



(51) International Patent Classification:

A61K 39/145 (2006.01) A61P 37/04 (2006.01)
A61P 31/16 (2006.01) C07K 14/11 (2006.01)

(21) International Application Number:

PCT/US2016/037595

(22) International Filing Date:

15 June 2016 (15.06.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/180,405 16 June 2015 (16.06.2015) US

(71) Applicants: ICAHN SCHOOL OF MEDICINE AT MOUNT SINAI [US/US]; One Gustave L. Levy Place, New York, NY 10029 (US). UNIVERSITAT FUR BODENKUL TUR WIEN [AT/AT]; Gregor-Mendel-Strasse 33, A-1180 Vienna (AT).

(72) Inventors: KRAMMER, Florian; 14 West 88th Street, Apt. 4A, New York, NY 10029 (US). JUNGBAUER, Alois; Ichagasse 31/1, 1210 Wien (AT). KLAUSBERGER, Miriam; Klopstockgass 30/25, 1170 Wien (AT). GRABHERR, Reingard; Ludwig Kaiserstrasse 11, 3021 Pressbaum (AT). TSCHELIESSNIG, Rupert; Lorbeer-gasse 10/10, 1030 Wien (AT).

(74) Agents: CHHEDA, Jennifer, J. et al.; Jones Day, 250 Vesey Street, New York, NY 10281-1047 (US).

(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, 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, KN, KP, KR, 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).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: INFLUENZA VIRUS VACCINES AND USES THEREOF

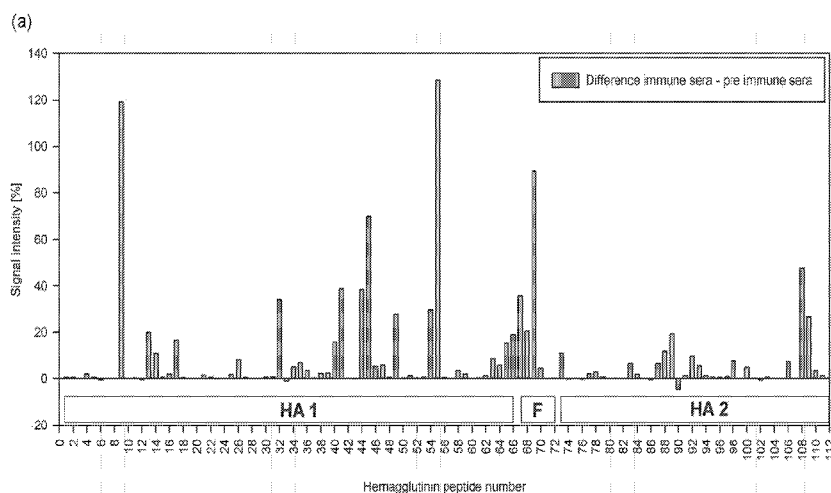


Fig. 1A

(57) Abstract: Provided herein are influenza virus mutated hemagglutinin polypeptides, including compositions comprising the same, vaccines comprising the same and methods of their use.

INFLUENZA VIRUS VACCINES AND USES THEREOF

[0001] This application claims the benefit of U.S. Provisional Application No. 62/180,405, filed June 16, 2015, which is incorporated herein by reference in its entirety.

[0002] This application incorporates by reference a Sequence Listing submitted with this application as text file entitled "Seqlisting_6923_239_228.txt" created on June 13, 2016 and having a size of 6,941 bytes.

1. INTRODUCTION

[0003] Provided herein are influenza virus hemagglutinin polypeptide mutants, and compositions comprising the same, vaccines comprising the same and methods of their use.

2. BACKGROUND

[0004] Influenza viruses are enveloped RNA viruses that belong to the family of Orthomyxoviridae (Palese and Shaw (2007) Orthomyxoviridae: The Viruses and Their Replication, 5th ed. Fields' Virology, edited by B.N. Fields, D.M. Knipe and P.M. Howley. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, USA, p1647-1689). The natural host of influenza A viruses are mainly avians, but influenza A viruses (including those of avian origin) also can infect and cause illness in humans and other animal hosts (bats, canines, pigs, horses, sea mammals, and mustelids). For example, the H5N1 avian influenza A virus circulating in Asia has been found in pigs in China and Indonesia and has also expanded its host range to include cats, leopards, and tigers, which generally have not been considered susceptible to influenza A (CIDRAP - Avian Influenza: Agricultural and Wildlife Considerations). The occurrence of influenza virus infections in animals could potentially give rise to human pandemic influenza strains.

[0005] Immunization is the only effective prophylaxis against seasonal influenza infection in humans, but high genetic variability of the main antibody targets of current vaccines makes annual re-formulation inevitable to date. Functionally, the hemagglutinin glycoprotein is composed of an immunodominant globular head domain involved in virus attachment to the host cell and the membrane proximal stalk domain mediating fusion of the viral and cell membrane in the host endosome. Yearly vaccination aims at inducing neutralizing antibodies against the HA

head domain; the prevalence of which impedes with receptor binding and is the current correlate of protection against influenza virus infection. This mode of action, however, is very strain-specific, and host immunity-driven evolution – reflected by the accumulation of genetic mutations in the head region – rapidly results in the generation of virus variants that are able to evade immune recognition (Carrat F, *et al.*, Vaccine 2007;25:6852–62; and Webster RG and EA Govorkova. Ann N Y Acad Sci 2014:n/a–n/a). To preserve vaccine efficacy, extensive surveillance and regular vaccine updates serve keep the vaccine-derived HAs antigenically similar to those of circulating viruses. In an effort to make annual vaccine updates dispensable, a trend in novel influenza vaccine design is to guide the immune response towards more conserved regions of the protein to broaden the protective efficacy of current vaccines (Krammer F, *et al.*, Curr Top Microbiol Immunol 2014). One such target is the HA stalk domain, which has received much attention in recent years because of its higher conservation compared to the head domain (Krystal M, *et al.*, Proc Natl Acad Sci U S A 1982;79:4800–4; Bhatt S, *et al.*, Mol Biol Evol 2011;28:2443–51; and Krammer F, *et al.*, Curr Top Microbiol Immunol 2014). Although sterically less accessible for immune recognition, anti-stalk antibodies have been shown to be elicited during natural infection in the mouse model and in humans, however at much lower levels as compared to antibodies directed towards the head region (Kostolanský F, *et al.*, Acta Virol 2002;46:229–36; Fislová T, *et al.*, Acta Virol 2005;49:243–50; Margine I, *et al.*, J Virol 2013;87:4728–37; and Styk B, *et al.*, Acta Virol 1979;23:1–8). New approaches are needed to redirect the immune response towards conserved antigenic regions and to avoid dominant responses against epitopes prone to antigenic drift.

3. SUMMARY

[0006] In one aspect, provided herein are influenza virus mutated hemagglutinin (HA) polypeptides comprising one or more influenza virus hemagglutinin (HA) stalk antigenic peptide(s) displayed in the globular head domain of an influenza virus hemagglutinin. In a specific embodiment, provided herein are influenza virus mutated HA polypeptides comprising a mutated influenza virus hemagglutinin (HA) globular head domain polypeptide, wherein the mutated influenza virus HA globular head domain polypeptide comprises one or more HA stalk antigenic peptides. In some embodiments, the one or more HA stalk antigenic peptides are inserted into an influenza virus HA globular head domain to form a mutated influenza virus HA

globular head domain. In other embodiments, the one or more HA stalk antigenic peptides replace one or more amino acid residues, one or more regions, or one or more epitopes in an influenza virus HA globular head domain to form a mutated influenza virus HA globular head domain. In certain embodiments, the one or more antigenic stalk peptides and the influenza virus HA globular head domain polypeptide are derived from hemagglutinin from different influenza virus strains or subtypes. For example, in some embodiments, the influenza virus HA globular head domain is from an H1 influenza virus and the one or more HA stalk antigenic peptides are from an H3 influenza virus. In another example, in certain embodiments, the influenza virus HA globular head domain is from one H1 influenza virus strain and the one or more HA stalk antigenic peptides are from another H1 influenza virus strain. In certain embodiments, an influenza virus mutated HA polypeptide described herein is soluble. In some embodiments, an influenza virus mutated HA polypeptide further comprises a cleavage site, such as a thrombin cleavage site, a trimerization domain, such as T4 foldon trimerization domain, and a peptide tag, such as a C-terminal hexahistidine-tag. In specific embodiments, influenza virus mutated hemagglutinin polypeptides elicit a cross-protective immune response (*e.g.*, an antibody response) against the HA stem domain epitopes displayed in the globular head domain of an influenza virus hemagglutinin. In certain embodiments, an influenza virus mutated HA polypeptide described herein is engineered into a vaccine formulation, such as a live influenza virus, an inactivated influenza virus, virus-like particle (“VLPs”), a subunit vaccine, or a split vaccine.

[0007] In certain embodiments, the influenza virus HA globular head domain is heterologous to the influenza virus HA stalk antigenic peptide. In certain embodiments, the influenza virus HA globular head domain is of a group, subtype, and/or strain that is heterologous to the group, subtype, and/or strain of the influenza virus HA stalk antigenic peptide. In certain embodiments, the influenza virus HA stalk antigenic peptide is of subtype H1. In certain embodiments, the influenza virus HA globular head domain is of subtype H3. In certain embodiments, the influenza virus HA stalk antigenic peptide is a B-cell epitope. In certain embodiments, the influenza virus HA stalk antigenic peptide is a T-cell epitope. In certain embodiments, the influenza virus HA stalk antigenic peptide comprises a continuous or discontinuous HA stalk domain sequence. In certain embodiments, the influenza virus HA stalk antigenic peptide has a length of 10-30 amino acids, 15-25 amino acids, 20-25 amino acids, or 25 amino acids. In

certain embodiments, the influenza virus HA stalk antigenic peptide is displayed in the antigenic site B loop of the influenza virus HA globular head domain.

[0008] In certain embodiments, the influenza virus HA stalk antigenic peptide comprises the sequence AKLRMVTGLRNIPSIQSRGLFGAIA (SEQ ID NO: 1), KVDDGFLDIWTYNAELLVLENERT (SEQ ID NO: 2), VYQILAIYSTVASSLVLLVSLGAIS (SEQ ID NO: 3), QSRGLFGAIA (SEQ ID NO: 4), VDGWYGYHHQ (SEQ ID NO: 5), IGNGCFEFYH (SEQ ID NO: 7) or a fragment or derivative thereof. In certain embodiments, two or more influenza virus HA stalk antigenic peptides are linked via a glycine linker, specifically said linker is of 5 amino acids length. In certain embodiments, the influenza virus HA stalk antigenic peptide comprises the sequence QSRGLFGAIAAGGGGGVDGWYGYHHQ (SEQ ID NO: 8) or VDGWYGYHHQGGGGGIGNGCFEFYH (SEQ ID NO: 9). In certain embodiments, the influenza HA stalk antigenic peptide comprises a charged serine before an N-terminal valine. In certain embodiment, the influenza HA stalk antigenic peptide further comprises up to 17 amino acids of the C-terminus of the homologous intersubunit or HA1 peptide. In certain embodiments, the influenza HA stalk antigenic peptide comprises or consists of any one of SEQ ID NOs: 1 to 5, SEQ ID NOs: 7 to 9, or SEQ ID NO:13.

[0009] In another aspect, provided herein is a nucleic acid sequence encoding an influenza virus mutated HA polypeptide. In certain embodiments, the nucleic acid sequence is complementary DNA (cDNA).

[0010] In another aspect, provided herein is a cell expressing a nucleic acid sequence encoding an influenza virus mutated HA polypeptide. In certain embodiments the cell is a eukaryotic or mammalian cell. In certain embodiments, the cell is isolated.

[0011] In another aspect, provided herein is a virus comprising a genome engineered to express a nucleic acid sequence encoding an influenza virus mutated HA polypeptide. In another aspect, provided herein is a virus comprising an influenza virus mutated HA polypeptide provided herein. In certain embodiments, the virus is influenza virus. In another aspect, provided herein is a virus-like particle comprising an influenza virus mutated HA polypeptide provided herein.

[0012] In another aspect, provided herein is a composition comprising an influenza virus mutated HA polypeptide provided herein. In another aspect, provided herein is a composition

comprising a virus provided herein. In another aspect, provided herein is a composition comprising a virus-like particle provided herein. In certain embodiments, a composition provided herein is for use in prevention and/or treatment of influenza virus disease in a subject. In certain embodiments, a composition provided herein is administered to a subject by intramuscular or intranasal route.

[0013] In another aspect, provided herein are methods for producing an influenza virus hemagglutinin stalk antigenic peptide. In certain embodiments, the method comprises (a) introducing synthetic peptides of 10 amino acid residues in length into the globular head domain of an influenza virus hemagglutinin polypeptide; and (b) selecting the influenza virus mutated hemagglutinin polypeptides which show strong reactivity to serum samples, wherein the synthetic peptides span the entire sequence of an influenza virus hemagglutinin stalk domain and the intersubunit region of the globular head domain, and wherein the serum samples are isolated from a subject exposed to influenza virus.

[0014] In certain embodiments, the method comprises (a) introducing synthetic peptides of 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11 or 10 amino acid residues in length into the globular head domain of an influenza virus hemagglutinin polypeptide; and (b) selecting the influenza virus mutated hemagglutinin polypeptides which show strong reactivity to serum samples, wherein the synthetic peptides span the entire sequence of an influenza virus hemagglutinin stalk domain or the intersubunit region, and wherein the serum samples are isolated from a subject exposed to influenza virus or a subject vaccinated against influenza.

[0015] In another aspect, provided herein is an isolated antibody directed against an influenza virus mutated HA polypeptide provided herein. In certain embodiments, the antibody is directed against an influenza virus HA stalk antigenic peptide provided herein.

[0016] In another aspect, provided herein is a method of eliciting cross-protective immunity against influenza virus in a subject, wherein the method comprises administering to the subject an influenza virus mutated HA polypeptide, or a nucleic acid sequence encoding an influenza virus mutated HA polypeptide, or a composition containing or expressing an influenza virus mutated HA polypeptide, or a vector containing or expressing an influenza virus mutated HA polypeptide, thereby eliciting an immune response in the subject.

[0017] In another aspect, provided herein is a method of eliciting cross-protective immunity against influenza virus in a subject, wherein the method comprises (a) administering to the subject a therapeutically effective amount of a nucleic acid sequence encoding a first influenza virus mutated HA polypeptide; (b) after a first period of time, administering a therapeutically effective amount of a second influenza virus mutated HA polypeptide; and (c) after a second period of time, administering a therapeutically effective amount of a third influenza virus mutated HA polypeptide. In certain embodiments, the routes of administration for the nucleic acid, and/or the second and/or third influenza virus mutated HA polypeptide may be the same or different. For example, in certain embodiments, the administration of the nucleic acid and the administration of the second and third influenza virus mutated HA polypeptide are via the same route (*e.g.*, intranasal or intramuscular); alternatively, in certain embodiments, the administration of the nucleic acid and the administration of the second and third influenza virus mutated HA polypeptide are different routes (*e.g.*, intranasal and intramuscular).

[0018] In another aspect, provided herein is a method of preventing influenza virus disease in a subject, wherein the method comprises administering to a subject a therapeutically effective amount of an influenza virus mutated HA polypeptide, or a nucleic acid sequence encoding an influenza virus mutated HA polypeptide, or a composition containing or expressing an influenza virus mutated HA polypeptide, or a vector containing or expressing an influenza virus mutated HA polypeptide, thereby preventing the influenza virus disease in a subject.

3.1 TERMINOLOGY

[0019] The terms “about” or “approximate,” when used in reference to an amino acid position refer to the particular amino acid position in a sequence or any amino acid that is within five, four, three, two, or one residues of that amino acid position, either in an N-terminal direction or a C-terminal direction.

[0020] As used herein, the term “about” or “approximately” when used in conjunction with a number refers to any number within 1, 5 or 10% of the referenced number. In certain embodiments, the term “about” encompasses the exact number recited.

[0021] The term “amino acid sequence identity” refers to the degree of identity or similarity between a pair of aligned amino acid sequences, usually expressed as a percentage. Percent identity is the percentage of amino acid residues in a candidate sequence that are identical (*i.e.*,

the amino acid residues at a given position in the alignment are the same residue) or similar (*i.e.*, the amino acid substitution at a given position in the alignment is a conservative substitution, as discussed below), to the corresponding amino acid residue in the peptide after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence homology. Sequence homology, including percentages of sequence identity and similarity, may be determined using sequence alignment techniques well-known in the art, preferably computer algorithms designed for this purpose, using the default parameters of said computer algorithms or the software packages containing them. Non-limiting examples of computer algorithms and software packages incorporating such algorithms include the following. The BLAST family of programs exemplify a particular, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences (*e.g.*, Karlin & Altschul, 1990, *Proc. Natl. Acad. Sci. USA* 87:2264-2268 (modified as in Karlin & Altschul, 1993, *Proc. Natl. Acad. Sci. USA* 90:5873-5877), Altschul *et al.*, 1990, *J. Mol. Biol.* 215:403-410, (describing NBLAST and XBLAST), Altschul *et al.*, 1997, *Nucleic Acids Res.* 25:3389-3402 (describing Gapped BLAST, and PSI-Blast). Another particular example is the algorithm of Myers and Miller (1988 *CABIOS* 4:11-17) which is incorporated into the ALIGN program (version 2.0) and is available as part of the GCG sequence alignment software package. Also particular is the FASTA program (Pearson W.R. and Lipman D.J., *Proc. Nat. Acad. Sci. USA*, 85:2444-2448, 1988), available as part of the Wisconsin Sequence Analysis Package. Additional examples include BESTFIT, which uses the “local homology” algorithm of Smith and Waterman (*Advances in Applied Mathematics*, 2:482-489, 1981) to find best single region of similarity between two sequences, and which is preferable where the two sequences being compared are dissimilar in length; and GAP, which aligns two sequences by finding a “maximum similarity” according to the algorithm of Neddleman and Wunsch (*J. Mol. Biol.* 48:443-354, 1970), and is preferable where the two sequences are approximately the same length and an alignment is expected over the entire length.

[0022] “Conservative substitution” refers to replacement of an amino acid of one class is with another amino acid of the same class. In particular embodiments, a conservative substitution does not alter the structure or function, or both, of a polypeptide. Classes of amino acids for the purposes of conservative substitution include hydrophobic (Met, Ala, Val, Leu, Ile), neutral hydrophilic (Cys, Ser, Thr), acidic (Asp, Glu), basic (Asn, Gln, His, Lys, Arg), conformation disrupters (Gly, Pro) and aromatic (Trp, Tyr, Phe).

[0023] As used herein, the term “derivative” in the context of an HA stalk peptide means (i) a peptide with 1, 2, 3, 4, or 5 amino acid changes as compared to an HA stalk peptide, for example, a conservative amino acid residue is substituted for one or more of the residues, and/or (ii) a peptide is shorter or longer at the N- and/or C-terminus by 1, 2, 3, 4, 5, 7, or 8 amino acid residues.

[0024] As used herein, the terms “disease” and “disorder” are used interchangeably to refer to a condition in a subject. In some embodiments, the condition is a viral infection. In specific embodiments, a term “disease” refers to the pathological state resulting from the presence of the virus in a cell or a subject, or by the invasion of a cell or subject by the virus. In certain embodiments, the condition is a disease in a subject, the severity of which is decreased by inducing an immune response in the subject through the administration of an immunogenic composition.

[0025] As used herein, the term “effective amount” in the context of administering a therapy to a subject refers to the amount of a therapy which has a prophylactic and/or therapeutic effect(s). In certain embodiments, an “effective amount” in the context of administration of a therapy to a subject refers to the amount of a therapy which is sufficient to achieve one, two, three, four, or more of the following effects: (i) reduce or ameliorate the severity of an influenza virus infection, disease or symptom associated therewith; (ii) reduce the duration of an influenza virus infection, disease or symptom associated therewith; (iii) prevent the progression of an influenza virus infection, disease or symptom associated therewith; (iv) cause regression of an influenza virus infection, disease or symptom associated therewith; (v) prevent the development or onset of an influenza virus infection, disease or symptom associated therewith; (vi) prevent the recurrence of an influenza virus infection, disease or symptom associated therewith; (vii) reduce or prevent the spread of an influenza virus from one cell to another cell, one tissue to another tissue, or one organ to another organ; (viii) prevent or reduce the spread of an influenza virus from one subject to another subject; (ix) reduce organ failure associated with an influenza virus infection; (x) reduce hospitalization of a subject; (xi) reduce hospitalization length; (xii) increase the survival of a subject with an influenza virus infection or disease associated therewith; (xiii) eliminate an influenza virus infection or disease associated therewith; (xiv) inhibit or reduce influenza virus replication; (xv) inhibit or reduce the entry of an influenza virus into a host cell(s); (xvi) inhibit or reduce replication of the influenza virus genome; (xvii) inhibit

or reduce synthesis of influenza virus proteins; (xviii) inhibit or reduce assembly of influenza virus particles; (xix) inhibit or reduce release of influenza virus particles from a host cell(s); (xx) reduce influenza virus titer; and/or (xxi) enhance or improve the prophylactic or therapeutic effect(s) of another therapy.

[0026] In certain embodiments, the effective amount does not result in complete protection from an influenza virus disease, but results in a lower titer or reduced number of influenza viruses compared to an untreated subject. In certain embodiments, the effective amount results in a 0.5 fold, 1 fold, 2 fold, 4 fold, 6 fold, 8 fold, 10 fold, 15 fold, 20 fold, 25 fold, 50 fold, 75 fold, 100 fold, 125 fold, 150 fold, 175 fold, 200 fold, 300 fold, 400 fold, 500 fold, 750 fold, or 1,000 fold or greater reduction in titer of influenza virus relative to an untreated subject. In some embodiments, the effective amount results in a reduction in titer of influenza virus relative to an untreated subject of approximately 1 log or more, approximately 2 logs or more, approximately 3 logs or more, approximately 4 logs or more, approximately 5 logs or more, approximately 6 logs or more, approximately 7 logs or more, approximately 8 logs or more, approximately 9 logs or more, approximately 10 logs or more, 1 to 3 logs, 1 to 5 logs, 1 to 8 logs, 1 to 9 logs, 2 to 10 logs, 2 to 5 logs, 2 to 7 logs, 2 logs to 8 logs, 2 to 9 logs, 2 to 10 logs 3 to 5 logs, 3 to 7 logs, 3 to 8 logs, 3 to 9 logs, 4 to 6 logs, 4 to 8 logs, 4 to 9 logs, 5 to 6 logs, 5 to 7 logs, 5 to 8 logs, 5 to 9 logs, 6 to 7 logs, 6 to 8 logs, 6 to 9 logs, 7 to 8 logs, 7 to 9 logs, or 8 to 9 logs. Benefits of a reduction in the titer, number or total burden of influenza virus include, but are not limited to, less severe symptoms of the infection, fewer symptoms of the infection and a reduction in the length of the disease associated with the infection.

[0027] As used herein, an “epitope” has the meaning known to one of skill in the art. Typically, the term “epitope” refers to a localized region of an antigen to which an antibody can specifically bind. An epitope can be a continuous epitope or a discontinuous epitope. A continuous epitope is a linear epitope. For example, in a polypeptide antigen, a continuous epitope consists of contiguous amino acids of the polypeptide. A discontinuous epitope is a conformation, non-linear epitope. For example, in a polypeptide antigen, a discontinuous epitope comprises amino acids from two or more non-contiguous regions of the polypeptide. One skilled in the art would understand that a linear epitope may or may not depend on secondary, tertiary, or quaternary structure. For example, in some embodiments, an anti-HA antibody recognizing an epitope described herein binds to a group of amino acid residues

regardless of their three dimensional protein structure. Alternatively, in some embodiments, an anti-HA antibody recognizing an epitope described herein does not recognize the individual amino acid residues making up the epitope, and, instead, requires a particular three-dimensional conformation (*e.g.*, bend, twist, turn, or fold) in order to recognize and bind the epitope.

[0028] As used herein, the term “fragment” in the context of a nucleic acid sequence refers to a nucleotide sequence comprising a portion of consecutive nucleotides from a parent sequence. In a specific embodiment, the term refers to a nucleotide sequence of 5 to 15, 5 to 25, 10 to 30, 15 to 30, 10 to 60, 25 to 100, 150 to 300 or more consecutive nucleotides from a parent sequence. In another embodiment, the term refers to a nucleotide sequence of at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 125, 150, 175, 200, 250, 275, 300, 325, 350, 375, 400, 425, 450 or 475 consecutive nucleotides of a parent sequence.

[0029] As used herein, the term “fragment” in the context of an amino acid sequence refers to an amino acid sequence comprising a portion of consecutive amino acid residues from a parent sequence. In a specific embodiment, the term refers to an amino acid sequence of 5 to 10, 10 to 15, 15 to 25, 5 to 30, 10 to 60, 25 to 100, 150 to 300 or more consecutive amino acid residues from a parent sequence. In another embodiment, the term refers to an amino acid sequence of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 125, 150, 175, or 200 consecutive amino acid residues of a parent sequence.

[0030] “Hemagglutinin” and “HA” refer to any influenza virus hemagglutinin. In certain embodiments, the hemagglutinin is influenza hemagglutinin, such as an influenza A hemagglutinin, an influenza B hemagglutinin, or an influenza C hemagglutinin. A typical hemagglutinin comprises domains known to those of skill in the art including a signal peptide (optional herein), a stem domain (also referred to as a “stalk domain”), a globular head domain, a luminal domain (optional herein), a transmembrane domain (optional herein) and a cytoplasmic domain (optional herein). In certain embodiments, a hemagglutinin consists of a single polypeptide chain, such as HA0. In certain embodiments, a hemagglutinin consists of more than one polypeptide chain in quaternary association, *e.g.* HA1 and HA2. Those of skill in the art will recognize that an immature HA0 might be cleaved to release a signal peptide (approximately 20 amino acids) yielding a mature hemagglutinin HA0. A hemagglutinin HA0 might be cleaved at another site to yield HA1 polypeptide (approximately 320 amino acids, including the globular

head domain and a portion of the stem domain) and HA2 polypeptide (approximately 220 amino acids, including the remainder of the stem domain, a luminal domain, a transmembrane domain and a cytoplasmic domain.

[0031] As used herein, the term “heterologous” in the context of a polypeptide, nucleic acid or virus refers to a polypeptide, nucleic acid or virus, respectively that is not normally found in nature or not normally associated in nature with a polypeptide, nucleic acid or virus of interest. For example, a “heterologous polypeptide” may refer to a polypeptide derived from a different virus, *e.g.*, a different influenza strain or subtype, or an unrelated virus or different species.

[0032] As used herein, the term “in combination,” in the context of the administration of two or more therapies to a subject, refers to the use of more than one therapy (*e.g.*, more than one prophylactic agent and/or therapeutic agent). The use of the term “in combination” does not restrict the order in which therapies are administered to a subject. For example, a first therapy (*e.g.*, a first prophylactic or therapeutic agent) can be administered prior to (*e.g.*, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 16 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (*e.g.*, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 16 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second therapy to a subject.

[0033] As used herein, the term “infection” means the invasion by, multiplication and/or presence of a virus in a cell or a subject. In one embodiment, an infection is an “active” infection, *i.e.*, one in which the virus is replicating in a cell or a subject. Such an infection is characterized by the spread of the virus to other cells, tissues, and/or organs, from the cells, tissues, and/or organs initially infected by the virus. An infection may also be a latent infection, *i.e.*, one in which the virus is not replicating. In certain embodiments, an infection refers to the pathological state resulting from the presence of the virus in a cell or a subject, or by the invasion of a cell or subject by the virus.

[0034] As used herein, the term “influenza virus disease” refers to the pathological state resulting from the presence of an influenza (*e.g.*, influenza A or B virus) virus in a cell or subject or the invasion of a cell or subject by an influenza virus. In specific embodiments, the term refers to a respiratory illness caused by an influenza virus.

[0035] As used herein, the terms “influenza virus hemagglutinin head domain polypeptide,” “influenza virus hemagglutinin head domain,” “HA globular head domain,” and “HA head domain” refer to the globular head domain of an influenza hemagglutinin polypeptide. The intervening amino acid sequence between residues C52 and C277, according to H3 numbering, of an influenza virus HA represents an exemplary influenza virus HA globular head domain.

[0036] As used herein, the term “intersubunit region,” in the context of influenza virus hemagglutinin, refers to the amino acid residues between the HA1 domain and HA2 domain of influenza virus hemagglutinin.

[0037] As used herein, the numeric term “log” refers to $\log_{10}$.

[0038] As used herein, the phrase “multiplicity of infection” or “MOI” is the average number of infectious virus particles per infected cell. The MOI is determined by dividing the number of infectious virus particles added (ml added x PFU/ml) by the number of cells added (ml added x cells/ml).

[0039] As used herein, the term “nucleic acid” is intended to include DNA molecules (*e.g.*, cDNA) and RNA molecules (*e.g.*, mRNA or pre-mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid can be single-stranded or double-stranded.

[0040] “Polypeptide” refers to a polymer of amino acids linked by amide bonds as is known to those of skill in the art. As used herein, the term can refer to a single polypeptide chain linked by covalent amide bonds. The term can also refer to multiple polypeptide chains associated by non-covalent interactions such as ionic contacts, hydrogen bonds, Van der Waals contacts and hydrophobic contacts. Those of skill in the art will recognize that the term includes polypeptides that have been modified, for example by post-translational processing such as signal peptide cleavage, disulfide bond formation, glycosylation (*e.g.*, *N*-linked glycosylation), protease cleavage and lipid modification (*e.g.* S-palmitoylation).

[0041] As used herein, the terms “prevent,” “preventing” and “prevention” in the context of the administration of a therapy(ies) to a subject to prevent an influenza virus disease refer to one or more of the prophylactic/beneficial effects resulting from the administration of a therapy or a combination of therapies. In a specific embodiment, the terms “prevent,” “preventing” and “prevention” in the context of the administration of a therapy(ies) to a subject to prevent an influenza virus disease refer to one or more of the following effects resulting from the administration of a therapy or a combination of therapies: (i) the inhibition of the development or

onset of an influenza virus disease or a symptom thereof; (ii) the inhibition of the recurrence of an influenza virus disease or a symptom associated therewith; and (iii) the reduction or inhibition in influenza virus infection and/or replication.

[0042] As used herein, the terms “purified” and “isolated” when used in the context of a polypeptide (including an antibody) that is obtained from a natural source, *e.g.*, cells, refers to a polypeptide which is substantially free of contaminating materials from the natural source, *e.g.*, soil particles, minerals, chemicals from the environment, and/or cellular materials from the natural source, such as but not limited to cell debris, cell wall materials, membranes, organelles, the bulk of the nucleic acids, carbohydrates, proteins, and/or lipids present in cells. Thus, a polypeptide that is isolated includes preparations of a polypeptide having less than about 30%, 20%, 10%, 5%, 2%, or 1% (by dry weight) of cellular materials and/or contaminating materials. As used herein, the terms “purified” and “isolated” when used in the context of a polypeptide (including an antibody) that is chemically synthesized refers to a polypeptide which is substantially free of chemical precursors or other chemicals which are involved in the syntheses of the polypeptide. In a specific embodiment, an influenza virus mutated hemagglutinin (HA) polypeptide is chemically synthesized. In another specific embodiment, an influenza virus mutated hemagglutinin (HA) polypeptide is isolated.

[0043] As used herein, the terms “replication,” “viral replication” and “virus replication” in the context of a virus refer to one or more, or all, of the stages of a viral life cycle which result in the propagation of virus. The steps of a viral life cycle include, but are not limited to, virus attachment to the host cell surface, penetration or entry of the host cell (*e.g.*, through receptor mediated endocytosis or membrane fusion), uncoating (the process whereby the viral capsid is removed and degraded by viral enzymes or host enzymes thus releasing the viral genomic nucleic acid), genome replication, synthesis of viral messenger RNA (mRNA), viral protein synthesis, and assembly of viral ribonucleoprotein complexes for genome replication, assembly of virus particles, post-translational modification of the viral proteins, and release from the host cell by lysis or budding and acquisition of a phospholipid envelope which contains embedded viral glycoproteins. In some embodiments, the terms “replication,” “viral replication” and “virus replication” refer to the replication of the viral genome. In other embodiments, the terms “replication,” “viral replication” and “virus replication” refer to the synthesis of viral proteins.

[0044] As used herein, the term “stalk antigenic peptide” in the context of an influenza virus hemagglutinin (HA) refers to an antigenic peptide comprising amino acids from or derived from an influenza virus hemagglutinin (HA) stalk domain, but less than the entire influenza virus hemagglutinin stalk domain. *See* Section 5.1, *infra*, for additional information regarding stalk antigenic peptides.

[0045] As used herein, the terms “stem domain,” “HA stem domain,” “stalk domain,” and “HA stalk domain” refer to a derivative, *e.g.* an engineered derivative, of a hemagglutinin polypeptide that comprises one or more polypeptide chains that make up a stem domain of hemagglutinin. A stem domain polypeptide might be a single polypeptide chain, two polypeptide chains or more polypeptide chains. Typically, a stem domain polypeptide is a single polypeptide chain (*i.e.*, corresponding to the stem domain of a hemagglutinin HA0 polypeptide) or two polypeptide chains (*i.e.*, corresponding to the stem domain of a hemagglutinin HA1 polypeptide in association with a hemagglutinin HA2 polypeptide). In a specific embodiment, the HA stem domain is all of the influenza virus HA amino acid residues excluding the Cys52 and Cys277 (according to H3 numbering) amino acid residues as well as the amino acid residues between these cysteines.

[0046] As used herein, the terms “subject” or “patient” are used interchangeably to refer to an animal (*e.g.*, birds, reptiles, and mammals). In a specific embodiment, a subject is a bird. In another embodiment, a subject is a mammal including a non-primate (*e.g.*, a camel, donkey, zebra, cow, pig, horse, goat, sheep, cat, dog, rat, and mouse) and a primate (*e.g.*, a monkey, chimpanzee, and a human). In certain embodiments, a subject is a non-human animal. In some embodiments, a subject is a farm animal or pet. In another embodiment, a subject is a human.

[0047] The terms “tertiary structure” and “quaternary structure” have the meanings understood by those of skill in the art. Tertiary structure refers to the three-dimensional structure of a single polypeptide chain. Quaternary structure refers to the three dimensional structure of a polypeptide having multiple polypeptide chains.

[0048] As used herein, the terms “therapies” and “therapy” can refer to any protocol(s), method(s), compound(s), composition(s), formulation(s), and/or agent(s) that can be used in the prevention or treatment of a viral infection or a disease or symptom associated therewith. In certain embodiments, the terms “therapies” and “therapy” refer to biological therapy, supportive therapy, and/or other therapies useful in treatment or prevention of a viral infection or a disease

or symptom associated therewith known to one of skill in the art. In some embodiments, the term “therapy” refers to (i) a nucleic acid encoding influenza virus mutated hemagglutinin (HA) polypeptide, (ii) influenza virus mutated hemagglutinin (HA) polypeptide, (iii) a vector or composition comprising a nucleic acid encoding influenza virus mutated hemagglutinin (HA) polypeptide, or (iv) a vector or composition comprising influenza virus mutated hemagglutinin (HA) polypeptide.

[0049] As used herein, the terms “treat,” “treatment,” and “treating” refer in the context of administration of a therapy(ies) to a subject to treat an influenza virus disease or infection to obtain a beneficial or therapeutic effect of a therapy or a combination of therapies. In specific embodiments, such terms refer to one, two, three, four, five or more of the following effects resulting from the administration of a therapy or a combination of therapies: (i) the reduction or amelioration of the severity of an influenza virus infection or a disease or a symptom associated therewith; (ii) the reduction in the duration of an influenza virus infection or a disease or a symptom associated therewith; (iii) the regression of an influenza virus infection or a disease or a symptom associated therewith; (iv) the reduction of the titer of an influenza virus; (v) the reduction in organ failure associated with an influenza virus infection or a disease associated therewith; (vi) the reduction in hospitalization of a subject; (vii) the reduction in hospitalization length; (viii) the increase in the survival of a subject; (ix) the elimination of an influenza virus infection or a disease or symptom associated therewith; (x) the inhibition of the progression of an influenza virus infection or a disease or a symptom associated therewith; (xi) the prevention of the spread of an influenza virus from a cell, tissue, organ or subject to another cell, tissue, organ or subject; (xii) the inhibition or reduction in the entry of an influenza virus into a host cell(s); (xiii) the inhibition or reduction in the replication of an influenza virus genome; (xiv) the inhibition or reduction in the synthesis of influenza virus proteins; (xv) the inhibition or reduction in the release of influenza virus particles from a host cell(s); and/or (xvi) the enhancement or improvement the therapeutic effect of another therapy.

[0050] As used herein, in some embodiments, the phrase “wild-type” in the context of a virus refers to the types of a virus that are prevalent, circulating naturally and producing typical outbreaks of disease.

4. **BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCES**

[0051] Fig. 1A and Fig. 1B. Stalk-derived synthetic peptides are bound by human immune serum IgG. Human sera from vaccinated (Fig. 1A) and placebo volunteers (Fig. 1B) were evaluated in a chemiluminescence binding assay for their reactivity with 112 synthetic peptides overlapping by 5 aa that cover the entire sequence of the H1N1-based vaccine strain HA (A/New Caledonia/20/1999). Peptides are assigned on basis of their location in the protein to hemagglutinin region 1 or 2 (HA1 or HA2, respectively) or the intersubunit/fusion peptide region (F). Bound purified and biotinylated human serum IgG was detected with a streptavidin-HRP conjugate. Bars display the difference in signal intensity between pre-immune sera and immune sera. Image spots were normalized and negative (deca-alanine) and positive control (Strep-tag) peptide spots were defined as 0 % and 100 % intensity respectively. Signal intensities (%) of bound serum antibodies to the panel of HA peptide spots were calculated.

[0052] Fig. 2A and Fig. 2B. Localization of linear HA epitopes and prediction of potential discontinuous epitopes. The spatial distribution of the peptides, and their relative orientation was assessed with a 3D model of the NC99 HA based on the available protein structure of a RCSB Protein Data Bank entry: 1HA0. Spheres indicate peptides that show increased binding with sera upon vaccination (Fig. 2A). Distances and angles between selected peptides were calculated to evaluate whether their spatial distribution allows for the formation of a putative discontinuous epitope (Fig. 2B). The abscissa gives the distances of the centre of masses of the epitopes, while the ordinate holds the relative angle of the end to end vectors of the individual epitopes. The figures shows peptides-pairs in a suitable distance from each (< 2 nm) other to form a discontinuous epitope (open grey circles); those of which located in the stalk region are indicated by black open circles and arrows. Distances exceeding 3.5 nm are not illustrated. While $\alpha = 0, \pi$ indicate a parallel orientation $\alpha = \pi / 2$ give a perpendicular orientation of these vectors. The first 22 and the last 49 amino acids of the experimental sequence had to be omitted to obtain the best structure match with the reference structure, affecting visualization of peptides 106, 108 and 109.

[0053] Fig. 3A and Fig. 3B. Sequence conservation of selected stalk epitopes and their exact location. Selected peptide sequences from NC99 were aligned to those derived from other H1 strains that were present in previous vaccine formulations or are of significance, as well as to a pandemic and seasonal representative of the closest phylogenetic HA subtype (H2).

Alignments were done with CLC Workbench using the ClustalW algorithm. The location of selected peptides is represented on basis on the trimeric structure of the HA of PR8, which showed an overall sequence identity of 88.3% (pdb: 1RU7) and visualized using PyMol.

[0054] Fig. 4A and Fig. 4B. Displayed H1 stalk epitopes partially protect from lethal heterologous H1 mouse challenge. BALB/c mice (N=4-5 per group) received a full-length HA DNA prime (i.m., 40 µg, day 0) and two truncated soluble poly(I.C)-adjuvanted HA protein booster immunizations (i.n. + i.m., 2.5 µg, day 21 and 42) of a H3 subtype HA (HIR05) with stalk peptides from a H1 subtype (NC99) virus engineered into its globular head: HIR05-NC99-Ep66-69 (triangles, point down), HIR05-NC99-Ep86-89 (squares), HIR05-NC99-Ep69+73 (diamonds) or HIR05-NC99-Ep73+96 (light grey circles). Control mice received a DNA prime and two soluble adjuvanted protein booster immunizations with wildtype H3-subtype HIR05 HA (black) or a single vaccination with inactivated whole virus vaccine Agriflu® intramuscularly on day 42 (grey). Three weeks later, all mice were intranasally challenged with 5 mLD₅₀ of pandemic NL09. Weight loss (Fig. 4A) and survival rates (Fig. 4B) were monitored for 14 days post challenge. The weight loss curves represent the mean percentage of the group initial body weight and error bars indicate the standard deviation.

[0055] Fig. 5. Displayed H1 stalk-derived epitopes elicit H1-specific antibodies. Pooled pre-challenge sera from vaccinated mice were assayed for antibody endpoint titers against divergent pandemic CAL09 HA. Samples were from mice (4-5 mice per group) vaccinated with displayed H1 HA NC99-derived stalk epitopes engineered into a H3 HA HIR05 head: HIR05-NC99-Ep66-69, HIR05-NC99-Ep69+73, HIR05-NC99-Ep73+96 or HIR05-NC99-Ep86-89 or were vaccinated with the negative control HIR05 vaccine (“(-) HIR05 (H3)”) or Afluria as positive control (“(+) CAL09 (pH1)”). Mice were bled 63 days post prime or 21 post second boost respectively. Values represent the geometric mean of the calculated end point titers (read-outs subtracted by the mean and three-fold standard deviation) and error bars depict the standard error of the mean.

[0056] Table 1. Description of the Sequences

SEQ ID NO	DESCRIPTION	SEQ
1	Peptide 66-69	AKLRMVTGLRNIPSIQSRGLFGAIA
2	Peptide 86-89	KVDDGFLDIWTYNAELLVLENERT
3	Peptide 106-109	VYQILAIYSTVASSLVLLVSLGAIS
4	Peptide 69	QSRGLFGAIA

SEQ ID NO	DESCRIPTION	SEQ
5	Peptide 73	VDGWYGYHHQ
6	Peptide 66-69 Derivative	VTGLRNIPSIQSRGLFGAIAGFIEG
7	Peptide 96	IGNGCFEFYH
8	Peptide 69+73	QSRGLFGAIAGGGGGVDGWYGYHHQ
9	Peptide 73+96	VDGWYGYHHQGGGGGIGNGCFEFYH
10	G5 linker	GGGGG
11	G5S Linker	GGGGGS
12	Streptavidin binding peptide	WSHPQFEK
13	Peptide 73+96	SVDGWYGYHHQGGGGGIGNGCFEFYH
14	Peptide 107	AIYSTVASSL
15	Thrombin cleavage site	LVPRGSP
16	His Tag	HHHHHH
17	Peptide 106	VYQILAIYST
18	Peptide 9	VTVTHSVNLL
19	Peptide 55	FALSRGFGSG
20	Peptide 66	AKLRMTGLR
21	Peptide 67	VTGLRNIPSI
22	Peptide 68	NIPSIQSRGL
23	Peptide 87	FLDIWTYNAE
24	Peptide 88	TYNAELLVLL
25	Peptide 89	LLVLENERT
26	Peptide 108	VASSLVLLVS
27	Peptide 109	VLLVSLGAIS
28	Peptide 25	QLSSVSSFER
29	Peptide 100	KNGTYDYPKY
30	Peptide 86	KVDDGFLDIW

5. DETAILED DESCRIPTION

[0057] In one aspect, provided herein are influenza virus mutated hemagglutinin (HA) polypeptides comprising one or more influenza virus hemagglutinin (HA) stalk antigenic peptide(s) displayed in the globular head domain of an influenza virus hemagglutinin. In a specific embodiment, provided herein are influenza virus mutated HA polypeptides comprising a mutated influenza virus hemagglutinin (HA) globular head domain polypeptide, wherein the mutated influenza virus HA globular head domain polypeptide comprises one or more HA stalk antigenic peptides. In some embodiments, the one or more HA stalk antigenic peptides are inserted into an influenza virus HA globular head domain to form a mutated influenza virus HA globular head domain. In other embodiments, the one or more HA stalk antigenic peptides

replace one or more amino acid residues, one or more regions, or one or more epitopes in an influenza virus HA globular head domain to form a mutated influenza virus HA globular head domain. In certain embodiments, the one or more antigenic stalk peptides and the influenza virus HA globular head domain polypeptide are derived from hemagglutinin from different influenza virus strains or subtypes. For example, in some embodiments, the influenza virus HA globular head domain is from an H1 influenza virus and the one or more HA stalk antigenic peptides are from an H3 influenza virus. In another example, in certain embodiments, the influenza virus HA globular head domain is from one H1 influenza virus strain and the one or more HA stalk antigenic peptides are from another H1 influenza virus strain. In certain embodiments, an influenza virus mutated HA polypeptide described herein is soluble. In some embodiments, an influenza virus mutated HA polypeptide further comprises a cleavage site, such as a thrombin cleavage site, a trimerization domain, such as T4 foldon trimerization domain, and a peptide tag, such as a C-terminal hexahistidine-tag. In specific embodiments, influenza virus mutated hemagglutinin polypeptides elicit a cross-protective immune response (*e.g.*, an antibody response) against the HA stem domain epitopes displayed in the globular head domain of an influenza virus hemagglutinin. In certain embodiments, an influenza virus mutated HA polypeptide described herein is engineered into a vaccine formulation, such as a live influenza virus, an inactivated influenza virus, virus-like particle ("VLPs"), a subunit vaccine, or a split vaccine.

[0058] The influenza virus mutated hemagglutinin (HA) polypeptides disclosed herein are based, in part, on the inventors' rational design strategies for influenza virus vaccines that elicit a cross-protective immune response (*e.g.*, an antibody response). In this regard, the influenza virus mutated hemagglutinin (HA) polypeptide is designed to display an influenza virus hemagglutinin stalk antigenic peptide in the hemagglutinin globular head domain. Without being bound by any particular theory of operation, it is believed that exposure to this polypeptide should provide protection against (i) the influenza virus subtype or strain of the stalk antigenic peptide, and (ii) the influenza virus subtype or strain of the globular head of the influenza virus mutated hemagglutinin (HA) polypeptide.

[0059] When designing the influenza virus mutated hemagglutinin (HA) polypeptides, care should be taken to maintain the stability of the resulting protein. In this regard, it is recommended that the size of the influenza virus stalk antigenic peptide displayed in the

influenza virus hemagglutinin globular head is approximately 10-30 amino acid residues in length (in specific embodiments, 25 amino acid residues in length). In addition, it is recommended that the influenza virus stalk antigenic peptide is displayed in a site within the influenza virus hemagglutinin globular head known to tolerate insertions, such as, for example, the antigenic site B. *See*, for example, Section 6, *infra*.

[0060] In another aspect, provided herein are nucleic acid sequences that encode an influenza virus mutated HA polypeptide and/or an influenza virus mutated HA globular head domain described herein (*see, e.g.*, Section 5.3, *infra*). In one embodiment, provided herein are nucleic acid sequences that encode an influenza virus mutated HA polypeptide. In another embodiment, provided herein are nucleic acid sequences that encode an influenza virus mutated HA globular head domain described herein. Such nucleic acids can be administered directly to a subject and/or utilized to produce to an immunogenic composition (*e.g.*, a vaccine).

[0061] In another aspect, provided herein are vectors, *e.g.*, expression vectors, containing a nucleic acid encoding an influenza virus mutated HA polypeptide described herein (*see, e.g.*, Section 5.3, *infra*). In a specific embodiment, the vector is a plasmid vector. In another specific embodiment, the vector is a viral vector (*see, e.g.*, Section 5.5, *infra*), *e.g.*, an influenza virus vector into which an influenza virus mutated hemagglutinin HA polypeptide described herein has been incorporated into the virions or an influenza virus vector comprising a genome engineered to express an influenza virus mutated hemagglutinin HA polypeptide described herein. In certain embodiments, the viral vector can be utilized in a subunit vaccine, a split vaccine, an inactivated vaccine, and/or a live, attenuated virus vaccine. In another specific embodiment, the vector is a baculovirus. The vectors provided herein can be designed for expression of an influenza virus mutated hemagglutinin HA polypeptide described herein using prokaryotic cells (*e.g.*, bacterial) or eukaryotic cells (*e.g.*, insect cells, yeast cells, plant cells, algae and mammalian cells). As such, also provided herein are cells (*e.g.*, prokaryotic and eukaryotic cells) comprising the vectors provided herein, which are capable of producing one or more influenza virus mutated HA polypeptide described herein.

[0062] In another aspect, provided herein are virus-like particles (VLPs) and virosomes into which influenza virus mutated HA polypeptides described herein have been incorporated (*see* Section 5.6, *infra*).

[0063] In another aspect, provided herein are compositions comprising one or more of influenza virus mutated HA polypeptides described herein, and/or one or more of the nucleic acids, vectors, VLPs, bacteria, or virosomes described herein (*see, e.g.,* Section 5.8, *infra*). In a specific embodiment, a composition provided herein comprises an influenza virus mutated HA polypeptide described herein. In another specific embodiment, a composition provided herein comprises a nucleic acid encoding an influenza virus mutated HA polypeptide described herein. In another specific embodiment, a composition provided herein comprises an expression vector comprising a nucleic acid encoding an influenza virus mutated HA polypeptide described herein. In another specific embodiment, a composition provided herein comprises an influenza virus or non-influenza virus having a genome engineered to express an influenza virus mutated HA polypeptide described herein.

[0064] In another aspect, provided herein are methods of using the influenza virus mutated hemagglutinin (HA) polypeptides described herein in the prevention and/or treatment of and/or immunization against influenza virus disease and/or infection in a subject. In one embodiment, provided herein are methods for immunizing a subject against an influenza virus comprising administering to the subject an effective amount of influenza virus mutated hemagglutinin (HA) polypeptide or an immunogenic composition described herein that provides such a polypeptide. In another embodiment, provided herein are methods for preventing an influenza virus disease in a subject comprising administering to the subject an effective amount of influenza virus mutated hemagglutinin (HA) polypeptide or an immunogenic composition described herein that provides such a polypeptide. In another embodiment, provided herein are methods for treating an influenza virus infection and/or influenza virus disease in a subject comprising administering to the subject an effective amount of influenza virus mutated hemagglutinin (HA) polypeptide or an immunogenic composition described herein that provides such a polypeptide.

[0065] In certain embodiments, one or more influenza virus mutated HA polypeptides or a composition thereof, and/or one or more of the nucleic acids, vectors, VLPs, or virosomes described herein, is administered to a subject to immunize the subject against multiple strains or subtypes of influenza virus. In a specific embodiment, said administration is sufficient to generate a host immune response in said individual against any one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen or seventeen known influenza A hemagglutinin subtypes or a later identified influenza A hemagglutinin subtype. In

another specific embodiment, said administration is sufficient to generate a host immune response in said individual against any influenza B hemagglutinin subtype now known or later identified.

[0066] In certain embodiments, one or more one or more influenza virus mutated HA polypeptides or a composition thereof and/or one or more of the nucleic acids, vectors, VLPs, or virosomes described herein, is administered to a subject once as a single dose. In certain embodiments, a first influenza virus mutated hemagglutinin HA polypeptide described herein or a composition, nucleic acid, vector, VLP, or virosome described herein, is administered to a subject as a single dose, followed by the administration of a second influenza virus mutated HA polypeptide described herein or a composition, nucleic acid, vector, VLP, or virosome described herein 3 to 6 weeks later.

[0067] In another embodiment, provided herein is a method of immunizing a subject against an influenza virus disease or infection comprising (i) priming said subject by administering to said subject an influenza virus mutated HA polypeptide (or a nucleic acid encoding said mutated HA polypeptide, a virus expressing said mutated HA polypeptide, a VLP containing said mutated HA polypeptide, etc.) and, after a period of time, and (ii) boosting said subject with the influenza virus mutated HA polypeptide described herein (or a nucleic acid encoding said influenza virus mutated HA polypeptide, a virus expressing said influenza virus mutated HA polypeptide, a VLP containing said influenza virus mutated HA polypeptide, etc.). In certain embodiments, the subject may be administered a second boost, comprising a second administration of the same or a different influenza virus mutated HA polypeptide described herein (or a nucleic acid encoding said influenza virus mutated HA polypeptide, a virus expressing said influenza virus mutated HA polypeptide, a VLP expressing said influenza virus mutated HA polypeptide, etc.). In certain embodiments, the period of time between the priming and boosting, or between the boosts if more than one boost is administered, of said subject may, for example, be 1 week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 1 month, 2 months, 3 months, or longer. In certain embodiments, the period of time between the priming and boosting (or between the first and second boosts) of said subject may, for example, range from 3-5 days, 7-10 days, 7-14 days, 14-21 days, 14-28 days, 21-28 days, 21 days to 1 month, 1 month to 2 months, 1 month to 3 months, 2 months to 3 months, 2 months to 4 months, or 4 months to 6 months.

[0068] Exemplary influenza virus mutated hemagglutinin (HA) polypeptides and the use of such polypeptides to induce a cross-protective immune response (*e.g.*, an antibody response) are illustrated in the working Example (*e.g.*, Section 6, *infra*). In particular, the working Example demonstrates the production of an influenza virus mutated hemagglutinin (HA) polypeptide which incorporates into an HA globular head domain an influenza virus hemagglutinin stalk antigenic peptide from a heterologous HA, and the induction of a cross-protective immune response in mice following an immunization regimen involving the administration of a nucleic acid construct encoding such a polypeptide and the polypeptide.

5.1 INFLUENZA VIRUS HEMAGGLUTININ STALK ANTIGENIC PEPTIDES

[0069] Provided herein are influenza virus hemagglutinin stalk antigenic peptides for use in the generation of influenza virus mutated HA polypeptides (*see*, Section 5.2 and Section 6, *infra*).

[0070] Generally, the influenza virus hemagglutinin stalk antigenic peptides provided herein comprise or consist of 10 to 50 amino acid residues, 10 to 40 amino acid residues, 10 to 30 amino acid residues, 20 to 30 amino acid residues, 20 to 40 amino acid residues, 20 to 50 amino acid residues, 10 to 25 amino acid residues, 10 to 15 amino acid residues, 15 to 25 amino acid residues, or 20 to 25 amino acid residues. In a specific embodiment, the influenza virus hemagglutinin stalk antigenic peptide consists of 25 amino acid residues. In certain embodiments, the influenza virus hemagglutinin stalk antigenic peptide comprises or consists of 10 to 30 amino acid residues, 10 to 25 amino acid residues, 15 to 25 amino acid residues, 10 to 15 amino acid residues, or 20 to 25 amino acid residues from an influenza virus hemagglutinin stalk domain. In a specific embodiment, the influenza virus hemagglutinin stalk antigenic peptide consists of 25 amino acid residues from an influenza virus HA stalk.

[0071] In certain embodiments, an influenza virus hemagglutinin stalk antigenic peptide provided herein comprises an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 98%, or 99% identical to a peptide of an influenza virus hemagglutinin stalk domain known to those of skill in the art.

[0072] In certain embodiments, an influenza virus hemagglutinin stalk antigenic peptide provided herein consists of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more influenza virus hemagglutinin HA

stalk domain epitopes of a same or different influenza virus strain, subtype, or group. In certain embodiments, the epitope is 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more amino acid residues in length. In certain embodiments, the epitope is 10 amino acid residues in length. In certain embodiments, the influenza virus hemagglutinin stalk domain epitope is a continuous epitope. In certain embodiments, the influenza virus hemagglutinin stalk antigenic peptide comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more influenza virus hemagglutinin stalk domain continuous epitopes. In certain embodiments, the continuous epitope is 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more amino acid residues in length. In certain embodiments, the continuous epitope is 10 amino acid residues in length. In certain embodiments, the influenza virus hemagglutinin stalk domain epitope is a discontinuous epitope. In certain embodiments, the influenza virus hemagglutinin stalk antigenic peptide comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more influenza virus hemagglutinin (HA) stalk domain discontinuous epitopes. In certain embodiments, a discontinuous epitope comprises 2, 3, or more influenza virus hemagglutinin stalk peptides, wherein each peptide comprises 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more contiguous influenza virus hemagglutinin stalk amino acid residues. In certain embodiments, a discontinuous epitope comprises 2 influenza virus hemagglutinin stalk peptides, wherein each peptide comprises 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more contiguous influenza virus hemagglutinin stalk amino acid residues. In certain embodiments, the discontinuous epitope comprises two influenza virus hemagglutinin stalk peptides, wherein the discontinuous epitope consists of 10 discontinuous amino acid residues from an influenza virus hemagglutinin stalk domain. In certain embodiments, the stalk antigenic peptide comprises one or more influenza virus HA stalk domain continuous epitopes and one or more influenza virus HA stalk domain discontinuous epitopes. In certain embodiments, the influenza virus HA stalk domain epitope is a B-cell epitope.

[0073] In some embodiments, an influenza virus hemagglutinin stalk antigenic peptide described herein comprises one or more influenza virus hemagglutinin stalk domain epitopes, wherein the one or more epitopes is an influenza A virus hemagglutinin epitope. In certain embodiments, an influenza virus hemagglutinin stalk antigenic peptide described herein comprises one or more influenza virus hemagglutinin stalk domain epitopes, wherein the one or

more epitopes is an influenza B virus hemagglutinin epitope. In some embodiments, an influenza virus hemagglutinin stalk antigenic peptide described herein comprises one or more influenza virus hemagglutinin stalk domain epitopes, wherein the one or more epitopes is an influenza C virus hemagglutinin epitope. In certain embodiments, the influenza virus HA stalk domain epitope is found in an influenza A virus hemagglutinin, influenza B virus hemagglutinin, and/or influenza C virus hemagglutinin. In some embodiments, the influenza virus HA stalk domain epitope is an H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, and/or H17 hemagglutinin epitope. In certain embodiments, the influenza virus HA stalk domain epitope found in one or more influenza virus hemagglutinin subtypes, *e.g.*, H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H3, H14, H15, H16, and/or H17 hemagglutinin. In some embodiments, the influenza virus HA stalk domain epitope is an influenza virus Group 1 hemagglutinin epitope, *e.g.*, H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16. In certain embodiments, the influenza virus HA stalk domain epitope is an influenza virus Group 2 hemagglutinin epitope, *e.g.*, H3, H4, H7, H10, H14, and H15. In some embodiments, the influenza virus HA stalk domain epitope is an H3 influenza virus hemagglutinin. In certain embodiments, the influenza virus HA stalk domain epitope is found both in Group 1 hemagglutinins, *e.g.*, H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16, and in Group 2 hemagglutinins, *e.g.*, H3, H4, H7, H10, H14, and H15. In certain embodiments, the influenza virus HA stalk domain epitope is an H1 influenza virus hemagglutinin epitope. In some embodiments, the H1 influenza virus is influenza A/New Caledonia/20/1999 virus. In certain embodiments, the influenza virus hemagglutinin HA stalk domain epitope is an H3 influenza virus hemagglutinin epitope. In some embodiments, the H3 influenza virus is influenza A/Hiroshima/52/2005 virus. In certain embodiments, the influenza virus HA stalk domain epitope is an epitope from a strain as described in Section 5.5.1, *infra*. In specific embodiments, an influenza virus HA stalk domain epitope is identified by techniques known to one of skill in the art or described herein (*e.g.*, Section 5.1.1, Section 5.1.2 and/or Section 6, *infra*).

[0074] In certain embodiments, the influenza virus hemagglutinin stalk antigenic peptides provided herein comprise one or more linkers, wherein the linker consists of between 1 and 10 heterologous amino acid residues. In certain embodiments, the one or more linker is positioned at one or both termini of the stalk antigenic peptide. In certain embodiments, the linker separates two influenza virus HA stalk domain epitopes. In certain embodiments, the linker consists of 1,

2, 3, 4, 5, or more glycine amino acid residues. In a specific embodiment, the linker consists of 5 glycine amino acid residues, *i.e.*, GGGGG (SEQ ID NO:10). In certain embodiments, the linker consists of glycine and serine amino acid residues. In a specific embodiment, the linker consists of the amino acid sequence: GGGGGS (SEQ ID NO:11). In certain embodiments, a heterologous charged serine amino acid residue is inserted directly N-terminal to a valine amino acid residue, wherein the valine amino acid residue is the N-terminal residue of an influenza virus HA stalk domain epitope. In certain embodiments, (i) a heterologous charged serine amino acid residue is inserted directly N-terminal to a valine amino acid residue, wherein the valine amino acid residue is the N-terminal residue of an influenza virus HA stalk domain epitope; and (ii) a linker is inserted directly N-terminal to the heterologous charged serine amino acid residue.

[0075] In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: AKLRMVTGLRNIPSIQSRGLFGAIA (SEQ ID NO:1) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: KVDDGFLDIWTYNAELLVLENERT (SEQ ID NO:2) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: VYQILAIYSTVASSLVLLVSLGAIS (SEQ ID NO:3) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: QSRGLFGAIA (SEQ ID NO:4) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: VDGWYGYHHQ (SEQ ID NO:5) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: IGNGCFEFYH (SEQ ID NO:7) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: QSRGLFGAIAAGGGGGVDGWYGYHHQ (SEQ ID NO:8) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: VDGWYGYHHQGGGGGIGNGCFEFYH (SEQ ID NO:9) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: SVDGWYGYHHQGGGGGIGNGCFEFYH (SEQ ID NO:13) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: AIYSTVASSL (SEQ ID NO:14) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino

acid sequence: VYQILAIYST (SEQ ID NO:17) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: VTVTHSVNLL (SEQ ID NO:18) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: FALSRGFGSG (SEQ ID NO:19) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: AKLRMVTGLR (SEQ ID NO:20) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: VTGLRNIPSI (SEQ ID NO:21) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: NIPSIQSRGL (SEQ ID NO:22) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: FLDIWTYNAE (SEQ ID NO:23) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: TYNAELLVLL (SEQ ID NO:24) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: LLVLLENERT (SEQ ID NO:25) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: VASSLVLLVS (SEQ ID NO:26) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: VLLVSLGAIS (SEQ ID NO:27) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: QLSSVSSFER (SEQ ID NO:28) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: KNGTYDYPKY (SEQ ID NO:29) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide comprises or consists of the amino acid sequence: KVDDGFLDIW (SEQ ID NO:30) or a derivative or fragment thereof. In certain embodiments, the stalk antigenic peptide is a peptide described in Section 6, *infra*.

5.1.1 Identification of linear influenza virus hemagglutinin epitopes

[0076] In another aspect, provided herein are methods for identifying linear stalk epitopes comprising screening serum for the ability to bind an influenza virus hemagglutinin peptide array, wherein the serum is from a subject that has been exposed to influenza virus. *See*, for example, Section 6, *infra*. For example, (i) overlapping peptides covering an influenza virus

hemagglutinin sequence are generated and immobilized to a membrane; (ii) labeled immunoglobulin Gs (IgGs) purified from sera from a subject exposed to influenza virus hemagglutinin are incubated on the peptide array membrane; and (iii) the ability of the labeled IgGs to bind peptides on the peptide array membrane is determined by, for example, chemiluminescence of the labeled IgG.

[0077] In certain embodiments, the peptide array consists of overlapping peptides spanning an influenza virus hemagglutinin sequence. In certain embodiments, the peptides are 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more amino acids in length. In certain embodiments, the peptides are 10 amino acids in length. In certain embodiments, there is overlap between consecutive peptides. In certain embodiments, the overlap between consecutive peptides is 5 amino acids. For example, peptide 1 consists of amino acid residues 1-10 of an influenza virus hemagglutinin, peptide 2 consists of amino acid residues 5-15 of the influenza virus hemagglutinin, peptide 3 consists of amino acid residues 10-20 of the influenza virus hemagglutinin, and so forth. The peptides can be synthesized by techniques known to one skilled in the art. For example, the peptides can be synthesized by conventional fmoc techniques.

[0078] In certain embodiments, the peptide array is synthesized based on the amino acid sequence of an influenza virus Group 1 hemagglutinin, *e.g.*, H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16. In certain embodiments, the peptide array is synthesized based on the amino acid sequence of an influenza virus Group 2 hemagglutinin, *e.g.*, H3, H4, H7, H10, H14, and H15. In certain embodiments, the peptide array is synthesized based on the amino acid sequence of an H3 influenza virus hemagglutinin. In certain embodiments, the peptide array is synthesized based on the amino acid sequence of an H1 influenza virus hemagglutinin. In certain embodiments, the H1 influenza is influenza A/New Caledonia/20/1999 virus.

[0079] In certain embodiments, the peptide array is immobilized on cellulose sheets. In certain embodiments, the peptides are c-terminally immobilized on the cellulose sheets via di- β -alanine anchors. In certain embodiments, the peptides are N-terminally acetylated and side chain protection groups are cleaved after immobilization on the membrane. In certain embodiments, the peptide membrane contains a negative control peptide, for example, peptide consisting only of alanine amino acid residues. In certain embodiments, the peptide membrane contains a

positive control peptide, for example, a peptide that binds streptavidin (*e.g.*, WSHQPFEK (SEQ ID NO:12)).

[0080] In certain embodiments, the serum is processed from a subject that has been infected with influenza virus. In certain embodiments, the serum is processed from a subject that has been vaccinated against influenza virus. In certain embodiments, the subject has been vaccinated with an influenza virus H1N1-based vaccine.

[0081] In certain embodiments, the IgGs are purified from the sera by an assay known to one skilled in the art. For example, immunoglobulins can be purified by a Protein G column, such as, for example, Protein G Fast Flow Sepharose (GE Health Care, Little Chalfont, UK). In certain embodiments, the IgGs are labeled with biotin. In certain embodiments, binding of the labeled IgG to the peptide array membrane is determined by secondary incubation with an antibody against the label of the IgG, wherein the antibody is conjugated to a detectable substance, such as, for example, horse radish peroxidase. In certain embodiments, the level of binding is normalized to the level of, for example, luminescence of a negative control peptide on the peptide array membrane and/or a positive control peptide on the peptide array membrane. In certain embodiments, binding of a peptide on the peptide array membrane to the labeled IgG indicates that the peptide is a linear epitope of influenza virus hemagglutinin.

5.1.2 Identification of discontinuous influenza virus hemagglutinin epitopes

[0082] In another aspect, provided herein are methods for identifying discontinuous stalk epitopes comprising 3-dimensional (3D) modeling of hemagglutinin (HA) sequences. *See*, for example, Section 6, *infra*. For example, a 3D model for an HA sequence, *e.g.*, the vaccine strain influenza A/New Caledonia/20/1999, can be utilized to identify the spatial distribution of epitopes and their relative orientation. For example, the spatial position of an epitope is characterized by the average of all coordinates of the contributing amino acids:

$\langle \zeta^{a,b} \rangle = 1/10 \sum_{i=1}^{10} \zeta_i^{a,b}$, wherein $\zeta_i^{a,b}$ resembles the mean position of an individual amino acid *a* or *b*, respectively, and wherein the mean position in return is calculated from the Cartesian coordinates of each individual site: $\zeta_i^{a,b} = \langle x_j^{a,b} + y_j^{a,b} + z_j^{a,b} \rangle_j$. In certain embodiments, the orientation of a 10 amino acid peptide in space is characterized by the end to end distance vector

$\langle \zeta^{a \rightarrow b} \rangle = \langle \zeta^b \rangle - \langle \zeta^a \rangle$. The relative angle of two epitopes is then calculated by:

$\langle \zeta^a \rangle \langle \zeta^a \rangle = \left| \langle \zeta^a \rangle \right| \left| \langle \zeta^a \rangle \right| \cos \theta$. Thus, the relation of two epitopes and their relative orientation

are given by their relative distances $\langle \zeta^{a \rightarrow b} \rangle$, and the angle θ . In certain embodiments, the RCSB Protein Data Bank: 1HA0 model is utilized. In certain embodiments, the first 22 and the last 49 amino acids in the experimental primary sequence are removed to facilitate 3D modeling. Algorithms to model the structure can be written in, for example, Mathematica (*see* website at wolfram.com/mathematica/?source=nav). The resulting 3D structure can be plotted in, for example, Visual Molecular Dynamics (VMD) (*see* website at ks.uiuc.edu/Research/vmd/), and can be rendered by, for example, POV-RAY (*see* website at povray.org/).

5.2 INFLUENZA VIRUS MUTATED HEMAGGLUTININ POLYPEPTIDE

[0083] In one aspect, provided herein are influenza virus mutated hemagglutinin (HA) polypeptides comprising one or more influenza virus hemagglutinin (HA) stalk antigenic peptide(s) displayed in the globular head domain of an influenza virus hemagglutinin. In a specific embodiment, provided herein are influenza virus mutated HA polypeptides comprising a mutated influenza virus hemagglutinin (HA) globular head domain polypeptide, wherein the mutated influenza virus HA globular head domain polypeptide comprises one or more HA stalk antigenic peptides. In specific embodiments, the influenza virus mutated HA polypeptides comprise an influenza virus HA stem domain and a mutated influenza virus HA globular head domain polypeptide. In certain specific embodiments, the influenza virus mutated HA polypeptides comprise a mutated influenza virus HA globular head domain and the remaining domains naturally found to be part of an influenza virus HA polypeptide. In some embodiments, an influenza virus mutated HA polypeptide is soluble. Techniques for producing soluble influenza virus HA polypeptides are known to one of skill in the art and are described herein (*see, e.g.,* Section 6, *infra*).

[0084] In certain embodiments, an influenza virus mutated HA polypeptide comprises a globular head domain, an influenza virus HA stem domain, and one or more other polypeptide domains. Useful polypeptide domains include domains that facilitate purification, folding and cleavage of portions of a polypeptide. For example, a His tag (His-His-His-His-His-His, SEQ ID

NO:16), FLAG epitope or other purification tag can facilitate purification of an influenza virus mutated HA polypeptide provided herein. In some embodiments, the His tag has the sequence, (His)*n*, wherein *n* is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or greater. A foldon, or trimerization, domain from bacteriophage T4 fibrin can facilitate trimerization of an influenza virus mutated HA polypeptide provided herein. In some embodiments, the trimerization domain comprises a wildtype GCN4pII trimerization heptad repeat or a modified GCN4pII trimerization heptad repeat that allows for the formation of trimeric or tetrameric coiled coils. *See, e.g.*, Weldon et al., 2010, PLoS ONE 5(9): e12466. The foldon domain can have any foldon sequence known to those of skill in the art (*see, e.g.*, Papanikolopoulou et al., 2004, J. Biol. Chem. 279(10):8991-8998, the contents of which are hereby incorporated by reference in their entirety. A foldon domain can be useful to facilitate trimerization of soluble polypeptides provided herein. Cleavage sites can be used to facilitate cleavage of a portion of a polypeptide, for example cleavage of a purification tag or foldon domain or both. Useful cleavage sites include a thrombin cleavage site, for example one with the sequence LVPRGSP (SEQ ID NO:15). In certain embodiments, the cleavage site is a cleavage site recognized by Tobacco Etch Virus (TEV) protease.

[0085] In certain embodiments, the mutated influenza virus HA globular head domain is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 98%, or 99% identical to the amino acid sequence an influenza virus HA globular head domain polypeptide known to those of skill in the art. In some embodiments, the mutated influenza virus HA globular head domain, prior to insertion one or more HA stalk antigenic peptides and/or the replacement one or more amino acid residues, one or more regions, or one or more epitopes in an influenza virus HA globular head domain with one or more HA stalk antigenic peptides is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 98%, or 99% identical to the amino acid sequence an influenza virus HA globular head domain polypeptide known to those of skill in the art.

[0086] In certain embodiments, an influenza virus mutated HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises 1, 2, 3, or more influenza virus HA stalk antigenic peptides. In some embodiments, the 1, 2, 3, or more influenza virus HA stalk antigenic peptides are of a same or different hemagglutinin group, subtype, and/or strain. In certain embodiments, an influenza virus mutated hemagglutinin HA polypeptide comprises a mutated influenza virus HA globular

head domain, wherein the mutated influenza virus HA globular head domain comprises 1 influenza virus HA stalk antigenic peptide. In some embodiments, an influenza virus mutated hemagglutinin HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises 2 influenza virus HA stalk antigenic peptides of the same or different hemagglutinin group, subtype, and/or strain. In certain embodiments, an influenza virus mutated hemagglutinin HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises 3 influenza virus HA stalk antigenic peptides of the same or different hemagglutinin group, subtype, and/or strain.

[0087] In certain embodiments, one or more influenza virus HA stalk antigenic peptides are of the same or different hemagglutinin group, subtype, and/or strain as the influenza virus globular head used as the basis to produce the mutated influenza virus HA globular head domain. In some embodiments, one or more influenza virus HA stalk antigenic peptides are of the same hemagglutinin group, subtype, and/or strain as the influenza virus globular head used as the basis to produce the mutated influenza virus HA globular head domain. In certain embodiments, one or more influenza virus HA stalk antigenic peptides are of a different hemagglutinin group, subtype, and/or strain as the influenza virus globular head used as the basis to produce the mutated influenza virus HA globular head domain.

[0088] In certain embodiments, an influenza virus mutated HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises (i) an influenza virus HA stalk antigenic peptide from an influenza A virus hemagglutinin; and (ii) an influenza virus HA globular head domain or a portion thereof from an influenza A virus hemagglutinin. In some embodiments, an influenza virus mutated HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises (i) an influenza virus HA stalk antigenic peptide from an influenza B virus hemagglutinin; and (ii) an influenza virus HA globular head domain or a portion thereof from an influenza B virus hemagglutinin. In certain embodiments, an influenza virus mutated HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises (i) an influenza virus HA stalk antigenic peptide from an influenza A virus hemagglutinin; and (ii) an influenza virus HA globular head domain or a portion thereof from an

influenza B virus hemagglutinin. In some embodiments, an influenza virus mutated HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises (i) an influenza virus HA stalk antigenic peptide from an influenza B virus hemagglutinin; and (ii) an influenza virus HA globular head domain or a portion thereof from an influenza A virus hemagglutinin. In certain embodiments, an influenza virus mutated HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises (i) an influenza virus HA stalk antigenic peptide from an influenza C virus hemagglutinin; and (ii) an influenza virus HA globular head domain or a portion thereof from an influenza C virus hemagglutinin.

[0089] In certain embodiments, an influenza virus mutated HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises (i) an influenza virus HA stalk antigenic peptide from an H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, and/or H17 hemagglutinin; and (ii) an influenza virus HA globular head domain or a portion thereof from an H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, or H17 hemagglutinin. In some embodiments, an influenza virus mutated HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises (i) an influenza virus HA stalk antigenic peptide from a Group 1 hemagglutinin, *e.g.*, H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16; and (ii) an influenza virus HA globular head domain or a portion thereof from a Group 1 hemagglutinin, *e.g.*, H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16. In certain embodiments, the an influenza virus mutated HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises (i) an influenza virus HA stalk antigenic peptide from a Group 2 hemagglutinin, *e.g.*, H3, H4, H7, H10, H14, and H15; and (ii) an influenza virus globular head domain or a portion thereof from a Group 2 hemagglutinin, *e.g.*, H3, H4, H7, H10, H14, and H15. In some embodiments, an influenza virus mutated HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises (i) an influenza virus HA stalk antigenic peptide from a Group 1 hemagglutinin, *e.g.*, H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16; and (ii) an influenza virus HA globular head domain or a portion thereof from a Group 2

hemagglutinin, *e.g.*, H3, H4, H7, H10, H14, and H15. In certain embodiments an influenza virus mutated HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises (i) an influenza virus HA stalk antigenic peptide from a Group 2 hemagglutinin, *e.g.*, H3, H4, H7, H10, H14, and H15; and (ii) an influenza virus HA globular head domain or a portion thereof from a Group 1 hemagglutinin, *e.g.*, H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16.

[0090] In certain embodiments, an influenza virus mutated HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises an influenza virus stalk antigenic peptide that is an H1 hemagglutinin peptide and an influenza virus HA globular head domain or a portion thereof that is an H3 hemagglutinin globular head domain. In some embodiments an influenza virus mutated HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises an influenza virus stalk antigenic peptide that is an H3 hemagglutinin peptide and an influenza virus HA globular head domain or a portion thereof that is an H1 hemagglutinin globular head domain or a portion thereof. In certain embodiments, the H1 hemagglutinin is the hemagglutinin from influenza A/New Caledonia/20/1999 virus. In certain embodiments, the H3 hemagglutinin is the hemagglutinin from influenza A/Hiroshima/52/2005 virus. In certain embodiments, the influenza virus stalk antigenic peptide is an influenza A/New Caledonia/20/1999 virus hemagglutinin peptide and the influenza virus HA globular head domain or a portion thereof is an influenza A/Hiroshima/52/2005 virus hemagglutinin globular head domain or a portion thereof. In certain embodiments, the influenza virus stalk antigenic peptide is an influenza A/Hiroshima/52/2005 virus hemagglutinin peptide and the influenza virus HA globular head domain or a portion thereof is an influenza A/New Caledonia/20/1999 virus hemagglutinin globular head domain or a portion thereof. In certain embodiments, the influenza virus mutated HA polypeptide comprises a mutated influenza virus HA globular head domain, wherein the mutated influenza virus HA globular head domain comprises (i) an influenza virus HA stalk antigenic peptide from an influenza virus strain as described in Section 5.5.1, *infra*; and (ii) an influenza virus globular head domain or a portion thereof from the same or different influenza virus strain as described in Section 5.5.1, *infra*. See, Section 5.1, *supra*, and Section 6, *infra*, for examples of influenza virus hemagglutinin stalk antigenic peptides.

[0091] As used herein, the term “portion thereof” in the context of an influenza virus HA globular head domain refers to an influenza virus HA globular head domain lacking certain amino acid residues of the head domain. For example, in certain embodiments, certain amino acid residues (*e.g.*, a region or an epitope) of the globular head domain are replaced with an influenza virus HA stalk antigenic peptide.

[0092] In certain embodiments, one or more influenza virus HA stalk antigenic peptides are inserted into an influenza virus HA globular head domain to form a mutated influenza virus HA globular head domain. In certain embodiments, the one or more influenza virus HA stalk antigenic peptides are inserted into one or more locations in an influenza virus HA globular head domain wherein the one or more locations are accessible, such that an immune response (*e.g.*, an antibody response) is generated against the one or more influenza virus HA stalk antigenic peptides. In some embodiments, one or more influenza virus HA stalk antigenic peptides are inserted into an antigenic region (*e.g.*, a region of the head domain known to comprise or consist of an epitope) associated with the influenza HA globular head domain (*e.g.*, antigenic sites A, B, C, and D, wherein the head domain is from subtype H3, or antigenic sites Sa, Sb, Ca and Cb, wherein the head domain is from subtype H1) to form a mutated influenza virus HA globular head domain. In certain embodiments, one or more influenza virus HA stalk antigenic peptides are inserted into the antigenic B site of an influenza virus HA globular head domain from subtype H3 to form a mutated influenza virus HA globular head domain. In some embodiments, one or more influenza virus HA stalk antigenic peptides are inserted into the antigenic A site of an influenza virus HA globular head domain from subtype H3 to form a mutated influenza virus HA globular head domain. In certain embodiments, one or more influenza virus HA stalk antigenic peptides are inserted into the antigenic C site of an influenza virus HA globular head domain from subtype H3 to form a mutated influenza virus HA globular head domain. In certain embodiments, one or more influenza virus HA stalk antigenic peptides are inserted into the antigenic D site of an influenza virus HA globular head domain from subtype H3 to form a mutated influenza virus HA globular head domain. In certain embodiments, the H3 subtype is influenza A/Hiroshima/52/2005.

[0093] In certain embodiments, one or more influenza virus HA stalk antigenic peptides are inserted into the antigenic Sa site of an influenza virus HA globular head domain from subtype H1 to form a mutated influenza virus HA globular head domain. In some embodiments, one or

more influenza virus HA stalk antigenic peptides are inserted into the antigenic Sb site of an influenza virus HA globular head domain from subtype H1 to form a mutated influenza virus HA globular head domain. In certain embodiments, one or more influenza virus HA stalk antigenic peptides are inserted into the antigenic Ca site of an influenza virus HA globular head domain from subtype H1 to form a mutated influenza virus HA globular head domain. In some embodiments, one or more influenza virus HA stalk antigenic peptides are inserted into the antigenic Cb site of an influenza virus HA globular head domain from subtype H1 to form a mutated influenza virus HA globular head domain. In certain embodiments, the H1 subtype is influenza A/New Caledonia/20/1999. In certain embodiments, the H3 subtype is influenza A/Hiroshima/52/2005. *See*, Section 5.1, *supra*, and Section 6, *infra*, for examples of influenza virus hemagglutinin stalk antigenic peptides.

[0094] In some embodiments, certain amino acid residues of an influenza virus HA globular head domain (*e.g.*, a region or epitope of an influenza virus HA globular head domain) are deleted and replaced with one or more influenza virus HA stalk antigenic peptides to form a mutated influenza virus HA globular head domain. In certain embodiments, certain amino acid residues of an influenza virus HA globular head domain (*e.g.*, a region or epitope of an influenza virus HA globular head domain) are deleted and replaced with 1, 2, 3, or more influenza virus HA stalk antigenic peptides (*see*, Section 5.1, *supra*) to form a mutated influenza virus HA globular head domain. In certain embodiments, about 150, 145, 140, 135, 130, 125, 120, 115, 110, 105, 100, 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid residues are deleted from an influenza virus HA globular head domain and replaced with 1, 2, 3, or more influenza virus HA stalk antigenic peptides (*see*, Section 5.1, *supra*) to form a mutated influenza virus HA globular head domain. In certain embodiments, 1-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, 100-110, 110-120, 120-130, 130-140, or 140-150 amino acid residues are deleted from an influenza virus HA globular head domain and replaced with 1, 2, 3, or more influenza virus HA stalk antigenic peptides (*see*, Section 5.1, *supra*) to form a mutated influenza virus HA globular head domain.

[0095] In certain embodiments, about 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid residues of an influenza virus HA globular head domain are substituted with 1, 2, 3, or more influenza virus HA stalk antigenic peptides to form a mutated influenza virus HA globular head domain. In some embodiments, about 1-10, 10-20, 20-30, 30-

40, 40-50, 50-60, 60-70, 70-80, 80-90, or 90-100 amino acid residues of an influenza virus HA globular head domain are substituted 1, 2, 3, or more influenza virus HA stalk antigenic peptides to form a mutated influenza virus HA globular head domain.

[0096] In certain embodiments, up to 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, or more amino acids are deleted from the N-terminus of an influenza virus HA head domain (as viewed from the primary amino acid sequence) and up to 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, and/or more amino acids are deleted from the C-terminus of an influenza virus HA globular head domain (as viewed from the primary amino acid sequence) in a mutated influenza virus HA globular head domain. In certain embodiments, 10-20, 10-25, 15-25, 20-40, 10-50, 20-50, 30-50, 40-60, 50-70 or 10-70 amino acids are deleted from the N-terminus of an influenza virus HA head domain (as viewed from the primary amino acid sequence) and 10-20, 10-25, 15-25, 20-40, 10-50, 20-50, 30-50, 40-60, 50-70, or 10-70, and/or amino acids are deleted from the C-terminus of an influenza virus HA globular head domain (as viewed from the primary amino acid sequence) in a mutated influenza virus HA globular head domain. *See*, Section 5.1, *supra*, and Section 6, *infra*, for examples of influenza virus HA stalk antigenic peptides.

[0097] In certain embodiments, one or more of the antigenic regions (*e.g.*, a region of the head domain known to comprise or consist of an epitope) associated with an influenza HA globular head domain (*e.g.*, antigenic sites A, B, C, and D, wherein the head domain is from subtype H3, or antigenic sites Sa, Sb, Ca and Cb, wherein the head domain is from subtype H1) are deleted and replaced with one or more influenza virus HA stalk antigenic peptides to form a mutated influenza virus HA globular head domain. In a specific embodiment, one antigenic region (*e.g.*, a region of the head domain known to comprise or consist of an epitope) associated with an influenza HA globular head domain (*e.g.*, antigenic site A, B, C, or D, wherein the head domain is from subtype H3, or antigenic site Sa, Sb, Ca or Cb, wherein the head domain is from subtype H1) are deleted and replaced with one or more influenza virus HA stalk antigenic peptides to form a mutated influenza virus HA globular head domain. In another specific embodiment, two antigenic regions (*e.g.*, 2 regions of the head domain known to comprise or consist of an epitope) associated with an influenza HA globular head domain (*e.g.*, antigenic sites A, B, C, or D, wherein the head domain is from subtype H3, or antigenic sites Sa, Sb, Ca or Cb, wherein the head domain is from subtype H1) are deleted and replaced with one or more influenza virus HA stalk antigenic peptides. In another specific embodiment, three antigenic

regions (*e.g.*, 3 regions of the head domain known to comprise or consist of an epitope) associated with an influenza HA globular head domain (*e.g.*, antigenic sites A, B, C, or D, wherein the head domain is from subtype H3, or antigenic sites Sa, Sb, Ca or Cb, wherein the head domain is from subtype H1) are deleted and replaced with one or more influenza virus HA stalk antigenic peptides to form a mutated influenza virus HA globular head domain. In another specific embodiment, four antigenic regions (*e.g.*, 4 regions of the head domain known to comprise or consist of an epitope) associated with an influenza HA globular head domain (*e.g.*, antigenic sites A, B, C, or D, wherein the head domain is from subtype H3, or antigenic sites Sa, Sb, Ca or Cb, wherein the head domain is from subtype H1) are deleted and replaced with one or more influenza virus HA stalk antigenic peptides to form a mutated influenza virus HA globular head domain. In another specific embodiment, five antigenic regions (*e.g.*, 5 regions of the head domain known to comprise or consist of an epitope) associated with an influenza HA globular head domain (*e.g.*, antigenic sites A, B, C, or D, wherein the head domain is from subtype H3, or antigenic sites Sa, Sb, Ca or Cb, wherein the head domain is from subtype H1) are deleted and replaced with one or more influenza virus HA stalk antigenic peptides to form a mutated influenza virus HA globular head domain.. Those of skill in the art can readily determine the antigenic regions (*e.g.*, epitopes) of an influenza virus HA globular head domain known in the art or later identified using techniques known to those of skill in the art and described herein. In certain embodiments, an influenza virus HA stalk antigenic peptide replaces the antigenic B site of an influenza virus HA globular head domain from subtype H3. In certain embodiments, an influenza virus HA stalk antigenic peptide replaces the antigenic A site of an influenza virus HA globular head domain from subtype H3. In certain embodiments, an influenza virus HA stalk antigenic peptide replaces the antigenic C site of an influenza virus head domain from subtype H3. In certain embodiments, an influenza virus hemagglutinin stalk antigenic peptide replaces the antigenic D site of an influenza virus HA globular head domain from subtype H3. In certain embodiments, an influenza virus hemagglutinin stalk antigenic peptide replaces the antigenic Sa site of an influenza virus HA globular head domain from subtype H1. In certain embodiments, an influenza virus hemagglutinin stalk antigenic peptide replaces the antigenic Sb site of an influenza virus HA globular head domain from subtype H1. In certain embodiments, an influenza virus hemagglutinin stalk antigenic peptide replaces the antigenic Ca site of an influenza virus HA globular head domain from subtype H1. In certain embodiments, an

influenza virus hemagglutinin stalk antigenic peptide replaces the antigenic Cb site of an influenza virus HA globular head domain from subtype H1. In certain embodiments, the H1 subtype is influenza A/New Caledonia/20/1999. In certain embodiments, the H3 subtype is influenza A/Hiroshima/52/2005. *See*, Section 5.1, *supra*, and Section 6, *infra*, for examples of influenza virus hemagglutinin stalk antigenic peptides.

[0098] In a specific embodiment, the influenza virus mutated HA polypeptides are immunogenic.

5.3 NUCLEIC ACIDS ENCODING INFLUENZA VIRUS MUTATED HEMAGGLUTININ POLYPEPTIDE

[0099] Provided herein are nucleic acid sequences that encode the influenza virus mutated HA polypeptides described herein. Due to the degeneracy of the genetic code, any nucleic acid sequence that encodes a influenza virus mutated HA polypeptides described herein is encompassed herein. In certain embodiments, nucleic acid sequences corresponding to naturally occurring influenza virus nucleic acid sequences encoding an HA polypeptide are used to produce an influenza virus mutated HA polypeptide.

[00100] Also provided herein are nucleic acid sequences capable of hybridizing to a nucleic acid sequence encoding an influenza virus mutated HA polypeptide. In certain embodiments, provided herein are nucleic acid sequences capable of hybridizing to a fragment of a nucleic acid sequence encoding an influenza virus mutated HA polypeptide. In other embodiments, provided herein are nucleic acid sequence capable of hybridizing to the full length of a nucleic acid sequence encoding an influenza virus mutated HA polypeptide. General parameters for hybridization conditions for nucleic acids are described in Sambrook *et al.*, Molecular Cloning - A Laboratory Manual (2nd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989), and in Ausubel *et al.*, Current Protocols in Molecular Biology, vol. 2, Current Protocols Publishing, New York (1994). Hybridization may be performed under high stringency conditions, medium stringency conditions, or low stringency conditions. Those of skill in the art will understand that low, medium and high stringency conditions are contingent upon multiple factors all of which interact and are also dependent upon the nucleic acids in question. For example, high stringency conditions may include temperatures within 5°C melting temperature of the nucleic acid(s), a low salt concentration (*e.g.*, less than 250 mM), and a high co-solvent

concentration (*e.g.*, 1-20% of co-solvent, *e.g.*, DMSO). Low stringency conditions, on the other hand, may include temperatures greater than 10°C below the melting temperature of the nucleic acid(s), a high salt concentration (*e.g.*, greater than 1000 mM) and the absence of co-solvents.

[00101] In some embodiments, a nucleic acid sequence encoding an influenza virus mutated HA polypeptide is isolated. In certain embodiments, an “isolated” nucleic acid sequence refers to a nucleic acid sequence which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid sequence. In other embodiments, an “isolated” nucleic acid sequence, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. The term “substantially free of cellular material” includes preparations of nucleic acid in which the nucleic acid sequence is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, nucleic acid that is substantially free of cellular material includes preparations of nucleic acid having less than about 30%, 20%, 10%, or 5% (by dry weight) of other nucleic acids. The term “substantially free of culture medium” includes preparations of nucleic acid in which the culture medium represents less than about 50%, 20%, 10%, or 5% of the volume of the preparation. The term “substantially free of chemical precursors or other chemicals” includes preparations in which the nucleic acid is separated from chemical precursors or other chemicals which are involved in the synthesis of the nucleic acid. In specific embodiments, such preparations of the nucleic acid have less than about 50%, 30%, 20%, 10%, 5% (by dry weight) of chemical precursors or compounds other than the nucleic acid of interest.

[00102] In addition, provided herein are nucleic acid sequences encoding the individual components of an influenza virus mutated HA. In specific embodiments, nucleic acid sequences encoding an HA stalk antigenic peptide and/or a mutated influenza virus HA globular head are provided. Nucleic acid sequences encoding components of an influenza virus mutated HA polypeptide may be assembled using standard molecular biology techniques known to the one of skill in the art.

5.4 EXPRESSION OF INFLUENZA VIRUS MUTATED HEMAGGLUTININ (HA) POLYPEPTIDE

[00103] Provided herein are vectors, including expression vectors, containing a nucleic acid encoding an influenza virus mutated HA polypeptide described herein. In a specific embodiment, the vector is an expression vector that is capable of directing the expression of a nucleic acid sequence encoding an influenza virus mutated HA polypeptide. Non-limiting examples of expression vectors include, but are not limited to, plasmids and viral vectors, such as replication defective retroviruses, adenoviruses, adeno-associated viruses and baculoviruses. Expression vectors also may include, without limitation, transgenic animals and non-mammalian cells/organisms, *e.g.*, mammalian cells/organisms that have been engineered to perform mammalian N-linked glycosylation. A nucleic acid or expression vector described herein may be used, for example, for *ex vivo* or *in vivo* gene therapy. Gene therapy techniques are known to those of skill in the art.

[00104] An expression vector comprises a nucleic acid sequence encoding an influenza virus mutated HA polypeptide described herein in a form suitable for expression of the nucleic acid sequence in a host cell. In a specific embodiment, an expression vector includes one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operably linked to the nucleic acid to be expressed. Within an expression vector, "operably linked" is intended to mean that a nucleic acid of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleic acid (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). Regulatory sequences include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Regulatory sequences include those which direct constitutive expression of a nucleic acid in many types of host cells, those which direct expression of the nucleic acid sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences), and those which direct the expression of the nucleic acid sequence upon stimulation with a particular agent (*e.g.*, inducible regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The term "host cell" is intended to include a particular subject cell transformed or transfected with a nucleic acid and the progeny or potential progeny of such a cell. Progeny of such a cell may not be identical to the parent cell transformed or transfected with the nucleic acid due to mutations or environmental influences

that may occur in succeeding generations or integration of the nucleic acid into the host cell genome.

[00105] Expression vectors can be designed for expression of an influenza virus mutated HA polypeptide described herein using prokaryotic (*e.g.*, *E. coli*) or eukaryotic cells (*e.g.*, insect cells (using baculovirus expression vectors, *see, e.g.*, Treanor *et al.*, 2007, JAMA, 297(14):1577-1582 incorporated by reference herein in its entirety), yeast cells, plant cells, algae or mammalian cells). Examples of yeast host cells include, but are not limited to *S. pombe* and *S. cerevisiae* and examples, *infra*. Examples of mammalian host cells include, but are not limited to, Crucell Per.C6 cells, Vero cells, CHO cells, VERY cells, BHK cells, HeLa cells, COS cells, MDCK cells, 293 cells, 3T3 cells or WI38 cells. In certain embodiments, the host cells are myeloma cells, *e.g.*, NS0 cells, 45.6 TG1.7 cells, AF-2 clone 9B5 cells, AF-2 clone 9B5 cells, J558L cells, MOPC 315 cells, MPC-11 cells, NCI-H929 cells, NP cells, NS0/1 cells, P3 NS1 Ag4 cells, P3/NS1/1-Ag4-1 cells, P3U1 cells, P3X63Ag8 cells, P3X63Ag8.653 cells, P3X63Ag8U.1 cells, RPMI 8226 cells, Sp20-Ag14 cells, U266B1 cells, X63AG8.653 cells, Y3.Ag.1.2.3 cells, and YO cells. Non-limiting examples of insect cells include *Sf9*, *Sf21*, *Trichoplusia ni*, *Spodoptera frugiperda* and *Bombyx mori*. In a particular embodiment, a mammalian cell culture system (*e.g.* Chinese hamster ovary or baby hamster kidney cells) is used for expression of an influenza virus mutated HA polypeptide. In another embodiment, a plant cell culture system is used for expression of an influenza virus mutated HA polypeptide. *See, e.g.*, U.S. Patent Nos. 7,504,560; 6,770,799; 6,551,820; 6,136,320; 6,034,298; 5,914,935; 5,612,487; and 5,484,719, and U.S. patent application publication Nos. 2009/0208477, 2009/0082548, 2009/0053762, 2008/0038232, 2007/0275014 and 2006/0204487 for plant cells and methods for the production of proteins utilizing plant cell culture systems. In specific embodiments, plant cell culture systems are not used for expression of an influenza virus mutated hemagglutinin HA polypeptide. The host cells comprising a nucleic acid sequence that encodes an influenza virus mutated HA polypeptides described herein can be isolated, *i.e.*, the cells are outside of the body of a subject. In certain embodiments, the cells are engineered to express a nucleic acid sequence that encodes an influenza virus mutated (HA polypeptide described herein).

[00106] An expression vector can be introduced into host cells via conventional transformation or transfection techniques. Such techniques include, but are not limited to, calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection,

lipofection, and electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook *et al.*, 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, New York, and other laboratory manuals. In certain embodiments, a host cell is transiently transfected with an expression vector containing a nucleic acid sequence encoding an influenza virus mutated HA polypeptide. In other embodiments, a host cell is stably transfected with an expression vector containing a nucleic acid sequence encoding an influenza virus mutated HA polypeptide.

[00107] For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a nucleic acid that encodes a selectable marker (*e.g.*, for resistance to antibiotics) is generally introduced into the host cells along with the nucleic acid of interest. Examples of selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

[00108] As an alternative to recombinant expression of an influenza virus mutated HA polypeptide using a host cell, an expression vector containing a nucleic acid sequence encoding an influenza virus mutated HA polypeptide can be transcribed and translated *in vitro* using, *e.g.*, T7 promoter regulatory sequences and T7 polymerase. In a specific embodiment, a coupled transcription/translation system, such as Promega TNT®, or a cell lysate or cell extract comprising the components necessary for transcription and translation may be used to produce an influenza virus mutated HA polypeptide.

[00109] Once an influenza virus mutated HA polypeptide has been produced, it may be isolated or purified by any method known in the art for isolation or purification of a protein, for example, by chromatography (*e.g.*, ion exchange, affinity, particularly by affinity for the specific antigen, by Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the isolation or purification of proteins. In certain embodiments, an influenza virus mutated HA polypeptide may be conjugated to a heterologous protein(s).

[00110] Accordingly, provided herein are methods for producing an influenza virus mutated HA polypeptide. In one embodiment, the method comprises culturing a host cell containing a

nucleic acid sequence encoding an influenza virus mutated HA polypeptide in a suitable medium such that the polypeptide is produced. In some embodiments, the method further comprises isolating the polypeptide from the medium or the host cell.

5.5 VIRAL VECTORS

5.5.1 Influenza Virus Vectors

[00111] In one aspect, provided herein are influenza viruses containing an influenza virus mutated HA polypeptide described herein. In a specific embodiment, the influenza virus mutated HA polypeptide is incorporated into the virions of the influenza virus. In some embodiments, the virions of the influenza virus have incorporated into them or express a heterologous polypeptide in addition to an influenza virus mutated HA polypeptide. The heterologous polypeptide may be a polypeptide that has immunopotentiating activity, or that targets the influenza virus to a particular cell type, such as an antibody that binds to an antigen on a specific cell type or a ligand that binds a specific receptor on a specific cell type.

[00112] Influenza viruses containing an influenza virus mutated HA polypeptide may be produced by supplying in *trans* the influenza virus mutated HA polypeptide during production of virions using techniques known to one skilled in the art, such as reverse genetics and helper-free plasmid rescue.

[00113] In another aspect, provided herein are influenza viruses comprising a genome engineered to express an influenza virus mutated HA polypeptide. In a specific embodiment, the genome of a parental influenza virus is engineered to encode an influenza virus mutated hemagglutinin HA polypeptide, which is expressed by progeny influenza virus. In another specific embodiment, the genome of a parental influenza virus is engineered to encode an influenza virus mutated HA polypeptide, which is expressed and incorporated into the virions of progeny influenza virus. Thus, the progeny influenza virus resulting from the replication of the parental influenza virus contains an influenza virus mutated HA polypeptide. In some embodiments, the virions of the parental influenza virus may have incorporated into them an influenza virus mutated hemagglutinin HA polypeptide, wherein the stalk antigenic peptide displayed in the HA globular head domain is from the same or a different type, subtype or strain of influenza virus as the parental influenza virus.

[00114] Since the genome of influenza A and B viruses consist of eight (8) single-stranded, negative sense segments (influenza C viruses consist of seven (7) single-stranded, negative sense segments), the genome of a parental influenza virus may be engineered to express an influenza virus mutated HA polypeptide using a recombinant segment and techniques known to one skilled in the art, such a reverse genetics and helper-free plasmid rescue. In one embodiment, the recombinant segment comprises a nucleic acid encoding the influenza virus mutated HA polypeptide as well as the 3' and 5' incorporation signals which are required for proper replication, transcription and packaging of the vRNAs (Fujii *et al.*, 2003, Proc. Natl. Acad. Sci. USA 100:2002-2007; Zheng, *et al.*, 1996, Virology 217:242-251, both of which are incorporated by reference herein in their entireties).

[00115] In some embodiments, the genome of a parental influenza virus may be engineered to express an influenza virus mutated HA polypeptide using a recombinant segment that is bicistronic. Bicistronic techniques allow the engineering of coding sequences of multiple proteins into a single mRNA through the use of internal ribosome entry site (IRES) sequences. IRES sequences direct the internal recruitment of ribosomes to the RNA molecule and allow downstream translation in a cap independent manner. Briefly, a coding region of one protein is inserted into the open reading frame (ORF) of a second protein. The insertion is flanked by an IRES and any untranslated signal sequences necessary for proper expression and/or function. The insertion must not disrupt the ORF, polyadenylation or transcriptional promoters of the second protein (*see, e.g.*, García-Sastre *et al.*, 1994, J. Virol. 68:6254-6261 and García-Sastre *et al.*, 1994 Dev. Biol. Stand. 82:237-246, each of which is hereby incorporated by reference in its entirety). *See also, e.g.*, U.S. Patent No. 6,887,699, U.S. Patent No. 6,001,634, U.S. Patent No. 5,854,037 and U.S. Patent No. 5,820,871, each of which is incorporated herein by reference in its entirety. Any IRES known in the art or described herein may be used in accordance with the invention (*e.g.*, the IRES of BiP gene, nucleotides 372 to 592 of GenBank database entry HUMGRP78; or the IRES of encephalomyocarditis virus (EMCV), nucleotides 1430-2115 of GenBank database entry CQ867238.). Thus, in certain embodiments, a parental influenza virus is engineered to contain a bicistronic RNA segment that expresses the influenza virus mutated HA polypeptide and another polypeptide, such as a gene expressed by the parental influenza virus. In some embodiments, the parental influenza virus gene is the HA gene.

[00116] Techniques known to one skilled in the art may be used to produce an influenza virus containing an influenza virus mutated hemagglutinin HA polypeptide and an influenza virus comprising a genome engineered to express an influenza virus mutated HA polypeptide. For example, reverse genetics techniques may be used to generate such an influenza virus. Briefly, reverse genetics techniques generally involve the preparation of synthetic recombinant viral RNAs that contain the non-coding regions of the negative-strand, viral RNA which are essential for the recognition by viral polymerases and for packaging signals necessary to generate a mature virion. The recombinant RNAs are synthesized from a recombinant DNA template and reconstituted *in vitro* with purified viral polymerase complex to form recombinant ribonucleoproteins (RNPs) which can be used to transfect cells. A more efficient transfection is achieved if the viral polymerase proteins are present during transcription of the synthetic RNAs either *in vitro* or *in vivo*. The synthetic recombinant RNPs can be rescued into infectious virus particles. The foregoing techniques are described in U.S. Patent No. 5,166,057 issued November 24, 1992; in U.S. Patent No. 5,854,037 issued December 29, 1998; in European Patent Publication EP 0702085A1, published February 20, 1996; in U.S. Patent Application Serial No. 09/152,845; in International Patent Publications PCT WO 97/12032 published April 3, 1997; WO 96/34625 published November 7, 1996; in European Patent Publication EP A780475; WO 99/02657 published January 21, 1999; WO 98/53078 published November 26, 1998; WO 98/02530 published January 22, 1998; WO 99/15672 published April 1, 1999; WO 98/13501 published April 2, 1998; WO 97/06270 published February 20, 1997; and EPO 780 475A1 published June 25, 1997, each of which is incorporated by reference herein in its entirety.

[00117] Alternatively, helper-free plasmid technology may be used to produce an influenza virus containing an influenza virus mutated HA polypeptide and an influenza virus comprising a genome engineered to express an influenza virus mutated HA polypeptide. Briefly, full length cDNAs of viral segments are amplified using PCR with primers that include unique restriction sites, which allow the insertion of the PCR product into the plasmid vector (Flandorfer *et al.*, 2003, J. Virol. 77:9116-9123; Nakaya *et al.*, 2001, J. Virol. 75:11868-11873; both of which are incorporated herein by reference in their entireties). The plasmid vector is designed so that an exact negative (vRNA sense) transcript is expressed. For example, the plasmid vector may be designed to position the PCR product between a truncated human RNA polymerase I promoter and a hepatitis delta virus ribozyme sequence such that an exact negative (vRNA sense)

transcript is produced from the polymerase I promoter. Separate plasmid vectors comprising each viral segment as well as expression vectors comprising necessary viral proteins may be transfected into cells leading to production of recombinant viral particles. In another example, plasmid vectors from which both the viral genomic RNA and mRNA encoding the necessary viral proteins are expressed may be used. For a detailed description of helper-free plasmid technology *see, e.g.*, International Publication No. WO 01/04333; U.S. Patent Nos. 6,951,754, 7,384,774, 6,649,372, and 7,312,064; Fodor *et al.*, 1999, J. Virol. 73:9679-9682; Quinlivan *et al.*, 2005, J. Virol. 79:8431-8439; Hoffmann *et al.*, 2000, Proc. Natl. Acad. Sci. USA 97:6108-6113; and Neumann *et al.*, 1999, Proc. Natl. Acad. Sci. USA 96:9345-9350, which are incorporated herein by reference in their entireties.

[00118] The influenza viruses described herein may be propagated in any substrate that allows the virus to grow to titers that permit their use in accordance with the methods described herein. In one embodiment, the substrate allows the viruses to grow to titers comparable to those determined for the corresponding wild-type viruses. In certain embodiments, the substrate is one which is biologically relevant to the influenza virus or to the virus from which the HA function is derived.

[00119] The influenza viruses described herein may be isolated and purified by any method known to those of skill in the art. In one embodiment, the virus is removed from cell culture and separated from cellular components, typically by well known clarification procedures, *e.g.*, such as gradient centrifugation and column chromatography, and may be further purified as desired using procedures well known to those skilled in the art, *e.g.*, plaque assays.

[00120] In certain embodiments, the influenza viruses, or influenza virus polypeptides, genes or genome segments for use as described herein are obtained or derived from an influenza A virus. In certain embodiments, the influenza viruses, or influenza virus polypeptides, genes or genome segments for use as described herein are obtained or derived from a single influenza A virus subtype or strain. In other embodiments, the influenza viruses, or influenza virus polypeptides, genes or genome segments for use as described herein are obtained or derived from two or more influenza A virus subtypes or strains.

[00121] In some embodiments, the influenza viruses, or influenza virus polypeptides, genes or genome segments for use as described herein are obtained or derived from an influenza B virus. In certain embodiments, the influenza viruses, or influenza virus polypeptides, genes or genome

segments for use as described herein are obtained or derived from a single influenza B virus subtype or strain. In other embodiments, the influenza viruses, or influenza virus polypeptides, genes or genome segments for use as described herein are obtained or derived from two or more influenza B virus subtypes or strains. In other embodiments, the influenza viruses, or influenza virus polypeptides, genes or genome segments for use as described herein are obtained or derived from a combination of influenza A and influenza B virus subtypes or strains.

[00122] In some embodiments, the influenza viruses, or influenza virus polypeptides, genes or genome segments for use as described herein are obtained or derived from an influenza C virus. In certain embodiments, the influenza viruses, or influenza virus polypeptides, genes or genome segments for use as described herein are obtained or derived from a single influenza C virus subtype or strain. In other embodiments, the influenza viruses, or influenza virus polypeptides, genes or genome segments for use as described herein are obtained or derived from two or more influenza C virus subtypes or strains. In other embodiments, the influenza viruses, or influenza virus polypeptides, genes or genome segments for use as described herein are obtained or derived from a combination of influenza C virus and influenza A virus and/or influenza B virus subtypes or strains.

[00123] Non-limiting examples of influenza A viruses include subtype H10N4, subtype H10N5, subtype H10N7, subtype H10N8, subtype H10N9, subtype H11N1, subtype H11N13, subtype H11N2, subtype H11N4, subtype H11N6, subtype H11N8, subtype H11N9, subtype H12N1, subtype H12N4, subtype H12N5, subtype H12N8, subtype H13N2, subtype H13N3, subtype H13N6, subtype H13N7, subtype H14N5, subtype H14N6, subtype H15N8, subtype H15N9, subtype H16N3, subtype H1N1, subtype H1N2, subtype H1N3, subtype H1N6, subtype H1N9, subtype H2N1, subtype H2N2, subtype H2N3, subtype H2N5, subtype H2N7, subtype H2N8, subtype H2N9, subtype H3N1, subtype H3N2, subtype H3N3, subtype H3N4, subtype H3N5, subtype H3N6, subtype H3N8, subtype H3N9, subtype H4N1, subtype H4N2, subtype H4N3, subtype H4N4, subtype H4N5, subtype H4N6, subtype H4N8, subtype H4N9, subtype H5N1, subtype H5N2, subtype H5N3, subtype H5N4, subtype H5N6, subtype H5N7, subtype H5N8, subtype H5N9, subtype H6N1, subtype H6N2, subtype H6N3, subtype H6N4, subtype H6N5, subtype H6N6, subtype H6N7, subtype H6N8, subtype H6N9, subtype H7N1, subtype H7N2, subtype H7N3, subtype H7N4, subtype H7N5, subtype H7N7, subtype H7N8, subtype

H7N9, subtype H8N4, subtype H8N5, subtype H9N1, subtype H9N2, subtype H9N3, subtype H9N5, subtype H9N6, subtype H9N7, subtype H9N8, and subtype H9N9.

[00124] Specific examples of strains of influenza A virus include, but are not limited to:

A/Victoria/361/2011 (H3N2); A/California/4/2009 (H1N1); A/California/7/2009 (H1N1); A/Perth/16/2009 (H3N2); A/Brisbane/59/2007 (H1N1); A/Brisbane/10/2007 ((H3N2); A/sw/Iowa/15/30 (H1N1); A/WSN/33 (H1N1); A/eq/Prague/1/56 (H7N7); A/PR/8/34; A/mallard/Potsdam/178-4/83 (H2N2); A/herring gull/DE/712/88 (H16N3); A/sw/Hong Kong/168/1993 (H1N1); A/mallard/Alberta/211/98 (H1N1); A/shorebird/Delaware/168/06 (H16N3); A/sw/Netherlands/25/80 (H1N1); A/sw/Germany/2/81 (H1N1); A/sw/Hannover/1/81 (H1N1); A/sw/Potsdam/1/81 (H1N1); A/sw/Potsdam/15/81 (H1N1); A/sw/Potsdam/268/81 (H1N1); A/sw/Finistere/2899/82 (H1N1); A/sw/Potsdam/35/82 (H3N2); A/sw/Cote d'Armor/3633/84 (H3N2); A/sw/Gent/1/84 (H3N2); A/sw/Netherlands/12/85 (H1N1); A/sw/Karrenzien/2/87 (H3N2); A/sw/Schwerin/103/89 (H1N1); A/turkey/Germany/3/91 (H1N1); A/sw/Germany/8533/91 (H1N1); A/sw/Belgium/220/92 (H3N2); A/sw/Gent/V230/92 (H1N1); A/sw/Leipzig/145/92 (H3N2); A/sw/Re220/92hp (H3N2); A/sw/Bakum/909/93 (H3N2); A/sw/Schleswig-Holstein/1/93 (H1N1); A/sw/Scotland/419440/94 (H1N2); A/sw/Bakum/5/95 (H1N1); A/sw/Best/5C/96 (H1N1); A/sw/England/17394/96 (H1N2); A/sw/Jena/5/96 (H3N2); A/sw/Oedenrode/7C/96 (H3N2); A/sw/Lohne/1/97 (H3N2); A/sw/Cote d'Armor/790/97 (H1N2); A/sw/Bakum/1362/98 (H3N2); A/sw/Italy/1521/98 (H1N2); A/sw/Italy/1553-2/98 (H3N2); A/sw/Italy/1566/98 (H1N1); A/sw/Italy/1589/98 (H1N1); A/sw/Bakum/8602/99 (H3N2); A/sw/Cotes d'Armor/604/99 (H1N2); A/sw/Cote d'Armor/1482/99 (H1N1); A/sw/Gent/7625/99 (H1N2); A/Hong Kong/1774/99 (H3N2); A/sw/Hong Kong/5190/99 (H3N2); A/sw/Hong Kong/5200/99 (H3N2); A/sw/Hong Kong/5212/99 (H3N2); A/sw/Ille et Villaine/1455/99 (H1N1); A/sw/Italy/1654-1/99 (H1N2); A/sw/Italy/2034/99 (H1N1); A/sw/Italy/2064/99 (H1N2); A/sw/Berlin/1578/00 (H3N2); A/sw/Bakum/1832/00 (H1N2); A/sw/Bakum/1833/00 (H1N2); A/sw/Cote d'Armor/800/00 (H1N2); A/sw/Hong Kong/7982/00 (H3N2); A/sw/Italy/1081/00 (H1N2); A/sw/Belzig/2/01 (H1N1); A/sw/Belzig/54/01 (H3N2); A/sw/Hong Kong/9296/01 (H3N2); A/sw/Hong Kong/9745/01 (H3N2); A/sw/Spain/33601/01 (H3N2); A/sw/Hong Kong/1144/02 (H3N2); A/sw/Hong Kong/1197/02 (H3N2); A/sw/Spain/39139/02 (H3N2); A/sw/Spain/42386/02 (H3N2); A/Switzerland/8808/2002 (H1N1); A/sw/Bakum/1769/03 (H3N2);

A/sw/Bissendorf/IDT1864/03 (H3N2); A/sw/Ehren/IDT2570/03 (H1N2);
A/sw/Gescher/IDT2702/03 (H1N2); A/sw/Haselünne/2617/03hp (H1N1);
A/sw/Löningen/IDT2530/03 (H1N2); A/sw/IVD/IDT2674/03 (H1N2);
A/sw/Nordkirchen/IDT1993/03 (H3N2); A/sw/Nordwalde/IDT2197/03 (H1N2);
A/sw/Norden/IDT2308/03 (H1N2); A/sw/Spain/50047/03 (H1N1); A/sw/Spain/51915/03
(H1N1); A/sw/Vechta/2623/03 (H1N1); A/sw/Visbek/IDT2869/03 (H1N2);
A/sw/Waltersdorf/IDT2527/03 (H1N2); A/sw/Damme/IDT2890/04 (H3N2);
A/sw/Geldern/IDT2888/04 (H1N1); A/sw/Granstedt/IDT3475/04 (H1N2);
A/sw/Greven/IDT2889/04 (H1N1); A/sw/Gudensberg/IDT2930/04 (H1N2);
A/sw/Gudensberg/IDT2931/04 (H1N2); A/sw/Lohne/IDT3357/04 (H3N2);
A/sw/Nortrup/IDT3685/04 (H1N2); A/sw/Seesen/IDT3055/04 (H3N2); A/sw/Spain/53207/04
(H1N1); A/sw/Spain/54008/04 (H3N2); A/sw/Stolzenau/IDT3296/04 (H1N2);
A/sw/Wedel/IDT2965/04 (H1N1); A/sw/Bad Griesbach/IDT4191/05 (H3N2);
A/sw/Cloppenburg/IDT4777/05 (H1N2); A/sw/Dötlingen/IDT3780/05 (H1N2);
A/sw/Dötlingen/IDT4735/05 (H1N2); A/sw/Eggldham/IDT5250/05 (H3N2);
A/sw/Harkenblek/IDT4097/05 (H3N2); A/sw/Hertzen/IDT4317/05 (H3N2);
A/sw/Kroegel/IDT4192/05 (H1N1); A/sw/Laer/IDT3893/05 (H1N1); A/sw/Laer/IDT4126/05
(H3N2); A/sw/Merzen/IDT4114/05 (H3N2); A/sw/Muesleringen-S./IDT4263/05 (H3N2);
A/sw/Osterhofen/IDT4004/05 (H3N2); A/sw/Sprengel/IDT3805/05 (H1N2);
A/sw/Stadtlöhne/IDT3853/05 (H1N2); A/sw/Vogelarn/IDT4096/05 (H1N1);
A/sw/Wohlerst/IDT4093/05 (H1N1); A/sw/Bad Griesbach/IDT5604/06 (H1N1);
A/sw/Herzlake/IDT5335/06 (H3N2); A/sw/Herzlake/IDT5336/06 (H3N2);
A/sw/Herzlake/IDT5337/06 (H3N2); and A/wild boar/Germany/R169/2006 (H3N2).

[00125] Other specific examples of strains of influenza A virus include, but are not limited to:
A/Toronto/3141/2009 (H1N1); A/Regensburg/D6/2009 (H1N1); A/Bayern/62/2009 (H1N1);
A/Bayern/62/2009 (H1N1); A/Bradenburg/19/2009 (H1N1); A/Bradenburg/20/2009 (H1N1);
A/Distrito Federal/2611/2009 (H1N1); A/Mato Grosso/2329/2009 (H1N1); A/Sao
Paulo/1454/2009 (H1N1); A/Sao Paulo/2233/2009 (H1N1); A/Stockholm/37/2009 (H1N1);
A/Stockholm/41/2009 (H1N1); A/Stockholm/45/2009 (H1N1); A/swine/Alberta/OTH-33-1/2009
(H1N1); A/swine/Alberta/OTH-33-14/2009 (H1N1); A/swine/Alberta/OTH-33-2/2009 (H1N1);
A/swine/Alberta/OTH-33-21/2009 (H1N1); A/swine/Alberta/OTH-33-22/2009 (H1N1);

A/swine/Alberta/OTH-33-23/2009 (H1N1); A/swine/Alberta/OTH-33-24/2009 (H1N1); A/swine/Alberta/OTH-33-25/2009 (H1N1); A/swine/Alberta/OTH-33-3/2009 (H1N1); A/swine/Alberta/OTH-33-7/2009 (H1N1); A/Beijing/502/2009 (H1N1); A/Firenze/10/2009 (H1N1); A/Hong Kong/2369/2009 (H1N1); A/Italy/85/2009 (H1N1); A/Santo Domingo/572N/2009 (H1N1); A/Catalonia/385/2009 (H1N1); A/Catalonia/386/2009 (H1N1); A/Catalonia/387/2009 (H1N1); A/Catalonia/390/2009 (H1N1); A/Catalonia/394/2009 (H1N1); A/Catalonia/397/2009 (H1N1); A/Catalonia/398/2009 (H1N1); A/Catalonia/399/2009 (H1N1); A/Sao Paulo/2303/2009 (H1N1); A/Akita/1/2009 (H1N1); A/Castro/JXP/2009 (H1N1); A/Fukushima/1/2009 (H1N1); A/Israel/276/2009 (H1N1); A/Israel/277/2009 (H1N1); A/Israel/70/2009 (H1N1); A/Iwate/1/2009 (H1N1); A/Iwate/2/2009 (H1N1); A/Kagoshima/1/2009 (H1N1); A/Osaka/180/2009 (H1N1); A/Puerto Montt/Bio87/2009 (H1N1); A/Sao Paulo/2303/2009 (H1N1); A/Sapporo/1/2009 (H1N1); A/Stockholm/30/2009 (H1N1); A/Stockholm/31/2009 (H1N1); A/Stockholm/32/2009 (H1N1); A/Stockholm/33/2009 (H1N1); A/Stockholm/34/2009 (H1N1); A/Stockholm/35/2009 (H1N1); A/Stockholm/36/2009 (H1N1); A/Stockholm/38/2009 (H1N1); A/Stockholm/39/2009 (H1N1); A/Stockholm/40/2009 (H1N1); A/Stockholm/42/2009 (H1N1); A/Stockholm/43/2009 (H1N1); A/Stockholm/44/2009 (H1N1); A/Utsunomiya/2/2009 (H1N1); A/WRAIR/0573N/2009 (H1N1); and A/Zhejiang/DTID-ZJU01/2009 (H1N1).

[00126] Non-limiting examples of influenza B viruses include strain Aichi/5/88, strain B/Brisbane/60/2008; Akita/27/2001, strain Akita/5/2001, strain Alaska/16/2000, strain Alaska/1777/2005, strain Argentina/69/2001, strain Arizona/146/2005, strain Arizona/148/2005, strain Bangkok/163/90, strain Bangkok/34/99, strain Bangkok/460/03, strain Bangkok/54/99, strain Barcelona/215/03, strain Beijing/15/84, strain Beijing/184/93, strain Beijing/243/97, strain Beijing/43/75, strain Beijing/5/76, strain Beijing/76/98, strain Belgium/WV106/2002, strain Belgium/WV107/2002, strain Belgium/WV109/2002, strain Belgium/WV114/2002, strain Belgium/WV122/2002, strain Bonn/43, strain Brazil/952/2001, strain Bucharest/795/03, strain Buenos Aires/161/00, strain Buenos Aires/9/95, strain Buenos Aires/SW16/97, strain Buenos Aires/VL518/99, strain Canada/464/2001, strain Canada/464/2002, strain Chaco/366/00, strain Chaco/R113/00, strain Cheju/303/03, strain Chiba/447/98, strain Chongqing/3/2000, strain clinical isolate SA1 Thailand/2002, strain clinical isolate SA10 Thailand/2002, strain clinical isolate SA100 Philippines/2002, strain clinical isolate SA101 Philippines/2002, strain clinical

isolate SA110 Philippines/2002), strain clinical isolate SA112 Philippines/2002, strain clinical isolate SA113 Philippines/2002, strain clinical isolate SA114 Philippines/2002, strain clinical isolate SA2 Thailand/2002, strain clinical isolate SA20 Thailand/2002, strain clinical isolate SA38 Philippines/2002, strain clinical isolate SA39 Thailand/2002, strain clinical isolate SA99 Philippines/2002, strain CNIC/27/2001, strain Colorado/2597/2004, strain Cordoba/VA418/99, strain Czechoslovakia/16/89, strain Czechoslovakia/69/90, strain Daeku/10/97, strain Daeku/45/97, strain Daeku/47/97, strain Daeku/9/97, strain B/Du/4/78, strain B/Durban/39/98, strain Durban/43/98, strain Durban/44/98, strain B/Durban/52/98, strain Durban/55/98, strain Durban/56/98, strain England/1716/2005, strain England/2054/2005), strain England/23/04, strain Finland/154/2002, strain Finland/159/2002, strain Finland/160/2002, strain Finland/161/2002, strain Finland/162/03, strain Finland/162/2002, strain Finland/162/91, strain Finland/164/2003, strain Finland/172/91, strain Finland/173/2003, strain Finland/176/2003, strain Finland/184/91, strain Finland/188/2003, strain Finland/190/2003, strain Finland/220/2003, strain Finland/WV5/2002, strain Fujian/36/82, strain Geneva/5079/03, strain Genoa/11/02, strain Genoa/2/02, strain Genoa/21/02, strain Genova/54/02, strain Genova/55/02, strain Guangdong/05/94, strain Guangdong/08/93, strain Guangdong/5/94, strain Guangdong/55/89, strain Guangdong/8/93, strain Guangzhou/7/97, strain Guangzhou/86/92, strain Guangzhou/87/92, strain Gyeonggi/592/2005, strain Hannover/2/90, strain Harbin/07/94, strain Hawaii/10/2001, strain Hawaii/1990/2004, strain Hawaii/38/2001, strain Hawaii/9/2001, strain Hebei/19/94, strain Hebei/3/94), strain Henan/22/97, strain Hiroshima/23/2001, strain Hong Kong/110/99, strain Hong Kong/1115/2002, strain Hong Kong/112/2001, strain Hong Kong/123/2001, strain Hong Kong/1351/2002, strain Hong Kong/1434/2002, strain Hong Kong/147/99, strain Hong Kong/156/99, strain Hong Kong/157/99, strain Hong Kong/22/2001, strain Hong Kong/22/89, strain Hong Kong/336/2001, strain Hong Kong/666/2001, strain Hong Kong/9/89, strain Houston/1/91, strain Houston/1/96, strain Houston/2/96, strain Hunan/4/72, strain Ibaraki/2/85, strain Incheon/297/2005, strain India/3/89, strain India/77276/2001, strain Israel/95/03, strain Israel/WV187/2002, strain Japan/1224/2005, strain Jiangsu/10/03, strain Johannesburg/1/99, strain Johannesburg/96/01, strain Kadoma/1076/99, strain Kadoma/122/99, strain Kagoshima/15/94, strain Kansas/22992/99, strain Khazkov/224/91, strain Kobe/1/2002, strain, strain Kouchi/193/99, strain Lazio/1/02, strain Lee/40, strain Leningrad/129/91, strain Lisbon/2/90), strain Los Angeles/1/02, strain Lusaka/270/99, strain Lyon/1271/96, strain

Malaysia/83077/2001, strain Maputo/1/99, strain Mar del Plata/595/99, strain Maryland/1/01, strain Memphis/1/01, strain Memphis/12/97-MA, strain Michigan/22572/99, strain Mie/1/93, strain Milano/1/01, strain Minsk/318/90, strain Moscow/3/03, strain Nagoya/20/99, strain Nanchang/1/00, strain Nashville/107/93, strain Nashville/45/91, strain Nebraska/2/01, strain Netherland/801/90, strain Netherlands/429/98, strain New York/1/2002, strain NIB/48/90, strain Ningxia/45/83, strain Norway/1/84, strain Oman/16299/2001, strain Osaka/1059/97, strain Osaka/983/97-V2, strain Oslo/1329/2002, strain Oslo/1846/2002, strain Panama/45/90, strain Paris/329/90, strain Parma/23/02, strain Perth/211/2001, strain Peru/1364/2004, strain Philippines/5072/2001, strain Pusan/270/99, strain Quebec/173/98, strain Quebec/465/98, strain Quebec/7/01, strain Roma/1/03, strain Saga/S172/99, strain Seoul/13/95, strain Seoul/37/91, strain Shangdong/7/97, strain Shanghai/361/2002), strain Shiga/T30/98, strain Sichuan/379/99, strain Singapore/222/79, strain Spain/WV27/2002, strain Stockholm/10/90, strain Switzerland/5441/90, strain Taiwan/0409/00, strain Taiwan/0722/02, strain Taiwan/97271/2001, strain Tehran/80/02, strain Tokyo/6/98, strain Trieste/28/02, strain Ulan Ude/4/02, strain United Kingdom/34304/99, strain USSR/100/83, strain Victoria/103/89, strain Vienna/1/99, strain Wuhan/356/2000, strain WV194/2002, strain Xuanwu/23/82, strain Yamagata/1311/2003, strain Yamagata/K500/2001, strain Alaska/12/96, strain GA/86, strain NAGASAKI/1/87, strain Tokyo/942/96, strain B/Wisconsin/1/2010; and strain Rochester/02/2001.

[00127] Non-limiting examples of influenza C viruses include strain Aichi/1/81, strain Ann Arbor/1/50, strain Aomori/74, strain California/78, strain England/83, strain Greece/79, strain Hiroshima/246/2000, strain Hiroshima/252/2000, strain Hyogo/1/83, strain Johannesburg/66, strain Kanagawa/1/76, strain Kyoto/1/79, strain Mississippi/80, strain Miyagi/1/97, strain Miyagi/5/2000, strain Miyagi/9/96, strain Nara/2/85, strain NewJersey/76, strain pig/Beijing/115/81, strain Saitama/3/2000), strain Shizuoka/79, strain Yamagata/2/98, strain Yamagata/6/2000, strain Yamagata/9/96, strain BERLIN/1/85, strain ENGLAND/892/8, strain GREAT LAKES/1167/54, strain JJ/50, strain PIG/BEIJING/10/81, strain PIG/BEIJING/439/82), strain TAYLOR/1233/47, and strain C/YAMAGATA/10/81.

[00128] In certain embodiments, the influenza viruses provided herein have an attenuated phenotype. In specific embodiments, the attenuated influenza virus is based on influenza A virus. In other embodiments, the attenuated influenza virus is based on influenza B virus. In yet other embodiments, the attenuated influenza virus is based on influenza C virus. In other

embodiments, the attenuated influenza virus may comprise genes or genome segments from one or more strains or subtypes of influenza A, influenza B, and/or influenza C virus. In some embodiments, the attenuated backbone virus comprises genes from an influenza A virus and an influenza B virus.

[00129] In specific embodiments, attenuation of influenza virus is desired such that the virus remains, at least partially, infectious and can replicate *in vivo*, but only generate low titers resulting in subclinical levels of infection that are non-pathogenic. Such attenuated viruses are especially suited for embodiments described herein wherein the virus or an immunogenic composition thereof is administered to a subject to induce an immune response. Attenuation of the influenza virus can be accomplished according to any method known in the art, such as, *e.g.*, selecting viral mutants generated by chemical mutagenesis, mutation of the genome by genetic engineering, selecting reassortant viruses that contain segments with attenuated function, or selecting for conditional virus mutants (*e.g.*, cold-adapted viruses). Alternatively, naturally occurring attenuated influenza viruses may be used as influenza virus backbones for the influenza virus vectors.

5.5.2 Non-Influenza Virus Vectors

[00130] In one aspect, provided herein are non-influenza viruses containing an influenza virus mutated HA polypeptide. In a specific embodiment, the influenza virus mutated HA polypeptide is incorporated into the virions of the non-influenza virus. In a specific embodiment, the influenza virus mutated HA polypeptide is contained in/expressed by a purified (*e.g.*, plaque purified) or isolated virus. The non-influenza viruses may be conjugated to moieties that target the viruses to particular cell types, such as immune cells. In some embodiments, the virions of the non-influenza virus have incorporated into them or express a heterologous polypeptide in addition to an influenza virus mutated HA polypeptide. The heterologous polypeptide may be a polypeptide that has immunopotentiating activity, or that targets the non-influenza virus to a particular cell type, such as an antibody that recognizes an antigen on a specific cell type or a ligand that binds a specific receptor on a specific cell type.

[00131] Non-influenza viruses containing/expressing an influenza virus mutated HA polypeptide can be produced using techniques known to those skilled in the art. Non-influenza viruses containing an influenza virus mutated HA polypeptide may be produced by supplying in

trans the influenza virus mutated HA polypeptide during production of virions using techniques known to one skilled in the art.

[00132] Any virus type, subtype or strain including, but not limited to, naturally occurring strains, variants or mutants, mutagenized viruses, reassortants and/or genetically modified viruses may be used as a non-influenza virus vector. In a specific embodiment, the parental non-influenza virus is not a naturally occurring virus. In another specific embodiment, the parental non-influenza virus is a genetically engineered virus. In certain embodiments, an enveloped virus is preferred for the expression of a membrane bound influenza virus mutated HA polypeptide described herein.

[00133] In an exemplary embodiment, the non-influenza virus vector is a Newcastle disease virus (NDV). In another embodiment, the non-influenza virus vector is a vaccinia virus. In other exemplary, non-limiting, embodiments, the non-influenza virus vector is adenovirus, adeno-associated virus (AAV), hepatitis B virus, retrovirus (such as, *e.g.*, a gammaretrovirus such as Mouse Stem Cell Virus (MSCV) genome or Murine Leukemia Virus (MLV), *e.g.*, Moloney murine leukemia virus, oncoretrovirus, or lentivirus), an alphavirus (*e.g.*, Venezuelan equine encephalitis virus), a rhabdovirus, such as vesicular stomatitis virus or papillomaviruses, poxvirus (such as, *e.g.*, vaccinia virus, a MVA-T7 vector, or fowlpox), metapneumovirus, measles virus, herpesvirus, such as herpes simplex virus, or foamyvirus. *See, e.g.*, Lawrie and Tumin, 1993, *Cur. Opin. Genet. Develop.* 3, 102-109 (retroviral vectors); Bett *et al.*, 1993, *J. Virol.* 67, 5911 (adenoviral vectors); Zhou *et al.*, 1994, *J. Exp. Med.* 179, 1867 (adeno-associated virus vectors); Dubensky *et al.*, 1996, *J. Virol.* 70, 508-519 (viral vectors from the pox family including vaccinia virus and the avian pox viruses and viral vectors from the alpha virus genus such as those derived from Sindbis and Semliki Forest Viruses); U.S. Pat. No. 5,643,576 (Venezuelan equine encephalitis virus); WO 96/34625 (VSV); Ohe *et al.*, 1995, *Human Gene Therapy* 6, 325-333; Woo *et al.*, WO 94/12629; Xiao & Brandsma, 1996, *Nucleic Acids. Res.* 24, 2630-2622 (papillomaviruses); and Bukreyev and Collins, 2008, *Curr Opin Mol Ther.* 10:46-55 (NDV), each of which is incorporated by reference herein in its entirety.

[00134] Methods of engineering non-influenza viruses to express influenza polypeptides are well known in the art, as are methods for attenuating, propagating, and isolating and purifying such viruses. For such techniques with respect to NDV vectors, *see, e.g.*, International Publication No. WO 01/04333; U.S. Patent Nos. 7,442,379, 6,146,642, 6,649,372, 6,544,785 and

7,384,774; Swayne *et al.* (2003). *Avian Dis.* 47:1047-1050; and Swayne *et al.* (2001). *J. Virol.* 11868-11873, each of which is incorporated by reference in its entirety. For such techniques with respect to poxviruses, *see, e.g.*, Piccini, *et al.*, *Methods of Enzymology* 153: 545-563, 1987; International Publication No. WO 96/11279; U.S. Pat. No. 4,769,330; U.S. Pat. No. 4,722,848; U.S. Pat. No. 4,769,330; U.S. Pat. No. 4,603,112; U.S. Pat. No. 5,110,587; U.S. Pat. No. 5,174,993; EP 83 286; EP 206 920; Mayr *et al.*, *Infection* 3: 6-14, 1975; and Sutter and Moss, *Proc. Natl. Acad. Sci. USA* 89: 10847-10851, 1992. In certain embodiments, the non-influenza virus is attenuated.

[00135] Exemplary considerations for the selection of a non-influenza virus vector, particularly for use in compositions for administration to a subject, are safety, low toxicity, stability, cell type specificity, and immunogenicity, particularly, antigenicity of the influenza virus mutated HA polypeptide expressed by the non-influenza virus vector.

5.6 VIRUS-LIKE PARTICLES AND VIROSOMES

[00136] The influenza virus mutated HA polypeptides described herein can be incorporated into virus-like particle (VLP) vectors, *e.g.*, purified/isolated VLPs. VLPs generally comprise a viral polypeptide(s) typically derived from a structural protein(s) of a virus. In some embodiments, the VLPs are not capable of replicating. In certain embodiments, the VLPs may lack the complete genome of a virus or comprise a portion of the genome of a virus. In some embodiments, the VLPs are not capable of infecting a cell. In some embodiments, the VLPs express on their surface one or more of viral (*e.g.*, virus surface glycoprotein) or non-viral (*e.g.*, antibody or protein) targeting moieties known to one skilled in the art or described herein.

[00137] Methods for producing and characterizing recombinantly produced VLPs have been described based on several viruses, including influenza virus (Bright *et al.* (2007) *Vaccine* 25:3871), human papilloma virus type 1 (Hagensee *et al.* (1991) *J. Virol.* 67:315), human papilloma virus type 16 (Kirnbauer *et al.* *Proc. Natl. Acad. Sci.* (1992)89:12180), HIV-1 (Haffer *et al.*, (1990) *J. Virol.* 64:2653), and hepatitis A (Winokur (1991) 65:5029), each of which is incorporated herein in its entirety. Methods for expressing VLPs that contain NDV proteins are provided by Pantua *et al.* (2006) *J. Virol.* 80:11062-11073, and in United States patent application Publication No. 20090068221, published March 12, 2009, each of which is

incorporated in its entirety herein. In a specific embodiment, the VLPs comprising influenza virus mutated HA polypeptides described herein are generated using baculovirus.

[00138] In specific embodiments, VLPs, *e.g.*, VLPs comprising an influenza virus mutated HA polypeptide, are expressed in cells (*e.g.*, 293T cells). In certain embodiments, the VLPs are expressed in cells that express surface glycoproteins that comprise sialic acid. In accordance with such embodiments, the cells are cultured in the presence of neuraminidase (*e.g.*, viral or bacterial neuraminidase). In certain embodiments, VLPs, *e.g.*, VLPs comprising an influenza virus mutated HA polypeptide, are expressed in cells that do not express surface glycoproteins that comprise sialic acid.

[00139] In a specific embodiment, an influenza virus mutated HA polypeptide may be incorporated into a virosome. A virosome containing an influenza virus mutated HA polypeptide may be produced using techniques known to those skilled in the art. For example, a virosome may be produced by disrupting a purified virus, extracting the genome, and reassembling particles with the viral proteins (*e.g.*, an influenza virus mutated HA polypeptide) and lipids to form lipid particles containing viral proteins.

5.7 GENERATION OF ANTIBODIES AGAINST INFLUENZA VIRUS MUTATED HEMAGGLUTININ (HA) POLYPEPTIDES

[00140] The influenza virus mutated HA polypeptides, nucleic acids encoding such polypeptides, or vectors comprising such nucleic acids or polypeptides described herein may be used to elicit neutralizing antibodies against influenza, for example, against the stalk region of an influenza virus hemagglutinin polypeptide. In a specific embodiment, the influenza virus mutated HA polypeptide, nucleic acids encoding such polypeptides, or vectors comprising such nucleic acids or polypeptides described herein may be administered to a non-human subject (*e.g.*, a mouse, rabbit, rat, guinea pig, etc.) to induce an immune response that includes the production of antibodies which may be isolated using techniques known to one of skill in the art (*e.g.*, immunoaffinity chromatography, centrifugation, precipitation, etc.).

[00141] Alternatively, the influenza virus mutated HA polypeptide described herein may be used to screen for antibodies from antibody libraries. For example, an isolated influenza virus mutated HA polypeptide may be immobilized to a solid support (*e.g.*, a silica gel, a resin, a derivatized plastic film, a glass bead, cotton, a plastic bead, a polystyrene bead, an alumina gel,

or a polysaccharide, a magnetic bead), and screened for binding to antibodies. As an alternative, the antibodies may be immobilized to a solid support and screened for binding to the isolated influenza virus mutated HA polypeptides. Any screening assay, such as a panning assay, ELISA, surface plasmon resonance, or other antibody screening assay known in the art may be used to screen for antibodies that bind to the influenza virus mutated HA polypeptide. The antibody library screened may be a commercially available antibody library, an *in vitro* generated library, or a library obtained by identifying and cloning or isolating antibodies from an individual infected with influenza. In particular embodiments, the antibody library is generated from a survivor of an influenza virus outbreak. Antibody libraries may be generated in accordance with methods known in the art. In a particular embodiment, the antibody library is generated by cloning the antibodies and using them in phage display libraries or a phagemid display library.

[00142] Antibodies identified in the methods described herein may be tested for neutralizing activity and lack of autoreactivity using the biological assays known in the art or described herein. In one embodiment, an antibody isolated from a non-human animal or an antibody library neutralizes a hemagglutinin polypeptide from more than one influenza subtype. In some embodiments, an antibody elicited or identified using an influenza virus mutated HA polypeptide, a nucleic acid encoding such a polypeptide, or a vector encoding such a nucleic acid or polypeptide neutralizes an influenza H3 virus. In some embodiments, an antibody elicited or identified using an influenza virus mutated HA polypeptide, a nucleic acid encoding such a polypeptide, or a vector comprising such a nucleic acid or polypeptide neutralizes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 or more subtypes or strains of influenza virus. In one embodiment, the neutralizing antibody neutralizes one or more influenza A viruses and one or more influenza B viruses. In particular embodiments, the neutralizing antibody is not, or does not bind the same epitope as CR6261, CR6325, CR6329, CR6307, CR6323, 2A, D7, D8, F10, G17, H40, A66, D80, E88, E90, H98, C179 (produced by hybridoma FERM BP-4517; clones sold by Takara Bio, Inc. (Otsu, Shiga, Japan)), and/or AI3C (FERM BP-4516); or any other antibody described in Ekiert DC *et al.* (2009) Antibody Recognition of a Highly Conserved Influenza Virus Epitope. Science (published in Science Express February 26, 2009); Kashyap *et al.* (2008) Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. Proc Natl Acad Sci U S A 105: 5986-5991; Sui *et*

al. (2009) Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat Struct Mol Biol* 16: 265-273; U.S. Patent Nos. 5,589,174, 5,631,350, 6,337,070, and 6,720,409; International Application No. PCT/US2007/068983 published as International Publication No. WO 2007/134237; International Application No. PCT/US2008/075998 published as International Publication No. WO 2009/036157; International Application No. PCT/EP2007/059356 published as International Publication No. WO 2008/028946; and International Application No. PCT/US2008/085876 published as International Publication No. WO 2009/079259. In other embodiments, the neutralizing antibody is not an antibody described in Wang *et al.* (2010) "Broadly Protective Monoclonal Antibodies against H3 Influenza Viruses following Sequential Immunization with Different Hemagglutinins," *PLOS Pathogens* 6(2):1-9. In particular embodiments, the neutralizing antibody does not use the Ig VH1-69 segment. In some embodiments, the interaction of the neutralizing antibody with the antigen is not mediated exclusively by the heavy chain.

[00143] Antibodies identified or elicited using an influenza virus mutated HA polypeptide, a nucleic acid encoding such a polypeptide, or a vector comprising such a nucleic acid or polypeptide include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site that specifically binds to a hemagglutinin polypeptide. The immunoglobulin molecules may be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), class (*e.g.*, IgG₁, IgG₂, IgG₃, IgG₄, IgA₁ and IgA₂) or subclass of immunoglobulin molecule. Antibodies include, but are not limited to, monoclonal antibodies, multispecific antibodies, human antibodies, humanized antibodies, chimeric antibodies, single-chain Fvs (scFv), single chain antibodies, Fab fragments, F(ab') fragments, disulfide-linked Fvs (sdFv), and anti-idiotypic (anti-Id) antibodies (including, *e.g.*, anti-Id antibodies to antibodies elicited or identified using a method described herein), and epitope-binding fragments of any of the above.

[00144] Antibodies elicited or identified using an influenza virus mutated HA polypeptide, nucleic acids encoding such a polypeptide or a vector comprising such a nucleic acid or polypeptide may be used in diagnostic immunoassays, passive immunotherapy, and generation of antiidiotypic antibodies. The antibodies before being used in passive immunotherapy may be modified, *e.g.*, the antibodies may be chimerized or humanized. *See, e.g.*, U.S. Patent Nos. 4,444,887 and 4,716,111; and International Publication Nos. WO 98/46645, WO 98/50433, WO

98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741, each of which is incorporated herein by reference in its entirety, for reviews on the generation of chimeric and humanized antibodies. In addition, the ability of the antibodies to neutralize hemagglutinin polypeptides and the specificity of the antibodies for the polypeptides may be tested prior to using the antibodies in passive immunotherapy. *See* Section 5.7, *infra*, for a discussion regarding use of neutralizing antibodies for the prevention or treatment of disease caused by influenza virus infection.

[00145] Antibodies elicited or identified using an influenza virus mutated HA polypeptide, a nucleic acid encoding such a polypeptide, or a vector comprising such a nucleic acid or polypeptide may be used to monitor the efficacy of a therapy and/or disease progression. Any immunoassay system known in the art may be used for this purpose including, but not limited to, competitive and noncompetitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assays), "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays and immunoelectrophoresis assays, to name but a few.

[00146] Antibodies elicited or identified using an influenza virus mutated HA polypeptide, a nucleic acid encoding such a polypeptide, or a vector comprising such a nucleic acid or polypeptide may be used in the production of antiidiotypic antibody. The antiidiotypic antibody can then in turn be used for immunization, in order to produce a subpopulation of antibodies that bind a particular antigen of influenza, *e.g.*, a neutralizing epitope of a hemagglutinin polypeptide (Jerne, 1974, *Ann. Immunol. (Paris)* 125c:373; Jerne *et al.*, 1982, *EMBO J.* 1:234, incorporated herein by reference in its entirety).

5.8 COMPOSITIONS

[00147] The nucleic acids, vectors, polypeptides, antibodies, or cells described herein (sometimes referred to herein as "active compounds") may be incorporated into compositions. In a specific embodiment, the compositions are pharmaceutical compositions, such as immunogenic compositions (*e.g.*, vaccine formulations). The pharmaceutical compositions provided herein can be in any form that allows for the composition to be administered to a subject. In a specific embodiment, the pharmaceutical compositions are suitable for veterinary

and/or human administration. The compositions may be used in methods of preventing or treating an influenza virus disease.

[00148] In one embodiment, a pharmaceutical composition comprises an influenza virus mutated HA polypeptide, in an admixture with a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical composition comprises a nucleic acid encoding an influenza virus mutated HA polypeptide described herein, in an admixture with a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical composition comprises an expression vector comprising a nucleic acid encoding an influenza virus mutated HA polypeptide, in an admixture with a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical composition comprises an influenza virus or non-influenza virus containing an influenza virus mutated HA polypeptide, in an admixture with a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical composition comprises an influenza virus or non-influenza virus having a genome engineered to express an influenza virus mutated HA polypeptide, in admixture with a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical composition comprises a virus-like particle or virosome containing an influenza virus mutated HA polypeptide, in an admixture with a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical composition comprises a cell(s) (*e.g.*, mammalian or bacteria cells) expressing or engineered to express an influenza virus mutated HA polypeptide, in an admixture with a pharmaceutically acceptable carrier.

[00149] In some embodiments, a pharmaceutical composition may comprise one or more other therapies in addition to a therapy that utilizes an influenza virus mutated HA polypeptide described herein.

[00150] As used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeiae for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition is administered. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Examples of suitable pharmaceutical carriers are

described in "Remington's Pharmaceutical Sciences" by E.W. Martin. The formulation should suit the mode of administration.

[00151] In a specific embodiment, pharmaceutical compositions are formulated to be suitable for the intended route of administration to a subject. For example, the pharmaceutical composition may be formulated to be suitable for parenteral, oral, intradermal, transdermal, colorectal, intraperitoneal, and rectal administration. In a specific embodiment, the pharmaceutical composition may be formulated for intravenous, oral, intraperitoneal, intranasal, intratracheal, subcutaneous, intramuscular, topical, intradermal, transdermal or pulmonary administration.

[00152] In certain embodiments, biodegradable polymers, such as ethylene vinyl acetate, polyanhydrides, polyethylene glycol (PEGylation), polymethyl methacrylate polymers, polylactides, poly(lactide-co-glycolides), polyglycolic acid, collagen, polyorthoesters, and polylactic acid, may be used as carriers. In some embodiments, the active compounds are prepared with carriers that increase the protection of the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Methods for preparation of such formulations will be apparent to those skilled in the art. Liposomes or micelles can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811. In certain embodiments, the pharmaceutical compositions comprise one or more adjuvants.

[00153] In specific embodiments, immunogenic compositions described herein are monovalent formulations. In other embodiments, immunogenic compositions described herein are multivalent formulations. In one example, a multivalent formulation comprises more than one vector expressing an influenza virus mutated HA polypeptide. In certain embodiments, a multivalent formulation may comprise one or more different influenza virus mutated HA polypeptides expressed using a single vector.

[00154] In certain embodiments, the pharmaceutical compositions described herein additionally comprise a preservative, *e.g.*, the mercury derivative thimerosal. In other embodiments, the pharmaceutical compositions described herein do not comprise a preservative.

[00155] In certain embodiments, the pharmaceutical compositions described herein additionally comprise egg protein (*e.g.*, ovalbumin or other egg proteins). In other embodiments, the pharmaceutical compositions described herein do not comprise egg protein.

[00156] In certain embodiments, the pharmaceutical compositions described herein additionally comprise one or more antimicrobial agents (*e.g.*, antibiotics) including, but not limited to gentamicin, neomycin, polymyxin (*e.g.*, polymyxin B), and kanamycin, streptomycin. In other embodiments, the pharmaceutical compositions described herein do not comprise any antibiotics.

[00157] In certain embodiments, the pharmaceutical compositions described herein additionally comprise one or more components used to inactivate a virus, *e.g.*, formalin or formaldehyde or a detergent such as sodium deoxycholate, octoxynol 9 (Triton X-100), and octoxynol 10. In other embodiments, the pharmaceutical compositions described herein do not comprise any components used to inactivate a virus.

[00158] In certain embodiments, the pharmaceutical compositions described herein additionally comprise gelatin. In other embodiments, the pharmaceutical compositions described herein do not comprise gelatin.

[00159] In certain embodiments, the pharmaceutical compositions described herein additionally comprise one or more buffers, *e.g.*, phosphate buffer and sucrose phosphate glutamate buffer. In other embodiments, the pharmaceutical compositions described herein do not comprise buffers.

[00160] In certain embodiments, the pharmaceutical compositions described herein additionally comprise one or more salts, *e.g.*, sodium chloride, calcium chloride, sodium phosphate, monosodium glutamate, and aluminum salts (*e.g.*, aluminum hydroxide, aluminum phosphate, alum (potassium aluminum sulfate), or a mixture of such aluminum salts). In other embodiments, the pharmaceutical compositions described herein do not comprise salts.

[00161] In specific embodiments, the pharmaceutical compositions described herein are low-additive influenza virus vaccines, *i.e.*, the pharmaceutical compositions do not comprise one or more additives commonly found in influenza virus vaccines. Low-additive influenza vaccines have been described (*see, e.g.*, International Application No. PCT/IB2008/002238 published as International Publication No. WO 09/001217 which is herein incorporated by reference in its entirety).

[00162] The pharmaceutical compositions described herein can be included in a container, pack, or dispenser together with instructions for administration.

[00163] The pharmaceutical compositions described herein can be stored before use, *e.g.*, the pharmaceutical compositions can be stored frozen (*e.g.*, at about -20°C or at about -70°C); stored in refrigerated conditions (*e.g.*, at about 4°C); or stored at room temperature (*see* International Application No. PCT/IB2007/001149 published as International Publication No. WO 07/110776, which is herein incorporated by reference in its entirety, for methods of storing compositions comprising influenza vaccines without refrigeration).

[00164] In certain embodiments, when the active compound in a pharmaceutical composition described herein is a cell engineered to an influenza virus mutated HA polypeptide, the cells in the pharmaceutical composition are not mammalian cells.

5.8.1 Vaccines and Immunogenic Compositions

[00165] In a specific embodiment, provided herein are subunit vaccines comprising an influenza virus mutated HA polypeptide described herein. In some embodiments, a subunit vaccine comprises an influenza virus mutated HA polypeptide and one or more surface glycoproteins, other targeting moieties, or adjuvants. In specific embodiments, a subunit vaccine comprises a single influenza virus mutated HA polypeptide. In other embodiments, a subunit vaccine comprises two, three, four or more influenza virus mutated HA polypeptides. In specific embodiments, the influenza virus mutated HA polypeptide(s) used in a subunit vaccine is not membrane-bound, *i.e.*, it is soluble.

[00166] In one embodiment, provided herein are immunogenic compositions (*e.g.*, vaccines) comprising live virus containing an influenza virus mutated HA polypeptide. In another embodiment, provided herein are immunogenic compositions (*e.g.*, vaccines) comprising live virus that is engineered to encode an influenza virus mutated hemagglutinin HA polypeptide, which is expressed by progeny virus produced in the subjects administered the compositions. In specific embodiments, the influenza virus mutated HA polypeptide is membrane-bound. In other specific embodiments, the influenza virus mutated HA polypeptide is not membrane-bound, *i.e.*, it is soluble. In particular embodiments, the live virus is an influenza virus, such as described in Section 5.5.1, *supra*. In other embodiments, the live virus is a non-influenza virus, such as described in Section 5.5.2, *supra*. In some embodiments, the live virus is attenuated. In some embodiments, an immunogenic composition comprises two, three, four or more live viruses

containing or engineered to express two, three, four or more different influenza virus mutated HA polypeptides.

[00167] In one embodiment, provided herein are immunogenic compositions (*e.g.*, vaccines) comprising an inactivated virus containing an influenza virus mutated HA polypeptide. In specific embodiments, the influenza virus mutated HA polypeptide is membrane-bound. In particular embodiments, the inactivated virus is an influenza virus, such as described in Section 5.5.1, *supra*. In other embodiments, the inactivated virus is a non-influenza virus, such as described in Section 5.5.2, *supra*. In some embodiments, an immunogenic composition comprises two, three, four or more inactivated viruses containing two, three, four or more different influenza virus mutated HA polypeptides. In certain embodiments, the inactivated virus immunogenic compositions comprise one or more adjuvants.

[00168] In one embodiment, an immunogenic composition comprising an influenza virus mutated HA polypeptide is a split virus vaccine. In some embodiments, split virus vaccine contains two, three, four or more different influenza virus mutated HA polypeptides. In certain embodiments, the influenza virus mutated HA polypeptide is/was membrane-bound. In certain embodiments, the split virus vaccines comprise one or more adjuvants.

5.8.2 Adjuvants

[00169] In certain embodiments, the compositions described herein comprise, or are administered in combination with, an adjuvant. The adjuvant for administration in combination with a composition described herein may be administered before, concomitantly with, or after administration of said composition. In some embodiments, the term “adjuvant” refers to a compound that when administered in conjunction with or as part of a composition described herein augments, enhances and/or boosts the immune response to an influenza virus mutated HA polypeptide, but when the compound is administered alone does not generate an immune response to the polypeptide. In some embodiments, the adjuvant generates an immune response to the polypeptide and does not produce an allergy or other adverse reaction. Adjuvants can enhance an immune response by several mechanisms including, *e.g.*, lymphocyte recruitment, stimulation of B and/or T cells, and stimulation of macrophages.

[00170] In certain embodiments, an adjuvant augments the intrinsic response to the influenza virus mutated HA polypeptide without causing conformational changes in the polypeptide that affect the qualitative form of the response. Specific examples of adjuvants include, but are not

limited to, aluminum salts (alum) (such as aluminum hydroxide, aluminum phosphate, and aluminum sulfate), 3 De-O-acylated monophosphoryl lipid A (MPL) (*see* GB 2220211), MF59 (Novartis), AS03 (GlaxoSmithKline), AS04 (GlaxoSmithKline), polysorbate 80 (Tween 80; ICL Americas, Inc.), imidazopyridine compounds (*see* International Application No. PCT/US2007/064857, published as International Publication No. WO2007/109812), imidazoquinoxaline compounds (*see* International Application No. PCT/US2007/064858, published as International Publication No. WO2007/109813) and saponins, such as QS21 (*see* Kensil *et al.*, in Vaccine Design: The Subunit and Adjuvant Approach (eds. Powell & Newman, Plenum Press, NY, 1995); U.S. Pat. No. 5,057,540). In some embodiments, the adjuvant is Freund's adjuvant (complete or incomplete). Other adjuvants are oil in water emulsions (such as squalene or peanut oil), optionally in combination with immune stimulants, such as monophosphoryl lipid A (*see* Stoute *et al.*, N. Engl. J. Med. 336, 86-91 (1997)). Another adjuvant is CpG (Bioworld Today, Nov. 15, 1998). Such adjuvants can be used with or without other specific immunostimulating agents such as MPL or 3-DMP, QS21, polymeric or monomeric amino acids such as polyglutamic acid or polylysine, or other immunopotentiating agents described in Section 5.8. It should be understood that different formulations of influenza virus mutated HA polypeptides may comprise different adjuvants or may comprise the same adjuvant.

5.9 PROPHYLACTIC AND THERAPEUTIC USES

[00171] In one aspect, provided herein are methods for inducing an immune response in a subject utilizing an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein. In a specific embodiment, a method for inducing an immune response to an influenza virus HA polypeptide in a subject comprises administering to a subject in need thereof an effective amount of an influenza virus mutated HA polypeptide described herein or an immunogenic composition thereof. In another embodiment, a method for inducing an immune response to an influenza virus HA polypeptide in a subject comprises administering to a subject in need thereof an effective amount of a nucleic acid encoding an influenza virus mutated HA polypeptide described herein or an immunogenic composition thereof. In another

embodiment, a method for inducing an immune response to an influenza virus HA polypeptide in a subject comprises administering to a subject in need thereof an effective amount of a viral vector containing or expressing an influenza virus mutated HA polypeptide described herein or an immunogenic composition thereof. In certain embodiments, an influenza virus mutated HA polypeptide described herein used in the method is a purified influenza virus mutated HA polypeptide described herein derived from a mammalian cell, a plant cell, or an insect cell.

[00172] In a specific embodiment, a method for inducing an immune response to an influenza virus HA polypeptide in a subject comprises administering to a subject in need thereof a subunit vaccine described herein. In another embodiment, a method for inducing an immune response to an influenza virus HA polypeptide in a subject comprises administering to a subject in need thereof a live virus vaccine described herein. In particular embodiments, the live virus vaccine comprises an attenuated virus. In another embodiment, a method for inducing an immune response to an influenza virus HA polypeptide in a subject comprises administering to a subject in need thereof an inactivated virus vaccine described herein. In another embodiment, a method for inducing an immune response to an influenza virus HA polypeptide in a subject comprises administering to a subject in need thereof a split virus vaccine described herein. In another embodiment, a method for inducing an immune response to an influenza virus HA polypeptide in a subject comprises administering to a subject in need thereof a virus-like particle vaccine described herein. In another embodiment, a method for inducing an immune response to an influenza HA polypeptide comprises administering to a subject in need thereof a virosome described herein. In another embodiment, a method for inducing an immune response to an influenza HA polypeptide comprises administering to a subject in need thereof a cell (*e.g.*, a bacterial or mammalian cell) expressing or engineered to express an influenza virus mutated HA polypeptide described herein or a composition thereof. In certain embodiments, an influenza virus mutated HA polypeptide described herein used in the method is a purified influenza virus mutated HA polypeptide described herein derived from a mammalian cell, a plant cell, or an insect cell.

[00173] In some embodiments, the immune response induced by an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein is effective

to prevent and/or treat an influenza virus infection caused by an influenza virus strain (*e.g.*, an influenza virus strain heterologous to the parental HA globular head domain of an influenza virus mutated HA polypeptide and/or an influenza virus strain heterologous to HA stalk antigenic peptide(s) of an influenza virus mutated HA polypeptide).

[00174] In another aspect, provided herein are immunization regimens involving a first immunization (*e.g.*, priming) with an immunogenic composition (*e.g.*, a vaccine) described herein followed by one, two, or more additional immunizations (*e.g.*, boostings) with an immunogenic composition (*e.g.*, a vaccine). In certain embodiments, each immunization administered to a subject is separated by a certain period of time, *e.g.*, 1-6 months, 3-6 months, 6-9 months, 6-9 months, 9-12 months, etc. In a specific embodiment, the immunogenic composition (*e.g.*, a vaccine) used in the first immunization is the same type of immunogenic composition (*e.g.*, a vaccine) used in one, two or more additional immunizations. For example, if the immunogenic composition (*e.g.*, a vaccine) used in the first immunization is a composition comprising nucleic acid sequence encoding an influenza virus mutated HA polypeptide, the vaccine formulation used for the one, two or more additional immunizations may be the same type of immunogenic composition (*e.g.*, a vaccine). In other specific embodiments, the immunogenic composition (*e.g.*, a vaccine) used in the first immunization is different from the type of immunogenic composition (*e.g.*, a vaccine) used in one, two or more additional immunizations. For example, if the immunogenic composition (*e.g.*, a vaccine) used in the first immunization is a composition comprising a nucleic acid sequence encoding an influenza virus mutated HA polypeptide, the immunogenic composition (*e.g.*, a vaccine) used in the one, two or more additional immunization is another type of immunogenic composition (*e.g.*, a vaccine, such as an immunogenic composition comprising an influenza virus mutated HA polypeptide. In certain embodiments, the immunogenic composition (*e.g.*, a vaccine) used in the additional immunizations changes. *See*, Example 6, *infra*, for an immunization regimen. In certain embodiments, the influenza virus mutated HA polypeptide used in the immunogenic composition (*e.g.*, vaccine) changes from one immunization to the next. For example, the one or more HA stalk antigenic peptides in an HA globular head of an influenza virus mutated HA polypeptide might be different in an immunogenic composition used to prime a subject than in an immunogenic composition used in one or more boosts. In other embodiments, the influenza virus mutated HA polypeptide used to prime and in one or more boosts is the same. Any route of

administration known to one of skill in the art can be used to administer an immunogenic composition (*e.g.*, a vaccine) described herein to a subject. In certain embodiments, the route of administration changes from one immunization to the next. For example, if intranasal immunization is used as one route of an immunogenic composition (*e.g.*, a vaccine) to a subject then intramuscular immunization might be used for the next route of administration of an immunogenic composition (*e.g.*, a vaccine) to the subject. *See, e.g.*, Example 6 for immunization regimens involving different routes of administration.

[00175] In one embodiment, provided herein is a method of immunizing a subject against influenza virus, comprising: (a) administering to the subject an immunogenic composition; and (b) after a certain period of time (*e.g.*, 1-6 months, 3-6 months, 6-9 months, 6-9 months, 9-12 months, etc.) administering to the subject the same or a different immunogenic composition. In certain embodiments, the method comprises administering to the subject one or more additional immunogenic compositions (*e.g.*, vaccines) described herein a certain period of time (*e.g.*, 1-6 months, 3-6 months, 6-9 months, 6-9 months, 9-12 months, etc.) after step (b).

[00176] In some embodiments, the immune response induced by an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein is effective to reduce symptoms resulting from an influenza virus disease/infection. Symptoms of influenza virus disease/infection include, but are not limited to, body aches (especially joints and throat), fever, nausea, headaches, irritated eyes, fatigue, sore throat, reddened eyes or skin, and abdominal pain.

[00177] In some embodiments, the immune response induced by an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein is effective to reduce the hospitalization of a subject suffering from an influenza virus disease/infection. In some embodiments, the immune response induced by an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an

influenza virus HA mutated protein) or a composition described herein is effective to reduce the duration of hospitalization of a subject suffering from an influenza virus disease/infection.

[00178] In another aspect, provided herein are methods for preventing and/or treating an influenza virus infection in a subject utilizing an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein. In one embodiment, a method for preventing or treating an influenza virus infection in a subject comprises administering to a subject in need thereof an influenza virus mutated HA polypeptide, a nucleic acid encoding such a polypeptide, a vector containing or expressing such a polypeptide, cell expressing such a polypeptide, or a composition of any one of the foregoing. In a specific embodiment, a method for preventing or treating an influenza virus infection in a subject comprises administering to a subject in need thereof a subunit vaccine, a live virus vaccine, an inactivated virus vaccine, a split virus vaccine or a virus-like particle vaccine.

[00179] In another aspect, provided herein are methods for preventing and/or treating an influenza virus disease in a subject utilizing an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector containing or expressing such a polypeptide, or cells expressing such a polypeptide, or a composition of any one of the foregoing. In a specific embodiment, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof an effective amount of an influenza virus mutated HA polypeptide or an immunogenic composition thereof. In another embodiment, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof an effective amount of a nucleic acid encoding an influenza virus mutated HA polypeptide or an immunogenic composition thereof. In another embodiment, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof an effective amount of a viral vector containing or expressing an influenza virus mutated HA polypeptide or an immunogenic composition thereof. In yet another embodiment, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof an effective amount of cells expressing an influenza virus mutated HA polypeptide or a pharmaceutical composition thereof.

[00180] In a specific embodiment, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof a subunit vaccine described herein. In another embodiment, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof a live virus vaccine described herein. In particular embodiments, the live virus vaccine comprises an attenuated virus. In another embodiment, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof an inactivated virus vaccine described herein. In another embodiment, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof a split virus vaccine described herein. In another embodiment, a method for preventing or treating an influenza virus disease comprises administering to a subject in need thereof a virus-like particle vaccine described herein. In another embodiment, a method for preventing or treating an influenza virus disease in a subject, comprising administering to a subject in need thereof a virosome described herein. In another embodiment, a method for preventing or treating an influenza virus disease in a subject comprising administering to a subject in need thereof a cell(s) (*e.g.*, bacterial or mammalian cells) expressing or engineered to express an influenza virus mutated HA polypeptide or a composition thereof.

[00181] In another aspect, provided herein are methods of preventing and/or treating an influenza virus disease in a subject by administering an antibody (*e.g.*, a neutralizing antibody(ies)) described herein. In a specific embodiment, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof an effective amount of a neutralizing antibody described herein, or a pharmaceutical composition thereof. In particular embodiments, the neutralizing antibody is a monoclonal antibody. In certain embodiments, the neutralizing antibody is not CR6261, CR6325, CR6329, CR6307, CR6323, 2A, D7, D8, F10, G17, H40, A66, D80, E88, E90, H98, C179 (FERM BP-4517), AI3C (FERM BP-4516) or any other antibody described in Ekiert DC *et al.* (2009) Antibody Recognition of a Highly Conserved Influenza Virus Epitope. *Science* (published in *Science Express* February 26, 2009); Kashyap *et al.* (2008) Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. *Proc Natl Acad Sci U S A* 105: 5986-5991; Sui *et al.* (2009) Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat Struct Mol Biol* 16:

265-273; U.S. Patent Nos. 5,589,174, 5,631,350, 6,337,070, and 6,720,409; International Application No. PCT/US2007/068983 published as International Publication No. WO 2007/134237; International Application No. PCT/US2008/075998 published as International Publication No. WO 2009/036157; International Application No. PCT/EP2007/059356 published as International Publication No. WO 2008/028946; and International Application No. PCT/US2008/085876 published as International Publication No. WO 2009/079259. In other embodiments, the neutralizing antibody is not an antibody described in Wang *et al.* (2010) “Broadly Protective Monoclonal Antibodies against H3 Influenza Viruses following Sequential Immunization with Different Hemagglutinins,” PLOS Pathogens 6(2):1-9.

[00182] In certain embodiments, the methods for preventing or treating an influenza virus disease or infection in a subject (*e.g.*, a human or non-human animal) provided herein result in a reduction in the replication of the influenza virus in the subject as measured by *in vivo* and *in vitro* assays known to those of skill in the art and described herein. In some embodiments, the replication of the influenza virus is reduced by approximately 1 log or more, approximately 2 logs or more, approximately 3 logs or more, approximately 4 logs or more, approximately 5 logs or more, approximately 6 logs or more, approximately 7 logs or more, approximately 8 logs or more, approximately 9 logs or more, approximately 10 logs or more, 1 to 3 logs, 1 to 5 logs, 1 to 8 logs, 1 to 9 logs, 2 to 10 logs, 2 to 5 logs, 2 to 7 logs, 2 logs to 8 logs, 2 to 9 logs, 2 to 10 logs 3 to 5 logs, 3 to 7 logs, 3 to 8 logs, 3 to 9 logs, 4 to 6 logs, 4 to 8 logs, 4 to 9 logs, 5 to 6 logs, 5 to 7 logs, 5 to 8 logs, 5 to 9 logs, 6 to 7 logs, 6 to 8 logs, 6 to 9 logs, 7 to 8 logs, 7 to 9 logs, or 8 to 9 logs.

[00183] Section 6, *infra*, sets forth how an influenza virus mutated HA may be used to vaccinate subjects against influenza virus infection.

5.9.1 Combination therapies

[00184] In various embodiments, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, cells expressing such a polypeptide, or a neutralizing antibody may be administered to a subject in combination with one or more other therapies (*e.g.*, antiviral, antibacterial, or immunomodulatory therapies). In some embodiments, a pharmaceutical composition (*e.g.*, an immunogenic composition) described herein may be administered to a subject in combination with one or more therapies. The one or more other

therapies may be beneficial in the treatment or prevention of an influenza virus disease or may ameliorate a symptom or condition associated with an influenza virus disease. In some embodiments, the one or more other therapies are pain relievers, anti-fever medications, or therapies that alleviate or assist with breathing. In certain embodiments, the therapies are administered less than 5 minutes apart, less than 30 minutes apart, 1 hour apart, at about 1 hour apart, at about 1 to about 2 hours apart, at about 2 hours to about 3 hours apart, at about 3 hours to about 4 hours apart, at about 4 hours to about 5 hours apart, at about 5 hours to about 6 hours apart, at about 6 hours to about 7 hours apart, at about 7 hours to about 8 hours apart, at about 8 hours to about 9 hours apart, at about 9 hours to about 10 hours apart, at about 10 hours to about 11 hours apart, at about 11 hours to about 12 hours apart, at about 12 hours to 18 hours apart, 18 hours to 24 hours apart, 24 hours to 36 hours apart, 36 hours to 48 hours apart, 48 hours to 52 hours apart, 52 hours to 60 hours apart, 60 hours to 72 hours apart, 72 hours to 84 hours apart, 84 hours to 96 hours apart, or 96 hours to 120 hours apart. In specific embodiments, two or more therapies are administered within the same patent visit.

[00185] Any anti-viral agents well-known to one of skill in the art may be used in combination with an active (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein. Non-limiting examples of anti-viral agents include proteins, polypeptides, peptides, fusion proteins, antibodies, nucleic acid molecules, organic molecules, inorganic molecules, and small molecules that inhibit and/or reduce the attachment of a virus to its receptor, the internalization of a virus into a cell, the replication of a virus, or release of virus from a cell. In particular, anti-viral agents include, but are not limited to, nucleoside analogs (*e.g.*, zidovudine, acyclovir, gancyclovir, vidarabine, idoxuridine, trifluridine, and ribavirin), foscarnet, amantadine, peramivir, rimantadine, saquinavir, indinavir, ritonavir, alpha-interferons and other interferons, AZT, zanamivir (Relenza®), and oseltamivir (Tamiflu®). Other anti-viral agents include influenza virus vaccines, *e.g.*, Fluarix® (GlaxoSmithKline), FluMist® (MedImmune Vaccines), Fluvirin® (Chiron Corporation), Flulaval® (GlaxoSmithKline), Afluria® (CSL Biotherapies Inc.), Agriflu® (Novartis) or Fluzone® (Aventis Pasteur).

[00186] In specific embodiments, the anti-viral agent is an immunomodulatory agent that is specific for a viral antigen. In particular embodiments, the viral antigen is an influenza virus

polypeptide other than an influenza virus mutated HA polypeptide. In other embodiments, the viral antigen is an influenza virus HA polypeptide.

[00187] Any anti-bacterial agents known to one of skill in the art may be used in combination with an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein. Non-limiting examples of anti-bacterial agents include Amikacin, Amoxicillin, Amoxicillin-clavulanic acid, Amphotericin-B, Ampicillin, Ampicillin-sulbactam, Apramycin, Azithromycin, Aztreonam, Bacitracin, Benzylpenicillin, Caspofungin, Cefaclor, Cefadroxil, Cefalexin, Cefalothin, Cefazolin, Cefdinir, Cefepime, Cefixime, Cefmenoxime, Cefoperazone, Cefoperazone-sulbactam, Cefotaxime, Cefoxitin, Cefpirome, Cefpodoxime, Cefpodoxime-clavulanic acid, Cefpodoxime-sulbactam, Cefprozil, Cefquinome, Ceftazidime, Ceftibutin, Ceftiofur, Ceftobiprole, Ceftriaxon, Cefuroxime, Chloramphenicol, Florfenicol, Ciprofloxacin, Clarithromycin, Clinafloxacin, Clindamycin, Cloxacillin, Colistin, Cotrimoxazole (Trimethoprim/sulphamethoxazole), Dalbavancin, Dalfopristin/Quinopristin, Daptomycin, Dibekacin, Dicloxacillin, Doripenem, Doxycycline, Enrofloxacin, Ertapenem, Erythromycin, Flucloxacillin, Fluconazole, Flucytosine, Fosfomycin, Fusidic acid, Garenoxacin, Gatifloxacin, Gemifloxacin, Gentamicin, Imipenem, Itraconazole, Kanamycin, Ketoconazole, Levofloxacin, Lincomycin, Linezolid, Loracarbef, Meropenem, Metronidazole, Mezlocillin, Mezlocillin-sulbactam, Minocycline, Moxifloxacin, Mupirocin, Nalidixic acid, Neomycin, Netilmicin, Nitrofurantoin, Norfloxacin, Ofloxacin, Oxacillin, Pefloxacin, Penicillin V, Piperacillin, Piperacillin-sulbactam, Piperacillin-tazobactam, Rifampicin, Roxithromycin, Sparfloxacin, Spectinomycin, Spiramycin, Streptomycin, Sulbactam, Sulfamethoxazole, Teicoplanin, Telavancin, Telithromycin, Temocillin, Tetracycline, Ticarcillin, Ticarcillin-clavulanic acid, Tigecycline, Tobramycin, Trimethoprim, Trovafloxacin, Tylosin, Vancomycin, Virginiamycin, and Voriconazole.

[00188] In some embodiments, a combination therapy comprises active immunization with an influenza virus mutated HA polypeptide described herein, or one or more vectors described in Sections 5.2–5.6, *supra*, and passive immunization with one or more neutralizing antibodies described in Section 5.7. In some embodiments, a combination therapy comprises administration of two or more different vectors described in Sections 5.2–5.6, *supra*.

[00189] In some embodiments, a combination therapy comprises active immunization with two or more influenza virus mutated HA polypeptides described herein.

5.9.2 Patient Populations

[00190] In certain embodiments, an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein may be administered to a naïve subject, *i.e.*, a subject that does not have a disease caused by influenza virus infection or has not been and is not currently infected with an influenza virus infection. In one embodiment, an active compound or composition described herein is administered to a naïve subject that is at risk of acquiring an influenza virus infection. In one embodiment, an active compound or composition described herein is administered to a subject that does not have a disease caused by the specific influenza virus, or has not been and is not infected with the specific influenza virus to which the influenza virus mutated HA polypeptide induces an immune response. An active compound or composition described herein may also be administered to a subject that is and/or has been infected with the influenza virus or another type, subtype or strain of the influenza virus to which the influenza virus mutated HA polypeptide induces an immune response.

[00191] In certain embodiments, an active (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein is administered to a patient who has been diagnosed with an influenza virus infection. In some embodiments, an active compound or composition described herein is administered to a patient infected with an influenza virus before symptoms manifest or symptoms become severe (*e.g.*, before the patient requires hospitalization). In some embodiments, an active compound or composition described herein is administered to a patient that is infected with or has been diagnosed with a different type of influenza virus than that of (i) the influenza virus from which the HA stalk antigenic peptides of the influenza virus mutated HA polypeptide of the active compound or composition was derived; and (ii) the influenza virus from which the HA globular head domain of the influenza virus mutated HA polypeptide of the active compound or composition was derived.

[00192] In certain embodiments, an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein is administered to a patient that may be or is infected with an influenza virus that belongs to (i) the same HA group as that of the HA globular head domain of an influenza virus mutated HA polypeptide, and/or (ii) the same HA group as that of the HA stalk antigenic peptides of an influenza virus mutated HA polypeptide. In certain embodiments, an active compound or composition described herein is administered to a patient that may be or is infected with an influenza virus of (i) the same subtype as that of the HA globular head domain of an influenza virus mutated HA polypeptide, and/or (ii) the same subtype as that of the HA stalk antigenic peptides of an influenza virus mutated HA polypeptide.

[00193] In some embodiments, a subject to be administered an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein or composition described herein is an animal. In certain embodiments, the animal is a bird. In certain embodiments, the animal is a canine. In certain embodiments, the animal is a feline. In certain embodiments, the animal is a horse. In certain embodiments, the animal is a cow. In certain embodiments, the animal is a mammal, *e.g.*, a horse, swine, mouse, or primate, preferably a human.

[00194] In certain embodiments, an immunogenic formulation comprising a live virus vector is not given concurrently with other live-virus vaccines.

5.10 MODES OF ADMINISTRATION

5.10.1 Routes of Delivery

[00195] An active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein may be delivered to a subject by a variety of routes. These include, but are not limited to, intranasal, intratracheal, oral, intradermal, intramuscular, intraperitoneal, transdermal, intravenous, conjunctival and subcutaneous routes. In some

embodiments, a composition is formulated for topical administration, for example, for application to the skin. In specific embodiments, the route of administration is nasal, *e.g.*, as part of a nasal spray. In certain embodiments, a composition is formulated for intramuscular administration. In some embodiments, a composition is formulated for subcutaneous administration. In certain embodiments, a composition is not formulated for administration by injection. In specific embodiments for live virus vaccines, the vaccine is formulated for administration by a route other than injection.

[00196] In cases where the antigen is a viral vector, a virus-like particle vector, or a bacterial vector, for example, it may be preferable to introduce an immunogenic composition via the natural route of infection of the backbone virus or bacteria from which the vector was derived. Alternatively, it may be preferable to introduce an influenza virus mutated HA polypeptide via the natural route of infection of the influenza virus from which polypeptide is derived. The ability of an antigen, particularly a viral vector, to induce a vigorous secretory and cellular immune response can be used advantageously. For example, infection of the respiratory tract by a viral vector may induce a strong secretory immune response, for example in the urogenital system, with concomitant protection against an influenza virus. In addition, in a preferred embodiment it may be desirable to introduce the pharmaceutical compositions into the lungs by any suitable route. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent for use as a spray.

[00197] In a specific embodiment, a subunit vaccine is administered intramuscularly. In another embodiment, a live influenza virus vaccine is administered intranasally. In another embodiment, an inactivated influenza virus vaccine, or a split influenza virus vaccine is administered intramuscularly. In another embodiment, a virus-like particle or composition thereof is administered intramuscularly.

5.10.2 Dosage and Frequency of Administration

[00198] The amount of an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein which will be effective in the treatment and/or prevention of an influenza virus infection or an influenza virus disease will depend on the nature of the disease, and can be determined by standard clinical techniques.

[00199] The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the infection or disease caused by it, and should be decided according to the judgment of the practitioner and each subject's circumstances. For example, effective doses may also vary depending upon means of administration, target site, physiological state of the patient (including age, body weight, health), whether the patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. Usually, the patient is a human but nonhuman mammals including transgenic mammals can also be treated. Treatment dosages are optimally titrated to optimize safety and efficacy.

[00200] In certain embodiments, an *in vitro* assay is employed to help identify optimal dosage ranges. Effective doses may be extrapolated from dose response curves derived from *in vitro* or animal model test systems.

[00201] Exemplary doses for nucleic acids encoding an influenza virus mutated HA polypeptide described herein range from about 10 ng to 1 g, 100 ng to 100 mg, 1 µg to 10 mg, or 30-300 µg nucleic acid, *e.g.*, DNA, per patient.

[00202] In certain embodiments, exemplary doses for an influenza virus mutated HA polypeptide described herein as provided in split virus vaccines and subunit vaccines) range from about 5 µg to 100 mg, 15 µg to 50 mg, 15 µg to 25 mg, 15 µg to 10 mg, 15 µg to 5 mg, 15 µg to 1 mg, 15 µg to 100 µg, 15 µg to 75 µg, 5 µg to 50 µg, 10 µg to 50 µg, 15 µg to 45 µg, 20 µg to 40 µg, or 25 to 35 µg per kilogram of the patient. In other embodiments, exemplary doses for influenza virus HA polypeptide range from about 1 µg to about 50 mg, about 5 µg to about 50 mg, about 1 µg to about 100 mg, about 5 µg to about 100 mg, about 15 µg to about 50 mg, about 15 µg to about 25 mg, about 15 µg to about 10 mg, about 15 µg to about 5 mg, about 15 µg to about 1 mg, about 15 µg to about 100 µg, about 15 µg to about 75 µg, about 5 µg to about 50 µg, about 10 µg to about 50 µg, about 15 µg to about 45 µg, about 20 µg to about 40 µg, or about 25 to about 35 µg of influenza virus mutated hemagglutinin HA polypeptide per dose, and can be administered to a subject once, twice, three or more times with intervals as often as needed.

[00203] Doses for infectious viral vectors may vary from 10-100, or more, virions per dose. In some embodiments, suitable dosages of a virus vector are 10^2 , 5×10^2 , 10^3 , 5×10^3 , 10^4 , 5×10^4 , 10^5 , 5×10^5 , 10^6 , 5×10^6 , 10^7 , 5×10^7 , 10^8 , 5×10^8 , 1×10^9 , 5×10^9 , 1×10^{10} , 5×10^{10} , $1 \times$

10^{11} , 5×10^{11} or 10^{12} pfu, and can be administered to a subject once, twice, three or more times with intervals as often as needed.

[00204] In certain embodiments, exemplary doses for VLPs range from about 0.01 μ g to about 100 mg, about 0.1 μ g to about 100 mg, about 5 μ g to about 100 mg, about 15 μ g to about 50 mg, about 15 μ g to about 25 mg, about 15 μ g to about 10 mg, about 15 μ g to about 5 mg, about 15 μ g to about 1 mg, about 15 μ g to about 100 μ g, about 15 μ g to about 75 μ g, about 5 μ g to about 50 μ g, about 10 μ g to about 50 μ g, about 15 μ g to about 45 μ g, about 20 μ g to about 40 μ g, or about 25 to about 35 μ g per kilogram of the patient.

[00205] In one embodiment, an inactivated vaccine is formulated such that it contains about 5 μ g to about 50 μ g, about 10 μ g to about 50 μ g, about 15 μ g to about 100 μ g, about 15 μ g to about 75 μ g, about 15 μ g to about 50 μ g, about 15 μ g to about 30 μ g, about 20 μ g to about 50 μ g, about 25 μ g to about 40 μ g, about 25 μ g to about 35 μ g of an influenza virus mutated HA polypeptide.

[00206] In certain embodiments, an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein is administered to a subject once as a single dose.

[00207] In certain embodiments, an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein is administered to a subject as a single dose followed by a second dose 3 to 6 weeks later. In certain embodiments, an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein is administered to a subject as a single dose followed by a second dose 3 to 6 weeks later, which is followed by administration of a third dose 3 to 6 weeks later. In certain embodiments, the second and/or third administrations may utilize a different active compound or composition. In accordance with these embodiments, booster inoculations may be administered to the subject at 6 to 12 month intervals following the second inoculation. In certain embodiments, the booster

inoculations may utilize a different active compound or composition. In certain embodiments, an active compound or composition is administered to a subject as a single dose once per year.

[00208] In particular embodiments, an active compound (*e.g.*, an influenza virus mutated HA polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector) containing or expressing such a polypeptide, or a cell expressing an influenza virus HA mutated protein) or a composition described herein is administered to a subject in the fall or winter, *i.e.*, prior to or during the influenza season in each hemisphere. In one embodiment, children are administered their first dose early in the season, *e.g.*, late September or early October in the Northern hemisphere, so that the second dose can be given prior to the peak of the influenza season.

[00209] For passive immunization with an antibody, the dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg, of the patient body weight. For example, dosages can be 1 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg or in other words, 70 mg or 700 mg or within the range of 70-700 mg, respectively, for a 70 kg patient. An exemplary treatment regime entails administration once per every two weeks or once a month or once every 3 to 6 months for a period of one year or over several years, or over several year-intervals. In some methods, two or more monoclonal antibodies with different binding specificities are administered simultaneously, in which case the dosage of each antibody administered falls within the ranges indicated. Antibody is usually administered on multiple occasions. Intervals between single dosages can be weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of antibody to the influenza virus mutated hemagglutinin (HA) polypeptide in the patient.

5.11 KITS

[00210] Provided herein is a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical/immunogenic compositions described herein, such as one or more active compounds provided herein. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

The kits encompassed herein can be used in accordance with the methods described herein. In one embodiment, a kit comprises an active compound described herein, preferably one or more influenza virus mutated hemagglutinin (HA) polypeptides, in one or more containers. In certain embodiments, a kit comprises a vaccine described herein, *e.g.*, a split virus vaccine, a subunit vaccine, an inactivated influenza virus vaccine, or a live influenza virus vaccine, wherein said vaccine comprises one or more influenza virus mutated hemagglutinin (HA) polypeptides. In a specific embodiment, provided herein are kits comprising an influenza virus mutated hemagglutinin polypeptide described herein and instructions for using the influenza virus mutated hemagglutinin polypeptide to assess the antibodies present in a subject. In another specific embodiment, provided herein are kits comprising an influenza virus mutated hemagglutinin polypeptide described herein for use in methods of assaying for the presence of HA stalk antigenic peptide-specific antibodies in a sample.

6. EXAMPLES

6.1 EXAMPLE 1: GLOBULAR HEAD-DISPLAYED CONSERVED INFLUENZA H1 HEMAGGLUTININ STALK EPITOPES CONFER PROTECTION AGAINST HETEROLOGOUS H1 VIRUS STRAIN

[00211] Significant genetic variability of the influenza A virus surface glycoprotein hemagglutinin, the main target of current vaccines, made it challenging to develop effective and long-lasting seasonal influenza virus prophylaxis so far. In an effort to overcome these hurdles which necessitate regular vaccine updates, the immune response was re-directed towards more conserved parts of the antigen to achieve broader cross-protective immunity against influenza. Hemagglutinin-reactivity of human serum samples from volunteers vaccinated with a monovalent group 1 H1 influenza A virus vaccine were mapped with special focus on its rather conserved stalk domain. A peptide-display format for improved accessibility of selected stalk epitopes was established by engineering such epitopes into the head domain of a phylogenetically distant group 2 H3 hemagglutinin. Vaccination of mice with an H1 vaccine containing a single 25 amino acid stalk-epitope in its head domain protected 75% of BALB/c mice from a lethal 5 mL_{D50} challenge dose with a heterologous H1 challenge strain. The identified conserved HA stalk epitopes in combination with the established HA-display format

are protective against drifted virus strains and are useful as vaccine candidates for seasonal influenza prophylaxis.

6.1.1 Materials and Methods

6.1.1.1 Synthesis of peptide membranes

[00212] A technique originally developed in Frank R. Tetrahedron 1992;48:9217–32 and modified in Pflegerl K, *et al.*, J Pept Res Off J Am Pept Soc 2002;59:174–82 was used for synthesis of peptide arrays with the MultiPep Multiple Peptide Synthesizer (Intavis Bioanalytical Instruments AG, Cologne, Germany). Peptides were C-terminally immobilized on cellulose sheets via di- β -alanine anchors and 112 decapeptides covering the entire HA sequence from Influenza A/New Caledonia/20/1999 (H1N1) were synthesized using conventional Fmoc technique. Peptides were designed to have five amino acid (aa) sequence overlaps. After the coupling reaction, all N-termini were acetylated and side chain protection groups were cleaved. Each peptide membrane included negative control peptide spots (deca-alanine) and positive control peptide spots (WSHPQFEK; SEQ ID NO:12) that bind streptavidin.

6.1.1.2 Preparation of human serum samples

[00213] Human serum samples were obtained from a clinical trial with healthy volunteers that received a novel type of monovalent influenza vaccine (Δ NS1-H1N1) or placebo (Wacheck V, *et al.*, J Infect Dis 2010;201:354–62). Frozen samples were thawed at 4°C, centrifuged, sterile-filtered and serum IgG was purified using Protein G Fast Flow Sepharose (GE Heath Care, Little Chalfont, UK) as affinity chromatography support and a FPLC pump system (GE Heath Care, Little Chalfont, UK) with an UV-detector. Before and after loading the serum samples, phosphate buffered saline (PBS) was applied to equilibrate the column and wash-off unbound proteins, respectively. Serum IgGs were eluted using 0.2 M acetic acid with 20% (v/v) ethylenglycol and fractions were collected by monitoring the absorption at 280 nm. Eluates of purified IgG were subsequently neutralized with 1 M sodium carbonate. Labeling of the purified IgG and a commercial anti-HA monoclonal antibody (mAb) (Clone C179, Takara Holdings Inc., Kyoto, Japan) was performed with a 20-fold molar excess of 20 mM biotinamidohexanoic acid N-hydroxysuccinimide ester in dimethylformamide. After one hour of incubation, biotinylated IgGs were dialyzed against PBS supplemented with 0.1 % (w/v) sodium azide. The samples were stored at 4°C.

6.1.1.3 Binding assays with purified human serum on peptide membranes

[00214] Binding assay conditions were evaluated for reduced unspecific binding of the streptavidin-HFP conjugate in blank assays without serum IgG incubation. Different concentrations of serum IgG (5 - 50 µg/mL), different incubation times with serum IgG (15 – 60 minutes) and the addition of urea (0.3 – 4 M) in the serum IgG incubation and washing buffer were tested. The optimized assay protocol was as follows: peptide membranes were re-equilibrated for 30 minutes in 20% (v/v) methanol and then washed with PBS containing 0.1% (v/v) Tween 20 (PBS-T). Membranes were blocked with PBS-T containing 3% (w/v) bovine serum albumin (BSA) for two hours, washed again with PBS-T and then incubated for one hour with 50 µg/mL biotin-labeled serum IgG diluted in incubation buffer (PBS-T with 1 % (w/v) BSA and 1.2 M urea). After a wash with PBS-T containing 1.2 M urea, membranes were incubated with a streptavidin-horseradish peroxidase (HRP) conjugate at a 1:3200 dilution in PBS-T containing 0.8 M NaCl and 1% (w/v) BSA for one hour. Membranes were finally washed with PBS-T with 0.8 M NaCl, incubated for 5 minutes with Super Signal Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and chemiluminescence of the peptides spots was measured. Imaged spots were normalized and negative and positive control peptide spots were defined as 0 % and 100 %, respectively. Signal intensities (%) of serum IgG binding to the hemagglutinin peptide spots were calculated.

6.1.1.4 Reconstruction of the HA 3D structure and prediction of potential discontinuous epitopes

[00215] A 3D model for the HA sequence of the vaccine strain NC99 was utilized to identify the spatial distribution of epitopes and their relative orientation. The spatial position of an epitope was characterized by the average of all coordinates of the contributing amino acids

$\langle \zeta^{a,b} \rangle = 1/10 \sum_{i=1}^{10} \zeta_i^{a,b}$. Therein, $\zeta_i^{a,b}$ resembles the mean position of an individual amino acid a or b, respectively. This mean position in return was calculated from the Cartesian coordinates of each individual site: $\zeta_i^{a,b} = \langle x_j^{a,b} + y_j^{a,b} + z_j^{a,b} \rangle_j$. Additionally, as each peptide is comprised of 10 aa, their orientation in space may be characterized by the end to end distance vector $\langle \zeta^{a \rightarrow b} \rangle = \langle \zeta^b \rangle - \langle \zeta^a \rangle$. The relative angle of two epitopes was then calculated by:

$\langle \zeta^a \rangle \langle \zeta^a \rangle = \left| \langle \zeta^a \rangle \right| \left| \langle \zeta^a \rangle \right| \cos \theta$. Thus, the relation of two epitopes and their relative orientation are given by their relative distances $\langle \zeta^{a \rightarrow b} \rangle$, and the angle θ . The sequence of the hemagglutinin precursor (RCSB Protein Data Bank: 1HA0) gave the best match to the sequence under investigation and was used as template for the 3D structure. The first 22 and the last 49 amino acids in the experimental primary sequence had to be neglected for proper modeling. The structure was obtained by algorithms written in Mathematica (*see* website at wolfram.com/mathematica/?source=nav), the 3D structure was plotted with Visual Molecular Dynamics (VMD) (*see* website at ks.uiuc.edu/Research/vmd/) and rendered by POV-RAY (*see* website at povray.org/).

6.1.1.5 Insect Cells

[00216] Sf9 insect cells (ATCC # CRL-1711) were routinely propagated in TNM-FH medium (Gemini Bio-Products, West Sacramento, CA) supplemented with 0.1% (v/v) Pluronic 68 (Sigma, St. Louis, MO), 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA) and a Penicillin-Streptomycin antibiotic mixture (Life Technologies, Carlsbad, CA) at 27 °C. Baculovirus amplification was performed in the presence of 3% (v/v) FBS. BTI-TN-5B1-4 (High Five - Vienna Institute of Biotechnology subclone; Krammer F, *et al.*, Mol Biotechnol 2010;45:226–34) cells were used for expression of soluble influenza A hemagglutinin antigens and maintained at 27°C in HyClone SFX serum free media (Fisher Scientific, Hampton, NH) at 27°C supplemented with Penicillin-Streptomycin antibiotic mixture.

6.1.1.6 Viruses

[00217] Pandemic virus A/Netherlands/602/2009 (NL09, pH1N1) was propagated in 8- to 10-day-old embryonated chicken eggs for 48 h at 37°C and titered on MDCK cells in the presence of tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin.

6.1.1.7 Generation of DNA and protein vaccine antigens

[00218] The nucleotide sequences of HA from A/New Caledonia/20/1999 (NC99, H1N1, GenBank: CY031336.1) and A/Hiroshima/52/2005 (HIR05, H3N2, GenBank: EU283414.1) were derived as described in Krammer F, *et al.*, Mol Biotechnol 2010;45:226–34 and Krammer F, *et al.*, J Virol Methods 2010;167:17–22. Selected H1 subtype HA2-derived epitopes of about 25 aa in size (peptide 66-69: AKLRMVTGLRNIPSIQSRGLFGAIA (SEQ ID NO:1), 86-89:

KVDDGFLDIWTYNAELLVLLNERT (SEQ ID NO:2), 106-109:
SVYQILAIYSTVASSLVLLVSLGAIS (SEQ ID NO:3), 69+73:
QSRGLFGAIAGGGGGVDGWYGYHHQ (SEQ ID NO:8) and 73+96:
SVDGWYGYHHQGGGGGIGNGCFEFYH (SEQ ID NO:13) were inserted into antigenic site B in the globular head domain of a phylogenetic group 2 H3 subtype influenza HA (HIR05) after L173 (H3 numbering including signal peptide) using overlap-extension PCR. Putative discontinuous epitopes were separated by a 5x-Glycine linker and a charged serine residue was introduced before hydrophobic valine residues for better epitope display (as for peptide 73 and 106 (SEQ ID NO:s 5 and 17, respectively)). Modified HA genes and wildtype negative control HA (HIR05) genes were cloned into a modified pFastBac vector (Invitrogen, Carlsbad, CA) under the control of the baculovirus very late polyhedrin promoter using BamHI and NotI restriction endonucleases (New England Biolabs, Ipswich, MA). The inserts were designed to yield soluble HA proteins with a thrombin cleavage site, a T4 foldon trimerisation domain and a C-terminal hexahistidine-tag for the generation of soluble HA protein antigens as described in Krammer F, *et al.*, PLoS ONE 2012;7:e43603, Krammer F, *et al.*, J Virol 2013;87:6542–50, Margine I, *et al.*, J Virol 2013;87:10435–46, and Margine I, *et al.*, J Vis Exp JoVE 2013:e51112. Soluble HA from pandemic CAL09 was expressed with a GCN4pII trimerisation domain and a C-terminal Strep-Tag II sequence to prevent background signals in serological assays as described in Krammer F, *et al.*, PLoS ONE 2012;7:e43603, Krammer F, *et al.*, J Virol 2013;87:6542–50, and Margine I, *et al.*, J Virol 2013;87:10435–46. Full-length proteins were cloned into a modified pCAGGS mammalian expression vector under the control of the cytomegalovirus (CMV) promoter for being utilized as DNA vaccine using SacI restriction endonuclease (New England Biolabs, Ipswich, MA). Plasmid DNA was isolated using the NucleoBond Xtra Endotoxin-free (EF) Maxiprep Kit (Macherey-Nagel GmbH & Co KG, Düren, DE) and DNA concentration was measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Recombinant bacmids for the expression of soluble proteins were generated using the Bac-to Bac System and E.coli DH10Bac and were isolated using a PureLink Plasmid Filter Midiprep Kit (all from Invitrogen, Carlsbad, CA). Recombinant baculovirus was generated using Cellfectin II transfection reagent (Invitrogen, Carlsbad, CA) and was rescued from Sf9 cells and amplified to a passage 3 virus stock. High Five cells were infected with the recombinant baculoviruses at a multiplicity of infection of approximately 10

and cells were cultured at 28°C shaking. The culture supernatant was harvested 3 days-post infection and clarified by low-speed centrifugation (5,000 g, 20 min, 4°C). The clarified culture supernatant was incubated with Nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Venlo, NL) (3 mL per 250 mL culture broth) for two hours at room temperature under continuous shaking at 75 rpm in a rotational shaker. Soluble protein was purified over 10 mL polypropylene columns (Qiagen, Venlo, NL). Captured resin was washed four times with ten bed volumes (=15 mL) washing buffer (50 mM Na₂HCO₃, 300 mM NaCl, 20 mM imidazole, pH 8) and protein was eluted in approximately five bed volumes (~ 8 mL) elution buffer (50 mM Na₂HCO₃, 300 mM NaCl, 300 mM imidazole, pH 8). Amicon Ultracell (Millipore Corporation, Billerica, MA) centrifugation devices (cut-off 30 kDa) were used for concentration and buffer exchange to PBS pH 7.4. Protein concentration was quantified using a Quickstart Bradford Dye Reagent (Bio-Rad Laboratories, Inc., Hercules, CA) with a BSA standard curve. Protein purity, integrity and identity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (4–20% polyacrylamide - Mini PROTEAN TGX gels, Bio-Rad Laboratories, Inc. Hercules, CA), Coomassie staining and Western blot.

6.1.1.8 Vaccine formulation

[00219] The vaccine prime consisted of 40 µg pCAGGS plasmid in water applied with an Ichor TriGrid *in vivo* electroporation system (Steel J, *et al.*, mBio 2010;1), encoding either full-length wildtype HIR05 HA (- control, H3) or one of the four HA2-epitope-display HAs (HIR05-NC99-Ep66-69, HIR05-NC99-Ep69+73, HIR05-NC99-Ep73+96 or HIR05-NC99-Ep86-89) under the control of the CMV promoter. The two booster immunizations were performed with 2.5 µg purified soluble trimeric HA protein of the wildtype HAs or one of the four epitope-display HA proteins in PBS. Each protein vaccine dose was adjuvanted with 2.5 µg poly(I.C) (Invitrogen, Carlsbad, CA). The positive control group (N=5) received a single intramuscular immunization with an aliquot of the unadjuvanted 2010-2011 trivalent inactivated vaccine Agriflu® (Novartis AG, Basel, CH) on day 42. The aliquot corresponded to 1 µg HA per strain and was from the 2010/11 influenza season (the H1 HA is from pandemic A/California/7/2009).

6.1.1.9 Mouse immunization and challenge

[00220] Animal experiments were performed in female 6 to 8 week-old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) under the guidelines of the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee (permit LA12-00028). Animals were

kept on a 12-hour light/dark cycle and had free access to food and water. Mice were anesthetized by intraperitoneal (i.p.) injection of 0.1 mL of a ketamine/xylazine mixture (0.15 mg/kg and 0.03 mg/kg) before intranasal or electroporation procedures. The four study groups (N=4 or 5 each) received a DNA prime with 40 µg plasmid in water (pCAGGS encoding full-length HA2-epitope-carrier HA) in the left calf muscle by in vivo electroporation using a TriGrid delivery system (Ichor Medical Systems, San Diego, CA). Three and six weeks later (day 21 and 42) animals were boosted by intranasal (i.n.) and intramuscular (i.m.) immunization with 2.5 µg of the respective purified soluble stalk-epitope-carrier HA protein in PBS adjuvanted with 2.5 µg poly(I.C) (Invitrogen, Carlsbad, CA). The negative control group (N=5) received a DNA prime encoding full-length wildtype HIR05 (H3) and two protein boosts (2.5 µg) with soluble wildtype HIR05 containing the same amount of adjuvant by i.n. and i.m. immunization, respectively. The positive control group (N=5) received a single immunization with unadjuvanted aliquot of the inactivated vaccine Agriflu® (Novartis AG, Basel, CH) intramuscularly on day 42. The aliquot corresponded to 1 µg HA per strain and was from the 2010/11 influenza season (the H1 HA is from pandemic A/California/7/2009). Three weeks after the last immunization (day 63) blood was drawn from anesthetized mice by submandibular bleeding for immunological assays. Immunized mice were challenged with 5 mLD₅₀ of heterologous pH1N1 NL09. Weight loss was monitored daily for up to 14 days. Animals that lost 20% or more of their initial body weight were scored dead and humanely euthanized, according to institutional guidelines.

6.1.1.10 Serological assay

[00221] A quantitative ELISA was performed to assess serum titers specific for the HA of pandemic CAL09 as in Klausberger M, *et al.*, Vaccine 2013. Pooled sera (N=5) from the different vaccine groups were assayed in duplicates. The plates were coated with rHA of CAL09 expressed with a GCN4pII trimerisation domain and a C-terminal Strep-Tag II sequence in order to avoid a background signal in ELISAs, as described in Krammer F, *et al.*, PLoS ONE 2012;7:e43603, Krammer F, *et al.*, J Virol 2013;87:6542–50, and Margine I, *et al.*, J Virol 2013;87:10435–46. Immulon 4HBX® ultra-high-binding polystyrene plates (Thermo Fisher Scientific, Waltham, MA) were coated with 2 µg/mL in coating buffer (0.1 M Na₂CO₃/NaHCO₃, pH 9.2, 50 µl/well) at 4 °C overnight. Plates were then blocked with PBS-T containing 3% (w/v) non-fat dry milk for two hours at room temperature. 3-fold dilutions to endpoint titer of pooled serum samples (100 µl per well in PBS-T with 1% non-fat dry milk

(starting concentration: 1:50 dilution) were then applied and incubated for two hours at room temperature. After three washes with 100 μ L PBS-T, plates were incubated for one hour with HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Dallas, TX) at a dilution of 1:3000 in PBS-T and non-fat dry milk (1%, v/v) at room temperature. Three washes with 100 μ L PBS-T removed unbound antibody and plates were developed using the SigmaFAST OPD substrate (Sigma, St. Louis, MO) (100 μ L/well) and stopped with 3 M HCl (50 μ L/well). The colorimetric change was measured as the change in optical density (OD 490 nm) on a Synergy 4 (BioTek, Winooski, VT) microplate reader. The endpoint titer was defined as the reciprocal of the highest dilution that yields an OD 490 nm value of greater than the mean plus three standard deviations of blank wells.

6.1.2 Results

6.1.2.1 HA2-derived synthetic peptides are recognized by human immune sera.

[00222] To identify immunogenic regions in the influenza HA protein, a chemiluminescence binding assay with purified biotinylated human serum IgG from vaccinated volunteers (Wacheck V, *et al.*, J Infect Dis 2010;201:354–62) was performed on peptide membranes. The membrane covered 112 synthetic decapeptides spanning the entire HA sequence of seasonal NC99 (sH1N1), on which the administered vaccine is based. A binding assay was established to evaluate pre-immune and immune serum samples from twelve vaccinated volunteers (Figure 1A) and four placebo controls (Figure 1B) for binding to the panel of synthetic peptides, whereby positive values indicate an increased immune response after vaccination. A large number of peptides located in the HA HA1 and HA2 domain were found to react well with the tested sera, with highest induction of antibodies (+ 120%) seen for two HA1-derived peptides (peptides 9 and 55 (SEQ ID NOs: 18 and 19, respectively)). Further studies focused on epitopes from the HA stem domain and, interestingly, a stretch covering the C-terminus of HA1 and the N-terminus of HA2 including the fusion peptide (peptides 65-69 (SEQ ID NOs: 20-22 and 4, respectively)) revealed induction of serum IgGs up to about 90% as compared to pre-immune sera. Two other HA2 regions, covering peptide pools 87-89 (SEQ ID NOs: 23-25, respectively) and 108-109 (SEQ ID NOs: 26 and 27, respectively) showed 20-40% higher signal intensities in immune compared to pre-immune sera. Besides two elevated reactivity patterns for HA1-derived peptides 9 and 25 (SEQ ID NOs: 18 and 28, respectively), the placebo group either maintained a rather consistent immune response or even a decreased response after vaccination.

In total, immunization success could be confirmed, as the sum of differences in signal intensities before and after vaccination for vaccinated volunteers amount for + 981 %, whereas for the placebo controls it was -72 %. To prove that binding of serum to the peptides is method-independent and specific, binding specificity was re-confirmed using Surface Plasmon Resonance with a number of peptides.

6.1.3 Localization of linear HA epitopes and prediction of potential discontinuous epitopes.

[00223] The peptides were mapped on the crystal structure of a closely related H1 subtype HA to visualize the location of the peptides (filled spheres, Figure 2A). The calculation of angles and distances between epitopes (Figure 2B) should serve to identify peptides, which, in combination, might form a discontinuous epitope. Putative discontinuous epitope pairs in the more conserved HA2 region comprise peptides 66-69 (SEQ ID NOs: 20-22 and 4, respectively), which cover the C-terminus of the HA1 and the fusion peptide, together with peptide 73 (SEQ ID NO:5). Moreover, the membrane-proximal peptide-pairs 73 and 96 (SEQ ID NOs: 5 and 7, respectively) as well as peptide 96 and 100 (SEQ ID NOs: 7 and 29, respectively) were found to be close enough to constitute a discontinuous epitope.

6.1.4 Design of a HA-based epitope-display vaccine

[00224] Peptides located in the more conserved region of HA, covering the fusion peptide region and stalk domain, were selected to generate a vaccine with broad reactivity. All selected peptides show a high degree of conservation among H1 subtypes covering two pandemic strains from 1918 and 2009, H1 strains with considerable antigenic drift (H1 vaccine strains) and even phylogenetic distant H2 strains (Figure 3). Antibody responses towards the proteins' stalk domain were generally shown to be underrepresented in the antibody repertoire during conventional vaccination (Krammer F, *et al.*, J Virol 2013;87:6542–50, Margine I, *et al.*, J Virol 2013;87:10435–46, and Krammer F, *et al.*, J Virol 2012;86:10302–7), a fact that might be explained by their poorer surface exposure, as seen with our selected epitopes in Figure 3. Thus, to make these epitopes more accessible to the immune system, they were transferred to the well exposed antigenic site B loop of the HA protein. This region has been demonstrated to tolerate insertions of heterologous epitopes of about 20 amino acids (Krammer F, *et al.*, J Virol Methods 2010;167:17–22, Muster T, *et al.*, J Virol 1995;69:6678–86, Ferko B, *et al.*, J Infect Dis 1998;178:1359–68, and Staczek J, *et al.*, Infect Immun 1998;66:3990–4); thus, insertions were

generated in that size range. The epitopes derived from a phylogenetic group 1 hemagglutinin (NC99, sH1N1) were genetically engineered into the head of a group 2 hemagglutinin (HIR05, H3N2). Given that humoral responses are generally known to be group-specific, a reduced background signal from the antigenically divergent carrier HA was expected in a serological assay. Peptides chosen as antigenic determinant in the stalk epitope-based HA-display vaccine are shown in Figure 3. They were either selected on basis of results obtained from the binding assay (as for peptide 66-69 (SEQ ID NO:1) and epitopes 87-89, 106, 108 and 109 (SEQ ID NOs: 23, 24, 25, 17, 26, and 27, respectively) or on basis of their spatial distribution which might allow for discontinuous epitope formation (as for peptides 69 + 73 (SEQ ID NO:8) or 73 + 96 (SEQ ID NO:9)). Peptides 86 and 107 (SEQ ID NOs: 30 and 14, respectively) were included as they are located next to or within an identified linear multi-epitope and were speculated to be involved in preservation of their immunogenicity. Low expression yields were obtained for the multi-peptide 106-109 (data not shown).

6.1.5 Displayed H1 HA2 epitopes partially protect from lethal heterologous H1 mouse challenge

[00225] To explore whether displayed H1-derived stalk epitopes are sufficient in serving as antigenic component for protection against a heterologous H1 challenge virus, immunized mice were intranasally infected with 5 mL_{D50} of pandemic NL09 virus three weeks after the last immunization. Interestingly, a multi-epitope of 25 amino acids in size and spanning the intersubunit region (HIR05-NC99-Ep66-69 (SEQ ID NO:1)) was antigenically sufficient in protecting three out of four mice (75%) from a lethal challenge with a heterologous H1 strain (Figure 4B). This group also experienced comparable weight-loss kinetics to the positive control group until day 6 post challenge (Figure 4A). One out of five mice (20%) that received an HA-displayed peptide from the long alpha-helix was also protected from the lethal challenge. Mice immunized with the two HA-carrier displaying putative discontinuous epitopes (HIR05-NC99-Ep69+73 (SEQ ID NO:8) or HIR05-NC99-Ep73+96 (SEQ ID NO:13)) all succumbed to infection between day 7 and day 9 post challenge, as did the negative control group (Figure 4B). None of the experimental groups, however, were protected from morbidity, reflected by weight-loss until day 8 or 9 (Figure 4A).

6.1.6 Displayed H1 HA2-derived epitopes elicit H1-specific antibodies

[00226] To evaluate whether the HA head-displayed stalk epitopes are able to elicit H1-specific antibodies, pre-challenge mouse serum were tested in an ELISA. Soluble HA from pH1N1 (containing a different trimerisation domain and purification tag from the HA used in the immunization of mice) was used as coating antigen. In general, all titers, including that of the positive control group, were very low, with less than a 1-log difference between positive and negative control (Figure 5). Highest seroreactivity was achieved in the group immunized with HIR05-NC99-Ep69+73 (SEQ ID NO:8), which reached endpoint titers almost 1 log higher than the positive control. Groups immunized with HIR05-NC99-Ep66-69 (SEQ ID NO:1) or HIR05-NC99-Ep73+96 (SEQ ID NO:13) showed comparable endpoint titers (2-fold higher than the positive control). Lowest titers were measured in mice vaccinated with HIR05-NC99-Ep86-89 (SEQ ID NO:2), which were in the range of titers obtained in the positive control group.

6.1.7 Conclusion

[00227] The prevalence of serum anti-HA head-directed neutralizing antibodies is the current correlate and major mediator of prevention of influenza virus infection and induction of these antibodies is goal of available vaccines. Although very potent, these antibodies have little cross-reactivity and therefore necessitate annual vaccine updates for seasonal influenza. Antibodies directed towards sequences that are highly conserved among subtypes could broaden immunity and contribute to longevity of immunity (Krammer F, *et al.*, Curr Top Microbiol Immunol 2014). Vaccine design oriented towards the conserved HA stalk domain has been the focus of research aiming for the development of a universal vaccine (Krammer F, *et al.*, J Virol 2013;87:6542–50, Margine I, *et al.*, J Virol 2013;87:10435–46, Steel J, *et al.*, mBio 2010;1, Krammer F, *et al.*, J Virol 2012;86:10302–7, and Eggink D, *et al.*, J Virol 2014;88:699–704). Stalk-directed antibodies reduce viral replication by hampering its fusion with the host endosomal membrane (Gocnik M, *et al.*, J Gen Virol 2007;88:951–5 and Okuno Y, *et al.*, J Virol 1993;67:2552–8). Natural infection in humans (Margine I, *et al.*, J Virol 2013;87:4728–37 and Styk B, *et al.*, Acta Virol 1979;23:1–8) and mice (Kostolansky F, *et al.*, Acta Virol 2002;46:229–36 and Margine I, *et al.*, J Virol 2013;87:4728–37) was shown to induce stalk-specific antibodies, however immunodominance of the head domain and structural constraints may be factors that render these antibodies scarce. In an effort to generate a stalk-directed vaccine, a soluble HA protein-display platform for conserved stalk epitopes was generated. The epitopes were identified by epitope mapping with human serum and peptides spanning the entire sequence of the H1 HA of the

replication-deficient virus vaccine strain NC99 (Wacheck V, *et al.*, J Infect Dis 2010;201:354–62). For rational design of prophylactic and therapeutic vaccines, knowledge about B-cell epitopes is a pre-requisite and in addition to X-ray crystallography and site-directed mutagenesis, peptide mapping is a method well appreciated due to its low costs and potential for high-throughput application as reviewed in Sivalingam and Shepherd (Sivalingam GN, *et al.*, Mol Immunol 2012;51:304–9). This approach has already been successfully applied in epitope identification and fine-mapping of B-cell epitopes with influenza antigens (Steel J, *et al.*, mBio 2010;1, Jackson DC, *et al.*, Pept Res 1991;4:114–24, Tang XL, *et al.*, J Virol 1988;62:4745–51, and Nagy Z, *et al.*, Scand J Immunol 1994;40:281–91), however there is only a scarce number of reports on the identification of antibody epitopes using human serum samples (Bui H-H, *et al.*, Proc Natl Acad Sci U S A 2007;104:246–51). Mapped H1 HA stalk-located multi-epitopes (20–25 aa) were made more accessible by displaying them in the HA globular head antigenic site B of a phylogenetically distant group 2 HA. BALB/c mice receiving a tripartite vaccine (DNA-prime and two protein boosts) were subjected to a heterologous H1 subtype challenge strain. A 25 aa multi-peptide encompassing the intersubunit region of the NC99 HA (18 aa from the HA1 C-terminus and 7 aa from the HA2 N-terminus, peptide NC99-Ep66-69 (SEQ ID NO:1)) was sufficient to protect 75% of BALB/c mice against a lethal 5 mLD₅₀ challenge dose with the pandemic NL09. This high degree of protection could be seen despite the fact that the challenge virus differed by 3 amino acids within this 25 aa the multi-epitope; the highest degree of genetic variation seen among all of the tested peptides. In contrast, Nagy (Nagy Z, *et al.*, Scand J Immunol 1994;40:281–91) as well as Horváth (Horváth A, *et al.*, Immunol Lett 1998;60:127–36) and co-workers performed challenge studies in BALB/c mice immunized with 100 µg H1 HA adjuvanted peptide of 25 aa with a sequence C-terminally shifted by 5 amino acids (VTGLRNIPSIQSRGLFGAIAGFIEG (SEQ ID NO:6)) as compared to a stalk antigenic peptide described herein. A polyvalent version of their vaccine protected roughly 50% of mice using a lower challenge dose (2 mLD₅₀) of the homologous mouse adapted H1N1 strain A/Puerto Rico/8/34 (Horváth A, *et al.*, Immunol Lett 1998;60:127–36). The stalk antigenic peptide described herein and Horvath's peptide comprise the intact intersubunit region of the uncleaved HA0 precursor of non-infectious virions, which is exposed on the surface on immature virus particles as reviewed by Steinhauer (Steinhauer DA. Virology 1999;258:1–20). The main functional relevance of these antibodies would be to prevent enzymatic cleavage at this region

and thereby render the virus unable to initiate an infectious cycle. In fact, it has been shown that a substantial amount of the virus present and transmitted via host droplets in humans is non-infectious (Fabian P, *et al.*, PLoS ONE 2008;3:e2691). Antibodies directed against the intersubunit region could prevent these particles from being activated by host extracellular proteases (Ekiert DC, *et al.*, Science 2009;324:246–51). Peptide specific antibodies were present in the serum of vaccinated mice at similar concentrations to mice that received the positive control. However, very low titers were observed for all vaccinated animals (including positive control mice) and the titers did not correlate with protection in the other challenge model. Inactivated vaccines were previously shown to be weakly immunogenic in mice, especially after single immunizations, which might explain the low antibody levels of our TIV-immunized positive control mice (Chen GL, *et al.*, J Infect Dis 2011;203:930–6). The animal with the lowest total IgG level, however, did not survive the challenge experiment. In addition to B-cell epitopes (Tang XL, *et al.*, J Virol 1988;62:4745–51, Nagy Z, *et al.*, Scand J Immunol 1994;40:281–91, Horváth A, *et al.*, Immunol Lett 1998;60:127–36, and Jackson DC, *et al.*, Virology 1986;155:625–32), the HA1 C-terminus was also shown to locate T-helper cell epitopes (Nagy Z, *et al.*, Scand J Immunol 1994;40:281–91, Horváth A, *et al.*, Immunol Lett 1998;60:127–36, Ffrench RA, *et al.*, J Virol 1989;63:3087–94, Rajnavölgyi E, *et al.*, Mol Immunol 1994;31:1403–14, and Gogolák P, *et al.*, Biochem Biophys Res Commun 2000;270:190–8). It is possible that the CD8⁺ T-cell response is involved in mediating protection in the heterologous challenge experiment described herein. Vaccination with neither of the head-displayed putative discontinuous epitopes (epitopes 69+73 (SEQ ID NO:8) and 73+96 (SEQ ID NO:13)) resulted in improved weight loss kinetics or protection against the close heterologous challenge strain. The weak signal with peptide 73 (SEQ ID NO:5) in the binding assay already might have been an indicator for very weak immunogenicity of this peptide. Interestingly, peptide 69 (SEQ ID NO:4) comprises 3 aa of the HA1 C-terminus and 7 aa of the N-terminal fusion peptide region of HA2. The fusion peptide is the most conserved sequence across all influenza subtypes (Krystal M, *et al.*, Proc Natl Acad Sci U S A 1982;79:4800–4). It was previously shown to be the target for monoclonal antibodies against subtype H3 and H5 HA (mAb CF2 and 1C9, respectively), showing prophylactic and therapeutic efficacy in mice (Gocnik M, *et al.*, J Gen Virol 2007;88:951–5, Varecková E, *et al.*, Arch Virol 2003;148:469–86, and Prabhu N, *et al.*, J Virol 2009;83:2553–62) and the induction of antibodies found in human

convalescent serum (Staneková Z, *et al.*, Influenza Other Respir Viruses 2012;6:389–95). Work done by Staneková (Staneková Z, *et al.*, Influenza Other Respir Viruses 2012;6:389–95) and Janulíková and colleagues evaluated the protective efficacy of the fusion peptide, comprising the first 38 aa of the HA2 N-terminus of a H3 subtype HA conjugated to Keyhole Limpet Hemocyanin (KLH) in an adjuvanted three-dose regimen and achieved 47% protection in a 2 mLD50 challenge with the homologous virus strain (Janulíková J, *et al.*, Acta Virol. 2012;56(3):169-76). Peptide 69 (SEQ ID NO:4) covers the first 7 aa of the HA2 N-terminus (GLFGAIA) only and might contain too little antigenic information to provide measurable protection. The minimum requirement for H5 HA mAb 1C9 comprises the first 9 aa of the HA2 N-terminus (Prabhu N, *et al.*, J Virol 2009;83:2553–62); thus inclusion of adjacent C-terminal residues could improve efficacy of the peptide. Conformational deviations of peptide 69 (SEQ ID NO:4) in the display-format from its native conformation in the protein are not likely to be responsible for the lack in protective efficacy; other mAbs that target the fusion peptide, such as 1C9 (Prabhu N, *et al.*, J Virol 2009;83:2553–62), CF2 (Varecková E, *et al.*, Arch Virol 2003;148:469–86), and the H1-subtype derived mAb Uni-1 (targeting 14 aa of the HA2 N-terminus) with cross-group in vitro neutralization capability (Hashem AM, *et al.*, Biochem Biophys Res Commun 2010;403:247–51) are linear antigenic determinants as well.

[00228] Another peptide that partially protected (20% of mice) from the close heterologous NL09 challenge was epitope 86-69 (SEQ ID NO:2), which falls within the HA2 long-alpha helix. This region was previously identified by Wang and co-workers to harbor the epitope of 12D1 (Wang TT, *et al.*, Proc Natl Acad Sci 2010;201013387), a monoclonal antibody (mAb) with broad reactive immunity to H3 viruses and cross-reactivity even with group 1 viruses. Dual vaccination with 25 µg KLH-conjugate with a 56 amino acid peptide that encompasses the entire helix did result in full protection of BALB/c mice. Also, this HA2-directed mAb 12D1 was shown to react with a continuous epitope on the long alpha helix. In fact, Kuo and colleagues (Kuo YC, *et al.*, Exp Cell Biol 1978;46:338–54) showed, that HA2-directed antibodies generally show improved binding with more denatured HA, which would additionally support eligibility of the peptide-based screening approach described herein for B-cell epitopes with special focus on the HA HA2 domain. Moreover, the conserved HA stalk epitopes identified herein in combination with the HA-display format are protective against drifted virus strains and are useful as vaccine candidates for seasonal influenza prophylaxis.

[00229] All publications, patents and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. An immunogenic influenza virus mutated hemagglutinin (HA) polypeptide comprising an influenza virus mutated hemagglutinin globular head domain, wherein the influenza virus mutated globular head domain comprises one or more influenza virus HA stalk antigenic peptides.

2. The immunogenic influenza virus mutated hemagglutinin polypeptide of claim 1, wherein said influenza virus HA stalk antigenic peptide is inserted into the influenza virus HA globular head domain.

3. The immunogenic influenza virus mutated hemagglutinin polypeptide of claim 1, wherein said influenza virus HA stalk antigenic peptide replaces one or more amino acids in the influenza virus HA globular head domain.

4. The immunogenic influenza virus mutated hemagglutinin polypeptide of any of claims 1 to 3, wherein said influenza virus HA globular head domain is heterologous to the influenza virus HA stalk antigenic peptide.

5. The immunogenic influenza virus mutated hemagglutinin polypeptide according to any one of claims 1 to 4, wherein said influenza virus HA stalk antigenic peptide is displayed in the antigenic site B loop of the influenza virus HA globular head domain.

6. The immunogenic influenza virus mutated hemagglutinin polypeptide according to any one of claims 1 to 5, wherein said influenza virus HA stalk antigenic peptide is of subtype H1.

7. The immunogenic influenza virus hemagglutinin polypeptide according to any one of claims 1 to 6, wherein said influenza virus HA globular head domain is of subtype H3.

8. The immunogenic influenza virus mutated hemagglutinin polypeptide according to any one of claims 1 to 7, wherein said influenza virus HA stalk antigenic peptide is a B-cell or T-cell epitope.

9. The immunogenic influenza virus mutated hemagglutinin polypeptide according to any one of claims 1 to 8, wherein said influenza virus HA stalk antigenic peptide comprises a continuous or discontinuous HA stalk domain sequence.

10. The immunogenic influenza virus mutated hemagglutinin polypeptide according to any one of claims 1 to 9, wherein said influenza virus HA stalk antigenic peptide has a length of 10-30 amino acids, 15-25 amino acids, 20-25 amino acids, or 25 amino acids.

11. The immunogenic influenza virus mutated hemagglutinin polypeptide according to any one of claims 1 to 10, wherein said influenza virus HA stalk antigenic peptide comprises the sequence AKLRMVTGLRNIPSIQSRGLFGAIA (SEQ ID NO: 1), KVDDGFLDIWTYNAELLVLENERT (SEQ ID NO: 2), VYQILAIYSTVASSLVLLVSLGAIS (SEQ ID NO: 3), QSRGLFGAIA (SEQ ID NO: 4), VDGWYGYHHQ (SEQ ID NO: 5), IGNGCFEFYH (SEQ ID NO: 7) or a fragment or derivative thereof.

12. The immunogenic influenza virus mutated hemagglutinin polypeptide according to any one of claims 1 to 11, wherein two or more influenza virus HA stalk antigenic peptides are linked via a glycine linker, specifically said linker is of 5 amino acids length.

13. The immunogenic influenza virus mutated hemagglutinin polypeptide according to any one of claims 1 to 12, wherein said influenza virus HA stalk antigenic peptide comprises the sequence QSRGLFGAIAAGGGGGVDGWYGYHHQ (SEQ ID NO: 8) or VDGWYGYHHQGGGGGIGNGCFEFYH (SEQ ID NO: 9).

14. The immunogenic influenza virus mutated hemagglutinin polypeptide according to any one of claims 1 to 13, wherein said influenza HA stalk antigenic peptide comprises a charged serine before an N-terminal valine.

15. The immunogenic influenza virus mutated hemagglutinin polypeptide according to any one of claims 1 to 14, wherein said influenza virus HA stalk antigenic peptide further comprises up to 17 amino acids of the C-terminus of the homologous intersubunit or HA1 peptide.

16. An influenza virus HA stalk antigenic peptide comprising or consisting of any one of SEQ ID NOs: 1 to 5, SEQ ID NOs: 7 to 9, or SEQ ID NO:13.

17. A nucleic acid encoding a peptide or polypeptide of according to any one of claims 1 to 16.

18. The nucleic acid of claim 17, which is complementary DNA (cDNA).

19. A cell expressing the nucleic acid of claim 17.

20. A virus comprising a genome engineered to express the nucleic acid of claim 17.

21. A virus comprising the polypeptide of any one of claims 1 to 15.

22. The virus of claim 20 or 21, wherein the virus is an influenza virus

23. A virus-like particle comprising the polypeptide of any one of claims 1 to 15.

24. A composition comprising the polypeptide of any one of claims 1 to 15.

25. A composition comprising the virus according to any one of claims 20 to 22.

26. A composition comprising the virus-like particle of claim 23.

27. The composition according to any one of claims 24 to 26 for use in prevention and/or treatment of influenza virus disease in a subject.

28. The composition according to claim 24 to 27, wherein said composition is administered by intramuscular or intranasal route.

29. A method for producing an influenza virus mutated hemagglutinin polypeptide, wherein the method comprises

(a) introducing synthetic peptides of 10 amino acid residues in length into the globular head domain of an influenza virus hemagglutinin polypeptide; and

(b) selecting the influenza virus mutated hemagglutinin polypeptide which show strong reactivity to serum samples,

wherein the synthetic peptides span the entire sequence of an influenza virus hemagglutinin stalk domain and the intersubunit region of the globular head domain, and wherein the serum samples are isolated from a subject exposed to influenza virus.

30. An isolated antibody directed against a polypeptide of any one of claims 1 to 15.

31. A method of eliciting cross-protective immunity against influenza virus in a subject, wherein the method comprises administering to the subject the polypeptide of any one of claims 1 to 15, a composition according to any one of claims 24 to 28, or the nucleic acid according to claim 17 or 18, thereby eliciting an immune response in the subject.

32. A method of preventing influenza virus disease in a subject, wherein the method comprises administering to a subject a therapeutically effective amount of the polypeptide according to any one of claims 1 to 15, a composition according to any one of claims 24 to 28, or the nucleic acid according to claim 17 or 18, thereby preventing the influenza virus disease in the subject.

33. A method of eliciting cross-protective immunity against influenza virus in a subject, wherein the method comprises

(a) administering to the subject a therapeutically effective amount of a nucleic acid according to claim 17 or 18;

(b) after a first period of time, administering a therapeutically effective amount of a first polypeptide according to any one of claims 1 to 15; and

(c) after a second period of time, administering a therapeutically effective amount of a second polypeptide according to any one of claims 1 to 15.

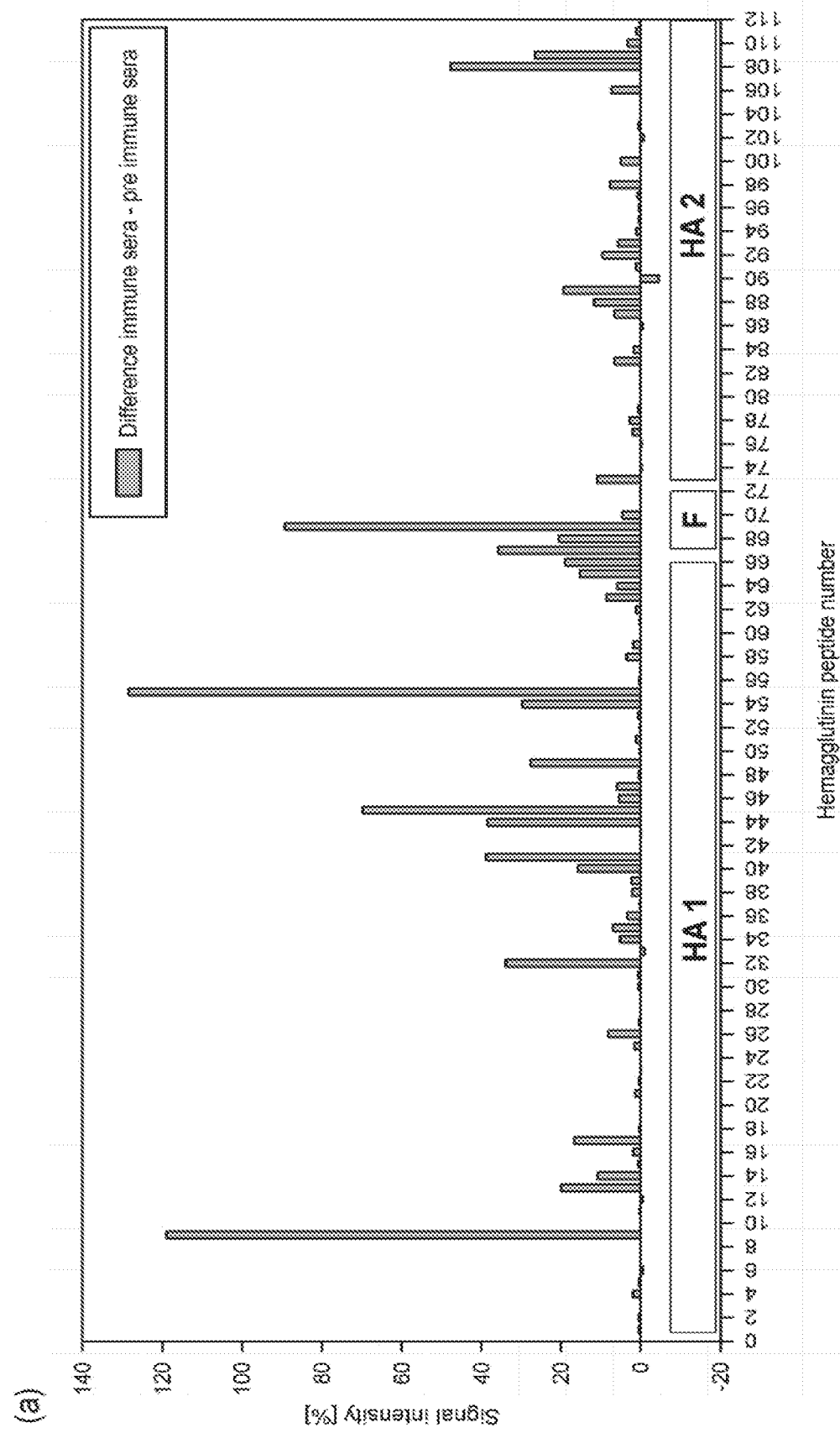


Fig. 1A

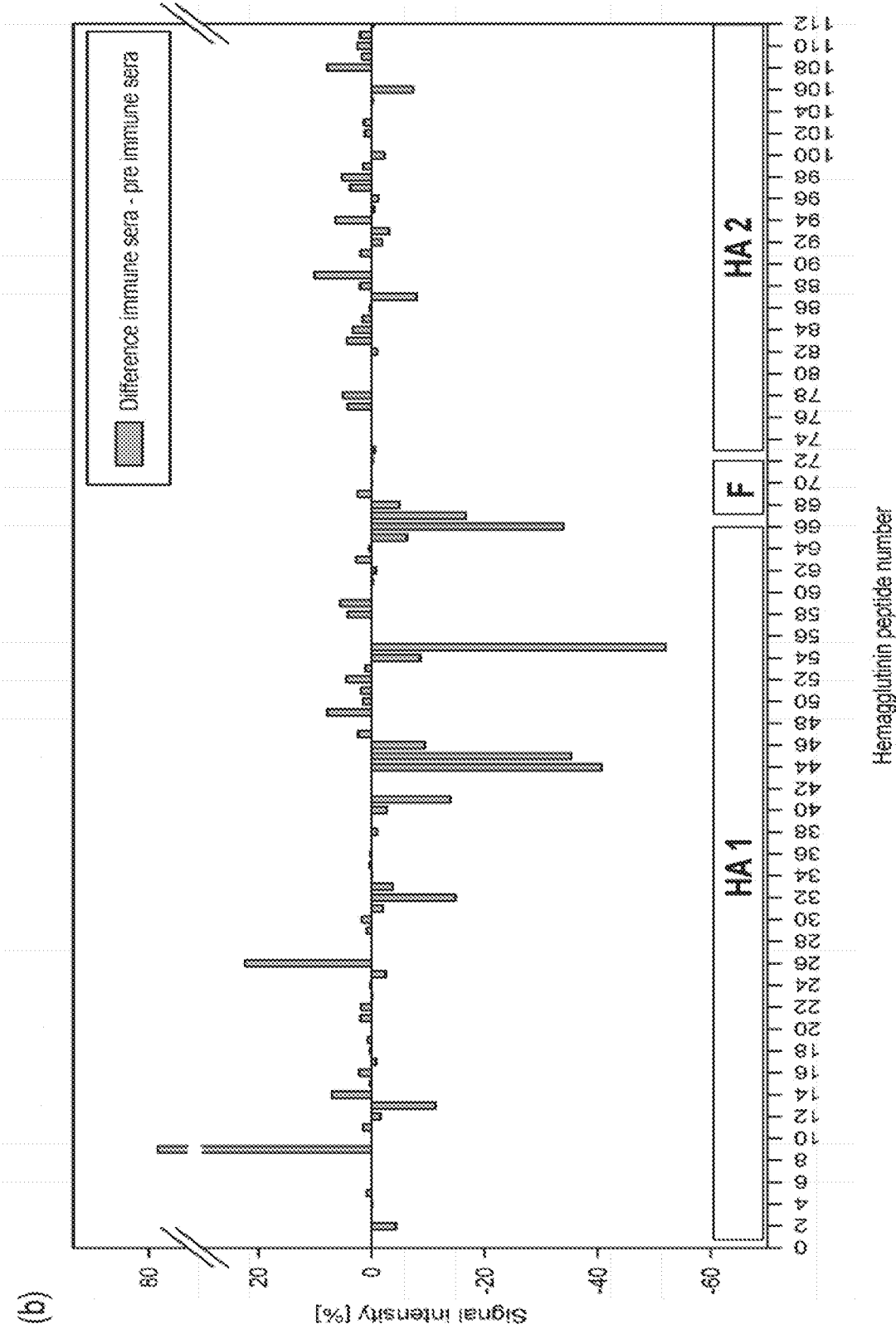


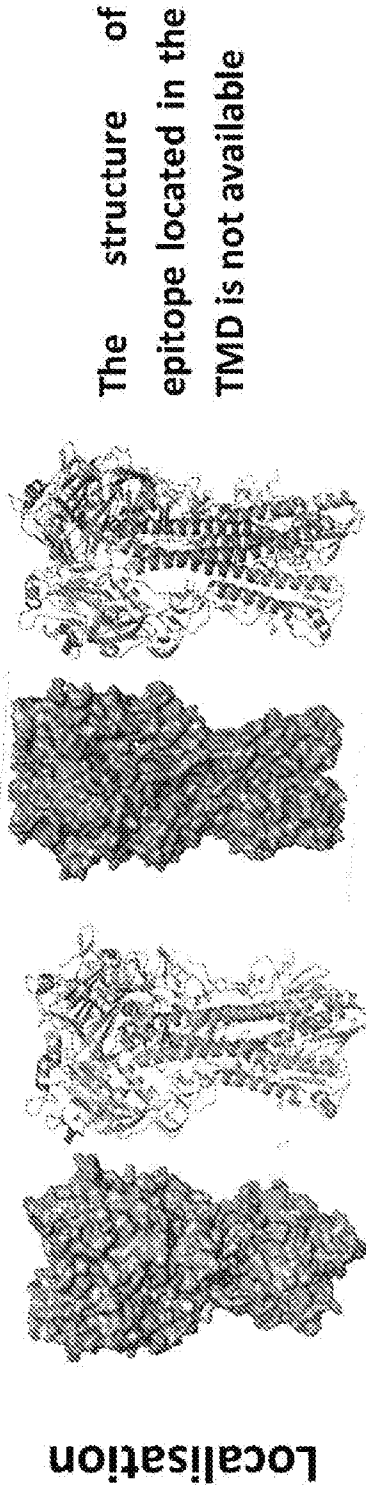
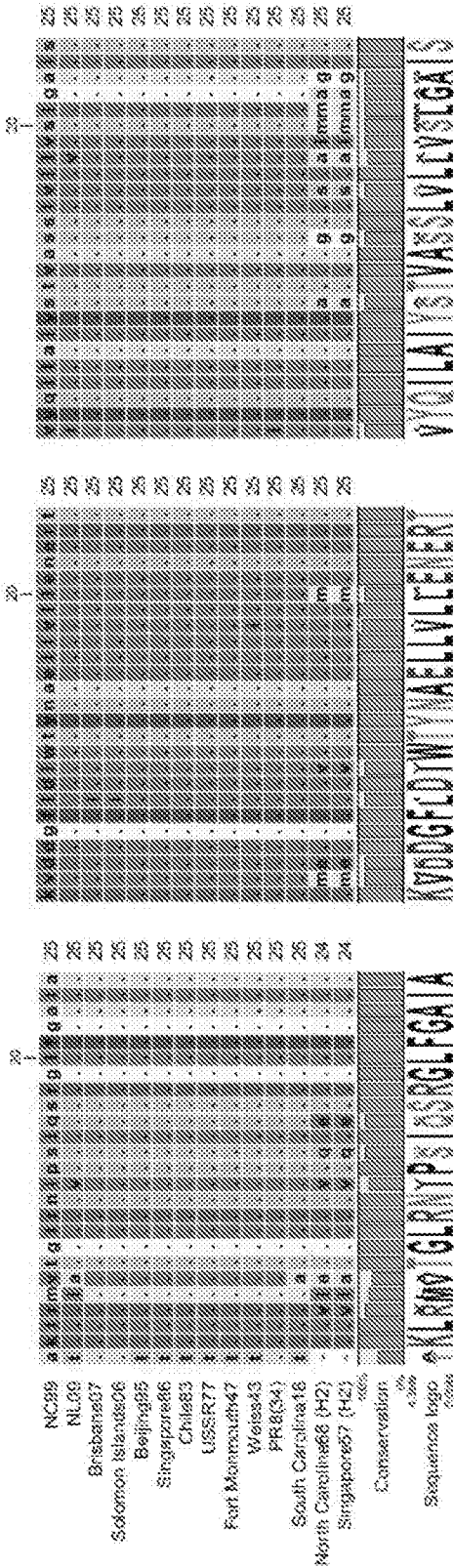
Fig. 1B

Putative linear epitopes

NC99-Ep 66-69

NC99-Ep 86-69

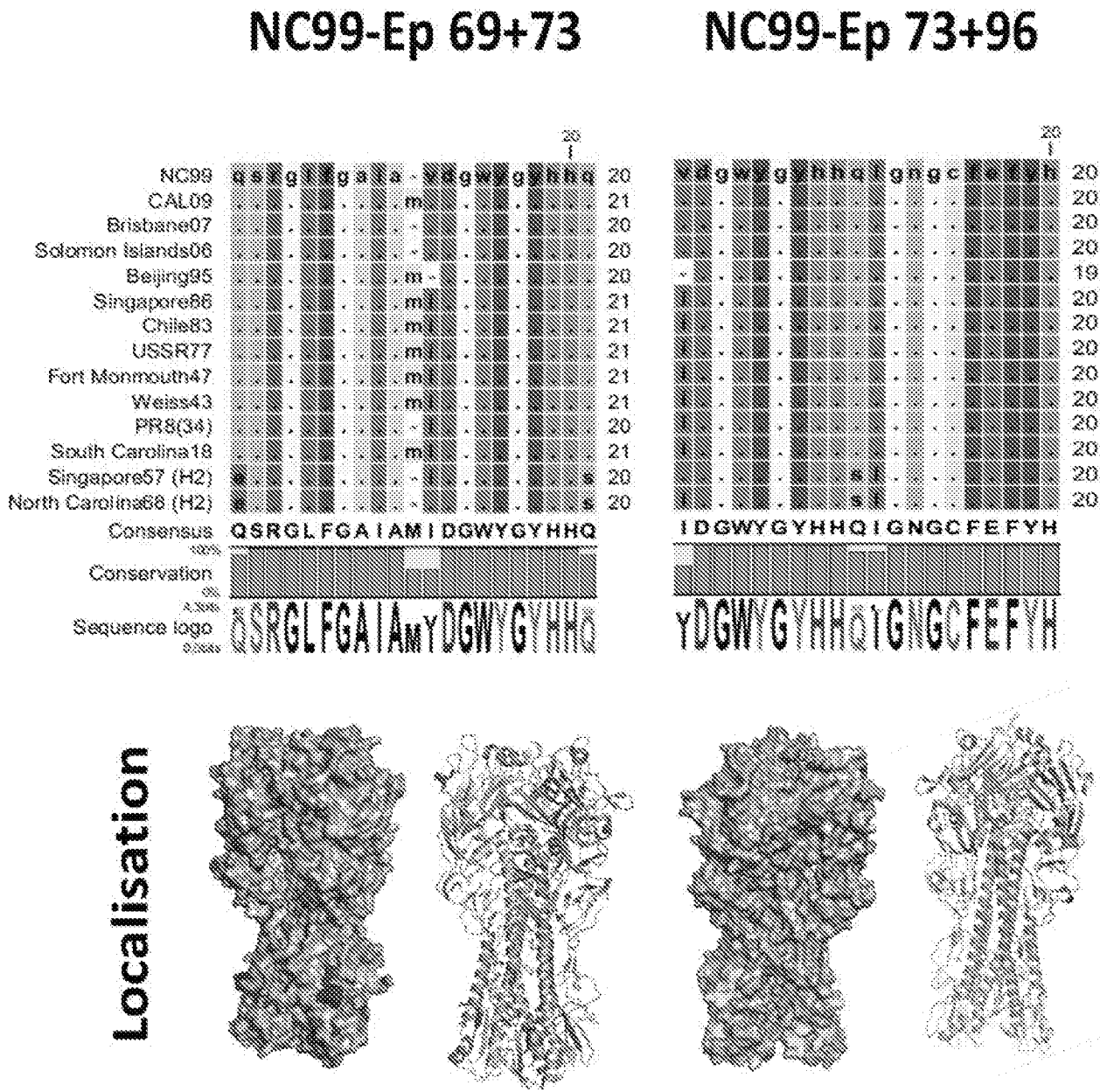
NC99-Ep 106-109



The structure of epitope located in the TMD is not available

Fig. 3A

Putative discontinuous epitopes



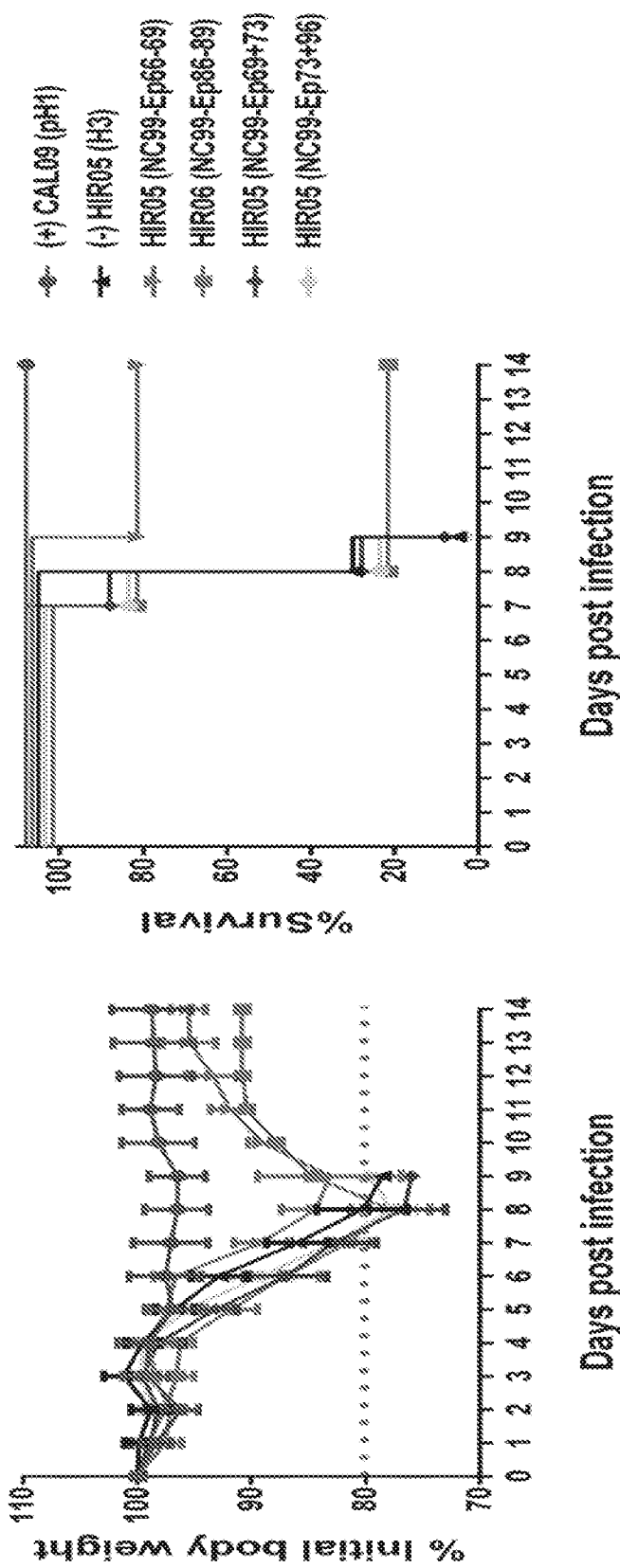


Fig. 4B

Fig. 4A

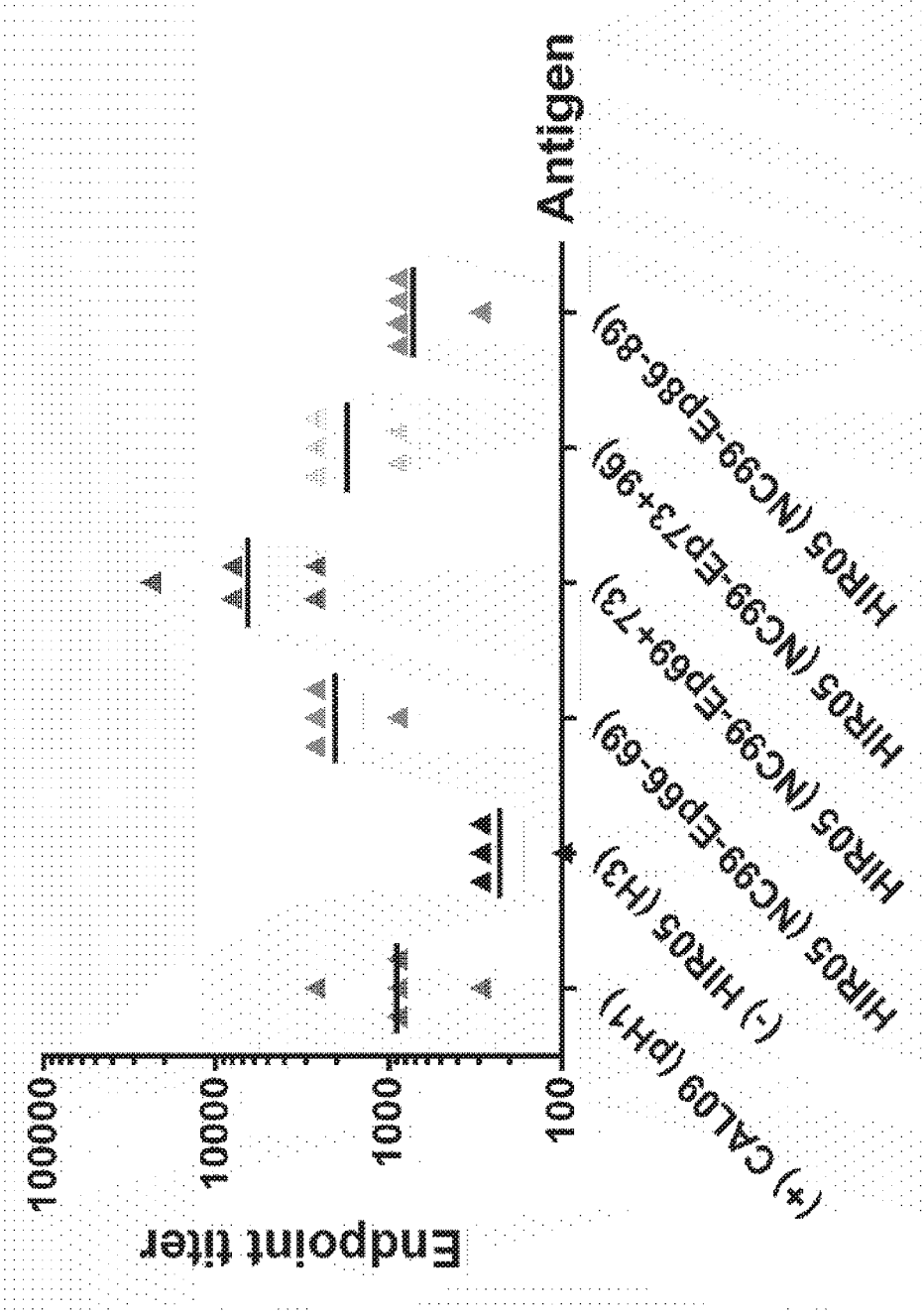


Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/37595

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/145, A61P 31/16, A61P 37/04, C07K 14/11 (2016.01)

CPC - A61K 39/145, A61K 2039/525, C12N 2760/16122, C12N 2760/16134

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)

IPC(8) - A61K 39/145, A61P 31/16, A61P 37/04, C07K 14/11 (2016.01)

CPC - A61K 39/145, A61K 2039/525, C12N 2760/16122, C12N 2760/16134

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

CPC - A61K 2039/5258, C07K 2319/00

(keyword limited; terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatBase, Google Patents, Google Scholar

Search terms: influenza, orthomyxovirus, hemagglutinin, head, globular, stalk, stem, insert, substitute, replace, mutate, modify, heterologous, antigenic, immunodominant, cross-reactive, cross-neutralizing, broadly-neutralizing

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Q0PZR5, UniProtKB Accession No. Q0PZR5, 29 October 2014 [online]. [Retrieved on 2 September 2016]. Retrieved from the internet <URL: http://www.uniprot.org/uniprot/Q0PZR5.txt?version=53 > Entire document	16
X	US 2015/0132330 A1 (MOUNT SINAI SCHOOL OF MEDICINE et al.) 14 May 2015 (14.05.2015) para [0101], [0132], [0133], [0135], [0137], [0145], [0215], [0218], [0219], [0221], [0223], [0587], [0588], [0786], [0787], [0974]	1-4, 29

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

* 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

2 September 2016 (02.09.2016)

Date of mailing of the international search report

15 SEP 2016

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/37595

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-15, 17-28, 30-33
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.