.0%	40.0%	10.0%	30.0%
Age group (%) 41-49	3.3%	15.0%	25.0%	10.0%	10.0%	5.0%	25.0%
Gender (%)							
Male	40.00%	50.00%	55.00%	35.00%	55.00%	25.00%	25.00%
Female	60.00%	50.00%	45.00%	65.00%	45.00%	75.00%	75.00%

Table 4. Subject Disposition (number (percentage of number of subjects))							
Randomized	30 (100.0)	20 (100.0)	20 (100.0)	20 (100.0)	20 (100.0)	20 (100.0)	20 (100.0)
Safety	30 (100.0)	20 (100.0)	20 (100.0)	20 (100.0)	20 (100.0)	20 (100.0)	20 (100.0)
Modified Intent-to-Treat (MiTT)	28 (93.3)	19 (95.0)	19 (95.0)	20 (100.0)	18	18	20 (100.0
Per-protocol	26 (86.7)	17 (85.0)	16 (80.0)	18 (90.0)	17 (85.0)	13 (63.0)	18 (90.0)

[0276] Adverse events (AEs, both local and systemic) were solicited 1-7 days post immunization.

[0277] Local pain was reported by 6.7% of patients administered placebo. 15% to 55% of patients in the vaccine groups reported pain. One subject reported severe pain (30 μg + alum treatment). There was no dose response effect.

[0278] Tenderness was reported by 10% of patients administered placebo. 20% to 55% of patients in the vaccine groups reported tenderness. One subject reported severe tenderness (60 μg + alum treatment). There was no dose response effect.

[0279] Headache was reported by 16.7% of patients administered placebo. 10% to 35% of patients in the vaccine groups reported tenderness. There was no dose response effect.

[0280] The vaccine was well tolerated overall. The majority of adverse events were local pain and tenderness and the majority were mild. Local adverse events were higher in the vaccine groups compared to placebo. There was no adverse event trend based on dose of vaccine. Additionally, there were no vaccine related severe adverse events.

Example 16

Immunogenicity of the RSV F Nanoparticle Vaccine

[0281] The Phase 1 trial was carried out, as provided in Example 15.

[0282] The immunogenicity of the RSV nanoparticle viruses was assessed. The scheme for various assays utilized to assess immunogenicity is provided in Figure 30.

RSV F/Synagis (Palivizumab) Peptide ELISA

Treatment Groups	Placebo (group 1)	5 μg + Alum (group 2)	15 μg + Alum (group 3)	30 μg + Alum (group 4)	30 μg (group 5)	60 μg + Alum (group 6)	60 μg (group 7)

[0283] ELISA plates were coated with streptavidin at 5 μg/mL. Palivizumab peptide at 1 μg/mL was bound to Streptavidin. Human sera from subjects treated with the vaccine of the invention was introduced to the plates. Secondary antibody (anti human HRP) was then added to detect anti-RSV F IgG against the Palivizumab peptide.

[0284] Figure 31 provides the results of this study. Each of the nanoparticle vaccine treatment groups produced significantly more RSV IgG than the control group, and the alum groups performed better than the non-alum groups. In Figure 31, each treatment group includes three measurements: (1) sera at day 1 (left bar), (2) sera at day 30 (middle bar), (3) sera at day 60 (right bar).

Anti-RSV F IgG ELISA

Treatment Groups	Placebo (group 1)	5 μg + Alum (group 2)	15 μg + Alum (group 3)	30 μg + Alum (group 4)	30 μg (group 5)	60 μg + Alum (group 6)	60 μg (group 7)

[0285] ELISA plates were coated with 2 μg/mL RSV F or RSV G antigen. Human sera from subjects treated with the vaccine of the invention followed by challenge with RSV was introduced to the plates. Secondary antibody (anti human HRP) was then added to detect anti-RSV F or anti-RSV G IgG in the human sera.

[0286] Each of the nanoparticle vaccine treatment groups produced IgG antibodies against RSV F at all time points tested (Figure 32A). In contrast, the vaccine treatment groups induced production of negligible amounts of anti-RSV G antibodies (Figure 32B). In Figure 32 A an B, each treatment group includes three measurements: (1) sera at day 1 (left bar), (2) sera at day 30 (middle bar), (3) sera at day 60 (right bar).

[0287] The results of this experiment are also provided in Tables 5A and 5B, below. Table 5A shows the geometric mean of IgG levels in all groups. The geometric mean fold rise in IgG levels was also plotted for the alum groups (Figure 33). A significant dose response was achieved ($p<0.05$). Table 5B shows the results of the ELISA (expressed as ELISA Units) for individual subjects in the 60 μg + Alum group.

Table 5A.

Vaccine Group	Placebo	5 µg + Alum	15 µg + Alum	30 µg + Alum	30 µg	60 µg + Alum	60 µg
# subjects (n)	26	17	16	18	17	13	18
Study day 1 (pre-vacc)							
GMT	482	830	559	586	519	485	530
95% CI	357, 651	530, 1302	355, 881	439, 782	286, 943	321, 733	307, 917
Study day 30 (pre-vacc)							
GMT	585	3627	2618	4695	4554	6795	5652
95% CI	423, 808	1750, 7517	1784, 3841	3622, 6086	2617, 7926	4813, 9592	3993, 8000
P-value [1]	<0.001	0.253	0.073	0.723		0.106	0.509
P-value [2]	<0.001	0.07	0.014	0.757	0.509	0.303	
P-value [3]		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Study day 60							
GMT	601	5913	4224	6521	4079	9256	5249
95% CI	438, 825	3653, 9569	3176, 5616	5282, 8051	2568, 6479	6899, 12419	3632, 7586
P-value [1]	<0.001	0.248	0.785	0.018		<0.001	0.323
P-value [2]	<0.001	0.848	0.487	0.154	0.323	0.005	
P-value [3]		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

GMT is the geometric mean titer.
[1] P-value is from t-test on log (base 10) transformed titer values comparing the specified treatment group to the RSV-F 30 µg unadjuvanted group.
[2] P-value is from t-test on log (base 10) transformed titer values comparing the specified treatment group to the RSV-F 60 µg unadjuvanted group.
[3] P-value is from t-test on log (base 10) transformed titer values comparing the specified treatment group to the Placebo group.

Table 5B.

	Anti F EU*	
Subject ID	**Day 30**	**Day 60**
1104	4,249	6,141
1105	4,492	6,300
1107	4,348	9,633
1108	9,400	7,360
1109	21,402	17,930
1110	7,969	8,555
1112	8,032	7,528
1113	11,037	9,058

1115	2,587	3,207
1117	9,505	16,923
1119	6,463	7,435
1120	5,047	8,205
11024	6,041	10,798
* ELISA unit		

RSV Plaque Reduction Neutralizing Titers (PRNTs)

[0288] Results from these experiments are provided in Figure 34 and Figure 35. Figure 34 shows that day 60 antibodies were significantly higher than placebo for all groups. Post-immunization PRNTs in vaccine groups exceeded levels that have been estimated to be protective in the elderly, children and infants.

[0289] Figure 35 shows the reverse cumulative distribution for Day 0, Day 30 and Day 60 PRNTs in the placebo and 30 μg + Alum groups. Minimum titers at day 0 were 5 $\log_2$ and minimum titer post-immunization with RSV F recombinant nanoparticles at day 60 was 8.5 $\log_2$.

Example 17

Avidity of Antibodies Induced by the RSV F Nanoparticle Vaccine

[0290] The Phase 1 trial was carried out, as provided in Example 15.

[0291] The avidity of antibodies in the human sera for RSV F was determined using a BIAcore SPR-based measurement system. RSV F protein was immobilized on a sensor surface, and sera were passed over the immobilized RSV F. Binding to the immobilized RSV F was measured based on the molecular mass on the sensor surface. Association and dissociation rates were measured as a function of time and plotted on a sensorgram, and the dissociation constant was calculated from the association and dissociation rates.

[0292] Figure 36 provides the controls for the BIAcore SPR-based assay. Figure 36A shows the association rates (k-On), dissociation rates (k-Off), and dissociation constants (KD) for positive controls palivizumab antibody and RSV reference sera (available from BEI Resources and described in Yang et al. 2007, *Biologicals* 35; 183-187). Figure 36B shows a sensorgram to demonstrate negative results from Day 0 and placebo control sera from the Phase 1 trial, in contrast to positive control palivizumab.

[0293] Figure 37 provides the binding curves for palivizumab antibody and a representative vaccinee (subject ID# 1112, day 30).

[0294] The results of the study for 13 subjects in the 60μg vaccine + adjuvant group are provided in Table 6, below. At Day 30, the KD for these 13 subjects ranged from 0.11 pmol to 992 pmol; at Day 60, the KD ranged from 0.00194 pmol to 675 pmol. Thus, the avidity of antibodies in the human sera for RSV F was, like the palivizumab antibody control, in the picomolar range.

Table 6

	KD	
Subject ID	**Day 30**	**Day 60**
1104	1.76 E-12	8.26 E-13
1105	3.92 E-13	8.23 E-14
1107	3.91 E-13	2.24 E-14
1108	6.80 E-13	1.94 E-15
1109	9.92 E-10	NA
1110	1.35 E-10	1.62 E-10
1112	2.29 E-12	3.64 E-13
1113	2.72 E-13	9.02 E-14
1115	4.21 E-13	6.75 E-10
1117	4.93 E-13	4.18 E-13
1119	1.11 E-13	1.55 E-13
1120	1.23 E-13	1.70 E-13
11024	6.18 E-13	4.79 E-13
Palivizumab	0.95 E-10	

Example 18

Palivizumab-like IgG antibodies Induced by the RSV F Nanoparticle Vaccine

[0295] The Phase 1 trial was carried out, as provided in Example 15.

[0296] Palivizumab-like IgG antibodies (antibodies that compete with palivizumab epitope peptide for binding RSV F) in the sera from 13 subjects in the 60μg + adjuvant group were measured via a competitive binding assay. The results, provided in Table 7 below, indicated that Palivizumab-like antibodies were well above protective levels (≥40μg/mL) in all subjects tested at both Day 30 and Day 60.

Table 7		
	Palivizumab-like IgG (µg/ml)	
Subject ID	**Day 30**	**Day 60**
1104	57	66
1105	85	106
1107	208	312
1108	169	116
1109	177	253
1110	106	134
1112	190	203
1113	337	315
1115	109	131
1117	76	261
1119	96	140
1120	77	122
11024	94	124

Example 18

Immunogenicity of a Recombinant RSV F Protein Nanoparticle Vaccine Manufactured in Insect Cells: Induction of Palivizumab-like Activity in Human Subjects

[0297] The vaccine administered in this example comprised nanoparticles comprising near-full length F protein, assembled into trimers. F protein was produced in Sf9 insect cells infected with a recombinant baculovirus. The cells were lysed and solubilized with detergent, and the F protein was purified chromatographically. The antigen was administered by intramuscular injection, with or without adsorption to $ALPO_4$.

[0298] Healthy adults (N = 150; mean age 31.3 years; 59% female) were enrolled in 6 cohorts, each including 20 active vaccine and 5 placebo recipients. Test articles were given as 2-dose series at a 30 day interval.

[0299] RSV F antigen was tested at doses of 5, 15, 30 and 60 µg adsorbed to $AlPO_4$, and 30 and 60µg without adjuvant. Safety was monitored by soliciting local and systemic symptoms for 7 days after each dose, and ascertainment of unsolicited adverse events for 6 months. Functional antibodies were assessed using the plaque reduction neutralization (PRN) and microneutralization (MN) assays (which gave similar results) and ELISA assays for multiple antigens as below.

[0300] The results of the trial indicated that MN responses appeared to lag anti-F responses (Figure 38). MN antibody responses occurred in the active groups, but anti-F responses were much more dynamic Whereas baseline anti-F IgG titers spanned a more limited range, baseline microneutralization (MN) titers varied over a >32-fold range. The vaccine induced substantial MN responses in subjects with low MN baselines (geometric mean 3.9-fold rises in those in the lowest 1/3 of the population), but overall fold- rises were limited by the 1/3 of subjects with high baseline values (where <2-fold responses were noted).

[0301] The results of the trial also showed that RSV F Nanoparticle Vaccine Elicited antibody Responses to antigenic site II Peptide 254-278 (Figure 39). Synthetic RSV-F peptide 254-278, which harbors the palivizumab and motavizumab epitopes, was biotinylated and immobilized to streptavidin-coated plates. Serial dilutions of sera were incubated with the peptide coated plates. Bound IgG was detected after washing with enzyme-conjugated goat anti-human IgG. Pre-immunization titers were uniformly low, but increased 5- to 15-fold with receipt of RSV-F nanoparticle vaccine.

[0302] Figure 40 and Table 8 show the results of a palivizumab competition ELISA with human serum before and after RSV-F nanoparticle immunization. ELISA plates were coated with RSV F protein antigen at 2µg/mL. Pre- and post-immunization sera were serially diluted, spiked with 50ng/mL biotinylated palivizumab, and then incubated in the coated plates. Enzyme-conjugated streptavidin was used to detect palivizumab bound to the plate. A four-parameter fit was generated and the serum dilution yielding 50% palivizumab binding inhibition was interpolated. Antibodies competitive with Palivizumab were present in low titer in normal adult sera, but were markedly increased by immunization with the RSV F nanoparticle vaccine (Figure 40A). Figure 40B shows the geometric-mean fold increase in palivizumab binding inhibition post dose 1 and post dose 2 in all subject groups. Adjuvant enhanced the palivizumab inhibition of vaccine-induced sera, and the 60ug + adjuvant group exhibited the highest levels of palivizumab inhibition.

Table 8. Concentration of Palivizumab-competitive antibody in study subject serum samples

		Placebo	**5 µg + $AlPO_4$**	**15 µg + $AlPO_4$**	**30 µg + $AlPO_4$**	**30 µg**	**60 µg + $AlPO_4$**	**60 µg**
		(N=26)	(N=17)	(N=16)	(N=18)	(N=17)	(N=13)	(N=18)
Study Day 1	N	26	17	16	18	17	13	18

	GMT	11	12	14	10	14	14	20
	95% CI	10, 12	10, 16	11, 20	NA , NA	21, 20	10, 20	13, 31
Study Day 30	N	26	17	16	18	17	13	18
	GMT	13	70	81	105	112	227	174
	95% CI	11, 17	41, 120	61, 107	80, 137	69, 180	167, 308	117, 260
Study Day 60	N	26	17	16	18	17	13	18
	GMT	14	111	128	142	100	337	157
	95% CI	11, 18	75, 162	103, 161	114, 175	69, 145	254, 448	108, 230

[0303] The results of the trial also indicated that the induction of anti-RSV F antibodies correlated with the presence of antibodies that competed for the palivizumab binding site (Figure 41). Despite anti-F titers present at Day 0, most healthy young adult sera yielded values at or near the palivizumab competition assay LLOQ. On Day 60, placebo recipients had an unchanged distribution, but active vaccinees (in red) showed an increase in anti-F antibodies with enrichment of palivizumab-competitive specificities.

[0304] Unlabeled palivizumab, added to normal serum, achieved 50% inhibition in the competitive ELISA at 2.1 µg/mL. This was used in Table 9 to calculate an approximate equivalent of "palivizumab-like" activity in vaccinee serum. In parallel, palivizumab was also spiked into 5 normal adult sera at three different levels, and its impact on MN titer was determined; increases in GMTs based on 2 replicates on each of 2 different days are shown in

Table 9. 50% inhibitory GMTs and calculated palivizumab-like activity in subject sera.

	Placebo	5 µg + Al	15 µg + Al	30 µg only	30 µg + Al	60 µg	60 µg + Al
Day 0 GMT (95% CI)	6 (5-6)	6 (5-8)	7 (5-10)	7 (5-10)	5 (-)	10 (7-15)	7 (5-10)
Day 0 "palivizumab equivalent"(µg/mL)	11	13	15	15	10	21	15
Day 60 GMT (95% CI)	7 (6-9)	55 (38-81)	64 (51-80)	50 (34-73)	71 (57-88)	79 (54-115)	169 (127-224)
Day 60 "palivizumab equivalent"(µg/mL)	15	116	135	106	149	165	337
Day 0 GMT (95% CI)	6 (5-6)	6 (5-8)	7 (5-10)	7 (5-10)	5 (-)	10 (7-15)	7 (5-10)

Table 10. Impact of defined palivizumab inputs on MN activity in human adult sera.				
	Baseline MN GMT	Fold-increase on MN GMT with added palivizumab		
Palivizumab	0	40µg/mL	80µg/mL	120µg/mL
Serum 1	64	2.2	3.8	4.8
Serum 2	136	1.3	2.1	2.7
Serum 3	192	1.5	2.0	2.1
Serum 4	342	1.1	1.3	1.4
Serum 5	456	0.9	1.0	1.3

[0305] The anti-F protein immune responses to the insect cell-derived RSV F protein nanoparticle were enriched in antibodies to the highly-conserved and clinically important F protein antigenic site II, which contains the palivizumab and motavizumab binding sites.

[0306] The levels of palivizumab-like activity achieved by the vaccine were commensurate with those that have been efficacious when passively administered to infants.

Example 19

RSV F-specific monoclonal antibodies bind the RSV F nanoparticle vaccine antigen

[0307] Various RSV F neutralizing monoclonal antibodies are known in the art and described, for example, in Crowe JE et al., Virology 252; 373 (1998), and in Beeler et al., Journal of Virology 63; 2941 (1989), both of which are incorprated herein by reference for all purposes. Figure 42 shows a schematic representation of the antibody recognition regions of the RSV F vaccine antigen, which are Site I, Site II, and Site IV/V/IV.

[0308] RSV F vaccine antigen was plated at 2µg/mL. RSV-specific monoclonal antibodies were serially diluted four fold and incubated with the RSV F vaccine antigen, and antibody binding was detected using anti-mouse HRP. As shown in Figure 42, the vaccine antigen was able to elicit binding of several neutralizing RSV F antibodies, including antibodies that bind at Site I, II, or IV/V/VI of the antigen.

Example 20

RSV F nanoparticle vaccine-induced immune responses in cotton rats

[0309] In this example, the efficacy of the RSV F vaccine was further tested in four groups of female cotton rats (5 per group). Group 1 animals received two intramuscular vaccinations, one on Day 0 and one on Day 28, with Formalin Inactivated RSV virus (FI-RSV) adsorbed onto alum at a 1:25 dilution. Animals in Group 2 received intramuscular vaccinations on Day

0 and Day 28 with 30μg of the recombinant RSV-F nanoparticle vaccine, formulated with adjuvant (2.4 mg/mL $AIP0_4$). Animals in Group 3 received the two vaccinations with 30 μg of the recombinant RSV-F nanoparticle vaccine in the absence of adjuvant. Animals in Group 4 were infected intranasally with 10^5 p.f.u of RSV-A2 (0.05 mL per nare) on Day 0 and Day 28. Sera were collected from all animals at Days 0, 28, and 49.

[0310] Polyclonal sera samples from immunized and infected cotton rats were serially diluted five fold and incubated with the RSV F antigen, plated at 2 μg/mL. Bound antibody was detected using anti-rat HRP. As shown in Figure 43, the RSV F vaccine induced robust anti-RSV IgG antibodies in cotton rats. Both RSV F vaccine groups elicited higher IgG titers compared to either immunization with FI-RSV or infection with RSV A2 at Days 28 and 49. The presence of adjuvant further increased the IgG titer at both Day 28 and Day 49 (Figure 43).

[0311] Neutralizing antibody responses in cotton rats were tested using a Hep-2 cell line infection assay. Serum dilutions were incubated with infectious RSV in plates, then the RSV/serum mixture was incubated with Hep-2 cells. The number of infectious virus plaques were counted, and neutralization titers were calculated as the inverse of the highest dilution of serum that produced 50% RSV inhibition of infection.

[0312] Day 0 serum did not exhibit measureable neutralization titers in any group. FI-RSV groups did not exhibit neutralization titers at any timepoint. At Day 49, mice infected with RSV A2 exhibited similar levels of neutralizing antibody to those immunized with RSV F in the absence of adjuvant. The highest neutralization titers were detected at Day 49 in the RSV F vaccine + adjuvant group, indicating that neutralization titers were induced most robustly in animals receiving RSV F vaccine in the presence of adjuvant (Figure 44).

[0313] To determine if neutralization is inhibited by the presence of RSV-F, cotton rat sera were pre-incubated with 20μg/mL of BSA, RSV F protein, RSV G protein, or both RSV F and RSV G protein, prior to incubation with Hep-2 cells in the neutralization assay. As shown in Table 11, pre-incubation with RSV F protein reduced neutralization titers to undetectable or nearly undetectable levels in sera from RSV A2-infected mice as well as mice immunized with the RSV F vaccine, with or without adjuvant.

Table 11. Neutralization of RSV is inhibited by the presence of RSV F protein

Cotton Rat Samples	**BSA +Sera**	**RSVF +Sera**	**RSVG +Sera**	**RSV G + RSV F + Sera**
FI-RSV	<20	<20	<20	<20

RSVF-Adj	1280	40	640	20
RSV F	320	<20	320	<20
RSV-A2 Infection	160	40	40	<20

[0314] A Hep-2 cell line fusion inhibition assay was used to test the ability of antibodies induced by FI-RSV, RSV F vaccine with or without adjuvant, or live RSV A2 to inhibit fusion-mediated RSV infection. Hep-2 cells were briefly pre-incubated with live RSV prior to incubation of the cells with serum dilutions from each group. The number of infectious virus plaques were counted, and fusion inhibition was expressed as the inverse of the dilution that resulted in 50% inhibition of plaque formation. Figure 45 shows that fusion-mediated infection of Hep-2 cells was inhibited by Day 49 sera from infected rats or rats immunized with RSV F vaccine, with or without adjuvant. However, fusion inhibition was enhanced by the presence of adjuvant. Further, at the Day 28 timepoint, only sera from rats immunized with RSV F vaccine with adjuvant inhibited fusion. Pre-incubation of rat sera with RSV F protein abrogated the fusion inhibition capacity in all groups, as shown in Table 12, indicating that the inhibition of fusion was mediated by RSV F-specific antibodies.

Table 12. Fusion inhibition is abrogated by the presence of RSV-F protein

Cotton Rat Samples	**BSA**	**RSVF**
FI-RSV	<20	<20
RSVF-Adj	2560	20
RSV F	40	<20
RSV-A2 Infection	20	20
RSV-A2/RSV F	80	20
RSVF/RSV-A2	160	20

[0315] Serum samples from individual animals were assessed for the capacity to compete with palivizumab monoclonal antibody for binding to RSV F. Serial dilutions of serum samples were incubated with biotinylated palivizumab prior to incubation with plate-bound RSV F, followed by detection of bound palivizumab with avidin-HRP. Data were expressed as the inverse of the geometric mean titer of antibodies that exhibited 50% inhibition of

palivizumab binding. Sera obtained from FI-RSV immunized animals did not inhibit palivizumab binding. Minimal inhibition of palivizumab binding was observed with sera from animals treated with live RSV. In contrast, sera from animals immunized with the RSV F vaccine exhibited competition with palivizumab for binding to RSV F nanoparticles, and competition was further enhanced with sera from animals that also received adjuvant (Figure 46).

[0316] Cotton rat sera from animals within each group were also assessed for the ability to inhibit the binding of other RSV-F-specific monoclonal antibodies. Serum-mediated inhibition of binding of antibodies 1107, 1153, 1243, 1112, or 1269, which bind to Sites I (1243), II (1107 and 1153), or IV, V, VI (1112 and 1269), of the RSV F protein, was determined. Serial dilutions of sera from each group were incubated with biotinylated antibodies 1107, 1153, 1112, 1269, or 1243 prior to incubation with plate-bound RSV F, followed by detection with avidin-HRP. 50% inhibition titers were calculated as the inverse of the highest dilution at which binding to RSV F was 50% inhibited. As shown in Figure 47, inhibition of neutralizing RSV F-specific monoclonal antibody binding to RSV F was enhanced with sera from rats immunized with RSV F vaccine compared to rats immunized with FI-RSV or live RSV-A2, and was further enhanced in the presence of adjuvant.

[0317] The avidity of anti-RSV antibodies induced by immunization with the RSV F nanoparticle vaccine was compared to the avidity of anti-RSV antibodies induced by immunization with FI-RSV. Direct ELISAs were conducted in which serial dilutions of cotton rat sera were incubated in plates with bound RSV antigen, and levels of bound antibody prior to or after a 7 molar urea wash step were measured. Antibodies in sera from RSV F nanoparticle vaccine immunized animals exhibited higher avidity for RSV in comparison to antibodies in sera from FI-RSV immunized animals (Figure 48). A larger percentage of the RSV-specific antibodies in sera from vaccine immunized animals was high avidity, as shown by the amount of antibody bound before and after the urea wash (Figure 48 and Table 13).

Table 13. O.D. 450 and percent high avidity antibodies in RSV F nanoparticle vaccine immunized versus FI-RSV immunized animals

Before Urea Wash	5630	603,000
After urea Wash	1014	275,690
% High Avidity	20	46

****

[0318] The foregoing detailed description has been given for clearness of understanding only and no unnecessary limitations should be understood therefrom as modifications will be obvious to those skilled in the art. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed inventions, or that any publication specifically or implicitly referenced is prior art.

[0319] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0320] Although the application has been broken into sections to direct the reader's attention to specific embodiments, such sections should be not be construed as a division amongst embodiments. The teachings of each section and the embodiments described therein are applicable to other sections.

[0321] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

CLAIMS

1. A respiratory syncytial virus (RSV) fusion (F) protein comprising at least one modification or mutation that increases the expression of said RSV F protein in a host cell.

2. A RSV F protein comprising at least one modification or mutation that reduces the cellular toxicity of said RSV F protein in a host cell.

3. A RSV F protein comprising at least one modification or mutation that enhances the immunogenic properties of said RSV F protein as compared to an unmodified RSV F protein.

4. The RSV F protein of any of claims 1-3, wherein said F protein further comprises an amino acid substitution at the amino acid position corresponding to the proline 102 residue of the wild-type RSV F protein (SEQ ID NO: 2).

5. The RSV F protein according to claim 4, wherein said proline 102 residue is replaced with an alanine residue.

6. The RSV F protein according to any of claims 4-5, further comprising an amino acid substitution at the amino acid position corresponding to the isoleucine 379 residue of the wild-type RSV F protein (SEQ ID NO: 2).

7. The RSV F protein according to claim 6, wherein said isoleucine 379 residue is replaced with a valine residue.

8. The RSV F protein according to any of claims 4-7, further comprising an amino acid substitution at the amino acid position corresponding to the methionine 447 residue of the wild-type RSV F protein (SEQ ID NO: 2).

9. The RSV F protein according to claim 8, wherein said methionine 447 residue is replaced with a valine residue.

10. The RSV F protein according to any of claims 1-9, wherein said RSV F protein assumes a lollipop morphology.

11. The RSV F protein according to any of claims 4-10, comprising a mutation that inactivates at least one furin cleavage site.

12. The RSV F protein according to claim 11, wherein said furin cleavage site is the primary cleavage site.

13. The RSV F protein according to any of claims 11-12, wherein said inactivation of at least one furin cleavage site is accomplished by introducing at least one amino acid substitution at positions corresponding to arginine 133, arginine 135, and arginine 136 of the wild-type RSV F protein (SEQ ID NO: 2).

14. The RSV F protein according to claim 13, wherein at least two amino acid substitutions are introduced at positions corresponding to arginine 133, arginine 135, and arginine 136 of the wild-type RSV F protein (SEQ ID NO: 2).

15. The RSV F protein according to any of claims 14, wherein three amino acid substitutions are introduced at positions corresponding to arginine 133, arginine 135, and arginine 136 of the wild-type RSV F protein (SEQ ID NO: 2).

16. The RSV F protein according to any of claims 13-15, wherein said arginine 133 residue is replaced with glutamine.

17. The RSV F protein according to any of claims 13-16, wherein said arginine 135 residue is replaced with glutamine.

18. The RSV F protein according to any of claims 13-17, wherein said arginine 136 residue is replaced with glutamine.

19. The RSV F protein according to any of claims 4-18, wherein said RSV F protein further comprises at least one modification of the cryptic poly(A) site of F2.

20. The RSV F protein according to any of claims 4-19, wherein said RSV F protein further comprises a deletion in the N-terminal half of the fusion domain corresponding to about amino acids 137-146 of the wild-type RSV F protein (SEQ ID NO: 2).

21. The RSV F protein of any of claims 1-3, wherein said modification or mutation is selected from the group consisting of:

(i) inactivation of at least one furin cleavage site;

(ii) a modification of the cryptic poly(A) site of F2; and

(iii) a deletion in the N-terminal half of the fusion domain corresponding to about amino acids 137-146 of the wild-type RSV F protein (SEQ ID NO: 2).

22. The RSV F protein of any of claims 1-3, wherein said RSV F protein harbors at least two mutations selected from the group consisting of:

(i) at least one amino acid substitution at positions corresponding to proline 102, isoleucine 379, and methionine 447 of the wild-type RSV F protein (SEQ ID NO: 2);

(ii) inactivation of at least one furin cleavage site;

(iii) a modification of the cryptic poly(A) site of F2; and

(iv) a deletion in the N-terminal half of the fusion domain corresponding to about amino acids 137-146 of the wild-type RSV F protein (SEQ ID NO: 2).

23. The RSV F protein of any of claims 1-3, wherein said RSV F protein harbors at least three mutations selected from the group consisting of:

(i) at least one amino acid substitution at positions corresponding to proline 102, isoleucine 379, and methionine 447 of the wild-type RSV F protein (SEQ ID NO: 2);

(ii) inactivation of at least one furin cleavage site;

(iii) a modification of the cryptic poly(A) site of F2; and

(iv) a deletion in the N-terminal half of the fusion domain corresponding to about amino acids 137-146 of the wild-type RSV F protein (SEQ ID NO: 2).

24. The RSV F protein of any of claims 1-3, wherein said RSV F protein harbors four mutations selected from the group consisting of:

(i) at least one amino acid substitution at positions corresponding to proline 102, isoleucine 379, and methionine 447 of the wild-type RSV F protein (SEQ ID NO: 2);

(ii) inactivation of at least one furin cleavage site;

(iii) a modification of the cryptic poly(A) site of F2; and

(iv) a deletion in the N-terminal half of the fusion domain corresponding to about amino acids 137-146 of the wild-type RSV F protein (SEQ ID NO: 2).

25. The RSV F protein of any of claims 21-24, wherein said RSV F protein harbors at least two amino acid substitutions at positions corresponding to proline 102, isoleucine 379, and methionine 447 of the wild-type RSV F protein (SEQ ID NO: 2).

26. The RSV F protein of any of claims 21-25, wherein said RSV F protein harbors three amino acid substitutions at positions corresponding to proline 102, isoleucine 379, and methionine 447 of the wild-type RSV F protein (SEQ ID NO: 2).

27. A RSV F protein encoded by the nucleic acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, and SEQ ID NO: 9.

28. A RSV F protein comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, and SEQ ID NO: 10.

29. The RSV F protein according to any of claims 1-28, wherein said RSV F protein exhibits increases expression in a host cell as compared to a wild-type RSV F protein.

30. The RSV F protein according to any of claims 1-29, wherein said RSV F protein exhibits enhanced immunogenic properties as compared to a wild-type RSV F protein.

31. The RSV F protein according to any of claims 1-30, wherein said host cell is a eukaryotic cell.

32. The RSV F protein according to claim 31, wherein said eukaryotic cell is an insect cell.

33. The RSV F protein according to claim 32, wherein said insect cell is an Sf9 cell.

34. The RSV F protein according to any of claims 1-33, wherein said RSV F protein is derived from an RSV strain selected from the group consisting of an A strain of human RSV, a B strain of human RSV, strains of bovine RSV, and strains of avian RSV.

35. A purified micelle comprising one or more RSV F proteins of any of claims 1-34.

36. A virus-like particle (VLP) comprising a RSV F protein of any of claims 1-34.

37. The VLP of claim 36, further comprising a matrix (M) protein.

38. The VLP of claim 37, wherein said M protein is derived from a human strain of RSV.

39. The VLP of claim 37, wherein said M protein is derived from a bovine strain of RSV.

40. The VLP of claim 37, wherein said M protein is M1 from an influenza virus strain.

41. The VLP of claim 40, wherein said influenza virus strain is an avian influenza virus strain.

42. The VLP of claim 41, wherein said avian influenza virus strain is an H5N1 strain.

43. The VLP of claim 42, wherein said H5N1 strain is A/Indonesia/5/05.

44. The VLP of claim 37, wherein said M protein is derived from a Newcastle Disease Virus (NDV) strain.

45. The VLP of any of claims 36-44, further comprising the RSV glycoprotein (G).

46. The VLP of any of claims 36-45, further comprising the RSV glycoprotein (SH).

47. The VLP of any of claims 36-46, further comprising the RSV nucleocapsid protein (N).

48. The VLP of any of claims 36-47, wherein the VLP is expressed in a eukaryotic cell under conditions which permit the formation of VLPs.

49. The VLP of claim 49, wherein the eukaryotic cell is selected from the group consisting of yeast, insect, amphibian, avian, mammalian, or plant cells.

50. An immunogenic composition comprising a RSV F protein of any of claims 1-34.

51. An immunogenic composition comprising a purified micelle according to claim 35.

52. An immunogenic composition comprising a VLP according to any of claims 36-49.

53. A pharmaceutically acceptable vaccine composition comprising a RSV F protein of any of claims 1-34, wherein the RSV F protein is capable of eliciting an immune response in a host.

54. A pharmaceutically acceptable vaccine composition comprising a purified micelle according to claim 35, wherein the micelle is capable of eliciting an immune response in a host.

55. A pharmaceutically acceptable vaccine composition comprising a VLP according to any of claims 36-49, wherein the VLP is capable of eliciting an immune response in a host.

56. A kit for immunizing a human subject against a viral infection comprising a RSV F protein of any of claims 1-34.

57. A kit for immunizing a human subject against a viral infection comprising a purified micelle according to claim 35.

58. A kit for immunizing a human subject against a viral infection comprising a VLP according to any of claims 36-49.

59. The kit according to any of claims 54-56, wherein the viral infection is a RSV infection.

60. A method of vaccinating a mammal against a viral infection comprising administering the RSV F protein of any of claims 1-34 in a pharmaceutically acceptable formulation to a human subject.

61. A method of vaccinating a mammal against a viral infection comprising administering a purified micelle of claim 35 in a pharmaceutically acceptable formulation to a human subject.

62. A method of vaccinating a mammal against a viral infection comprising administering a VLP according to any of claims 36-49 in a pharmaceutically acceptable formulation to a human subject.

63. The method according to any of claims 60-62, wherein the pharmaceutically acceptable formulation comprises an adjuvant.

64. The method according to claim 63, wherein the adjuvant is a non-phospholipid liposome.

65. A method of generating an immune response against a viral infection comprising the RSV F protein of any of claims 1-34 in a pharmaceutically acceptable formulation to a human subject.

66. A method of generating an immune response against a viral infection comprising administering a purified micelle of claim 35 in a pharmaceutically acceptable formulation to a human subject.

67. A method of generating an immune response against a viral infection comprising administering a VLP according to any of claims 36-49 in a pharmaceutically acceptable formulation to a human subject.

68. An isolated nucleic acid encoding a RSV F protein according to any of claims 1-30.

69. An isolated cell comprising a nucleic acid of claim 68.

70. A vector comprising the nucleic acid of claim 68.

71. A method of making a RSV F protein, comprising:

(a) transforming a host cell to express a nucleic acid of claim 68; and

(b) culturing said host cell under conditions conducive to the production of said RSV F protein.

72. A method of making a RSV F protein micelle, comprising:

(a) transforming a host cell to express a nucleic acid of claim 68; and

(b) culturing said host cell under conditions conducive to the production of said RSV F protein micelle.

73. The method according to any of claims 71-72, wherein said host cell is an insect cell.

74. The method according to claim 73, wherein said insect cell is an insect cell transfected with a baculovirus vector comprising a nucleic acid of claim 68.

75. A method of preventing viral replication in the lung of an animal, comprising administering a RSV F protein of any of claims 1-34 to said animal.

76. The method of claim 75, wherein the RSV F protein is administered intramuscularly.