conformation (Battles *et al*. 2017. *Nat. Commun.* 8(1): 1528) and mediates fusion of the virion envelope and the host cell plasma membrane. During the fusion process, the F protein undergoes irreversible refolding from the labile pre-fusion conformation to the stable post-fusion conformation (see Figure 2).

Neutralizing antibodies specifically recognizing the pre-fusion hMPV F protein structure were found in human sera (Wen *et al*. 20012. *Nat. Struct. Mol. Biol.* 19: 461–463; Ngwuta *et al.* 2015. *Science Translational Medicine* 7(309): 309; Rossey *et al.* 2018. *Trends in Microbiology* 26(3): 209–19) indicating that the pre-fusion F protein could be a favorable vaccine candidate (Melero & Mas. 2015. *Virus Res.* 209: 128-35). One clear disadvantage of the pre-fusion over post-fusion F protein conformation is its instability. Previous attempts to produce stabilized pre-fusion F protein employed thorough structural analysis and computer modeling. In particular, one group described design of a highly stable pre-fusion RSV F protein, capable to provide protective response in rats (see *Krarup et al*. 2015. *Nat. Commun.* 6: 8143). Another group disclosed construction of stabilized pre-fusion forms of the hMPV F protein, which elicited neutralizing antibodies in mice immunized with those proteins (see WO2016/103238).

Crystal structures of the F protein in pre-fusion and post-fusion conformations were determined for hMPV, RSV and other paramyxoviruses (see e.g. Battles *et al.* 2017. *Nat. Commun.* 8(1): 1528). In spite of general structural similarities, it was revealed that the pre-fusion hMPV F protein possesses unique structural features that confer substantial functional and immunological differences between the F proteins of hMPV and RSV.

Despite significant progress in understanding a mechanism of action, structure and immunogenic properties of the hMPV F protein, no F protein based vaccine is on the market. Therefore, the objective of this invention is to provide novel modified pre-fusion hMPV F protein candidates for development of a human vaccine against a respiratory tract infection.

SUMMARY

The present disclosure provides recombinant immunogenic human metapneumovirus (hMPV) F proteins and fragments thereof (herein referred to as the hMPV F proteins) capable to elicit neutralizing antibodies and protect against hMPV infection. A native coding sequence of the hMPV F protein was modified to generate stable pre-fusion conformation. Such modifications were designed based on three-dimensional (3D) homology models included as a part of the present disclosure. The disclosure further includes methods of producing the recombinant immunogenic proteins and methods of using the immunogenic proteins for prevention and/or treatment of hMPV infection in humans.

In one example, the present disclosure provides a modified hMPV F protein or a fragment thereof, stabilized in the pre-fusion conformation, comprising a single-chain polypeptide composed of an F2 domain, a heterologous peptide linker and an F1 domain lacking a fusion peptide (FP), wherein the linker is positioned between the F2 and F1 domains and contains one or more cysteine residue(s) each of which forms a non-natural disulfide bond with a cysteine residue present in the F1 domain. In one aspect, the present disclosure refers to an immunogenic human metapneumovirus (hMPV) modified F protein or fragment thereof, stabilized in a pre-fusion conformation, comprising a recombinant single-chain polypeptide lacking a fusion peptide (FP) and comprising an F2 domain, a heterologous peptide linker and an F1 domain, wherein the linker is positioned between the F2 and F1 domains and contains a cysteine residue which forms a disulfide bond with a non-natural cysteine residue present in the F1 domain.

In one example, the single-chain F protein comprises F2 domain and F1 domains connected so that the C-terminus of F2 is proximal to the N-terminus of F1. A protease cleavage site between F2 and F1 may be mutated to eliminate the cleavage of the protein precursor. In some examples, the F1 domain may be a truncated F1 domain, e.g. so that it lacks the fusion peptide (FP) spanning the amino acid residues at positions 103-118 of the native hMPV F protein sequence of SEQ ID NO: 1, corresponding to residues 1 to 16 of the native F1 domain sequence of SEQ ID NO: 3. Thus, the F1 domain may comprise a fragment corresponding to residues 119 to 539 of SEQ ID NO: 1 or residues 17 to 437 of SEQ ID NO: 3. In some examples, the single-chain polypeptide may comprise a fragment of the F1 domain corresponding to residues 119 to 490 of SEQ ID NO: 1 or residues 17 to 338 of SEQ ID NO: 3, which does not contain an anchor transmembrane (TM) domain and a cytoplasmic tail at its C-terminus. Additionally, the F1 and F2 domains may be joined via a heterologous peptide linker containing e.g. five residues comprising at least one cysteine residue, preferably the linker of SEQ ID NO: 4.

In yet one example, the single-chain F protein of the present disclosure has a stable pre-fusion conformation. On one side, the pre-fusion conformation is stabilized by abolished protease cleavage between F1 and F2 domains and lack of the free N-terminus of F1. Another feature that confers stabilization is the presence of at least one additional (including a non-natural) disulfide bond, which fixes the HRA domain inside the cavity formed by trimerization (see Figure 3). In one example, a non-natural disulfide bond can be formed between a cysteine residue of the heterologous peptide linker inserted between F2 and F1 and a cysteine residue located in the F1 domain, preferably in the C-terminal region thereof and located within said cavity. For instance, a non-natural disulfide bond can be formed between a cysteine residue in the peptide linker and a non-natural cysteine residue present in the F1 domain at position 338 of the native hMPV F protein sequence of SEQ ID NO: 1, corresponding to position 236 of the native F1 domain sequence of SEQ ID NO: 3. In such examples, the F1 domain present in the single-chain F protien (e.g. a truncated F1 domain lacking the fusion peptide of residues 1-16 of SEQ ID NO: 3) may comprise a mutation such as A236C in the sequence of SEQ ID NO: 3 (corresponding to A338C in SEQ ID NO: 1). The cysteine residue in the peptide linker may, for example, be immediately adjacent to the F2 domain, e.g. may be the first residue at the N-terminus of the heterologous linker and adjacent to the C-terminus of the F2 domain. For instance, the cysteine residue in the peptide linker may be present in the recombinant polypeptide at a position equivalent to residue 103 of SEQ ID NO: 1.

In a further example, the single-chain F protein may comprise one or more additional modification(s) that compensate for an altered geometry of the single-chain-containing F trimer. Preferably, said modification(s) is(are) substitution(s) at positions corresponding to positions 49, 51, 67, 80, 97, 137, 147, 159, 160, 161, 166, 177, 185, 258, 266, 294, 480 and/or 481 of the native hMPV F protein sequence of SEQ ID NO: 1. In particular, an asparagine at position 97 can be substituted for a glutamate (N97Q) or an alanine at position 185 can be substituted for a proline (A185P). Thus, the recombinant polypeptide may comprise the F2 domain comprising one or more substitution(s) at positions 31, 33, 49, 62 and/or 79 of SEQ ID NO: 2. The recombinant polypeptide may comprise an F1 domain (e.g. a truncated F1 domain lacking residues 1-16 of SEQ ID NO: 3) comprising one or more substitution(s) at positions 35, 45, 57, 58, 59, 64, 75, 83, 156, 164, 192, 378 and/or 379 of SEQ ID NO: 3.

Furthermore, some mutation(s) can compensate for a deficiency of cavity filling. Particularly, the cavity filling mutations can be selected from the list comprising the amino acid substitutions at positions 49, 67, 80, 137, 147, 159, 160, 161, 177 and 258 of the native hMPV F protein sequence of SEQ ID NO: 1. In one example, the cavity filling mutations include a T49M substitution, an I67L substitution, an I137W substitution, an A147V substitution, an A159V substitution, a T160F substitution, an A161M substitution and/or an I177L substitution in SEQ ID NO: 1. Thus in some examples, the recombinant polypeptide may comprise an F2 domain comprising a T31M or I49L substitution in the sequence of SEQ ID NO: 2. In other examples, the recombinant polypeptide may comprise an (e.g. truncated) F1 domain comprising one or more substitutions in SEQ ID NO: 3 selected from I35W, A45V, A57V, T58F, A59M, I75L and/or F156I.

In another example, the recombinant single-chain F protein may comprise one or more substitution(s) leading to formation of non-natural hydrogen bond(s) or salt bridge(s). For example, such substitutions include an E80N and S266D in SEQ ID NO: 1. Thus the recombinant polypeptide may comprise the F2 domain comprising a E62N substitution in the sequence of SEQ ID NO: 2. In other examples, the recombinant polypeptide may comprise an (e.g. truncated) F1 domain comprising the substitution S164D in SEQ ID NO: 3.

In some examples, the recombinant single-chain F protein may comprise further cysteine substitution(s) for creation an additional stabilizing disulfide bond(s). For example, substitutions E51C and K166C can form a non-natural disulfide bond between a cysteine residues at position 51 of the β-strand of the F2 domain and a cysteine at position 166 of the HRA α4 element of the native hMPV F protein sequence of SEQ ID NO: 1. This modification impairs a possible salt-bridge between E51 and K138 from a helix in HRA. Mutation S266D introduces a non-natural salt-bridge to K138 to compensate

loss of attachment for this helix. Additionally, substitution of the vicinal residues I480 and L481 of SEQ ID NO: 1 for cysteine allows introduction of three disulfide bonds across three protomers to make the covalently linked trimer protein. Thus in one example, the recombinant polypeptide may comprise an F2 domain comprising a E33C substitution in the sequence of SEQ ID NO: 2. In other examples, the recombinant polypeptide may comprise an (e.g. truncated) F1 domain comprising the substitution(s) K64C, S164D, I378C and/or L379C in SEQ ID NO: 3.

In another example, the recombinant single-chain F protein may comprise a modification(s) helpful for expression of a soluble recombinant protein. For instance, a substitution of a glycine for a glutamic acid residue may be present at position 294 (G294E) of the native hMPV F protein sequence of SEQ ID NO: 1, which may lead to a higher yield of the protein expression. Thus in one example, the recombinant polypeptide may comprise a (e.g. truncated) F1 domain comprising a substitution G192E in SEQ ID NO: 3.

In some examples, the recombinant single-chain F protein may comprise combinations of two, three, four, five, six, seven, eight, nine, ten or more amino acid substitutions and/or other modifications.

In some examples, the recombinant single-chain F protein may comprise a trimerization helper, so called foldon domain, e.g. linked to the C-terminus of the recombinant F protein subunit, that allows formation of a protein trimer. The foldon domain may derive from fibritin of T4 bacteriophage. The recombinant hMPV F protein of the present disclosure may be produced as mono- or hetero-trimer.

In some examples, the recombinant hMPV F protein may comprise or consist of an amino acid sequence having at least 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the amino acid sequence of any one of SEQ ID NOs: 5 to 9 or 24 to 28. The recombinant single-chain F protein may comprise an F2 domain comprising or consisting of an amino acid sequence having at least 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the amino acid sequence of SEQ ID NO: 2. The recombinant single-chain F protein may comprise an F1 domain comprising or consisting of an amino acid sequence having at least 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the amino acid sequence of SEQ ID NO: 3. In some examples, the recombinant single-chain F protein may comprise a truncated F1 domain comprising or consisting of an amino acid sequence having at least 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to residues 17-437 or 17-388 of the amino acid sequence of SEQ ID NO: 3.

The recombinant hMPV proteins of the present disclosure are immunogenic and can induce neutralizing antibodies recognizing the native hMPV F protein. The present disclosure also includes immunogenic fragments of the recombinant hMPV proteins and the immunogenic proteins having at least 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the sequence of anyone of SEQ ID NOs: 5 to 9 or 24 to 28.

In one aspect, the present disclosure refers to an immunogenic protein comprising or consisting of the amino acid sequence as set forth in SEQ ID NO: 5 or SEQ ID NO: 25, or a variant thereof having at least 85%, 90%, 95%, 98% or 99% sequence identity thereto.
In one aspect, the present disclosure refers to an SEQ ID NO: 6 or SEQ ID NO: 24, or a variant thereof having at least 85%, 90%, 95%, 98% or 99% sequence identity thereto.
In one aspect, the present disclosure refers to an immunogenic protein comprising or consisting of the amino acid sequence as set forth in SEQ ID NO: 7 or SEQ ID NO: 26, or a variant thereof having at least 85%, 90%, 95%, 98% or 99% sequence identity thereto.
In one aspect, the present disclosure refers to an immunogenic protein comprising or consisting of the amino acid sequence as set forth in SEQ ID NO: 8 or SEQ ID NO: 27, or a variant thereof having at least 85%, 90%, 95%, 98% or 99% sequence identity thereto.
In one aspect, the present disclosure refers to an immunogenic protein comprising or consisting of the amino acid sequence as set forth in SEQ ID NO: 9 or SEQ ID NO: 28, or a variant thereof having at least 85%, 90%, 95%, 98% or 99% sequence identity thereto.
The present disclosure also provides isolated nucleic acid molecules encoding the modified hMPV F-proteins, vectors comprising the isolated nucleic acid molecules, host cells for recombinant expression of the modified hMPV F proteins.

The present disclosure also provides immunogenic compositions or vaccines comprising the recombinant hMPV F proteins, or isolated DNA molecules encoding the hMPV F protein or vectors of the disclosure, further comprising a pharmaceutically acceptable carrier and/or excipient, used with or without an adjuvant. Particularly, the disclosure provides immunogenic compositions or vaccines for stimulating an immune response in a subject, particularly an immune response, which can neutralize hMPV viruses and protect against hMPV infections. In one aspect, the present disclosure refers to an immunogenic composition or vaccine comprising the immunogenic protein disclosed herein, further comprising a pharmaceutically acceptable carrier and/or excipient, optionally further comprising an adjuvant, for prevention and/or treatment of the hMPV infection in a human subject. In another aspect, the present disclosure refers to use of an immunogenic composition or vaccine comprising the immunogenic protein disclosed herein, further comprising a pharmaceutically acceptable carrier and/or excipient, optionally further comprising an adjuvant, in the manufacture of a medicament for prevention and/or treatment of the hMPV infection in a human subject. The disclosure further provides immunogenic compositions or vaccines comprising additional antigens derived from hMPV, RSV or PIV3 (parainfluenza virus type 3). The immunogenic proteins, isolated DNA molecules, vectors and immunogenic compositions or vaccines disclosed herein are suitable for use as a medicament, particularly for the prophylactic and/or therapeutic treatment of viral respiratory tract infections and associated diseases, especially infections and disease caused by hMPV.

Methods of production the recombinant hMPV F proteins, or isolated DNA molecules encoding the hMPV F protein or immunogenic compositions (vaccines) are encompassed in the present disclosure. Methods of generating an immune response in a subject, and methods of treating, inhibiting or preventing hMPV infections are also included.

In a further example, the present disclosure provides an immunogenic human metapneumovirus (hMPV) modified F protein or fragment thereof, stabilized in a pre-fusion conformation by one or more amino acid substitutions relative to a native hMPV F protein sequence; wherein the modified F protein or fragment thereof comprises a glutamine residue substituted for an asparagine residue at a position corresponding to position 97 of the native hMPV F protein sequence of SEQ ID NO: 1 (N97Q).

In a further example, the present disclosure provides an immunogenic human metapneumovirus (hMPV) modified F protein or fragment thereof, stabilized in a pre-fusion conformation by one or more amino acid substitutions relative to a native hMPV F protein sequence; wherein the modified F protein or fragment thereof comprises a glycine residue substituted for a glutamic acid residue at a position corresponding to position 294 of the native hMPV F protein sequence of SEQ ID NO: 1 (G294E).

In a further example, the present disclosure provides an immunogenic human metapneumovirus (hMPV) modified F protein or fragment thereof, stabilized in a pre-fusion conformation by one or more amino acid substitutions relative to a native hMPV F protein sequence; wherein the modified F protein or

fragment thereof comprises one or more substitution(s) at positions corresponding to positions 49, 51, 67, 80, 137, 147, 159, 160, 161, 166, 177, 258, 266, 480 and/or 481 of the native hMPV F protein sequence of SEQ ID NO: 1.

In a further example, the present disclosure provides an immunogenic human metapneumovirus (hMPV) modified F protein or fragment thereof, stabilized in a pre-fusion conformation by two or more amino acid substitutions relative to a native hMPV F protein sequence; wherein the modified F protein or fragment thereof comprises the substitutions E51C and K166C relative to the native hMPV F protein sequence of SEQ ID NO: 1, and wherein the substituted cysteine residues form a non-native disulfide bond.

In a further example, the present disclosure provides an immunogenic human metapneumovirus (hMPV) modified F protein or fragment thereof, stabilized in a pre-fusion conformation by one or more amino acid substitutions relative to a native hMPV F protein sequence; wherein the modified F protein or fragment thereof comprises one or more substitutions selected from the group consisting of T49M, E80N, I137W, A147V, A159V, T160F, A161M, I67L, I177L, F258I, S266D, I480C and/or L481C relative to the native hMPV F protein sequence of SEQ ID NO: 1.

In a further example, the present disclosure provides an immunogenic human metapneumovirus (hMPV) modified F protein or fragment thereof, stabilized in a pre-fusion conformation by three or more amino acid substitutions relative to a native hMPV F protein sequence; wherein the modified F protein or fragment thereof comprises at least the substitutions T49M, A161M and I67L or I177L relative to the native hMPV F protein sequence of SEQ ID NO: 1.

In a further example, the present disclosure provides an immunogenic human metapneumovirus (hMPV) modified F protein or fragment thereof, stabilized in a pre-fusion conformation by three or more amino acid substitutions relative to a native hMPV F protein sequence; wherein the modified F protein or fragment thereof comprises one of the following substitution combinations relative to the native hMPV F protein sequence of SEQ ID NO: 1:

N97Q, R102G and G294E;

N97Q, R102G, T160F, I177L and G294E;

N97Q, R102G, T49M, I67L, A161M, E80N, F258I and G294E;

N97Q, R102G, T49M, I67L, A161M, E51C, K166C, S266D, G294E, I480C and L481C; or

N97Q, R102G, T49M, A161M, I137W, A159V, A147V, I177L and G294E.

Unless otherwise specified herein, all amino acid positions mentioned in the present specification correspond to the amino acid positions of the native hMPV F protein sequence of SEQ ID NO: 1. The

CLAIMS

1. An immunogenic human metapneumovirus (hMPV) modified F protein or fragment thereof, stabilized in a pre-fusion conformation, comprising a recombinant single-chain polypeptide lacking a fusion peptide (FP) and comprising an F2 domain, a heterologous peptide linker and an F1 domain, wherein the linker is positioned between the F2 and F1 domains and contains a cysteine residue which forms a disulfide bond with a non-natural cysteine residue present in the F1 domain.

2. The protein or fragment thereof of claim 1, wherein the F2 domain comprises or consists of an amino acid sequence having at least 85%, at least 90%, at least 95% or at least 99% sequence identity to SEQ ID NO: 2.

3. The protein or fragment thereof of claim 1 or 2, wherein the F1 domain comprises or consists of an amino acid sequence having at least 85%, at least 90%, at least 95% or at least 99% sequence identity to SEQ ID NO: 3 or to residues 17-388 thereof.

4. The protein or fragment thereof of any preceding claim, wherein:
(a) the F1 domain comprises a cysteine residue substituted at position 236 of SEQ ID NO: 3;
(b) the recombinant single-chain polypeptide comprises a cysteine residue substituted at position 338 relative to the amino acid positions of a native hMPV F protein sequence of SEQ ID NO: 1;
(c) the linker comprises a cysteine residue which forms a disulfide bond with a non-natural cysteine residue substituted at position 236 in the F1 domain of SEQ ID NO: 3; and/or
(d) the linker comprises a cysteine residue which forms a disulfide bond with a non-natural cysteine residue substituted at position 338 relative to the amino acid positions of the native hMPV F protein sequence of SEQ ID NO: 1.

5. The protein of any preceding claim, wherein the linker consists of 1 to 5, preferably 5 amino acids, preferably selected from the group consisting of CGAGA, CGAGV, CGAAV, AGCGA, CAAAV, and CAAFV sequences.

6. The protein of any preceding claim, wherein the single-chain polypeptide further comprises one or more substitution(s) relative to the native hMPV F protein sequence of SEQ ID NO: 1 that stabilize the pre-fusion conformation.

7. The protein of any proceeding claim, wherein the single-chain polypeptide comprises one of the following substitution combinations:

N97Q, R102G and G294E;

N97Q, R102G, T160F, I177L and G294E;

T49M, I67L, E80N, N97Q, R102G, A161M, F258I and G294E;

T49M, E51C, I67L, N97Q, R102G, A161M, K166C, S266D, G294E, I480C and L481C; or

T49M, N97Q, R102G, I137W, A147V, A159V, A161M, I177L and G294E ,

wherein the positions are relative to the amino acid positions of the native hMPV F protein sequence of SEQ ID NO: 1.

8. The protein of any preceding claim, wherein the single-chain polypeptide comprises a trimerization helper domain (foldon), consisting of the amino acid sequence of SEQ ID NO: 10 or any of SEQ ID NOs 29 to 33, and optionally further comprising the amino acid sequence of SEQ ID NO: 34 or SEQ ID NO: 35 upstream of the foldon domain.

9. The protein of any preceding claim, wherein the single-chain polypeptide comprises or consists of an amino acid sequence of any of SEQ ID NOs: 5 to 9 or 24 to 28.

10. An immunogenic protein comprising or consisting of the amino acid sequence as set forth in SEQ ID NO: 5 or SEQ ID NO: 25, or a variant thereof having at least 85%, 90%, 95%, 98% or 99% sequence identity thereto.

11. An immunogenic protein comprising or consisting of the amino acid sequence as set forth in SEQ ID NO: 6 or SEQ ID NO: 24, or a variant thereof having at least 85%, 90%, 95%, 98% or 99% sequence identity thereto.

12. An immunogenic protein comprising or consisting of the amino acid sequence as set forth in SEQ ID NO: 7 or SEQ ID NO: 26, or a variant thereof having at least 85%, 90%, 95%, 98% or 99% sequence identity thereto.

13. An immunogenic protein comprising or consisting of the amino acid sequence as set forth in SEQ ID NO: 8 or SEQ ID NO: 27, or a variant thereof having at least 85%, 90%, 95%, 98% or 99% sequence identity thereto.

14. An immunogenic protein comprising or consisting of the amino acid sequence as set forth in SEQ ID NO: 9 or SEQ ID NO: 28, or a variant thereof having at least 85%, 90%, 95%, 98% or 99% sequence identity thereto.

15. An immunogenic composition or vaccine comprising the immunogenic protein of any preceding claim, further comprising a pharmaceutically acceptable carrier and/or excipient, optionally further comprising an adjuvant, for prevention and/or treatment of the hMPV infection in a human subject.

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16. Use of an immunogenic composition or vaccine comprising the immunogenic protein of any of claims 1-14, further comprising a pharmaceutically acceptable carrier and/or excipient, optionally further comprising an adjuvant, in the manufacture of a medicament for prevention and/or treatment of the hMPV infection in a human subject.