

METHODS FOR ELICITING AN IMMUNE RESPONSE TO RESPIRATORY SYNCYTIAL VIRUS AND STREPTOCOCCUS PNEUMONIAE INFECTION

FIELD OF THE INVENTION

5 The present invention relates to novel methods for eliciting an immune response to respiratory syncytial virus (RSV) and *S. pneumoniae* (*Streptococcus pneumoniae*) infection.

BACKGROUND TO THE INVENTION

10 Respiratory syncytial virus (RSV) can cause severe lower respiratory tract infection in older adults and adults with chronic medical conditions including cardiopulmonary and immunocompromising conditions. In older adults (e.g., >60 years), RSV infection may progress to bronchiolitis or pneumonia. Symptoms in children are often more severe. In 2015, an estimated 1.5 million episodes of RSV-related acute respiratory illness occurred in
15 older adults in industrialized countries; approximately 14.5% of these episodes involved a hospital admission. Currently, only one vaccine (AREXVY®) has been approved to prevent lower respiratory tract disease caused by RSV in older adults.

 Like RSV, pneumococcal disease also presents high disease burden among older adults, and the seasonality of RSV disease and invasive pneumococcal disease is similar. In
20 Europe and the United States, pneumococcal pneumonia is the most common community-acquired bacterial pneumonia, estimated to affect approximately 100 per 100,000 adults each year. The risk for pneumococcal disease is much higher in infants and elderly people, as well as immune compromised persons of any age. Even in economically developed regions, invasive pneumococcal disease carries high mortality; for adults with pneumococcal
25 pneumonia the mortality rate averages 10%-20%, while it may exceed 50% in the high- risk groups. Pneumonia is by far the most common cause of pneumococcal death worldwide. There are currently five pneumococcal conjugate vaccines (PCV) available on the global market: PREVNAR (PREVENAR in some countries) (heptavalent vaccine), SYNFLORIX (a decavalent vaccine), PREVNAR 13 (PREVENAR 13 in some countries) (tridecavalent
30 vaccine) VAXNEUVANCE (a fifteen valent vaccine) and PREVNAR-20 / APEXXNAR (a 20-valent vaccine).

 Accordingly, there is a need to address remaining unmet medical need for patients to receive an RSV vaccine and a PCV vaccine, while retaining the benefits of the RSV vaccine.

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SUMMARY OF THE INVENTION

To meet these and other needs, the present invention relates to novel methods for eliciting an immune response to respiratory syncytial virus (RSV) and *S. pneumoniae* (*Streptococcus pneumoniae*) infection. The following clauses describe some aspects and
5 embodiments of the invention:

In an aspect, there is provided a method of inducing an immune response to respiratory syncytial virus (RSV) in a human comprising co-administration of (a) an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F
10 protein comprising at least one modification that stabilizes the prefusion conformation of the F protein; and (b) an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F.

In an aspect, there is provided an immunogenic combination for use in a method of
15 preventing respiratory syncytial virus (RSV) infection in a human comprising (a) an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein, and an adjuvant; and (b) an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A,
20 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, wherein the method comprises co-administering the compositions to the human.

In an aspect, there is provided use of an immunogenic combination for preventing respiratory syncytial virus (RSV) infection in a human comprising (a) administering an immunogenic composition comprising an immunogenic composition comprising a
25 recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein, and an adjuvant; and (b) administering an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, wherein a) and b) are co-administered to the human.

In an aspect, there is provided an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein, for use in raising an immune response to RSV in a human, wherein an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8,
30 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F is co-administered.

In an aspect, there is provided an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, for use in raising an immune response to *S. pneumoniae* in a human, wherein an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein is co-administered.

In an aspect, there is provided an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein, and an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, for use in raising an immune response to RSV and *S. pneumoniae* in a human comprising co-administering to the human said immunogenic compositions.

In an aspect, there is provided use of immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F in raising an immune response to *S. pneumoniae* in a human, wherein an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein is co-administered.

In an aspect, there is provided use of immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein, and an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, for use in raising an immune response to RSV and *S. pneumoniae* in a human comprising co-administering to the human said immunogenic compositions.

In embodiments of each of the aspects herein, the at least one modification is selected from the group consisting of (i) an addition of an amino acid sequence comprising a heterologous trimerization domain; (ii) a deletion of at least one furin cleavage site; (iii) a deletion of at least one non-furin cleavage site; (iv) a deletion of one or more amino acids of the pep27 domain; and (v) at least one substitution or addition of a hydrophilic amino acid in a hydrophobic domain of the F protein extracellular domain.

In embodiments of each of the aspects herein, the at least one modification comprises the addition of an amino acid sequence comprising a heterologous trimerization domain.

5 In embodiments of each of the aspects herein, the recombinant RSV antigen comprises an F2 domain and an F1 domain of an RSV F protein with no intervening furin cleavage site wherein the polypeptide further comprises a heterologous trimerization domain positioned C-terminal to the F1 domain.

In embodiments of each of the aspects herein, the recombinant RSV antigen comprises a pre-fusion RSV F polypeptide having an HRA region (residues 137-239 of
10 reference RSV F protein of SEQ ID NO:1) and a DIII region (residues 51-98 and 206-308 of reference RSV F protein of SEQ ID NO:1), wherein a cysteine residue is introduced into the HRA region and a cysteine residue is introduced into the DIII region, and a disulfide bond is formed between the introduced cysteine residue in the HRA region and the introduced
15 cysteine residue in the DIII region that prevents a post-fusion HRA-HRB six-helix bundle from forming.

In embodiments of each of the aspects herein, the immunogenic composition comprising a recombinant RSV antigen further comprises an adjuvant.

In embodiments of each of the aspects herein, the adjuvant comprises MPL and QS-21.

20 In embodiments of each of the aspects herein, the MPL is present in the immunogenic composition in an amount of about 25 µg.

In embodiments of each of the aspects herein, the QS-21 is present in the immunogenic composition in an amount of about 25 µg.

In embodiments of each of the aspects herein, the human is 60 years old and above.
25 In an embodiment, the human is 60 to 69 years old. In an embodiment, the human is 70 to 79 years old. In an embodiment the human is ≥80 years old.

In embodiments of each of the aspects herein, the glycoconjugates are individually conjugated to CRM197.

In embodiments of each of the aspects herein, the immunogenic composition
30 comprising at least one glycoconjugate further comprises an adjuvant.

In embodiments of each of the aspects herein, the adjuvant is an aluminum salt selected from the group consisting of aluminum phosphate, aluminum sulfate, and aluminum hydroxide.

The present invention also provides the use of an immunogenic composition in the manufacture of a medicament for inducing an immune response in a subject (e.g. human).

In an aspect, there is provided the use of an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein polypeptide comprising at least one modification that stabilizes the prefusion conformation of the F protein, in the manufacture of a medicament for raising an immune response to RSV in a human, wherein an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F is co-administered.

In an aspect, there is provided the use of an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, in the manufacture of a medicament for raising an immune response to *S. pneumoniae* in a human, wherein an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein polypeptide comprising at least one modification that stabilizes the prefusion conformation of the F protein is co-administered.

In an aspect, there is provided the use of an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein polypeptide comprising at least one modification that stabilizes the prefusion conformation of the F protein, and an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, in the manufacture of a medicament for raising an immune response to RSV and *S. pneumoniae* in a human comprising co-administering to the human said immunogenic compositions.

In an aspect, there is provided the use of an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F in the manufacture of a medicament for raising an immune response to *S. pneumoniae* in a human, in co-administration with an immunogenic composition comprising a recombinant RSV antigen

comprising a soluble F protein polypeptide comprising at least one modification that stabilizes the prefusion conformation of the F protein.

In an aspect, there is provided the use of an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein polypeptide comprising at least one modification that stabilizes the prefusion conformation of the F protein in the manufacture of a medicament for raising an immune response to RSV in a human in co-administration with an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F

DESCRIPTION OF DRAWINGS/FIGURES

FIG. 1A The wildtype RSV F protein sequence is depicted in schematic form: signal peptide (25 residues), 529 residue ectodomain including 3 heptad repeats (HRA, HRB, and HRC), transmembrane region (TM), short cytoplasmic tail, glycosylation sites (circled G), and furin cleavage sites (scissors). The signal peptide (SP) and p27 peptide are removed during protein maturation, resulting in the F1 and F2 chains (diamond symbol=post-cleavage site left after p27 removal).

FIG. 1B The recombinant RSV soluble F protein is shown with the S155C, S290C, S190F, and V207L substitutions (vertical lettering) and foldon; the other figure labels are as described in FIG. 1A.

FIG. 2 depicts the study design overview of the Ph3 open-label, randomized, controlled, multi-country study to evaluate the immune response, safety, and reactogenicity of RSVPreF3 OA investigational vaccine when co-administered with 20-valent pneumococcal conjugate vaccine (PCV) in adults aged 60 years and older.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art. Throughout this specification and the claims that follow, unless the context requires otherwise, the term "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions, etc.), whether supra or infra, are hereby

incorporated by reference in their entirety. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention. All definitions provided herein in the context of one aspect of the invention also apply to other aspects of the invention.

5 As used herein, the term “co-administer” or “co-administration” or variations thereon, refers to administering separate immunogenic compositions on the same day. Co-administration of separate immunogenic compositions may occur via the same route of administration (e.g., subcutaneous or intramuscular injection.) Alternatively, co-administration of separate immunogenic compositions may occur via different routes of
10 administration (e.g., intramuscular and intradermal; intramuscular and intranasal; inhalation and subcutaneous; etc.). In one aspect of the invention, co-administration is via the same route, intramuscular injection.

 As used herein, “immunogenic combination” refers to a plurality of separately formulated immunogenic compositions administered in a single immunization regimen, e.g.,
15 co-administration.

 As used herein, “elicit an immunogenic response” or “induce an immune response” is understood to mean generate an immune response, preferably an antibody response in a human. In an embodiment, the immune response is an antibody response and a B cell and/or T cell response. In an embodiment, the immune response is an antibody response to
20 *S. pneumoniae* (*Streptococcus pneumoniae*). In an embodiment, the immune response is an opsonophagocytic (OP) antibody (Ab) response to *S. pneumoniae*. In an embodiment, the immune response is an antibody response to RSV (respiratory syncytial virus). In an embodiment, the immune response is in terms of RSV-A neutralization antibodies. In an embodiment, the immune response is in terms of RSV-B neutralization antibodies.
25 Opsonophagocytic Assays are described for example in WO2018/134693. In an embodiment, the immune response is in terms of both an antibody response to *S. pneumoniae* and an antibody response to RSV.

 As used herein, the term “glycoconjugate” refers to a capsular saccharide linked covalently to a carrier protein, wherein the capsular saccharide is derived from a serotype of
30 *Streptococcus pneumoniae*.

 As used herein, the terms “soluble F protein” or “recombinant RSV soluble F protein” are used interchangeably and refer to an RSV F protein from any subtype of RSV. In an embodiment, the RSV F protein is from RSV subgroup A, RSV subgroup P, or a combination thereof. In an embodiment, the recombinant RSV soluble F protein comprises S155C,
35 S290C, S190F, and V207L substitutions and include a trimerization foldon domain. An example of such a substituted RSV F protein is disclosed in WO2014160463 and the

substituted amino acid positions are described with reference to the wildtype RSV F protein sequence set forth as sequence identifier 124 therein. As used herein, a soluble F protein is expressed as a single protein (F0) that is eventually cleaved internally by furin. An exemplary F0 recombinant RSV soluble F protein is depicted in FIG.1B and an exemplary F0 protein sequence is set forth in SEQ ID NO:1. Furin proteolysis separates the protein into two chains, one comprising an F1 amino acid sequence and one comprising an F2 amino acid sequence, and thus a recombinant RSV soluble F protein will comprise at least an F1 chain and an F2 chain. Because of the presence of two furin cleavage sites, proteolysis can result in the two chains, F1 and F2, as well as the release of the p27 peptide however, some mature recombinant RSV soluble F protein may be cleaved at only one furin cleavage site and the p27 peptide is not released.

By "F1 chain" is intended the C-terminal portion of the cleaved F0 molecule. An exemplary F1 chain is indicated in FIG.1B and exemplary F1 chain polypeptide sequences are set forth in SEQ ID NO:2 and SEQ ID NO:8.

By "F2 chain" is intended the N-terminal portion of the cleaved F0 molecule from which the signal protein has been removed. An exemplary F2 chain is indicated in FIG.1B and an exemplary F2 chain polypeptide sequence is set forth in SEQ ID NO:3.

By "p27 peptide" is intended an amino acid sequence corresponding to the full-length of amino acids of the polypeptide (F0) that would be released through complete proteolysis at both of the two furin sites. In some embodiments, furin proteolysis releases a 27 amino acid p27 peptide which may vary RSV strain to RSV strain. In some embodiments, a p27 peptide has the amino acid sequence of SEQ ID NO:7.

By "trimer" is intended a protein complex of three recombinant RSV soluble F proteins wherein each recombinant RSV soluble F protein comprises an F1 chain and a F2 chain. In an embodiment, each recombinant RSV soluble F protein of the complex is independently selected from the group consisting of: F' and F protomer; such that structure of the trimer is described by the formula

$$nF + (3-n)F' \quad (\text{Formula I})$$

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wherein n is an integer from 0-3, F is an F protomer and F' is an F' protomer.

By "uncleaved furin cleavage site" is intended the minimal amino acid sequence ...RXRRX₁... (SEQ ID NO. 9) where X and X₁ are any amino acids (see, e.g., Moehring et al. (1993) "Strains of CHO-K1 cells resistant to Pseudomonas exotoxin A and cross-resistant to diphtheria toxin and viruses," *Infect. Immun.* 41:998-1009.)

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By “antigen” is intended a compound, composition, or substance that can stimulate the production of antibodies and/or a T cell response in an animal, including compositions that are injected, absorbed or otherwise introduced into an animal. Accordingly, by the term “antigenic polypeptide” is intended a polypeptide that can stimulate the production of antibodies and/or a T cell response in an animal. The term “antigen” includes all related antigenic epitopes. The term “epitope” or “antigenic determinant” refers to a site on an antigen to which B and/or T cells respond. The “dominant antigenic epitopes” or “dominant epitope” are those epitopes to which a functionally significant host immune response, e.g., an antibody response or a T-cell response, is made. Thus, with respect to a protective immune response against a pathogen, the dominant antigenic epitopes are those antigenic epitopes that when recognized by the host immune system result in protection from disease caused by the pathogen. The term “T-cell epitope” refers to an epitope that when bound to an appropriate MHC molecule is specifically bound by a T cell (via a T cell receptor). A “B-cell epitope” is an epitope that is specifically bound by an antibody (or B cell receptor molecule).

By “immunogenic composition” is intended a composition of matter suitable for administration to a human or animal subject (e.g., in an experimental setting) that is capable of eliciting a specific immune response, e.g., against a pathogen, such as RSV and/or *S. pneumoniae*. As such, an immunogenic composition includes one or more antigens (for example, polypeptide antigens) or antigenic epitopes. An immunogenic composition can also include one or more additional components capable of eliciting or enhancing an immune response, such as an excipient, carrier, and/or adjuvant. In certain instances, immunogenic compositions are administered to elicit an immune response that protects the subject, wholly or partially, against symptoms or conditions induced by a pathogen. In some cases, symptoms or disease caused by a pathogen is prevented (or reduced or ameliorated) by inhibiting replication of the pathogen (e.g., RSV) following exposure of the subject to the pathogen. In the context of this disclosure, the term immunogenic composition will be understood to encompass compositions that are intended for administration to a subject or population of subjects for the purpose of eliciting a protective pre-exposure immune response against RSV or palliative post-exposure immune response against RSV (that is, vaccine compositions or vaccines).

By “adjuvant” is intended an agent that enhances the production of an immune response in a non-specific manner. Common adjuvants include suspensions of minerals (alum, aluminium hydroxide, aluminium phosphate) onto which antigen is adsorbed; emulsions, including water-in-oil, and oil-in-water (and variants thereof, including double emulsions and reversible emulsions), liposaccharides, lipopolysaccharides,

immunostimulatory nucleic acids (such as CpG oligonucleotides), liposomes, Toll-like Receptor agonists (particularly, TLR2, TLR4, TLR7/8 and TLR9 agonists), and various combinations of such components.

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Immunogenic Composition - RSV

RSV F, the primary target of neutralizing antibodies against the virus, is a Class I fusion glycoprotein abundant on the viral envelope. Like other viral fusion proteins, F exists in a trimeric "prefusion" state on the viral surface. Once entry is triggered, it inserts
10 hydrophobic fusion loops into the cell membrane and undergoes extensive conformational change to fold into an energetically favorable trimeric "postfusion" state, fusing the viral and cell membranes together in the process.

RSV exists as a single serotype but has two antigenic subgroups: A and B. The F glycoproteins of the two groups are about 90% identical. The A subgroup, the B subgroup, or
15 a combination or hybrid of both can be used herein. An example sequence for the A subgroup is SEQ ID NO: 11 (A2 strain; GenBank GI: 138251; Swiss Prot P03420), and for the B subgroup is SEQ ID NO: 12 (18537 strain; GI: 138250; Swiss Prot P13843). SEQ ID NO:11 and SEQ ID NO:12 are both 573 amino acid sequences. The signal peptide in A2 strain is a.a. 1-21, but in 18537 strain it is 1-22. In both sequences the TM domain is from
20 about a.a. 530-550 but has alternatively been reported as 525-548.

The precursor RSV F protein sequence contains a signal peptide (25 residues), a 529 residue ectodomain including 3 heptad repeats (HRA, HRB, and HRC), a transmembrane region and a short cytoplasmic tail. The wildtype protein is expressed as a single polypeptide (F0) that is eventually cleaved internally at two furin sites. Furin
25 proteolysis releases a 27 amino acid peptide (p27) and separates the protein into two chains, F1 and F2, which remain linked by two disulfide bonds (C37-C439; C69-C212). These cleavages free the N-terminus of the hydrophobic fusion peptide (F1 N-terminus), making it available for eventual membrane insertion, which allows F to function as a fusogen. The final mature RSV F protein also contains glycosylations at three N-linked
30 glycosylation sites (N27, N70 and N500).

Exogenously expressed F ectodomain spontaneously folds into a highly-stable trimer in the postfusion conformation. The postfusion F crystal structure has been solved and shows the preservation of two important, surface exposed, neutralizing epitopes, Site A and Site C.

The prefusion F structure was solved by co-expressing the F ectodomain with the Fab fragment of a prefusion F-specific antibody. It and two related antibodies recognize an epitope on F that is unique to its prefusion conformation, designated Site Ø. This epitope is near the top of the molecule and is formed by residues 60-75 (F2) and the HRA helix residues 196-209 (F1). In the extensive structural rearrangement that occurs when F flips to its postfusion conformation, this epitope is destroyed, with ~180° change in the relative position of the loop with respect to the HRA helix in the pre- and postfusion molecules. By contrast, the other two neutralizing sites in F, Site A and Site C, are preserved in both the prefusion and postfusion conformations. The quaternary epitope site V overlaps with a β_3 - β_4 motif and is present on the trimeric form of prefusion RSV-F. See McLellan et al (2015) "Characterization of a Prefusion-Specific Antibody That Recognizes a Quaternary, Cleavage-Dependent Epitope on the RSV Fusion Glycoprotein," *PLOS Pathog*, 11:e1005035. Site V is shown in FIG. 2.

To stabilize the F ectodomain in its prefusion conformation, three main changes to the ectodomain sequence are required. A C-terminal trimerization domain is needed. An example is the foldon which is a 27-residue domain from the C-terminus of the T4 bacteriophage fibritin molecule that spontaneously trimerizes upon expression. In the absence of this domain but with the additional changes, the F protein will take up the prefusion conformation, but fails to form trimers, and is not highly immunogenic. The foldon has been linked to the F C-terminus by a short 4-residue non-cleavable linker, Ser-Ala-Ile-Gly.

The other two changes are stabilizing mutations designed based on the antibody-bound structure to help maintain F in its prefusion state without antibody addition. Following several testing/design iterations, a combination of two sets of mutations gave the best results. This molecule, termed DS-Cav1 in McLellan et. al. *Science* (2013) vol. 342 p. 592), contains a disulfide ("DS") introduced by mutating Ser residues at 155 and 290 to Cys (S155C-S290C) to prevent it from flipping to the postfusion state. It also contains cavity-filling mutations, S190F and V207L, to stabilize Site Ø at the apex. This RSV soluble F protein comprising S155C, S290C, S190F, and V207L substitutions raises neutralizing antibodies much more potently than postfusion F and is able to deplete over 85% of the RSV-specific neutralizing antibodies in human sera.

In an aspect, the immunogenic composition comprising a recombinant RSV antigen comprises a soluble F protein comprising at least one modification that stabilizes the

prefusion conformation of the F protein. In an embodiment, the at least one modification is selected from the group consisting of:

- i) an addition of an amino acid sequence comprising a heterologous trimerization domain;
- 5 ii) a deletion of at least one furin cleavage site;
- iii) a deletion of at least one non-furin cleavage site;
- iv) a deletion of one or more amino acids of the pep27 domain; and
- v) at least one substitution or addition of a hydrophilic amino acid in a hydrophobic domain of the F protein extracellular domain.

10 In an embodiment, the at least one modification comprises the addition of an amino acid sequence comprising a heterologous trimerization domain. In an embodiment, the recombinant RSV antigen comprises an F2 domain and an F1 domain of an RSV F protein with no intervening furin cleavage site wherein the polypeptide further comprises a heterologous trimerization domain positioned C-terminal to the F1 domain. In another
15 embodiment, the recombinant RSV antigen comprises an F2 domain and an F1 domain of an RSV F protein with furin cleavage sites wherein the protein further comprises a heterologous trimerization domain positioned C-terminal to the F1 domain. In a particular embodiment, the recombinant RSV antigen comprises a pre-fusion RSV F polypeptide having an HRA region (residues 137-239 of reference RSV F protein of SEQ ID NO:11) and
20 a DIII region (residues 51-98 and 206-308 of reference RSV F protein of SEQ ID NO:11), wherein a cysteine residue is introduced into the HRA region and a cysteine residue is introduced into the DIII region, and a disulfide bond is formed between the introduced cysteine residue in the HRA region and the introduced cysteine residue in the DIII region that prevents a post-fusion HRA-HRB six-helix bundle from forming.

25 In an aspect, an immunogenic composition herein comprises at least one recombinant RSV soluble F protein selected from the group consisting of: (a) a recombinant RSV soluble F protein comprising a F1 chain having S155C, S290C, S190F, and V207L substitutions ("F protomer"); (b) a recombinant RSV soluble F protein comprising a F1 chain having S155C, S290C, S190F, and V207L substitutions, further comprising at least 10
30 amino acids of the p27 peptide ("F' protomer"); and (c) a recombinant RSV soluble F protein comprising (i) a F1 chain having S190I and D486S substitutions, (ii) an F2 chain having 103C substitution, and (iii) a fusion peptide having 148C substitution.

In an aspect, an immunogenic composition herein comprises a population of trimers of recombinant RSV soluble F proteins, wherein each trimer of said population is comprised

- of three recombinant RSV soluble F protein selected independently from the group consisting of: (a) a recombinant RSV soluble F protein comprising a F1 chain having S155C, S290C, S190F, and V207L substitutions ("F protomer"); (b) a recombinant RSV soluble F protein comprising a F1 chain having S155C, S290C, S190F, and V207L substitutions, further comprising at least 10 amino acids of the p27 peptide ("F' protomer"); and (c) a recombinant RSV soluble F protein comprising (i) a F1 chain having S190I and D486S substitutions, (ii) an F2 chain having 103C substitution, and (iii) a fusion peptide having 148C substitution. In such embodiments the trimers are described by the formula

$$nF+(3-n)F' \quad (\text{Formula I})$$

- wherein n is an integer from 0-3, F is an F protomer and F' is an F' protomer. In some embodiments, n is an integer from 1-3. In some embodiments, n is an integer from 2-3. In some embodiments, n is 3. In some embodiments, at least 1%, such as at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, at least 12%, at least 13%, at least 14%, at least 15%, at least 20%, or at least 25% of the recombinant RSV soluble F proteins in the population of trimers are F' protomers. The separation of RSV soluble F proteins into F' protomers and F protomers and determination of their quantitation may be carried out by reversed-phase liquid chromatography (RP-HPLC) under denaturing conditions. As described elsewhere herein, by using RP-HPLC, trimers are denatured into F and F' protomers and the protomers are then resolved based on their differences in hydrophobicity.

- In some embodiments, at least 1%, such as at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, at least 12%, at least 13%, at least 14%, at least 15%, at least 20%, at least 25%, at least 30%, at least 31%, at least 32%, at least 33%, at least 34%, at least 35%, at least 36%, at least 37%, at least 38%, at least 39%, at least 40%, at least 41%, at least 42%, at least 43%, at least 44%, at least 45% of said trimers of recombinant RSV soluble F proteins comprise at least one F' protomer. For instance, in one embodiment it was observed that when roughly 20% of protomers were F', roughly 40% of trimers in a population comprised at least one F' protomer.

- In some embodiments, the F' protomer comprises a F1 chain comprising an uncleaved furin cleavage site. In some embodiments, the F' protomer has a molecular mass at least 1.0 kDa, such as at least 1.5 kDa, at least 2.0 kDa, at least 2.5 kDa, at least 3.0 kDa greater than the molecular mass of the F protomer. In some embodiments, the ratio of trimers of recombinant RSV soluble F proteins comprising at least one F' protomer to trimers

of recombinant RSV soluble F proteins comprising zero F' protomers is at least 1:1.2; such as at least 1:1.3; at least 1:1.4; at least 1:1.5; at least 1:1.6; at least 1:1.7; at least 1:1.8; at least 1:1.9; at least 1:2.0; at least 1:2.1; at least 1:2.2; at least 1:2.3; at least 1:2.4; at least 1:2.5; at least 1:2.6; at least 1:2.7; at least 1:2.8; at least 1:2.9; at least 1:3.0; at least 1:3.5; at least 1:4; at least 1:9; at least 1:19; or at least 1:29.

In some embodiments, a recombinant RSV soluble F protein comprises a F1 chain and a F2 chain wherein the F1 chain comprises an amino acid sequence having at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity to the amino acid sequence of SEQ ID NO:2. Suitably the F1 chain comprises the amino acid sequence of SEQ ID NO:2. Both an F and F' protomer are described by this embodiment. In some embodiments, the F1 chain consists of the amino acid sequence of SEQ ID NO:2, for example in the case of a F protomer.

In some embodiments, a F' protomer comprises a F1 chain comprising the amino acid sequence of SEQ ID NO:6. In some embodiments, an F' protomer comprises a F1 chain having a N-terminal p27 peptide. In some embodiments, an F' protomer comprises a F1 chain comprising the amino acid sequence of SEQ ID NO:7.

In some embodiments, a recombinant RSV soluble F protein comprises a F1 chain and a F2 chain wherein the F2 chain comprises an amino acid sequence having at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity to the amino acid sequence of SEQ ID NO:3. Suitably the F2 chain comprises, such as consists of, the amino acid sequence of SEQ ID NO:3. Both an F and F' protomer are described by this embodiment.

In some embodiments, a recombinant RSV soluble F protein comprises a F1 chain and a F2 chain wherein the F1 chain comprises an amino acid sequence having at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity to the amino acid sequence of SEQ ID NO:2 and the F2 chain comprises an amino acid sequence having at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity to the amino acid sequence of SEQ ID NO:3. Suitably the F1 chain comprises the amino acid sequence of SEQ ID NO:2 and the F2 chain comprises, such as consists of, the amino acid sequence of SEQ ID NO:3. Both an F and F' protomer are described by this embodiment. In such embodiments the F1 chain may suitably consist of the amino acid sequence of SEQ ID NO:2, for example in the case of a F protomer.

In some embodiments, a recombinant RSV soluble F protein comprises a F1 chain comprising an amino acid sequence having at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity to SEQ ID NO:8 and a F2 chain comprising an amino acid sequence having at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity to SEQ ID NO:3, for example in the case of a F' protomer. Suitably the F1 chain comprises, such as consists of, the amino acid sequence of SEQ ID NO:8 and the F2 chain comprises, such as consists of, the amino acid sequence of SEQ ID NO:3.

In some embodiments, a recombinant RSV soluble F protein comprises the entirety of the F0 molecule, such as before processing during expression. In some embodiments, the F0 molecule comprises an amino acid sequence having at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO: 10. Suitably the F0 chain comprises, such as consists of, the amino acid sequence of SEQ ID NO:1 or SEQ ID NO: 10. In an embodiment, the recombinant RSV soluble F protein comprises an amino acid sequence having at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity to the amino acid sequence of SEQ ID NO:1. In an embodiment, the recombinant RSV soluble F protein comprises an amino acid sequence having at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity to the amino acid sequence of SEQ ID NO:10. In another embodiment, the recombinant RSV soluble F protein has 100% identity to the amino acid sequence of SEQ ID NO:1. In another embodiment, the recombinant RSV soluble F protein has 100% identity to the amino acid sequence of SEQ ID NO:10.

Engineered variants of a recombinant RSV soluble F protein that share sequence similarity with the aforementioned sequences can also be employed in the context of the embodiments described above. It will be understood by those of skill in the art, that the similarity between a recombinant RSV soluble F protein (and polynucleotide) sequences as described below, can be expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity); the higher the percentage, the more similar are the primary structures of the two sequences. In general, the more similar the primary structures of two amino acid (or polynucleotide) sequences, the more similar are the higher order structures resulting from folding and assembly. Variants of a recombinant RSV soluble F protein (and polynucleotide) sequences typically have one or a small number of amino acid deletions, additions or substitutions but will nonetheless share a very high percentage of their amino acid, and generally their polynucleotide sequence. More importantly, the variants

retain the structural and, thus, conformational attributes of the reference sequences disclosed herein.

Methods of determining sequence identity are well known in the art, and are applicable to recombinant RSV soluble F proteins, as well as the nucleic acids that encode them (e.g., as described below). Various programs and alignment algorithms are described in: Smith and Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman and Wunsch, *J. Mol. Biol.* 48:443, 1970; Higgins and Sharp, *Gene* 73:237, 1988; Higgins and Sharp, *CABIOS* 5:151, 1989; Corpet et al., *Nucleic Acids Research* 16:10881, 1988; and Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988. Altschul et al., *Nature Genet.* 6:119, 1994, presents a detailed consideration of sequence alignment methods and homology calculations. The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., *J. Mol. Biol.* 215:403, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, Md.) and on the internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. A description of how to determine sequence identity using this program is available on the NCBI website.

In some instances, the recombinant RSV soluble F protein has one or more amino acid modification relative to the amino acid sequence of the naturally occurring strain from which it is derived (e.g., in addition to the aforementioned stabilizing modifications). Such differences can be an addition, deletion or substitution of one or more amino acids. A variant typically differs by no more than 20%, such as no more than 15%, 10%, 5%, 2%, or 1% of the amino acid residues. For example, those having deletions of up to 5 (such as 1 or 2) residues at 0-5 locations (such as 0, 1 or 2), insertions of up to 5 residues (such as 1 or 2) at 0-5 five locations (such as 0, 1 or 2) and substitution of up to 20 residues (such as up to 10 residues, in particular up to 5 residues) as compared to the exemplary F1 chain, F2 chain, F0 molecule, and p27 peptide sequences of SEQ ID NOs:1, 2, 3, 6, 7, and 8 that do not alter the conformation or immunogenic epitopes of the resulting recombinant RSV soluble F proteins. Thus, a variant in the context of recombinant RSV soluble F protein, typically shares at least 80%, or 85%, more commonly, at least 90% or more, such as 95%, or even 98% or 99% sequence identity with a reference protein, e.g., the reference sequences illustrated in the exemplary F1 chain, F2 chain, F0 molecule, and p27 peptide sequences of SEQ ID NOs:1, 2, 3, 6, 7, and 8 or any of the exemplary recombinant RSV soluble F protein disclosed herein. Additional variants included as a feature of this disclosure include the variants disclosed in WO2014160463.

In some embodiments, the antigen composition comprises a trimer of said soluble F protein. Thus, in some embodiments, the antigen composition comprises a trimer comprising three identical RSV soluble F protein. In some embodiments, the antigen composition comprises a trimer comprising at least one recombinant RSV soluble F protein that differs at the sequence level from another recombinant RSV soluble F protein member of the trimer. In some embodiments, the antigen composition comprises a trimer of two identical recombinant RSV soluble F protein and one recombinant RSV soluble F protein that differs at the sequence level from the other two recombinant RSV soluble F protein. In some embodiments, the antigen composition comprises three different recombinant RSV soluble F protein (in which each recombinant RSV soluble F protein differs at the amino acid sequence level).

Another aspect of this disclosure concerns recombinant nucleic acids that encode recombinant RSV soluble F proteins as described above. In certain embodiments, the recombinant nucleic acids are codon optimized for expression in a selected prokaryotic or eukaryotic host-cell. To facilitate replication and expression, the nucleic acids can be incorporated into a vector, such as a prokaryotic or a eukaryotic expression vector. Host-cells including recombinant RSV soluble F protein-encoding nucleic acids are also a feature of this disclosure. Favorable host-cells include prokaryotic (i.e., bacterial) host-cells, such as *E. coli*, as well as numerous eukaryotic host-cells, including fungal (e.g., yeast) cells, insect cells, and mammalian cells (such as CHO, VERO and HEK293cells).

Exemplary procedures sufficient to guide one of ordinary skill in the art through the production of recombinant RSV soluble F protein-encoding nucleic acids can be found in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 3d ed., Cold Spring Harbor Press, 2001; Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates, 1992 (and Supplements to 2003); and Ausubel et al., *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, 4th ed., Wiley & Sons, 1999.

Recombinant RSV soluble F proteins disclosed herein are produced using well established procedures for the expression and purification of recombinant proteins. Procedures sufficient to guide one of skill in the art can be found in the following references: Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 200; and Ausubel et al. *Short Protocols in Molecular Biology*, 4th ed., John Wiley & Sons, Inc., 999. Additional and specific details are provided

hereinbelow. The nucleic acids previously disclosed can suitably be introduced into host-cells and used to produce the recombinant RSV soluble F protein described herein.

Host-cells that include recombinant RSV soluble F protein-encoding nucleic acids are, thus, also a feature of this disclosure. Favorable host-cells include prokaryotic (i.e.,
5 bacterial) host-cells, such as *E. coli*, as well as numerous eukaryotic host-cells, including fungal (e.g., yeast, such as *Saccharomyces cerevisiae* and *Picchia pastoris*) cells, insect cells, plant cells, and mammalian cells (such as CHO and HEK293 cells). Recombinant RSV soluble F protein nucleic acids are introduced (e.g., transduced, transformed or transfected) into host-cells, for example, via a vector, such as an expression vector. As described above,
10 the vector is most typically a plasmid, but such vectors can also be, for example, a viral particle, a phage, etc. Examples of appropriate expression hosts include: bacterial cells, such as *E. coli*, *Streptomyces*, and *Salmonella typhimurium*; fungal cells, such as *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Neurospora crassa*; insect cells such as *Drosophila* and *Spodoptera frugiperda*; mammalian cells such as 3T3, COS, CHO, BHK,
15 HEK 293 or Bowes melanoma; plant cells, including algae cells, etc.

In some aspects, the RSV vaccines of the present disclosure include a RNA comprising an open reading frame (ORF) encoding a RSV antigen. Some aspects of the present disclosure provide respiratory syncytial virus (RSV) vaccines comprising a
ribonucleic acid (RNA) that comprises an open reading frame (ORF) encoding an RSV
20 antigen, wherein the ORF comprises (or consists of, or consists essentially of) a sequence that is at least 95% (e.g., at least 96%, at least 97%, at least 98%, at least 99%) or 95%-99% identical to a sequence identified by any one of SEQ ID NOS: 1-9.

In some aspects, the vaccine is formulated in a lipid nanoparticle. In some embodiments, the ORF comprises a sequence that is at least 98% identical to a sequence
25 identified by any one of SEQ ID NOS: 1-9. In some embodiments, the RNA comprises an ORF encoding an RSV antigen, wherein the ORF comprises a sequence identified by (is 100% identical to) any one of SEQ ID NOS: 1-9.

In some embodiments, the ORF comprises (or consists of, or consists essentially of) a sequence that is identified by (is 100% identical to) SEQ ID NO: 1. In some embodiments,
30 the ORF comprises a sequence that is identified by (is 100% identical to) SEQ ID NO: 1. In some embodiments, the ORF comprises (or consists of, or consists essentially of) a sequence that is identified by (is 100% identical to) SEQ ID NO: 3. In some embodiments, the ORF comprises (or consists of, or consists essentially of) a sequence that is identified by (is 100% identical to) SEQ ID NO: 52. In some embodiments, the ORF comprises a
35 sequence that is identified by (is 100% identical to) SEQ ID NO: 54. In some embodiments,

the ORF comprises a sequence that is identified by (is 100% identical to) SEQ ID NO: 56. In some embodiments, the ORF comprises a sequence that is identified by (is 100% identical to) SEQ ID NO: 58.

Other aspects of the present disclosure provide a RSV vaccine comprising (or consisting of, or consisting essentially of) a RNA that comprises an ORF encoding an RSV antigen, wherein the ORF encodes a sequence identified by any one of SEQ ID NOS: 5, 8, 11, 14, 17, 20, 22, 35, 38, 41, 44, 47, 50, 61, 65, 67, 70, 73, 76. In some aspects, said RSV vaccine is formulated in a lipid nanoparticle.

In some embodiments, the RNA comprises (or consists of, or consists essentially of) an ORF encoding a single chain recombinant RSV F peptide comprising a deletion of wild type RSV F amino acid positions 98-146 and a linker of eight to fourteen amino acids between wildtype RSV F amino acid positions 97 and 147, wherein the recombinant F peptide comprises the following amino acid modifications to stabilize the recombinant RSV F peptide when oligomerized to form a trimer in a prefusion conformation: (i) 190F and 207L amino acid substitutions, (ii) 155C and 290C amino acid substitutions, and (iii) one (or more) of (a) 486C and 490C amino acid substitutions; (b) 180C and 186C amino acid substitutions; (c) 486C and 489C amino acid substitutions; (d) 512C and 513C amino acid substitutions; and (e) an 505C amino acid substitution. The amino acid substitutions that introduce additional cysteine amino acid residues may result in either intra-peptide or inter-peptide disulfide bonds, specifically between the recited substitution pairs (e.g., a non-native intra peptide disulfide bond between 155C and 290C or between 180C and 186C; a non-native inter-peptide disulfide bond between 486C and 490C, between 486C and 489C or between 512C and 513C).

In some embodiments, intramuscular (IM) administration of a therapeutically effective amount of the immunogenic composition to a human induces in the human an immune response to RSV, e.g. a neutralizing antibody titer to (or against) RSV F.

In some embodiments, the neutralizing antibody titer is at least 5-fold to at least 100-fold (e.g., at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, or at least 50-fold) higher relative to control. In some embodiments the control is a RNA vaccine encoding a membrane-bound DS-CAV1 -stabilized prefusion F protein of RSV. In some embodiments, the control RNA vaccine encoding a membrane -bound DS-CAV1- stabilized prefusion F protein of RSV comprises (or consists of, or consists essentially of) a sequence identified by SEQ ID NO: 90 or SEQ ID NO: 92. In some embodiments, the control is a live attenuated RSV vaccine, an inactivated RSV vaccine, or a protein subunit RSV vaccine. In some embodiments, the at least 5-fold to at least 100-fold (e.g., at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, or at least 50-fold) higher neutralizing

antibody titer is induced in the subject following administration of a dose of the vaccine that is at least 5-fold (e.g., at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, at least 10-fold) lower relative to the control.

5 In some embodiments, IM administration of a therapeutically effective amount of the vaccine to a subject induces in the subject at least 10-fold, at least 15-fold, at least 20-fold, or at least 25-fold higher prefusion RSV F-specific neutralizing antibody titers relative to the control.

10 In some embodiments, IM administration of a therapeutically effective amount of the vaccine to a subject confers prophylactic protection at a 5-fold (e.g., at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, at least 10-fold) lower dose relative to the control.

In some embodiments, the RSV F neutralizing antibody titer is induced in the subject following fewer than three (e.g., one or two) doses of the vaccine.

Adjuvants

15 Immunogenic compositions for use in the methods disclosed herein may include, in addition to the recombinant RSV antigen, an adjuvant system. The term "adjuvant" refers to an agent that augments, stimulates, activates, potentiates, or modulates the immune response to an antigen of the composition at either the cellular or humoral level, e.g. immunologic adjuvants stimulate the response of the immune system to the antigen(s), but have no immunological effect by themselves. Examples of suitable adjuvants include but are not limited to inorganic adjuvants (e.g. inorganic metal salts such as aluminium phosphate or 20 aluminium hydroxide), organic non-peptide adjuvants (e.g. saponins, such as QS21, or squalene), oil-based adjuvants (e.g. Freund's complete adjuvant and Freund's incomplete adjuvant), cytokines (e.g. IL-1 β , IL-2, IL-7, IL-12, IL-18, GM-CSF, 5 and INF- γ) particulate adjuvants (e.g. immuno-stimulatory complexes (ISCOMS), liposomes, or biodegradable 25 microspheres), virosomes, bacterial adjuvants (e.g. monophosphoryl lipid A (MPL), such as 3-de-O-acylated monophosphoryl lipid A (3D-MPL), or muramyl peptides), synthetic adjuvants (e.g. non-ionic block copolymers, muramyl peptide analogues, or synthetic lipid A), synthetic polynucleotides adjuvants (e.g. polyarginine or polylysine) and immunostimulatory oligonucleotides containing unmethylated CpG dinucleotides ("CpG").

30 In particular, the adjuvant(s) may be organic non-peptide adjuvants (e.g. saponins, such as QS21, or squalene) and/or bacterial adjuvants (e.g. monophosphoryl lipid A (MPL), such as 3-de-O-acylated monophosphoryl lipid A (3DMPL). One suitable adjuvant is monophosphoryl lipid A (MPL), in particular 3-de-O-acylated monophosphoryl lipid A (3D-MPL). Chemically it is often supplied as a mixture of 3-de-O-acylated monophosphoryl lipid 35 A with either 4, 5, or 6 acylated chains. It can be purified and prepared by the methods

taught in GB 2122204B, which reference also discloses the preparation of diphosphoryl lipid A, and 3-O-deacylated variants thereof. Other purified and synthetic lipopolysaccharides have been described [U.S. Pat. No. 6,005,099 and EP0729473B1; Hilgers, 1986; Hilgers, 1987; and 20 EP0549074B1].

5 Saponins are also suitable adjuvants [Lacaille-Dubois, 1996]. For example, the saponin Quil A (derived from the bark of the South American tree *Quillaja saponaria* Molina), and fractions thereof, are described in U.S. Pat. No. 5,057,540 and Kensil, 1996; and EP 0 362 279 B1. Purified fractions of Quil A are also known as immunostimulants, such as QS21 and QS17; methods of their production are disclosed in U.S. Pat. No. 5,057,540 and EP 0 362 279 B1. Use of QS21 is further described in Kensil, 1991. Combinations of QS21 and
10 polysorbate or cyclodextrin are also known (WO 99/10008). Particulate adjuvant systems comprising fractions of QuilA, such as QS21 and QS7 are described in WO 96/33739 and WO 96/11711.

 Adjuvants such as those described above may be formulated together with carriers,
15 such as liposomes, oil in water emulsions, and/or metallic salts (including aluminum salts such as aluminum hydroxide). For example, 3D-MPL may be formulated with aluminum hydroxide (EP 0 689 454) or oil in water emulsions (WO 95/17210); QS21 may be formulated with cholesterol containing liposomes (WO 96/33739), oil in water emulsions (WO 95/17210) or alum (WO 98/15287).

20 Combinations of adjuvants may be utilized in the disclosed compositions, in particular a combination of a monophosphoryl lipid A and a saponin derivative (see, e.g., WO 94/00153; WO 95/17210; WO 96/33739; WO 98/56414; WO 99/12565; WO 99/11241), more particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a
25 composition where the QS21 is quenched in cholesterol-containing liposomes (DQ) as disclosed in WO 96/33739. A potent adjuvant formulation involving QS21, 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210 and is another formulation which may find use in the disclosed compositions. Thus, suitable adjuvant systems include, for example, a combination of monophosphoryl lipid A, preferably 3D-MPL,
30 together with an aluminium salt (e.g. as described in WO00/23105). A further exemplary adjuvant comprises QS21 and/or MPL and/or CpG. QS21 may be quenched in cholesterol-containing liposomes as disclosed in WO 96/33739.

 Accordingly, a suitable adjuvant for use in the disclosed compositions is AS01, a
35 liposome based adjuvant containing 3D MPL and QS-21. The liposomes, which are the vehicles for the MPL and QS-21 immuno-enhancers, are composed of dioleoyl

phosphatidylcholine (DOPC) and cholesterol in a phosphate buffered saline solution. A particularly suitable adjuvant for use in the disclosed immunogenic compositions is AS01E, a particularly preferred variant of the AS01 adjuvant. The AS01E adjuvant comprises dioleoyl phosphatidylcholine (DOPC), cholesterol and 3D MPL [in an amount of 500µg DOPC, 125 µg cholesterol and 25µg 3D-MPL, each value given approximately per vaccine dose], QS21 [25µg/dose], phosphate NaCl buffer and water to a volume of 0.5ml.

Immunogenic composition - PCV

10 In an aspect, one of the immunogenic compositions of the present invention will typically comprise conjugated capsular saccharide antigens (also named glycoconjugates), wherein the saccharides are derived from serotypes of *S. pneumoniae* (*Streptococcus pneumoniae*).

The immunogenic composition comprises glycoconjugates from different *S.*
15 *pneumoniae* serotypes. In one embodiment there are 15 or more different serotypes. In an embodiment, there are 20 or more different serotypes. In an embodiment there are 15 to 20 different serotypes. In an embodiment there are 15 or 20 different serotypes. In an embodiment there are 15 to 25 different serotypes. In an embodiment there are 15 or 25 different serotypes.

20 In one embodiment there are 15 different serotypes. In one embodiment there are 16 different serotypes. In an embodiment there are 17 different serotypes. In an embodiment there are 18 different serotypes. In an embodiment there are 19 different serotypes. In an embodiment there are 20 different serotypes. In an embodiment there are 21 different serotypes. In an embodiment there are 22 different serotypes. In an embodiment there are
25 23 different serotypes. In an embodiment there are 24 different serotypes. In an embodiment there are 25 different serotypes. The capsular saccharides are conjugated to a carrier protein to form glycoconjugates as described here below.

In an embodiment, the serotypes can be selected from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F. In an
30 embodiment, the serotypes can be selected from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F.

If the protein carrier is the same for 2 or more capsular saccharides in the composition, the capsular saccharides could be conjugated to the same molecule of the

protein carrier (carrier molecules having 2 or more different capsular saccharides conjugated to it) [see for instance WO 2004/083251].

In a preferred embodiment though, the capsular saccharides are each individually conjugated to different molecules of the protein carrier (each molecule of protein carrier only having one type of capsular saccharide conjugated to it). In said embodiment, the capsular saccharides are said to be individually conjugated to the carrier protein.

In one embodiment a capsular saccharide is linked directly to a carrier protein. In a second embodiment a bacterial saccharide is linked to a protein through a spacer/linker.

Carrier protein

A component of the glycoconjugate of the invention is a carrier protein to which the capsular saccharide is conjugated. The terms "protein carrier" or "carrier protein" or "carrier" may be used interchangeably herein. Carrier proteins should be amenable to standard conjugation procedures.

In an embodiment, the carrier protein of the glycoconjugates is selected in the group consisting of: DT (Diphtheria toxin), TT (tetanus toxoid) or fragment C of TT, CRM₁₉₇ (a nontoxic but antigenically identical variant of diphtheria toxin), the A chain of diphtheria toxin mutant CRM₁₉₇ (CN103495161), other DT mutants (such as CRM176, CRM228, CRM45 (Uchida et al. (1973) J. Biol. Chem. 218:3838-3844), CRM9, CRM102, CRM103 or CRM107; and other mutations described by Nicholls and Youle in Genetically Engineered Toxins, Ed: Frankel, Maecel Dekker Inc. (1992); deletion or mutation of Glu-148 to Asp, Gln or Ser and/or Ala 158 to Gly and other mutations disclosed in U.S. Patent Nos. 4,709,017 and 4,950,740 ; mutation of at least one or more residues Lys 516, Lys 526, Phe 530 and/or Lys 534 and other mutations disclosed in U.S. Patent Nos. 5,917,017 and 6,455,673 ; or fragment disclosed in U.S. Patent No. 5,843,711 , pneumococcal pneumolysin (ply) (Kuo et al. (1995) Infect Immun 63:2706-2713) including ply detoxified in some fashion, for example dPLY-GMBS (WO 2004/081515 and WO 2006/032499) or dPLY-formol, PhtX, including PhtA, PhtB, PhtD, PhtE (sequences of PhtA, PhtB, PhtD or PhtE are disclosed in WO 00/37105 and WO 00/39299) and fusions of Pht proteins for example PhtDE fusions, PhtBE fusions, Pht A-E (WO 01/98334 , WO 03/054007 , WO 2009/000826), OMPC (meningococcal outer membrane protein - usually extracted from *Neisseria meningitidis* serogroup B (EP0372501), PorB (from *N. meningitidis*), PD (*Haemophilus influenzae* protein D; see, e.g., EP0594610 B), or immunologically functional equivalents thereof, synthetic peptides

(EP0378881 , EP0427347), heat shock proteins (WO 93/17712 , WO 94/03208), pertussis proteins (WO 98/58668 , EP0471177), cytokines, lymphokines, growth factors or hormones (WO 91/01146), artificial proteins comprising multiple human CD4+ T cell epitopes from various pathogen derived antigens (Falugi et al. (2001) Eur J Immunol 31:3816-3824) such as N19 protein (Baraldoi et al. (2004) Infect Immun 72:4884-4887) pneumococcal surface protein PspA (WO 02/091998), iron uptake proteins (WO 01/72337), toxin A or B of *Clostridium difficile* (WO 00/61761), transferrin binding proteins, pneumococcal adhesion protein (PsaA), recombinant *Pseudomonas aeruginosa* exotoxin A (in particular non-toxic mutants thereof (such as exotoxin A bearing a substitution at glutamic acid 553 (Douglas et al. (1987) J. Bacteriol. 169(11):4967-4971)). Other proteins, such as ovalbumin, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or purified protein derivative of tuberculin (PPD) also can be used as carrier proteins. Other suitable carrier proteins include inactivated bacterial toxins such as cholera toxoid (e.g., as described in WO 2004/083251), *Escherichia coli* LT, *E. coli* ST, and exotoxin A from *P. aeruginosa*.

15 In an embodiment, the carrier protein of the glycoconjugates is independently selected from the group consisting of TT, DT, DT mutants (such as CRM₁₉₇), *H. influenzae* protein D, PhtX, PhtD, PhtDE fusions (particularly those described in WO 01/98334 and WO 03/054007), detoxified pneumolysin, PorB, N19 protein, PspA, OMPC, toxin A or B of *C. difficile* and PsaA. In an embodiment, the carrier protein of each serotype of the glycoconjugates is independently selected from the group consisting of TT, DT, DT mutants (such as CRM₁₉₇ or derivatives thereof), and *H. influenzae* protein D. In an embodiment, the carrier protein of each serotype of the glycoconjugates is independently selected from the group consisting of TT, DT and CRM₁₉₇. In an embodiment, the carrier protein of each serotype of the glycoconjugates is independently selected from the group consisting of TT, DT and CRM₁₉₇. In an embodiment, the carrier protein of each serotype of the glycoconjugates is independently selected from the group consisting of TT, DT and CRM₁₉₇. In an embodiment, the carrier protein of each serotype of the glycoconjugates is CRM₁₉₇ or derivatives thereof. In an embodiment, the carrier protein of each serotype of the glycoconjugates is CRM₁₉₇.

30 In an embodiment, the carrier protein of the glycoconjugates of the invention is DT (Diphtheria toxoid). In another embodiment, the carrier protein of the glycoconjugates of the invention is TT (tetanus toxoid).

In another embodiment, the carrier protein of the glycoconjugates of the invention is PD (*H. influenzae* protein D; see, e.g., EP0594610 B).

In another embodiment, the carrier protein of the glycoconjugates is CRM₁₉₇. The CRM₁₉₇ protein is a nontoxic form of diphtheria toxin but is immunologically indistinguishable from the diphtheria toxin. CRM₁₉₇ is produced by *Corynebacterium diphtheriae* infected by the nontoxigenic phage β 197^{tox} created by nitrosoguanidine mutagenesis of the toxigenic corynephage beta (Uchida et al. (1971) Nature New Biology 233:8-11). The CRM₁₉₇ protein has the same molecular weight as the diphtheria toxin but differs therefrom by a single base change (guanine to adenine) in the structural gene. This single base change causes an amino acid substitution (glutamic acid for glycine) in the mature protein and eliminates the toxic properties of diphtheria toxin. The CRM₁₉₇ protein is a safe and effective T-cell dependent carrier for saccharides. Further details about CRM₁₉₇ and production thereof can be found, e.g., in U.S. Patent No. 5,614,382 . In an embodiment, the capsular saccharides of the invention are conjugated to CRM₁₉₇ protein or the A chain of CRM₁₉₇ (see CN103495161). In an embodiment, the capsular saccharides of the invention are conjugated the A chain of CRM₁₉₇ obtained via expression by genetically recombinant *E. coli* (see CN103495161). In an embodiment, the capsular saccharides of the invention are all conjugated to CRM₁₉₇. In an embodiment, the capsular saccharides of the invention are all conjugated to the A chain of CRM₁₉₇.

Accordingly, in frequent embodiments, the glycoconjugates of the invention comprise CRM₁₉₇ as the carrier protein, wherein the capsular polysaccharide is covalently linked to CRM₁₉₇.

Capsular saccharide

The term "saccharide" throughout this specification may indicate polysaccharide or oligosaccharide and includes both. In frequent embodiments, the saccharide is a polysaccharide, in particular a *S. pneumoniae* capsular polysaccharide.

Capsular polysaccharides may be prepared by standard techniques known to those of ordinary skill in the art.

In an embodiment, capsular polysaccharides may be prepared, e.g., from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F of *S. pneumoniae*. In an embodiment, capsular polysaccharides may be prepared, e.g., from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F.

Typically capsular polysaccharides are produced by growing each *S. pneumoniae* serotype in a medium (e.g., in a soy-based medium), the polysaccharides are

then prepared from the bacteria culture. Bacterial strains of *S. pneumoniae* used to make the respective polysaccharides that are used in the glycoconjugates of the invention may be obtained from established culture collections or clinical specimens.

5 The population of the organism (each *S. pneumoniae* serotype) is often scaled up from a seed vial to seed bottles and passaged through one or more seed fermentors of increasing volume until production scale fermentation volumes are reached. At the end of the growth cycle the cells are lysed and the lysate broth is then harvested for downstream (purification) processing (see for example WO 2006/110381, WO 2008/118752, and U.S. Patent App. Pub. Nos. 2006/0228380, 2006/0228381, 2008/0102498 and 2008/0286838).

10 The individual polysaccharides are typically purified through centrifugation, precipitation, ultra-filtration, and/or column chromatography (see for example WO 2006/110352 and WO 2008/118752).

Purified polysaccharides may be activated (e.g., chemically activated) to make them capable of reacting (e.g., either directly to the carrier protein or via a linker such as an eTEC
15 spacer) and then incorporated into glycoconjugates of the invention, as further described herein.

In an embodiment, the capsular saccharide may be shorter than native length saccharide chain of repeating oligosaccharide units.

In an embodiment, the capsular saccharides may be oligosaccharides.
20 Oligosaccharides have a low number of repeat units (typically 5-15 repeat units) and are typically derived synthetically or by hydrolysis of polysaccharides.

Preferably, the capsular saccharides used in the immunogenic compositions herein are polysaccharides. High molecular weight capsular polysaccharides are able to induce certain antibody immune responses due to the epitopes present on the antigenic surface.
25 The isolation and purification of high molecular weight capsular polysaccharides is preferably contemplated for use in the conjugates, compositions and methods of the present invention.

In some embodiments, the purified capsular polysaccharides before conjugation have a molecular weight of between 5 kDa and 4,000 kDa. In other such embodiments, the capsular polysaccharide has a molecular weight of between 10 kDa and 4,000 kDa; between
30 50 kDa and 4,000 kDa; between 50 kDa and 3,000 kDa; between 50 kDa and 2,000 kDa; between 50 kDa and 1,500 kDa; between 50 kDa and 1,000 kDa; between 50 kDa and 750

- kDa; between 50 kDa and 500 kDa; between 100 kDa and 4,000 kDa; between 100 kDa and 3,000 kDa; 100 kDa and 2,000 kDa; between 100 kDa and 1,500 kDa; between 100 kDa and 1,000 kDa; between 100 kDa and 750 kDa; between 100 kDa and 500 kDa; between 100 and 400 kDa; between 200 kDa and 4,000 kDa; between 200 kDa and 3,000 kDa;
- 5 between 200 kDa and 2,000 kDa; between 200 kDa and 1,500 kDa; between 200 kDa and 1,000 kDa; or between 200 kDa and 500 kDa.

In further embodiments, the capsular polysaccharide has a molecular weight of between 70 kDa to 150 kDa; 80 kDa to 160 kDa; 90 kDa to 250 kDa; 100 kDa to 1,000; 100 kDa to 500 kDa; 100 kDa to 400 kDa; 100 kDa to 160 kDa; 150 kDa to 600 kDa; 200 kDa to

10 1,000 kDa; 200 kDa to 600 kDa; 200 kDa to 400 kDa; 300 kDa to 1,000 kDa; 300 kDa to 600 kDa; 300 kDa to 500 kDa or 500 kDa to 600 kDa. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

A capsular polysaccharide can become slightly reduced in size during normal purification procedures. Additionally, as described herein, capsular polysaccharide can be

15 subjected to sizing techniques before conjugation. Mechanical or chemical sizing maybe employed. Chemical hydrolysis maybe conducted using acetic acid. Mechanical sizing maybe conducted using High Pressure Homogenization Shearing. The molecular weight ranges mentioned above refer to purified capsular polysaccharides before conjugation (e.g., before activation).

In an embodiment, the capsular polysaccharide from each of the serotypes of *S. pneumoniae*, has a molecular weight falling within one of the molecular weight ranges as described here above. In an embodiment, the capsular polysaccharide from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F of *S. pneumoniae*, has a molecular weight falling within one of the molecular weight ranges as

25 described here above. In an embodiment, the capsular polysaccharide from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of *S. pneumoniae*, has a molecular weight falling within one of the molecular weight ranges as described here above. In a preferred embodiment, the capsular polysaccharide from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F or 33F of *S. pneumoniae* has a

30 molecular weight falling within one of the molecular weight ranges as described here above. In a preferred embodiment, the capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of *S. pneumoniae*, has a molecular weight falling within one of the molecular weight ranges as described here above.

As used herein, the term "molecular weight" of polysaccharide or of carrier protein-polysaccharide conjugate refers to molecular weight calculated by size exclusion chromatography (SEC) combined with multiangle laser light scattering detector (MALLS).

In an embodiment, some of the capsular saccharides in the immunogenic composition are O-acetylated. In some embodiments, the capsular saccharides from serotypes 9V, 18C, 11A, 15B, 22F and/or 33F of the invention are O-acetylated. In some embodiments, the capsular saccharides from serotypes 9V, 11A, 15B, 22F and/or 33F of the disclosure are O-acetylated.

In some embodiments, the glycoconjugate from *S. pneumoniae* serotypes 1, 7F, 9V and/or 18C of the invention are O-acetylated. In some embodiments, the glycoconjugate from *S. pneumoniae* serotypes 1, 7F and 9V is O-acetylated and the glycoconjugate from *S. pneumoniae* serotype 18C is de-O-acetylated.

The degree of O-acetylation of the polysaccharide can be determined by any method known in the art, for example, by proton NMR (see for example Lemercinier et al. (1996) Carbohydrate Research 296:83-96, Jones et al. (2002) J. Pharmaceutical and Biomedical Analysis 30:1233-1247, WO 2005/033148 and WO 00/56357). Another commonly used method is described in Hestrin (1949) J. Biol. Chem. 180:249-261. Preferably, the presence of O-acetyl groups is determined by ion-HPLC analysis.

In some embodiments, the glycoconjugate from *S. pneumoniae* serotype 1 comprise a saccharide which has a degree of O-acetylation of between 10 and 100%, between 20 and 100%, between 30 and 100%, between 40 and 100%, between 50 and 100%, between 60 and 100%, between 70 and 100%, between 75 and 100%, 80 and 100%, 90 and 100%, 50 and 90%, 60 and 90%, 70 and 90% or 80 and 90%. In other embodiments, the degree of O-acetylation is $\geq 10\%$, $\geq 20\%$, $\geq 30\%$, $\geq 40\%$, $\geq 50\%$, $\geq 60\%$, $\geq 70\%$, $\geq 80\%$, $\geq 90\%$, or about 100%.

In some embodiments, the glycoconjugate from *S. pneumoniae* serotype 7F comprise a saccharide which has a degree of O-acetylation of between 10 and 100%, between 20 and 100%, between 30 and 100%, between 40 and 100%, between 50 and 100%, between 60 and 100%, between 70 and 100%, between 75 and 100%, 80 and 100%, 90 and 100%, 50 and 90%, 60 and 90%, 70 and 90% or 80 and 90%. In other embodiments, the degree of O-acetylation is $\geq 10\%$, $\geq 20\%$, $\geq 30\%$, $\geq 40\%$, $\geq 50\%$, $\geq 60\%$, $\geq 70\%$, $\geq 80\%$, $\geq 90\%$, or about 100%.

In some embodiments, the glycoconjugate from *S. pneumoniae* serotype 9V comprise a saccharide which has a degree of O-acetylation of between 10 and 100%, between 20 and 100%, between 30 and 100%, between 40 and 100%, between 50 and 100%, between 60 and 100%, between 70 and 100%, between 75 and 100%, 80 and 100%, 90 and 100%, 50 and 90%, 60 and 90%, 70 and 90% or 80 and 90%. In other embodiments, the degree of O-acetylation is $\geq 10\%$, $\geq 20\%$, $\geq 30\%$, $\geq 40\%$, $\geq 50\%$, $\geq 60\%$, $\geq 70\%$, $\geq 80\%$, $\geq 90\%$, or about 100%.

In some embodiments, the glycoconjugate from *S. pneumoniae* serotype 18C comprise a saccharide which has a degree of O-acetylation of between 10 and 100%, between 20 and 100%, between 30 and 100%, between 40 and 100%, between 50 and 100%, between 60 and 100%, between 70 and 100%, between 75 and 100%, 80 and 100%, 90 and 100%, 50 and 90%, 60 and 90%, 70 and 90% or 80 and 90%. In other embodiments, the degree of O-acetylation is $\geq 10\%$, $\geq 20\%$, $\geq 30\%$, $\geq 40\%$, $\geq 50\%$, $\geq 60\%$, $\geq 70\%$, $\geq 80\%$, $\geq 90\%$, or about 100%. Preferably though, the glycoconjugate from *S. pneumoniae* serotype 18C is de-O-acetylated. In some said embodiments, the glycoconjugate from *S. pneumoniae* serotype 18C comprise a saccharide which has a degree of O-acetylation of between 0 and 50%, between 0 and 40%, between 0 and 30%, between 0 and 20%, between 0 and 10%, between 0 and 5%, or between 0 and 2%. In other embodiments, the degree of O-acetylation is $\leq 50\%$, $\leq 40\%$, $\leq 30\%$, $\leq 20\%$, $\leq 10\%$, $\leq 5\%$, $\leq 2\%$, or $\leq 1\%$.

By % of O-acetylation it is meant the percentage of a given capsular saccharide relative to 100% (where each repeat unit is fully acetylated relative to its acetylated structure).

The purified polysaccharides described herein are chemically activated to make the saccharides capable of reacting with the carrier protein. These pneumococcal conjugates are prepared by separate processes and formulated into a single dosage formulation as described below.

Glycoconjugates

The purified saccharides are chemically activated to make the saccharides (i.e., activated saccharides) capable of reacting with the carrier protein, either directly or via a linker. Once activated, each capsular saccharide is separately conjugated to a carrier protein to form a glycoconjugate. In one embodiment, each capsular saccharide is conjugated to the same carrier protein. The chemical activation of the saccharides and subsequent

conjugation to the carrier protein can be achieved by the activation and conjugation methods disclosed herein.

Capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F of *S. pneumoniae* may be prepared as disclosed above. Capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of *S. pneumoniae* may also be prepared as disclosed above.

In an embodiment, the polysaccharides are activated with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide is then coupled directly or via a spacer (linker) group to an amino group on the carrier protein (preferably CRM₁₉₇). For example, the spacer could be cystamine or cysteamine to give a thiolated polysaccharide which could be coupled to the carrier via a thioether linkage obtained after reaction with a maleimide-activated carrier protein (for example using N-[γ-maleimidobutyroxy]succinimide ester (GMBS)) or a haloacetylated carrier protein (for example using iodoacetimide, N-succinimidyl bromoacetate (SBA; SIB), N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (sulfo-SIAB), N-succinimidyl iodoacetate (SIA), or succinimidyl 3-[bromoacetamido]propionate (SBAP)). Preferably, the cyanate ester (optionally made by CDAP chemistry) is coupled with hexane diamine or adipic acid dihydrazide (ADH) and the amino-derivatised saccharide is conjugated to the carrier protein (e.g., CRM₁₉₇) using carbodiimide (e.g., EDAC or EDC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described for example in WO 93/15760, WO 95/08348 and WO 96/129094.

In an embodiment of the present invention, the glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F are prepared using CDAP chemistry. In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 14, 18C, 19F, and 23F are prepared using CDAP chemistry. In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 4, 5, 6B, 7F, 8, 9V, 14, 18C, 19A, 19F, and 23F are prepared using CDAP chemistry. In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 14, 18C, 19A, 19F, and 23F are prepared using CDAP chemistry. In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 14, 18C, 19A, 19F, and 23F are prepared using CDAP chemistry.

In an embodiment, at least one glycoconjugate selected from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F is prepared using CDAP chemistry. In an embodiment, at least one glycoconjugate selected from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F is prepared using CDAP chemistry. In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F are all prepared using CDAP chemistry. In an embodiment, glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F are all prepared using CDAP chemistry.

Other suitable techniques for conjugation use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S-NHS, EDC, TSTU. Many are described in International Patent Application Publication No. WO 98/42721. Conjugation may involve a carbonyl linker which may be formed by reaction of a free hydroxyl group of the saccharide with CDI (see Bethell et al. (1979) 1. Biol. Chern. 254:2572-2574; Hearn et al. (1981) J. Chromatogr. 218:509-518) followed by reaction with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group, reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

In a preferred embodiment, at least one of capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F of *S. pneumoniae* is conjugated to the carrier protein by reductive amination (such as described in U.S. Patent Appl. Pub. Nos. 2006/0228380, 2007/184072, 2007/0231340, 2007/0184071; WO 2006/110381; WO2008079653; and WO 2008143709). In a preferred embodiment, at least one of capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of *S. pneumoniae* is conjugated to the carrier protein by reductive amination.

In an embodiment of the present invention, the glycoconjugate from *S. pneumoniae* serotype 6A is prepared by reductive amination. In an embodiment, the glycoconjugate from *S. pneumoniae* serotype 19A is prepared by reductive amination. In an embodiment, the glycoconjugate from *S. pneumoniae* serotype 3 is prepared by reductive amination. In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 6A and 19A are prepared by reductive amination. In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 3, 6A and 19A are prepared by reductive amination.

In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F and 23F are prepared by reductive amination. In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 4, 6B, 9V, 14, 18C, 19F and 23F are prepared by reductive amination. In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 4, 5, 6B, 9V, 14, 18C, 19F and 23F are prepared by reductive amination. In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F are prepared by reductive amination. In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F and 23F are prepared by reductive amination. In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F are prepared by reductive amination. In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F are all prepared by reductive amination.

In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F and 23F are all prepared by reductive amination.

In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 15B, 18C, 19A, 19F, 22F and 23F are all prepared by reductive amination.

In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F and 23F are all prepared by reductive amination.

In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 2, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F are all prepared by reductive amination.

In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 4, 5, 6A, 6B, 7F, 9V, 12F, 14, 15C, 18C, 19A, 19F, 22F, 23F and 33F are all prepared by reductive amination.

In an embodiment, the glycoconjugates from *S. pneumoniae* serotypes 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 15C, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F are all prepared by reductive amination.

In an embodiment, at least one glycoconjugate selected from the *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F is prepared by reductive amination. In a preferred embodiment, the glycoconjugates from *S. pneumoniae*

serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F are all prepared by reductive amination.

In an embodiment, at least one glyconjugate selected from the *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F is prepared by reductive amination. In a preferred embodiment, the glyconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F are all prepared by reductive amination.

Reductive amination involves two steps, (1) oxidation of the polysaccharide, (2) reduction of the activated polysaccharide and a carrier protein to form a conjugate. Before oxidation, the polysaccharide is optionally hydrolyzed. Mechanical or chemical hydrolysis maybe employed. Chemical hydrolysis maybe conducted using acetic acid. The oxidation step may involve reaction with periodate. For the purpose of the present invention, the term "periodate" includes both periodate and periodic acid; the term also includes both metaperiodate (IO_4^-) and orthoperiodate (IO_5^{5-}) and includes the various salts of periodate (e.g., sodium periodate and potassium periodate). In an embodiment the capsular polysaccharide is oxidized in the presence of metaperiodate, preferably in the presence of sodium periodate (NaIO_4). In another embodiment the capsular polysaccharide is oxidized in the presence of orthoperiodate, preferably in the presence of periodic acid.

In an embodiment, the oxidizing agent is a stable nitroxyl or nitroxide radical compound, such as piperidine-N-oxy or pyrrolidine-N-oxy compounds, in the presence of an oxidant to selectively oxidize primary hydroxyls (as described in WO 2014/097099). In said reaction, the actual oxidant is the N-oxoammonium salt, in a catalytic cycle. In an aspect, said stable nitroxyl or nitroxide radical compound are piperidine-N-oxy or pyrrolidine-N-oxy compounds. In an aspect, said stable nitroxyl or nitroxide radical compound bears a TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy) or a PROXYL (2,2,5,5-tetramethyl-1-pyrrolidinyloxy) moiety. In an aspect, said stable nitroxyl radical compound is TEMPO or a derivative thereof. In an aspect, said oxidant is a molecule bearing a N-halo moiety. In an aspect, said oxidant is selected from the group consisting of N-Chlorosuccinimide, N-Bromosuccinimide, N-Iodosuccinimide, Dichloroisocyanuric acid, 1,3,5-trichloro-1,3,5-triazine-2,4,6-trione, Dibromoisocyanuric acid, 1,3,5-tribromo-1,3,5-triazine-2,4,6-trione, Diiodoisocyanuric acid and 1,3,5-triiodo-1,3,5-triazine-2,4,6-trione. Preferably said oxidant is N-Chlorosuccinimide.

In an embodiment, capsular polysaccharides from serotypes 12F *S. pneumoniae* are conjugated to the carrier protein by reductive amination, wherein the oxidizing agent is

2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO) free radical and N-Chlorosuccinimide (NCS) as the cooxidant (as described in WO 2014/097099). Therefore in one aspect, the glycoconjugates from *S. pneumoniae* serotype 12F are obtainable by a method comprising the steps of: a) reacting a 12F saccharide with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and N-chlorosuccinimide (NCS) in an aqueous solvent to produce an activated saccharide; and b) reacting the activated saccharide with a carrier protein comprising one or more amine groups (said method is designated "TEMPO/NCS-reductive amination" thereafter).

Optionally the oxidation reaction is quenched by addition of a quenching agent. The quenching agent maybe selected from vicinal diols, 1,2-aminoalcohols, amino acids, glutathione, sulfite, bisulfate, dithionite, metabisulfite, thiosulfate, phosphites, hypophosphites or phosphorous acid (such as glycerol, ethylene glycol, propan-1,2-diol, butan-1,2-diol or butan-2,3-diol, ascorbic acid).

Following the oxidation step of the polysaccharide, the polysaccharide is said to be activated and is referred to an "activated polysaccharide" here below. The activated polysaccharide and the carrier protein may be lyophilised (freeze-dried), either independently (discrete lyophilization) or together (co-lyophilized). In one embodiment the activated polysaccharide and the carrier protein are co-lyophilized. In another embodiment the activated polysaccharide and the carrier protein are lyophilized independently.

In one embodiment the lyophilization takes place in the presence of a non-reducing sugar, possible non-reducing sugars include sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinin.

The second step of the conjugation process is the reduction of the activated polysaccharide and a carrier protein to form a conjugate (so-called reductive amination), using a reducing agent. Reducing agents which are suitable include the cyanoborohydrides (such as sodium cyanoborohydride, sodium triacetoxyborohydride or sodium or zinc borohydride in the presence of Bronsted or Lewis acids), amine boranes such as pyridine borane, 2-Picoline Borane, 2,6-diborane-methanol, dimethylamine-borane, t-BuMeⁱPrN-BH₃, benzylamine-BH₃ or 5-ethyl-2-methylpyridine borane (PEMB) or borohydride exchange resin. In one embodiment the reducing agent is sodium cyanoborohydride.

In an embodiment, the reduction reaction is carried out in aqueous solvent (e.g., selected from PBS, MES, HEPES, Bis-tris, ADA, PIPES, MOPSO, BES, MOPS, DIPSO, MOBS, HEPPSO, POPSO, TEA, EPPS, Bicine or HEPB, at a pH between 6.0 and 8.5, 7.0 and 8.0, or 7.0 and 7.5), in another embodiment the reaction is carried out in aprotic solvent.

In an embodiment, the reduction reaction is carried out in DMSO (dimethylsulfoxide) or in DMF (dimethylformamide) solvent. The DMSO or DMF solvent may be used to reconstitute the activated polysaccharide and carrier protein which has been lyophilized.

At the end of the reduction reaction, there may be unreacted aldehyde groups remaining in the conjugates, these may be capped using a suitable capping agent. In one embodiment this capping agent is sodium borohydride (NaBH_4). Following the conjugation (the reduction reaction and optionally the capping), the glycoconjugates may be purified (enriched with respect to the amount of polysaccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis, concentration/diafiltration operations, tangential flow filtration precipitation/elution, column chromatography (DEAE or hydrophobic interaction chromatography), and depth filtration. In an embodiment, the glycoconjugates are purified by diafiltration or ion exchange chromatography or size exclusion chromatography.

In one embodiment the glycoconjugates are sterile filtered.

In an embodiment, the glycoconjugates of the invention are prepared using the eTEC conjugation, such as described in WO 2014/027302. Said glycoconjugates comprise a saccharide covalently conjugated to a carrier protein through one or more eTEC spacers, wherein the saccharide is covalently conjugated to the eTEC spacer through a carbamate linkage, and wherein the carrier protein is covalently conjugated to the eTEC spacer through an amide linkage. The eTEC linked glycoconjugates of the invention may be represented by the general formula (I): where the atoms that comprise the eTEC spacer are contained in the central box.

The eTEC spacer includes seven linear atoms (i.e., $-\text{C}(\text{O})\text{NH}(\text{CH}_2)_2\text{SCH}_2\text{C}(\text{O})-$) and provides stable thioether and amide bonds between the saccharide and carrier protein.

Synthesis of the eTEC linked glycoconjugate involves reaction of an activated hydroxyl group of the saccharide with the amino group of a thioalkylamine reagent, e.g., cystamine or cysteinamine or a salt thereof, forming a carbamate linkage to the saccharide to provide a thiolated saccharide. Generation of one or more free sulfhydryl groups is accomplished by reaction with a reducing agent to provide an activated thiolated saccharide. Reaction of the free sulfhydryl groups of the activated thiolated saccharide with an activated carrier protein having one or more α -haloacetamide groups on amine containing residues generates a thioether bond to form the conjugate, wherein the carrier protein is attached to the eTEC spacer through an amide bond.

In said glycoconjugates, the saccharide may be a polysaccharide or an oligosaccharide. The carrier protein may be selected from any suitable carrier as described herein or known to those of skill in the art. In an embodiment, the saccharide is a polysaccharide. In some such embodiments, the carrier protein is CRM₁₉₇. In some such
5 embodiments, the eTEC linked glycoconjugate comprises a pneumococcal serotype capsular polysaccharide, which is covalently conjugated to CRM₁₉₇ through an eTEC spacer.

In some embodiments, the glycoconjugates of the present invention comprise a capsular saccharide having a molecular weight of between 10 kDa and 2,000 kDa. In other such embodiments, the capsular saccharide has a molecular weight of between 50 kDa and
10 1,000 kDa. In other such embodiments, the capsular saccharide has a molecular weight of between 70 kDa and 900 kDa. In other such embodiments, the capsular saccharide has a molecular weight of between 100 kDa and 800 kDa. In other such embodiments, the capsular saccharide has a molecular weight of between 200 kDa and 600 kDa. In further such embodiments, the capsular saccharide has a molecular weight of 100 kDa to 1000
15 kDa; 100 kDa to 900 kDa; 100 kDa to 800 kDa; 100 kDa to 700 kDa; 100 kDa to 600 kDa; 100 kDa to 500 kDa; 100 kDa to 400 kDa; 100 kDa to 300 kDa; 150 kDa to 1,000 kDa; 150 kDa to 900 kDa; 150 kDa to 800 kDa; 150 kDa to 700 kDa; 150 kDa to 600 kDa; 150 kDa to 500 kDa; 150 kDa to 400 kDa; 150 kDa to 300 kDa; 200 kDa to 1,000 kDa; 200 kDa to 900
20 kDa; 200 kDa to 800 kDa; 200 kDa to 700 kDa; 200 kDa to 600 kDa; 200 kDa to 500 kDa; 200 kDa to 400 kDa; 200 kDa to 300; 250 kDa to 1,000 kDa; 250 kDa to 900 kDa; 250 kDa to 800 kDa; 250 kDa to 700 kDa; 250 kDa to 600 kDa; 250 kDa to 500 kDa; 250 kDa to 400 kDa; 250 kDa to 350 kDa; 300 kDa to 1,000 kDa; 300 kDa to 900 kDa; 300 kDa to 800 kDa; 300 kDa to 700 kDa; 300 kDa to 600 kDa; 300 kDa to 500 kDa; 300 kDa to 400 kDa; 400
25 kDa to 1,000 kDa; 400 kDa to 900 kDa; 400 kDa to 800 kDa; 400 kDa to 700 kDa; 400 kDa to 600 kDa; 500 kDa to 600 kDa. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure. In some such embodiments, the glycoconjugate is prepared using reductive amination.

In some embodiments, the glycoconjugate of the invention has a molecular weight of between 400 kDa and 15,000 kDa; between 500 kDa and 10,000 kDa; between 2,000 kDa
30 and 10,000 kDa; between 3,000 kDa and 8,000 kDa; or between 3,000 kDa and 5,000 kDa. In other embodiments, the glycoconjugate has a molecular weight of between 500 kDa and 10,000 kDa. In other embodiments, glycoconjugate has a molecular weight of between 1,000 kDa and 8,000 kDa. In still other embodiments, the glycoconjugate has a molecular weight of between 2,000 kDa and 8,000 kDa or between 3,000 kDa and 7,000 kDa. In
35 further embodiments, the glycoconjugate of the invention has a molecular weight of between

200 kDa and 20,000 kDa; between 200 kDa and 15,000 kDa; between 200 kDa and 10,000 kDa; between 200 kDa and 7,500 kDa; between 200 kDa and 5,000 kDa; between 200 kDa and 3,000 kDa; between 200 kDa and 1,000 kDa; between 500 kDa and 20,000 kDa; between 500 kDa and 15,000 kDa; between 500 kDa and 12,500 kDa; between 500 kDa and 10,000 kDa; between 500 kDa and 7,500 kDa; between 500 kDa and 6,000 kDa; between 500 kDa and 5,000 kDa; between 500 kDa and 4,000 kDa; between 500 kDa and 3,000 kDa; between 500 kDa and 2,000 kDa; between 500 kDa and 1,500 kDa; between 500 kDa and 1,000 kDa; between 750 kDa and 20,000 kDa; between 750 kDa and 15,000 kDa; between 750 kDa and 12,500 kDa; between 750 kDa and 10,000 kDa; between 750 kDa and 7,500 kDa; between 750 kDa and 6,000 kDa; between 750 kDa and 5,000 kDa; between 750 kDa and 4,000 kDa; between 750 kDa and 3,000 kDa; between 750 kDa and 2,000 kDa; between 750 kDa and 1,500 kDa; between 1,000 kDa and 15,000 kDa; between 1,000 kDa and 12,500 kDa; between 1,000 kDa and 10,000 kDa; between 1,000 kDa and 7,500 kDa; between 1,000 kDa and 6,000 kDa; between 1,000 kDa and 5,000 kDa; between 1,000 kDa and 4,000 kDa; between 1,000 kDa and 2,500 kDa; between 2,000 kDa and 15,000 kDa; between 2,000 kDa and 12,500 kDa; between 2,000 kDa and 10,000 kDa; between 2,000 kDa and 7,500 kDa; between 2,000 kDa and 6,000 kDa; between 2,000 kDa and 5,000 kDa; between 2,000 kDa and 4,000 kDa; or between 2,000 kDa and 3,000 kDa.

In further embodiments, the glycoconjugate of the invention has a molecular weight of between 3,000 kDa and 20,000 kDa; between 3,000 kDa and 15,000 kDa; between 3,000 kDa and 10,000 kDa; between 3,000 kDa and 7,500 kDa; between 3,000 kDa and 5,000 kDa; between 4,000 kDa and 20,000 kDa; between 4,000 kDa and 15,000 kDa; between 4,000 kDa and 12,500 kDa; between 4,000 kDa and 10,000 kDa; between 4,000 kDa and 7,500 kDa; between 4,000 kDa and 6,000 kDa; or between 4,000 kDa and 5,000 kDa.

In further embodiments, the glycoconjugate of the invention has a molecular weight of between 5,000 kDa and 20,000 kDa; between 5,000 kDa and 15,000 kDa; between 5,000 kDa and 10,000 kDa; between 5,000 kDa and 7,500 kDa; between 6,000 kDa and 20,000 kDa; between 6,000 kDa and 15,000 kDa; between 6,000 kDa and 12,500 kDa; between 6,000 kDa and 10,000 kDa or between 6,000 kDa and 7,500 kDa.

The molecular weight of the glycoconjugate is measured by SEC-MALLS. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

In an embodiment, the serotype 22F glycoconjugate of the invention comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 or 0.7 or about 0.8 mM acetate per mM serotype 22F

polysaccharide. In an embodiment, the glycoconjugate comprises at least 0.5, 0.6 or 0.7 mM acetate per mM serotype 22F polysaccharide. In an embodiment, the glycoconjugate comprises at least 0.6 mM acetate per mM serotype 22F polysaccharide. In an embodiment, the glycoconjugate comprises at least 0.7 mM acetate per mM serotype 22F polysaccharide.

5 In an embodiment, the serotype 33F glycoconjugate of the invention comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 mM acetate per mM serotype 33F capsular polysaccharide. In an embodiment, the glycoconjugate comprises at least 0.5, 0.6 or 0.7 mM acetate per mM serotype 33F capsular polysaccharide. In an embodiment, the glycoconjugate comprises at least 0.6 mM acetate per mM serotype 33F capsular polysaccharide. In an embodiment, the glycoconjugate comprises at least 0.7 mM acetate per mM serotype 33F capsular polysaccharide. In an embodiment, the presence of O-acetyl groups is determined by ion-HPLC analysis.

15 In an embodiment, the serotype 15B glycoconjugate of the invention comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 mM acetate per mM serotype 15B capsular polysaccharide. In an embodiment, the glycoconjugate comprises at least 0.5, 0.6 or 0.7 mM acetate per mM serotype 15B capsular polysaccharide. In an embodiment, the glycoconjugate comprises at least 0.6 mM acetate per mM serotype 15B capsular polysaccharide. In an embodiment, the glycoconjugate comprises at least 0.7 mM acetate per mM serotype 15B capsular polysaccharide. In an embodiment, the presence of O-acetyl groups is determined by ion-HPLC analysis.

25 In an embodiment, the serotype 15B glycoconjugate of the invention comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 mM glycerol per mM serotype 15B capsular polysaccharide. In an embodiment, the serotype 15B glycoconjugate of the invention comprises at least 0.5, 0.6 or 0.7 mM glycerol per mM serotype 15B capsular polysaccharide. In an embodiment, the serotype 15B glycoconjugate of the invention comprises at least 0.6 mM glycerol per mM serotype 15B capsular polysaccharide. In an embodiment, the serotype 15B glycoconjugate of the invention comprises at least 0.7 mM glycerol per mM serotype 15B capsular polysaccharide.

30 In an embodiment, the serotype 11A glycoconjugate of the invention comprises at least 0.3, 0.5, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6, 3.0, 3.4, 3.8, 4.2, 4.6 or about 5.0 mM acetate per mM serotype 11A polysaccharide. In an embodiment, the serotype 11A glycoconjugate comprises at least 1.8, 2.2 or 2.6 mM acetate per mM serotype 11A polysaccharide. In an embodiment, the glycoconjugate comprises at least 0.6 mM acetate per mM serotype 11A polysaccharide. In an embodiment, the serotype 11A glycoconjugate of the invention

comprises at least 0.6, 1.0, 1.4, 1.8, 2.2, 2.6, 3.0, 3.4, 3.8, 4.2 or about 4.6 mM acetate per mM serotype 11A polysaccharide and less than about 5.0 mM acetate per mM serotype 11A polysaccharide. In an embodiment, the serotype 11A glycoconjugate of the invention comprises at least 0.6, 1.0, 1.4, 1.8, 2.2, 2.6, or about 3.0 mM acetate per mM serotype 11A polysaccharide and less than about 3.4 mM acetate per mM serotype 11A polysaccharide. In an embodiment, the serotype 11A glycoconjugate of the invention comprises at least 0.6, 1.0, 1.4, 1.8, 2.2, 2.6, or about 3.0 mM acetate per mM serotype 11A polysaccharide and less than about 3.3 mM acetate per mM serotype 11A polysaccharide. Any of the above number is contemplated as an embodiment of the disclosure.

10 In an embodiment, the serotype 11A glycoconjugate of the invention comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or about 1.0 mM glycerol per mM serotype 11A polysaccharide. In an embodiment, the serotype 11A glycoconjugate comprises at least 0.2, 0.3 or 0.4 mM glycerol per mM serotype 11A polysaccharide. In an embodiment, the serotype 11A glycoconjugate of the invention comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 15 0.7, 0.8 or about 0.9 mM glycerol per mM serotype 11A polysaccharide and less than about 1.0 mM glycerol per mM serotype 11A polysaccharide. In an embodiment, the serotype 11A glycoconjugate of the invention comprises at least 0.3, 0.4, 0.5, 0.6, or about 0.7 mM glycerol per mM serotype 11A polysaccharide and less than about 0.8 mM glycerol per mM serotype 11A polysaccharide. Any of the above number is contemplated as an embodiment of the disclosure.

Another way to characterize the glycoconjugates is by the number of lysine residues in the carrier protein (e.g., CRM₁₉₇) that become conjugated to the saccharide which can be characterized as a range of conjugated lysines (degree of conjugation). The evidence for lysine modification of the carrier protein, due to covalent linkages to the polysaccharides, 25 can be obtained by amino acid analysis using routine methods known to those of skill in the art. Conjugation results in a reduction in the number of lysine residues recovered, compared to the carrier protein starting material used to generate the conjugate materials. In an embodiment, the degree of conjugation of the glycoconjugate of the invention is between 2 and 15, between 2 and 13, between 2 and 10, between 2 and 8, between 2 and 6, between 2 and 5, between 2 and 4, between 3 and 15, between 3 and 13, between 3 and 10, 30 between 3 and 8, between 3 and 6, between 3 and 5, between 3 and 4, between 5 and 15, between 5 and 10, between 8 and 15, between 8 and 12, between 10 and 15 or between 10 and 12. In an embodiment, the degree of conjugation of the glycoconjugate of the invention is about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, 35 about 12, about 13, about 14 or about 15. In an embodiment, the degree of conjugation of

the glycoconjugate of the invention is between 4 and 7. In some such embodiments, the carrier protein is CRM₁₉₇.

The glycoconjugates of the invention may also be characterized by the ratio (weight/weight) of saccharide to carrier protein. In some embodiments, the ratio of polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.5 and 3 (e.g., about 0.5, about 0.6, about 0.7, about 0.8, about 0.9, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.1, about 2.2, about 2.3, about 2.4, about 2.5, about 2.6, about 2.7, about 2.8, about 2.9, or about 3.0). In other embodiments, the saccharide to carrier protein ratio (w/w) is between 0.5 and 2.0, between 0.5 and 1.5, between 0.8 and 1.2, between 0.5 and 1.0, between 1.0 and 1.5 or between 1.0 and 2.0. In further embodiments, the saccharide to carrier protein ratio (w/w) is between 0.8 and 1.2. In a preferred embodiment, the ratio of capsular polysaccharide to carrier protein in the conjugate is between 0.9 and 1.1. In some such embodiments, the carrier protein is CRM₁₉₇.

The glycoconjugates and immunogenic compositions used herein may contain free saccharide that is not covalently conjugated to the carrier protein, but is nevertheless present in the glycoconjugate composition. The free saccharide may be non-covalently associated with (i.e., non-covalently bound to, adsorbed to, or entrapped in or with) the glycoconjugate.

In a preferred embodiment, the glycoconjugate comprises less than about 50%, 45%, 40%, 35%, 30%, 25%, 20% or 15% of free polysaccharide compared to the total amount of polysaccharide. In a preferred embodiment the glycoconjugate comprises less than about 25% of free polysaccharide compared to the total amount of polysaccharide. In a preferred embodiment the glycoconjugate comprises less than about 20% of free polysaccharide compared to the total amount of polysaccharide. In a preferred embodiment the glycoconjugate comprises less than about 15% of free polysaccharide compared to the total amount of polysaccharide.

The glycoconjugates may also be characterized by their molecular size distribution (K_d). Size exclusion chromatography media (CL-4B) can be used to determine the relative molecular size distribution of the conjugate. Size Exclusion Chromatography (SEC) is used in gravity fed columns to profile the molecular size distribution of conjugates. Large molecules excluded from the pores in the media elute more quickly than small molecules. Fraction collectors are used to collect the column eluate. The fractions are tested colorimetrically by saccharide assay. For the determination of K_d , columns are calibrated to

establish the fraction at which molecules are fully excluded (V_0), ($K_d=0$), and the fraction representing the maximum retention (V_i), ($K_d=1$). The fraction at which a specified sample attribute is reached (V_e), is related to K_d by the expression, $K_d = (V_e - V_0) / (V_i - V_0)$.

In an embodiment, at least 30% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In an embodiment, at least 40% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In an embodiment, at least 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, or 85% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In an embodiment, at least 60% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In an embodiment, between 50% and 80% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In an embodiment, between 65% and 80% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. The frequency of attachment of the saccharide chain to a lysine on the carrier protein is another parameter for characterizing the glycoconjugates of the invention. For example, in some embodiments, at least one covalent linkage between the carrier protein and the polysaccharide occurs for every 4 saccharide repeat units of the polysaccharide. In another embodiment, the covalent linkage between the carrier protein and the polysaccharide occurs at least once in every 10 saccharide repeat units of the polysaccharide. In another embodiment, the covalent linkage between the carrier protein and the polysaccharide occurs at least once in every 15 saccharide repeat units of the polysaccharide. In a further embodiment, the covalent linkage between the carrier protein and the polysaccharide occurs at least once in every 25 saccharide repeat units of the polysaccharide.

In frequent embodiments, the carrier protein is CRM₁₉₇ and the covalent linkage via an eTEC spacer between the CRM₁₉₇ and the polysaccharide occurs at least once in every 4, 10, 15 or 25 saccharide repeat units of the polysaccharide.

In other embodiments, the conjugate comprises at least one covalent linkage between the carrier protein and saccharide for every 5 to 10 saccharide repeat units; every 2 to 7 saccharide repeat units; every 3 to 8 saccharide repeat units; every 4 to 9 saccharide repeat units; every 6 to 11 saccharide repeat units; every 7 to 12 saccharide repeat units; every 8 to 13 saccharide repeat units; every 9 to 14 saccharide repeat units; every 10 to 15 saccharide repeat units; every 2 to 6 saccharide repeat units, every 3 to 7 saccharide repeat units; every 4 to 8 saccharide repeat units; every 6 to 10 saccharide repeat units; every 7 to 11 saccharide repeat units; every 8 to 12 saccharide repeat units; every 9 to 13 saccharide repeat units; every 10 to 14 saccharide repeat units; every 10 to 20 saccharide repeat units;

every 4 to 25 saccharide repeat units or every 2 to 25 saccharide repeat units. In frequent embodiments, the carrier protein is CRM₁₉₇.

5 In another embodiment, at least one linkage between carrier protein and saccharide occurs for every 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 saccharide repeat units of the polysaccharide. In an embodiment, the carrier protein is CRM₁₉₇. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

The immunogenic composition of the invention comprises at least one glycoconjugate of each of the thirteen following *S. pneumoniae* serotypes: 9V, 1, 3, 4, 5, 6A,
10 6B, 7F, 14, 18C, 19A, 19F, and 23F.

In an embodiment, any of the immunogenic composition comprising at least one glycoconjugate above comprises in addition at least one glycoconjugate of *S. pneumoniae* serotype 15B.

15 In an embodiment, any of the immunogenic composition comprising at least one glycoconjugate above comprises in addition at least one glycoconjugate of *S. pneumoniae* serotype 22F.

In an embodiment, any of the immunogenic composition comprising at least one glycoconjugate above comprises in addition at least one glycoconjugate of *S. pneumoniae* serotype 33F.

20 In an embodiment, any of the immunogenic composition comprising at least one glycoconjugate above comprises in addition at least one glycoconjugate of *S. pneumoniae* serotype 8.

In an embodiment, any of the immunogenic composition comprising at least one glycoconjugate above comprises in addition at least one glycoconjugate of *S. pneumoniae* serotype 10A.
25

In an embodiment, any of the immunogenic composition comprising at least one glycoconjugate above comprises in addition at least one glycoconjugate of *S. pneumoniae* serotype 11A.

In an embodiment, any of the immunogenic composition comprising at least one glycoconjugate above comprises in addition at least one glycoconjugate of *S. pneumoniae* serotype 12F.

5 In an embodiment, any of the immunogenic composition comprising at least one glycoconjugate above comprises in addition at least one glycoconjugate of each of the two following *S. pneumoniae* serotypes:

- 10
 - 15B and 22F,
 - 15B and 33F,
 - 15B and 12F,
 - 15B and 10A,
 - 15B and 11A,
 - 15B and 8,
 - 22F and 33F,
 - 22F and 12F,
 - 15
 - 22F and 10A,
 - 22F and 11A,
 - 22F and 8,
 - 33F and 12F,
 - 33F and 10A,
 - 20
 - 33F and 11A,
 - 33F and 8,
 - 12F and 10A,
 - 12F and 11A,
 - 12F and 8,
 - 25
 - 10A and 11A,
 - 10A and 8, or
 - 11A and 8.

In an embodiment, any of the immunogenic composition comprising at least one glycoconjugate above comprises in addition at least one glycoconjugate of each of the three following *S. pneumoniae* serotypes:

- 35
 - 15B and 22F and 33F,
 - 15B and 22F and 12F,
 - 15B and 22F and 10A,
 - 15B and 22F and 11A,
 - 15B and 22F and 8,
 - 15B and 33F and 12F,
 - 15B and 33F and 10A,
 - 15B and 33F and 11A,
 - 15B and 33F and 8,
 - 40
 - 15B and 12F and 10A,
 - 15B and 12F and 11A,
 - 15B and 12F and 8,
 - 15B and 10A and 11A,
 - 15B and 10A and 8,
 - 45
 - 15B and 11A and 8,
 - 22F and 33F and 12F,

- 5
 - 22F and 33F and 10A,
 - 22F and 33F and 11A,
 - 22F and 33F and 8,
 - 22F and 12F and 10A,
 - 22F and 12F and 11A,
 - 22F and 12F and 8,
 - 22F and 10A and 11A,
 - 22F and 10A and 8,
 - 22F and 11A and 8,
 - 10
 - 33F and 12F and 10A,
 - 33F and 12F and 11A,
 - 33F and 12F and 8,
 - 33F and 10A and 11A,
 - 33F and 10A and 8,
 - 15
 - 33F and 11A and 8,
 - 12F and 10A and 11A,
 - 12F and 10A and 8,
 - 12F and 11A and 8, or
 - 10A and 11A and 8.

20 In an embodiment, any of the immunogenic composition comprising at least one glycoconjugate above comprises in addition at least one glycoconjugate of each of the four following *S. pneumoniae* serotypes:

- 25
 - 15B and 22F and 33F and 12F,
 - 15B and 22F and 33F and 10A,
 - 15B and 22F and 33F and 11A,
 - 15B and 22F and 33F and 8,
 - 15B and 22F and 12F and 10A,
 - 15B and 22F and 12F and 11A,
 - 15B and 22F and 12F and 8,
 - 30
 - 15B and 22F and 10A and 11A,
 - 15B and 22F and 10A and 8,
 - 15B and 22F and 11A and 8,
 - 15B and 33F and 12F and 10A,
 - 15B and 33F and 12F and 11A,
 - 35
 - 15B and 33F and 12F and 8,
 - 15B and 33F and 10A and 11A,
 - 15B and 33F and 10A and 8,
 - 15B and 33F and 11A and 8,
 - 15B and 12F and 10A and 11A,
 - 40
 - 15B and 12F and 10A and 8,
 - 15B and 12F and 11A and 8,
 - 15B and 10A and 11A and 8,
 - 22F and 33F and 12F and 10A,
 - 22F and 33F and 12F and 11A,
 - 45
 - 22F and 33F and 12F and 8,
 - 22F and 33F and 10A and 11A,
 - 22F and 33F and 10A and 8,
 - 22F and 33F and 11A and 8,
 - 22F and 12F and 10A and 11A,
 - 50
 - 22F and 12F and 10A and 8,
 - 22F and 12F and 11A and 8,

- 22F and 10A and 11A and 8,
- 33F and 12F and 10A and 11A,
- 33F and 12F and 10A and 8,
- 33F and 12F and 11A and 8,
- 33F and 10A and 11A and 8 or
- 12F and 10A and 11A and 8.

In an embodiment, any of the immunogenic composition comprising at least one glycoconjugate above comprises in addition at least one glycoconjugate of each of the five following *S. pneumoniae* serotypes:

- 15B and 22F and 33F and 12F and 10A,
- 15B and 22F and 33F and 12F and 11A,
- 15B and 22F and 33F and 12F and 8,
- 15B and 22F and 33F and 10A and 11A,
- 15B and 22F and 33F and 10A and 8,
- 15B and 22F and 33F and 11A and 8,
- 15B and 22F and 12F and 10A and 11A,
- 15B and 22F and 12F and 10A and 8,
- 15B and 22F and 12F and 11A and 8,
- 15B and 22F and 10A and 11A and 8,
- 15B and 33F and 12F and 10A and 11A,
- 15B and 33F and 12F and 10A and 8,
- 15B and 33F and 12F and 11A and 8,
- 15B and 33F and 10A and 11A and 8,
- 15B and 12F and 10A and 11A and 8,
- 22F and 33F and 12F and 10A and 11A,
- 22F and 33F and 12F and 10A and 8,
- 22F and 33F and 12F and 11A and 8,
- 22F and 33F and 10A and 11A and 8,
- 22F and 12F and 10A and 11A and 8 or
- 33F and 12F and 10A and 11A and 8.

In an embodiment, any of the immunogenic composition comprising at least one glycoconjugate above comprises in addition at least one glycoconjugate of each of the six following *S. pneumoniae* serotypes:

- 15B and 22F and 33F and 12F and 10A and 11A,
- 15B and 22F and 33F and 12F and 10A and 8,
- 15B and 22F and 33F and 12F and 11A and 8,
- 15B and 22F and 33F and 10A and 11A and 8,
- 15B and 22F and 12F and 10A and 11A and 8,
- 15B and 33F and 12F and 10A and 11A and 8 or
- 22F and 33F and 12F and 10A and 11A and 8.

In an embodiment, any of the immunogenic composition comprising at least one glycoconjugate above comprises in addition at least one glycoconjugate of each of the seven following *S. pneumoniae* serotypes: 15B and 22F and 33F and 12F and 10A and 11A and 8.

In an embodiment, any of the immunogenic composition above comprises in addition glycoconjugates from *S. pneumoniae* serotype 2.

In an embodiment, any of the immunogenic composition above comprises in addition glycoconjugates from *S. pneumoniae* serotype 17F.

5 In an embodiment, any of the immunogenic composition above comprises in addition glycoconjugates from *S. pneumoniae* serotype 20.

In an embodiment, any of the immunogenic composition above comprises in addition glycoconjugates from *S. pneumoniae* serotype 15C.

10 In an embodiment, the immunogenic composition may further comprise at least one glycoconjugate selected from the group consisting of *S. pneumoniae* serotype 6C, 7C, 9N, 15A, 15B, 15C, 16F, 17F, 20, 23A, 23B, 31, 34, 35B, 35F, and 38.

Preferably, all the glycoconjugates of the above immunogenic composition are individually conjugated to the carrier protein.

In an embodiment of any of the above immunogenic composition, the
15 glycoconjugates from *S. pneumoniae* serotype 9V is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic compositions, the glycoconjugates from *S. pneumoniae* serotype 22F is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic composition, the glycoconjugates from *S. pneumoniae* serotype 33F is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic composition, the
20 glycoconjugates from *S. pneumoniae* serotype 15B is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic composition, the glycoconjugates from *S. pneumoniae* serotype 12F is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic composition, the glycoconjugates from *S. pneumoniae* serotype 10A is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic composition, the
25 glycoconjugates from *S. pneumoniae* serotype 11A is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic composition, the glycoconjugates from *S. pneumoniae* serotype 8 is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic composition, the glycoconjugates from *S. pneumoniae* serotypes 4, 6B, 14, 18C, 19F and 23F are conjugated to CRM₁₉₇. In an embodiment of any of the above
30 immunogenic composition, the glycoconjugates from *S. pneumoniae* serotypes 1, 5 and 7F are conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic composition, the glycoconjugates from *S. pneumoniae* serotypes 6A and 19A are conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic composition, the glycoconjugates from *S. pneumoniae* serotype 3 is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic compositions, the glycoconjugates from *S. pneumoniae* serotype 2 is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic compositions, the glycoconjugates from *S. pneumoniae* serotype 17F is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic compositions, the glycoconjugates from *S. pneumoniae* serotype 20 is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic compositions, the glycoconjugates from *S. pneumoniae* serotype 15C is conjugated to CRM₁₉₇.

10 In an embodiment, the glycoconjugates of the above immunogenic compositions are all individually conjugated to CRM₁₉₇.

In one embodiment the above immunogenic composition comprises glycoconjugates from 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 different serotypes.

15 In one embodiment the above immunogenic composition comprises glycoconjugates from 16 or 20 different serotypes.

In an embodiment the above immunogenic composition is a 14, 15, 16, 17, 18, 19 or 20 valent pneumococcal conjugate composition. In an embodiment the above immunogenic composition is a 15-valent pneumococcal conjugate composition. In an embodiment the above immunogenic composition is a 20-valent pneumococcal conjugate composition.

20 In an embodiment, the immunogenic composition comprises glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F.

In an embodiment, the immunogenic composition comprises conjugated *S. pneumoniae* saccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F.

25 In an embodiment, the glycoconjugates of the immunogenic composition consists of glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F. In an embodiment, the glycoconjugates of the immunogenic composition of the invention consists of glycoconjugates from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F.

Preferably, all the glycoconjugates of the immunogenic composition of the invention are individually conjugated to the carrier protein. In an embodiment, the glycoconjugates of the immunogenic composition above are individually conjugated to CRM₁₉₇.

In an embodiment, any of the immunogenic compositions above do not comprise
5 capsular saccharide from *S. pneumoniae* serotypes 9N, 9A and 9L.

After conjugation of the capsular polysaccharide to the carrier protein, the glycoconjugates are purified (enriched with respect to the amount of polysaccharide-protein conjugate) by a variety of techniques. These techniques include concentration/diafiltration operations, precipitation/elution, column chromatography, and depth filtration. See, e.g., U.S.
10 Appl. Publication No. 2007/0184072 and WO 2008/079653 . After the individual glycoconjugates are purified, they are compounded to formulate the immunogenic composition used herein.

Adjuvants

In an embodiment, the immunogenic compositions comprise aluminum salts (alum)
15 as adjuvant (e.g., aluminum phosphate, aluminum sulfate or aluminum hydroxide). In a preferred embodiment, the immunogenic compositions comprise aluminum phosphate or aluminum hydroxide as adjuvant. In a preferred embodiment, the immunogenic compositions comprise aluminum phosphate as adjuvant

In an embodiment of the present invention, the immunogenic compositions comprise
20 a CpG Oligonucleotide as adjuvant. A CpG oligonucleotide as used herein refers to an immunostimulatory CpG oligodeoxynucleotide (CpG ODN), and accordingly these terms are used interchangeably unless otherwise indicated. Immunostimulatory CpG oligodeoxynucleotides contain one or more immunostimulatory CpG motifs that are unmethylated cytosine-guanine dinucleotides, optionally within certain preferred base
25 contexts. The methylation status of the CpG immunostimulatory motif generally refers to the cytosine residue in the dinucleotide. An immunostimulatory oligonucleotide containing at least one unmethylated CpG dinucleotide is an oligonucleotide which contains a 5' unmethylated cytosine linked by a phosphate bond to a 3' guanine, and which activates the immune system through binding to Toll-like receptor 9 (TLR-9). In another embodiment the
30 immunostimulatory oligonucleotide may contain one or more methylated CpG dinucleotides, which will activate the immune system through TLR9 but not as strongly as if the CpG motif(s) was/were unmethylated. CpG immunostimulatory oligonucleotides may comprise one or more palindromes that in turn may encompass the CpG dinucleotide. CpG

oligonucleotides have been described in a number of issued patents, published patent applications, and other publications, including U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; and 6,339,068. 5. Uses of the immunogenic compositions of the invention

5 **METHODS OF USE**

Disclosed herein are methods of inducing an immune response to respiratory syncytial virus (RSV) in a human, and use of an immunogenic combination or immunogenic composition(s) for inducing an immune response to respiratory syncytial virus (RSV) in a human.

10 An immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F may also be used in each of the aspects herein. For example, in an aspect, the method of inducing an immune response to respiratory syncytial virus (RSV) in a human comprises co-administration of (a) an immunogenic composition comprising a recombinant RSV antigen
15 comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein; and (b) an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F.

In an aspect, the method of inducing an immune response to respiratory syncytial
20 virus (RSV) in a human comprises co-administration of (a) an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein; and (b) an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F,
25 22F, 23F and 33F.

In an aspect, there is provided an immunogenic combination for use in a method of method of preventing respiratory syncytial virus (RSV) infection in a human comprising (a) an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the
30 F protein, and an adjuvant; and (b) an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, wherein the method comprises co-administering the compositions to the human.

In an aspect, there is provided the use of an immunogenic combination for preventing respiratory syncytial virus (RSV) infection in a human comprising (a) administering an immunogenic composition comprising an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one
5 modification that stabilizes the prefusion conformation of the F protein, and an adjuvant; and (b) administering an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, wherein a) and b) are co-administered to the human.

Disclosed herein are methods of inducing an immune response to *S. pneumoniae* in
10 a human, and use of an immunogenic combination or immunogenic composition(s) for inducing an immune response to *S. pneumoniae* in a human.

In an aspect, the method of inducing an immune response to *S. pneumoniae* in a human comprises co-administration of (a) an immunogenic composition comprising a
15 recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein; and (b) an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F.

In an aspect, there is provided an immunogenic combination for use in a method of
20 method of preventing an *S. pneumoniae* infection in a human comprising (a) an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein, and an adjuvant; and (b) an immunogenic composition comprising at least one
25 glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, wherein the method comprises co-administering the compositions to the human.

In an aspect, there is provided use of an immunogenic combination for preventing an
30 *S. pneumoniae* infection in a human comprising (a) administering an immunogenic composition comprising an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein, and an adjuvant; and (b) administering an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F,
35 22F, 23F and 33F, wherein a) and b) are co-administered to the human.

In an aspect, there is provided an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein, for use in raising an immune response to RSV in a human, wherein an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F is co-administered.

In an aspect, there is provided an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, for use in raising an immune response to *S. pneumoniae* in a human, wherein an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein is co-administered.

In an aspect, there is provided an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein, and an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, for use in raising an immune response to RSV and *S. pneumoniae* in a human comprising co-administering to the human said immunogenic compositions.

In an aspect, there is provided use of an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F in raising an immune response to *S. pneumoniae* in a human, wherein an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein is co-administered.

In an aspect, there is provided use of an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein in raising an immune response to RSV in a human, wherein an immunogenic composition at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F is co-administered. In an aspect, there is provided use of an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein, and an immunogenic composition comprising at

least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, for use in raising an immune response to RSV and *S. pneumoniae* in a human comprising co-administering to the human said immunogenic compositions.

- 5 The present invention also provides the use of an immunogenic composition in the manufacture of a medicament for inducing an immune response in a subject (e.g. human).

 In an aspect, there is provided the use of an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein polypeptide comprising at least one modification that stabilizes the prefusion conformation of the F protein, in the manufacture of
10 a medicament for raising an immune response to RSV in a human, wherein an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F is co-administered.

 In an aspect, there is provided the use of an immunogenic composition comprising at
15 least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, in the manufacture of a medicament for raising an immune response to *S. pneumoniae* in a human, wherein an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein polypeptide comprising at least one modification that stabilizes the prefusion
20 conformation of the F protein is co-administered.

 In an aspect, there is provided the use of an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein polypeptide comprising at least one modification that stabilizes the prefusion conformation of the F protein, and an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes
25 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, in the manufacture of a medicament for raising an immune response to RSV and *S. pneumoniae* in a human comprising co-administering to the human said immunogenic compositions.

 In an aspect, there is provided the use of an immunogenic composition comprising at
30 least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F in the manufacture of a medicament for raising an immune response to *S. pneumoniae* in a human, in co-administration with an immunogenic composition comprising a recombinant RSV antigen

comprising a soluble F protein polypeptide comprising at least one modification that stabilizes the prefusion conformation of the F protein.

In an aspect, there is provided the use of an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein polypeptide comprising at least one modification that stabilizes the prefusion conformation of the F protein, in the manufacture of a medicament for raising an immune response to RSV in a human in co-administration with an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F.

In an embodiment, the immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F is an immunogenic composition in which all of the glycoconjugates are individually conjugated to CRM₁₉₇ and the glycoconjugates are adsorbed onto aluminium phosphate. In an embodiment, the immunogenic composition comprises approximately 2.0 µg of saccharides from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F, approximately 4.0 µg of saccharides from serotype 6B, in which all of the glycoconjugates are individually conjugated to CRM197, L-histidine, polysorbate 20, sodium chloride, and aluminum phosphate adjuvant. In an embodiment, the immunogenic composition is a 0.5 mL dose comprising (or consisting) of approximately 2.0 µg of saccharides from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F, approximately 4.0 µg of saccharides from serotype 6B, 30 µg of CRM197 carrier protein, 1.55 mg L-histidine, 1 mg of polysorbate 20, 4.50 mg sodium chloride, and 125 µg of aluminum as aluminum phosphate adjuvant. In particular, such immunogenic composition comprises 2.0 µg of saccharides from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F, and 4.0 µg of saccharides from serotype 6B.

In a preferred embodiment, the immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F is an immunogenic composition in which all of the glycoconjugates are individually conjugated to CRM₁₉₇ and the glycoconjugates are adsorbed onto aluminium phosphate. In an embodiment, the immunogenic composition comprises approximately 2.2 µg of saccharides from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, approximately 4.4 µg of saccharides from serotype 6B, in which all of the glycoconjugates are individually conjugated to CRM197, polysorbate 80, succinate buffer,

sodium chloride, and aluminum phosphate adjuvant. In an embodiment, the immunogenic composition is a 0.5 mL dose comprising (or consisting) of approximately 2.2 µg of saccharides from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, approximately 4.4 µg of saccharides from serotype 6B, 51 µg CRM197 carrier protein, 100 µg polysorbate 80, 295 µg succinate buffer, 4.4 mg sodium chloride, and 125 µg aluminium as aluminium phosphate adjuvant. In particular, such immunogenic composition comprises 2.2 µg of saccharides from each of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, and 4.4 µg of saccharides from serotype 6B.

10 In an embodiment, the method, the immunogenic combination, the use, the immunogenic compositions of the invention demonstrates non-inferiority of the immunogenic composition comprising glycoconjugates of *S. pneumoniae* serotypes (i.e. PCV vaccine, e.g. PCV20) when co-administered with the RSV vaccine (e.g. RSVPreF3 OA investigational vaccine) compared to the immunogenic composition comprising glycoconjugates of *S. pneumoniae* serotypes (e.g. PCV20) administered alone. The opsonophagocytic (OP) antibody (Ab) titers for each of the pneumococcal vaccine serotype (ST) expressed as between groups geometric mean titer (GMT) ratio, 1 month after the PCV20 dose. The true Group GMT ratio between the Control group (PCV20 vaccine) divided by Co-ad group (RSV vaccine when co-administered with the immunogenic composition comprising glycoconjugates of *S. pneumoniae* serotypes (i.e. PCV vaccine, e.g. PCV20)) in OP Ab titer for each PCV serotypes one month after the PCV vaccine is above 2. The Opsonophagocytic antibodies are determined by multiplexed Opsonophagocytosis Assay (MOPA).

25 In an embodiment, the method, the immunogenic combination, the use, the immunogenic compositions of the invention demonstrates non-inferiority of RSV Vaccine (e.g. RSVPreF3 OA investigational vaccine) in terms of RSV-A neutralization antibodies when co-administered with the immunogenic composition comprising glycoconjugates of *S. pneumoniae* serotypes (i.e. PCV vaccine, e.g. PCV20) compared to the RSV vaccine (e.g. RSVPreF3 OA investigational vaccine) administered alone. The true GMT ratio between Control group (the RSV vaccine e.g. the RSVPreF3 OA investigational vaccine) divided by Co-ad group (the RSV vaccine (e.g. RSVPreF3 OA investigational vaccine) when co-administered with the immunogenic composition comprising glycoconjugates of *S. pneumoniae* serotypes (i.e. PCV vaccine, e.g. PCV20) vaccine) in RSV-A neutralizing antibody (Ab) titers one month after the RSVPreF3 OA investigational vaccine dose is above 1.5.

In an embodiment, the method, the immunogenic combination, the use, the immunogenic compositions of the invention demonstrates non-inferiority of the RSV vaccine (e.g. RSVPreF3 OA investigational vaccine) in terms of RSV-B neutralization antibodies when co-administered with the immunogenic composition comprising glycoconjugates of *S. pneumoniae* serotypes (i.e. PCV vaccine, e.g. PCV20) compared to the RSV vaccine (e.g. RSVPreF3 OA investigational vaccine) administered alone. The true GMT ratio between Control group (the RSV vaccine e.g. RSVPreF3 OA investigational vaccine) divided by Co-ad group (the RSV vaccine e.g. RSVPreF3 OA investigational vaccine when co-administered with the immunogenic composition comprising glycoconjugates of *S. pneumoniae* serotypes (i.e. PCV vaccine, e.g. PCV20) vaccine) in RSV-B neutralizing Ab titers one month after the RSV vaccine (e.g. RSVPreF3 OA investigational vaccine) dose is above 1.5.

EXAMPLE

Vaccination against RSV and against pneumococcal disease can be beneficial in older adults (i.e., ≥ 60 years old) since they are at high risk of complications. However, there is a risk that co-administration of vaccines could reduce the efficacy of one vaccine or the other – e.g., co-administration of an RSV vaccine and a pneumococcal conjugate vaccine could result in the pneumococcal conjugate vaccine interfering with the immune response against the RSV vaccine, or vice versa. Accordingly, non-inferiority of each vaccine when co-administered together should be explored. A Phase 3 open label, randomized, controlled, multi-country study will be conducted to assess the immunogenicity, safety, and reactogenicity of the RSVPreF3 OA investigational vaccine when co-administered with 20-valent pneumococcal conjugate vaccine (referred to as *PREVNAR 20* in the US or *Apexxnar* in Europe, also referred to as PCV20) in adults ≥ 60 years old.

Approximately 1090 eligible participants will be randomly (1:1) assigned to 2 study groups using a centralized randomization system at Visit 1 (Day 1). The randomization algorithm will use a minimization procedure accounting for age (60 to 69, 70 to 79 or ≥ 80 years) and center. Overall, participants will be enrolled in 3 age categories with a balance between males and females. It is intended to enroll:

- Approximately 40% of participants 60 to 69 years old, approximately 30% of participants 70 to 79 years old, and approximately 10% of participants ≥ 80 years old. The remaining 20% can be distributed freely across the 3 age categories.
- Approximately 40% of participants from each sex; the remaining 20% can be distributed freely between the 2 sexes.

The total duration of the study, per participant, will be approximately 6 months for the co-administration (“co-ad”) group, and 7 months for the control. In the co-ad group, participants will receive RSVPreF3 OA investigational vaccine [RSVPreF3 120 μ g + AS01E

(25µg QS-21, 25µg MPL)] and PCV20 vaccine (0.5mL single dose pre-filled syringe as per Prescribing Information) on Visit 1 (Day 1) and follow-up for 6 months, until end of study (EoS). In the control group, participants will receive PCV20 vaccine on Visit 1 (Day 1) and RSVPreF3 OA investigational vaccine on Visit 2 (Day 31) and follow-up for 6 months, until EoS.

Primary Objectives

- To demonstrate the non-inferiority of PCV20 when co-administered with the RSVPreF3 OA investigational vaccine compared to PCV20 administered alone.
- Opsonophagocytic (OP) antibody (Ab) titers for each of the pneumococcal vaccine serotype (ST) expressed as between groups geometric mean titer (GMT) ratio, 1 month after the PCV20 dose. The opsonophagocytic antibodies will be determined by multiplexed opsonophagocytosis assay (MOPA).
- To demonstrate the non-inferiority of RSVPreF3 OA investigational vaccine in terms on RSV-A neutralization antibodies when co-administered with PCV20 compared to RSVPreF3 OA investigational vaccine administered alone.
- RSV-A neutralizing Ab titers expressed as between groups GMT ratio, 1 month after the RSVPreF3 OA investigational vaccine dose.
- To demonstrate the non-inferiority of RSVPreF3 OA investigational vaccine in terms of RSV-B neutralization antibodies when co-administered with the PCV20 vaccine compared to RSVPreF3 OA investigational vaccine administered alone.

RSV-A and RSV-B neutralization assays

The serum neutralization assay is a functional assay that measures the ability of serum antibodies to neutralize RSV entry and replication in a host cell line. Virus neutralization is performed by incubating a fixed amount of RSV-A strain (Long, ATCC No. VR-26) or RSV-B strain (18537, ATCC No. VR-1580) with serial dilutions of the test serum. The serum-virus mixture is then transferred onto a Vero cells culture (African Green Monkey, kidney, Cercopithecus aethiops, ATCC CCL 81) and incubated for 2 days to allow infection of the Vero cells by non-neutralized virus and the formation of plaques in the cell monolayer. Following a fixation step, RSV-infected cells are detected using a primary antibody directed against RSV (Polyclonal anti-RSV-A/B IgG) and a secondary antibody conjugated to horse-radish peroxidase (HRP), allowing the visualization of plaques after coloration with TrueBlue peroxidase substrate.

Viral plaques are counted using an automated microscope coupled to an image analyzer (Scanlab system with a Reading software or equivalent). For each serum dilution, a ratio, expressed as a percentage, is calculated between the number of plaques at each serum dilution and the number of plaques in the virus control wells (no serum added). The serum neutralizing antibody titer is expressed in Estimated Dilution 60 (ED60) and corresponds to the inverse of the interpolated serum dilution that yields a 60% reduction in the number of plaques compared to the virus control wells, as described by others. For the testing of Phase III studies, secondary standards calibrated against the international reference will be included in every run to allow conversion into international units.

10

Inclusion Criteria

All participants must satisfy all the following criteria at study entry:

- A male or female ≥ 60 YOA at the time of the first study intervention administration.
- Participants who, in the opinion of the investigator, can and will comply with the requirements of the protocol (e.g., completion of the eDiary, return for follow-up visits, ability to access and utilize a phone or other electronic communications).
- Written or witnessed informed consent obtained from the participant prior to any study-specific procedure being performed.
- Participants living in the general community or in an assisted-living facility that provides minimal assistance, such that the participant is primarily responsible for self-care and activities of daily living.
- Participants who are medically stable in the opinion of the investigator at the time of first study intervention administration. Participants with chronic stable medical conditions with or without specific treatment, such as diabetes mellitus, hypertension, or cardiac disease, are allowed to participate in this study if considered by the investigator as medically stable.

Exclusion Criteria

The following criteria should be checked at the time of study entry. The potential participant MAY NOT be included in the study if ANY exclusion criterion applies:

Medical Conditions

- Any confirmed or suspected immunosuppressive or immunodeficient condition resulting from disease (e.g., current malignancy, human immunodeficiency virus) or immunosuppressive/cytotoxic therapy (e.g., medication used during cancer chemotherapy, organ transplantation, or to treat autoimmune disorders), based on medical history and physical examination (no laboratory testing required).
 - History of any reaction or hypersensitivity (e.g., anaphylaxis) likely to be exacerbated by the study interventions, in particular any history of severe allergic reaction to any vaccine containing diphtheria toxoid (for example, diphtheria-tetanus-pertussis [DTaP]), or PPSV23.
 - Participants considered by investigator as suffering from serious or unstable chronic illness.
 - Any history of dementia or any medical condition that moderately or severely impairs cognition.
 - Recurrent or uncontrolled neurological disorders or seizures.
- Participants with medically-controlled chronic neurological diseases can be enrolled in the study as per investigator assessment, provided that their condition will allow them to comply with the requirements of the protocol (e.g., completion of the eDiary, attend regular phone calls/study site visits).
- Significant underlying illness that in the opinion of the investigator would be expected to prevent completion of the study (e.g., life-threatening disease likely to limit survival up to EoS).
 - Any medical condition that in the judgment of the investigator would make intramuscular injection unsafe.
- Prior and Concomitant Therapy
- History of previous vaccination with any licensed or investigational pneumococcal conjugate vaccine, or planned receipt through study participation.
 - History of previous vaccination with any licensed or investigational pneumococcal

polysaccharide vaccine in the last 5 years from enrollment, or planned receipt through study participation.

- Previous vaccination with any licensed or investigational RSV vaccine
- 5 • Use of any investigational or non-registered product (drug, vaccine or medical device) other than the study interventions during the period beginning 30 days before the first dose of study interventions and ending 30 days after the last study intervention administration,
- 10 or their planned use during the study period.
- Planned or actual administration of a vaccine not foreseen by the study protocol in the period starting 30 days before the first study intervention administration and ending
- 15 30 days after the last study intervention administration.
- Planned or actual administration of adjuvanted quadrivalent influenza vaccine or live influenza vaccine not foreseen by the study protocol in the period starting
- 20 30 days before the first study intervention administration and ending 30 days after the last study intervention administration.
- Administration of long-acting immune-modifying drugs during the period starting 180 days before the administration of first dose of study interventions or planned administration at any time during the study period (e.g., infliximab).
- 25 • Administration of immunoglobulins and/or any blood products or plasma derivatives during the period starting 90 days before the administration of first dose of study interventions or planned administration during the study period.
- Chronic administration (defined as more than 14 consecutive days in
- 30 total) of immunosuppressants or other immune-modifying drugs during the period starting 90 days prior to the first study intervention dose or planned administration during the study period.
- 35 For corticosteroids, this will mean prednisone ≥ 20 mg/day, or equivalent. Inhaled and topical steroids are allowed.

Prior/Concurrent Clinical Study Experience

- Concurrently participating in another clinical study, at any time during the study period, in which the participant has been or will be exposed to an investigational or a non-investigational vaccine/product (drug or invasive medical device).

Other Exclusions

- History of chronic alcohol consumption and/or drug abuse as deemed by the investigator to render the potential participant unable/unlikely to provide accurate safety reports or comply with study procedures.
- Bedridden participants.
- Planned move during the study conduct that prohibits participation until study end.
- Participation of any study personnel or their immediate dependents, family, or household members.

SEQUENCE LISTINGS

- SEQ ID NO:1 (precursor recombinant RSV soluble F protein comprising S155C, S290C, S190F, and V207L substitutions)
- 5 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKENKCNGTDAKVLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNYTLNNA
KKTNTVLSKKRKRRLGFLGVGSAIASGVAVCKVLHLEGEVNIKSALLSTNKAVVSLSNG
VSVLTFKVLDLKNYIDKQLLPILNKQSCSISNIETVIEFQQKNNRLEITREFSVNAGVTPVST
YMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMCIIKEEVLAYVVQLPLYGVIDTPC
10 WKLHTSPLCTTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTMNSL
TLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNRGIKTF
GCDYVSNKGVDTVSGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKI
NQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDG EWVLLSTFL
- 15 SEQ ID NO:2 (F1 chain of mature recombinant RSV soluble F protein comprising S155C, S290C, S190F, and V207L substitutions)
- FLGFLGVGSAIASGVAVCKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTFKVL
LKNYIDKQLLPILNKQSCSISNIETVIEFQQKNNRLEITREFSVNAGVTPVSTYMLTNSELLS
LINDMPITNDQKKLMSNNVQIVRQQSYSIMCIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLC
20 TTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLC
NVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNRGIKTFSGCDYVSNK
GVDTVSGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRK
SDELLSAIGGYIPEAPRDGQAYVRKDG EWVLLSTFL
- 25 SEQ ID NO:3 (F2 chain of mature recombinant RSV soluble F protein comprising S155C, S290C, S190F, and V207L substitutions)
- QNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIKENKCNGTDAKVLIKQEL
DKYKNAVTELQLLMQSTPATNNRARR
- 30 SEQ ID NO:4(representing non-glycosylated N500)
KINQSLAFIRK
- SEQ ID NO:5(representing N500 with glycan removed by PNGase F)
KIDQSLAFIRK
- 35 SEQ ID NO:6(fragment of p27 peptide detectable by mass spec)

FMNYTLNNAK

SEQ ID NO:7(p27)

ELPRFMNYTLNNAKKTNTLSKKRKRR

5

SEQ ID NO:8 (F1 chain of F' protomer-containing p27)

ELPRFMNYTLNNAKKTNTLSKKRKRRFLGFLGVGSAIASGVAVCKVLHLEGEVVK
IKSALLSTNKAVVSLSNVSVLTFKVLDLKNIYDKQLLPILNKQSCSISNIETVIEFQQKNNRLL
EITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMCIIKEE
10 VLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAE
TCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYG
KTKCTASNKNRGIKTFSNNGCDYVSNKGVDTVSVGNTLYYVVKQEGKSLYVKGEPIINFYDP
LVFPSDEFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGEWVLLSTF
L

15

SEQ ID NO:9 (uncleaved furin cleavage site, X and X₁ are independently any amino acid)

RXRRX₁

20

SEQ ID NO: 10 (expressed recombinant RSV soluble F protein comprising S155C, S290C, S190F, and V207L substitutions)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKENKCGTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNYTLNNA
KKTNTLSKKRKRRFLGFLGVGSAIASGVAVCKVLHLEGEVNIKSALLSTNKAVVSLSNV
25 VSVLTFKVLDLKNIYDKQLLPILNKQSCSISNIETVIEFQQKNNRLL EITREFSVNAGVTPVST
YMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMCIIKEEVLAYVVQLPLYGVIDTPC
WKLHTSPLCTTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTMNSL
TLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNRGIKTFN
GCDYVSNKGVDTVSVGNTLYYVVKQEGKSLYVKGEPIINFYDPLVFPSEFDASISQVNEKI
30 NQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGEWVLLSTFLGGLVPRGGSAGSGWSH
PQFEKGGGSGGGSGGGSWSHPQFEKSGKGGHHHHHH

SEQ ID NO: 11 (RSV WT F protein; A2 strain; GenBank GI: 138251; Swiss Prot P03420)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIK
ENKCGTDAKVKLIKQELDKYKNAVTELQLLMQSTPPTNNRARRELPRFMNYTLNNAKKTNT
VLSKKRKRRFLGFLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNVSVLT
SKVLDLKNYIDKQLLPVNVKQSCSISNIETVIEFQQKNNRLL EITREFSVNAGVTPVSTYMLT
NSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIKEEVLAYVVQLPLYGVIDTPCWKLH
40 TSPLCTTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLP
SEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNRGIKTFNNGCDY

VSNKGMDTVSVGNTLYYVНКQEGKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSL
AFIRKSDELLHNVNAGKSTTNIMITIIIVIVILLS
LIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN

- 5 SEQ ID NO: 12 (RSV WT F protein; subgroup B; 18537 strain; GI: 138250; Swiss
Prot P13843)
MELLIHRSSAIFLTLAVNALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIELSNIK
ETKCNGTDTKVKLIKQELDKYKNAVTELQLLMQNTPAANNRARRREAPQYMNNTINTTKNLN
VSISKRRRFLGFLGVSASGIAVSKVLHLEGEVKNKIKNALLSTNKAVVSLNGVSVLTS
10 KVLDLKNYINNRLPIVNQQSCRISNIETVIEFQQMNSRLLEITREFSVNAGVTTPLSTYMLTN
SELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIIKEEVLAYVVQLPIYGVIDTPCWKLHT
SPLCTTNIKEGSNICLTRTDRGWYCDNAGSVSFFPQADTCKVQSNRVFCDTMNSLTLPSEV
SLCNTDIFNSKYDCKIMTSKTDISSSVITSLGAIVSCYGKTKCTASNKNRGIKTFNSGCDYVS
NKGVDTVSVGNTLYYVНКLEGKNLYVKGEPIINYDPLVFPSDEFDASISQVNEKINQSLAFI
15 RRSDELLHNVNTGKSTTNIMITIIIVIVVLLSLAIGLLLYCKAKNTPVTLSKDQLSGINNIAFSK

CLAIMS

1. A method of inducing an immune response to respiratory syncytial virus (RSV) in a human comprising co-administration of:
 - a) an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein; and
 - b) an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* (*Streptococcus pneumoniae*) serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F.
2. An immunogenic combination for use in a method of preventing respiratory syncytial virus (RSV) infection in a human comprising:
 - a) an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein, and an adjuvant; and
 - b) an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* (*Streptococcus pneumoniae*) serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F,wherein the method comprises co-administering the compositions to the human.
3. Use of an immunogenic combination for preventing respiratory syncytial virus (RSV) infection in a human comprising:
 - a) administering an immunogenic composition comprising an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein; and
 - b) administering an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* (*Streptococcus pneumoniae*) serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F,wherein a) and b) are co-administered to the human.
4. An immunogenic composition comprising a recombinant RSV (Respiratory syncytial virus) antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein, for

use in raising an immune response to RSV in a human, wherein an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* (*Streptococcus pneumoniae*) serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F is co-administered.

5. An immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* (*Streptococcus pneumoniae*) serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, for use in raising an immune response to *S. pneumoniae* in a human, wherein an immunogenic composition comprising a recombinant RSV antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein is co-administered.

6. An immunogenic composition comprising a recombinant RSV (Respiratory syncytial virus) antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein, and an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* (*Streptococcus pneumoniae*) serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, for use in raising an immune response to RSV and *S. pneumoniae* in a human comprising co-administering to the human said immunogenic compositions.

7. Use of an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* (*Streptococcus pneumoniae*) serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F in raising an immune response to *S. pneumoniae* in a human, wherein an immunogenic composition comprising a recombinant RSV (respiratory syncytial virus) antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein is co-administered.

8. Use of an immunogenic composition comprising a recombinant RSV (respiratory syncytial virus) antigen comprising a soluble F protein comprising at least one modification that stabilizes the prefusion conformation of the F protein, and an immunogenic composition comprising at least one glycoconjugate from each of *S. pneumoniae* (*Streptococcus pneumoniae*) serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, for use in raising an

immune response to RSV and *S. pneumoniae* in a human comprising co-administering to the human said immunogenic compositions.

9. The method of claim 1, the immunogenic combination of claim 2, the use of claims 3, 7, or 8, or the immunogenic compositions of claims 4-6, wherein the at least one modification is selected from the group consisting of:

- i) an addition of an amino acid sequence comprising a heterologous trimerization domain;
- ii) a deletion of at least one furin cleavage site;
- iii) a deletion of at least one non-furin cleavage site;
- iv) a deletion of one or more amino acids of the pep27 domain; and
- v) at least one substitution or addition of a hydrophilic amino acid in a hydrophobic domain of the F protein extracellular domain.

10. The method of claim 1, the immunogenic combination of claim 2, the use of claims 3, 7, or 8, or the immunogenic compositions of claims 4-6, wherein the at least one modification comprises the addition of an amino acid sequence comprising a heterologous trimerization domain.

11. The method of claim 1, the immunogenic combination of claim 2, the use of claims 3, 7, or 8, or the immunogenic compositions of claims 4-6, wherein the recombinant RSV antigen comprises an F2 domain and an F1 domain of an RSV F protein with no intervening furin cleavage site wherein the polypeptide further comprises a heterologous trimerization domain positioned C-terminal to the F₁ domain.

12. The method of claim 1, the immunogenic combination of claim 2, the use of claims 3, 7, or 8, or the immunogenic compositions of claims 4-6, wherein the recombinant RSV antigen comprises a pre-fusion RSV F polypeptide having an HRA region (residues 137-239 of reference RSV F protein of SEQ ID NO:1) and a DIII region (residues 51-98 and 206-308 of reference RSV F protein of SEQ ID NO:1), wherein a cysteine residue is introduced into the HRA region and a cysteine residue is introduced into the DIII region, and a disulfide bond is formed between the introduced cysteine residue in the HRA region and the introduced cysteine residue in the DIII region that prevents a post-fusion HRA-HRB six-helix bundle from forming.

13. The method of claim 1, the immunogenic combination of claim 2, the use of claims 3, 7, or 8, or the immunogenic compositions of claims 4-6, wherein the immunogenic composition comprises a recombinant RSV antigen further comprises an adjuvant.

14. The method of claim 13, the immunogenic combination of claim 13, or the use of claim 13, wherein the adjuvant comprises MPL and QS-21.

15. The method of claim 14, the immunogenic combination of claim 14, or the use of claim 14, wherein the MPL is present in the immunogenic composition in an amount of about 25 µg.

16. The method of claim 14, the immunogenic combination of claim 14, or the use of claim 14, wherein the QS-21 is present in the immunogenic composition in an amount of about 25 µg.

17. The method of claim 1, the immunogenic combination of claim 2, the use of claims 3, 7, or 8, or the immunogenic compositions of claims 4-6, wherein the human is 60 years old and above.

18. The method of claim 1, the immunogenic combination of claim 2, the use of claims 3, 7, or 8, or the immunogenic compositions of claims 4-6, wherein said glycoconjugates are individually conjugated to CRM197.

19. The method of claim 1, the immunogenic combination of claim 2, the use of claims 3, 7, or 8, or the immunogenic compositions of claims 4-6, wherein the immunogenic composition comprising at least one glycoconjugate further comprises an adjuvant.

20. The method of claim 19, the immunogenic combination of claim 19, or the use of claim 19, wherein the adjuvant is an aluminum salt selected from the group consisting of aluminum phosphate, aluminum sulfate, and aluminum hydroxide.