(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
07 December 2017 (07.12.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/207477 A1

(51) International Patent Classification:
C07K 14/135 (2006.01)

(21) International Application Number:
PCT/EP2017/062870

(22) International Filing Date:
29 May 2017 (29.05.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
16171997.6 30 May 2016 (30.05.2016) EP

(71) Applicant: **JANSSEN VACCINES & PREVENTION B.V.** [NL/NL]; Archimedesweg 4, 2333 CN Leiden (NL).

(72) Inventor: **LANGEDIJK, Johannes, Petrus, Maria**; Archimedesweg 4-6, 2333 CN Leiden (NL).

(74) Agent: **MANTEN, Annemieke** et al.; Archimedesweg 4-6, 2333 CN Leiden (NL).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report (Art. 21(3))*

WO 2017/207477 A1

(54) Title: STABILIZED PRE-FUSION RSV F PROTEINS

(57) Abstract: The present invention provides stable pre-fusion respiratory syncitial virus (RSV) F proteins, immunogenic compositions comprising said proteins and uses thereof for the prevention and/or treatment of RSV infection.

Stabilized pre-fusion RSV F proteins

The present invention relates to the field of medicine. The invention in particular relates to recombinant pre-fusion RSV F proteins, to nucleic acid molecules encoding the RSV F proteins, and uses thereof, e.g. in vaccines.

Background of the invention

After discovery of the respiratory syncytial virus (RSV) in the 1950s, the virus soon became a recognized pathogen associated with lower and upper respiratory tract infections in humans. Worldwide, it is estimated that 64 million RSV infections occur each year resulting in 160.000 deaths (WHO Acute Respiratory Infections Update September 2009). The most severe disease occurs particularly in premature infants, the elderly and immunocompromised individuals. In children younger than 2 years, RSV is the most common respiratory tract pathogen, accounting for approximately 50% of the hospitalizations due to respiratory infections, and the peak of hospitalization occurs at 2-4 months of age. It has been reported that almost all children have been infected by RSV by the age of two. Repeated infection during lifetime is attributed to ineffective natural immunity. In the elderly, the RSV disease burden is similar to those caused by non-pandemic influenza A infections.

RSV is a paramyxovirus, belonging to the subfamily of pneumovirinae. Its genome encodes for various proteins, including the membrane proteins known as RSV Glycoprotein (G) and RSV fusion (F) protein which are the major antigenic targets for neutralizing antibodies. Antibodies against the fusion-mediating part of the F1 protein can prevent virus uptake in the cell and thus have a neutralizing effect.

RSV F fuses the viral and host-cell membranes by irreversible protein refolding from the labile pre-fusion conformation to the stable post-fusion conformation. Structures of both conformations have been determined for RSV F (McLellan JS, *et al. Science* **342**, 592-598

(2013); McLellan JS, *et al. Nat Struct Mol Biol* **17**, 248-250 (2010); McLellan JS, *et al. Science* **340**, 1113-1117 (2013); Swanson KA, *et al. Proceedings of the National Academy of Sciences of the United States of America* **108**, 9619-9624 (2011)), as well as for the fusion proteins from related paramyxoviruses, providing insight into the mechanism of this complex fusion machine. Like other type I fusion proteins, the inactive precursor, RSV F_0, requires cleavage during intracellular maturation by a furin-like protease. RSV F contains two furin sites, which leads to three proteins: F2, p27 and F1, with the latter containing a hydrophobic fusion peptide (FP) at its N-terminus. In order to refold from the pre-fusion to the post-fusion conformation, the refolding region 1 (RR1) between residue 137 and 216, that includes the FP and heptad repeat A (HRA) has to transform from an assembly of helices, loops and strands to a long continuous helix. The FP, located at the N-terminal segment of RR1, is then able to extend away from the viral membrane and insert into the proximal membrane of the target cell. Next, the refolding region 2 (RR2), which forms the C-terminal stem in the pre-fusion F spike and includes the heptad repeat B (HRB), relocates to the other side of the RSV F head and binds the HRA coiled-coil trimer with the HRB domain to form the six-helix bundle. The formation of the RR1 coiled-coil and relocation of RR2 to complete the six-helix bundle are the most dramatic structural changes that occur during the refolding process.

A vaccine against RSV infection is currently not available, but is desired due to the high disease burden. The RSV fusion glycoprotein (RSV F) is an attractive vaccine antigen it is the principal target of neutralizing antibodies in human sera. Most neutralizing antibodies in human sera are directed against the pre-fusion conformation, but due to its instability the pre-fusion conformation has a propensity to prematurely refold into the post-fusion conformation, both in solution and on the surface of the virions. As indicated above, crystal structures have revealed a large conformational change between the pre-fusion and post-fusion states. The magnitude of the rearrangement suggested that only a portion of antibodies

directed to the post-fusion conformation of RSV-F will be able to cross react with the native conformation of the pre-fusion spike on the surface of the virus. Accordingly, efforts to produce a vaccine against RSV have focused on developing vaccines that contain pre-fusion forms of RSV F protein (see, e.g., WO20101149745, WO2010/1149743, WO2009/1079796, WO2012/158613). However, these efforts have not yet yielded stable pre-fusion RSV F proteins that could be used as candidates for testing in humans.

Therefore, a need remains for efficient vaccines against RSV, in particular vaccines comprising RSV F proteins in the pre-fusion conformation. The present invention aims at providing such stable pre-fusion RSV F proteins for use in vaccinating against RSV in a safe and efficacious manner.

Summary of the invention

The present invention provides stable, recombinant, pre-fusion respiratory syncytial virus (RSV) fusion (F) proteins, i.e. recombinant RSV F proteins that are stabilized in the pre-fusion conformation, and fragments thereof. The RSV F proteins, or fragments thereof, comprise at least one epitope that is specific to the pre-fusion conformation F protein. In certain embodiments, the pre-fusion RSV F proteins are soluble proteins. In certain embodiments the RSV F proteins are trimeric. In certain embodiments, the RSV F proteins are multimers of trimeric RSV F proteins. The invention also provides nucleic acid molecules encoding the pre-fusion RSV F proteins, or fragments thereof, as well as vectors comprising such nucleic acid molecules.

The invention also relates to compositions, preferably immunogenic compositions, comprising a RSV F protein, a nucleic acid molecule and/or a vector, and to the use thereof in inducing an immune response against RSV F protein, in particular to the use thereof as a vaccine. The invention also relates to methods for inducing an anti-respiratory syncytial virus

(RSV) immune response in a subject, comprising administering to the subject an effective amount of a pre-fusion RSV F protein, a nucleic acid molecule encoding said RSV F protein, and/or a vector comprising said nucleic acid molecule. Preferably, the induced immune response is characterized by neutralizing antibodies to RSV and/or protective immunity against RSV. In particular aspects, the invention relates to a method for inducing anti-respiratory syncytial virus (RSV) F antibodies in a subject, comprising administering to the subject an effective amount of an immunogenic composition comprising a pre-fusion RSV F protein, a nucleic acid molecule encoding said RSV F protein, and/or a vector comprising said nucleic acid molecule.

Brief description of the Figures

FIG.1: Purification of protein F with mutations N67I, S155C, S215P, S290C, D486N. Superdex200 gel filtration chromatogram of the eluate from the ion-exchange column. The arrow indicates the collected peak.

FIG.2: A) SDS-PAGE analysis of the F N67I, S155C, S215P, S290C, D486N protein sample containing peak from the SEC chromatogram under reducing (R) and non-reducing (NR) conditions. The gels are stained with Coomassie Brilliant Blue.

FIG. 3: The protein concentration of purified RSV F protein F N67I, S155C, S215P, S290C, D486N was measured by Q Octet assay with CR9501 and CR9503 monoclonal antibodies. CR9501 only binds to RSV F in the pre-fusion conformation. CR9503 binds RSV F both in the pre-fusion conformation and the post-fusion conformation. Plotted as Mean±SE.

FIG. 4: Temperature stability of RSV F protein F N67I, S155C, S215P, S290C, D486N. Melting temperature (Tm °C) determined by differential scanning fluorimetry (DSF) assay with SyproOrange fluorescent dye. Introduction of the disulfide bridge by the S155C, S290C substitutions results in an additional Tm. The first conformational transition occurs at

65 °C and the second one occurs at 70 °C, which is probably the result of the disulfide. The disulfide bridge can prevent the complete irreversible folding even when the protein is heated over 65 °C (data not shown).

Detailed description of the invention

The fusion protein (F) of the respiratory syncytial virus (RSV) is involved in fusion of the viral membrane with a host cell membrane, which is required for infection. RSV F mRNA is translated into a 574 amino acid precursor protein designated F0, which contains a signal peptide sequence at the N-terminus (e.g. amino acid residues 1-26 of SEQ ID NO: 13) which is removed by a signal peptidase in the endoplasmic reticulum. F0 is cleaved at two sites (between amino acid residues 109/110 and 136/137) by cellular proteases (in particular furin, or furin-like)) removing a short glycosylated intervening sequence (also referred to a p27 region, comprising the amino acid residues 110 to 136, and generating two domains or subunits designated F1 and F2. The F1 domain (amino acid residues 137-574) contains a hydrophobic fusion peptide at its N-terminus and the C-terminus contains the transmembrane (TM) (amino acid residues 530-550) and cytoplasmic region (amino acid residues 551-574). The F2 domain (amino acid residues 27-109) is covalently linked to F1 by two disulfide bridges. The F1-F2 heterodimers are assembled as homotrimers in the virion.

A vaccine against RSV infection is currently not yet available. One potential approach to producing a vaccine is a subunit vaccine based on purified RSV F protein. However, for this approach it is desirable that the purified RSV F protein is in a conformation which resembles the conformation of the pre-fusion state of RSV F protein, which is stable over time and can be produced in sufficient quantities. In addition, for a soluble, subunit-based vaccine, the RSV F protein needs to be truncated by deletion of the transmembrane (TM) and the cytoplasmic region to create a soluble secreted F protein (sF). Because the TM region is responsible for membrane anchoring and stability, the anchorless soluble F protein is

considerably more labile than the full-length protein and will readily refold into the post-fusion end-state. In order to obtain soluble F protein in the stable pre-fusion conformation that shows high expression levels and high stability, the pre-fusion conformation thus needs to be stabilized. Because also the full length (membrane-bound) RSV F protein is metastable, the stabilization of the pre-fusion conformation is also desirable for the full length RSV F protein, e.g. for any life attenuated or vector based vaccine approach.

For the stabilization of soluble RSV F, that is cleaved into the F1 and F2 subunit, in the pre-fusion conformation, a fibritin – based trimerization domain was fused to the C-terminus of the soluble RSV-F C-terminal end (McLellan et al., Nature Struct. Biol.17: 2-248-250 (2010); McLellan et al., Science 340(6136):1113-7 (2013)). This fibritin domain or 'Foldon' is derived from T4 fibritin and was described earlier as an artificial natural trimerization domain (Letarov et al., Biochemistry Moscow 64: 817-823 (1993); S-Guthe et al., J. Mol. Biol. 337: 905-915. (2004)). However, the trimerization domain does not result in stable pre-fusion RSV-F protein (Krarup et al., Nature Comm. 6:8143, (2015)). Moreover, these efforts have not yet resulted in candidates suitable for testing in humans.

Recently, we described combinations of several mutations that are capable of stabilizing the RSV F protein in the pre-fusion conformation (WO2014/174018 and WO2014/202570). Thus, stable pre-fusion RSV F proteins have been described comprising a mutation of the amino acid residue on position 67 and/or a mutation of the amino acid residue on position 215, preferably a mutation of amino acid residue N/T on position 67 into I and/or a mutation of amino acid residue S on position 215 into P. In addition, soluble pre-fusion RSV F proteins have been described comprising a truncated F1 domain, and comprising a mutation of the amino acid residue on position 67 and/or a mutation of the amino acid residue on position 215, preferably a mutation of amino acid residue N/T on position 67 into I and/or a mutation of amino acid residue S on position 215 into P, wherein the protein comprises a

heterologous trimerization domain linked to said truncated F1 domain. Additional pre-fusion RSV F proteins have been described, wherein the proteins comprise at least one further mutation, such as a mutation of the amino acid residue D on position 486 into N.

According to the present invention it has been found that the introduction of a disulphide bridge between amino acid residue 155 and 290 (numbering according to SEQ ID NO: 13) further stabilizes the protein in the pre-fusion conformation. The present invention thus provides recombinant pre-fusion F proteins further comprising a mutation of the amino acid residue S on position 155 into C (S155C) and of the amino acid S on position 290 into C (S290C).

The present invention provides novel stable recombinant pre-fusion respiratory syncytial virus (RSV) Fusion (F) proteins, or fragments thereof, comprising

(a) a mutation of the amino acid residue N/T on position 67 into I;

(b) a mutation of the amino acid residue S on position 215 into P;

(c) a mutation of the amino acid residue D on position 486 into N; and

wherein the protein further comprises a mutation of the amino acid residue S on position 155 into C (S155C) and of the amino acid S on position 290 into C (S290C).

The present invention thus provides a unique combination of mutations to provide recombinant stable pre-fusion RSV F proteins, i.e. RSV F proteins that are stabilized in the pre-fusion conformation, or fragments thereof. The stable pre-fusion RSV F proteins of the invention, or fragments thereof, are in the pre-fusion conformation, i.e. they comprise (display) at least one epitope that is specific to the pre-fusion conformation F protein. An epitope that is specific to the pre-fusion conformation F protein is an epitope that is not presented in the post-fusion conformation. Without wishing to be bound by any particular theory, it is believed that the pre-fusion conformation of RSV F protein may contain epitopes

that are the same as those on the RSV F protein expressed on natural RSV virions, and therefore may provide advantages for eliciting protective neutralizing antibodies.

In certain embodiments, the pre-fusion RSV F proteins of the invention, or fragments thereof, comprise at least one epitope that is recognized by a pre-fusion specific monoclonal antibody, comprising a heavy chain CDR1 region of SEQ ID NO: 1, a heavy chain CDR2 region of SEQ ID NO: 2, a heavy chain CDR3 region of SEQ ID NO: 3 and a light chain CDR1 region of SEQ ID NO: 4, a light chain CDR2 region of SEQ ID NO: 5, and a light chain CDR3 region of SEQ ID NO: 6 (hereafter referred to as CR9501) and/or a pre-fusion specific monoclonal antibody, comprising a heavy chain CDR1 region of SEQ ID NO: 7, a heavy chain CDR2 region of SEQ ID NO: 8, a heavy chain CDR3 region of SEQ ID NO: 9 and a light chain CDR1 region of SEQ ID NO: 10, a light chain CDR2 region of SEQ ID NO: 11, and a light chain CDR3 region of SEQ ID NO: 12 (referred to as CR9502). CR9501 and CR9502 comprise the heavy and light chain variable regions, and thus the binding specificities, of the antibodies 58C5 and 30D8, respectively, which have previously been shown to bind specifically to RSV F protein in its pre-fusion conformation and not to the post-fusion conformation (see WO2012/006596).

In certain embodiments, the recombinant pre-fusion RSV F proteins are trimeric.

As used throughout the present application nucleotide sequences are provided from 5' to 3' direction, and amino acid sequences from N-terminus to C-terminus, as custom in the art.

As indicated above, fragments of the pre-fusion RSV F protein are also encompassed by the present invention. The fragment may result from either or both of amino-terminal (e.g. by cleaving off the signal sequence) and carboxy-terminal deletions (e.g. by deleting the transmembrane region and/or cytoplasmic tail). The fragment may be chosen to comprise an immunologically active fragment of the F protein, i.e. a part that will give rise to an immune

response in a subject. This can be easily determined using in silico, in vitro and/or in vivo methods, all routine to the skilled person.

In certain embodiments, the encoded proteins or fragments thereof according to the invention comprise a signal sequence, also referred to as leader sequence or signal peptide, corresponding to amino acids 1-26 of SEQ ID NO: 13. Signal sequences typically are short (e.g. 5-30 amino acids long) amino acid sequences present at the N-terminus of the majority of newly synthesized proteins that are destined towards the secretory pathway, and are typically cleaved by signal peptidase to generate a free signal peptide and a mature protein.

In certain embodiments, the proteins or fragments thereof according to the invention do not comprise a signal sequence.

In certain embodiments, the (fragments of the) pre-fusion RSV F proteins are soluble. In certain embodiments, the stable pre-fusion RSV F proteins or fragments thereof according to the invention comprise a truncated F1 domain, and comprise a heterologous trimerization domain linked to said truncated F1 domain. According to the invention, it was shown that by linking a heterologous trimerization domain to the C-terminal amino acid residue of a truncated F1 domain, combined with the stabilizing mutation(s), soluble RSV F proteins are provided that show high expression and that bind to pre-fusion-specific antibodies, indicating that the proteins are in the pre-fusion conformation. In addition, the RSV F proteins are stabilized in the pre-fusion conformation, i.e. even after processing of the proteins they still bind to the pre-fusion specific antibodies CR9501 and/or CR9502, indicating that the pre-fusion specific epitope is retained.

In certain embodiments, the RSV F proteins are multimers of trimeric RSV F proteins. Thus, in some embodiments, the RSV F proteins may comprise an assembly domain for higher order assemblies of trimers.

It is known that RSV exists as a single serotype having two antigenic subgroups: A and B. The amino acid sequences of the mature processed F proteins of the two groups are about 93% identical. As used throughout the present application, the amino acid positions are given in reference to the sequence of an RSV F protein of subgroup A (SEQ ID NO: 13). As used in the present invention, the wording "the amino acid at position "x" of the RSV F protein thus means the amino acid corresponding to the amino acid at position "x" in the RSV F protein of the RSV of SEQ ID NO: 13. Note that, in the numbering system used throughout this application 1 refers to the N-terminal amino acid of an immature F0 protein (SEQ ID NO: 13). When another RSV strain is used, the amino acid positions of the F protein are to be numbered with reference to the numbering of the F protein of SEQ ID NO: 13 by aligning the sequences of the other RSV strain with the F protein of SEQ ID NO: 13 with the insertion of gaps as needed. Sequence alignments can be done using methods well known in the art, e.g. by CLUSTALW, Bioedit or CLC Workbench.

In certain embodiments, the RSV strain is the RSV strain of SEQ ID NO: 20.

In certain embodiments, the RSV strain is an RSV B strain. In certain embodiments, the RSV strain is the RSV B strain of SEQ ID NO: 15.

An amino acid according to the invention can be any of the twenty naturally occurring (or 'standard' amino acids) or variants thereof, such as e.g. D-amino acids (the D-enantiomers of amino acids with a chiral center), or any variants that are not naturally found in proteins, such as e.g. norleucine. The standard amino acids can be divided into several groups based on their properties. Important factors are charge, hydrophilicity or hydrophobicity, size and functional groups. These properties are important for protein structure and protein–protein interactions. Some amino acids have special properties such as cysteine, that can form covalent disulfide bonds (or disulfide bridges) to other cysteine residues, proline that induces turns of the

protein backbone, and glycine that is more flexible than other amino acids. Table 1 shows the abbreviations and properties of the standard amino acids.

It will be appreciated by a skilled person that the mutations can be made to the protein by routine molecular biology procedures. The mutations according to the invention preferably result in increased expression levels and/or increased stabilization of the pre-fusion RSV F proteins as compared RSV F proteins that do not comprise these mutation(s).

In certain embodiments, the pre-fusion RSV F proteins or fragments thereof comprise at least four mutations (as compared to a wild-type RV F protein, e.g. the comprising the amino acid sequence of SEQ ID NO : 13). In certain embodiments, the proteins or fragments thereof comprise at least five mutations.

In certain embodiments, the proteins or fragments thereof comprise at least six mutations.

In certain embodiments, the pre-fusion RSV F polypeptides thus comprise at least one further mutation selected from the group consisting of:

(a) a mutation of the amino acid residue on position 46;

(b) a mutation of the amino acid residue on position 83;

(c) a mutation of the amino acid residue on position 92;

(d) a mutation of the amino acid residue on position 184;

(e) a mutation of the amino acid residue on position 203;

(f) a mutation of the amino acid residue on position 207; and

(g) a mutation of the amino acid residue on position 487.

In certain embodiments, the at least one further mutation is selected from the group consisting of:

(a) a mutation of the amino acid residue S on position 46 into G;

(b) a mutation of the amino acid residue L on position 83 into M:

(c) a mutation of the amino acid residue E on position 92 into D;

(d) a mutation of the amino acid residue G on position 184 into N;

(e) a mutation of the amino acid residue L on position 203 into I;

(f) a mutation of the amino acid residue V on position 207 into I: and

(g) a mutation of the amino acid residue E on position 487 into Q, N or I.

In certain other embodiments, the heterologous trimerization domain comprises the amino acid sequence GYIPEAPRDGQAYVRKDGEWVLLSTFL (SEQ ID NO: 14).

As described above, in certain embodiments, the proteins of the invention or fragments thereof comprise a truncated F1 domain. As used herein a "truncated" F1 domain refers to a F1 domain that is not a full length F1 domain, i.e. wherein either N-terminally or C-terminally one or more amino acid residues have been deleted. According to the invention, at least the transmembrane domain and cytoplasmic tail have been deleted to permit expression as a soluble ectodomain.

In certain other embodiments, the trimerization domain is linked to amino acid residue 513 of the RSV F1 domain. In certain embodiments, the trimerization domain thus comprises SEQ ID NO: 14 and is linked to amino acid residue 513 of the RSV F1 domain.

In certain embodiments, the RSV F protein of the invention comprises the amino acid sequence of SEQ ID NO: 21.

In certain embodiments, the level of expression of the pre-fusion RSV F proteins of the invention is increased, as compared to a wild-type RSV F protein. In certain embodiments the level of expression is increased at least 5-fold, preferably up to 10-fold. In certain embodiments, the level of expression is increased more than 10-fold.

The pre-fusion RSV F proteins according to the invention are stable, i.e. do not readily change into the post-fusion conformation upon processing of the proteins, such as e.g. purification, freeze-thaw cycles, and/or storage etc.

In certain embodiments, the pre-fusion RSV F proteins according to the invention have an increased stability upon storage a 4°C as compared to a RSV F protein without the mutation(s). In certain embodiments, the proteins are stable upon storage at 4°C for at least 30 days, preferably at least 60 days, preferably at least 6 months, even more preferably at least 1 year. With "stable upon storage", it is meant that the proteins still display the at least one epitope specific for the a pre-fusion specific antibody (e.g. CR9501) upon storage of the protein in solution (e.g. culture medium) at 4° C for at least 30 days. In certain embodiments, the proteins display the at least one pre-fusion specific epitope for at least 6 months, preferably for at least 1 year upon storage of the pre-fusion RSV F proteins at 4°C.

In certain embodiments, the pre-fusion RSV F proteins according to the invention have an increased stability when subjected to heat, as compared to RSV F proteins without said mutation(s). In certain embodiments, the pre-fusion REV F proteins are heat stable for at least 30 minutes at a temperature of 55° C, preferably at 58° C, more preferably at 60° C With "heat stable" it is meant that the proteins still display the at least one pe-fusion specific epitope after having been subjected for at least 30 minutes to an increased temperature (i.e. a temperature of 55°C or above), e.g. as determined using a method as described in the Examples (see Fig. 4).

In certain embodiments, the proteins display the at least one pre-fusion specific epitope after being subjected to 1 to 6 freeze-thaw cycles in an appropriate formulation buffer.

As used throughout the present application nucleotide sequences are provided from 5' to 3' direction, and amino acid sequences from N-terminus to C-terminus, as custom in the art.

In certain embodiments, the encoded proteins according to the invention further comprise a leader sequence, also referred to as signal sequence or signal peptide,

corresponding to amino acids 1-26 of SEQ ID NO: 13. This is a short (typically 5-30 amino acids long) peptide present at the N-terminus of the majority of newly synthesized proteins that are destined towards the secretory pathway. In certain embodiments, the proteins according to the invention do not comprise a leader sequence.

In certain embodiments, the proteins comprise a HIS-Tag. A His-Tag or polyhistidine-tag is an amino acid motif in proteins that consists of at least five histidine (H) residues, often at the N- or C-terminus of the protein, which is generally used for purification purposes.

The present invention further provides nucleic acid molecules encoding the RSV F proteins according to the invention.

In preferred embodiments, the nucleic acid molecules encoding the proteins according to the invention are codon-optimized for expression in mammalian cells, preferably human cells. Methods of codon-optimization are known and have been described previously (e.g. WO 96/09378). A sequence is considered codon-optimized if at least one non-preferred codon as compared to a wild type sequence is replaced by a codon that is more preferred. Herein, a non-preferred codon is a codon that is used less frequently in an organism than another codon coding for the same amino acid, and a codon that is more preferred is a codon that is used more frequently in an organism than a non-preferred codon. The frequency of codon usage for a specific organism can be found in codon frequency tables, such as in http://www.kazusa.or.jp/codon. Preferably more than one non-preferred codon, preferably most or all non-preferred codons, are replaced by codons that are more preferred. Preferably the most frequently used codons in an organism are used in a codon-optimized sequence. Replacement by preferred codons generally leads to higher expression.

It will be understood by a skilled person that numerous different polynucleotides and nucleic acid molecules can encode the same protein as a result of the degeneracy of the

genetic code. It is also understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the protein sequence encoded by the nucleic acid molecules to reflect the codon usage of any particular host organism in which the proteins are to be expressed. Therefore, unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may or may not include introns.

Nucleic acid sequences can be cloned using routine molecular biology techniques, or generated de novo by DNA synthesis, which can be performed using routine procedures by service companies having business in the field of DNA synthesis and/or molecular cloning (e.g. GeneArt, GenScripts, Invitrogen, Eurofins).

The invention also provides vectors comprising a nucleic acid molecule as described above. In certain embodiments, a nucleic acid molecule according to the invention thus is part of a vector. Such vectors can easily be manipulated by methods well known to the person skilled in the art, and can for instance be designed for being capable of replication in prokaryotic and/or eukaryotic cells. In addition, many vectors can be used for transformation of eukaryotic cells and will integrate in whole or in part into the genome of such cells, resulting in stable host cells comprising the desired nucleic acid in their genome. The vector used can be any vector that is suitable for cloning DNA and that can be used for transcription of a nucleic acid of interest. Suitable vectors according to the invention are e.g. adenovectors, alphavirus, paramyxovirus, vaccinia virus, herpes virus, retroviral vectors etc. The person skilled in the art is capable of choosing suitable expression vectors, and inserting the nucleic acid sequences of the invention in a functional manner.

Host cells comprising the nucleic acid molecules encoding the pre-fusion RSV F proteins form also part of the invention. The pre-fusion RSV F proteins may be produced

through recombinant DNA technology involving expression of the molecules in host cells, e.g. Chinese hamster ovary (CHO) cells, tumor cell lines, BHK cells, human cell lines such as HEK293 cells, PER.C6 cells, or yeast, fungi, insect cells, and the like, or transgenic animals or plants. In certain embodiments, the cells are from a multicellular organism, in certain embodiments they are of vertebrate or invertebrate origin. In certain embodiments, the cells are mammalian cells. In certain embodiments, the cells are human cells. In general, the production of a recombinant proteins, such the pre-fusion RSV F proteins of the invention, in a host cell comprises the introduction of a heterologous nucleic acid molecule encoding the protein in expressible format into the host cell, culturing the cells under conditions conducive to expression of the nucleic acid molecule and allowing expression of the protein in said cell. The nucleic acid molecule encoding a protein in expressible format may be in the form of an expression cassette, and usually requires sequences capable of bringing about expression of the nucleic acid, such as enhancer(s), promoter, polyadenylation signal, and the like. The person skilled in the art is aware that various promoters can be used to obtain expression of a gene in host cells. Promoters can be constitutive or regulated, and can be obtained from various sources, including viruses, prokaryotic, or eukaryotic sources, or artificially designed.

Cell culture media are available from various vendors, and a suitable medium can be routinely chosen for a host cell to express the protein of interest, here the pre-fusion RSV F proteins. The suitable medium may or may not contain serum.

A “heterologous nucleic acid molecule” (also referred to herein as ‘transgene’) is a nucleic acid molecule that is not naturally present in the host cell. It is introduced into for instance a vector by standard molecular biology techniques. A transgene is generally operably linked to expression control sequences. This can for instance be done by placing the nucleic acid encoding the transgene(s) under the control of a promoter. Further regulatory sequences may be added. Many promoters can be used for expression of a transgene(s), and

are known to the skilled person, e.g. these may comprise viral, mammalian, synthetic promoters, and the like. A non-limiting example of a suitable promoter for obtaining expression in eukaryotic cells is a CMV-promoter (US 5,385,839), e.g. the CMV immediate early promoter, for instance comprising nt. –735 to +95 from the CMV immediate early gene enhancer/promoter. A polyadenylation signal, for example the bovine growth hormone polyA signal (US 5,122,458), may be present behind the transgene(s). Alternatively, several widely used expression vectors are available in the art and from commercial sources, e.g. the pcDNA and pEF vector series of Invitrogen, pMSCV and pTK-Hyg from BD Sciences, pCMV-Script from Stratagene, etc, which can be used to recombinantly express the protein of interest, or to obtain suitable promoters and/or transcription terminator sequences, polyA sequences, and the like.

The cell culture can be any type of cell culture, including adherent cell culture, e.g. cells attached to the surface of a culture vessel or to microcarriers, as well as suspension culture. Most large-scale suspension cultures are operated as batch or fed-batch processes because they are the most straightforward to operate and scale up. Nowadays, continuous processes based on perfusion principles are becoming more common and are also suitable. Suitable culture media are also well known to the skilled person and can generally be obtained from commercial sources in large quantities, or custom-made according to standard protocols. Culturing can be done for instance in dishes, roller bottles or in bioreactors, using batch, fed-batch, continuous systems and the like. Suitable conditions for culturing cells are known (see e.g. Tissue Culture, Academic Press, Kruse and Paterson, editors (1973), and R.I. Freshney, Culture of animal cells: A manual of basic technique, fourth edition (Wiley-Liss Inc., 2000, ISBN 0-471-34889-9)).

The invention further provides compositions comprising a pre-fusion RSV F protein and/or a nucleic acid molecule, and/or a vector, as described above. The invention thus

provides compositions comprising a pre-fusion RSV F protein that displays an epitope that is present in a pre-fusion conformation of the RSV F protein but is absent in the post-fusion conformation. The invention also provides compositions comprising a nucleic acid molecule and/or a vector, encoding such pre-fusion RSV F protein. The invention further provides immunogenic compositions comprising a pre-fusion RSV F protein, and/or a nucleic acid molecule, and/or a vector, as described above. The invention also provides the use of a stabilized pre-fusion RSV F protein, a nucleic acid molecule, and/or a vector, according to the invention, for inducing an immune response against RSV F protein in a subject. Further provided are methods for inducing an immune response against RSV F protein in a subject, comprising administering to the subject a pre-fusion RSV F protein, and/or a nucleic acid molecule, and/or a vector, according to the invention. Also provided are pre-fusion RSV F proteins, nucleic acid molecules, and/or vectors, according to the invention for use in inducing an immune response against RSV F protein in a subject. Further provided is the use of the pre-fusion RSV F proteins, and/or nucleic acid molecules, and/or vectors according to the invention for the manufacture of a medicament for use in inducing an immune response against RSV F protein in a subject.

The pre-fusion RSV F proteins, nucleic acid molecules, or vectors of the invention may be used for prevention (prophylaxis) and/or treatment of RSV infections. In certain embodiments, the prevention and/or treatment may be targeted at patient groups that are susceptible RSV infection. Such patient groups include, but are not limited to e.g., the elderly (e.g. $\geq$ 50 years old, $\geq$ 60 years old, and preferably $\geq$ 65 years old), the young (e.g. $\leq$ 5 years old, $\leq$ 1 year old), hospitalized patients and patients who have been treated with an antiviral compound but have shown an inadequate antiviral response.

The pre-fusion RSV F proteins, nucleic acid molecules and/or vectors according to the invention may be used e.g. in stand-alone treatment and/or prophylaxis of a disease or

condition caused by RSV, or in combination with other prophylactic and/or therapeutic treatments, such as (existing or future) vaccines, antiviral agents and/or monoclonal antibodies.

The invention further provides methods for preventing and/or treating RSV infection in a subject utilizing the pre-fusion RSV F proteins, nucleic acid molecules and/or vectors according to the invention. In a specific embodiment, a method for preventing and/or treating RSV infection in a subject comprises administering to a subject in need thereof an effective amount of a pre-fusion RSV F protein, nucleic acid molecule and/or a vector, as described above. A therapeutically effective amount refers to an amount of a protein, nucleic acid molecule or vector, that is effective for preventing, ameliorating and/or treating a disease or condition resulting from infection by RSV. Prevention encompasses inhibiting or reducing the spread of RSV or inhibiting or reducing the onset, development or progression of one or more of the symptoms associated with infection by RSV. Amelioration as used in herein may refer to the reduction of visible or perceptible disease symptoms, viremia, or any other measurable manifestation of influenza infection.

For administering to subjects, such as humans, the invention may employ pharmaceutical compositions comprising a pre-fusion RSV F protein, a nucleic acid molecule and/or a vector as described herein, and a pharmaceutically acceptable carrier or excipient. In the present context, the term "pharmaceutically acceptable" means that the carrier or excipient, at the dosages and concentrations employed, will not cause any unwanted or harmful effects in the subjects to which they are administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press

[2000]). The RSV F proteins, or nucleic acid molecules, preferably are formulated and administered as a sterile solution although it may also be possible to utilize lyophilized preparations. Sterile solutions are prepared by sterile filtration or by other methods known per se in the art. The solutions are then lyophilized or filled into pharmaceutical dosage containers. The pH of the solution generally is in the range of pH 3.0 to 9.5, e.g. pH 5.0 to 7.5. The RSV F proteins typically are in a solution having a suitable pharmaceutically acceptable buffer, and the composition may also contain a salt. Optionally stabilizing agent may be present, such as albumin. In certain embodiments, detergent is added. In certain embodiments, the RSV F proteins may be formulated into an injectable preparation.

In certain embodiments, a composition according to the invention further comprises one or more adjuvants. Adjuvants are known in the art to further increase the immune response to an applied antigenic determinant. The terms “adjuvant” and "immune stimulant" are used interchangeably herein, and are defined as one or more substances that cause stimulation of the immune system. In this context, an adjuvant is used to enhance an immune response to the RSV F proteins of the invention. Examples of suitable adjuvants include aluminium salts such as aluminium hydroxide and/or aluminium phosphate; oil-emulsion compositions (or oil-in-water compositions), including squalene-water emulsions, such as MF59 (see e.g. WO 90/14837); saponin formulations, such as for example QS21 and Immunostimulating Complexes (ISCOMS) (see e.g. US 5,057,540; WO 90/03184, WO 96/11711, WO 2004/004762, WO 2005/002620); bacterial or microbial derivatives, examples of which are monophosphoryl lipid A (MPL), 3-O-deacylated MPL (3dMPL), CpG-motif containing oligonucleotides, ADP-ribosylating bacterial toxins or mutants thereof, such as *E. coli* heat labile enterotoxin LT, cholera toxin CT, and the like; eukaryotic proteins (e.g. antibodies or fragments thereof (e.g. directed against the antigen itself or CD1a, CD3, CD7, CD80) and ligands to receptors (e.g. CD40L, GMCSF, GCSF, etc), which stimulate immune

response upon interaction with recipient cells. In certain embodiments the compositions of the invention comprise aluminium as an adjuvant, e.g. in the form of aluminium hydroxide, aluminium phosphate, aluminium potassium phosphate, or combinations thereof, in concentrations of 0.05 – 5 mg, e.g. from 0.075-1.0 mg, of aluminium content per dose.

The pre-fusion RSV F proteins may also be administered in combination with or conjugated to nanoparticles, such as e.g. polymers, liposomes, virosomes, virus-like particles. The pre-fusion F proteins may be combined with, encapsidated in or conjugated to the nanoparticles with or without adjuvant. Encapsulation within liposomes is described, e.g. in US 4,235,877. Conjugation to macromolecules is disclosed, for example in US 4,372,945 or US 4,474,757. In other embodiments, the RSV F proteins are assembled in higher order assemblies of multimers.

In other embodiments, the compositions do not comprise adjuvants.

In certain embodiments, the invention provides methods for making a vaccine against respiratory syncytial virus (RSV), comprising providing a composition according to the invention and formulating it into a pharmaceutically acceptable composition. The term "vaccine" refers to an agent or composition containing an active component effective to induce a certain degree of immunity in a subject against a certain pathogen or disease, which will result in at least a decrease (up to complete absence) of the severity, duration or other manifestation of symptoms associated with infection by the pathogen or the disease. In the present invention, the vaccine comprises an effective amount of a pre-fusion RSV F protein and/or a nucleic acid molecule encoding a pre-fusion RSV F protein, and/or a vector comprising said nucleic acid molecule, which results in an immune response against the F protein of RSV. This provides a method of preventing serious lower respiratory tract disease leading to hospitalization and the decrease in frequency of complications such as pneumonia and bronchiolitis due to RSV infection and replication in a subject. The term “vaccine”

according to the invention implies that it is a pharmaceutical composition, and thus typically includes a pharmaceutically acceptable diluent, carrier or excipient. It may or may not comprise further active ingredients. In certain embodiments it may be a combination vaccine that further comprises other components that induce an immune response, e.g. against other proteins of RSV and/or against other infectious agents. The administration of further active components may for instance be done by separate administration or by administering combination products of the vaccines of the invention and the further active components.

Compositions may be administered to a subject, e.g. a human subject. The total dose of the RSV F proteins in a composition for a single administration can for instance be about 0.01 μg to about 10 mg, e.g. 1 μg – 1 mg, e.g. 10 μg – 100 μg. Determining the recommended dose will be carried out by experimentation and is routine for those skilled in the art.

Administration of the compositions according to the invention can be performed using standard routes of administration. Non-limiting embodiments include parenteral administration, such as intradermal, intramuscular, subcutaneous, transcutaneous, or mucosal administration, e.g. intranasal, oral, and the like. In one embodiment a composition is administered by intramuscular injection. The skilled person knows the various possibilities to administer a composition, e.g. a vaccine in order to induce an immune response to the antigen(s) in the vaccine.

A subject as used herein preferably is a mammal, for instance a rodent, e.g. a mouse, a cotton rat, or a non-human-primate, or a human. Preferably, the subject is a human subject.

The proteins, nucleic acid molecules, vectors, and/or compositions may also be administered, either as prime, or as boost, in a homologous or heterologous prime-boost regimen. If a boosting vaccination is performed, typically, such a boosting vaccination will be administered to the same subject at a time between one week and one year, preferably between

two weeks and four months, after administering the composition to the subject for the first time (which is in such cases referred to as 'priming vaccination'). In certain embodiments, the administration comprises a prime and at least one booster administration.

In addition, the proteins of the invention may be used as diagnostic tool, for example to test the immune status of an individual by establishing whether there are antibodies in the serum of such individual capable of binding to the protein of the invention. The invention thus also relates to an in vitro diagnostic method for detecting the presence of an RSV infection in a patient said method comprising the steps of a) contacting a biological sample obtained from said patient with a protein according to the invention; and b) detecting the presence of antibody-protein complexes.

Stabilized pre-fusion RSV F proteins obtainable and/or obtained by such method also form part of the invention, as well as uses thereof as described above.

Examples

EXAMPLE 1: *Preparation of stable pre-fusion RSV F polypeptide of SEQ ID NO: 21*

To increase the stability of RSV F in the pre-fusion conformation two additional amino acid substitutions were introduced in a pre-fusion RSV F variant that was described previously (WO2014/174018 and WO2014/202570). The constructs were synthesized and codon-optimized at Gene Art (Life Technologies, Carlsbad, CA). The constructs were cloned into pCDNA2004 or generated by standard methods widely known within the field involving site-directed mutagenesis and PCR and sequenced. The expression platform used was the 293Freestyle cells (Life Technologies). The cells were transiently transfected using 293Fectin (Life Technologies) according to the manufacturer's instructions and cultured for 5 days at 37°C and 10% CO_2. The culture supernatant was harvested and spun for 5 minutes at 300 g to

remove cells and cellular debris. The spun supernatant was subsequently sterile filtered using a 0.22 um vacuum filter and stored at 4°C until use.

EXAMPLE 3: *Purification of pre-fusion RSV F protein*

The recombinant polypeptide of SEQ ID NO: 21 was purified by a 2-step purification protocol applying a cat-ion exchange column for the initial purification and subsequently a superdex200 column for the polishing step to remove residual contaminants. For the initial ion-exchange step the culture supernatant was diluted with 2 volumes of 50 mM NaOAc pH 5.0 and passed over a 5 ml HiTrap Capto S column at 5 ml per minute. Subsequently the column was washed with 10 column volumes (CV) of 20 mM NaOAc, 50mM NaCl, 0.01% (v/v) tween20, pH 5 and eluted 2 CV of 20 mM NaOAc, 1M NaCl, 0.01% (v/v) tween20, pH 5. The eluate was concentrated using a spin concentrator and the protein was further purified using a superdex200 column using 40mM Tris, 500mM NaCl, 0.01% (v/v) tween20, pH 7.4 as running buffer. In Figure 1 the chromatogram of the gel filtration column is shown. The dominant peak contained the pre-fusion RSV F protein. The fractions containing this peak were again pooled and the protein concentration was determined using OD280 and stored a 4°C until use. In Figure 2 a non-reduced and reduced SDS-PAGE analysis of the final protein preparation is shown and as can be seen the purity was >95%. The identity of the band was verified using western blotting and protein F specific antibodies (not shown).

Quantitative Octet (BioLayer Interferometry) was used for measuring protein concentration in the supernatants. CR9501 (an antibody specifically recognizing pre-fusion RSV F protein) and CR9503 (recognizing post-fusion RSV F protein) were biotinylated by standard protocols and immobilized on Streptavidin biosensors (ForteBio, Portsmouth, UK). Afterwards, the coated biosensors were blocked in mock cell culture supernatant. A

quantitative experiment was performed as follows: temperature 30C, shaking speed 1000 rpm, time of the assay 300 sec.

The concentration of the protein was calculated using a standard curve. The standard curve was prepared for each coated antibody using the pre-fusion RSV F protein (Krarup et. al., 2015, *supra*) diluted in mock medium (Figure 3). The data analysis was done using the ForteBio Data Analysis 6.4 software (ForteBio).

EXAMPLE 3: *Temperature stability of the RSV F protein*

Temperature stability of the purified protein was determined by differential scanning fluorometry (DSF). The purified pre-fusion F protein was mixed with SYPRO orange fluorescent dye (Life Technologies S6650) in a 96-well optical qPCR plate. The optimal dye and protein concentration was determined experimentally (data not shown). Protein dilutions were performed in PBS, and a negative control sample containing the dye only was used as a reference subtraction. The measurement was performed in a qPCR instrument (Applied Biosystems ViiA 7) using the following parameters: a temperature ramp from 25–95 °C with a rate of 0.015 °C per second. Data was collected continuously. The melting curves were plotted using GraphPad PRISM software (version 5.04). Melting temperatures were calculated at the 50% maximum of fluorescence using a non-linear EC50 shift equation. The melting temperature of the RSV F protein of SEQ ID NO: 21 was 68.5 degrees (Fig. 4). A reference pre-fusion RSV F without substitutions at position 357 and 371 had a melting temperature of 65.0 which means the double mutation increased the melting temperature by 3.5 degrees.

Table 1. Standard amino acids, abbreviations and properties

Amino Acid	3-Letter	1-Letter	Side chain polarity	Side chain charge (pH 7.4)
alanine	Ala	A	non-polar	Neutral
arginine	Arg	R	polar	Positive
asparagine	Asn	N	polar	Neutral
aspartic acid	Asp	D	polar	Negative
cysteine	Cys	C	non-polar	Neutral
glutamic acid	Glu	E	polar	Negative
glutamine	Gln	Q	polar	Neutral
glycine	Gly	G	non-polar	Neutral
histidine	His	H	polar	positive(10%) neutral(90%)
isoleucine	Ile	I	non-polar	Neutral
leucine	Leu	L	non-polar	Neutral
lysine	Lys	K	polar	Positive
methionine	Met	M	non-polar	Neutral
phenylalanine	Phe	F	non-polar	Neutral
proline	Pro	P	non-polar	Neutral
serine	Ser	S	polar	Neutral
threonine	Thr	T	polar	Neutral
tryptophan	Trp	W	non-polar	Neutral
tyrosine	Tyr	Y	polar	Neutral
valine	Val	V	non-polar	Neutral

Table 2. Amino acid sequences of antibodies CR9501 and CR9502

Ab	VH domain	VH CDR1	VH CDR2	VH CDR3
CR9501	Amino acids 1-125 of SEQ ID NO: 16	GASINSDNYYWT (SEQ ID NO:1)	HISYTGNTYYTPSLKS (SEQ ID NO:2)	CGAYVLISNCGWFDS (SEQ ID NO:3)
CR9502	Amino acids 1-121 of SEQ ID NO:18	GFTFSGHTIA (SEQ ID NO:7)	WVSTNNGNTEYAQKIQG (SEQ ID NO:8)	EWLVMGGFAFDH (SEQ ID NO:9)

Ab	VL domain	VL CDR1	VL CDR2	VL CDR3
CR9501	Amino acids 1-107 of SEQ ID NO: 17	QASQDISTYLN (SEQ ID NO: 4)	GASNLET (SEQ ID NO:5)	QQYQYLPYT (SEQ ID NO:6)
CR9502	Amino acids 1-110 of SEQ ID NO: 19	GANNIGSQNVH (SEQ ID NO:10)	DDRDRPS (SEQ ID NO:11)	QVWDSSRDQAVI (SEQ ID NO:12)

Sequences

RSV F protein full length sequence (SEQ ID NO: 13)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKENKCNGTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMN
YTLNNAKKTNVTLSKKRKRRFLGFLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLS
TNKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPIVNKQSCSISNIETVIEFQQKNNRLLE
ITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSI
IKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSV
ITSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNKQE
GKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAVKST
TNIMITTIIIVIIVILLSLIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN

RSV F protein B1 full length sequence (SEQ ID NO: 15)

MELLIHRLSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
LSNIKETKCNGTDTKVKLIKQELDKYKNAVTELQLLMQNTPAANNRARREAPQYMN
YTINTTKNLNVSISKKRKRRFLGFLLGVGSAIASGIAVSKVLHLEGEVNKIKNALLSTN
KAVVSLSNGVSVLTSKVLDLKNYINNQLLPIVNQQSCRISNIETVIEFQQKNSRLLEIN
REFSVNAGVTTPLSTYMLTNSELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIIK
EEVLAYVVQLPIYGVIDTPCWKLHTSPLCTTNIKEGSNICLTRTDRGWYCDNAGSVSF
FPQADTCKVQSNRVFCDTMNSLTLPSEVSLCNTDIFNSKYDCKIMTSKTDISSSVITSL
GAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNKLEGKN
LYVKGEPIINYYDPLVFPSDEFDASISQVNEKINQSLAFIRRSDELLHNVNTGKSTTNI
MITTIIIVIIVVLLSLIAIGLLLYCKAKNTPVTLSKDQLSGINNIAFSK

SEQ ID NO: 14 (fibritin)

GYIPEAPRDGQAYVRKDGEWVLLSTFL

RSV F protein CL57-v224 full length sequence (SEQ ID NO: 20)

MELPILKTNAITTILAAVTLCFASSQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKENKCNGTDAKVKLIKQELDKYKNAVTELQLLMQSTPAANNRARRELPRFMN
YTLNNTKNNNVTLSKKRKRRFLGFLLGVGSAIASGIAVSKVLHLEGEVNKIKSALLST
NKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPIVNKQSCSISNIETVIEFQQKNNRLLEI
TREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSII
KEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNIDIFNPKYDCKIMTSKTDVSSSVI
TSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNKQEG
KSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNVGKSTT
NIMITTIIIVIIVILLLLIAVGLFLYCKARSTPVTLSKDQLSGINNIAFSN

RSV F, N67I, S215P, D486N, and additional cys-bridge 155-290 (SEQ ID NO: 21)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKEIKCNGTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNY
TLNNAKKTNVTLSKKRKRRFLGFLLGVGSAIASGVAVCKVLHLEGEVNKIKSALLST
NKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPIVNKQSCSIPNIETVIEFQQKNNRLLEI
TREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMCI
IKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSV
ITSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNKQE
GKSLYVKGEPIINFYDPLVFPSNEFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAP
RDGQAYVRK
DGEWVLLSTFL

CR9501 heavy chain (SEQ ID NO: 16):

QVQLVQSGPGLVKPSQTLALTCNVSGASINSDNYYWTWIRQRPGGGLEWIGHISYTG
NTYYTPSLKSRLSMSLETSQSQFSLRLTSVTAADSAVYFCAACGAYVLISNCGWFDS
WGQGTQVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC

CR9501 light chain (SEQ ID NO: 17):

EIVMTQSPSSLSASIGDRVTITCQASQDISTYLNWYQQKPGQAPRLLIYGASNLETGVP
SRFTGSGYGTDFSVTISSLQPEDIATYYCQQYQYLPYTFAPGTKVEIKRTVAAPSVFIF
PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS
LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

CR9502 heavy chain (SEQ ID NO: 18):

EVQLLQSGAELKKPGASVKISCKTSGFTFSGHTIAWVRQAPGQGLEWMGWVSTNNG
NTEYAQKIQGRVTMTMDTSTSTVYMELRSLTSDDTAVYFCAREWLVMGGFAFDHW
GQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSC

CR9502 light chain (SEQ ID NO: 19):

QSVLTQASSVSVAPGQTARITCGANNIGSQNVHWYQQKPGQAPVLVVYDDRDRPSG
IPDRFSGSNSGNTATLTISRVEAGDEADYYCQVWDSSRDQAVIFGGGTKLTVLGQPK
AAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQS
NNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTIAPTECS

Claims

1. A recombinant pre-fusion respiratory syncitial virus (RSV) Fusion (F) protein comprising (a) a mutation of the amino acid residue N/T on position 67 into I; (b) a mutation of the amino acid residue S on position 215 into P; (c) a mutation of the amino acid residue D on position 486 into N; and wherein the protein further comprises a mutation of the amino acid residue S on position 155 into C (S155C) and of the amino acid S on position 290 into C (S290C).

2. Pre-fusion RSV F protein according to claim 1, wherein the protein comprises at least one epitope that is specific to the pre-fusion conformation F protein, wherein the at least one epitope is recognized by a pre-fusion specific monoclonal antibody, comprising a heavy chain CDR1 region of SEQ ID NO: 1, a heavy chain CDR2 region of SEQ ID NO: 2, a heavy chain CDR3 region of SEQ ID NO: 3 and a light chain CDR1 region of SEQ ID NO: 4, a light chain CDR2 region of SEQ ID NO: 5, and a light chain CDR3 region of SEQ ID NO: 6 and/or a pre-fusion specific monoclonal antibody, comprising a heavy chain CDR1 region of SEQ ID NO: 7, a heavy chain CDR2 region of SEQ ID NO: 8, a heavy chain CDR3 region of SEQ ID NO: 9 and a light chain CDR1 region of SEQ ID NO: 10, a light chain CDR2 region of SEQ ID NO: 67, and a light chain CDR3 region of SEQ ID NO: 11.

3. Pre-fusion RSV F protein according to claim 1 or 2, wherein the protein is trimeric.

4. Pre-fusion RSV F protein according to any one of the preceding claims 1-3, comprising a truncated F1 domain and a heterologous trimerization domain linked to said truncated F1 domain.

5. Pre-fusion RSV F protein according to claim 4, wherein the heterologous trimerization domain comprises the amino acid sequence GYIPEAPRDGQAYVRKDGEWVLLSTFL (SEQ ID NO: 14).

6. Pre-fusion RSV F protein according to claim 4 or 5, wherein the trimerization domain is linked to amino acid residue 513 of the RSV F protein.

7. Pre-fusion RSV F protein according to any one of the preceding claims, wherein the RSV F protein comprises the amino acid sequence of SEQ ID NO: 21.

8. Nucleic acid molecule encoding a pre-fusion RSV F protein according to any one of the preceding claims 1-7.

9. Nucleic acid molecule according to claim 8, wherein the nucleic acid molecule has been codon-optimized for expression in mammalian cells.

10. Vector comprising a nucleic acid molecule according to claim 8 or claim 9.

11. Composition comprising a pre-fusion RSV F protein according to any of the claims 1-7, a nucleic acid molecule according to claim 8 or claim 9 and/or a vector according to claim 10.

12. Pre-fusion RSV F protein according to any of the claims 1-7, a nucleic acid molecule according to claim 8 or claim 9, and/or a vector according to claim 10 for use in inducing an immune response against RSV F protein.

13. Pre-fusion RSV F protein according to any of the claims 1-7, a nucleic acid molecule according to claim 8 or claim 9, and/or a vector according to claim 10 for use as a vaccine.

14. Pre-fusion RSV F protein according to any of the claims 1-7, a nucleic acid molecule according to claim 8 or claim 9, and/or a vector according to claim 10 for use in the prophylaxis and/or treatment of RSV infection.

Column:	Superdex200 16/600
Buffer:	40 mM TRIS, 150 mM NaCl, pH 7.5
Loop (ml):	4
Sample volume (ml):	
Flow (ml / min):	1,0
Fractions (ml):	1 ml
Temperature °C:	18
Comments:	

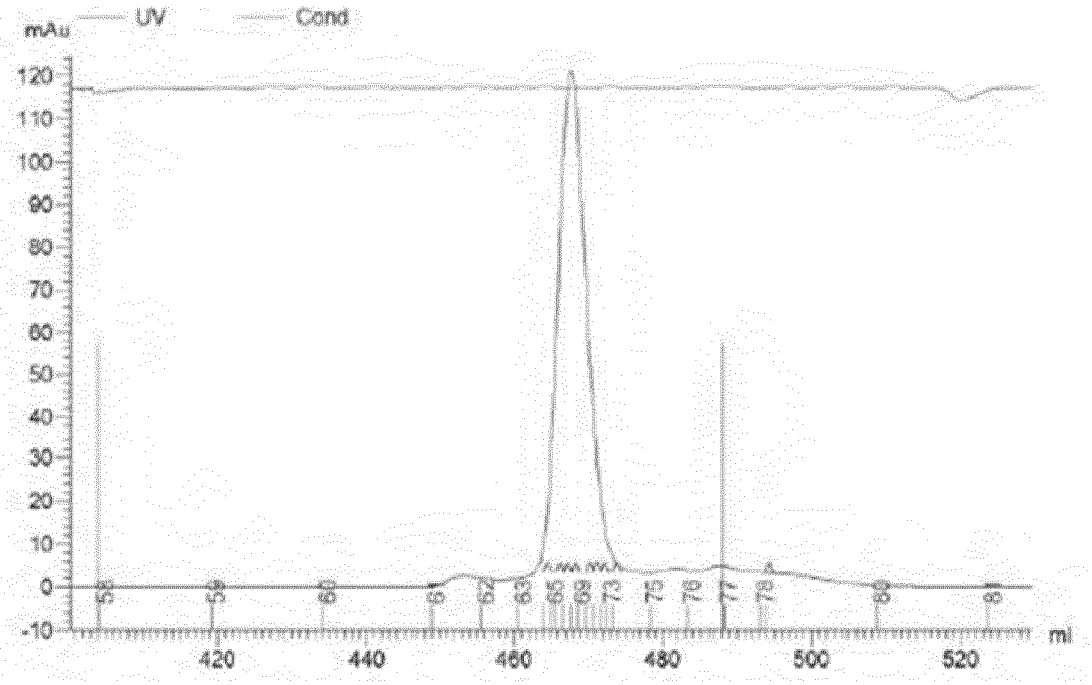

FIG.1

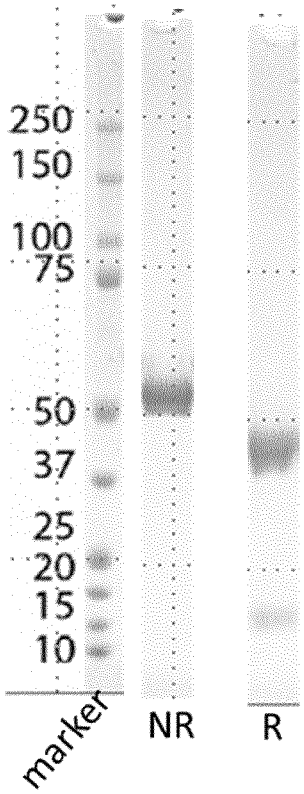

FIG. 2

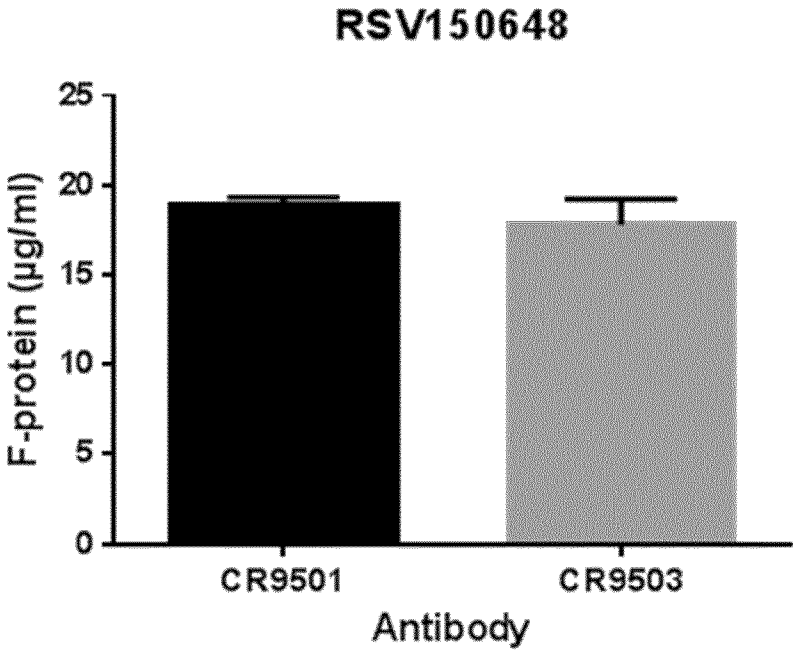
RSV150648
25
20
15
10
5
0
F-protein (µg/ml)
CR9501
CR9503
Antibody

FIG. 3

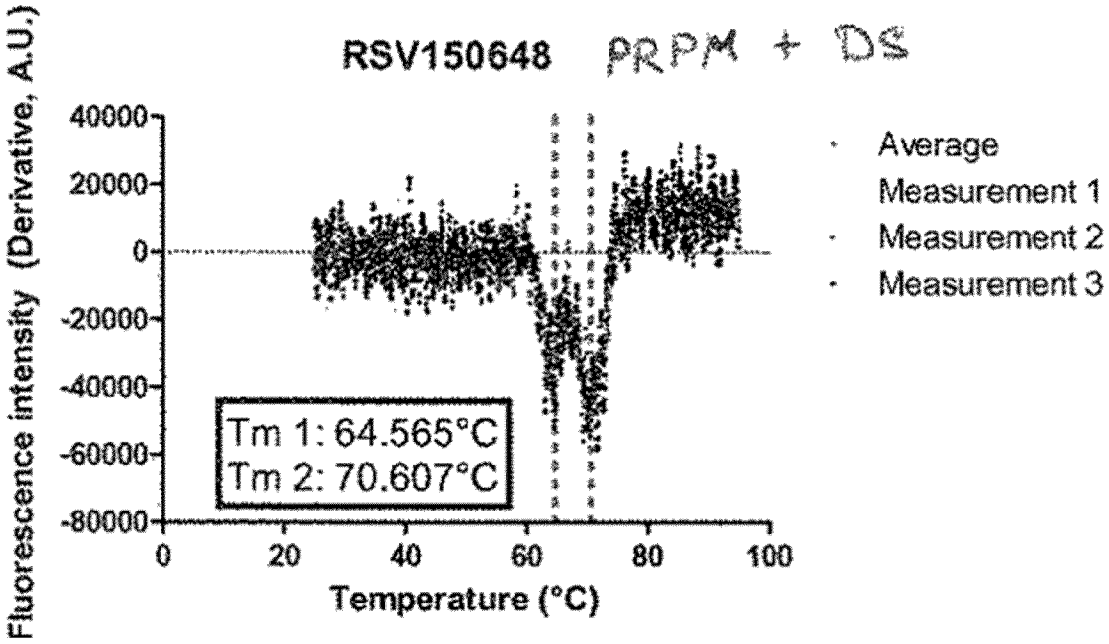
RSV150648 PRPM + DS
Fluorescence intensity (Derivative, A.U.)
40000
20000
0
-20000
-40000
-60000
-80000
0
20
40
60
80
100
Temperature (°C)
Tm 1: 64.565°C
Tm 2: 70.607°C
Average
Measurement 1
Measurement 2
Measurement 3

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/062870

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/135
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/174018 A1 (CRUCELL HOLLAND BV [NL]) 30 October 2014 (2014-10-30) cited in the application sequence 79 table 11 claim 30 -----	1-14
X	WO 2015/013551 A1 (MARSHALL CHRISTOPHER PATRICK [US]; MCLELLAN JASON SCOTT [US]; ALFF PET) 29 January 2015 (2015-01-29) sequence 9 claim 13 -----	1-14

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 August 2017	14/08/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Herrmann, Klaus

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/062870

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014174018 A1	30-10-2014	AR 096113 A1	09-12-2015
		AU 2014259474 A1	05-11-2015
		CA 2910067 A1	30-10-2014
		CL 2015003124 A1	13-05-2016
		CN 105188745 A	23-12-2015
		EA 201592048 A1	29-04-2016
		EP 2988780 A1	02-03-2016
		HK 1220124 A1	28-04-2017
		JP 2016520039 A	11-07-2016
		KR 20160002938 A	08-01-2016
		PE 18672015 A1	07-01-2016
		PH 12015502377 A1	22-02-2016
		SG 11201508567X A	27-11-2015
		TW 201520221 A	01-06-2015
		US 2016102123 A1	14-04-2016
		WO 2014174018 A1	30-10-2014
WO 2015013551 A1	29-01-2015	CA 2919353 A1	29-01-2015
		EP 3024483 A1	01-06-2016
		JP 2016527253 A	08-09-2016
		US 2015030622 A1	29-01-2015
		WO 2015013551 A1	29-01-2015