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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 October 2010 (14.10.2010)

PCT

(10) International Publication Number
WO 2010/117786 A1

(51) International Patent Classification:
A61K 39/145 (2006.01) *C07K 14/11* (2006.01)

(21) International Application Number:
PCT/US2010/029202

(22) International Filing Date:
30 March 2010 (30.03.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/164,896 30 March 2009 (30.03.2009) US
61/299,084 28 January 2010 (28.01.2010) US

(71) Applicant *(for all designated States except US)*: **MOUNT SINAI SCHOOL OF MEDICINE OF NEW YORK UNIVERSITY** [US/US]; One Gustave L. Levy Place, New York, NY 10029 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LOWEN, Anice, C.** [CA/US]; 134 West 93rd Street, Apt. 8D, New York, NY 10025 (US). **STEEL, John** [GB/US]; 134 West 93rd Street, Apt. 8D, New York, NY 10025 (US). **GARCIA-SASTRE, Adolfo** [ES/US]; 16 East 96th Street, Apt. 3G, New York, NY 10128 (US). **PALESE, Peter** [US/US]; 414 Highwood Avenue, Leonia, NJ 07605 (US).

(74) Agents: **CORUZZI, Laura, A.** et al.; Jones Day, 222 East 41st Street, New York, NY 10017-6702 (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, 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, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, 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, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (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, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

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

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: INFLUENZA VIRUS VACCINES AND USES THEREOF

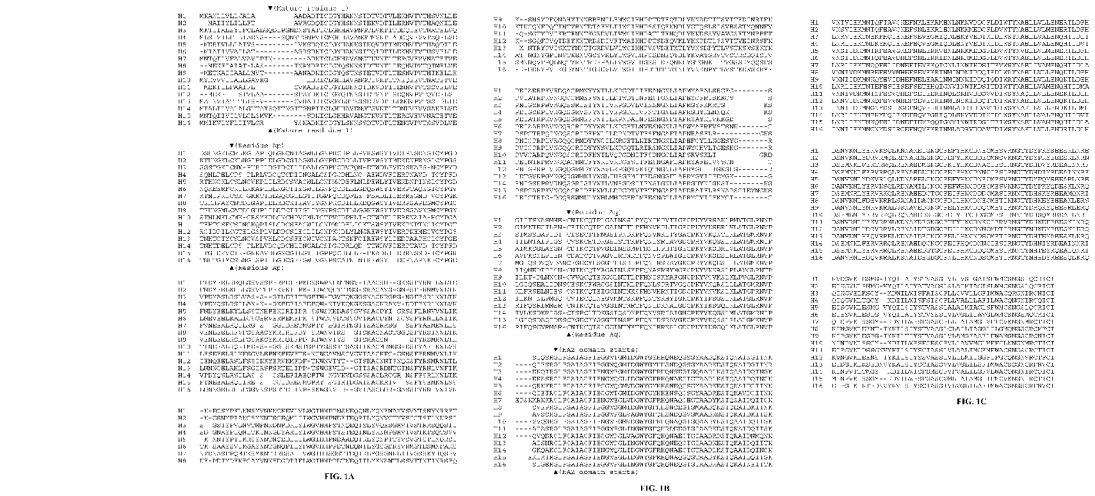

FIG. 1A

FIG. 1B

FIG. 1C

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

WO 2010/117786 A1

INFLUENZA VIRUS VACCINES AND USES THEREOF

[0001] This application claims priority benefit of U.S. Provisional Application No. 61/164,896, filed March 30, 2009 and U.S. Provisional Application No. 61/299,084, filed January 28, 2010, each of which is incorporated by reference in its entirety herein.

[0002] This invention was made, in part, with United States Government support under award number RC1 AI086061 from the National Institutes of Health (NIH) National Institute of Allergy and Infectious Diseases, award number U54 AI057158 from the NIH, award number HHSN266200700010C from the United States Department of Health and Human Services, and award number U01 AI070469 from the NIH. The United States Government may have certain rights in this invention.

1. INTRODUCTION

[0003] Provided herein are influenza hemagglutinin stem domain polypeptides, 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 viruses are avians, but influenza viruses (including those of avian origin) also can infect and cause illness in humans and other animal hosts (canines, pigs, horses, sea mammals, and mustelids). For example, the H5N1 avian influenza 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] Influenza A and B viruses are major human pathogens, causing a respiratory disease that ranges in severity from sub-clinical infection to primary viral pneumonia which can result in death. The clinical effects of infection vary with the virulence of the

influenza strain and the exposure, history, age, and immune status of the host. The cumulative morbidity and mortality caused by seasonal influenza is substantial due to the relatively high attack rate. In a normal season, influenza can cause between 3-5 million cases of severe illness and up to 500,000 deaths worldwide (World Health Organization (2003) Influenza: Overview; http://www.who.int/mediacentre/factsheets/fs211/en/; March 2003). In the United States, influenza viruses infect an estimated 10-15% of the population (Glezen and Couch RB (1978) Interpandemic influenza in the Houston area, 1974-76. N Engl J Med 298: 587-592; Fox *et al.* (1982) Influenza virus infections in Seattle families, 1975-1979. II. Pattern of infection in invaded households and relation of age and prior antibody to occurrence of infection and related illness. Am J Epidemiol 116: 228-242) and are associated with approximately 30,000 deaths each year (Thompson WW *et al.* (2003) Mortality Associated with Influenza and Respiratory Syncytial Virus in the United States. JAMA 289: 179-186; Belshe (2007) Translational research on vaccines: influenza as an example. Clin Pharmacol Ther 82: 745-749).

[0006] In addition to annual epidemics, influenza viruses are the cause of infrequent pandemics. For example, influenza A viruses can cause pandemics such as those that occurred in 1918, 1957, 1968, and 2009. Due to the lack of pre-formed immunity against the major viral antigen, hemagglutinin (HA), pandemic influenza can affect greater than 50% of the population in a single year and often causes more severe disease than epidemic influenza. A stark example is the pandemic of 1918, in which an estimated 50-100 million people were killed (Johnson and Mueller (2002) Updating the Accounts: Global Mortality of the 1918-1920 "Spanish" Influenza Pandemic Bulletin of the History of Medicine 76: 105-115). Since the emergence of the highly pathogenic avian H5N1 influenza virus in the late 1990s (Claas *et al.* (1998) Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 351: 472-7), there have been concerns that it may be the next pandemic virus.

[0007] An effective way to protect against influenza virus infection is through vaccination; however, current vaccination approaches rely on achieving a good match between circulating strains and the isolates included in the vaccine. Such a match is often difficult to attain due to a combination of factors. First, influenza viruses are constantly undergoing change: every 3-5 years the predominant strain of influenza A virus is replaced by a variant that has undergone sufficient antigenic drift to evade existing antibody responses. Isolates to be included in vaccine preparations must

therefore be selected each year based on the intensive surveillance efforts of the World Health Organization (WHO) collaborating centers. Second, to allow sufficient time for vaccine manufacture and distribution, strains must be selected approximately six months prior to the initiation of the influenza season. Often, the predictions of the vaccine strain selection committee are inaccurate, resulting in a substantial drop in the efficacy of vaccination.

[0008] The possibility of a novel subtype of influenza A virus entering the human population also presents a significant challenge to current vaccination strategies. Since it is impossible to predict what subtype and strain of influenza virus will cause the next pandemic, current, strain-specific approaches cannot be used to prepare a pandemic influenza vaccine.

3. SUMMARY

[0009] In one aspect, provided herein are influenza hemagglutinin stem domain polypeptides. In certain embodiments, the influenza hemagglutinin stem domain polypeptides lack globular head domains as described herein.

[0010] While not intending to be bound by any particular theory of operation, it is believed that the globular head domain of an influenza hemagglutinin comprises one or more highly immunogenic regions. These highly immunogenic regions might generate a host immune response. However, the highly immunogenic regions might also vary from strain to strain of influenza virus. Embodiments presented herein are based on, in part, the discovery that residues in influenza hemagglutinin stem domains are relatively conserved and immunogenic, and that antibodies binding to this region may be neutralizing. An influenza hemagglutinin stem domain polypeptide, lacking all or substantially all of an influenza hemagglutinin globular head domain, may be used to generate an immune response to one or more conserved epitopes of the stem domain polypeptide. Removal of the highly immunogenic regions of the globular head domain might expose one or more epitopes of the stem domain polypeptide to a host immune system. In addition, in certain embodiments, elimination of the glycosylation of the influenza hemagglutinin stem domain through alteration of glycosylation sites present therein may render the conserved regions of the stem domain more accessible to the host immune response.

[0011] If the one or more epitopes of the stem domain polypeptide are less immunogenic than the highly immunogenic regions of a globular head domain, the absence of a globular head domain in the stem domain polypeptide might allow an immune response against the one or more epitopes of the stem domain polypeptide to develop. Advantageously, since the amino acid sequences of influenza hemagglutinin stem domain polypeptides might be conserved or highly conserved across viral subtypes, an immune response against an influenza hemagglutinin stem domain polypeptide provided herein might cross react with one or more viral subtypes other than the subtype corresponding to the stem domain polypeptide. Accordingly, the influenza hemagglutinin stem domain polypeptides provided herein may be useful for immunogenic compositions (*e.g.* vaccines) capable of generating immune responses against a plurality of influenza virus strains.

[0012] Without being bound by any theory, influenza hemagglutinin stem domain polypeptides described herein are based, in part, on the inventors' discovery of polypeptides that lack the globular head domain of influenza hemagglutinin and maintain the stability of the pre-fusion conformation of influenza hemagglutinin. In one aspect, without being bound by theory, the inventors have discovered that the maintenance of cysteine residues identified as A_p and A_q in influenza hemagglutinin polypeptides in FIG. 1 contributes the stability of the stalk region of influenza hemagglutinin. In another aspect, without being bound by theory, the inventors have discovered that influenza hemagglutinin stem domain polypeptides that maintain the pre-fusion conformation of influenza hemagglutinin polypeptides are more effective at inducing a protective effect in subjects. In certain aspects, the stability of the pre-fusion conformation can be conferred by introducing amino acid substitutions at certain residues, such as HA1 H17Y (H3 numbering).

3.1 TERMINOLOGY

[0013] 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.

[0014] 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.

[0015] 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, are 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.

[0016] "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).

[0017] 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.

[0018] 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; (ix) prevent or reduce the spread of an influenza virus from one subject to another subject; (x) reduce organ failure associated with an influenza virus infection; (xi) reduce hospitalization of a subject; (xii) reduce hospitalization length; (xiii) increase the survival of a subject with an influenza virus infection or disease associated therewith; (xiv) eliminate an influenza virus infection or disease associated therewith; (xv) inhibit or reduce influenza virus replication; (xvi) inhibit or reduce the entry of an influenza virus into a host cell(s);

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

[0019] 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.

[0020] "Hemagglutinin" and "HA" refer to any hemagglutinin known to those of skill in the art. 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, 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). In certain embodiments, a hemagglutinin comprises a signal peptide, a transmembrane domain and a cytoplasmic domain. In certain embodiments, a hemagglutinin lacks a signal peptide, *i.e.* the hemagglutinin is a mature hemagglutinin. In certain embodiments, a hemagglutinin lacks a transmembrane domain or cytoplasmic domain, or both. As used herein, the terms "hemagglutinin" and "HA" encompass hemagglutinin polypeptides that are modified 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).

[0021] "HA1 N-terminal stem segment" refers to a polypeptide segment that corresponds to the amino-terminal portion of the stem domain of an influenza hemagglutinin HA1 polypeptide. In certain embodiments, an HA1 N-terminal stem segment consists of amino acid residues corresponding approximately to amino acids $A_{N\text{-}term}$ through A_p of an HA1 domain. $A_{N\text{-}term}$ is the N-terminal amino acid of HA1 as recognized by those of skill in the art. A_p is the cysteine residue in the HA1 N-terminal stem segment that forms or is capable of forming a disulfide bond with a cysteine residue in an HA1 C-terminal stem segment. Residue A_p is identified in influenza A hemagglutinin polypeptides in FIG. 1. Exemplary HA1 N-terminal stem segments are described herein. In certain embodiments, an HA1 N-terminal stem segment consists of amino acid residues corresponding approximately to amino acids 1-52 of HA1 from an H3 hemagglutinin. Note that, in this numbering system, 1 refers to the N-terminal amino acid of the mature HA0 protein, from which the signal peptide has been removed.

[0022] "HA1 C-terminal stem segment" refers to a polypeptide segment that corresponds to the carboxy-terminal portion of the stem domain of an influenza hemagglutinin HA1 polypeptide. In certain embodiments, an HA1 C-terminal stem segment consists of amino acid residues corresponding approximately to amino acids A_q through $A_{C\text{-}term}$ of an HA1 domain. A_q is the cysteine residue in the HA1 C-terminal stem segment that forms or is capable of forming a disulfide bond with a cysteine residue in an HA1 N-terminal stem segment. $A_{C\text{-}term}$ is the C-terminal amino acid of the

HA1 domain as recognized by those of skill in the art. Residue A_q is identified in influenza A hemagglutinin polypeptides in FIG. 1. Exemplary HA1 C-terminal stem segments are described herein. In certain embodiments, an HA1 C-terminal stem segment consists of amino acid residues corresponding approximately to amino acids 277-346 of HA1 from an H3 hemagglutinin. Note that, in this numbering system, 1 refers to the N-terminal amino acid of the mature HA0 protein, from which the signal peptide has been removed.

[0023] "HA2" refers to a polypeptide domain that corresponds to the HA2 domain of an influenza hemagglutinin polypeptide known to those of skill in the art. In certain embodiments, an HA2 consists of a stem domain, a luminal domain, a transmembrane domain and a cytoplasmic domain (*see, e.g.,* Scheiffle *et al.,* 2007, *EMBO J.* 16(18):5501-5508, the contents of which are incorporated by reference in their entirety). In certain embodiments, an HA2 consists of a stem domain, a luminal domain and a transmembrane domain. In certain embodiments, an HA2 consists of a stem domain and a luminal domain; in such embodiments, the HA2 might be soluble. In certain embodiments, an HA2 consists of a stem domain; in such embodiments, the HA2 might be soluble.

[0024] 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.

[0025] 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.

[0026] 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.

[0027] 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.

[0028] As used herein, the phrases "IFN deficient system" or "IFN-deficient substrate" refer to systems, *e.g.*, cells, cell lines and animals, such as pigs, mice, chickens, turkeys, rabbits, rats, etc., which do not produce IFN or produce low levels of IFN (*i.e.*, a reduction in IFN expression of 5-10%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90% or more when compared to IFN-competent systems under the same conditions), do not respond or respond less efficiently to IFN, and/or are deficient in the activity of one or more antiviral genes induced by IFN.

[0029] As used herein, the numeric term "log" refers to $\log_{10}$.

[0030] 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).

[0031] As used herein, the term "nucleic acid" is intended to include DNA molecules (*e.g.*, cDNA or genomic DNA) and RNA molecules (*e.g.*, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid can be single-stranded or double-stranded.

[0032] "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).

[0033] 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 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.

[0034] As used herein, the terms "purified" and "isolated" when used in the context of a polypeptide (including 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 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 hemagglutinin stem domain polypeptide is chemically synthesized. In another specific embodiment, an influenza hemagglutinin stem domain polypeptide is isolated.

[0035] 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.

[0036] "Stem domain polypeptide" refers 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 certain embodiments, a stem domain polypeptide is derived from an influenza hemagglutinin. Engineered stem domain polypeptides can comprise one or more linkers as described below.

[0037] 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. In another embodiment, a subject is a human infant. In another embodiment, a subject is a human child. In another embodiment, a subject is a human adult. In another embodiment, a subject is an elderly human. In another embodiment, a subject is a premature human infant.

[0038] As used herein, the term "premature human infant" refers to a human infant born at less than 37 weeks of gestational age.

[0039] As used herein, the term "human infant" refers to a newborn to 1 year old human.

[0040] As used herein, the term "human child" refers to a human that is 1 year to 18 years old.

[0041] As used herein, the term "human adult" refers to a human that is 18 years or older.

[0042] As used herein, the term "elderly human" refers to a human 65 years or older.

[0043] 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.

[0044] 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 a nucleic acid encoding an influenza virus hemagglutinin stem domain polypeptide, an influenza virus hemagglutinin stem domain polypeptide, or a vector or composition comprising said nucleic acid encoding an influenza virus hemagglutinin stem domain polypeptide or an influenza hemagglutinin stem domain polypeptide. In some embodiments, the term "therapy" refers to an antibody that specifically binds to an influenza virus hemagglutinin polypeptide or an influenza virus hemagglutinin stem domain polypeptide.

[0045] As used herein, the terms "treat," "treatment," and "treating" refer in the context of administration of a therapy(ies) to a subject to treating an influenza virus disease 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.

[0046] 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. In other embodiments, the term "wild-type" in the context of a virus refers to a parental virus.

4. BRIEF DESCRIPTION OF THE DRAWINGS

[0047] **Fig. 1** presents a sequence alignment by CLUSTALW of representative sequences of 16 subtypes of influenza virus A hemagglutinin (SEQ ID NOS:1-16, respectively).

[0048] **Fig. 2** presents a sequence alignment by CLUSTALW of a representative sequence of influenza virus B hemagglutinin (SEQ ID NO:17) aligned with influenza A HK68-H3N2 (SEQ ID NO:3) and PR8-H1N1 (SEQ ID NO:1) hemagglutinins.

[0049] **Fig. 3** provides exemplary nucleotide constructs encoding wild type HA and influenza HA stem domain polypeptides.

[0050] **Fig. 4** provides the putative structure of an influenza HA stem domain polypeptide.

[0051] **Figs. 5A and 5B** provide protein expression of exemplary influenza HA stem domain polypeptides.

[0052] **Fig. 6** provides an exemplary construct for expressing an influenza HA stem domain polypeptide with nucleotide (SEQ ID NO:169) and amino acid (SEQ ID NO:170) sequences. The glycine linker is underlined.

[0053] **Fig. 7** provides an exemplary construct for expressing an influenza HA stem domain polypeptide with nucleotide (SEQ ID NO:171) and amino acid (SEQ ID NO:172) sequences. The glycine linker is underlined.

[0054] **Fig. 8** provides an exemplary construct for expressing an influenza HA stem domain polypeptide with nucleotide (SEQ ID NO:173) and amino acid (SEQ ID NO:174) sequences. The proline-glycine linker is underlined.

[0055] **Fig. 9** provides an exemplary construct for expressing an influenza HA stem domain polypeptides with nucleotide (SEQ ID NO:175) and amino acid (SEQ ID NO:176) sequences. The glycine linker, thrombin cleavage site, foldon domain and HIS tag are underlined.

[0056] **Figs. 10A-10B.** Schematic of headless HA constructs. (A) Schematic of the linear structure of the full length influenza virus HA protein (top) and a generalized headless HA protein (bottom). Linker peptides tested in the context of the PR8 and HK68 HA sequences are shown. Inserted amino acids are shown in bold face font, while amino acids present in the native HA sequence are in regular font. (B) Schematic of the folded structures of the full length and headless HAs of PR8 virus (left panel) and HK68 virus (right panel). In both cases headless HAs carrying the 4G linker bridge are depicted. The HA1 subunit is colored dark grey and the HA2 subunit is light grey. The location of 4G linker sequences is indicated with an arrow in each panel. The full length HA structures were downloaded from the Protein Database (PDB): PR8 HA, PDB ID 1rvx and HK68 HA, PDB ID 1mgn. Schematics of headless HAs were generated using the full length HA coordinates as a starting point and 4G loops were manually docked into the headless HA carbon to close the discontinuous alpha carbon amino acid chain. Final images were generated by PyMol (Delano Scientific).

[0057] **Figs. 11A -11B.** Expression of headless HA constructs in transiently transfected cells. Headless HA constructs were expressed in 293T cells by plasmid transfection in the absence of exogenous trypsin. At 24 hours post-transfection, whole cell lysates were prepared and subjected to SDS-PAGE followed by Western blotting. HA proteins were detected using the polyclonal 3951 antiserum (for PR8) or the monoclonal 12D1 (for HK68). Molecular weight markers in kDa are shown to the left of each blot and transfected constructs are identified above the appropriate lane. "Mock" indicates untransfected cells; "Full" indicates the full length HA protein; for the headless HA constructs, the amino acid sequence bridging the N and C terminal strands of HA1 is shown. Letters in bold font indicate inserted amino acids, while letters in regular font represent residues present in the wild-type HA. In the region of the cys52 to cys277 disulfide bond, the wild-type sequences are as follows. PR8: K50L51C52…C277N278T279K280. HK68: K50I51C52…C277I278S279E280. PR8

based constructs are shown in panel (A) and HK68 based constructs are shown in panel (B). In (B) the molecular weight of the full length HK68 HA0 protein is indicated by an arrowhead.

[0058] **Figs. 12A-12B.** Detection of headless HA proteins on the surface of transfected cells. Full length and headless HA constructs were expressed in 293T cells by plasmid transfection. At 24 hours post-transfection, cells were trypsinized and HA proteins on the cell surface were stained using the polyclonal 3951 antiserum (for PR8) or the monoclonal 12D1 (for HK68) prior to analysis by flow cytometry. (A) Mock transfected cells stained with 3951 immune sera are compared to cells transfected with pDZ PR8 HA or cells transfected with pCAGGS PR8 2G, 4G or PG headless HA constructs. (B) Mock transfected cells stained with mAb 12D1 are compared to cells transfected with pCAGGS HK68 HA or cells transfected with pCAGGS HK68 2G, 4G or PG headless HA constructs.

[0059] **Figs. 13A-13B.** Incorporation of headless HA proteins into virus-like particles. The HA content of VLPs generated by co-transfection of HA constructs with pGagEGFP was assessed by Western blotting. (A) PR8 based VLPs were probed with the polyclonal 3951 antiserum. (B) HK68 based proteins were detected with the monoclonal 12D1. Bands are identified to the right of each blot. Note that VLPs were produced in the presence of exogenous trypsin resulting in the cleavage of HA0 to produce HA1 (not visualized here) and HA2. Ramps above the lanes indicate a 1/3 dilution of the sample: for each VLP, the left lane shows VLPs harvested from the equivalent of three 10 cm dishes of 293T cells, while the right lane shows VLPs harvested from one 10 cm dish.

[0060] **Fig. 14.** Vaccination of mice with headless HA constructs provides protection from death. The average body weight loss in each group of vaccinated mice following challenge with PR8 virus is shown. Error bars represent standard deviation. * indicates the death of a mouse.

[0061] **Figs. 15A-15F.** Anti-sera from mice vaccinated with the PR8 4G headless HA shows broad cross-reactivity by ELISA. The vaccine groups from which sera are derived are identified at the top of each column and the ELISA substrate used is indicated to the right of each row. Sera from vaccinated mice are shown in black with filled symbols. Each mouse is represented by a unique symbol which is the same in each panel. A rabbit anti-serum raised against whole PR8 virus is shown in grey with open triangles and a serum sample taken from a naïve mouse is shown in grey with open

squares. Reactivity of mouse sera to (A) whole PR8 virus, (B) purified recombinant A/New Caledonia/20/1999 HA protein, (C) purified recombinant A/California/04/2009 HA, (D) purified recombinant A/Singapore/1/1957 HA, (E) purified recombinant A/Viet Nam/1203/2004 HA, and (F) purified recombinant A/Hong Kong/1/1968 HA are shown.

[0062] **Fig. 16A-16B.** present schematic diagrams of representative headless molecules. (A) Headless HA construct based on the A/Hong Kong/68 hemagglutinin protein with the linker bridge positioned between amino acids 52 and 277 of the HA1 domain. (B) Headless HA construct based on the A/PR/8/34 hemagglutinin protein, with the linker bridge positioned between amino acids 46 and 276 of the HA1 domain.

[0063] **Fig. 17A-17B.** present schematic diagrams of the primary protein sequences of representative headless molecules. (A) Headless HA construct based on the A/Hong Kong/68 hemagglutinin protein with the linker bridge positioned between amino acids 52 and 277 of the HA1 domain. (B) Headless HA construct based on the A/PR/8/34 hemagglutinin protein, with the linker bridge positioned between amino acids 46 and 276 of the HA1 domain.

5. DETAILED DESCRIPTION

5.1 POLYPEPTIDES

[0064] Provided herein are influenza hemagglutinin stem domain polypeptides. While not intending to be bound by any particular theory of operation, it is believed that the influenza hemagglutinin stem domain polypeptides are useful for presenting one or more relatively conserved antigenic regions to a host immune system in order to generate an immune response that is capable of cross-reacting with a plurality of influenza strains. Since the one or more antigenic regions are well conserved across influenza hemagglutinin subtypes, such an immune response might cross-react with several subtypes of full-length influenza hemagglutinin polypeptides.

[0065] It is believed that full-length influenza hemagglutinin presents several highly antigenic segments in its globular head domain. These highly antigenic segments might be more accessible to a host immune system or more immunogenic in structure, or both. It is believed that a host immune system responds preferentially to these highly immunogenic segments compared to one or more epitopes in the stem domain of an influenza hemagglutinin. Further, since a globular head domain of an influenza hemagglutinin might be variable across subtypes and viral strains, an immune response

against one globular head domain subtype might be limited to the specific highly antigenic segments of that globular head domain. Strains with different globular head domains might not cross react with the same immune response. As such, the effectiveness of vaccines presenting hemagglutinin polypeptides might be limited to the specific strains presented in the vaccine. Hence, a given conventional influenza vaccine is likely only effective against the influenza strains predicted to be virulent during a given flu season.

[0066] Advantageously, influenza hemagglutinin stem domain polypeptides provided herein might be useful to generate an immune response against multiple influenza strains. The influenza hemagglutinin stem domain polypeptides generally do not comprise the highly antigenic, variable globular head domains of conventional influenza vaccine polypeptides. Thus, they should not generate immune responses limited to the variable segments of the globular head domains. Instead, they present one or more epitopes in the relatively conserved stem domain of influenza hemagglutinin. As such, they might be used to generate a host immune response against multiple influenza strains that carry the relatively conserved epitopes. Accordingly, the influenza hemagglutinin stem domain polypeptides find use as antigens in the compositions, vaccines and methods described in detail below. The influenza hemagglutinin stem domain polypeptides might be useful for generating a host immune response against any one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen or sixteen known influenza A hemagglutinin subtypes or a later identified influenza A hemagglutinin subtype. The influenza hemagglutinin stem domain polypeptides might also be useful for generating a host immune response against any influenza B hemagglutinin subtype now known or later identified.

[0067] Generally, the influenza hemagglutinin stem domain polypeptides provided herein are polypeptides that comprise or consist essentially of the stem domain of an influenza hemagglutinin polypeptide. The stem domain of an influenza hemagglutinin polypeptide is the stem domain that is generally recognized by those of skill in the art.

[0068] As is known to those of skill in the art, a full-length influenza hemagglutinin typically comprises an HA1 domain and an HA2 domain. The stem domain is formed by two segments of the HA1 domain and most or all of the HA2 domain. The two segments of the HA1 domain are separated, in primary sequence, by a globular head domain.

[0069] In certain embodiments, influenza hemagglutinin stem domain polypeptides comprise little or no globular head domain of an influenza hemagglutinin polypeptide. In certain embodiments, an influenza hemagglutinin stem domain polypeptides is an influenza hemagglutinin that has had its globular head domain deleted by any technique deemed suitable by one of skill in the art.

[0070] In certain embodiments, influenza hemagglutinin stem domain polypeptides described herein maintain the cysteine residues identified in influenza hemagglutinin polypeptides as A_p and A_q in FIG. 1. In certain embodiments, influenza hemagglutinin stem domain polypeptides described herein have greater stability at a pH lower than the hemagglutinin of a wild-type influenza viruse (e.g., a pH less than 5.2, less than 5.1, less than 5.0, or less than 4.9, such as 4.8, 4.7, 4.6, 4.5, 4.4., 4.3, 4.2, 4.1, 4.0, 3.9, 3.8, etc.). In particular embodiments, influenza hemagglutinin stem domain polypeptides described herein undergo conformational changes from the pre-fusion to the fusion conformation at a pH lower than the hemagglutinin of wild-type influenza viruses. In some embodiments, influenza hemagglutinin stem domain polypeptides described herein comprise one or more amino acid substitutions, such as HA1 H17Y (H3 numbering) that increases the stability of the polypeptides at a low pH (e.g., a pH of between 4.9 to 5.2, 4.5 to 3.5, 3.5 to 2.5, 2.5 to 1.5, 1.5 to 0.5). The stability of influenza hemagglutinin stem domain polypeptides can be assessed using techniques known in the art, such as sensitivity of the hemagglutininmolecules to trypsin digestion, as described in, e.g., Thoennes et al., 2008, Virology 370: 403-414.

[0071] The influenza hemagglutinin stem domain polypeptides can be prepared according to any technique deemed suitable to one of skill, including techniques described below. In certain embodiments, the stem domain polypeptides are isolated.

[0072] The typical primary structure of an influenza hemagglutinin stem domain polypeptide provided herein comprises, in the following order, an HA1 N-terminal stem segment, a linker, an HA1 C-terminal stem segment and an HA2. The primary sequence might be formed by a single polypeptide, or it might be formed by multiple polypeptides. Typically, a single polypeptide is expressed by any technique deemed suitable by one of skill in the art. In single polypeptide embodiments, the HA1 segments and the HA2 are in tertiary association. As is known to those of skill in the art, a single HA polypeptide might be cleaved, for example by a protease, under appropriate expression conditions to yield two polypeptides in quaternary association. The cleavage

is typically between the HA1 C-terminal stem segment and the HA2. In certain embodiments, provided herein are multiple polypeptide, for example two polypeptide, influenza hemagglutinin stem domains. In multiple polypeptide embodiments, the HA1 segments and HA2 are in quaternary association.

[0073] In certain embodiments, an influenza hemagglutinin stem domain polypeptide provided herein is monomeric. In certain embodiments, an influenza hemagglutinin stem domain polypeptide provided herein is multimeric. In certain embodiments, an influenza hemagglutinin stem domain polypeptide provided herein is trimeric. Those of skill in the art will recognize that native influenza hemagglutinin polypeptides are capable of trimerization *in vivo* and that certain influenza hemagglutinin stem domain polypeptides provided herein are capable of trimerization. In particular embodiments described below, influenza hemagglutinin stem domain polypeptides provided herein comprise trimerization domains to facilitate trimerization.

[0074] In certain embodiments, an influenza hemagglutinin stem domain polypeptide comprises a signal peptide. Typically, the signal peptide is cleaved during or after polypeptide expression and translation to yield a mature influenza hemagglutinin stem domain polypeptide. The signal peptide might be advantageous for expression of the influenza hemagglutinin stem domain polypeptides. In certain embodiments, also provided herein are mature influenza hemagglutinin stem domain polypeptides that lack a signal peptide.

[0075] Influenza hemagglutinin HA2 typically comprises a stem domain, transmembrane domain and a cytoplasmic domain. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides that comprise an HA2 stem domain, an HA2 luminal domain, an HA2 transmembrane domain and an HA2 cytoplasmic domain. Such influenza hemagglutinin stem domain polypeptides might be expressed as membrane-bound antigens. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides that comprise an HA2 stem domain, an HA2 luminal domain, and an HA2 transmembrane domain but lack some or all of the typical cytoplasmic domain. Such influenza hemagglutinin stem domain polypeptides might be expressed as membrane-bound antigens. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides that comprise an HA2 stem domain and an HA2 luminal domain but lack both an HA2 transmembrane domain and an HA2 cytoplasmic domain. Such influenza hemagglutinin stem domain polypeptides might advantageously be expressed as soluble polypeptides. In certain

embodiments, provided herein are influenza hemagglutinin stem domain polypeptides that comprise an HA2 stem domain but lack an HA2 luminal domain, an HA2 transmembrane domain and an HA2 cytoplasmic domain. Such influenza hemagglutinin stem domain polypeptides might advantageously be expressed as soluble polypeptides. In certain embodiments, the influenza hemagglutinin stem domain polypeptides comprise an HA2 stem domain having at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% amino acid sequence identity to an influenza HA2 stem domain known to those of skill in the art. Exemplary known HA2 stem domains from known influenza A and influenza B hemagglutinins are provided in the tables below.

[0076] Also provided herein are influenza hemagglutinin stem domain polypeptides comprising deleted forms of HA2 stem domains wherein up to 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid residues are deleted from either or both termini of the HA2 stem domain. Further provided herein are influenza hemagglutinin stem domain polypeptides comprising altered forms of HA2 stem domains wherein up to 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid residues are conservatively substituted with other amino acids. Further provided are influenza hemagglutinin stem domain polypeptides comprising deleted and altered HA2 stem domains.

[0077] The HA1 N-terminal stem segment might be any HA1 N-terminal stem segment recognized by one of skill in the art based on the definition provided herein. Typically, an HA1 N-terminal stem segment corresponds to a polypeptide consisting of the N-terminal amino acid of a mature HA1 (*i.e.* an HA1 lacking a signal peptide) through the cysteine residue located in sequence at approximately the 52^{nd} residue of the HA1. This cysteine residue, termed A_p herein, is generally capable of forming a disulfide bridge with a cysteine residue in the C-terminal stem segment of HA1. Sequences of 16 representative influenza A hemagglutinins are presented in FIG. 1, and residue A_p is identified in each.

[0078] In certain embodiments, the HA1 N-terminal stem segment does not end exactly at A_p (*e.g.*, Cys_{52} of an HA1 subunit from an H3 hemagglutinin), but at a residue in sequence and structure vicinity to A_p. For example, in certain embodiments, the HA1 N-terminal stem segment ends at A_{p-1}, A_{p-2}, A_{p-3}, or A_{p-4}. In other embodiments, the HA1 N-terminal stem segment ends at A_{p+1}, A_{p+2}, A_{p+3}, A_{p+4} or A_{p+5}. The end of an HA1 N-terminal stem segment should be selected in conjunction with the end of the HA1 C-terminal stem segment and the linker so that the resulting linked HA1 stem

domain is capable of forming a three-dimensional structure similar, as described below, to an influenza hemagglutinin stem domain.

[0079] In certain embodiments, the influenza hemagglutinin stem domain polypeptides comprise an HA1 N-terminal stem segment having at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% amino acid sequence identity to an influenza HA1 N-terminal stem segment known to those of skill in the art. Exemplary known HA1 N-terminal stem segments are provided in the tables below.

[0080] Also provided herein are influenza hemagglutinin stem domain polypeptides comprising deleted forms of HA1 N-terminal stem segments wherein up to 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid residues are deleted from either or both termini of the HA1 N-terminal stem segment. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides that comprise expanded forms of HA1 N-terminal stem segments wherein 1, 2 or 3 residues are added to the C-terminus of the HA1 N-terminal stem segments; these added residues might be derived from the amino acid sequence of a globular head domain adjacent to an HA1 N-terminal stem segment. Further provided herein are influenza hemagglutinin stem domain polypeptides comprising altered forms of HA1 N-terminal stem segments wherein up to 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid residues are conservatively substituted with other amino acids. Further provided are influenza hemagglutinin stem domain polypeptides comprising deleted and altered HA1 N-terminal stem segments.

[0081] The HA1 C-terminal stem segment might be any HA1 C-terminal stem segment recognized by one of skill in the art based on the definition provided herein. Typically, an HA1 C-terminal stem segment corresponds to a polypeptide consisting of the cysteine residue located in sequence at approximately the 277^{th} residue of an HA1 (using H3 numbering) through the C-terminal amino acid of the HA1. This cysteine residue, termed A_q herein, is generally capable of forming a disulfide bridge with cysteine residue A_p in the N-terminal stem segment of HA1. Sequences of 16 representative influenza A hemagglutinins are presented in FIG. 1, and residue A_q is identified in each.

[0082] In certain embodiments, the HA1 C-terminal stem segment does not start at A_q (*e.g.*, Cys_{277} of an HA1 subunit from an H3 hemagglutinin), but at a residue in sequence and structure vicinity to A_q. For example, in certain embodiments, the HA1 C-terminal stem segment starts at A_{q-1}, A_{q-2}, A_{q-3}, or A_{q-4}. In other embodiments, the HA1 C-terminal stem segment starts at A_{q+1}, A_{q+2}, A_{q+3}, A_{q+4} or A_{q+5}. The end of an HA1 N-

terminal stem segment should be selected in conjunction with the start of the HA1 C-terminal stem segment and the linker so that the resulting HA1 stem domain is capable of forming a three-dimensional structure similar, as described below, to an influenza hemagglutinin.

[0083] In certain embodiments, the influenza hemagglutinin stem domain polypeptides comprise an HA1 C-terminal stem segment having at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% amino acid sequence identity to an influenza HA1 C-terminal stem segment known to those of skill in the art. Exemplary known HA1 C-terminal stem segments are provided in the tables below.

[0084] In certain embodiments, the end of the N-terminal stem segment is A_{p-1}, and the start of the C-terminal stem segment is A_{q-1}. In certain embodiments, the end of the N-terminal stem segment is A_{p-2}, and the start of the C-terminal stem segment is A_{q-2}. In certain embodiments, the end of the N-terminal stem segment is A_{p-3}, and the start of the C-terminal stem segment is A_{q-3}. In certain embodiments, the end of the N-terminal stem segment is A_{p-4}, and the start of the C-terminal stem segment is A_{q-4}. In certain embodiments, the end of the N-terminal stem segment is A_{p-5}, and the start of the C-terminal stem segment is A_{q-5}.

[0085] In certain embodiments, the end of the N-terminal stem segment is A_{p+1}, and the start of the C-terminal stem segment is A_{q+1}. In certain embodiments, the end of the N-terminal stem segment is A_{p+2}, and the start of the C-terminal stem segment is A_{q+2}. In certain embodiments, the end of the N-terminal stem segment is A_{p+3}, and the start of the C-terminal stem segment is A_{q+3}. In certain embodiments, the end of the N-terminal stem segment is A_{p+4}, and the start of the C-terminal stem segment is A_{q+4}. In certain embodiments, the end of the N-terminal stem segment is A_{p+5}, and the start of the C-terminal stem segment is A_{q+5}.

[0086] In certain embodiments, the end of the N-terminal stem segment is A_{p-1}, and the start of the C-terminal stem segment is A_{q+1}. In certain embodiments, the end of the N-terminal stem segment is A_{p-2}, and the start of the C-terminal stem segment is A_{q+2}. In certain embodiments, the end of the N-terminal stem segment is A_{p-3}, and the start of the C-terminal stem segment is A_{q+3}. In certain embodiments, the end of the N-terminal stem segment is A_{p-4}, and the start of the C-terminal stem segment is A_{q+4}. In certain embodiments, the end of the N-terminal stem segment is A_{p-5}, and the start of the C-terminal stem segment is A_{q+5}.

[0087] In certain embodiments, the end of the N-terminal stem segment is A_p (i.e., the end of the N-terminal stem segment is Cysteine), and the start of the C-terminal stem segment is A_q (i.e., the start of the C-terminal stem segment is Cysteine). In certain embodiments, the end of the N-terminal stem segment is A_{p+1}, and the start of the C-terminal stem segment is A_{q-1}. In certain embodiments, the end of the N-terminal stem segment is A_{p+2}, and the start of the C-terminal stem segment is A_{q-2}. In certain embodiments, the end of the N-terminal stem segment is A_{p+3}, and the start of the C-terminal stem segment is A_{q-3}. In certain embodiments, the end of the N-terminal stem segment is A_{p+4}, and the start of the C-terminal stem segment is A_{q-4}. In certain embodiments, the end of the N-terminal stem segment is A_{p+5}, and the start of the C-terminal stem segment is A_{q-5}.

[0088] Also provided herein are influenza hemagglutinin stem domain polypeptides comprising deleted forms of HA1 C-terminal stem segments wherein up to 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid residues are deleted from either or both termini of the HA1 C-terminal stem segment. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides that comprise expanded forms of HA1 C-terminal stem segments wherein 1, 2 or 3 residues are added to the N-terminus of the HA1 C-terminal stem segments; these added residues might be derived from the amino acid sequence of a globular head domain adjacent to an HA1 C-terminal stem segment. In particular embodiments, if one residue is added to the C-terminal stem segment, then one residue is added to the N-terminal stem segment; if two residues are added to the C-terminal stem segment, then two residues are added to the N-terminal stem segment; if three residues are added to the C-terminal stem segment, then three residues are added to the N-terminal stem segment. Further provided herein are influenza hemagglutinin stem domain polypeptides comprising altered forms of HA1 C-terminal stem segments wherein up to 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid residues are conservatively substituted with other amino acids. Further provided are influenza hemagglutinin stem domain polypeptides comprising deleted and altered HA1 C-terminal stem segments.

[0089] The influenza hemagglutinin stem domain polypeptides might be based on (*i.e.* might have sequence identity, as described above) any influenza hemagglutinin known to those of skill or later discovered. In certain embodiments, influenza hemagglutinin stem domain polypeptides are based on an influenza A hemagglutinin. In certain embodiments, the influenza hemagglutinin stem domain polypeptides are based on an influenza A hemagglutinin selected from the group consisting of H1, H2, H3, H4,

H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16. In certain embodiments, influenza hemagglutinin stem domain polypeptides are based on an influenza B hemagglutinin, as described in detail below.

[0090] The HA1 N-terminal stem segments might be based on (*i.e.* might have sequence identity, as described above) any HA1 N-terminal stem segments known to those of skill or later discovered. In certain embodiments, the HA1 N-terminal stem segments are based on influenza A HA1 N-terminal stem segments. In certain embodiments, the HA1 N-terminal stem segments are based on an influenza A hemagglutinin selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16. In certain embodiments, the HA1 N-terminal stem segment is selected from SEQ ID NOS:34-49. In certain embodiments, the HA1 N-terminal stem segment is selected from SEQ ID NOS:34-49, each having one amino acid deleted from its C-terminus. In certain embodiments, the HA1 N-terminal stem segment is selected from SEQ ID NOS:34-49, each having two amino acids deleted from its C-terminus. In certain embodiments, the HA1 N-terminal stem segment is selected from SEQ ID NOS:34-49, each having three amino acids deleted from its C-terminus. In certain embodiments, the HA1 N-terminal stem segment is selected from SEQ ID NOS:34-49, each having four amino acids deleted from its C-terminus. In certain embodiments, the HA1 N-terminal stem segment is selected from SEQ ID NOS:34-49, each having five amino acids deleted from its C-terminus. In certain embodiments, the HA1 N-terminal stem segment is selected from SEQ ID NOS:177-224.

[0091] The HA1 C-terminal stem segments might be based on (*i.e.* might have sequence identity, as described above) any HA1 C-terminal stem segments known to those of skill or later discovered. In certain embodiments, the HA1 C-terminal stem segments are based on influenza A HA1 C-terminal stem segments. In certain embodiments, the HA1 C-terminal stem segments are based on an influenza A hemagglutinin selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16. In certain embodiments, the HA1 C-terminal stem segment is selected from SEQ ID NOS:50-65. In certain embodiments, the HA1 C-terminal stem segment is selected from SEQ ID NOS: 50-65, each having one amino acid deleted from its N-terminus. In certain embodiments, the HA1 C-terminal stem segment is selected from SEQ ID NOS: 50-65, each having two amino acids deleted from its N-terminus. In certain embodiments, the HA1 C-terminal stem

segment is selected from SEQ ID NOS: 50-65, each having three amino acids deleted from its N-terminus. In certain embodiments, the HA1 C-terminal stem segment is selected from SEQ ID NOS: 50-65, each having four amino acids deleted from its N-terminus. In certain embodiments, the HA1 C-terminal stem segment is selected from SEQ ID NOS: 50-65, each having five amino acids deleted from its N-terminus. In certain embodiments, the HA1 C-terminal stem segment is selected from SEQ ID NOS:226-273.

[0092] The HA2 stem domains might be based on (*i.e.* might have sequence identity, as described above) any HA2 stem domains known to those of skill or later discovered. In certain embodiments, the HA2 stem domains are based on influenza A HA2 stem domains. In certain embodiments, the HA2 stem domains are based on an influenza A hemagglutinin selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16. In certain embodiments, the HA2 stem domain is selected from SEQ ID NOS:66-97.

[0093] In embodiments comprising a signal peptide, the signal peptide might be based on any influenza virus signal peptide known to those of skill in the art. In certain embodiments, the signal peptides are based on influenza A signal peptides. In certain embodiments, the signal peptides are based on an influenza A hemagglutinin selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16. In certain embodiments, the signal peptide might be any signal peptide deemed useful to one of skill in the art. In certain embodiments, the signal peptide is selected from SEQ ID NOS:18-33.

[0094] In embodiments comprising a luminal domain, the luminal domain might be based on any influenza luminal domain known to those of skill in the art. In certain embodiments, the luminal domains are based on influenza A luminal domains. In certain embodiments, the HA2 luminal domains are based on an influenza A hemagglutinin selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16. In certain embodiments, the luminal domain might be any luminal domain deemed useful to one of skill in the art. In certain embodiments, the luminal domain is selected from SEQ ID NOS:98-113.

[0095] In embodiments comprising a transmembrane domain, the transmembrane domain might be based on any influenza transmembrane domain known to those of skill in the art. In certain embodiments, the transmembrane domains are based on influenza A transmembrane domains. In certain embodiments, the HA2 transmembrane domains

are based on an influenza A hemagglutinin selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16. In certain embodiments, the transmembrane domain might be any transmembrane domain deemed useful to one of skill in the art. In certain embodiments, the transmembrane domain is selected from SEQ ID NOS:114-129.

[0096] In embodiments comprising a cytoplasmic domain, the cytoplasmic domain might be based on any influenza cytoplasmic domain known to those of skill in the art. In certain embodiments, the cytoplasmic domains are based on influenza A cytoplasmic domains. In certain embodiments, the HA2 cytoplasmic domains are based on an influenza A hemagglutinin selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16. In certain embodiments, the cytoplasmic domain might be any cytoplasmic domain deemed useful to one of skill in the art. In certain embodiments, the cytoplasmic domain is selected from SEQ ID NOS:130-145.

[0097] In certain embodiments, one or more of the glycosylation sites in the hemagglutinin stem domain are altered or deleted such that glycosylation at these sites will not occur during processing and maturation of the polypeptide. Those of skill in the art will recognize that influenza HA typically comprises one or more glycosylation sequences (*e.g.* Asn-Xaa-Ser/Thr/Cys, wherein Xaa is any amino acid other than Pro). In certain embodiments, one or more amino acid residues in a glycosylation sequence is conservatively substituted with an amino acid residue that disrupts the glycosylation sequence. In certain embodiments, one or more amino acid residues in a glycosylation sequence is substituted with any amino acid residue that disrupts the glycosylation sequence. In certain embodiments, one or more asparagine residues in a glycosylation sequence is substituted with alanine. In a particular embodiment, the asparagine at position 38 of an H3 hemagglutinin is changed to an alanine.

[0098] Table 1, below, identifies signal peptides, HA1 N-terminal stem segments, HA1 C-terminal stem segments and HA2 domains of influenza A hemagglutinin polypeptides. These signal peptides, stem segments and domains are useful in the polypeptides and methods described herein.

TABLE 1. Exemplary Influenza A Hemagglutinin Sequences

HA Subtype (Genbank No.)	Signal peptide	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment	HA2 Domain

HA Subtype (Genbank No.)	Signal peptide	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment	HA2 Domain
H1 PR8-H1N1 (EF467821.1)	MKAN LLVLL CALAA ADA [SEQ ID NO.:18]	DTICIGYHANN STDTVDTVLE KNVTVTHSVN LLEDSHNGKL C [SEQ ID NO.:34]	CNTKCQTPLG AINSSLPYQNI HPVTIGECPKY VRSAKLRMVT GLRNNPSIQSR [SEQ ID NO.:50]	GLFGAIAGFIEGGW TGMIDGWYGYHHQ NEQGSGYAADQKST QNAINGITNKVNTVI EKMNIQFTAVGKEF NKLEKRMENLNKK VDDGFLDIWTYNAE LLVLLENERTLDFH DSNVKNLYEKVKSQ LKNNAKEIGNGCFE FYHKCDNECMESVR NGTYDYPKYSEESK LNREKVDGVKLES MGIYQILAIYSTVAS SLVLLVSLGAISFW MCSNGSLQCRICI [SEQ ID NO.:66]
H2 (L11136)	MAIIY LILLFT AVRG [SEQ ID NO.:19]	DQICIGYHSNN STEKVDTILER NVTVTHAQNI LEKTHNGKLC [SEQ ID NO.:35]	CETKCQTPLG AINTTLPFHNV HPLTIGECPKY VKSERLVLAT GLRNVPQIESR [SEQ ID NO.:51]	GLFGAIAGFIEGGW QGMIDGWYGYHHS NDQGSGYAADKEST QKAIDGITNRVNSVI EKMNTQFEAVGKEF SNLEKRLENLNKKM EDGFLDVWTYNAE LLVLMENERTLDFH DSNVKNLYDRVRM QLRDNAKELGNGCF EFYHKCDDECMNS VKNGTYDYPKYEEE SKLNRNEIKGVKLS NMGVYQILAIYATV AGSLSLAIMIAGISL WMCSNGSLQCRICI [SEQ ID NO.:67]

HA Subtype (Genbank No.)	Signal peptide	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment	HA2 Domain
H3 HK68-H3N2 (EF409245) PDB: 1HGJ	MKTII ALSYIF CLALG [SEQ ID NO.:20]	QDLPGNDNST ATLCLGHHAV PNGTLVKTITD DQIEVTNATEL VQSSSTGKIC [SEQ ID NO.:36]	CISECITPNGSI PNDKPFQNVN KITYGACPKY VKQNTLKLAT GMRNVPEKQT R [SEQ ID NO.52]	GLFGAIAGFIENGW EGMIDGWYGFRHQ NSEGTGQAADLKST QAAIDQINGKLNRVI EKTNEKFHQIEKEFS EVEGRIQDLEKYVE DTKIDLWSYNAELL VALENQHTIDLTDS EMNKLFEKTRRQLR ENAEDMGNGCFKIY HKCDNACIESIRNGT YDHDVYRDEALNN RFQIKGVELKSGYK DWILWISFAISCFLL CVVLLGFIMWACQR GNIRCNICI [SEQ ID NO.:68]
H4 (D90302)	MLSIVI LFLLIA ENSS [SEQ ID NO.:21]	QNYTGNPVIC MGHHAVANG TMVKTLADDQ VEVVTAQELV ESQNLPELC [SEQ ID NO.:37]	CVSKCHTDKG SLSTTKPFQNI SRIAVGDCPRY VKQGSLKLAT GMRNIPEKAS R [SEQ ID NO.:53]	GLFGAIAGFIENGW QGLIDGWYGFRHQ NAEGTGTAADLKST QAAIDQINGKLNRLI EKTNDKYHQIEKEF EQVEGRIQDLENYV EDTKIDLWSYNAEL LVALENQHTIDVTD SEMNKLFERVRRQL RENAEDKGNGCFEI FHKCDNNCIESIRNG TYDHDIYRDEAINN RFQIQGVKLTQGYK DIILWISFSISCFLLV ALLLAFILWACQNG NIRCQICI [SEQ ID NO.:69]

HA Subtype (Genbank No.)	Signal peptide	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment	HA2 Domain
H5 (X07826)	MERIV LLLAI VSLVK S [SEQ ID NO.:22]	DQICIGYHAN KSTKQVDTIM EKNVTVTHAQ DILERTHNGKL C [SEQ ID NO.:38]	CDTKCQTPVG EINSSMPFHNI HPHTIGECPKY VKSDRLVLAT GLRNVPQRKK R [SEQ ID NO.:54]	GLFGAIAGFIEGGW QGMVDGWYGYHH SNEQGSGYAADKES TQKAIDGITNKVNSI IDKMNTRFEAVGKE FNNLERRVENLNKK MEDGFLDVWTYNV ELLVLMENERTLDF HDSNVNNLYDKVR LQLKDNARELGNGC FEFYHKCDNECMES VRNGTYDYPQYSEE ARLNREEISGVKLES MGVYQILSIYSTVAS SLALAIMIAGLSFW MCSNGSLQCRICI [SEQ ID NO.:70]
H6 (D90303)	MIAIIV VAILA TAGRS [SEQ ID NO.:23]	DKICIGYHAN NSTTQIDTILE KNVTVTHSVE LLENQKEERF C [SEQ ID NO.:39]	CDATCQTVAG VLRTNKTFQN VSPLWIGECPK YVKSESLRLA TGLRNVPQIET R [SEQ ID NO.:55]	GLFGAIAGFIEGGW TGMIDGWYGYHHE NSQGSGYAADREST QKAVDGITNKVNSII DKMNTQFEAVDHE FSNLERRIDNLNKR MEDGFLDVWTYNA ELLVLLENERTLDL HDANVKNLYERVK SQLRDNAMILGNGC FEFWHKCDDECMES VKNGTYDYPKYQD ESKLNRQEIESVKLE SLGVYQILAIYSTVS SSLVLVGLIIAVGLW MCSNGSMQCRICI [SEQ ID NO.:71]

HA Subtype (Genbank No.)	Signal peptide	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment	HA2 Domain
H7 (M24457)	MNTQI LVFAL VAVIP TNA [SEQ ID NO.:24]	DKICLGHHAV SNGTKVNTLT ERGVEVVNAT ETVERTNIPKI C [SEQ ID NO.:40]	CEGECYHSGG TITSRLPFQNIN SRAVGKCPRY VKQESLLLAT GMKNVPEPSK KRKKR [SEQ ID NO.:56]	GLFGAIAGFIENGW EGLVDGWYGFRHQ NAQGEGTAADYKS TQSAIDQITGKLNRL IEKTNQQFELIDNEF TEVEKQIGNLINWT KDSITEVWSYNAELI VAMENQHTIDLADS EMNRLYERVRKQL RENAEEDGTGCFEIF HKCDDDCMASIRNN TYDHSKYREEAMQ NRIQIDPVKLSSGYK DVILWFSFGASCFLL LAIAMGLVFICVKN GNMRCTICI [SEQ ID NO.:72]
H8 (D90304)	MEKFI AIATL ASTNA Y [SEQ ID NO.:25]	DRICIGYQSNN STDTVNTLIEQ NVPVTQTMEL VETEKHPAYC [SEQ ID NO.:41]	CNTKCQTYAG AINSSKPFQNA SRHYMGECPK YVKKASLRLA VGLRNTPSVEP R [SEQ ID NO.:57]	GLFGAIAGFIEGGWS GMIDGWYGFHHSN SEGTGMAADQKST QEAIDKITNKVNNIV DKMNREFEVVNHEF SEVEKRINMINDKID DQIEDLWAYNAELL VLLENQKTLDEHDS NVKNLFDEVKRRLS ANAIDAGNGCFDIL HKCDNECMETIKNG TYDHKEYEEEAKLE RSKINGVKLEENTT YKILSIYSTVAASLC LAILIAGGLILGMQN GSCRCMFCI [SEQ ID NO.:73]

HA Subtype (Genbank No.)	Signal peptide	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment	HA2 Domain
H9 (D90305)	METK AIIAAL LMVTA ANA [SEQ ID NO.:26]	DKICIGYQSTN STETVDTLTES NVPVTHTKEL LHTEHNGMLC [SEQ ID NO.:42]	CVVQCQTEKG GLNTTLPFHNI SKYAFGNCPK YVGVKSLKLP VGLRNVPAVS SR [SEQ ID NO.:58]	GLFGAIAGFIEGGWP GLVAGWYGFQHSN DQGVGMAADKGST QKAIDKITSKVNNII DKMNKQYEVIDHEF NELEARLNMINNKI DDQIQDIWAYNAEL LVLLENQKTLDEHD ANVNNLYNKVKRA LGSNAVEDGNGCFE LYHKCDDQCMETIR NGTYDRQKYQEESR LERQKIEGVKLESEG TYKILTIYSTVASSL VLAMGFAAFLFWA MSNGSCRCNICI [SEQ ID NO.:74]
H10 (M21647)	MYKV VVIIAL LGAVK G [SEQ ID NO.:27]	LDRICLGHHA VANGTIVKTL TNEQEEVTNA TETVESTNLN KLC [SEQ ID NO.:43]	CESKCFWRGG SINTKLPFQNL SPRTVGQCPK YVNQRSLLLA TGMRNVPEVV QGR [SEQ ID NO.:59]	GLFGAIAGFIENGW EGMVDGWYGFRHQ NAQGTGQAADYKS TQAAIDQITGKLNRL IEKTNTEFESIESEFS ETEHQIGNVINWTK DSITDIWTYNAELLV AMENQHTIDMADSE MLNLYERVRKQLR QNAEEDGKGCFEIY HTCDDSCMESIRNN TYDHSQYREEALLN RLNINPVKLSSGYK DIILWFSFGESCFVL LAVVMGLVFFCLKN GNMRCTICI [SEQ ID NO.:75]

HA Subtype (Genbank No.)	Signal peptide	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment	HA2 Domain
H11 (D90306)	MEKTL LFAAIF LCVKA [SEQ ID NO.:28]	DEICIGYLSNN STDKVDTIIEN NVTVTSSVEL VETEHTGSFC [SEQ ID NO.:44]	CSTKCQTEIGG INTNKSFHNV HRNTIGDCPK YVNVKSLKLA TGPRNVPAIAS R [SEQ ID NO.:60]	GLFGAIAGFIEGGWP GLINGWYGFQHRDE EGTGIAADKESTQK AIDQITSKVNNIVDR MNTNFESVQHEFSEI EERINQLSKHVDDS VVDIWSYNAQLLVL LENEKTLDLHDSNV RNLHEKVRRMLKD NAKDEGNGCFTFYH KCDNKCIERVRNGT YDHKEFEEESKINR QEIEGVKLDSSGNV YKILSIYSCIASSLVL AALIMGFMFWACS NGSCRCTICI [SEQ ID NO.:76]
H12 (D90307)	MEKFII LSTVL AASFA Y [SEQ ID NO.:29]	DKICIGYQTNN STETVNTLSEQ NVPVTQVEEL VHRGIDPILC [SEQ ID NO.:45]	CVTECQLNEG VMNTSKPFQN TSKHYIGKCPK YIPSGSLKLAI GLRNVPQVQD R [SEQ ID NO.:61]	GLFGAIAGFIEGGWP GLVAGWYGFQHQN AEGTGIAADRDSTQ RAIDNMQNKLNNVI DKMNKQFEVVNHE FSEVESRINMINSKI DDQITDIWAYNAEL LVLLENQKTLDEHD ANVRNLHDRVRRV LRENAIDTGDGCFEI LHKCDNNCMDTIRN GTYNHKEYEEESKI ERQKVNGVKLEENS TYKILSIYSSVASSL VLLLMIIGGFIFGCQ NGNVRCTFCI [SEQ ID NO.:77]

HA Subtype (Genbank No.)	Signal peptide	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment	HA2 Domain
H13 (D90308)	MALN VIATL TLISVC VHA [SEQ ID NO.:30]	DRICVGYLSTN SSERVDTLLEN GVPVTSSIDLIE TNHTGTYC [SEQ ID NO.:46]	CNTKCQTSVG GINTNRTFQNI DKNALGDCPK YIKSGQLKLAT GLRNVPAISNR [SEQ ID NO.:62]	GLFGAIAGFIEGGWP GLINGWYGFQHQNE QGTGIAADKESTQK AIDQITTKINNIIDKM NGNYDSIRGEFNQV EKRINMLADRIDDA VTDIWSYNAKLLVL LENDKTLDMHDAN VKNLHEQVRRELKD NAIDEGNGCFELLH KCNDSCMETIRNGT YDHTEYAEESKLKR QEIDGIKLKSEDNVY KALSIYSCIASSVVL VGLILSFIMWACSSG NCRFNVCI [SEQ ID NO.:78]
H14 (M35997)	MIALIL VALAL SHTAY S [SEQ ID NO.:31]	QITNGTTGNPII CLGHHAVENG TSVKTLTDNH VEVVSAKELV ETNHTDELC [SEQ ID NO.:47]	CTSPCLTDKGS IQSDKPFQNVS RIAIGNCPKYV KQGSLMLATG MRNIPGKQAK [SEQ ID NO.:63]	GLFGAIAGFIENGW QGLIDGWYGFRHQ NAEGTGTAADLKST QAAIDQINGKLNRLI EKTNEKYHQIEKEF EQVEGRIQDLEKYV EDTKIDLWSYNAEL LVALENQHTIDVTD SEMNKLFERVRRQL RENAEDQGNGCFEI FHQCDNNCIESIRNG TYDHNIYRDEAINN RIKINPVTLTMGYK DIILWISFSMSCFVF VALILGFVLWACQN GNIRCQICI [SEQ ID NO.:79]

HA Subtype (Genbank No.)	Signal peptide	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment	HA2 Domain
H15 (L43917)	MNTQI IVILVL GLSMV KS [SEQ ID NO.:32]	DKICLGHHAV ANGTKVNTLT ERGVEVVNAT ETVEITGIDKV C [SEQ ID NO.:48]	CEGECFYSGG TINSPLPFQNID SRAVGKCPRY VKQSSLPLAL GMKNVPEKIR TR [SEQ ID NO.:64]	GLFGAIAGFIENGW EGLIDGWYGFRHQN AQGQGTAADYKST QAAIDQITGKLNRLI EKTNKQFELIDNEFT EVEQQIGNVINWTR DSLTEIWSYNAELL VAMENQHTIDLADS EMNKLYERVRRQL RENAEEDGTGCFEIF HRCDDQCMESIRNN TYNHTEYRQEALQN RIMINPVKLSSGYKD VILWFSFGASCVML LAIAMGLIFMCVKN GNLRCTICI [SEQ ID NO.:80]
H16 (EU293865)	MMIK VLYFLI IVLGR YSKA [SEQ ID NO.:33]	DKICIGYLSNN SSDTVDTLTEN GVPVTSSVDL VETNHTGTYC [SEQ ID NO.:49]	CNTKCQTSLG GINTNKTFQNI ERNALGDCPK YIKSGQLKLAT GLRNVPSIGER [SEQ ID NO.:65]	GLFGAIAGFIEGGWP GLINGWYGFQHQNE QGTGIAADKASTQK AINEITTKINNIIEKM NGNYDSIRGEFNQV EKRINMLADRVDDA VTDIWSYNAKLLVL LENDRTLDLHDANV RNLHDQVKRALKS NAIDEGDGCFNLLH KCNDSCMETIRNGT YNHEDYREESQLKR QEIEGIKLKTEDNVY KVLSIYSCIASSIVLV GLILAFIMWACSNG SCRFNVCI [SEQ ID NO.:81]

[0099] Table 1A, below, identifies useful HA1 N-terminal stem segments and HA1 C-terminal stem segments for the polypeptides and methods described herein.

TABLE 1A. Exemplary Influenza A Hemagglutinin Sequences

HA Subtype (Genbank No.)	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment
H1 PR8-H1N1 (EF467821.1) No Cys	DTICIGYHANNSTDTVDT VLEKNVTVTHSVNLLED SHNGKL [SEQ ID NO.:177]	NTKCQTPLGAINSSLPYQNIHPVTIGEC PKYVRSAKLRMVTGLRNNPSIQSR [SEQ ID NO.:226]
H1 PR8-H1N1 (EF467821.1) No Cys Δ1	DTICIGYHANNSTDTVDT VLEKNVTVTHSVNLLED SHNGKL [SEQ ID NO.:178]	TKCQTPLGAINSSLPYQNIHPVTIGECP KYVRSAKLRMVTGLRNNPSIQSR [SEQ ID NO.:227]
H1 PR8-H1N1 (EF467821.1) No Cys Δ3	DTICIGYHANNSTDTVDT VLEKNVTVTHSVNLLED SHNGK [SEQ ID NO.:179]	KCQTPLGAINSSLPYQNIHPVTIGECPK YVRSAKLRMVTGLRNNPSIQSR [SEQ ID NO.:228]
H1 PR8-H1N1 (EF467821.1) PR8-CON-A	DTICIGYHANNSTDTVDT VLEKNVTVTHSVNLLED SHNGKLCRLKC [SEQ ID NO.:309]	CKCQTPLGAINSSLPYQNIHPVTIGECP KYVRSAKLRMVTGLRNNPSIQSRG [SEQ ID NO.:310]
H2 (L11136) No Cys	DQICIGYHSNNSTEKVDT ILERNVTVTHAQNILEKT HNGKL [SEQ ID NO.:180]	ETKCQTPLGAINTTLPFHNVHPLTIGE CPKYVKSERLVLATGLRNVPQIESR [SEQ ID NO.:229]
H2 (L11136) No Cys Δ1	DQICIGYHSNNSTEKVDT ILERNVTVTHAQNILEKT HNGKL [SEQ ID NO.:181]	TKCQTPLGAINTTLPFHNVHPLTIGECP KYVKSERLVLATGLRNVPQIESR [SEQ ID NO.:230]
H2 (L11136) No Cys Δ3	DQICIGYHSNNSTEKVDT ILERNVTVTHAQNILEKT HNGK [SEQ ID NO.:182]	KCQTPLGAINTTLPFHNVHPLTIGECP KYVKSERLVLATGLRNVPQIESR [SEQ ID NO.:231]
H3 HK68-H3N2 (EF409245) PDB: 1HGJ No Cys	QDLPGNDNSTATLCLGH HAVPNGTLVKTITDDQIE VTNATELVQSSSTGKI [SEQ ID NO.:183]	ISECITPNGSIPNDKPFQNVNKITYGAC PKYVKQNTLKLATGMRNVPEKQTR [SEQ ID NO.:232]
H3 HK68-H3N2 (EF409245) PDB: 1HGJ No Cys Δ1	QDLPGNDNSTATLCLGH HAVPNGTLVKTITDDQIE VTNATELVQSSSTGKI [SEQ ID NO.:184]	SECITPNGSIPNDKPFQNVNKITYGACP KYVKQNTLKLATGMRNVPEKQTR [SEQ ID NO.:233]

HA Subtype (Genbank No.)	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment
H3 HK68-H3N2 (EF409245) PDB: 1HGJ No Cys Δ3	QDLPGNDNSTATLCLGH HAVPNGTLVKTITDDQIE VTNATELVQSSSTGK [SEQ ID NO.:185]	ECITPNGSIPNDKPFQNVNKITYGACP KYVKQNTLKLATGMRNVPEKQTR [SEQ ID NO.:234]
H3 HK68-H3N2 PDB: 1HGJ (EF409245) HK68-CON-A	STATLCLGHHAVPNGTL VKTITDDQIEVTNATELV QSSSTGKIC [SEQ ID NO.:308]	CISECITPNGSIPNDKPFQNVNKITYGA CPKYVKQNTLKLATGMRNVPEKQTR [SEQ ID NO.:52]
H4 (D90302) No Cys	QNYTGNPVICMGHHAV ANGTMVKTLADDQVEV VTAQELVESQNLPEL [SEQ ID NO.:186]	VSKCHTDKGSLSTTKPFQNISRIAVGD CPRYVKQGSLKLATGMRNIPEKASR [SEQ ID NO.:235]
H4 (D90302) No Cys Δ1	QNYTGNPVICMGHHAV ANGTMVKTLADDQVEV VTAQELVESQNLPEL [SEQ ID NO.:187]	SKCHTDKGSLSTTKPFQNISRIAVGDC PRYVKQGSLKLATGMRNIPEKASR [SEQ ID NO.:236]
H4 (D90302) No Cys Δ3	QNYTGNPVICMGHHAV ANGTMVKTLADDQVEV VTAQELVESQNLPE [SEQ ID NO.:188]	KCHTDKGSLSTTKPFQNISRIAVGDCP RYVKQGSLKLATGMRNIPEKASR [SEQ ID NO.:237]
H5 (X07826) No Cys	DQICIGYHANKSTKQVD TIMEKNVTVTHAQDILE RTHNGKL [SEQ ID NO.:189]	DTKCQTPVGEINSSMPFHNIHPHTIGE CPKYVKSDRLVLATGLRNVPQRKKR [SEQ ID NO.:238]
H5 (X07826) No Cys Δ1	DQICIGYHANKSTKQVD TIMEKNVTVTHAQDILE RTHNGKL [SEQ ID NO.:190]	TKCQTPVGEINSSMPFHNIHPHTIGECP KYVKSDRLVLATGLRNVPQRKKR [SEQ ID NO.:239]
H5 (X07826) No Cys Δ3	DQICIGYHANKSTKQVD TIMEKNVTVTHAQDILE RTHNGK [SEQ ID NO.:191]	KCQTPVGEINSSMPFHNIHPHTIGECPK YVKSDRLVLATGLRNVPQRKKR [SEQ ID NO.:240]
H6 (D90303) No Cys	DKICIGYHANNSTTQIDT ILEKNVTVTHSVELLENQ KEERF [SEQ ID NO.:192]	DATCQTVAGVLRTNKTFQNVSPLWIG ECPKYVKSESLRLATGLRNVPQIETR [SEQ ID NO.:241]
H6 (D90303) No Cys Δ1	DKICIGYHANNSTTQIDT ILEKNVTVTHSVELLENQ KEERF [SEQ ID NO.:193]	ATCQTVAGVLRTNKTFQNVSPLWIGE CPKYVKSESLRLATGLRNVPQIETR [SEQ ID NO.:242]

HA Subtype (Genbank No.)	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment
H6 (D90303) No Cys Δ3	DKICIGYHANNSTTQIDT ILEKNVTVTHSVELLENQ KEER [SEQ ID NO.:194]	TCQTVAGVLRTNKTFQNVSPLWIGEC PKYVKSESLRLATGLRNVPQIETR [SEQ ID NO.:243]
H7 (M24457) No Cys	DKICLGHHAVSNGTKVN TLTERGVEVVNATETVE RTNIPKI [SEQ ID NO.:195]	EGECYHSGGTITSRLPFQNINSRAVGK CPRYVKQESLLLATGMKNVPEPSKKR KKR [SEQ ID NO.:244]
H7 (M24457) No Cys Δ1	DKICLGHHAVSNGTKVN TLTERGVEVVNATETVE RTNIPKI [SEQ ID NO.:196]	GECYHSGGTITSRLPFQNINSRAVGKC PRYVKQESLLLATGMKNVPEPSKKRK KR [SEQ ID NO.:245]
H7 (M24457) No Cys Δ3	DKICLGHHAVSNGTKVN TLTERGVEVVNATETVE RTNIPK [SEQ ID NO.:197]	ECYHSGGTITSRLPFQNINSRAVGKCP RYVKQESLLLATGMKNVPEPSKKRKK R [SEQ ID NO.:246]
H8 (D90304) No Cys	DRICIGYQSNNSTDTVNT LIEQNVPVTQTMELVET EKHPAY [SEQ ID NO.:198]	NTKCQTYAGAINSSKPFQNASRHYMG ECPKYVKKASLRLAVGLRNTPSVEPR [SEQ ID NO.:247]
H8 (D90304) No Cys Δ1	DRICIGYQSNNSTDTVNT LIEQNVPVTQTMELVET EKHPAY [SEQ ID NO.:199]	TKCQTYAGAINSSKPFQNASRHYMGE CPKYVKKASLRLAVGLRNTPSVEPR [SEQ ID NO.:248]
H8 (D90304) No Cys Δ3	DRICIGYQSNNSTDTVNT LIEQNVPVTQTMELVET EKHPA [SEQ ID NO.:200]	KCQTYAGAINSSKPFQNASRHYMGEC PKYVKKASLRLAVGLRNTPSVEPR [SEQ ID NO.:249]
H9 (D90305) No Cys	DKICIGYQSTNSTETVDT LTESNVPVTHTKELLHTE HNGML [SEQ ID NO.:201]	VVQCQTEKGGLNTTLPFHNISKYAFG NCPKYVGVKSLKLPVGLRNVPAVSSR [SEQ ID NO.:250]
H9 (D90305) No Cys Δ1	DKICIGYQSTNSTETVDT LTESNVPVTHTKELLHTE HNGML [SEQ ID NO.:202]	VQCQTEKGGLNTTLPFHNISKYAFGN CPKYVGVKSLKLPVGLRNVPAVSSR [SEQ ID NO.:251]
H9 (D90305) No Cys Δ3	DKICIGYQSTNSTETVDT LTESNVPVTHTKELLHTE HNGM [SEQ ID NO.:203]	QCQTEKGGLNTTLPFHNISKYAFGNCP KYVGVKSLKLPVGLRNVPAVSSR [SEQ ID NO.:252]

HA Subtype (Genbank No.)	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment
H10 (M21647) No Cys	LDRICLGHHAVANGTIV KTLTNEQEEVTNATETV ESTNLNKL [SEQ ID NO.:204]	ESKCFWRGGSINTKLPFQNLSPRTVGQ CPKYVNQRSLLLATGMRNVPEVVQG R [SEQ ID NO.:253]
H10 (M21647) No Cys Δ1	LDRICLGHHAVANGTIV KTLTNEQEEVTNATETV ESTNLNKL [SEQ ID NO.:205]	SKCFWRGGSINTKLPFQNLSPRTVGQC PKYVNQRSLLLATGMRNVPEVVQGR [SEQ ID NO.:254]
H10 (M21647) No Cys Δ3	LDRICLGHHAVANGTIV KTLTNEQEEVTNATETV ESTNLNK [SEQ ID NO.:206]	KCFWRGGSINTKLPFQNLSPRTVGQCP KYVNQRSLLLATGMRNVPEVVQGR [SEQ ID NO.:255]
H11 (D90306) No Cys	DEICIGYLSNNSTDKVDT IIENNVTVTSSVELVETE HTGSF [SEQ ID NO.:207]	STKCQTEIGGINTNKSFHNVHRNTIGD CPKYVNVKSLKLATGPRNVPAIASR [SEQ ID NO.:256]
H11 (D90306) No Cys Δ1	DEICIGYLSNNSTDKVDT IIENNVTVTSSVELVETE HTGSF [SEQ ID NO.:208]	TKCQTEIGGINTNKSFHNVHRNTIGDC PKYVNVKSLKLATGPRNVPAIASR [SEQ ID NO.:257]
H11 (D90306) No Cys Δ3	DEICIGYLSNNSTDKVDT IIENNVTVTSSVELVETE HTGS [SEQ ID NO.:209]	KCQTEIGGINTNKSFHNVHRNTIGDCP KYVNVKSLKLATGPRNVPAIASR [SEQ ID NO.:258]
H12 (D90307) No Cys	DKICIGYQTNNSTETVNT LSEQNVPVTQVEELVHR GIDPIL [SEQ ID NO.:210]	VTECQLNEGVMNTSKPFQNTSKHYIG KCPKYIPSGSLKLAIGLRNVPQVQDR [SEQ ID NO.:259]
H12 (D90307) No Cys Δ1	DKICIGYQTNNSTETVNT LSEQNVPVTQVEELVHR GIDPIL [SEQ ID NO.:211]	TECQLNEGVMNTSKPFQNTSKHYIGK CPKYIPSGSLKLAIGLRNVPQVQDR [SEQ ID NO.:260]
H12 (D90307) No Cys Δ3	DKICIGYQTNNSTETVNT LSEQNVPVTQVEELVHR GIDPI [SEQ ID NO.:212]	ECQLNEGVMNTSKPFQNTSKHYIGKC PKYIPSGSLKLAIGLRNVPQVQDR [SEQ ID NO.:261]
H13 (D90308) No Cys	DRICVGYLSTNSSERVDT LLENGVPVTSSIDLIETN HTGTY [SEQ ID NO.:213]	NTKCQTSVGGINTNRTFQNIDKNALG DCPKYIKSGQLKLATGLRNVPAISNR [SEQ ID NO.:262]

HA Subtype (Genbank No.)	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment
H13 (D90308) No Cys Δ1	DRICVGYLSTNSSERVDT LLENGVPVTSSIDLIETN HTGTY [SEQ ID NO.:214]	TKCQTSVGGINTNRTFQNIDKNALGD CPKYIKSGQLKLATGLRNVPAISNR [SEQ ID NO.:263]
H13 (D90308) No Cys Δ3	DRICVGYLSTNSSERVDT LLENGVPVTSSIDLIETN HTGT [SEQ ID NO.:215]	KCQTSVGGINTNRTFQNIDKNALGDC PKYIKSGQLKLATGLRNVPAISNR [SEQ ID NO.:264]
H14 (M35997) No Cys	QITNGTTGNPIICLGHHA VENGTSVKTLTDNHVEV VSAKELVETNHTDEL [SEQ ID NO.:216]	TSPCLTDKGSIQSDKPFQNVSRIAIGNC PKYVKQGSLMLATGMRNIPGKQAK [SEQ ID NO.:265]
H14 (M35997) No Cys Δ1	QITNGTTGNPIICLGHHA VENGTSVKTLTDNHVEV VSAKELVETNHTDEL [SEQ ID NO.:217]	SPCLTDKGSIQSDKPFQNVSRIAIGNCP KYVKQGSLMLATGMRNIPGKQAK [SEQ ID NO.:266]
H14 (M35997) No Cys Δ3	QITNGTTGNPIICLGHHA VENGTSVKTLTDNHVEV VSAKELVETNHTDE [SEQ ID NO.:218]	PCLTDKGSIQSDKPFQNVSRIAIGNCPK YVKQGSLMLATGMRNIPGKQAK [SEQ ID NO.:267]
H15 (L43917) No Cys	DKICLGHHAVANGTKV NTLTERGVEVVNATETV EITGIDKV [SEQ ID NO.:219]	EGECFYSGGTINSPLPFQNIDSRAVGK CPRYVKQSSLPLALGMKNVPEKIRTR [SEQ ID NO.:268]
H15 (L43917) No Cys Δ1	DKICLGHHAVANGTKV NTLTERGVEVVNATETV EITGIDKV [SEQ ID NO.:220]	GECFYSGGTINSPLPFQNIDSRAVGKC PRYVKQSSLPLALGMKNVPEKIRTR [SEQ ID NO.:269]
H15 (L43917) No Cys Δ3	DKICLGHHAVANGTKV NTLTERGVEVVNATETV EITGIDK [SEQ ID NO.:221]	ECFYSGGTINSPLPFQNIDSRAVGKCP RYVKQSSLPLALGMKNVPEKIRTR [SEQ ID NO.:270]
H16 (EU293865) No Cys	DKICIGYLSNNSSDTVDT LTENGVPVTSSVDLVET NHTGTY [SEQ ID NO.:222]	NTKCQTSLGGINTNKTFQNIERNALGD CPKYIKSGQLKLATGLRNVPSIGER [SEQ ID NO.:271]
H16 (EU293865) No Cys Δ1	DKICIGYLSNNSSDTVDT LTENGVPVTSSVDLVET NHTGTY [SEQ ID NO.:223]	TKCQTSLGGINTNKTFQNIERNALGDC PKYIKSGQLKLATGLRNVPSIGER [SEQ ID NO.:272]

HA Subtype (Genbank No.)	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment
H16 (EU293865) No Cys Δ3	DKICIGYLSNNSSDTVDT LTENGVPVTSSVDLVET NHTGT [SEQ ID NO.:224]	KCQTSLGGINTNKTFQNIERNALGDCP KYIKSGQLKLATGLRNVPSIGER [SEQ ID NO.:273]

[00100] Table 2, below, identifies putative stem domains, luminal domains, transmembrane domains and cytoplasmic domains of HA2 polypeptides.

TABLE 2 Exemplary Influenza A Hemagglutinin Sequences

HA2 domain Subtype (Genbank No.)	Stem domain	Luminal domain	Transmembrane domain	Cytoplasmic domain
H1 PR8-H1N1 (EF467821.1)	GLFGAIAGFIEGGWT GMIDGWYGYHHQNE QGSGYAADQKSTQN AINGITNKVNTVIEK MNIQFTAVGKEFNKL EKRMENLNKKVDDG FLDIWTYNAELLVLL ENERTLDFHDSNVKN LYEKVKSQLKNNAK EIGNGCFEFYHKCDN ECMESVRNGTYDYP KYSEESKLNREKVDG VKLES [SEQ ID NO.:82]	MGIYQ [SEQ ID NO.:98]	ILAIYSTVASSL VLLVSLGAISF WMCS [SEQ ID NO.:114]	NGSLQCRI CI [SEQ ID NO.:130]
H2 (L11136)	GLFGAIAGFIEGGWQ GMIDGWYGYHHSND QGSGYAADKESTQK AIDGITNRVNSVIEK MNTQFEAVGKEFSNL EKRLENLNKKMEDG FLDVWTYNAELLVL MENERTLDFHDSNV KNLYDRVRMQLRDN AKELGNGCFEFYHKC DDECMNSVKNGTYD YPKYEEESKLNRNEI KGVKLSN [SEQ ID NO.:83]	MGVYQ [SEQ ID NO.:99]	ILAIYATVAGSL SLAIMIAGISLW MCS [SEQ ID NO.:115]	NGSLQCRI CI [SEQ ID NO.:131]
H3 HK68-H3N2	GLFGAIAGFIENGWE GMIDGWYGFRHQNS	SGYKD	WILWISFAISCF LLCVVLLGFIM	RGNIRCNI CI

HA2 domain Subtype (Genbank No.)	Stem domain	Luminal domain	Transmembrane domain	Cytoplasmic domain
(EF409245) PDB: 1HGJ	EGTGQAADLKSTQA AIDQINGKLNRVIEKT NEKFHQIEKEFSEVE GRIQDLEKYVEDTKI DLWSYNAELLVALE NQHTIDLTDSEMNKL FEKTRRQLRENAED MGNGCFKIYHKCDN ACIESIRNGTYDHDV YRDEALNNRFQIKGV ELK [SEQ ID NO.:84]	[SEQ ID NO.:100]	WACQ [SEQ ID NO.:116]	[SEQ ID NO.:132]
H4 (D90302)	GLFGAIAGFIENGWQ GLIDGWYGFRHQNA EGTGTAADLKSTQA AIDQINGKLNRLIEKT NDKYHQIEKEFEQVE GRIQDLENYVEDTKI DLWSYNAELLVALE NQHTIDVTDSEMNKL FERVRRQLRENAEDK GNGCFEIFHKCDNNC IESIRNGTYDHDIYRD EAINNRFQIQGVKLT [SEQ ID NO.:85]	QGYKD [SEQ ID NO.:101]	IILWISFSISCFLL VALLLAFILWA CQ [SEQ ID NO.:117]	NGNIRCQI CI [SEQ ID NO.:133]
H5 (X07826)	GLFGAIAGFIEGGWQ GMVDGWYGYHHSN EQGSGYAADKESTQ KAIDGITNKVNSIIDK MNTRFEAVGKEFNN LERRVENLNKKMED GFLDVWTYNVELLV LMENERTLDFHDSNV NNLYDKVRLQLKDN ARELGNGCFEFYHKC DNECMESVRNGTYD YPQYSEEARLNREEIS GVKLES [SEQ ID NO.:86]	MGVYQ [SEQ ID NO.:102]	ILSIYSTVASSL ALAIMIAGLSF WMCS [SEQ ID NO.:118]	NGSLQCRI CI [SEQ ID NO.:134]
H6 (D90303)	GLFGAIAGFIEGGWT GMIDGWYGYHHENS QGSGYAADRESTQK AVDGITNKVNSIIDK MNTQFEAVDHEFSNL ERRIDNLNKRMEDGF LDVWTYNAELLVLL	LGVYQ [SEQ ID NO.:103]	ILAIYSTVSSSL VLVGLIIAVGL WMCS [SEQ ID NO.:119]	NGSMQCR ICI [SEQ ID NO.:135]

HA2 domain Subtype (Genbank No.)	Stem domain	Luminal domain	Transmembrane domain	Cytoplasmic domain
	ENERTLDLHDANVK NLYERVKSQLRDNA MILGNGCFEFWHKC DDECMESVKNGTYD YPKYQDESKLNRQEI ESVKLES [SEQ ID NO.:87]			
H7 (M24457)	GLFGAIAGFIENGWE GLVDGWYGFRHQNA QGEGTAADYKSTQS AIDQITGKLNRLIEKT NQQFELIDNEFTEVE KQIGNLINWTKDSITE VWSYNAELIVAMEN QHTIDLADSEMNRLY ERVRKQLRENAEED GTGCFEIFHKCDDDC MASIRNNTYDHSKYR EEAMQNRIQIDPVKL S [SEQ ID NO.:88]	SGYKD [SEQ ID NO.:104]	VILWFSFGASCF LLLAIAMGLVFI CVK [SEQ ID NO.:120]	NGNMRCT ICI [SEQ ID NO.:136]
H8 (D90304)	GLFGAIAGFIEGGWS GMIDGWYGFHHSNS EGTGMAADQKSTQE AIDKITNKVNNIVDK MNREFEVVNHEFSEV EKRINMINDKIDDQIE DLWAYNAELLVLLE NQKTLDEHDSNVKN LFDEVKRRLSANAID AGNGCFDILHKCDNE CMETIKNGTYDHKE YEEEAKLERSKINGV KLEE [SEQ ID NO.:89]	NTTYK [SEQ ID NO.:105]	ILSIYSTVAASL CLAILIAGGLIL GMQ [SEQ ID NO.:121]	NGSCRCM FCI [SEQ ID NO.:137]
H9 (D90305)	GLFGAIAGFIEGGWP GLVAGWYGFQHSND QGVGMAADKGSTQK AIDKITSKVNNIIDKM NKQYEVIDHEFNELE ARLNMINNKIDDQIQ DIWAYNAELLVLLEN QKTLDEHDANVNNL YNKVKRALGSNAVE DGNGCFELYHKCDD QCMETIRNGTYDRQ	EGTYK [SEQ ID NO.:106]	ILTIYSTVASSL VLAMGFAAFLF WAMS [SEQ ID NO.:122]	NGSCRCNI CI [SEQ ID NO.:138]

HA2 domain Subtype (Genbank No.)	Stem domain	Luminal domain	Transmembrane domain	Cytoplasmic domain
	KYQEESRLERQKIEG VKLES [SEQ ID NO.:90]			
H10 (M21647)	GLFGAIAGFIENGWE GMVDGWYGFRHQN AQGTGQAADYKSTQ AAIDQITGKLNRLIEK TNTEFESIESEFSETEH QIGNVINWTKDSITDI WTYNAELLVAMENQ HTIDMADSEMLNLYE RVRKQLRQNAEEDG KGCFEIYHTCDDSCM ESIRNNTYDHSQYRE EALLNRLNINPVKLS [SEQ ID NO.:91]	SGYKD [SEQ ID NO.:107]	IILWFSFGESCF VLLAVVMGLV FFCLK [SEQ ID NO.:123]	NGNMRCT ICI [SEQ ID NO.:139]
H11 (D90306)	GLFGAIAGFIEGGWP GLINGWYGFQHRDE EGTGIAADKESTQKA IDQITSKVNNIVDRM NTNFESVQHEFSEIEE RINQLSKHVDDSVVD IWSYNAQLLVLLENE KTLDLHDSNVRNLHE KVRRMLKDNAKDEG NGCFTFYHKCDNKCI ERVRNGTYDHKEFEE ESKINRQEIEGVKLDS S [SEQ ID NO.:92]	GNVYK [SEQ ID NO.:108]	ILSIYSCIASSLV LAALIMGFMFW ACS [SEQ ID NO.:124]	NGSCRCTI CI [SEQ ID NO.:140]
H12 (D90307)	GLFGAIAGFIEGGWP GLVAGWYGFQHQNA EGTGIAADRDSTQRA IDNMQNKLNNVIDK MNKQFEVVNHEFSE VESRINMINSKIDDQI TDIWAYNAELLVLLE NQKTLDEHDANVRN LHDRVRRVLRENAID TGDGCFEILHKCDNN CMDTIRNGTYNHKE YEEESKIERQKVNGV KLEE [SEQ ID NO.:93]	NSTYK [SEQ ID NO.:109]	ILSIYSSVASSLV LLLMIIGGFIFG CQN [SEQ ID NO.:125]	GNVRCTF CI [SEQ ID NO.:141]
H13 (D90308)	GLFGAIAGFIEGGWP GLINGWYGFQHQNE	DNVYK [SEQ ID	ALSIYSCIASSV VLVGLILSFIM	GNCRFNV CI

HA2 domain Subtype (Genbank No.)	Stem domain	Luminal domain	Transmembrane domain	Cytoplasmic domain
	QGTGIAADKESTQKA IDQITTKINNIIDKMN GNYDSIRGEFNQVEK RINMLADRIDDAVTD IWSYNAKLLVLLEND KTLDMHDANVKNLH EQVRRELKDNAIDEG NGCFELLHKCNDSC METIRNGTYDHTEYA EESKLKRQEIDGIKLK SE [SEQ ID NO.:94]	NO.:110]	WACSS [SEQ ID NO.:126]	[SEQ ID NO.:142]
H14 (M35997)	GLFGAIAGFIENGWQ GLIDGWYGFRHQNA EGTGTAADLKSTQA AIDQINGKLNRLIEKT NEKYHQIEKEFEQVE GRIQDLEKYVEDTKI DLWSYNAELLVALE NQHTIDVTDSEMNKL FERVRRQLRENAEDQ GNGCFEIFHQCDNNC IESIRNGTYDHNIYRD EAINNRIKINPVTLT [SEQ ID NO.:95]	MGYKD [SEQ ID NO.:111]	IILWISFSMSCF VFVALILGFVL WACQ [SEQ ID NO.:127]	NGNIRCQI CI [SEQ ID NO.:143]
H15 (L43917)	GLFGAIAGFIENGWE GLIDGWYGFRHQNA QGQGTAADYKSTQA AIDQITGKLNRLIEKT NKQFELIDNEFTEVE QQIGNVINWTRDSLT EIWSYNAELLVAME NQHTIDLADSEMNKL YERVRRQLRENAEED GTGCFEIFHRCDDQC MESIRNNTYNHTEYR QEALQNRIMINPVKL S [SEQ ID NO.:96]	SGYKD [SEQ ID NO.:112]	VILWFSFGASC VMLLAIAMGLI FMCVKN [SEQ ID NO.:128]	GNLRCTIC I [SEQ ID NO.:144]
H16 (EU293865)	GLFGAIAGFIEGGWP GLINGWYGFQHQNE QGTGIAADKASTQKA INEITTKINNIIEKMNG NYDSIRGEFNQVEKR INMLADRVDDAVTDI WSYNAKLLVLLEND	DNVYK [SEQ ID NO.:113]	VLSIYSCIASSIV LVGLILAFIMW ACS [SEQ ID NO.:129]	NGSCRFN VCI [SEQ ID NO.:145]

HA2 domain Subtype (Genbank No.)	Stem domain	Luminal domain	Transmembrane domain	Cytoplasmic domain
	RTLDLHDANVRNLH DQVKRALKSNAIDEG DGCFNLLHKCNDSC METIRNGTYNHEDYR EESQLKRQEIEGIKLK TE [SEQ ID NO.:97]			

[00101] In certain embodiments, the influenza hemagglutinin stem domain polypeptides comprise one or more immunogenic epitopes in the tertiary or quaternary structure of an influenza hemagglutinin polypeptide.

[00102] In certain embodiments, the HA1 N-terminal stem segment comprises the amino acid sequence A_{17}-A_{18}-$(Xaa)_n$-A_{38} (SEQ ID NO:146), wherein

A_{17} is Y or H;

A_{18} is H, L, or Q;

$(Xaa)_n$ represents a sequence of 18-20 amino acid residues; and

A_{38} is H, S, Q, T or N.

[00103] In certain embodiments, the HA1 C-terminal stem segment comprises the amino acid sequence A_{291}-A_{292} (SEQ ID NO:147), wherein

A_{291} is T, S, N, D, P or K; and

A_{292} is L, M, K or R.

[00104] In certain embodiments, the HA2 domain comprises the amino acid sequence A_{18}-A_{19}-A_{20}-A_{21} (SEQ ID NO:148), wherein

A_{18} is V or I;

A_{19} is D, N or A;

A_{20} is G, and

A_{21} is W.

[00105] In certain embodiments, the HA2 domain comprises the amino acid sequence A_{38}-A_{39}-A_{40}-A_{41}-A_{42}-A_{43}-A_{44}-A_{45}-A_{46}-A_{47}-A_{48}-A_{49}-A_{50}-A_{51}-A_{52}-A_{53}-A_{54}-A_{55}-A_{56} (SEQ ID NO:149), wherein

A_{38} is K, Q, R, L or Y;

A_{39} is any amino acid residue;

A_{40} is any amino acid residue;

A_{41} is T;

A_{42} is Q;

A_{43} is any amino acid residue;

A_{44} is A;

A_{45} is I;

A_{46} is D;

A_{47} is any amino acid residue;

A_{48} is I, V or M;

A_{49} is T, Q or N;

A_{50} is any amino acid residue;

A_{51} is K;

A_{52} is V or L;

A_{53} is N;

A_{54} is any amino acid residue;

A_{55} is V, I or L; and

A_{56} is V or I.

[00106] In certain embodiments, the influenza stem domain polypeptides comprise two amino acid sequences selected from SEQ ID NOS:146-149. In certain embodiments, the influenza stem domain polypeptides comprise three amino acid sequences selected from SEQ ID NOS:146-149. In certain embodiments, the influenza stem domain polypeptides comprise four amino acid sequences selected from SEQ ID NOS:146-149.

[00107] In certain embodiments, the HA1 N-terminal stem segments are based on an influenza B hemagglutinin. In certain embodiments, the HA1 N-terminal stem segment is selected from SEQ ID NOS:154-157, presented in Table 3 below.

[00108] In certain embodiments, the HA1 C-terminal stem segments are based on an influenza B hemagglutinin. In certain embodiments, the HA1 C-terminal stem segment is selected from SEQ ID NOS:158-159, presented in Table 3 below.

[00109] In certain embodiments, the HA2 stem domains are based on an influenza B hemagglutinin. Exemplary residues for the end of an N-terminal stem segment and the end of a C-terminal stem segment of an influenza B hemagglutinin are indicated in FIG. 2. In certain embodiments, the HA2 stem domain is according to SEQ ID NO:160, presented in Tables 3 and 4 below.

[00110] In particular embodiments, the boundaries of the influenza B virus HA1 N-terminal stem segment and influenza B virus HA1 C-terminal segment are defined with respect to three pairs of amino acid residues: Arg_{50} and Ser_{277}; Ala_{66} and Trp_{271}; and Lys_{80} and Ser_{277}. The residue numbers are based on the numbering of the B-HA from influenza virus B as described in Protein Data Bank accession No. 3BT6. The amino acid sequence corresponding to the X-ray crystal structure of the B-HA protein in Protein Data Bank accession No. 3BT6 is aligned with representative H1 and H3 amino acid sequence and shown in FIG. 2. Positions of the three pairs of residues are also highlighted in FIG. 2.

[00111] In certain embodiments, an influenza B virus HA1 N-terminal stem segment starts at residue 1 (based on numbering of an influenza B virus HA1 subunit as in PDB file 3BT6) and ends at Arg_{50}. In certain embodiments, an influenza B virus HA1 N-terminal stem segment starts at residue 1 and ends at Ala_{66}. In some embodiments, an influenza B virus HA1 N-terminal stem segment starts at residue 1 and ends at Lys_{80}. In some embodiments, an influenza B virus N-terminal stem segment starts at residue 1 and ends at Arg_{80}.

[00112] In some embodiments, an influenza B virus HA1 N-terminal stem segment has an amino acid sequence according to any one of SEQ ID NOS:154-157, as illustrated in TABLE 3. In some embodiments, an influenza B virus HA1 N-terminal stem segment has an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to any one of the amino acid sequences of any one of SEQ ID NOS:154-157.

[00113] In some embodiments, an influenza B virus HA1 N-terminal stem segment has an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to the amino acid sequence SEQ ID NO:154, which corresponds to residues 1-50 of the influenza B virus HA1.

[00114] In some embodiments, an influenza B virus HA1 N-terminal stem segment has an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to the amino acid sequence SEQ ID NO:155, which corresponds to residues 1-66 of the influenza B virus HA1.

[00115] In some embodiments, an influenza B virus HA1 N-terminal stem segment has an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to the amino acid sequence SEQ ID NO:156, which corresponds to residues 1-80 of the influenza B virus HA1.

[00116] In some embodiments, an influenza B virus HA1 N-terminal stem segment has an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to the amino acid sequence SEQ ID NO:157, which corresponds to residues 1-80 of the influenza B virus HA1.

[00117] In some embodiments, an influenza B virus HA1 C-terminal stem segment has an amino acid sequence that starts at Ser_{277} residue or Trp_{271}, or corresponding residues in other influenza B virus HA subtypes.

[00118] In some embodiments, an influenza B virus HA1 C-terminal stem segment has an amino acid sequence according to any one of SEQ ID NOS:158-159, as illustrated in TABLE 3. In some embodiments, an influenza B virus HA1 C-terminal stem segment has an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to SEQ ID NO:158, which correspond to residues 277-344 of influenza B virus HA1. In some embodiments, an influenza B virus HA1 C-terminal stem segment has an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to SEQ ID NO.:159, which correspond to residues 271-344 of influenza B virus HA1.

[00119] In some embodiments, an influenza B virus HA1 C-terminal stem segment starts at residue-276, residue-275, residue-274, residue-273, or residue-272. In other embodiments, an influenza B virus HA1 C-terminal stem segment starts at residue-278, residue-279, residue-280, residue-281, or residue-282.

[00120] In certain embodiments, the influenza B virus HA2 domain is in tertiary or quaternary association with the influenza B virus HA1 domain through the influenza B virus HA1 N-terminal segment, the influenza B virus HA1 C-terminal segment, or both.

[00121] In some embodiments, the influenza B virus HA1 C-terminal segment and the influenza B virus HA2 subunit are covalently linked. For example, at its C-terminus (*e.g.*, at the ending residue of the second sequence), the influenza B virus HA1 C-terminal segment is covalently linked to the influenza B virus HA2 domain in such embodiments. In some embodiments, the influenza B virus HA1 C-terminal segment and influenza B virus HA2 domain form a continuous polypeptide chain.

[00122] In some embodiments, the influenza B virus HA2 domain has the amino acid sequence of SEQ ID NO:160 or 161, as illustrated in TABLE 3 or 4. In some embodiments, the amino acid sequence of the HA2 domain is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to any one of SEQ ID NOS:160-161.

[00123] In certain embodiments, the influenza B stem domain polypeptides comprise a signal peptide. The signal peptide can be any signal peptide deemed suitable to those of skill in the art, including any signal peptide described herein. In certain embodiments, the signal peptide is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to any of SEQ ID NOS:150-153. In certain embodiments, the signal peptide is according to any of SEQ ID NOS:150-153.

[00124] In certain embodiments, the influenza B stem domain polypeptides comprise a luminal domain. The luminal domain can be any luminal domain deemed suitable to those of skill in the art, including any luminal domain described herein. In certain embodiments, the luminal is at least 60% or 80%, identical to SEQ ID NO:162. In certain embodiments, the luminal domain is according to SEQ ID NO:162.

[00125] In certain embodiments, the influenza B stem domain polypeptides comprise a transmembrane domain. The transmembrane domain can be any transmembrane domain deemed suitable to those of skill in the art, including any transmembrane domain described herein. In certain embodiments, the transmembrane domain is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to SEQ ID NO:163. In certain embodiments, the transmembrane domain is according to SEQ ID NO:163.

[00126] In certain embodiments, the influenza B stem domain polypeptides comprise a cytoplasmic domain. The cytoplasmic domain can be any cytoplasmic domain deemed suitable to those of skill in the art, including any cytoplasmic domain described herein. In certain embodiments, the cytoplasmic domain is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to SEQ ID NO:164. In certain embodiments, the cytoplasmic domain is according to SEQ ID NO:164.

TABLE 3: Exemplary Influenza B Hemagglutinin Sequences

HA construct variants	Signal peptide	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment	HA2 Domain
Arg50-Ser277	MKAIIVILMV VTSNA [SEQ ID NO.:150]	DRICTGITSSNS PHVVKTATQG EVNVTGVIPLT TTPTKSHFANL KGTETR [SEQ ID NO.:154]	SKVIKGSLPLI GEADCLHEKY GGLNKSKPYY TGEHAKAIGN CPIWVKTPLKL ANGTKYRPPA KLLKER [SEQ ID NO.:158]	GFFGAIAGFLEGG WEGMIAGWHGY TSHGAHGVAVAA DLKSTQEAINKIT KNLNSLSELEVKN LQRLSGAMDELH NEILELDEKVDDL RADTISSQIELAVL LSNEGIINSEDEHL

HA construct variants	Signal peptide	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment	HA2 Domain
				LALERKLKKMLG PSAVEIGNGCFET KHKCNQTCLDRI AAGTFDAGEFSLP TFDSLNITAASLN DDGLDNHTILLYY STAASSLAVTLMI AIFVVYMVSRDN VSCSICL [SEQ ID NO.:160]
Ala66-Trp271	MKAIIVILMV VTSNA [SEQ ID NO.:151]	DRICTGITSSNS PHVVKTATQG EVNVTGVIPLT TTPTKSHFANL KGTETRGKLC PKCLNCTDLD VA [SEQ ID NO.:155]	WCASGRSKVI KGSLPLIGEAD CLHEKYGGLN KSKPYYTGEH AKAIGNCPIW VKTPLKLANG TKYRPPAKLL KER [SEQ ID NO.:159]	GFFGAIAGFLEGG WEGMIAGWHGY TSHGAHGVAVAA DLKSTQEAINKIT KNLNSLSELEVKN LQRLSGAMDELH NEILELDEKVDDL RADTISSQIELAVL LSNEGIINSEDEHL LALERKLKKMLG PSAVEIGNGCFET KHKCNQTCLDRI AAGTFDAGEFSLP TFDSLNITAASLN DDGLDNHTILLYY STAASSLAVTLMI AIFVVYMVSRDN VSCSICL [SEQ ID NO.:160]
Lys80-Ser277	MKAIIVILMV VTSNA [SEQ ID NO.:152]	DRICTGITSSNS PHVVKTATQG EVNVTGVIPLT TTPTKSHFANL KGTETRGKLC PKCLNCTDLD VALGRPKCTG KIPSAK [SEQ ID NO.:156]	SKVIKGSLPLI GEADCLHEKY GGLNKSKPYY TGEHAKAIGN CPIWVKTPLKL ANGTKYRPPA KLLKER [SEQ ID NO.:158]	GFFGAIAGFLEGG WEGMIAGWHGY TSHGAHGVAVAA DLKSTQEAINKIT KNLNSLSELEVKN LQRLSGAMDELH NEILELDEKVDDL RADTISSQIELAVL LSNEGIINSEDEHL LALERKLKKMLG PSAVEIGNGCFET KHKCNQTCLDRI AAGTFDAGEFSLP TFDSLNITAASLN DDGLDNHTILLYY STAASSLAVTLMI AIFVVYMVSRDN VSCSICL

HA construct variants	Signal peptide	HA1 N-terminal Stem Segment	HA1 C-terminal Stem Segment	HA2 Domain
				[SEQ ID NO.:160]
Arg80-Ser277	MKAIIVILMV VTSNA [SEQ ID NO.:153]	DRICTGITSSNS PHVVKTATQG EVNVTGVIPLT TTPTKSHFANL KGTETRGKLC PKCLNCTDLD VALGRPKCTG KIPSAR [SEQ ID NO.:157]	SKVIKGSLPLI GEADCLHEKY GGLNKSKPYY TGEHAKAIGN CPIWVKTPLKL ANGTKYRPPA KLLKER [SEQ ID NO.:158]	GFFGAIAGFLEGG WEGMIAGWHGY TSHGAHGVAVAA DLKSTQEAINKIT KNLNSLSELEVKN LQRLSGAMDELH NEILELDEKVDDL RADTISSQIELAVL LSNEGIINSEDEHL LALERKLKKMLG PSAVEIGNGCFET KHKCNQTCLDRI AAGTFDAGEFSLP TFDSLNITAASLN DDGLDNHTILLYY STAASSLAVTLMI AIFVVYMVSRDN VSCSICL [SEQ ID NO.:160]

[00127] Table 4 provides the putative stem domain, luminal domain, transmembrane domain and cytoplasmic domain of HA from influenza B.

TABLE 4: Exemplary Influenza B Hemagglutinin Sequences

HA2 domain Subtype (Genbank No.)	Stem domain	Luminal domain	Transmembrane domain	Cytoplasmic domain
HA2 (AY096185)	GFFGAIAGFLEG GWEGMIAGWH GYTSHGAHGV AVAADLKSTQE AINKITKNLNSL SELEVKNLQRL SGAMDELHNEI LELDEKVDDLR ADTISSQIELAV LLSNEGIINSED EHLLALERKLK KMLGPSAVEIG NGCFETKHKCN QTCLDRIAAGT FDAGEFSLPTFD	DGLDN [SEQ ID NO.:162]	HTILLYYSTAAS SLAVTLMIAIFV VYMV [SEQ ID NO.:163]	SRDNVSCSIC L [SEQ ID NO.:164]

HA2 domain Subtype (Genbank No.)	Stem domain	Luminal domain	Transmembrane domain	Cytoplasmic domain
	SLNITAASLND [SEQ ID NO.:161]			

[00128] As illustrated in FIGS. 1 and 2, HA1 N-terminal stem segments share sequence identity between influenza A and influenza B and additionally across influenza A subtypes. Similarly, HA1 C-terminal stem segments also share sequence identity between influenza A and influenza B and additionally across influenza A subtypes. Further, HA2 domains also share sequence identity between influenza A and influenza B and additionally across influenza A subtypes.

[00129] In some embodiments, the influenza hemagglutinin stem domain polypeptide is a hybrid polypeptide that comprises or consists essentially of segments and/or domains from a plurality of influenza strains or subtypes. For example, an influenza hemagglutinin stem domain polypeptide might comprise HA1 N-terminal and HA1 C-terminal stem segments from different influenza A virus HA subtypes. In some embodiments, the HA1 N-terminal stem segment is from influenza A virus while the HA1 C-terminal stem segment is from influenza B virus. Similarly, HA2 may also be from influenza A virus while the HA1 N-terminal and/or C-terminal stem segment is from influenza B virus.

[00130] It will be understood that any combination of the sequence elements listed in Tables 1-4 or the variants thereof may be used to form the hemagglutinin HA stem domain polypeptides of the present invention.

[00131] In an influenza stem domain polypeptide provided herein, a linker covalently connects the HA1 N-terminal stem segment to the HA1 C-terminal stem segment. In certain embodiments, the linker is a direct bond. In certain embodiments, the linker is a peptide that comprises one amino acid residue, two or fewer amino acid residues, three or fewer amino acid residues, four or fewer amino acid residues, five or fewer amino acid residues, ten or fewer amino acid residues, 15 or fewer amino acid residues, 20 or fewer amino acid residues, 30 or fewer amino acid residues, 40 or fewer amino acid residues, or 50 or fewer amino acid residues. In certain embodiments, the linker peptide comprises 50 or more amino acid residues. In certain embodiments the linker substantially lacks a globular head domain. In other words, the linker comprises no

more than 10, 9, 8, 7, 6, 5 or 4 contiguous, sequential amino acid residues from the amino acid sequence of an influenza globular head domain. In certain embodiments, the linker is other than Lys-Leu-Asn-Gly-Ser-Gly-Ile-Met-Lys-Thr-Glu-Gly-Thr-Leu-Glu-Asn (SEQ ID NO:311). In certain embodiments, the linker is other than Asn-Asn-Ile-Asp-Thr or Lys-Leu-Asn-Gly-Ser-Gly-Ile-Met-Lys-Thr-Glu-Gly-Thr-Leu-Glu-Asn (SEQ ID NO:312). In certain embodiments, the linker is other than Asn-Asn-Ile-Asp-Thr (SEQ ID NO:315).

[00132] In certain embodiments, the linker is covalently connected, at one end, to the C-terminus of the HA1 N-terminal stem segment. The linker peptide is also covalently connected, at the other end, to the N-terminus of the HA1 C-terminal stem segment. In certain embodiments, one of the covalent links is an amide bond. In certain embodiments, both covalent links are amide bonds.

[00133] The linker might be any linker deemed suitable by one of skill in the art. In certain embodiments, the linker is selected based on the HA1 N-terminal stem segment and the HA1 C-terminal stem segment. In these embodiments, the linker might be selected with molecular modeling programs such as InsightII and Quanta, both from Accelrys. In certain embodiments, the linker is a structural motif that allows structural alignment of the HA1 N-terminal stem segment and the HA1 C-terminal stem segment that is consistent with the structure of a hemagglutinin stem domain as recognized by those of skill in the art. In certain embodiments, the linker is selected from a library of candidate linkers. In certain embodiments, the library includes three dimensional polypeptide structures in a publicly available database such as the Protein Data Bank (PDB) or the Macromolecular Structure Database at the European Molecular Biology Laboratory (EMBL) or European Bioinformatics Institute (EBI). In certain embodiments, the library includes proprietary three-dimensional polypeptide structures associated with commercial programs such as InsightII and Quanta, both from Accelrys. Additionally, any databases or collections of protein structures or structural elements can be used to select the linker. Exemplary database or collections of protein structural elements include but are not limited to the Structural Classification of Proteins (SCOP, maintained by and available through Cambridge University); the database of protein families (Pfam, maintained by and available through the Wellcome Trust Sanger Institute); the Universal Protein Resource (UniProt, maintained by and available through the UniProt Consortium); the Integrated resource for protein families (InterPro; maintained by and available through EMBL-EBI); the Class Architecture Topology

Homologous superfamily (CATH, maintained by and available through Institute of Structural and Molecular Biology at the University College London); and the families of structurally similar proteins (FSSP, maintained by and available through EBI). Any algorithm deemed suitable by one of skill in the art may be used to select the linker, including but not limited by those used by SCOP, CATH and FSSP. Useful examples include but are not limited to Pymol (Delano Scientific LLC), InsightII and Quanta (both from Accelrys), MIDAS (University of California, San Francisco), SwissPDB viewer (Swiss Institute of Bioinformatics), TOPOFIT (Northeastern University), CBSU LOOPP (Cornell University), and SuperPose (University of Alberta, Edmonton).

[00134] In certain embodiments, the linker is a direct bond. In certain embodiments, the linker is selected from the group consisting of Gly, Gly-Gly, Gly-Gly-Gly, Gly-Gly-Gly-Gly and Gly-Gly-Gly-Gly-Gly. In certain embodiments, the linker is selected from the group consisting of Gly-Pro and Pro-Gly. In certain embodiments, the linker is a 281 turn loop, *e.g.* having the sequence ITPNGSIPNDKPFQNVNKITYGA (SEQ ID NO:165).

[00135] In certain embodiments the linker comprises a glycosylation sequence. In certain embodiments, the linker comprises an amino acid sequence according to Asn-Xaa-Ser/Thr where Xaa is any amino acid other than proline and Ser/Thr is serine or threonine. In certain embodiments, the linker comprises the amino acid sequence Asn-Ala-Ser. In certain embodiments the linker is a glycosylation sequence. In certain embodiments, the linker is an amino acid sequence according to Asn-Xaa-Ser/Thr where Xaa is any amino acid other than proline and Ser/Thr is serine or threonine. In certain embodiments, the linker is the amino acid sequence Asn-Ala-Ser.

[00136] In certain embodiments, influenza hemagglutinin stem domain polypeptides are capable of forming a three dimensional structure that is similar to the three dimensional structure of the stem domain of a native influenza hemagglutinin. Structural similarity might be evaluated based on any technique deemed suitable by those of skill in the art. For instance, reaction, *e.g.* under non-denaturing conditions, of an influenza hemagglutinin stem domain polypeptide with a neutralizing antibody or antiserum that recognizes a native influenza hemagglutinin might indicate structural similarity. Useful neutralizing antibodies or antisera are described in, *e.g.,* Sui, *et al.,* 2009, *Nat. Struct. Mol. Biol.* 16(3):265-273, Ekiert *et al.*, February 26, 2009, *Science* [DOI: 10.1126/science.1171491], Wang *et al.* (2010) "Broadly Protective Monoclonal Antibodies against H3 Influenza Viruses following Sequential Immunization with

Different Hemagglutinins," PLOS Pathogens 6(2):1-9, and Kashyap *et al.*, 2008, *Proc. Natl. Acad. Sci. USA* 105(16):5986-5991, the contents of which are hereby incorporated by reference in their entireties. In certain embodiments, the antibody or antiserum is an antibody or antiserum that reacts with a non-contiguous epitope (*i.e.*, not contiguous in primary sequence) that is formed by the tertiary or quaternary structure of a hemagglutinin.

[00137] In certain embodiments, structural similarity might be assessed by spectroscopic techniques such as circular dichroism, Raman spectroscopy, NMR, 3D NMR and X-ray crystallography. Known influenza hemagglutinin structures determined by X-ray crystallography are described in structural coordinates in Protein Data Bank files including but not limited to 1HGJ (an HA H3N2 strain) and 1RUZ (an HA H1N1 strain).

[00138] In certain embodiments, structural similarity is evaluated by RMS deviation between corresponding superimposed portions of two structures. In order to create a meaningful superimposition, in certain embodiments the coordinates of at least 20 corresponding atoms, 25 corresponding atoms, 30 corresponding atoms, 40 corresponding atoms, 50 corresponding atoms, 60 corresponding atoms, 70 corresponding atoms, 80 corresponding atoms, 90 corresponding atoms, 100 corresponding atoms, 120 corresponding atoms, 150 corresponding atoms, 200 corresponding atoms, or 250 corresponding atoms are used to calculate an RMS deviation.

[00139] In certain embodiments, the coordinates of all corresponding atoms in amino acid backbones are used to calculate an RMS deviation. In certain embodiments, the coordinates of all corresponding alpha carbon-atoms in the amino acid backbones are used to calculate an RMS deviation. In certain embodiments, the coordinates of all corresponding identical residues, including side chains, are used to calculate an RMS deviation.

[00140] In certain embodiments, coordinates of all or a portion of the corresponding atoms in a HA1 N-terminal segment are used to calculate an RMS deviation. In certain embodiments, coordinates of all or a portion of the corresponding atoms in a HA1 C-terminal segment are used to calculate an RMS deviation. In certain embodiments, coordinates of all or a portion of the corresponding atoms in both a HA1 N-terminal segment and a C-terminal segment are used to calculate an RMS deviation. In certain

embodiments, coordinates of all or a portion of corresponding atoms in HA2 domains are used to calculate an RMS deviation.

[00141] In certain embodiments, the RMS deviation between the structures of a influenza hemagglutinin stem domain polypeptide and corresponding portions of a known influenza A virus hemagglutinin stem domain (*e.g.*, from 1HGJ or 1RUZ) is 5 Å or less, 4 Å or less, 3 Å or less, 2.5 Å or less, 2 Å or less, 1.5 Å or less, 1 Å or less, 0.75 Å or less, 0.5 Å or less, 0.3 Å or less, 0.2 Å or less, or 0.1 Å or less. Commercially available or open source software might be used to perform the structural superimpositions and/or RMS deviation calculations. Useful examples include but are not limited to Pymol (Delano Scientific LLC), InsightII and Quanta (both from Accelrys), MIDAS (University of California, San Francisco), SwissPDB viewer (Swiss Institute of Bioinformatics), TOPOFIT (Northeastern University), CBSU LOOPP (Cornell University), and SuperPose (University of Alberta, Edmonton).

[00142] In certain embodiments, any influenza hemagglutinin stem domain polypeptide provided herein can further comprise one or more polypeptide domains deemed suitable to those of skill in the art. 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:166), FLAG epitope or other purification tag can facilitate purification of a polypeptide provided herein. A foldon, or trimerization, domain from bacteriophage T4 fibritin can facilitate trimerization of polypeptides provided herein. 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. Examples include GSGYIPEAPRDGQAYVRKDGEWVLLSTFL (SEQ ID NO:167). 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:168).

[00143] In certain embodiments, provided are influenza hemagglutinin stem domain polypeptides comprising an elastase cleavage site. Those of skill in the art will recognize that the trypsin cleavage site at the linkage between HA1 and HA2 can be mutated to an elastase cleavage site by substituting valine for the arginine or lysine at the HA1-HA2 cleavage site in a hemagglutinin sequence (*see*, *e.g.*, Stech *et al.*, 2005,

Nature Med. 11(6):683-689). Accordingly, provided herein are influenza hemagglutinin stem domain polypeptides having a valine substitution at the C-terminus of the C-terminal stem segment (i.e., the C-terminus of the HA1 domain). In particular embodiments, provided herein are influenza hemagglutinin stem domain polypeptides comprising any of SEQ ID NOS:50-65 or 158-159 wherein the C-terminal amino acid residue, *e.g.* arginine or lysine, of SEQ ID NOS:50-65 or 158-159 is substituted with a valine residue.

[00144] In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides that are predicted to be resistant to protease cleavage at the junction between HA1 and HA2. Those of skill in the art should recognize that the Arg-Gly sequence spanning HA1 and HA2 is a recognition site for trypsin and is typically cleaved for hemagglutinin activiation. Since the stem domain polypeptides described herein need not be activated, provided herein are influenza hemagglutinin stem domain polypeptides that are predicted to be resistant to protease cleavage. In certain embodiments, provided is any influenza hemagglutinin stem domain polypeptide described herein wherein the protease site spanning HA1 and HA2 is mutated to a sequence that is resistant to protease cleavage. In certain embodiments, provided is any influenza hemagglutinin stem domain polypeptide described herein wherein the C-terminal residue of the HA1 C-terminal stem segment is any residue other than Lys or Arg. In certain embodiments, provided is any influenza hemagglutinin stem domain polypeptide described herein wherein the N-terminal residue of the HA2 domain is proline. In certain embodiments, provided is any influenza hemagglutinin stem domain polypeptide described herein wherein the C-terminal residue of the HA1 C-terminal stem segment is Ala and the N-terminal residue of the HA2 domain is also Ala.

[00145] In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment in binding association with an HA2 stem domain. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment, in turn covalently linked to an HA2 stem domain. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of a signal peptide covalently linked to an HA1 N-terminal stem segment

covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment, in turn covalently linked to an HA2 stem domain.

[00146] In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment in binding association with an HA2 stem domain that is covalently linked to an HA2 luminal domain. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment, in turn covalently linked to an HA2 stem domain that is covalently linked to an HA2 luminal domain. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of a signal peptide covalently linked to an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment, in turn covalently linked to an HA2 stem domain that is covalently linked to an HA2 luminal domain.

[00147] In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment in binding association with an HA2 stem domain that is covalently linked to, in sequence, a thrombin cleavage site, a foldon domain and a His tag. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment, in turn covalently linked to an HA2 stem domain that is covalently linked to, in sequence, a thrombin cleavage site, a foldon domain and a His tag. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of a signal peptide covalently linked to an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment, in turn covalently linked to an HA2 stem domain that is covalently linked to, in sequence, a thrombin cleavage site, a foldon domain and a His tag.

[00148] In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment in binding association with an HA2 stem domain that is covalently linked to an HA2 luminal domain that is covalently linked to, in sequence, a thrombin cleavage site, a foldon

domain and a His tag. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment, in turn covalently linked to an HA2 stem domain that is covalently linked to an HA2 luminal domain that is covalently linked to, in sequence, a thrombin cleavage site, a foldon domain and a His tag. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of a signal peptide covalently linked to an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment, in turn covalently linked to an HA2 stem domain that is covalently linked to an HA2 luminal domain that is covalently linked to, in sequence, a thrombin cleavage site, a foldon domain and a His tag.

[00149] In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment in binding association with an HA2 stem domain that is covalently linked to an HA2 luminal domain that is in turn covalently linked to an HA2 transmembrane domain. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment, in turn covalently linked to an HA2 stem domain that is covalently linked to an HA2 luminal domain that is in turn covalently linked to an HA2 transmembrane domain. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of a signal peptide covalently linked to an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment, in turn covalently linked to an HA2 stem domain that is covalently linked to an HA2 luminal domain that is in turn covalently linked to an HA2 transmembrane domain.

[00150] In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment in binding association with an HA2 stem domain that is covalently linked to an HA2 luminal domain that is in turn covalently linked to an HA2 transmembrane domain that is in turn covalently linked to an HA2 cytoplasmic domain. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of an HA1 N-

terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment, in turn covalently linked to an HA2 stem domain that is covalently linked to an HA2 luminal domain that is in turn covalently linked to an HA2 transmembrane domain that is in turn covalently linked to an HA2 cytoplasmic domain. In certain embodiments, provided herein are influenza hemagglutinin stem domain polypeptides consisting of a signal peptide covalently linked to an HA1 N-terminal stem segment covalently linked to a linker, in turn covalently linked to an HA1 C-terminal stem segment, in turn covalently linked to an HA2 stem domain that is covalently linked to an HA2 luminal domain that is in turn covalently linked to an HA2 transmembrane domain that is in turn covalently linked to an HA2 cytoplasmic domain.

[00151] In certain embodiments, provided herein is an influenza hemagglutinin stem domain polypeptide having a sequence selected from the group consisting of:

(SEQ ID NO:34)-LL-(SEQ ID NO:50)-(SEQ ID NO:66),
(SEQ ID NO:35)-LL-(SEQ ID NO:51)-(SEQ ID NO:67),
(SEQ ID NO:36)-LL-(SEQ ID NO:52)-(SEQ ID NO:68),
(SEQ ID NO:37)-LL-(SEQ ID NO:53)-(SEQ ID NO:69),
(SEQ ID NO:38)-LL-(SEQ ID NO:54)-(SEQ ID NO:70),
(SEQ ID NO:39)-LL-(SEQ ID NO:55)-(SEQ ID NO:71),
(SEQ ID NO:40)-LL-(SEQ ID NO:56)-(SEQ ID NO:72),
(SEQ ID NO:41)-LL-(SEQ ID NO:57)-(SEQ ID NO:73),
(SEQ ID NO:42)-LL-(SEQ ID NO:58)-(SEQ ID NO:74),
(SEQ ID NO:43)-LL-(SEQ ID NO:59)-(SEQ ID NO:75),
(SEQ ID NO:44)-LL-(SEQ ID NO:60)-(SEQ ID NO:76),
(SEQ ID NO:45)-LL-(SEQ ID NO:61)-(SEQ ID NO:77),
(SEQ ID NO:46)-LL-(SEQ ID NO:62)-(SEQ ID NO:78),
(SEQ ID NO:47)-LL-(SEQ ID NO:63)-(SEQ ID NO:79),
(SEQ ID NO:48)-LL-(SEQ ID NO:64)-(SEQ ID NO:80), and
(SEQ ID NO:49)-LL-(SEQ ID NO:65)-(SEQ ID NO:81),

wherein each sequence above is linked to the adjacent sequence as described herein and wherein LL is a linker as described herein. In particular, the HA1 C-terminal segments can be covalently or non-covalently linked to the HA2 domains. In certain embodiments, LL is selected from the group consisting of a direct bond, Gly, Gly-Gly, Gly-Gly-Gly, Gly-Gly-Gly-Gly, (Gly)n (wherein n is any number of Glycine residues so long as there is flexibility in the peptide linker; in certain embodiments, n is 2, 3, 4, 5, 6,

or 7 Glycine residues), Gly-Pro, ITPNGSIPNDKPFQNVNKITYGA (SEQ ID NO:165) and Asn-Ala-Ser.

[00152] In certain embodiments, provided herein is an influenza hemagglutinin stem domain polypeptide having a sequence selected from the group consisting of:
(SEQ ID NO:34)-LL-(SEQ ID NO:50)-(SEQ ID NO:82),
(SEQ ID NO:35)-LL-(SEQ ID NO:51)-(SEQ ID NO:83),
(SEQ ID NO:36)-LL-(SEQ ID NO:52)-(SEQ ID NO:84),
(SEQ ID NO:37)-LL-(SEQ ID NO:53)-(SEQ ID NO:85),
(SEQ ID NO:38)-LL-(SEQ ID NO:54)-(SEQ ID NO:86),
(SEQ ID NO:39)-LL-(SEQ ID NO:55)-(SEQ ID NO:87),
(SEQ ID NO:40)-LL-(SEQ ID NO:56)-(SEQ ID NO:88),
(SEQ ID NO:41)-LL-(SEQ ID NO:57)-(SEQ ID NO:89),
(SEQ ID NO:42)-LL-(SEQ ID NO:58)-(SEQ ID NO:90),
(SEQ ID NO:43)-LL-(SEQ ID NO:59)-(SEQ ID NO:91),
(SEQ ID NO:44)-LL-(SEQ ID NO:60)-(SEQ ID NO:92),
(SEQ ID NO:45)-LL-(SEQ ID NO:61)-(SEQ ID NO:93),
(SEQ ID NO:46)-LL-(SEQ ID NO:62)-(SEQ ID NO:94),
(SEQ ID NO:47)-LL-(SEQ ID NO:63)-(SEQ ID NO:95),
(SEQ ID NO:48)-LL-(SEQ ID NO:64)-(SEQ ID NO:96), and
(SEQ ID NO:49)-LL-(SEQ ID NO:65)-(SEQ ID NO:97),
wherein each sequence above is linked to the adjacent sequence as described herein and wherein LL is a linker as described herein. In particular, the HA1 C-terminal segments can be covalently or non-covalently linked to the HA2 domains. In certain embodiments, LL is selected from the group consisting of a direct bond, Gly, Gly-Gly, Gly-Gly-Gly, Gly-Gly-Gly-Gly, (Gly)n, Gly-Pro, ITPNGSIPNDKPFQNVNKITYGA (SEQ ID NO:165) and Asn-Ala-Ser.

[00153] In certain embodiments, provided herein is an influenza hemagglutinin stem domain polypeptide having a sequence selected from the group consisting of:
(SEQ ID NO:34)-LL-(SEQ ID NO:50)-(SEQ ID NO:82)-(SEQ ID NO:98),
(SEQ ID NO:35)-LL-(SEQ ID NO:51)-(SEQ ID NO:83)-(SEQ ID NO:99),
(SEQ ID NO:36)-LL-(SEQ ID NO:52)-(SEQ ID NO:84)-(SEQ ID NO:100),
(SEQ ID NO:37)-LL-(SEQ ID NO:53)-(SEQ ID NO:85)-(SEQ ID NO:101),
(SEQ ID NO:38)-LL-(SEQ ID NO:54)-(SEQ ID NO:86)-(SEQ ID NO:102),
(SEQ ID NO:39)-LL-(SEQ ID NO:55)-(SEQ ID NO:87)-(SEQ ID NO:103),

(SEQ ID NO:40)-LL-(SEQ ID NO:56)-(SEQ ID NO:88)-(SEQ ID NO:104),
(SEQ ID NO:41)-LL-(SEQ ID NO:57)-(SEQ ID NO:89)-(SEQ ID NO:105),
(SEQ ID NO:42)-LL-(SEQ ID NO:58)-(SEQ ID NO:90)-(SEQ ID NO:106),
(SEQ ID NO:43)-LL-(SEQ ID NO:59)-(SEQ ID NO:91)-(SEQ ID NO:107),
(SEQ ID NO:44)-LL-(SEQ ID NO:60)-(SEQ ID NO:92)-(SEQ ID NO:108),
(SEQ ID NO:45)-LL-(SEQ ID NO:61)-(SEQ ID NO:93)-(SEQ ID NO:109),
(SEQ ID NO:46)-LL-(SEQ ID NO:62)-(SEQ ID NO:94)-(SEQ ID NO:110),
(SEQ ID NO:47)-LL-(SEQ ID NO:63)-(SEQ ID NO:95)-(SEQ ID NO:111),
(SEQ ID NO:48)-LL-(SEQ ID NO:64)-(SEQ ID NO:96)-(SEQ ID NO:112), and
(SEQ ID NO:49)-LL-(SEQ ID NO:65)-(SEQ ID NO:97)-(SEQ ID NO:113),
wherein each sequence above is linked to the adjacent sequence as described herein and wherein LL is a linker as described herein. In particular, the HA1 C-terminal segments can be covalently or non-covalently linked to the HA2 domains. In certain embodiments, LL is selected from the group consisting of a direct bond, Gly, Gly-Gly, Gly-Gly-Gly, Gly-Gly-Gly-Gly, (Gly)n, Gly-Pro, ITPNGSIPNDKPFQNVNKITYGA (SEQ ID NO:165) and Asn-Ala-Ser.

[00154] In certain embodiments, provided herein is an influenza hemagglutinin stem domain polypeptide having a sequence selected from the group consisting of:
(SEQ ID NO:34)-LL-(SEQ ID NO:50)-(SEQ ID NO:82)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:35)-LL-(SEQ ID NO:51)-(SEQ ID NO:83)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:36)-LL-(SEQ ID NO:52)-(SEQ ID NO:84)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:37)-LL-(SEQ ID NO:53)-(SEQ ID NO:85)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:38)-LL-(SEQ ID NO:54)-(SEQ ID NO:86)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:39)-LL-(SEQ ID NO:55)-(SEQ ID NO:87)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:40)-LL-(SEQ ID NO:56)-(SEQ ID NO:88)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:41)-LL-(SEQ ID NO:57)-(SEQ ID NO:89)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),

(SEQ ID NO:42)-LL-(SEQ ID NO:58)-(SEQ ID NO:90)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:43)-LL-(SEQ ID NO:59)-(SEQ ID NO:91)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:44)-LL-(SEQ ID NO:60)-(SEQ ID NO:92)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:45)-LL-(SEQ ID NO:61)-(SEQ ID NO:93)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:46)-LL-(SEQ ID NO:62)-(SEQ ID NO:94)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:47)-LL-(SEQ ID NO:63)-(SEQ ID NO:95)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:48)-LL-(SEQ ID NO:64)-(SEQ ID NO:96)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166), and
(SEQ ID NO:49)-LL-(SEQ ID NO:65)-(SEQ ID NO:97)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
wherein each sequence above is linked to the adjacent sequence as described herein and wherein LL is a linker as described herein. In particular, the HA1 C-terminal segments can be covalently or non-covalently linked to the HA2 domains. In certain embodiments, LL is selected from the group consisting of a direct bond, Gly, Gly-Gly, Gly-Gly-Gly, Gly-Gly-Gly-Gly, (Gly)n, Gly-Pro, ITPNGSIPNDKPFQNVNKITYGA (SEQ ID NO:165) and Asn-Ala-Ser.

[00155] In certain embodiments, provided herein is an influenza hemagglutinin stem domain polypeptide having a sequence selected from the group consisting of:
(SEQ ID NO:34)-LL-(SEQ ID NO:50)-(SEQ ID NO:82)-(SEQ ID NO:98)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:35)-LL-(SEQ ID NO:51)-(SEQ ID NO:83)-(SEQ ID NO:99)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:36)-LL-(SEQ ID NO:52)-(SEQ ID NO:84)-(SEQ ID NO:100)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:37)-LL-(SEQ ID NO:53)-(SEQ ID NO:85)-(SEQ ID NO:101)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:38)-LL-(SEQ ID NO:54)-(SEQ ID NO:86)-(SEQ ID NO:102)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),

(SEQ ID NO:39)-LL-(SEQ ID NO:55)-(SEQ ID NO:87)-(SEQ ID NO:103)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:40)-LL-(SEQ ID NO:56)-(SEQ ID NO:88)-(SEQ ID NO:104)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:41)-LL-(SEQ ID NO:57)-(SEQ ID NO:89)-(SEQ ID NO:105)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:42)-LL-(SEQ ID NO:58)-(SEQ ID NO:90)-(SEQ ID NO:106)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:43)-LL-(SEQ ID NO:59)-(SEQ ID NO:91)-(SEQ ID NO:107)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:44)-LL-(SEQ ID NO:60)-(SEQ ID NO:92)-(SEQ ID NO:108)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:45)-LL-(SEQ ID NO:61)-(SEQ ID NO:93)-(SEQ ID NO:109)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:46)-LL-(SEQ ID NO:62)-(SEQ ID NO:94)-(SEQ ID NO:110)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:47)-LL-(SEQ ID NO:63)-(SEQ ID NO:95)-(SEQ ID NO:111)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
(SEQ ID NO:48)-LL-(SEQ ID NO:64)-(SEQ ID NO:96)-(SEQ ID NO:112)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166), and
(SEQ ID NO:49)-LL-(SEQ ID NO:65)-(SEQ ID NO:97)-(SEQ ID NO:113)-(SEQ ID NO:168)-(SEQ ID NO:167)-(SEQ ID NO:166),
wherein each sequence above is linked to the adjacent sequence as described herein and wherein LL is a linker as described herein. In particular, the HA1 C-terminal segments can be covalently or non-covalently linked to the HA2 domains. In certain embodiments, LL is selected from the group consisting of a direct bond, Gly, Gly-Gly, Gly-Gly-Gly, Gly-Gly-Gly-Gly, Gly-Pro, ITPNGSIPNDKPFQNVNKITYGA (SEQ ID NO:165) and Asn-Ala-Ser.

[00156] In certain embodiments, provided herein is an influenza hemagglutinin stem domain polypeptide having a sequence selected from the group consisting of:
(SEQ ID NO:177)-LL-(SEQ ID NO:226)-(SEQ ID NO:66),
(SEQ ID NO:178)-LL-(SEQ ID NO:227)-(SEQ ID NO:66),
(SEQ ID NO:179)-LL-(SEQ ID NO:228)-(SEQ ID NO:66),
(SEQ ID NO:180)-LL-(SEQ ID NO:229)-(SEQ ID NO:67),

(SEQ ID NO:181)-LL-(SEQ ID NO:230)-(SEQ ID NO:67),
(SEQ ID NO:182)-LL-(SEQ ID NO:231)-(SEQ ID NO:67),
(SEQ ID NO:183)-LL-(SEQ ID NO:232)-(SEQ ID NO:68),
(SEQ ID NO:184)-LL-(SEQ ID NO:233)-(SEQ ID NO:68),
(SEQ ID NO:185)-LL-(SEQ ID NO:234)-(SEQ ID NO:68),
(SEQ ID NO:186)-LL-(SEQ ID NO:235)-(SEQ ID NO:69),
(SEQ ID NO:187)-LL-(SEQ ID NO:236)-(SEQ ID NO:69),
(SEQ ID NO:188)-LL-(SEQ ID NO:237)-(SEQ ID NO:69),
(SEQ ID NO:189)-LL-(SEQ ID NO:238)-(SEQ ID NO:70),
(SEQ ID NO:190)-LL-(SEQ ID NO:239)-(SEQ ID NO:70),
(SEQ ID NO:191)-LL-(SEQ ID NO:240)-(SEQ ID NO:70),
(SEQ ID NO:192)-LL-(SEQ ID NO:241)-(SEQ ID NO:71),
(SEQ ID NO:193)-LL-(SEQ ID NO:242)-(SEQ ID NO:71),
(SEQ ID NO:194)-LL-(SEQ ID NO:243)-(SEQ ID NO:71),
(SEQ ID NO:195)-LL-(SEQ ID NO:244)-(SEQ ID NO:72),
(SEQ ID NO:196)-LL-(SEQ ID NO:245)-(SEQ ID NO:72),
(SEQ ID NO:197)-LL-(SEQ ID NO:246)-(SEQ ID NO:72),
(SEQ ID NO:198)-LL-(SEQ ID NO:247)-(SEQ ID NO:73),
(SEQ ID NO:199)-LL-(SEQ ID NO:248)-(SEQ ID NO:73),
(SEQ ID NO:200)-LL-(SEQ ID NO:249)-(SEQ ID NO:73),
(SEQ ID NO:201)-LL-(SEQ ID NO:250)-(SEQ ID NO:74),
(SEQ ID NO:202)-LL-(SEQ ID NO:251)-(SEQ ID NO:74),
(SEQ ID NO:203)-LL-(SEQ ID NO:252)-(SEQ ID NO:74),
(SEQ ID NO:204)-LL-(SEQ ID NO:253)-(SEQ ID NO:75),
(SEQ ID NO:205)-LL-(SEQ ID NO:254)-(SEQ ID NO:75),
(SEQ ID NO:206)-LL-(SEQ ID NO:255)-(SEQ ID NO:75),
(SEQ ID NO:207)-LL-(SEQ ID NO:256)-(SEQ ID NO:76),
(SEQ ID NO:208)-LL-(SEQ ID NO:257)-(SEQ ID NO:76),
(SEQ ID NO:209)-LL-(SEQ ID NO:258)-(SEQ ID NO:76),
(SEQ ID NO:210)-LL-(SEQ ID NO:259)-(SEQ ID NO:77),
(SEQ ID NO:211)-LL-(SEQ ID NO:260)-(SEQ ID NO:77),
(SEQ ID NO:212)-LL-(SEQ ID NO:261)-(SEQ ID NO:77),
(SEQ ID NO:213)-LL-(SEQ ID NO:262)-(SEQ ID NO:78),
(SEQ ID NO:214)-LL-(SEQ ID NO:263)-(SEQ ID NO:78),

(SEQ ID NO:215)-LL-(SEQ ID NO:264)-(SEQ ID NO:78),
(SEQ ID NO:216)-LL-(SEQ ID NO:265)-(SEQ ID NO:79),
(SEQ ID NO:217)-LL-(SEQ ID NO:266)-(SEQ ID NO:79),
(SEQ ID NO:218)-LL-(SEQ ID NO:267)-(SEQ ID NO:79),
(SEQ ID NO:219)-LL-(SEQ ID NO:268)-(SEQ ID NO:80),
(SEQ ID NO:220)-LL-(SEQ ID NO:269)-(SEQ ID NO:80),
(SEQ ID NO:221)-LL-(SEQ ID NO:270)-(SEQ ID NO:80),
(SEQ ID NO:222)-LL-(SEQ ID NO:271)-(SEQ ID NO:81),
(SEQ ID NO:223)-LL-(SEQ ID NO:272)-(SEQ ID NO:81),
(SEQ ID NO:224)-LL-(SEQ ID NO:273)-(SEQ ID NO:81),
(SEQ ID NO:309)-LL-(SEQ ID NO:310)-(SEQ ID NO:66), and
(SEQ ID NO:308)-LL-(SEQ ID NO:52)-(SEQ ID NO:68),
(wherein each sequence above is linked to the adjacent sequence as described herein and wherein LL is a linker as described herein. In particular, the HA1 C-terminal segments can be covalently or non-covalently linked to the HA2 domains. In certain embodiments, LL is selected from the group consisting of a direct bond, Gly, Gly-Gly, Gly-Gly-Gly, Gly-Gly-Gly-Gly, (Gly)n, Gly-Pro, ITPNGSIPNDKPFQNVNKITYGA (SEQ ID NO:165) and Asn-Ala-Ser.

[00157] In certain embodiments, provided herein is an influenza hemagglutinin stem domain polypeptide having a sequence selected from the group consisting of:
(SEQ ID NO:154)-LL-(SEQ ID NO:158)-(SEQ ID NO:160),
(SEQ ID NO:155)-LL-(SEQ ID NO:159)-(SEQ ID NO:160),
(SEQ ID NO:156)-LL-(SEQ ID NO:158)-(SEQ ID NO:160), and
(SEQ ID NO:157)-LL-(SEQ ID NO:159)-(SEQ ID NO:160),
wherein each sequence above is linked to the adjacent sequence as described herein and wherein LL is a linker as described herein. In particular, the HA1 C-terminal segments can be covalently or non-covalently linked to the HA2 domains. In certain embodiments, LL is selected from the group consisting of a direct bond, Gly, Gly-Gly, Gly-Gly-Gly, Gly-Gly-Gly-Gly, (Gly)n, Gly-Pro, ITPNGSIPNDKPFQNVNKITYGA (SEQ ID NO:165) and Asn-Ala-Ser.

[00158] In certain embodiments, provided herein is an influenza hemagglutinin stem domain polypeptide having a sequence selected from the group consisting of:
(SEQ ID NO:154)-LL-(SEQ ID NO:158)-(SEQ ID NO:161),
(SEQ ID NO:155)-LL-(SEQ ID NO:159)-(SEQ ID NO:161),

(SEQ ID NO:156)-LL-(SEQ ID NO:158)-(SEQ ID NO:161), and
(SEQ ID NO:157)-LL-(SEQ ID NO:159)-(SEQ ID NO:161),
wherein each sequence above is linked to the adjacent sequence as described herein and wherein LL is a linker as described herein. In particular, the HA1 C-terminal segments can be covalently or non-covalently linked to the HA2 domains. In certain embodiments, LL is selected from the group consisting of a direct bond, Gly, Gly-Gly, Gly-Gly-Gly, Gly-Gly-Gly-Gly, (Gly)n, Gly-Pro, ITPNGSIPNDKPFQNVNKITYGA (SEQ ID NO:165) and Asn-Ala-Ser.

[00159] In certain embodiments, provided herein is an influenza hemagglutinin stem domain polypeptide having a sequence selected from the group consisting of:
(SEQ ID NO:154)-LL-(SEQ ID NO:158)-(SEQ ID NO:161)-(SEQ ID NO:162),
(SEQ ID NO:155)-LL-(SEQ ID NO:159)-(SEQ ID NO:161)-(SEQ ID NO:162),
(SEQ ID NO:156)-LL-(SEQ ID NO:158)-(SEQ ID NO:161)-(SEQ ID NO:162), and
(SEQ ID NO:157)-LL-(SEQ ID NO:159)-(SEQ ID NO:161)-(SEQ ID NO:162),
wherein each sequence above is linked to the adjacent sequence as described herein and wherein LL is a linker as described herein. In particular, the HA1 C-terminal segments can be covalently or non-covalently linked to the HA2 domains. In certain embodiments, LL is selected from the group consisting of a direct bond, Gly, Gly-Gly, Gly-Gly-Gly, Gly-Gly-Gly-Gly, (Gly)n, Gly-Pro, ITPNGSIPNDKPFQNVNKITYGA (SEQ ID NO:165) and Asn-Ala-Ser.

[00160] In certain embodiments, provided herein is an influenza hemagglutinin stem domain polypeptide having a sequence selected from the group consisting of:
(SEQ ID NO:154)-LL-(SEQ ID NO:158)-(SEQ ID NO:161)-(SEQ ID NO:168)-(SEQ ID NO:167)-SEQ ID NO:166),
(SEQ ID NO:155)-LL-(SEQ ID NO:159)-(SEQ ID NO:161)-(SEQ ID NO:168)-(SEQ ID NO:167)-SEQ ID NO:166),
(SEQ ID NO:156)-LL-(SEQ ID NO:158)-(SEQ ID NO:161)-(SEQ ID NO:168)-(SEQ ID NO:167)-SEQ ID NO:166), and
(SEQ ID NO:157)-LL-(SEQ ID NO:159)-(SEQ ID NO:161)-(SEQ ID NO:168)-(SEQ ID NO:167)-SEQ ID NO:166),
wherein each sequence above is linked to the adjacent sequence as described herein and wherein LL is a linker as described herein. In particular, the HA1 C-terminal segments can be covalently or non-covalently linked to the HA2 domains. In certain embodiments, LL is selected from the group consisting of a direct bond, Gly, Gly-Gly,

Gly-Gly-Gly, Gly-Gly-Gly-Gly, (Gly)n, Gly-Pro, ITPNGSIPNDKPFQNVNKITYGA (SEQ ID NO:165) and Asn-Ala-Ser.

[00161] In certain embodiments, provided herein is an influenza hemagglutinin stem domain polypeptide having a sequence selected from the group consisting of:
(SEQ ID NO:154)-LL-(SEQ ID NO:158)-(SEQ ID NO:161)-(SEQ ID NO:162)-(SEQ ID NO:168)-(SEQ ID NO:167)-SEQ ID NO:166),
(SEQ ID NO:155)-LL-(SEQ ID NO:159)-(SEQ ID NO:161)-(SEQ ID NO:162)-(SEQ ID NO:168)-(SEQ ID NO:167)-SEQ ID NO:166),
(SEQ ID NO:156)-LL-(SEQ ID NO:158)-(SEQ ID NO:161)-(SEQ ID NO:162)-(SEQ ID NO:168)-(SEQ ID NO:167)-SEQ ID NO:166), and
(SEQ ID NO:157)-LL-(SEQ ID NO:159)-(SEQ ID NO:161)-(SEQ ID NO:162)-(SEQ ID NO:168)-(SEQ ID NO:167)-SEQ ID NO:166),
wherein each sequence above is linked to the adjacent sequence as described herein and wherein LL is a linker as described herein. In particular, the HA1 C-terminal segments can be covalently or non-covalently linked to the HA2 domains. In certain embodiments, LL is selected from the group consisting of a direct bond, Gly, Gly-Gly, Gly-Gly-Gly, Gly-Gly-Gly-Gly, (Gly)n, Gly-Pro, ITPNGSIPNDKPFQNVNKITYGA (SEQ ID NO:165) and Asn-Ala-Ser.

[00162] In certain embodiments, the influenza hemagglutinin polypeptides described herein do not comprise polypeptides having the amino acid sequence of either Thr-Gly-Leu-Arg-Asn (SEQ ID NO:313) or Gly-Ile-Thr-Asn-Lys-Val-Asn-Ser-Val-Ile-Glu-Lys (SEQ ID NO:314). In certain embodiments, the influenza hemagglutinin polypeptides described herein do not comprise polypeptides having the amino acid sequence of Thr-Gly-Leu-Arg-Asn (SEQ ID NO:313) and Gly-Ile-Thr-Asn-Lys-Val-Asn-Ser-Val-Ile-Glu-Lys (SEQ ID NO:314). In certain embodiments, the influenza hemagglutinin polypeptides described herein do not comprise polypeptides having the amino acid sequence of either Thr-Gly-Met-Arg-Asn (SEQ ID NO:316) or Gln-Ile-Asn-Gly-Lys-Leu-Asn-Arg-Leu-Ile-Glu-Lys (SEQ ID NO:317). In certain embodiments, the influenza hemagglutinin polypeptides described herein do not comprise polypeptides having the amino acid sequence of Thr-Gly-Met-Arg-Asn (SEQ ID NO:316) and Gln-Ile-Asn-Gly-Lys-Leu-Asn-Arg-Leu-Ile-Glu-Lys (SEQ ID NO:317). In certain embodiments, the influenza hemagglutinin polypeptides described herein do not comprise polypeptides having the amino acid sequence of either Thr-Gly-Met-Arg-Asn (SEQ ID NO:316) or Gln-Ile-Asn-Gly-Lys-Leu-Asn-Arg-Val-Ile-Glu-Lys (SEQ ID

NO:318). In certain embodiments, the influenza hemagglutinin polypeptides described herein do not comprise polypeptides having the amino acid sequence of Thr-Gly-Met-Arg-Asn (SEQ ID NO:316) and Gln-Ile-Asn-Gly-Lys-Leu-Asn-Arg-Val-Ile-Glu-Lys (SEQ ID NO:318).

[00163] In certain embodiments, the influenza hemagglutinin polypeptides described herein are not recognized or bound by the antibody C179 (produced by hybridoma FERM BP-4517; clones sold by Takara Bio, Inc. (Otsu, Shiga, Japan)) or by the antibody AI3C (FERM BP-4516).

5.2 NUCLEIC ACIDS ENCODING INFLUENZA HEMAGGLUTININ STEM DOMAIN POLYPEPTIDES

[00164] Provided herein are nucleic acids that encode an influenza hemagglutinin stem domain polypeptide. In a specific embodiment, provided herein is a nucleic acid that encodes an influenza virus hemagglutinin stem domain polypeptide. Due to the degeneracy of the genetic code, any nucleic acid that encodes an influenza hemagglutinin stem domain polypeptide described herein is encompassed herein. In certain embodiments, nucleic acids corresponding to naturally occurring influenza virus nucleic acids encoding an HA1 N-terminal stem segment, an HA1 C-terminal stem segment, HA2 domain, luminal domain, transmembrane domain, and/or cytoplasmic domain are used to produce an influenza hemagglutinin stem domain polypeptide.

[00165] Also provided herein are nucleic acids capable of hybridizing to a nucleic acid encoding an influenza hemagglutinin stem domain polypeptide. In certain embodiments, provided herein are nucleic acids capable of hybridizing to a fragment of a nucleic acid encoding an influenza hemagglutinin stem domain polypeptide. In other embodiments, provided herein are nucleic acids capable of hybridizing to the full length of a nucleic acid encoding an influenza hemagglutinin stem domain 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.

[00166] In some embodiments, a nucleic acid encoding an influenza virus hemagglutinin stem domain polypeptide is isolated. In certain embodiments, an "isolated" nucleic acid refers to a nucleic acid molecule which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. In other words, the isolated nucleic acid can comprise heterologous nucleic acids that are not associated with it in nature. In other embodiments, an "isolated" nucleic acid, 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 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.

[00167] In addition, provided herein are nucleic acids encoding the individual components of an influenza hemagglutinin stem domain polypeptide. In specific embodiments, nucleic acids encoding an HA1 N-terminal stem segment, an HA1 C-terminal stem segment and/or HA2 domain are provided. Nucleic acids encoding components of an influenza hemagglutinin stem domain polypeptide may be assembled using standard molecular biology techniques known to the one of skill in the art.

5.3 EXPRESSION OF INFLUENZA HEMAGGLUTININ STEM DOMAIN POLYPEPTIDES

[00168] Provided herein are vectors, including expression vectors, containing a nucleic acid encoding an influenza hemagglutinin stem domain polypeptide. In a specific embodiment, the vector is an expression vector that is capable of directing the expression of a nucleic acid encoding an influenza hemagglutinin stem domain 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.

[00169] In some embodiments, provided herein are expression vectors encoding components of an influenza hemagglutinin stem domain polypeptide (*e.g.*, HA1 N-terminal stem segment, an HA1 C-terminal stem segment and/or an HA2). Such vectors may be used to express the components in one or more host cells and the components may be isolated and conjugated together with a linker using techniques known to one of skill in the art.

[00170] An expression vector comprises a nucleic acid encoding an influenza hemagglutinin stem domain polypeptide in a form suitable for expression of the nucleic acid 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 only in certain host cells (*e.g.*, tissue-specific regulatory sequences), and those which direct the expression of the nucleic acid 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.

[00171] Expression vectors can be designed for expression of an influenza hemagglutinin stem domain polypeptide 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 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 hosts 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 *Sf*9, *Sf*21, *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 hemagglutinin stem domain polypeptide. In another embodiment, a plant cell culture sytem is used for expression of an influenza hemagglutinin stem domain 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.

[00172] 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 encoding an influenza hemagglutinin stem domain polypeptide. In other embodiments, a host cell is stably transfected with an

expression vector containing a nucleic acid encoding an influenza hemagglutinin stem domain polypeptide.

[00173] 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).

[00174] As an alternative to recombinant expression of an influenza hemagglutinin stem domain polypeptide using a host cell, an expression vector containing a nucleic acid encoding an influenza hemagglutinin stem domain 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 hemagglutinin stem domain polypeptide.

[00175] Once an influenza hemagglutinin stem domain 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 hemagglutinin stem domain polypeptide may be conjugated to heterologous proteins, e.g., a major histocompatibility complex (MHC) with or without heat shock proteins (e.g., Hsp10, Hsp20, Hsp30, Hsp40, Hsp60, Hsp70, Hsp90, or Hsp100). In certain embodiments, an influenza hemagglutinin stem domain polypeptide may be conjugated to immunomodulatory molecules, such as proteins which would target the influenza hemagglutinin stem domain polypeptide to immune cells such as B cells (e.g., C3d) or T cells. In certain embodiments, an influenza hemagglutinin stem domain polypeptide may be conjugated to proteins which stimulate the innate immune system such as interferon type 1, alpha, beta, or gamma interferon, colony stimulating factors

such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, IL-15, IL-18, IL-21, IL-23, tumor necrosis factor (TNF)-β, TNFα, B7.1, B7.2, 4-1BB, CD40 ligand (CD40L), and drug-inducible CD40 (iCD40).

[00176] Accordingly, provided herein are methods for producing an influenza hemagglutinin stem domain polypeptide. In one embodiment, the method comprises culturing a host cell containing a nucleic acid encoding the 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.

5.4 INFLUENZA VIRUS VECTORS

[00177] In one aspect, provided herein are influenza viruses containing an influenza hemagglutinin stem domain polypeptide. In a specific embodiment, the influenza hemagglutinin stem domain polypeptide is incorporated into the virions of the influenza virus. The 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 influenza virus have incorporated into them or express a heterologous polypeptide in addition to an influenza hemagglutinin stem domain 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.

[00178] Influenza viruses containing an influenza hemagglutinin stem domain polypeptide may be produced by supplying in *trans* the influenza hemagglutinin stem domain polypeptide during production of virions using techniques known to one skilled in the art, such as reverse genetics and helper-free plasmid rescue. Alternatively, the replication of a parental influenza virus comprising a genome engineered to express an influenza hemagglutinin stem domain polypeptide in cells susceptible to infection with the virus wherein hemagglutinin function is provided in *trans* will produce progeny influenza viruses containing the influenza hemagglutinin stem domain polypeptide.

[00179] In another aspect, provided herein are influenza viruses comprising a genome engineered to express an influenza hemagglutinin stem domain polypeptide. In a specific embodiment, the genome of a parental influenza virus is engineered to encode an influenza hemagglutinin stem domain 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 hemagglutinin stem domain 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 contain an influenza hemagglutinin stem domain polypeptide. The virions of the parental influenza virus may have incorporated into them an influenza virus hemagglutinin polypeptide that is from the same or a different type, subtype or strain of influenza virus. Alternatively, the virions of the parental influenza virus may have incorporated into them a moiety that is capable of functionally replacing one or more of the activities of influenza virus hemagglutinin polypeptide (*e.g.*, the receptor binding and/or fusogenic activities of influenza virus hemagglutinin). In certain embodiments, one or more of the activities of the influenza virus hemagglutinin polypeptide is provided by a fusion protein comprising (i) an ectodomain of a polypeptide heterologous to influenza virus fused to (ii) a transmembrane domain, or a transmembrane domain and a cytoplasmic domain of an influenza virus hemagglutinin polypeptide. In a specific embodiment, the virions of the parental influenza virus may have incorporated into them a fusion protein comprising (i) an ectodomain of a receptor binding/fusogenic polypeptide of an infectious agent other than influenza virus fused to (ii) a transmembrane domain, or a transmembrane domain and a cytoplasmic domain of an influenza virus hemagglutinin. For a description of fusion proteins that provide one or more activities of an influenza virus hemagglutinin polypeptide and methods for the production of influenza viruses engineered to express such fusion proteins, see, *e.g.*, International patent application Publication No. WO 2007/064802, published June 7, 2007, which is incorporated herein by reference in its entirety.

[00180] In some embodiments, the virions of the parental influenza virus have incorporated into them a heterologous polypeptide. In certain embodiments, the genome of a parental influenza virus is engineered to encode a heterologous polypeptide and an influenza virus hemagglutinin stem domain polypeptide, which are expressed by progeny influenza virus. In specific embodiments, the influenza hemagglutinin stem domain polypeptide, the heterologous polypeptide or both are incorporated into virions of the progeny influenza virus.

[00181] The heterologous polypeptide may be a polypeptide that targets the 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. In

some embodiments, the targeting polypeptide replaces the target cell recognition function of the virus. In a specific embodiment, the heterologous polypeptide targets the influenza virus to the same cell types that influenza virus infects in nature. In other specific embodiments, the heterologous polypeptide targets the progeny influenza virus to immune cells, such as B cells, T cells, macrophages or dendritic cells. In some embodiments, the heterologous polypeptide recognizes and binds to cell-specific markers of antigen presenting cells, such as dendritic cells (*e.g.*, such as CD44). In one embodiment, the heterologous polypeptide is DC-SIGN which targets the virus to dendritic cells. In another embodiment, the heterologous polypeptide is an antibody (*e.g.*, a single-chain antibody) that targets the virus to an immune cell, which may be fused with a transmembrane domain from another polypeptide so that it is incorporated into the influenza virus virion. In some embodiments, the antibody is a CD20 antibody, a CD34 antibody, or an antibody against DEC-205. Techniques for engineering viruses to express polypeptides with targeting functions are known in the art. See, *e.g.*, Yang *et al.*, 2006, PNAS 103: 11479-11484 and United States patent application Publication No. 20080019998, published January 24, 2008, and No. 20070020238, published January 25, 2007, the contents of each of which are incorporated herein in their entirety.

[00182] In another embodiment, the heterologous polypeptide is a viral attachment protein. Non-limiting examples of viruses whose attachment protein(s) can be used in this aspect are viruses selected from the group of: Lassa fever virus, Hepatitis B virus, Rabies virus, Newcastle disease virus (NDV), a retrovirus such as human immunodeficiency virus, tick-borne encephalitis virus, vaccinia virus, herpesvirus, poliovirus, alphaviruses such as Semliki Forest virus, Ross River virus, and Aura virus (which comprise surface glycoproteins such as E1, E2, and E3), Borna disease virus, Hantaan virus, foamyvirus, and SARS-CoV virus.

[00183] In one embodiment, a flavivirus surface glycoprotein may be used, such as Dengue virus (DV) E protein. In some embodiments, a Sindbis virus glycoprotein from the alphavirus family is used (K. S. Wang, R. J. Kuhn, E. G. Strauss, S. Ou, J. H. Strauss, J. Virol. 66, 4992 (1992)). In certain embodiments, the heterologous polypeptide is derived from an NDV HN or F protein; a human immunodeficiency virus (HIV) gp160 (or a product thereof, such as gp41 or gp120); a hepatitis B virus surface antigen (HBsAg); a glycoprotein of herpesvirus (e.g., gD, gE); or VP1 of poliovirus.

[00184] In another embodiment, the heterologous polypeptide is derived from any non-viral targeting system known in the art. In certain embodiments, a protein of a

nonviral pathogen such as an intracellular bacteria or protozoa is used. In some embodiments, the bacterial polypeptide is provided by, *e.g.*, Chlamydia, Rikettsia, Coxelia, Listeria, Brucella, or Legionella. In some embodiments, protozoan polypeptide is provided by, *e.g.*, Plasmodia species, *Leishmania spp.*, *Toxoplasma gondii*, or *Trypanosoma cruzi*. Other exemplary targeting systems are described in Waehler *et al.*, 2007, "Engineering targeted viral vectors for gene therapy," Nature Reviews Genetics 8: 573-587, which is incorporated herein in its entirety.

[00185] In certain embodiments, the heterologous polypeptide expressed by an influenza virus has immunopotentiating (immune stimulating) activity. Non-limiting examples of immunopotentiating polypeptides include, but are not limited to, stimulation molecules, cytokines, chemokines, antibodies and other agents such as Flt-3 ligands. Specific examples of polypeptides with immunopotentiating activity include: interferon type 1, alpha, beta, or gamma interferon, colony stimulating factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, IL-15, IL-18, IL-21, IL-23, tumor necrosis factor (TNF)-β, TNFα., B7.1, B7.2, 4-1BB, CD40 ligand (CD40L), and drug-inducible CD40 (iCD40) (see, *e.g.*, Hanks, B. A., *et al.* 2005. Nat Med 11:130-137, which is incorporated herein by reference in its entirety.)

[00186] 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 hemagglutinin stem domain polypeptide (and any other polypeptide, such as a heterologous 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 hemagglutinin stem domain 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). In a specific embodiment, the recombinant segment uses the 3' and 5' noncoding and/or nontranslated sequences of segments of influenza viruses that are from a different or the same type, subtype or strain as the parental influenza virus. In some embodiments, the recombinant segment comprises the 3' noncoding region of an influenza virus hemagglutinin polypeptide, the untranslated regions of an

influenza virus hemagglutinin polypeptide, and the 5' non-coding region of an influenza virus hemagglutinin polypeptide. In specific embodiments, the recombinant segment comprises the 3' and 5' noncoding and/or nontranslated sequences of the HA segment of an influenza virus that is the same type, subtype or strain as the influenza virus type, subtype or strain as the HA1 N-terminal stem segment, the HA1 C-terminal stem segment and/or the HA2 of an influenza hemagglutinin stem domain polypeptide. In certain embodiments, the recombinant segment encoding the influenza hemagglutinin stem domain polypeptide may replace the HA segment of a parental influenza virus. In some embodiments, the recombinant segment encoding the influenza hemagglutinin stem domain polypeptide may replace the NS1 gene of the parental influenza virus. In some embodiments, the recombinant segment encoding the influenza hemagglutinin stem domain polypeptide may replace the NA gene of the parental influenza virus. Exemplary influenza virus strains that can be used to express the influenza hemagglutinin stem domain polypeptides include Ann Arbor/1/50, A/Puerto Rico/8/34, A/South Dakota/6/2007, A/Uruguay/716/2007, and B/Brisbane/60/2008.

[00187] In some embodiments, the genome of a parental influenza virus may be engineered to express an influenza hemagglutinin stem domain 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 hemagglutinin stem domain polypeptide and another polypeptide, such as gene expressed by the parental influenza virus. In some embodiments, the parental influenza virus gene is the HA gene. In some embodiments, the parental influenza virus gene is the NA gene. In some embodiments, the parental influenza virus gene is the NS1 gene.

[00188] Techniques known to one skilled in the art may be used to produce an influenza virus containing an influenza hemagglutinin stem domain polypeptide and an influenza virus comprising a genome engineered to express an influenza hemagglutinin stem domain 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.

[00189] Alternatively, helper-free plasmid technology may be used to produce an influenza virus containing an influenza hemagglutinin stem domain polypeptide and an influenza virus comprising a genome engineered to express an influenza hemagglutinin stem domain 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.

[00190] 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. In a specific embodiment, an attenuated influenza virus by virtue of, *e.g.*, a mutation in the NS1 gene, may be propagated in an IFN-deficient substrate. For example, a suitable IFN-deficient substrate may be one that is defective in its ability to produce or respond to interferon, or is one which An IFN-deficient substrate may be used for the growth of any number of viruses which may require interferon-deficient growth environment. See, for example, U.S. Patent Nos. 6,573,079, issued June 3, 2003, 6,852,522, issued February 8, 2005, and 7,494,808, issued February 24, 2009, the entire contents of each of which is incorporated herein by reference in its entirety.

[00191] 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.

[00192] 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.

[00193] 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.

[00194] 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.

[00195] 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.

[00196] Specific examples of strains of influenza A virus include, but are not limited to: 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/Egglham/IDT5250/05 (H3N2); A/sw/Harkenblek/IDT4097/05 (H3N2); A/sw/Hertzen/IDT4317/05 (H3N2); A/sw/Krogel/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/Sprenge/IDT3805/05 (H1N2); A/sw/Stadtlohn/IDT3853/05 (H1N2); A/sw/Voglarn/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).

[00197] 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 (H1 N1); 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).

[00198] Non-limiting examples of influenza B viruses include strain Aichi/5/88, strain 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 ncheon/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 Lissabon/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, and strain Rochester/02/2001.

[00199] 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.

[00200] 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.

[00201] 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.

[00202] In one embodiment, an influenza virus may be attenuated, at least in part, by virtue of substituting the HA gene of the parental influenza virus with an influenza hemagglutinin stem domain polypeptide described herein. In some embodiments, an influenza virus may be attenuated, at least in part, by engineering the influenza virus to express a mutated NS1 gene that impairs the ability of the virus to antagonize the cellular interferon (IFN) response. Examples of the types of mutations that can be

introduced into the influenza virus NS1 gene include deletions, substitutions, insertions and combinations thereof. One or more mutations can be introduced anywhere throughout the NS1 gene (*e.g.*, the N-terminus, the C-terminus or somewhere in between) and/or the regulatory element of the NS1 gene. In one embodiment, an attenuated influenza virus comprises a genome having a mutation in an influenza virus NS1 gene resulting in a deletion consisting of 5, preferably 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 75, 80, 85, 90, 95, 99, 100, 105, 110, 115, 120, 125, 126, 130, 135, 140, 145, 150, 155, 160, 165, 170 or 175 amino acid residues from the C-terminus of NS1, or a deletion of between 5-170, 25-170, 50-170, 100-170, 100-160, or 105-160 amino acid residues from the C-terminus. In another embodiment, an attenuated influenza virus comprises a genome having a mutation in an influenza virus NS1 gene such that it encodes an NS1 protein of amino acid residues 1-130, amino acid residues 1-126, amino acid residues 1-120, amino acid residues 1-115, amino acid residues 1-110, amino acid residues 1-100, amino acid residues 1-99, amino acid residues 1-95, amino acid residues 1-85, amino acid residues 1-83, amino acid residues 1-80, amino acid residues 1-75, amino acid residues 1-73, amino acid residues 1-70, amino acid residues 1-65, or amino acid residues 1-60, wherein the N-terminus amino acid is number 1. For examples of NS1 mutations and influenza viruses comprising a mutated NS1, see, *e.g.*, U.S. Patent Nos. 6,468,544 and 6,669,943; and Li *et al.*, 1999, J. Infect. Dis. 179:1132-1138, each of which is incorporated by reference herein in its entirety.

5.5 NON-INFLUENZA VIRUS VECTORS

[00203] In one aspect, provided herein are non-influenza viruses containing an influenza hemagglutinin stem domain polypeptide. In a specific embodiment, the influenza hemagglutinin stem domain polypeptide is incorporated into the virions of the non-influenza 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 hemagglutinin stem domain 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. See Section 5.4 *supra* for examples of such heterologous polypeptides.

[00204] Non-influenza viruses containing an influenza hemagglutinin stem domain polypeptide may be produced by supplying in *trans* the influenza hemagglutinin stem domain polypeptide during production of virions using techniques known to one skilled in the art. Alternatively, the replication of a parental non-influenza virus comprising a genome engineered to express an influenza hemagglutinin stem domain polypeptide in cells susceptible to infection with the virus wherein hemagglutinin function is provided in *trans* will produce progeny viruses containing the influenza hemagglutinin stem domain polypeptide.

[00205] 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 hemagglutinin stem domain polypeptide described herein.

[00206] 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.

[00207] In a specific embodiment, the non-influenza virus vector is NDV. Any NDV type, subtype or strain may serve as the backbone that is engineered to express an influenza hemagglutinin stem domain polypeptide, including, but not limited to, naturally-occurring strains, variants or mutants, mutagenized viruses, reassortants and/or genetically engineered viruses. In a specific embodiment, the NDV that serves as the backbone for genetic engineering is a naturally-occurring strain. In certain embodiments, the NDV that serves as the backbone for genetic engineering is a lytic strain. In other embodiments, the NDV that serves as the backbone for genetic engineering is a non-lytic strain. In certain embodiments, the NDV that serves as the backbone for genetic engineering is lentogenic strain. In some embodiments, the NDV that serves as the backbone for genetic engineering is a mesogenic strain. In other embodiments, the NDV that serves as the backbone for genetic engineering is a velogenic strain. Specific examples of NDV strains include, but are not limited to, the 73-T strain, Ulster strain, MTH-68 strain, Italien strain, Hickman strain, PV701 strain, Hitchner B1 strain, La Sota strain, YG97 strain, MET95 strain, and F48E9 strain. In a specific embodiment, the NDV that serves as the backbone for genetic engineering is the Hitchner B1 strain. In another specific embodiment, the NDV that serves as the backbone for genetic engineering is the La Sota strain.

[00208] In one embodiment, the NDV used as the backbone for a non-influenza virus vector is engineered to express a modified F protein in which the cleavage site of the F protein is replaced with one containing one or two extra arginine residues, allowing the mutant cleavage site to be activated by ubiquitously expressed proteases of the furin family. Specific examples of NDVs that express such a modified F protein include, but are not limited to, rNDV/F2aa and rNDV/F3aa. For a description of mutations introduced into a NDV F protein to produce a modified F protein with a mutated cleavage site, see, e.g., Park *et al.* (2006) "Engineered viral vaccine constructs with dual specificity: Avian influenza and Newcastle disease." PNAS USA 103: 8203-2808, which is incorporated herein by reference in its entirety.

[00209] In one embodiment, the non-influenza virus vector is a poxvirus. A poxvirus vector may be based on any member of the poxviridae, in particular, a vaccinia virus or

an avipox virus (*e.g.*, such as canarypox, fowlpox, etc.) that provides suitable sequences for vaccine vectors. In a specific embodiment, the poxviral vector is a vaccinia virus vector. Suitable vaccinia viruses include, but are not limited to, the Copenhagen (VC-2) strain (Goebel, *et al.*, Virol 179: 247-266, 1990; Johnson, *et al.*, Virol. 196: 381-401, 1993), modified Copenhagen strain (NYVAC) (U.S. Pat. No. 6,265,189), the WYETH strain and the modified Ankara (MVA) strain (Antoine, *et al.*, Virol. 244: 365-396, 1998). Other suitable poxviruses include fowlpox strains such as ALVAC and TROVAC vectors that provide desirable properties and are highly attenuated (*see*, *e.g.*, U.S. Pat. No. 6,265,189; Tartaglia *et al.*, In AIDS Research Reviews, Koff, *et al.*, eds., Vol. 3, Marcel Dekker, N.Y., 1993; and Tartaglia *et al.*, 1990, Reviews in Immunology 10: 13-30, 1990).

[00210] Methods of engineering non-influenza viruses to express an influenza hemagglutinin stem domain polypeptide 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.

[00211] 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 hemagglutinin stem domain polypeptide expressed by the non-influenza virus vector.

5.6 VIRAL-LIKE PARTICLES AND VIROSOMES

[00212] Influenza hemagglutinin stem domain polypeptides can be incorporated into viral-like particle (VLP) vectors. 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. In some embodiments, the VLPs comprise an influenza hemagglutinin stem domain polpeptide and a viral structural protein, such as HIV gag. In a specific embodiment, the VLPs comprise an influenza hemagglutinin stem domain polypeptide and an HIV gag polypeptide, such as described in Example 2 in Section 6.2 below.

[00213] 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 (Hagnesee *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.

[00214] In a specific embodiment, an influenza hemagglutinin stem domain polypeptide may be incorporated into a virosome. A virosome containing an influenza hemagglutinin stem domain 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 hemagglutinin stem domain polypeptide) and lipids to form lipid particles containing viral proteins.

5.7 BACTERIAL VECTORS

[00215] In a specific embodiment, bacteria may be engineered to express an influenza hemagglutinin stem domain polypeptide described herein. Suitable bacteria for expression of an influenza virus hemagglutinin stem domain include, but are not limited to, *Listeria*, *Salmonella*, *Shigella sp.*, *Mycobacterium tuberculosis*, *E. coli*, *Neisseria*

meningitides, *Brucella abortus*, *Brucella melitensis*, *Borrelia burgdorferi*, and *Francisella tularensis*. In a specific embodiment, the bacteria engineered to express an influenza hemagglutinin stem domain polypeptide are attenuated. Techniques for the production of bacteria engineered to express a heterologous polypeptide are known in the art and can be applied to the expression of an influenza hemagglutinin stem domain polypeptide. *See*, *e.g.*, United States Patent Application Publication No. 20080248066, published October 9, 2008, and United States Patent Application Publication No. 20070207171, published September 6, 2007, each of which are incorporated by reference herein in their entirety.

5.8 PLANT AND ALGAE VECTORS

[00216] In certain embodiments, plants (e.g., plants of the genus *Nicotiana*) may be engineered to express an influenza hemagglutinin stem domain polypeptide described herein. In specific embodiments, plants are engineered to express an influenza hemagglutinin stem domain polypeptide described herein via an agroinfiltration procedure using methods known in the art. For example, nucleic acids encoding a gene of interest, e.g., a gene encoding influenza hemagglutinin stem domain polypeptide described herein, are introduced into a strain of Agrobacterium. Subsequently the strain is grown in a liquid culture and the resulting bacteria are washed and suspended into a buffer solution. The plants are then exposed (e.g., via injection or submersion) to the Agrobacterium that comprises the nucleic acids encoding an influenza hemagglutinin stem domain polypeptide described herein such that the Agrobacterium transforms the gene of interest to a portion of the plant cells. The influenza hemagglutinin stem domain polypeptide is then transiently expressed by the plant and can isolated using methods known in the art and described herein. (For specific examples see Shoji et al., 2008, Vaccine, 26(23):2930-2934; and D'Aoust et al., 2008, J. Plant Biotechnology, 6(9):930-940). In a specific embodiment, the plant is a tobacco plant (i.e., *Nicotiana tabacum*). In another specific embodiment, the plant is a relative of the tobacco plant (e.g., *Nicotiana benthamiana*).

[00217] In other embodiments, algae (e.g., *Chlamydomonas reinhardtii*) may be engineered to express an influenza hemagglutinin stem domain polypeptide described herein (see, e.g., Rasala et al., 2010, Plant Biotechnology Journal (Published online March 7, 2010)).

5.9 GENERATION OF ANTIBODIES AGAINST INFLUENZA HEMAGGLUTININ STEM DOMAIN POLYPEPTIDE

[00218] The influenza hemagglutinin stem domain 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 influenza virus hemagglutinin polypeptide. In a specific embodiment, the influenza hemagglutinin stem domain polypeptides, 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.).

[00219] Alternatively, influenza hemagglutinin stem domain polypeptides described herein may be used to screen for antibodies from antibody libraries. For example, an isolated influenza hemagglutinin stem domain 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 hemagglutinin stem domain polypeptide. 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 hemagglutinin stem domain. 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.

[00220] 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 hemagglutinin stem domain 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 hemagglutinin stem domain 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)), AI3C (produced by hybridoma 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.

[00221] Antibodies identified or elicited using an influenza hemagglutinin stem domain 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.*, IgG_1, IgG_2, IgG_3, IgG_4, IgA_1 and IgA_2) 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.

[00222] Antibodies elicited or identified using an influenza hemagglutinin stem domain 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 5.11 *infra* for a discussion regarding use of neutralizing antibodies for the prevention or treatment of disease caused by influenza virus infection.

[00223] Antibodies elicited or identified using an influenza hemagglutinin stem domain 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.

[00224] Antibodies elicited or identified using an influenza hemagglutinin stem domain 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).

5.10 STIMULATION OF CELLS WITH INFLUENZA HEMAGGLUTININ STEM DOMAIN PEPTIDE

[00225] In another aspect, provided herein are methods for stimulating cells *ex vivo* with an influenza hemagglutinin stem domain polypeptide described herein. Such cells, *e.g.*, dendritic cells, may be used *in vitro* to generate antibodies against the influenza hemagglutinin stem domain polypeptide or may themselves be administered to a subject by, *e.g.*, an adoptive transfer technique known in the art. *See*, *e.g.*, United States patent application Publication No. 20080019998, published January 24, 2008, which is incorporated herein by reference in its entirety, for a description of adoptive transfer techniques. In certain embodiments, when cells that have been stimulated *ex vivo* with an influenza hemagglutinin stem domain polypeptide described herein are administered to a subject, the cells are not mammalian cells (e.g., CB-1 cells).

[00226] In one non-limiting example, a vector, *e.g.*, an influenza virus vector, engineered to express an influenza hemagglutinin stem domain polypeptide described herein can be used to generate dendritic cells (DCs) that express the influenza hemagglutinin stem domain polypeptide and display immunostimulatory properties directed against an influenza virus hemagglutinin polypeptide. Such DCs may be used to expand memory T cells and are potent stimulators of T cells, including influenza hemagglutinin stem domain polypeptide-specific cytotoxic T lymphocyte clones. See Strobel *et al.*, 2000, Human Gene Therapy 11:2207-2218, which is incorporated herein by reference in its entirety.

[00227] An influenza hemagglutinin stem domain polypeptide described herein may be delivered to a target cell in any way that allows the polypeptide to contact the target cell, *e.g.*, a DC, and deliver the polypeptide to the target cell. In certain embodiments, the influenza hemagglutinin stem domain polypeptide is delivered to a subject, as

described herein. In some such embodiments, cells contacted with the polypeptide may be isolated and propagated.

[00228] In certain embodiments, an influenza hemagglutinin stem domain polypeptide is delivered to a target cell *in vitro*. Techniques known to one of skill in the art may be used to deliver the polypeptide to target cells. For example, target cells may be contacted with the polypeptide in a tissue culture plate, tube or other container. The polypeptide may be suspended in media and added to the wells of a culture plate, tube or other container. The media containing the polypeptide may be added prior to plating of the cells or after the cells have been plated. The target cells are preferably incubated with the polypeptide for a sufficient amount of time to allow the polypeptide to contact the cells. In certain embodiments, the cells are incubated with the polypeptide for about 1 hour or more, about 5 hours or more, about 10 hours or more, about 12 hours or more, about 16 hours or more, about 24, hours or more, about 48 hours or more, about 1 hour to about 12 hours, about 3 hours to about 6 hours, about 6 hours to about 12 hours, about 12 hours to about 24 hours, or about 24 hours to about 48 hours. In certain embodiments, wherein the influenza hemagglutinin stem domain polypeptide is in a virus, the contacting of the target cells comprises infecting the cells with the virus.

[00229] The target cells may be from any species, including, e.g., humans, mice, rats, rabbits and guinea pigs. In some embodiments, target cells are DCs obtained from a healthy subject or a subject in need of treatment. In certain embodiments, target cells are DCs obtained from a subject in whom it is desired to stimulate an immune response to the polypeptide. Methods of obtaining cells from a subject are well known in the art.

5.11 COMPOSITIONS

[00230] The nucleic acids, vectors, polypeptides, bacteria, 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.

[00231] In one embodiment, a pharmaceutical composition comprises an influenza hemagglutinin stem domain polypeptide, in an admixture with a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical composition comprises a nucleic acid encoding an influenza hemagglutinin stem domain 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 hemagglutinin stem domain 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 hemagglutinin stem domain 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 hemagglutinin stem domain polypeptide, in admixture with a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical composition comprises a viral-like particle or virosome containing an influenza hemagglutinin stem domain polypeptide, in an admixture with a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical composition comprises a bacteria expressing or engineered to express an influenza hemagglutinin stem domain polypeptide, in an admixture with a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical composition comprises cells stimulated with an influenza hemagglutinin stem domain polypeptide, in an admixture with a pharmaceutically acceptable carrier.

[00232] In some embodiments, a pharmaceutical composition may comprise one or more other therapies in addition to an active compound.

[00233] 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.

[00234] 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.

[00235] 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.

[00236] 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 one or more vectors expressing an influenza hemagglutinin stem domain polypeptide derived from an influenza A virus hemagglutinin polypeptide and one or more vectors expressing an influenza hemagglutinin stem domain polypeptide derived from an influenza B virus hemagglutinin polypeptide. In another example, a multivalent formulation comprises a vector expressing an influenza hemagglutinin stem domain polypeptide derived from an influenza A virus H3 antigen and a vector expressing an influenza hemagglutinin stem domain polypeptide derived from an influenza A virus H1 antigen. In another example, a multivalent formulation comprises a vector expressing an influenza hemagglutinin stem domain polypeptide derived from an influenza A virus H3 antigen, a vector expressing an influenza hemagglutinin stem domain polypeptide

derived from an influenza A virus H1 antigen, and a vector expressing an influenza hemagglutinin stem domain polypeptide derived from an influenza B virus HA antigen. In certain embodiments, a multivalent formulation may comprise one or more different influenza hemagglutinin stem domain polypeptides expressed using a single vector.

[00237] In certain embodiments, the pharmaceutical compositions described herein additionally comprise a preservative, e.g., the mercury derivative thimerosal. In a specific embodiment, the pharmaceutical compositions described herein comprises 0.001% to 0.01% thimerosal. In other embodiments, the pharmaceutical compositions described herein do not comprise a preservative. In a specific embodiment, thimerosal is used during the manufacture of a pharmaceutical composition described herein and the thimerosal is removed via purification steps following production of the pharmaceutical composition, i.e., the pharmaceutical composition contains trace amounts of thimerosal (<0.3 μg of mercury per dose after purification; such pharmaceutical compositions are considered thimerosal-free products).

[00238] In certain embodiments, the pharmaceutical compositions described herein additionally comprise egg protein (e.g., ovalbumin or other egg proteins). The amount of egg protein in the pharmaceutical compositions described herein may range from about 0.0005 to about 1.2. μg of egg protein to 1 ml of pharmaceutical composition. In other embodiments, the pharmaceutical compositions described herein do not comprise egg protein.

[00239] 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.

[00240] 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.

[00241] In certain embodiments, the pharmaceutical compositions described herein additionally comprise gelatin. In other embodiments, the pharmaceutical compositions described herein do not comprise gelatin.

[00242] 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.

[00243] 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.

[00244] In specific embodiments, the 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 Aplication No. PCT/IB2008/002238 published as International Publication No. WO 09/001217 which is herein incorporated by reference in its entirety).

[00245] The pharmaceutical compositions described herein can be included in a container, pack, or dispenser together with instructions for administration.

[00246] 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 Aplication 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).

[00247] In certain embodiments, when the active compound in a pharmaceutical composition described herein is a cell engineered to express an influenza hemagglutinin stem domain polypeptide, the cells in the pharmaceutical composition are not mammalian cells (e.g., CB-1 cells).

5.11.1 Subunit Vaccines

[00248] In a specific embodiment, provided herein are subunit vaccines comprising an influenza hemagglutinin stem domain polypeptide described herein. In some embodiments, a subunit vaccine comprises an influenza hemagglutinin stem domain

polypeptide and one or more surface glycoproteins (*e.g.*, influenza virus neuraminidase), other targeting moieties or adjuvants. In specific embodiments, a subunit vaccine comprises a single influenza hemagglutinin stem domain polypeptide. In other embodiments, a subunit vaccine comprises two, three, four or more influenza hemagglutinin stem domain polypeptides. In specific embodiments, the influenza hemagglutinin stem domain polypeptide(s) used in a subunit vaccine is not membrane-bound, *i.e.*, it is soluble.

[00249] In certain embodiments, provided herein are subunit vaccines comprising about 10 μg to about 60 μg of one or more influenza hemagglutinin stem domain polypeptides described herein, about 0.001% to 0.01% thimerosal, about 0.1 μg to about 1.0 μg chicken egg protein, about 1.0 μg to about 5.0 μg polymyxin, about 1.0 μg to about 5.0 μg neomycin, about 0.1 μg to about 0.5 μg betapropiolactone, and about .001 to about .05 % w/v of nonylphenol ethoxylate per dose.

[00250] In a specific embodiment, a subunit vaccine provided herein comprises or consists of a 0.5 ml dose that comprises 45 μg of influenza hemagglutinin stem domain polypeptide(s) provided herein, ≤ 1.0 μg of mercury (from thimerosal), ≤ 1.0 μg chicken egg protein (i.e., ovalbumin), ≤ 3.75 μg polymyxin, and ≤ 2.5 μg neomycin. In some embodiments, a subunit vaccine provided herein additionally comprises or consists of not more than 0.5 μg betapropiolactone, and not more than 0.015 % w/v of nonylphenol ethoxylate per dose. In some embodiments, the 0.5 ml dose subunit vaccine is packaged in a pre-filled syringe.

[00251] In a specific embodiment, a subunit vaccine provided herein consists of a 5.0 ml multidose vial (0.5 ml per dose) that comprises 45 μg of influenza hemagglutinin stem domain polypeptide(s) provided herein, 25.0 μg of mercury (from thimerosal), ≤ 1.0 μg chicken egg protein (i.e., ovalbumin), ≤ 3.75 μg polymyxin, and ≤ 2.5 μg neomycin. In some embodiments, a subunit vaccine provided herein additionally comprises or consists of not more than 0.5 μg betapropiolactone, and not more than 0.015 % w/v of nonylphenol ethoxylate per dose.

[00252] In a specific embodiment, the subunit vaccine is prepared using influenza virus that was propagated in embryonated chicken eggs (i.e., the components of the subunit vaccine (e.g., a hemagglutinin stem domain polypeptide) are isolated from virus that was propagated in embryonated chicken eggs). In another specific embodiment, the subunit vaccine is prepared using influenza virus that was not propagated in embryonated chicken eggs (i.e., the components of the subunit vaccine (e.g., a

hemagglutinin stem domain polypeptide) are isolated from virus that was not propagated in embryonated chicken eggs). In another specific embodiment, the subunit vaccine is prepared using influenza virus that was propagated in mammalian cells, e.g., immortalized human cells (see, e.g., International Application No. PCT/EP2006/067566 published as International Publication No. WO 07/045674 which is herein incorporated by reference in its entirety) or canine kidney cells such as MDCK cells (see, e.g., International Application No. PCT/IB2007/003536 published as International Publication No. WO 08/032219 which is herein incorporated by reference in its entirety) (i.e., the components of the subunit vaccine (e.g., a hemagglutinin stem domain polypeptide) are isolated from virus that was propagated in mammalian cells). In another specific embodiment, the hemagglutinin stem domain polypeptide(s) in a subunit vaccine are prepared using an expression vector, *e.g.*, a viral vector, plant vector or a bacterial vector (i.e., the hemagglutinin stem domain polypeptide(s) in the subunit vaccine are obtained/isolated from an expression vector).

5.11.2 Live Virus Vaccines

[00253] In one embodiment, provided herein are immunogenic compositions (*e.g.*, vaccines) comprising live virus containing an influenza hemagglutinin stem domain polypeptide. In another embodiment, provided herein are immunogenic compositions (*e.g.*, vaccines) comprising live virus that is engineered to encode an influenza hemagglutinin stem domain polypeptide, which is expressed by progeny virus produced in the subjects administered the compositions. In specific embodiments, the influenza hemagglutinin stem domain polypeptide is membrane-bound. In other specific embodiments, the influenza virus hemagglutinin stem domain polypeptide is not membrane-bound, *i.e.*, soluble. In particular embodiments, the live virus is an influenza virus, such as described in Section 5.4, *supra*. In other embodiments, the live virus is a non-influenza virus, such as described in Section 5.5, *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 hemagglutinin stem domain polypeptides.

[00254] In certain embodiments, provided herein are immunogenic compositions (*e.g.*, vaccines) comprising about 10^5 to about 10^{10} fluorescent focus units (FFU) of live attenuated influenza virus containing one or more influenza hemagglutinin stem domain polypeptides described herein, about 0.1 to about 0.5 mg monosodium glutamate, about

1.0 to about 5.0 mg hydrolyzed procine gelatin, about 1.0 to about 5.0 mg arginine, about 10 to about 15 mg sucrose, about 1.0 to about 5.0 mg dibasic potassium phosphate, about 0.5 to about 2.0 mg monobasic potassium phosphate, and about 0.001 to about 0.05 μg/ml gentamicin sulfate per dose. In some embodiments, the immunogenic compositions (*e.g.*, vaccines) are packaged as pre-filled sprayers containing single 0.2 ml doses.

[00255] In a specific embodiment, provided herein are immunogenic compositions (*e.g.*, vaccines) comprising $10^{6.5}$ to $10^{7.5}$ FFU of live attenuated influenza virus containing one or more influenza hemagglutinin stem domain polypeptides described herein, 0.188 mg monosodium glutamate, 2.0 mg hydrolyzed procine gelatin, 2.42 mg arginine, 13.68 mg sucrose, 2.26 mg dibasic potassium phosphate, 0.96 mg monobasic potassium phosphate, and < 0.015 μg/ml gentamicin sulfate per dose. In some embodiments, the immunogenic compositions (*e.g.*, vaccines) are packaged as pre-filled sprayers containing single 0.2 ml doses.

[00256] In a specific embodiment, the live virus that contains an influenza hemagglutinin stem domain polypeptide is propagated in embryonated chicken eggs before its use in an immunogenic composition described herein. In another specific embodiment, the live virus that contains an influenza hemagglutinin stem domain polypeptide is not propagated in embryonated chicken eggs before its use in an immunogenic composition described herein. In another specific embodiment, the live virus that contains an influenza hemagglutinin stem domain polypeptide is propagated in mammalian cells, e.g., immortalized human cells (see, e.g., International Application No. PCT/EP2006/067566 published as International Publication No. WO 07/045674 which is herein incorporated by reference in its entirety) or canine kidney cells such as MDCK cells (see, e.g., International Application No. PCT/IB2007/003536 published as International Publication No. WO 08/032219 which is herein incorporated by reference in its entirety) before its use in an immunogenic composition described herein.

[00257] An immunogenic composition comprising a live virus for administration to a subject may be preferred because multiplication of the virus in the subject may lead to a prolonged stimulus of similar kind and magnitude to that occurring in natural infections, and therefore, confer substantial, long lasting immunity.

5.11.3 Inactivated Virus Vaccines

[00258] In one embodiment, provided herein are immunogenic compositions (*e.g.*, vaccines) comprising an inactivated virus containing an influenza hemagglutinin stem domain polypeptide. In specific embodiments, the influenza hemagglutinin stem domain polypeptide is membrane-bound. In particular embodiments, the inactivated virus is an influenza virus, such as described in Section 5.4, *supra*. In other embodiments, the inactivated virus is a non-influenza virus, such as described in Section 5.5, *supra*. In some embodiments, an immunogenic composition comprises two, three, four or more inactivated viruses containing two, three, four or more different influenza hemagglutinin stem domain polypeptides. In certain embodiments, the inactivated virus immunogenic compositions comprise one or more adjuvants.

[00259] Techniques known to one of skill in the art may be used to inactivate viruses containing an influenza hemagglutinin stem domain polypeptide. Common methods use formalin, heat, or detergent for inactivation. *See*, *e.g.*, U.S. Patent No. 6,635,246, which is herein incorporated by reference in its entirety. Other methods include those described in U.S. Patent Nos. 5,891,705; 5,106,619 and 4,693,981, which are incorporated herein by reference in their entireties.

[00260] In certain embodiments, provided herein are immunogenic compositions (*e.g.*, vaccines) comprising inactivated influenza virus such that each dose of the immunogenic composition comprises about 15 to about 60 μg of influenza hemagglutinin stem domain polypeptide described herein, about 1.0 to about 5.0 mg sodium chloride, about 20 to about 100 μg monobasic sodium phosphate, about 100 to about 500 μg dibasic sodium phosphate, about 5 to about 30 μg monobasic potassium phosphate, about 5 to about 30 μg potassium chloride, and about .5 to about 3.0 μg calcium chloride. In some embodiments, the immunogenic compositions (*e.g.*, vaccines) are packaged as single 0.25 ml or single 0.5 ml doses. In other embodiments, the immunogenic compositions (*e.g.*, vaccines) are packaged as multi-dose formulations.

[00261] In certain embodiments, provided herein are immunogenic compositions (*e.g.*, vaccines) comprising inactivated influenza virus such that each dose of the immunogenic composition comprises about 15 to about 60 μg of influenza hemagglutinin stem domain polypeptide described herein, about 0.001% to 0.01% thimerosal, about 1.0 to about 5.0 mg sodium chloride, about 20 to about 100 μg monobasic sodium phosphate, about 100 to about 500 μg dibasic sodium phosphate, about 5 to about 30 μg monobasic potassium phosphate, about 5 to about 30 μg potassium chloride, and about 0.5 to about 3.0 μg calcium chloride per dose. In some

embodiments, the immunogenic compositions (*e.g.*, vaccines) are packaged as single 0.25 ml or single 0.5 ml doses. In other embodiments, the immunogenic compositions (*e.g.*, vaccines) are packaged as multi-dose formulations.

[00262] In a specific embodiment, immunogenic compositions (*e.g.*, vaccines) provided herein are packaged as single 0.25 ml doses and comprise 22.5 μg of influenza hemagglutinin stem domain polypeptide described herein, 2.05 mg sodium chloride, 40 μg monobasic sodium phosphate, 150 μg dibasic sodium phosphate, 10 μg monobasic potassium phosphate, 10 μg potassium chloride, and 0.75 μg calcium chloride per dose.

[00263] In a specific embodiment, immunogenic compositions (*e.g.*, vaccines) provided herein are packaged as single 0.5 ml doses and comprise 45 μg of influenza hemagglutinin stem domain polypeptide described herein, 4.1 mg sodium chloride, 80 μg monobasic sodium phosphate, 300 μg dibasic sodium phosphate, 20 μg monobasic potassium phosphate, 20 μg potassium chloride, and 1.5 μg calcium chloride per dose.

[00264] In a specific embodiment, immunogenic compositions (*e.g.*, vaccines) are packaged as multi-dose formulations comprising or consisting of 5.0 ml of vaccine (0.5 ml per dose) and comprise 24.5 μg of mercury (from thimerosal), 45 μg of influenza hemagglutinin stem domain polypeptide described herein, 4.1 mg sodium chloride, 80 μg monobasic sodium phosphate, 300 μg dibasic sodium phosphate, 20 μg monobasic potassium phosphate, 20 μg potassium chloride, and 1.5 μg calcium chloride per dose.

[00265] In a specific embodiment, the inactivated virus that contains an influenza hemagglutinin stem domain polypeptide was propagated in embryonated chicken eggs before its inactivation and subsequent use in an immunogenic composition described herein. In another specific embodiment, the inactivated virus that contains an influenza hemagglutinin stem domain polypeptide was not propagated in embryonated chicken eggs before its inactivation and subsequent use in an immunogenic composition described herein. In another specific embodiment, the inactivated virus that contains an influenza hemagglutinin stem domain polypeptide was propagated in mammalian cells, e.g., immortalized human cells (see, e.g., International Application No. PCT/EP2006/067566 published as International Publication No. WO 07/045674 which is herein incorporated by reference in its entirety) or canine kidney cells such as MDCK cells (see, e.g., International Application No. PCT/IB2007/003536 published as International Publication No. WO 08/032219 which is herein incorporated by reference in its entirety) before its inactivation and subsequent use in an immunogenic composition described herein.

5.11.4 Split Virus Vaccines

[00266] In one embodiment, an immunogenic composition comprising an influenza hemagglutinin stem domain polypeptide is a split virus vaccine. In some embodiments, split virus vaccine contains two, three, four or more different influenza hemagglutinin stem domain polypeptides. In certain embodiments, the influenza hemagglutinin stem domain polypeptide is/was membrane-bound. In certain embodiments, the split virus vaccines comprise one or more adjuvants.

[00267] Techniques for producing split virus vaccines are known to those skilled in the art. By way of non-limiting example, an influenza virus split vaccine may be prepared using inactivated particles disrupted with detergents. One example of a split virus vaccine that can be adapted for use in accordance with the methods described herein is the Fluzone®, Influenza Virus Vaccine (Zonal Purified, Subvirion) for intramuscular use, which is formulated as a sterile suspension prepared from influenza viruses propagated in embryonated chicken eggs. The virus-containing fluids are harvested and inactivated with formaldehyde. Influenza virus is concentrated and purified in a linear sucrose density gradient solution using a continuous flow centrifuge. The virus is then chemically disrupted using a nonionic surfactant, octoxinol-9, (Triton® X-100 - A registered trademark of Union Carbide, Co.) producing a "split virus." The split virus is then further purified by chemical means and suspended in sodium phosphate-buffered isotonic sodium chloride solution.

[00268] In certain embodiments, provided herein are split virus vaccines comprising about 10 μg to about 60 μg of one or more influenza hemagglutinin stem domain polypeptides described herein, about 0.01 to about 1.0 mg octoxynol-10 (TRITON X-100®, about 0.5 to 0.5 mg α-tocopheryl hydrogen succinate, about 0.1 to 1.0 mg polysorbate 80 (Tween 80), about 0.001 to about 0.003 μg hydrocortisone, about 0.05 to about 0.3 μg gentamcin sulfate, about 0.5 to about 2.0 μgchicken egg protein (ovalbumin), about 25 to 75 μg formaldehyde, and about 25 to 75 μg sodium deoxycholate.

[00269] In a specific embodiment, a split virus vaccine provided herein comprises or consists of a 0.5 ml dose that comprises 45 μg of influenza hemagglutinin stem domain polypeptide(s) provided herein, ≤ 0.085 mg octoxynol-10 (TRITON X-100®, ≤ 0.1 mg α-tocopheryl hydrogen succinate, ≤ .415 mg polysorbate 80 (Tween 80), ≤ 0.0016 μg hydrocortisone, ≤ 0.15 μg gentamcin sulfate, ≤ 1.0 chicken egg protein (ovalbumin), ≤

50 μg formaldehyde, and ≤ 50 μg sodium deoxycholate. In some embodiments, the 0.5 ml dose subunit vaccine is packaged in a pre-filled syringe.

[00270] In a specific embodiment, the split virus vaccine is prepared using influenza virus that was propagated in embryonated chicken eggs. In another specific embodiment, the split virus vaccine is prepared using influenza virus that was not propagated in embryonated chicken eggs. In another specific embodiment, the split virus vaccine is prepared using influenza virus that was propagated in mammalian cells, e.g., immortalized human cells (see, e.g., PCT/EP2006/067566 published as WO 07/045674 which is herein incorporated by reference in its entirety) or canine kidney cells such as MDCK cells (see, e.g., PCT/IB2007/003536 published as WO 08/032219 which is herein incorporated by reference in its entirety).

5.11.5 Adjuvants

[00271] 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, concommitantly 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 hemagglutinin stem domain 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.

[00272] In certain embodiments, an adjuvant augments the intrinsic response to the influenza hemagglutinin stem domain 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 5.4, *supra*. It should be understood that different formulations of influenza hemagglutinin stem domain polypeptide may comprise different adjuvants or may comprise the same adjuvant.

5.12 PROPHYLACTIC AND THERAPEUTIC USES

[00273] In one aspect, provided herein are methods for inducing an immune response in a subject utilizing an active compound, i.e., an influenza hemagglutinin stem domain polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector, or a bacteria) containing or expressing such a polypeptide, or cells stimulated with such a polypeptide. In a specific embodiment, a method for inducing an immune response to an influenza virus hemagglutinin polypeptide in a subject comprises administering to a subject in need thereof an effective amount of an influenza virus hemagglutinin stem domain polypeptide or an immunogenic composition thereof. In another embodiment, a method for inducing an immune response to an influenza virus hemagglutinin polypeptide in a subject comprises administering to a subject in need thereof an effective amount of a nucleic acid encoding an influenza hemagglutinin stem domain polypeptide or an immunogenic composition thereof. In another embodiment, a method for inducing an immune response to an influenza virus hemagglutinin polypeptide in a subject comprises administering to a subject in need thereof an effective amount of a viral vector containing or expressing an influenza hemagglutinin stem domain polypeptide or an immunogenic composition thereof. In yet another embodiment, a method for inducing an immune response to an influenza virus

hemagglutinin polypeptide in a subject comprises administering to a subject in need thereof an effective amount of cells stimulated with an influenza hemagglutinin stem domain polypeptide or a pharmaceutical composition thereof. In certain embodiments, an influenza hemagglutinin stem domain polypeptide used in the method is a purified influenza hemagglutinin stem domain polypeptide derived from a mammalian cell, a plant cell, or an insect cell.

[00274] In a specific embodiment, a method for inducing an immune response to an influenza virus hemagglutinin 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 hemagglutinin 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 hemagglutinin 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 hemagglutinin 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 hemagglutinin polypeptide in a subject comprises administering to a subject in need thereof a viral-like particle vaccine described herein. In another embodiment, a method for inducing an immune response to an influenza hemagglutinin 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 hemagglutinin polypeptide comprises administering to a subject in need thereof a bacteria expressing or engineered to express an influenza hemagglutinin stem domain polypeptide or a composition thereof. In certain embodiments, an influenza hemagglutinin stem domain polypeptide used in the method is a purified influenza hemagglutinin stem domain polypeptide derived from a mammalian cell, a plant cell, or an insect cell.

[00275] In some embodiments, the immune response induced by an active compound or a composition described herein is effective to prevent and/or treat an influenza virus infection caused by any subtype or strain of influenza virus. In certain embodiments, the immune response induced by an active compound or a composition described herein is effective to prevent and/or treat an influenza virus infection caused by a subtype of

influenza virus that belongs to one HA group (e.g., Group 1, which comprises H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16) and not the other HA group (e.g., Group 2, which comprises H3, H4, H7, H10, H14, and H15). For example, the immune response induced may be effective to prevent and/or treat an influenza virus infection caused by an influenza virus that belongs to the HA group consisting of H11, H13, H16, H9, H8, H12, H6, H1, H5 and H2. Alternatively, the immune response induced may be effective to prevent and/or treat an influenza virus infection caused by an influenza virus that belongs to the HA group consisting of H3, H4, H14, H10, H15 and H7. In some embodiments, the immune response induced by an active compound or a composition described herein is effective to prevent and/or treat an influenza virus infection caused by one, two, three, four or five subtypes of influenza virus. In certain embodiments, the immune response induced by an active compound or a composition described herein is effective to prevent and/or treat an influenza virus infection caused by six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen or fifteen subtypes of influenza virus. In some embodiments, the immune response induced by an active compound or a composition described herein is effective to prevent and/or treat an influenza virus infection caused by one or more variants within the same subtype of influenza virus.

[00276] In some embodiments, the immune response induced by an active compound or a composition described herein is effective to prevent and/or treat an influenza virus infection caused by both H1N1 and H2N2 subtypes. In other embodiments, the immune response induced by an active compound or a composition described herein is not effective to prevent and/or treat an influenza virus infection caused by both H1N1 and H2N2 subtypes. In some embodiments, the immune response induced by an active compound or a composition described herein is effective to prevent and/or treat an influenza virus infection caused by H1N1, H2N2, and H3N2 subtypes. In some embodiments, the immune response induced by an active compound or a composition described herein is effective to prevent and/or treat an influenza virus infection caused by H3N2 subtypes. In other embodiments, the immune response induced by an active compound or a composition described herein is not effective to prevent and/or treat an influenza virus infection caused by H3N2 subtypes.

[00277] In some embodiments, the immune response induced by an active compound or a composition described herein is effective to prevent and/or treat an influenza virus disease caused by any subtype or strain of influenza virus. In certain embodiments, the immune response induced by an active compound or a composition described herein is

effective to prevent and/or treat an influenza virus disease caused by a subtype of influenza virus that belongs to one HA group and not the other HA group. For example, the immune response induced may be effective to prevent and/or treat an influenza virus disease caused by an influenza virus that belongs to the HA group consisting of H11, H13, H16, H9, H8, H12, H6, H1, H5 and H2. Alternatively, the immune response induced may be effective to prevent and/or treat an influenza virus disease caused by an influenza virus that belongs to the HA group consisting of H3, H4, H14, H10, H15 and H7. In some embodiments, the immune response induced by an active compound or a composition described herein is effective to prevent and/or treat an influenza virus disease caused by any of one, two, three, four or five subtypes of influenza virus. In certain embodiments, the immune response induced by an active compound or a composition described herein is effective to prevent and/or treat an influenza virus disease caused by any of six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen or fifteen subtypes of influenza virus. In some embodiments, the immune response induced by an active compound or a composition described herein is effective to prevent and/or treat an influenza virus disease caused by one or more variants within the same subtype of influenza virus.

[00278] In some embodiments, the immune response induced by an active compound 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.

[00279] In some embodiments, the immune response induced by an active compound 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 or a composition described herein is effective to reduce the duration of hospitalization of a subject suffering from an influenza virus disease/infection.

[00280] 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 hemagglutinin stem domain polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector containing or expressing such a polypeptide, or cells stimulated with such a polypeptide) 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 hemagglutinin stem domain polypeptide, a nucleic acid encoding such a polypeptide, a vector containing or 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 viral-like particle vaccine.

[00281] In another aspect, provided herein are methods for preventing and/or treating an influenza virus disease in a subject utilizing an influenza hemagglutinin stem domain polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector containing or expressing such a polypeptide, or cells stimulated with such a polypeptide. 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 hemagglutinin stem domain 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 hemagglutinin stem domain 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 hemagglutinin stem domain 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 stimulated with an influenza hemagglutinin stem domain polypeptide or a pharmaceutical composition thereof.

[00282] 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 viral-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 bacteria expressing or engineered to express an influenza hemagglutinin stem domain polypeptide or a composition thereof.

[00283] In another aspect, provided herein are methods of preventing and/or treating an influenza virus disease in a subject by administering neutralizing antibodies 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.

[00284] 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.

5.12.1 Combination therapies

[00285] In various embodiments, an influenza hemagglutinin stem domain polypeptide described herein, a nucleic acid encoding such a polypeptide, a vector (*e.g.*, a viral vector or a bacteria) containing or expressing such a polypeptide, cells stimulated with 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 part. In specific embodiments, two or more therapies are administered within the same patent visit.

[00286] Any anti-viral agents well-known to one of skill in the art may used in combination with an active compound or pharmaceutical 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, gangcyclovir, 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).

[00287] 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 a hemagglutinin polypeptide. In other embodiments, the viral antigen is an influenza virus hemagglutinin polypeptide.

[00288] Any anti-bacterial agents known to one of skill in the art may used in combination with an active compound or pharmaceutical composition described herein. Non-limiting examples of anti-bacterial agents include Amikacin, Amoxicillin, Amoxicillin-clavulanic acid, Amphothericin-B, Ampicillin, Ampicllin-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, Chloramphenicole, Florfenicole, Ciprofloxacin, Clarithromycin,

Clinafloxacin, Clindamycin, Cloxacillin, Colistin, Cotrimoxazol (Trimthoprim/sulphamethoxazole), Dalbavancin, Dalfopristin/Quinopristin, Daptomycin, Dibekacin, Dicloxacillin, Doripenem, Doxycycline, Enrofloxacin, Ertapenem, Erythromycin, Flucloxacillin, Fluconazol, Flucytosin, Fosfomycin, Fusidic acid, Garenoxacin, Gatifloxacin, Gemifloxacin, Gentamicin, Imipenem, Itraconazole, Kanamycin, Ketoconazole, Levofloxacin, Lincomycin, Linezolid, Loracarbef, Mecillnam (amdinocillin), Meropenem, Metronidazole, Meziocillin, Mezlocillin-sulbactam, Minocycline, Moxifloxacin, Mupirocin, Nalidixic acid, Neomycin, Netilmicin, Nitrofurantoin, Norfloxacin, Ofloxacin, Oxacillin, Pefloxacin, Penicillin V, Piperacillin, Piperacillin-sulbactam, Piperacillin-tazobactam, Rifampicin, Roxythromycin, Sparfloxacin, Spectinomycin, Spiramycin, Streptomycin, Sulbactam, Sulfamethoxazole, Teicoplanin, Telavancin, Telithromycin, Temocillin, Tetracyklin, Ticarcillin, Ticarcillin-clavulanic acid, Tigecycline, Tobramycin, Trimethoprim, Trovafloxacin, Tylosin, Vancomycin, Virginiamycin, and Voriconazole.

[00289] In some embodiments, a combination therapy comprises active immunization with an influenza hemagglutinin stem domain polypeptide, or one or more vectors described in Sections 5.2–5.7 and passive immunization with one or more neutralizing antibodies described in Section 5.9. In some embodiments, a combination therapy comprises immunization with one or more vectors described in Sections 5.2–5.7 and administration of cells (*e.g.*, by adoptive transfer) described in Section 5.9.

[00290] In some embodiments, a combination therapy comprises administration of two or more different vectors described in Sections 5.2–5.7. In one example, one or more vectors expressing an influenza hemagglutinin stem domain polypeptide derived from an influenza A virus hemagglutinin polypeptide and one or more vectors expressing an influenza hemagglutinin stem domain polypeptide derived from an influenza B virus hemagglutinin polypeptide are administered in combination. In some embodiments, a combination therapy comprises administration of a vector expressing an influenza hemagglutinin stem domain polypeptide derived from an influenza A virus H3 antigen and a vector expressing an influenza hemagglutinin stem domain polypeptide derived from an influenza A virus H1 antigen. In some embodiments, the combination therapy comprises administration of a vector expressing an influenza hemagglutinin stem domain polypeptide derived from an influenza A virus H3 antigen, a vector expressing an influenza hemagglutinin stem domain polypeptide derived from an

influenza A virus H1 antigen, and a vector expressing an influenza hemagglutinin stem domain polypeptide derived from an influenza B virus hemagglutinin polypeptide.

[00291] In some embodiments, a combination therapy comprises active immunization with an active compound that induces an immune response to one, two, three, or more HA subtypes in one HA group (e.g., Group 1) in combination with an active compound that induces an immune response to one, two, three, or more HA subtypes in the other HA group (e.g., Group 2).

[00292] In some embodiments, a combination therapy comprises active immunization with two or more influenza hemagglutinin stem domain polypeptides described in Section 5.1.

[00293] In certain embodiments, a combination therapy comprises active immunization with one, two, or more influenza hemagglutinin stem domain polypeptides derived from an influenza A virus and one or more influenza hemagglutinin stem domain polypeptides derived from an influenza B virus.

5.12.2 Patient Populations

[00294] In certain embodiments, an active compound or 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 hemagglutinin stem domain 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 hemagglutinin stem domain polypeptide induces an immune response.

[00295] In certain embodiments, an active compound or 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 the influenza virus from which the HA stem domain polypeptide of the active compound or composition was derived.

[00296] 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 that belongs to the same HA group as that of the influenza hemagglutinin stem domain 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 the same subtype as that of the influenza hemagglutinin stem domain polypeptide.

[00297] In some embodiments, a subject to be administered an active compound 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.

[00298] In certain embodiments, a subject to be administered an active compound or composition described herein is a human adult. In certain embodiments, a subject to be administered an active compound or composition described herein is a human adult more than 50 years old. In certain embodiments, a subject to be administered an active compound or composition described herein is an elderly human subject.

[00299] In certain embodiments, a subject to be administered an active compound or composition described herein is a human child. In certain embodiments, a subject to be administered an active compound or composition described herein is a human infant. In certain embodiments, a subject to whom an active compound or composition described herein is administered is not an infant of less than 6 months old. In a specific embodiment, a subject to be administered an active compound or composition described herein is 2 years old or younger.

[00300] In specific embodiments, a subject to be administered an active compound or composition described herein is any infant or child more than 6 months of age and any adult over 50 years of age. In other embodiments, the subject is an individual who is pregnant. In another embodiment, the subject is an individual who may or will be pregnant during the influenza season (*e.g.*, November to April). In specific

embodiments, a subject to be administered an active compound or composition described herein is a woman who has given birth 1, 2, 3, 4, 5, 6, 7, or 8 weeks earlier.

[00301] In some embodiments, the human subject to be administered an active compound or composition described herein is any individual at increased risk of influenza virus infection or disease resulting from influenza virus infection (*e.g.*, an immunocompromised or immunodeficient individual). In some embodiments, the human subject to be administered an active compound or composition described herein is any individual in close contact with an individual with increased risk of influenza virus infection or disease resulting from influenza virus infection (*e.g.*, immunocompromised or immunosuppressed individuals).

[00302] In some embodiments, the human subject to be administered an active compound or composition described herein is an individual affected by any condition that increases susceptibility to influenza virus infection or complications or disease resulting from influenza virus infection. In other embodiments, an active compound or composition described herein is administered to a subject in which an influenza virus infection has the potential to increase complications of another condition that the individual is affected by, or for which they are at risk. In particular embodiments, such conditions that increase susceptibility to influenza virus complications or for which influenza virus increases complications associated with the condition are, *e.g.*, conditions that affect the lung, such as cystic fibrosis, emphysema, asthma, or bacterial infections (e.g., infections caused by *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Legionella pneumophila*, and *Chlamydia trachomatus*); cardiovascular disease (e.g., congenital heart disease, congestive heart failure, and coronary artery disease); endocrine disorders (e.g., diabetes), neurological and neuron-developmental conditions (e.g., disorders of the brain, the spinal cord, the peripheral nerve, and muscle (such as cerebral palsy, epilepsy (seizure disorders), stroke, intellectual disability (e,g, mental retardation), muscular dystrophy, and spinal cord injury)).

[00303] In some embodiments, the human subject to be administered an active compound or composition described herein is an individual that resides in a group home, such as a nursing home. In some embodiments, the human subject to be administered an active compound or composition described herein works in, or spends a significant amount of time in, a group home, *e.g.*, a nursing home. In some embodiments, the human subject to be administered an active compound or composition described herein is a health care worker (*e.g.*, a doctor or nurse). In some embodiments, the human

subject to be administered an active compound or composition described herein is a smoker. In a specific embodiment, the human subject to be administered an active compound or composition described herein is immunocompromised or immunosuppressed.

[00304] In addition, subjects at increased risk of developing complications from influenza who may be administered an active compound or composition described herein include: any individual who can transmit influenza viruses to those at high risk for complications, such as, *e.g.*, members of households with high-risk individuals, including households that will include infants younger than 6 months, individuals coming into contact with infants less than 6 months of age, or individuals who will come into contact with individuals who live in nursing homes or other long-term care facilities; individuals with long-term disorders of the lungs, heart, or circulation; individuals with metabolic diseases (*e.g.*, diabetes); individuals with kidney disorders; individuals with blood disorders (including anemia or sickle cell disease); individuals with weakened immune systems (including immunosuppression caused by medications, malignancies such as cancer, organ transplant, or HIV infection); children who receive long-term aspirin therapy (and therefore have a higher chance of developing Reye syndrome if infected with influenza).

[00305] In other embodiments, subjects for administration of an active compound or composition described herein include healthy individuals six months of age or older, who: plan to travel to foreign countries and areas where flu outbreaks may be occurring, such, *e.g.*, as the tropics and the Southern Hemisphere from April through September; travel as a part of large organized tourist groups that may include persons from areas of the world where influenza viruses are circulating; attend school or college and reside in dormitories, or reside in institutional settings; or wish to reduce their risk of becoming ill with influenza.

[00306] In some embodiments, a subject for whom administration of an active compound or composition described herein is contraindicated include any individual for whom influenza vaccination is contraindicated, such as: infants younger than six months of age; and individuals who have had an anaphylactic reaction (allergic reactions that cause difficulty breathing, which is often followed by shock) to eggs, egg products, or other components used in the production of the immunogenic formulation. In certain embodiments, when administration of an active compound or composition described herein is contraindicated due to one or more components used in the production of the

immunogenic formulation (e.g., due to the presence of egg or egg products), the active compound or composition may be produced in a manner that does not include the component that causes the administration of an active compound or composition to be contraindicated (e.g., the active compound or composition may be produced without the use of eggs or egg products).

[00307] In some embodiments, it may be advisable not to administer a live virus vaccine to one or more of the following patient populations: elderly humans; infants younger than 6 months old; pregnant individuals; infants under the age of 1 years old; children under the age of 2 years old; children under the age of 3 years old; children under the age of 4 years old; children under the age of 5 years old; adults under the age of 20 years old; adults under the age of 25 years old; adults under the age of 30 years old; adults under the age of 35 years old; adults under the age of 40 years old; adults under the age of 45 years old; adults under the age of 50 years old; elderly humans over the age of 70 years old; elderly humans over the age of 75 years old; elderly humans over the age of 80 years old; elderly humans over the age of 85 years old; elderly humans over the age of 90 years old; elderly humans over the age of 95 years old; children and adolescents (2-17 years of age) receiving aspirin or aspirin-containing medications, because of the complications associated with aspirin and wild-type influenza virus infections in this age group; individuals with a history of asthma or other reactive airway diseases; individuals with chronic underlying medical conditions that may predispose them to severe influenza infections; individuals with a history of Guillain-Barre syndrome; individuals with acute serious illness with fever; or individuals who are moderately or severely ill. For such individuals, administration of inactivated virus vaccines, split virus vaccines, subunit vaccines, virosomes, viral-like particles or the non-viral vectors described herein may be preferred. In certain embodiments, subjects preferably administered a live virus vaccine may include healthy children and adolescents, ages 2-17 years, and healthy adults, ages 18-49.

[00308] In certain embodiments, an immunogenic formulation comprising a live virus vector is not given concurrently with other live-virus vaccines.

5.13 MODES OF ADMINISTRATION

5.13.1 Routes of Delivery

[00309] An active compound or 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.

[00310] 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 hemagglutinin stem domain 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.

[00311] In a specific embodiment, a subunit vaccine is administered intramuscularly. In another embodiment, a live influenza virus or live NDV vaccine is administered intranasally. In another embodiment, an inactivated influenza virus vaccine, or a split influenza virus vaccine is administered intramuscularly. In another embodiment, an inactivated NDV virus vaccine or a split NDV virus vaccine is administered intramuscularly. In another embodiment, a viral-like particle or composition thereof is administered intramuscularly.

[00312] In some embodiments, cells stimulated with an influenza hemagglutinin stem domain polypeptide *in vitro* may be introduced (or re-introduced) into a subject using

techniques known to one of skill in the art. In some embodiments, the cells can be introduced into the dermis, under the dermis, or into the peripheral blood stream. In some embodiments, the cells introduced into a subject are preferably cells derived from that subject, to avoid an adverse immune response. In other embodiments, cells also can be used that are derived from a donor host having a similar immune background. Other cells also can be used, including those designed to avoid an adverse immunogenic response.

5.13.2 Dosage and Frequency of Administration

[00313] The amount of an active compound or composition 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.

[00314] 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.

[00315] 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.

[00316] Exemplary doses for nucleic acids encoding influenza hemagglutinin stem domain polypeptides 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.

[00317] In certain embodiments, exemplary doses for influenza hemagglutinin stem domain polypeptides (e.g., 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 hemagglutinin stem domain

polypeptides range from about 1 μg to 50 mg, 5 μg to 50 mg, 1 μg to 100 mg, 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 of influenza hemagglutinin stem domain polypeptides per dose.

[00318] 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 x 10^2, 10^3, 5 x 10^3, 10^4, 5 x 10^4, 10^5, 5 x 10^5, 10^6, 5 x 10^6, 10^7, 5 x 10^7, 10^8, 5 x 10^8, 1 x 10^9, 5 x 10^9, 1 x 10^{10}, 5 x 10^{10}, 1 x 10^{11}, 5 x 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.

[00319] In certain embodiments, exemplary doses for VLPs range from about 0.01 μg to about 100 mg, about 0.1 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 per kilogram of the patient. In other embodiments, exemplary doses for influenza hemagglutinin stem domain polypeptides 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 hemagglutinin stem domain polypeptides per dose, and can be administered to a subject once, twice, three or more times with intervals as often as needed.

[00320] In one embodiment, an inactivated vaccine is formulated such that it contains about 5 μg to about 50 μg, about 10 μg to about 50 μg, about about 15 μg to about 100 μg, about 15 μg to about 75 μg, about 15 μg to about 50 μg, about 15 μg to about 30 μg, about 20 μg to about 50 μg, about 25 μg to about 40 μg, about 25 μg to about 35 μg of an influenza hemagglutinin stem domain polypeptide. Such a vaccine may contain a combination of one or more different influenza hemagglutinin stem domain polypeptides, for example, one or more influenza hemagglutinin stem domain polypeptides from an influenza A virus and one or more influenza hemagglutinin stem domain polypeptides from an influenza B virus. In some embodiments, influenza hemagglutinin stem domain polypeptides derived from, *e.g.*, A/H1N1, A/H3N2, and B

hemagglutinin polypeptides are included in a trivalent inactivated vaccine (TIV), formulated such that a 0.5-mL dose contains 15 μg each of influenza hemagglutinin stem domain polypeptide. In one embodiment, a live attenuated influenza vaccine (LAIV) is formulated such that a 0.2-mL dose contains $10^{6.5–7.5}$ fluorescent focal units of live attenuated influenza viruses from three strains expressing at least one influenza hemagglutinin stem domain polypeptide.

[00321] In certain embodiments, an active compound or composition is administered to a subject once as a single dose. In certain embodiments, an active compound or composition is administered to a subject as a single dose followed by a second dose 3 to 6 weeks later. 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 some embodiments, the administration of the same active compound or composition may be repeated and the administrations may be separated by at least 1 day, 2 days, 3 days, 5 days, 10 days, 15 days, 30 days, 45 days, 2 months, 75 days, 3 months, or at least 6 months. In certain embodiments, an active compound or composition is administered to a subject as a single dose once per year.

[00322] In specific embodiments for administration to children, two doses of an active compound or composition, given at least one month apart, are administered to a child. In specific embodiments for administration to adults, a single dose is given. In another embodiment, two doses of an active compound or composition, given at least one month apart, are administered to an adult. In another embodiment, a young child (six months to nine years old) may be administered an active compound or composition for the first time in two doses given one month apart. In a particular embodiment, a child who received only one dose in their first year of vaccination should receive two doses in the following year. In some embodiments, two doses administered 4 weeks apart are preferred for children 2 -8 years of age who are administered an influenza vaccine, *e.g.*, an immunogenic formulation described herein, for the first time. In certain embodiments, for children 6-35 months of age, a half dose (0.25 ml) may be preferred, in contrast to 0.5 ml which may be preferred for subjects over three years of age.

[00323] In particular embodiments, an active compound or composition 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, so that the second dose can be given prior to the peak of the influenza season.

[00324] 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 hemagglutinin stem domain polypeptide in the patient.

5.14 BIOLOGICAL ASSAYS

5.14.1 Assays for Testing Activity of Influenza Hemagglutinin Stem Domain Polypeptide

[00325] Assays for testing the expression of a influenza hemagglutinin stem domain polypeptide in a vector disclosed herein may be conducted using any assay known in the art. For example, an assay for incorporation into a viral vector comprises growing the virus as described in this section or Sections 5.4 or 5.5, purifying the viral particles by centrifugation through a sucrose cushion, and subsequent analysis for influenza hemagglutinin stem domain polypeptide expression by an immunoassay, such as Western blotting, using methods well known in the art.

[00326] In one embodiment, an influenza hemagglutinin stem domain polypeptide disclosed herein is assayed for proper folding and functionality by testing its ability to bind specifically to a neutralizing antibody directed to an influenza virus hemagglutinin polypeptide, such as the stalk region of the polypeptide, using any assay for antibody-antigen interaction known in the art. Neutralizing antibodies for use in such assays include, for example, the neutralizing antibodies described in Ekiert *et al.*, 2009, *Science Express*, 26 February 2009; Kashyap *et al.*, 2008, *Proc Natl Acad Sci U S A* 105: 5986-5991; Sui *et al.* 2009, *Nature Structural and Molecular Biology*, 16:265-273; Wang *et*

al., 2010, *PLOS Pathogens* 6(2):1-9; 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.. These antibodies include CR6261, CR6325, CR6329, CR6307, CR6323, 2A, D7, D8, F10, G17, H40, A66, D80, E88, E90, H98, C179 (FERM BP-4517), AI3C (FERM BP-4516), among others.

[00327] In another embodiment, an influenza hemagglutinin stem domain polypeptide disclosed herein is assayed for proper folding by determination of the structure or conformation of the influenza hemagglutinin stem domain polypeptide using any method known in the art such as, *e.g.*, NMR, X-ray crystallographic methods, or secondary structure prediction methods, *e.g.*, circular dichroism.

5.14.2 Assays for Testing Activity of Antibodies Generated using Influenza Hemagglutinin Stem Domain Polypeptide

[00328] Antibodies described herein may be characterized in a variety of ways known to one of skill in the art (e.g. ELISA, Surface Plasmon resonance display (BIAcore), Western blot, immunofluorescence, immunostaining and/or microneutralization assays). In some embodiments, antibodies are assayed for the ability to specifically bind to an influenza virus hemagglutinin polypeptide, or a vector comprising said polypeptide. Such an assay may be performed in solution (e.g., Houghten, 1992, Bio/Techniques 13:412 421), on beads (Lam, 1991, Nature 354:82 84), on chips (Fodor, 1993, Nature 364:555 556), on bacteria (U.S. Patent No. 5,223,409), on spores (U.S. Patent Nos. 5,571,698; 5,403,484; and 5,223,409), on plasmids (Cull *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:1865 1869) or on phage (Scott and Smith, 1990, Science 249:386 390; Cwirla *et al.*, 1990, Proc. Natl. Acad. Sci. USA 87:6378 6382; and Felici, 1991, J. Mol. Biol. 222:301 310) (each of these references is incorporated herein in its entirety by reference).

[00329] Specific binding of an antibody to the influenza virus hemagglutinin polypeptide and cross-reactivity with other antigens can be assessed by any method known in the art. Immunoassays which can be used to analyze specific binding and cross-reactivity include, but are not limited to, competitive and non-competitive assay

systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art (see, e.g., Ausubel *et al.*, eds., 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein in its entirety).

[00330] The binding affinity of an antibody to an influenza virus hemagglutinin polypeptide and the off-rate of an antibody-antigen interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled antigen (e.g., ^{3}H or ^{125}I) with the antibody of interest in the presence of increasing amounts of unlabeled antigen, and the detection of the antibody bound to the labeled antigen. The affinity of the antibody for an influenza virus hemagglutinin polypeptide and the binding off-rates can be determined from the data by Scatchard plot analysis. Competition with a second antibody can also be determined using radioimmunoassays. In this case, an influenza virus hemagglutinin polypeptide is incubated with the test antibody conjugated to a labeled compound (e.g., ^{3}H or ^{125}I) in the presence of increasing amounts of an unlabeled second antibody.

[00331] In certain embodiments, antibody binding affinity and rate constants are measured using the KinExA 3000 System (Sapidyne Instruments, Boise, ID). In some embodiments, surface plasmon resonance (*e.g.*, BIAcore kinetic) analysis is used to determine the binding on and off rates of the antibodies to an influenza virus hemagglutinin polypeptide. BIAcore kinetic analysis comprises analyzing the binding and dissociation of influenza virus hemagglutinin polypeptide from chips with immobilized antibodies to an influenza virus hemagglutinin polypeptide on their surface. A typical BIAcore kinetic study involves the injection of 250 μL of an antibody reagent (mAb, Fab) at varying concentration in HBS buffer containing 0.005% Tween-20 over a sensor chip surface, onto which has been immobilized the influenza virus hemagglutinin polypeptide. The flow rate is maintained constant at 75 μL/min. Dissociation data is collected for 15 min or longer as necessary. Following each injection/dissociation cycle, the bound antibody is removed from the influenza virus hemagglutinin polypeptide surface using brief, 1 min pulses of dilute acid, typically 10-100 mM HCl, though other

regenerants are employed as the circumstances warrant. More specifically, for measurement of the rates of association, k_{on}, and dissociation, k_{off}, the polypeptide is directly immobilized onto the sensor chip surface through the use of standard amine coupling chemistries, namely the EDC/NHS method (EDC= N-diethylaminopropyl)-carbodiimide). Briefly, a 5-100 nM solution of the polypeptide in 10 mM NaOAc, pH 4 or pH 5 is prepared and passed over the EDC/NHS-activated surface until approximately 30-50 RU's worth of polypeptide are immobilized. Following this, the unreacted active esters are "capped" off with an injection of 1M Et-NH_2. A blank surface, containing no polypeptide, is prepared under identical immobilization conditions for reference purposes. Once an appropriate surface has been prepared, a suitable dilution series of each one of the antibody reagents is prepared in HBS/Tween-20, and passed over both the polypeptide and reference cell surfaces, which are connected in series. The range of antibody concentrations that are prepared varies, depending on what the equilibrium binding constant, K_D, is estimated to be. As described above, the bound antibody is removed after each injection/dissociation cycle using an appropriate regenerant.

[00332] The neutralizing activity of an antibody can be determined utilizing any assay known to one skilled in the art. Antibodies described herein can be assayed for their ability to inhibit the binding of an influenza virus, or any other composition comprising influenza virus hemagglutinin polypeptide (*e.g.*, a VLP, liposome, or detergent extract), to its host cell receptor (*i.e.*, sialic acid) using techniques known to those of skill in the art. For example, cells expressing influenza virus receptors can be contacted with a composition comprising influenza virus hemagglutinin polypeptide in the presence or absence of the antibody and the ability of the antibody to inhibit the antigen's binding can measured by, for example, flow cytometry or a scintillation assay. The composition comprising an influenza virus hemagglutinin polypeptide or the antibody can be labeled with a detectable compound such as a radioactive label (e.g., ^{32}P, ^{35}S, and ^{125}I) or a fluorescent label (e.g., fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine) to enable detection of an interaction between the composition comprising an influenza virus hemagglutinin polypeptide and a cell receptor. Alternatively, the ability of antibodies to inhibit an influenza virus hemagglutinin polypeptide from binding to its receptor can be determined in cell-free assays. For example, a composition comprising an influenza virus hemagglutinin polypeptide can be contacted with an antibody and the ability of the antibody to inhibit the composition comprising an influenza virus hemagglutinin

polypeptide from binding to a cell receptor can be determined. In a specific embodiment, the antibody is immobilized on a solid support and the composition comprising an influenza virus hemagglutinin polypeptide is labeled with a detectable compound. Alternatively, a composition comprising an influenza virus hemagglutinin polypeptide is immobilized on a solid support and the antibody is labeled with a detectable compound. In certain embodiments, the ability of an antibody to inhibit an influenza virus hemagglutinin polypeptide from binding to a cell receptor is determined by assessing the percentage of binding inhibition of the antibody relative to a control (e.g., an antibody known to inhibit the influenza virus hemagglutinin polypeptide from binding to the cell receptor).

[00333] In other embodiments, an antibody suitable for use in the methods described herein does not inhibit influenza virus receptor binding, yet is still found to be neutralizing in an assay described herein. In some embodiments, an antibody suitable for use in accordance with the methods described herein reduces or inhibits virus-host membrane fusion in an assay known in the art or described herein.

[00334] In one embodiment, virus-host membrane fusion is assayed in an *in vitro* assay using an influenza virus containing a reporter and a host cell capable of being infected with the virus. An antibody inhibits fusion if reporter activity is inhibited or reduced compared to a negative control (*e.g.*, reporter activity in the presence of a control antibody or in the absence of antibody).

[00335] In one embodiment, virus-host membrane fusion is detected using a model system of cell fusion. In an exemplary cell fusion assay, cells (*e.g.*, HeLa cells) are transfected with a plasmid encoding an influenza hemagglutinin polypeptide and contacted and exposed to a buffer that allows the hemagglutinin polypeptide fusion function (*e.g.*, pH 5.0 buffer) in the presence of an antibody. An antibody is neutralizing if it reduces or inhibits syncytia formation compared to a negative control (*e.g.*, syncytia formation in the presence of a control antibody or in the absence of antibody).

[00336] In other embodiments, virus-host membrane fusion is assayed using an *in vitro* liposome-based assay. In an exemplary assay, the host cell receptor is reconstituted into liposomes containing one half of a reporter. Influenza hemagglutinin polypeptide is reconstituted into another set of liposomes containing another half of a reporter. When the two liposome populations are mixed together, fusion is detected by reconstitution of the reporter, for example, an enzymatic reaction that can be detected colorimetrically. The antibody inhibits fusion if reporter activity is reduced or inhibited

compared to reporter activity in an assay conducted in the absence of antibody or in the presence of a control antibody. In certain embodiments, the ability of an antibody to inhibit fusion is determined by assessing the percentage of fusion in the presence of the antibody relative to the percentage of fusion in the presence a control.

5.14.3 Assays for Testing Activity of Stimulated Cells

[00337] Cells stimulated in accordance with the methods described herein may be analyzed, for example, for integration, transcription and/or expression of the polynucleotide or gene(s) of interest, the number of copies of the gene integrated, and the location of the integration. Such analysis may be carried out at any time and may be carried out by any methods known in the art. In other embodiments, successful stimulation of the target cell with an influenza hemagglutinin stem domain polypeptide described herein is determined by detecting production of neutralizing antibodies against the influenza hemagglutinin stem domain polypeptide using methods known in the art or described herein.

[00338] In certain embodiments, subjects in which the stimulated cells, e.g., DCs, are administered can be analyzed for location of the cells, expression of a vector-delivered polynucleotide or gene encoding the influenza hemagglutinin stem domain polypeptide, stimulation of an immune response (e.g., production of neutralizing antibodies against the influenza hemagglutinin stem domain polypeptide), and/or monitored for symptoms associated with influenza virus infection or a disease associated therewith by any methods known in the art or described herein.

[00339] Reporter assays can be used to determine the specificity of the targeting of the influenza hemagglutinin stem domain polypeptide. For example, a mixed population of bone marrow cells can be obtained from a subject and cultured in vitro. The influenza hemagglutinin stem domain polypeptide can be administered to the mixed population of bone marrow cells, and expression of a reporter gene associated with the influenza hemagglutinin stem domain polypeptide can be assayed in the cultured cells. In some embodiments, at least about 50%, more preferably at least about 60%, 70%, 80% or 90%, still more preferably at least about 95% of stimulated cells in the mixed cell population are dendritic cells.

5.14.4 Antiviral Activity Assays

[00340] Antibodies described herein or compositions thereof can be assessed *in vitro* for antiviral activity. In one embodiment, the antibodies or compositions thereof are tested *in vitro* for their effect on growth of an influenza virus. Growth of influenza virus can be assessed by any method known in the art or described herein (e.g. in cell culture). In a specific embodiment, cells are infected at a MOI of 0.0005 and 0.001, 0.001 and 0.01, 0.01 and 0.1, 0.1 and 1, or 1 and 10, or a MOI of 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5 or 10 and incubated with serum free media supplemented. Viral titers are determined in the supernatant by hemagglutinin plaques or any other viral assay described herein. Cells in which viral titers can be assessed include, but are not limited to, EFK-2 cells, Vero cells, MDCK cells, primary human umbilical vein endothelial cells (HUVEC), H292 human epithelial cell line and HeLa cells. *In vitro* assays include those that measure altered viral replication (as determined, e.g., by plaque formation) or the production of viral proteins (as determined, e.g., by Western blot analysis) or viral RNAs (as determined, e.g., by RT-PCR or northern blot analysis) in cultured cells in vitro using methods which are well known in the art or described herein.

[00341] In one non-limiting example, a monolayer of the target mammalian cell line is infected with different amounts (*e.g.*, multiplicity of 3 plaque forming units (pfu) or 5 pfu) of virus (*e.g.*, influenza) and subsequently cultured in the presence or absence of various dilutions of antibodies (*e.g.*, 0.1 μg/ml, 1 μg/ml, 5 μg/ml, or 10 μg/ml). Infected cultures are harvested 48 hours or 72 hours post infection and titered by standard plaque assays known in the art on the appropriate target cell line (*e.g.*, Vero cells).

[00342] In a non-limiting example of a hemagglutination assay, cells are contacted with an antibody and are concurrently or subsequently infected with the virus (*e.g.*, at an MOI of 1) and the virus is incubated under conditions to permit virus replication (*e.g.*, 20-24 hours). The antibodies are preferably present throughout the course of infection. Viral replication and release of viral particles is then determined by hemagglutination assays using 0.5% chicken red blood cells. See, *e.g.*, Kashyap *et al.*, PNAS USA 105: 5986-5991. In some embodiments, a compound is considered an inhibitor of viral replication if it reduces viral replication by at least 2 wells of HA, which equals approximately a 75% reduction in viral titer. In specific embodiments, an inhibitor reduces viral titer in this assay by 50% or more, by 55% or more, by 60% or more, by 65% or more, by 70% or more, by 75% or more, by 80% or more, by 85% or more, by 90% or more, or by 95% or more. In other specific embodiments an inhibitor results in a reduction 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 in influenza virus titer in the subject. The log-reduction in Influenza virus titer may be as compared to a negative control, as compared to another treatment, or as compared to the titer in the patient prior to antibody administration.

5.14.5 Cytotoxicity Assays

[00343] Many assays well-known in the art can be used to assess viability of cells (infected or uninfected) or cell lines following exposure to an active compound or a composition thereof and, thus, determine the cytotoxicity of the compound or composition. For example, cell proliferation can be assayed by measuring Bromodeoxyuridine (BrdU) incorporation (*See*, *e.g.*, Hoshino *et al.*, 1986, Int. J. Cancer 38, 369; Campana *et al.*, 1988, J. Immunol. Meth. 107:79), (3H) thymidine incorporation (*See*, *e.g.*, Chen, J., 1996, Oncogene 13:1395-403; Jeoung, J., 1995, J. Biol. Chem. 270:18367 73), by direct cell count, or by detecting changes in transcription, translation or activity of known genes such as proto-oncogenes (*e.g.*, fos, myc) or cell cycle markers (Rb, cdc2, cyclin A, D1, D2, D3, E, etc). The levels of such protein and mRNA and activity can be determined by any method well known in the art. For example, protein can be quantitated by known immunodiagnostic methods such as ELISA, Western blotting or immunoprecipitation using antibodies, including commercially available antibodies. mRNA can be quantitated using methods that are well known and routine in the art, for example, using northern analysis, RNase protection, or polymerase chain reaction in connection with reverse transcription. Cell viability can be assessed by using trypan-blue staining or other cell death or viability markers known in the art. In a specific embodiment, the level of cellular ATP is measured to determined cell viability.

[00344] In specific embodiments, cell viability is measured in three-day and seven-day periods using an assay standard in the art, such as the CellTiter-Glo Assay Kit (Promega) which measures levels of intracellular ATP. A reduction in cellular ATP is

indicative of a cytotoxic effect. In another specific embodiment, cell viability can be measured in the neutral red uptake assay. In other embodiments, visual observation for morphological changes may include enlargement, granularity, cells with ragged edges, a filmy appearance, rounding, detachment from the surface of the well, or other changes. These changes are given a designation of T (100% toxic), PVH (partially toxic–very heavy–80%), PH (partially toxic–heavy–60%), P (partially toxic–40%), Ps (partially toxic–slight–20%), or 0 (no toxicity–0%), conforming to the degree of cytotoxicity seen. A 50% cell inhibitory (cytotoxic) concentration (IC_{50}) is determined by regression analysis of these data.

[00345] In a specific embodiment, the cells used in the cytotoxicity assay are animal cells, including primary cells and cell lines. In some embodiments, the cells are human cells. In certain embodiments, cytotoxicity is assessed in one or more of the following cell lines: U937, a human monocyte cell line; primary peripheral blood mononuclear cells (PBMC); Huh7, a human hepatoblastoma cell line; 293T, a human embryonic kidney cell line; and THP-1, monocytic cells. In certain embodiments, cytotoxicity is assessed in one or more of the following cell lines: MDCK, MEF, Huh 7.5, Detroit, or human tracheobronchial epithelial (HTBE) cells.

[00346] Active compounds or compositions thereof can be tested for *in vivo* toxicity in animal models. For example, animal models, described herein and/or others known in the art, used to test the activities of active compounds can also be used to determine the *in vivo* toxicity of these compounds. For example, animals are administered a range of concentrations of active compounds. Subsequently, the animals are monitored over time for lethality, weight loss or failure to gain weight, and/or levels of serum markers that may be indicative of tissue damage (*e.g.*, creatine phosphokinase level as an indicator of general tissue damage, level of glutamic oxalic acid transaminase or pyruvic acid transaminase as indicators for possible liver damage). These *in vivo* assays may also be adapted to test the toxicity of various administration mode and/or regimen in addition to dosages.

[00347] The toxicity and/or efficacy of an active compound can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. An active compound that exhibits large therapeutic indices is preferred.

While an active compound that exhibits toxic side effects may be used, care should be taken to design a delivery system that targets such agents to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[00348] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage of an active compound for use in humans. The dosage of such agents lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any active compound used in a method described herein, the effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high-performance liquid chromatography. Additional information concerning dosage determination is provided herein.

[00349] Further, any assays known to those skilled in the art can be used to evaluate the prophylactic and/or therapeutic utility of the active compounds and compositions described herein, for example, by measuring viral infection or a condition or symptoms associated therewith.

5.14.6 In vivo Antiviral Activity

[00350] Active compounds and compositions thereof are preferably assayed *in vivo* for the desired therapeutic or prophylactic activity prior to use in humans. For example, *in vivo* assays can be used to determine whether it is preferable to administer an active compound or composition thereof and/or another thereapy. For example, to assess the use of an active compound or composition thereof to prevent an influenza virus disease, the composition can be administered before the animal is infected with influenza virus. Alternatively, or in addition, an active compound or composition thereof can be administered to the animal at the same time that the animal is infected with influenza virus. To assess the use of an active compound or composition thereof to treat an influenza virus infection or disease associated therewith, the compound or composition may be administered after infecting the animal with influenza virus. In a specific

embodiment, an active compound or composition thereof is administered to the animal more than one time.

[00351] Active compounds and compositions thereof can be tested for antiviral activity in animal model systems including, but are not limited to, rats, mice, chicken, cows, monkeys, pigs, goats, sheep, dogs, rabbits, guinea pigs, etc. In a specific embodiment, active compounds and compositions thereof are tested in a mouse model system. Such model systems are widely used and well-known to the skilled artisan. In a specific embodiment, active compounds and compositions thereof are tested in a mouse model system. Non-limiting examples of animal models for influenza virus are provided in this section.

[00352] In general, animals are infected with influenza virus and concurrently or subsequently treated with an active compound or composition thereof, or placebo. Alternatively, animals are treated with an active compound or composition thereof or placebo and subsequently infected with influenza virus. Samples obtained from these animals (*e.g.*, serum, urine, sputum, semen, saliva, plasma, or tissue sample) can be tested for viral replication via well known methods in the art, *e.g.*, those that measure altered viral titers (as determined, *e.g.*, by plaque formation), the production of viral proteins (as determined, *e.g.*, by Western blot, ELISA, or flow cytometry analysis) or the production of viral nucleic acids (as determined, *e.g.*, by RT-PCR or northern blot analysis). For quantitation of virus in tissue samples, tissue samples are homogenized in phosphate-buffered saline (PBS), and dilutions of clarified homogenates are adsorbed for 1 hour at 37°C onto monolayers of cells (*e.g.*, Vero, CEF or MDCK cells). In other assays, histopathologic evaluations are performed after infection, preferably evaluations of the organ(s) the virus is known to target for infection. Virus immunohistochemistry can be performed using a viral-specific monoclonal antibody.

[00353] The effect of an active compound or composition thereof on the virulence of a virus can also be determined using *in vivo* assays in which the titer of the virus in an infected subject administered an active compound or composition thereof, the length of survival of an infected subject administered an active compound or composition thereof, the immune response in an infected subject administered an active compound or composition thereof, the number, duration and/or severity of the symptoms in an infected subject administered an active compound or composition thereof, and/or the time period before onset of one or more symptoms in an infected subject administered an active compound or composition thereof, is assessed. Techniques known to one of

skill in the art can be used to measure such effects. In certain embodiments, an active compound or composition thereof 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, an active compound or composition thereof 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.

[00354] Influenza virus animal models, such as ferret, mouse, guinea pig, squirrel monkey, macaque, and chicken, developed for use to test antiviral agents against influenza virus have been described. *See*, *e.g.*, Sidwell *et al.*, Antiviral Res., 2000, 48:1-16; Lowen A.C. *et al.* PNAS., 2006, 103: 9988-92; and McCauley *et al.*, Antiviral Res., 1995, 27:179-186 and Rimmelzwann *et al.*, Avian Diseases, 2003, 47:931-933. For mouse models of influenza, non-limiting examples of parameters that can be used to assay antiviral activity of active compounds administered to the influenza-infected mice include pneumonia-associated death, serum α1-acid glycoprotein increase, animal weight, lung virus assayed by hemagglutinin, lung virus assayed by plaque assays, and histopathological change in the lung. Statistical analysis is carried out to calculate significance (*e.g.*, a P value of 0.05 or less).

[00355] In other assays, histopathologic evaluations are performed after infection of an animal model subject. Nasal turbinates and trachea may be examined for epithelial changes and subepithelial inflammation. The lungs may be examined for bronchiolar epithelial changes and peribronchiolar inflammation in large, medium, and small or terminal bronchioles. The alveoli are also evaluated for inflammatory changes. The medium bronchioles are graded on a scale of 0 to 3+ as follows: 0 (normal: lined by medium to tall columnar epithelial cells with ciliated apical borders and basal pseudostratified nuclei; minimal inflammation); 1+ (epithelial layer columnar and even in outline with only slightly increased proliferation; cilia still visible on many cells); 2+

(prominent changes in the epithelial layer ranging from attenuation to marked proliferation; cells disorganized and layer outline irregular at the luminal border); 3+ (epithelial layer markedly disrupted and disorganized with necrotic cells visible in the lumen; some bronchioles attenuated and others in marked reactive proliferation).

[00356] The trachea is graded on a scale of 0 to 2.5+ as follows: 0 (normal: Lined by medium to tall columnar epithelial cells with ciliated apical border, nuclei basal and pseudostratified. Cytoplasm evident between apical border and nucleus. Occasional small focus with squamous cells); 1+ (focal squamous metaplasia of the epithelial layer); 2+ (diffuse squamous metaplasia of much of the epithelial layer, cilia may be evident focally); 2.5+ (diffuse squamous metaplasia with very few cilia evident).

[00357] Virus immunohistochemistry is performed using a viral-specific monoclonal antibody (*e.g.* NP-, N- or HN-specific monoclonal antibodies). Staining is graded 0 to 3+ as follows: 0 (no infected cells); 0.5+ (few infected cells); 1+ (few infected cells, as widely separated individual cells); 1.5+ (few infected cells, as widely separated singles and in small clusters); 2+ (moderate numbers of infected cells, usually affecting clusters of adjacent cells in portions of the epithelial layer lining bronchioles, or in small sublobular foci in alveoli); 3+ (numerous infected cells, affecting most of the epithelial layer in bronchioles, or widespread in large sublobular foci in alveoli).

[00358] In one example, the ability to induce lung lesions and cause infection in an animal model of virus infection is compared using wild-type virus and mock virus. Lung lesions can be assessed as a percentage of lung lobes that are healthy by visual inspection. Animals are euthanized 5 days p.i. by intravenous administration of pentobarbital, and their lungs are removed in toto. The percentage of the surface of each pulmonary lobe that is affected by macroscopic lesions is estimated visually. The percentages are averaged to obtain a mean value for the 7 pulmonary lobes of each animal. In other assays, nasal swabs can be tested to determine virus burden or titer. Nasal swabs can be taken during necropsy to determine viral burden post-infection.

[00359] In one embodiment, virus is quantified in tissue samples. For example, tissue samples are homogenized in phosphate-buffered saline (PBS), and dilutions of clarified homogenates adsorbed for 1 h at 37°C onto monolayers of cells (e.g., MDCK cells). Infected monolayers are then overlaid with a solution of minimal essential medium containing 0.1% bovine serum albumin (BSA), 0.01% DEAE-dextran, 0.1% $NaHCO_3$, and 1% agar. Plates are incubated 2 to 3 days until plaques could be visualized. Tissue culture infectious dose (TCID) assays to titrate virus from PR8-infected samples are

carried out as follows. Confluent monolayers of cells (e.g., MDCK cells) in 96-well plates are incubated with log dilutions of clarified tissue homogenates in media. Two to three days after inoculation, 0.05-ml aliquots from each well are assessed for viral growth by hemagglutination assay (HA assay).

5.14.6.1.1 Assays in Humans

[00360] In one embodiment, an active compound or composition thereof that modulates replication an influenza virus are assessed in infected human subjects. In accordance with this embodiment, an active compound or composition thereof is administered to the human subject, and the effect of the active compound or composition on viral replication is determined by, *e.g.*, analyzing the level of the virus or viral nucleic acids in a biological sample (*e.g.*, serum or plasma). An active compound or composition thereof that alters virus replication can be identified by comparing the level of virus replication in a subject or group of subjects treated with a control to that in a subject or group of subjects treated with an active compound or composition thereof. Alternatively, alterations in viral replication can be identified by comparing the level of the virus replication in a subject or group of subjects before and after the administration of an active compound or composition thereof. Techniques known to those of skill in the art can be used to obtain the biological sample and analyze the mRNA or protein expression.

[00361] In another embodiment, the effect of an active compound or composition thereof on the severity of one or more symptoms associated with an influenza virus infection/disease are assessed in an infected subject. In accordance with this embodiment, an active compound or composition thereof or a control is administered to a human subject suffering from influenza virus infection and the effect of the active compound or composition on one or more symptoms of the virus infection is determined. An active compound or composition thereof that reduces one or more symptoms can be identified by comparing the subjects treated with a control to the subjects treated with the active compound or composition. In another embodiment, an active compound or composition thereof is administered to a healthy human subject and monitored for efficacy as a vaccine (e.g., the subject is monitored for the onset of symptoms of influenza virus infection; the ability of influenza virus to infect the subject; and/or a reduction in/absence of one or more symptoms associated with influenza virus infection). Techniques known to physicians familiar with infectious diseases can be

used to determine whether an ative compound or composition thereof reduces one or more symptoms associated with the influenza virus disease.

5.15 KITS

[00362] Provided herein is a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical 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.

[00363] The kits encompassed herein can be used in the above methods. In one embodiment, a kit comprises an active compound described herein, preferably one or more influenza hemagglutinin stem domain 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.

6. EXAMPLES

6.1 EXAMPLE 1: INFLUENZA HEMAGGLUTININ STEM DOMAIN POLYPEPTIDES

[00364] This example describes the generation of constructs that express influenza hemagglutinin stem domain polypeptides. The influenza hemagglutinin stem domain polypeptides lack the globular head domain of influenza virus hemagglutinin and maintain the structural integrity of the stalk region of the influenza virus hemagglutinin. Since the stalk region of influenza virus hemagglutinin is relatively conserved among influenza viruses, the influenza hemagglutinin stem domain polypeptides should induce neutralizing antibodies against the stalk region of hemagglutinin that are cross-reactive with influenza virus hemagglutinin from different influenza virus subtypes and strains.

[00365] FIG. 3 depicts two schematic nucleotide constructs for expressing an influenza HA stem domain polypeptide from influenza A HK68-H3N2. FIG. 3 also depicts a schematic of a construct (WT HA) for expressing full length influenza HA. The first construct ("Membrane Bound HA") provides a nucleotide sequence encoding

the N-terminal and C-terminal segments, linker peptides, and an HA2 domain. The first construct also encodes a transmembrane (TM) domain and a cytoplasmic (CT) domain. The first construct also encodes a signal peptide (SP).

[00366] Additionally, a second construct ("Soluble HA") provides a nucleotide sequence not encoding the SP, TM or CT in order to generate soluble form of the influenza HA stem domain polypeptide. The second construct includes, after the sequence encoding the HA2 domain, a nucleotide sequence encoding a thrombin cleavage site, a trimerization domain, and a His-tag.

[00367] FIG. 4 illustrates the location of the linker peptide in a putative three dimensional structure of an influenza HA stem domain polypeptide.

[00368] **Constructs:**

[00369] Construct #1: The nucleotide sequence encoding amino acids 53 to 276 of the influenza HA1 domain was deleted from the full-length influenza virus A/Puerto Rico/8/34 (PR8; H1N1) hemagglutinin and replaced by a linker sequence encoding two glycine residues (GG). Fig. 6 provides the PR8 HA construct (PR8 HAΔGHD (2G)) with a GG linker with both nucleotide (SEQ ID NO:169) and amino acid (SEQ ID NO:170) sequences. A similar construct was made using the full-length influenza virus A/Hong Kong/1/68 (HK68; H3N2) hemagglutinin polypeptide (HK68 HAΔGHD (2G)) and the construct was inserted in the pCAGGS expression vector. The PR8 HAΔGHD (2G) and HK68 HAΔGHD (2G) constructs were each inserted into a pPol1 vector for use in the rescue of recombinant influenza virus.

[00370] Construct #2: The nucleotide sequence encoding amino acids 53 to 276 of the influenza HA1 domain was deleted from the full-length PR8 hemagglutinin and replaced by a linker sequence encoding four glycine residues (GGGG). FIG. 7 provides the PR8 HA construct (PR8 HAΔGHD (4G)) with a GGGG linker with both nucleotide (SEQ ID NO:171) and amino acid (SEQ ID NO:172) sequences. A similar construct was made using the full-length influenza virus HK68, H3N2 hemagglutinin polypeptide (HK68 HAΔGHD (4G)). The PR8 HAΔGHD (4G) and HK68 HAΔGHD (4G) constructs were each inserted in the pCAGGS expression vector. The PR8 HAΔGHD (4G) and HK68 HAΔGHD (4G) constructs were each inserted into a pPol1 vector for use in the rescue of recombinant influenza virus.

[00371] Construct #3: The nucleotide sequence encoding amino acids 53 to 276 of the influenza HA1 domain was deleted from the full-length PR8 hemagglutinin and replaced by a linker sequence encoding a proline residue immediately followed by a

glycine residue (PG). Fig. 8 provides the PR8 HA construct (PR8 HAΔGHD (PG)) with a PG linker with both nucleotide (SEQ ID NO:173) and amino acid (SEQ ID NO:174) sequences. A similar construct was made using the full-length influenza virus HK68, H3N2 hemagglutinin polypeptide (HK68 HAΔGHD (PG)). The PR8 HAΔGHD (PG) and HK68 HAΔGHD (PG) constructs were each inserted in the pCAGGS expression vector. The PR8 HAΔGHD (PG) and HK68 HAΔGHD (PG) constructs were each inserted into a pPol1 vector for use in the rescue of recombinant influenza virus.

[00372] Construct #4: The nucleotide sequence encoding amino acids 53 to 276 of the influenza HA1 domain is deleted from the full-length PR8 and replaced by a linker sequence encoding four glycine residues (GGGG). FIG. 9 provides the PR8 HA construct with a GGGG linker with both nucleotide (SEQ ID NO:175) and amino acid (SEQ ID NO:176) sequences. The PR8 HA construct in Fig. 9 also encodes, after the influenza HA2 domain, a thrombin cleavage site, a foldon domain for trimerization and a HIS_6 tag. A similar construct may be made using the influenza virus HK68, H3N2 hemagglutinin polypeptide.

[00373] **Expression of Constructs:**

[00374] The pCAGGS expression vectors containing either the HK68 HAΔGHD (2G) construct, PR8 HAΔGHD (4G) construct, HK68 HAΔGHD (4G) construct, PR8 HAΔGHD (PG) construct, or HK68 HAΔGHD (PG) construct were transiently transfected into 293T cells in the absence of exogenous trypsin. Influenza HA stem domain polypeptides HAΔGHD were shown to be expressed in human 293T cell cultures. At 24 hours post-transfection, cells were lysed and lysates were subjected to SDS-PAGE followed by Western blotting. Either a rabbit polyclonal antiserum raised against a HK68 influenza A virus HA2 preparation or a mouse monoclonal raised against multiple H3 HA proteins was used as a primary antibody, as indicated at the bottom of each blot shown in FIGS. 5A and 5B.

[00375] As shown in FIG. 5A, polyclonal antibodies against a HK68 influenza A virus HA2 preparation recognized full length HA0 expressed by the control construct (lane 2) and truncated HA0 (HA0ΔGHD) expressed by the PR8 HAΔGHD (4G) construct (lane 3) and PR8 HAΔGHD (PG) construct (lane 4).

[00376] As shown in FIG. 5B, monoclonal antibodies against multiple H3 HA proteins also recognized full length HA0 expressed by the control construct (lane 2) and truncated HA0 (HA0ΔGHD) expressed by the HK68 HAΔGHD (2G) construct (lane 3), HK68 HAΔGHD (4G) construct (lane 4) and HK68 HAΔGHD (PG) construct (lane 5).

6.2 EXAMPLE 2: INFLUENZA VIRUS VACCINE BASED ON CONSERVED HEMAGGLUTININ STALK DOMAIN

[00377] This example describes the effectiveness of an influenza hemagglutinin stem domain polypeptide (sometimes referred to herein as a "headless HA") vaccine in inducing an immune response that provides full protection against death and partial protection against disease following lethal viral challenge.

6.2.1 Materials and Methods

[00378] **Plasmids**

[00379] pGag-EGFP was generously provided by Carol Carter, Stonybrook University (Hermida-Matsumoto, L., and M. D. Resh. 2000. Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. J Virol 74:8670-9). The pCAGGS expression plasmid was kindly provided by J. Miyazaki, Osaka University (Miyazaki, J., S. Takaki, K. Araki, F. Tashiro, A. Tominaga, K. Takatsu, and K. Yamamura. 1989. Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5. Gene 79:269-77). The plasmids pDZ PR8 HA and pDZ PR8 NA were constructed previously as described in reference (Quinlivan, M., D. Zamarin, A. Garcia-Sastre, A. Cullinane, T. Chambers, and P. Palese. 2005. Attenuation of equine influenza viruses through truncations of the NS1 protein. J Virol 79:8431-9). For the construction of pCAGGS HK68 HA and pCAGGS HK68 NA, viral genes were reverse transcribed (Transcriptor RT, Roche) from purified virion RNA, amplified (PFU turbo, Stratagene) and cloned into the vector pPOL1 (Fodor, E., L. Devenish, O. G. Engelhardt, P. Palese, G. G. Brownlee, and A. Garcia-Sastre. 1999. Rescue of influenza A virus from recombinant DNA. J Virol 73:9679-82) following the recombinational protocol described by Wang et al. (Wang, S., Q. Liu, J. Pu, Y. Li, L. Keleta, Y. W. Hu, J. Liu, and E. G. Brown. 2008. Simplified recombinational approach for influenza A virus reverse genetics. J Virol Methods 151:74-8). Protein coding regions were then amplified with primers carrying the appropriate restriction enzyme sites and subcloned into the multiple cloning site of pCAGGS between the Not1 and Nhe1 sites. Headless HA constructs were generated by either excise or fusion PCR methods. Excise PCR was performed on the pPOL1 PR8 HA or pPOL1 HK68 HA plasmids. The resulting PCR products were circularized by ligation and the open reading frame of the headless HA was then subcloned into pCAGGS at the Not1 and Nhe1 sites. Fusion PCR was performed on pDZ PR8 HA or

pCAGGS HK68 plasmid templates and products were inserted into pCAGGS at the Not1 and Nru1 sites. Primer sequences used are as follows Table 5 below.

Table 5: Summary of Sequences of Primers Used in the Construction of Headless HAs Listed in Figure 10

Construct	PCR method[a]	Upstream primer[b]	Downstream primer[c]
PR8 2G	Excise	ACATAGTTTTCCGTTGTGGCTGTCTTCGAGC [SEQ ID NO: 274]	GGAGGCTGTAACACGAAGTGTCAAACACCCCTGGGAGCTATA AACA [SEQ ID NO: 291]
PR8 4G	Excise	ACATAGTTTTCCGTTGTGGCTGTCTTCGAGC [SEQ ID NO: 275]	GGAGGCGGAGGTTGTAACACGAAGTGTCAAACACCCCTGGGA GCTATAAACA [SEQ ID NO: 292]
PR8 PG	Excise	ACATAGTTTTCCGTTGTGGCTGTCTTCGAGC [SEQ ID NO: 276]	CCAGGCTGTAACACGAAGTGTCAAACACCCCTGGGAGCTATA AACA [SEQ ID NO: 293]
PR8 No Cys 1G	Fusion	TGACACTTCGTGTTACCTAGTTTTCCGTTGTGGCTG [SEQ ID NO: 277]	GGTAACACGAAGTGTCAAACAC [SEQ ID NO: 294]
PR8 No Cys 2G	Fusion	ACTTCGTGTTTCCGCCTAGTTTTCCGTTGTGGCTG [SEQ ID NO: 278]	GGCGGAAACACGAAGTGTCAAACAC [SEQ ID NO: 295]
PR8 No Cys 3G	Fusion	CGTGTTACCTCCGCCTAGTTTTCCGTTGTGGCTG [SEQ ID NO: 279]	GGCGGAGGTAACACGAAGTGTCAAACAC [SEQ ID NO: 296]
PR8 No Cys	Fusion	TGTTTGACACTTCGTGTTTAGTTTTCCGTTGTGGCTGTC [SEQ ID NO: 280]	GCCACAACGGAAAACTAAACACGAAGTGTCAAACACCC [SEQ ID NO: 297]
PR8 No Cys Δ1	Fusion	GGTGTTTGACACTTCGTTAGTTTTCCGTTGTGGCTGTC [SEQ ID NO: 281]	GCCACAACGGAAAACTAACGAAGTGTCAAACACCCCTG [SEQ ID NO: 298]
PR8 No Cys Δ3	Fusion	AGGGGTGTTTGACACTTTTTTCCGTTGTGGCTGTCTTC [SEQ ID NO: 282]	ACAGCCACAACGGAAAAAAGTGTCAAACACCCCTGGGA [SEQ ID NO: 299]

PR8 No Cys NAS	Fusion	ACTTCGTGTTGGAGGCGTTTAGTTTTCCGTTGTGGCTG [SEQ ID NO: 283]	AACGCCTCCAACACGAAGTGTCAAACAC [SEQ ID NO: 300]
HK68 2G	Excise	GCUCCUCAACGGGGAAAAUAUGCGGA [SEQ ID NO: 284]	GGCTGTATTTCTGAATGCATCACTCC [SEQ ID NO: 301]
HK68 4G	Excise	GCUCCUCAACGGGGAAAAUAUGCGGAGGC [SEQ ID NO: 285]	GGAGGTTGTATTTCTGAATGCATCACTCC [SEQ ID NO: 302]
HK68 PG	Excise	GCUCCUCAACGGGGAAAAUAUGCCCA [SEQ ID NO: 286]	GGCTGTATTTCTGAATGCATCACTCC [SEQ ID NO: 303]
HK68 No Cys	Fusion	GAGTGATGCATTCAGAAATTATTTTCCCCGTTGAGGAGC [SEQ ID NO: 287]	CCTCAACGGGGAAAATAATTTCTGAATGCATCACTCCA [SEQ ID NO: 304]
HK68 No Cys Δ1	Fusion	TGGAGTGATGCATTCAGATATTTTCCCCGTTGAGGAGC [SEQ ID NO: 288]	CCTCAACGGGGAAAATATCTGAATGCATCACTCCAAAT [SEQ ID NO: 305]
HK68 No Cys Δ3	Fusion	ATTTGGAGTGATGCATTCTTTCCCCGTTGAGGAGCTC [SEQ ID NO: 289]	CTCCTCAACGGGGAAAGAATGCATCACTCCAAATGG [SEQ ID NO: 306]
HK68 No Cys NAS	Fusion	TTCAGAAATGGAGGCGTTTATTTTCCCCGTTGAGGAG [SEQ ID NO: 290]	AACGCCTCCATTTCTGAATGCATCACTCC [SEQ ID NO: 307]

[a] In the Excise PCR method, primers flanking the sequence targeted for deletion are used to amplify the remainder of the gene and plasmid vector. Thus, the downstream primer is the forward primer in the PCR reaction and the upstream primer is the reverse primer. A linear fragment is produced which lacks the intervening sequence. A short foreign sequence can be introduced into the deletion site by adding it to the 5' end of one of the primers. Once generated, the PCR product is purified and self-ligated in order to produce a circular plasmid carrying the modified gene of interest.

In the Fusion PCR method two fragments of the desired product are PCR amplified independently. One fragment corresponds to the sequence upstream of the introduced mutation and the second corresponds to the sequence downstream of the mutation. The upstream fragment is amplified using the upstream primer in combination with a 5' outside primer, while the downstream fragment is produced using the downstream primer together with a 3' outside primer. The nucleotide sequence to be introduced at the site of mutation is added to both fragments through inclusion in both the upstream and downstream primers. The two pieces are then fused in a subsequent PCR reaction using only the 5' and 3' outside primers. The PR8 outside primers used for all PR8 fusion PCRs were PR8 5': cgagctcatgaaggcaaacctactgg and PR8 3': cgcagatcttcagatgcatattctgcact. The HK68 outside primers used for all HK68 fusion PCRs were HK68 5'notI: ggaagcggccgcatgaagaccatcattgctttgag and HK68 3'nruI: gcggcgtcgcgatcaaatgcaaatgttgcacctaa.

[b] These primers anneal immediately upstream of the mutation site in both Excise and Fusion PCR methods.

[c] These primers anneal immediately downstream of the mutation site in both Excise and Fusion PCR method

[00380] **Antibodies**

[00381] Monoclonal antibody (mAb) 12D1 was generated by sequential intramuscular immunization of Balb/C mice with plasmid DNAs encoding the HK68 HA, the A/Alabama/1/1981 (H3N2) HA, and the A/Beijing/46/1992 (H3N2) HA, followed by a boost with whole A/Wyoming/03/2003 (H3N2) virus. See U.S. provisional application Serial Nos. 61/181,263 and 61/224,302, filed May 26, 2009 and July 9, 2009, which are incorporated herein by reference in their entirety, for a description of the 12D1 mAb. This mAb binds multiple H3 HA proteins and maps to the HA2 subunit. Rabbit polyclonal serum 3951 was raised against PR8 virus from which the HA1 subunit had been removed by treatment with acid and DTT (Graves, P. N., J. L. Schulman, J. F. Young, and P. Palese. 1983. Preparation of influenza virus subviral particles lacking the HA1 subunit of hemagglutinin: unmasking of cross-reactive HA2 determinants. Virology 126:106-16).

[00382] **Cells and Viruses**

[00383] 293T cells were obtained from the ATCC and were maintained in Dulbecco's modified Eagles medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Clontech).

[00384] Influenza A/Puerto Rico/8/1934 (H1N1) virus was obtained by reverse genetics as previously described (Steel, J., S. V. Burmakina, C. Thomas, E. Spackman, A. Garcia-Sastre, D. E. Swayne, and P. Palese. 2008. A combination in-ovo vaccine for avian influenza virus and Newcastle disease virus. Vaccine 26:522-31) using plasmids encoding the eight genes defined by accession numbers AF389115 to AF189122 (A/Puerto Rico/8/34/Mount Sinai) in the NCBI database. The virus was amplified in 10-11 day old embryonated chickens eggs and titrated by plaque assay.

[00385] **Western blotting**

[00386] To assess expression levels of HA-based proteins, 293T cells were transfected with 2 μg of the appropriate plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 24 h post-transfection, cells were lysed in 2x protein loading buffer (125 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate, 20% glycerol, 10% β-mercaptoethanol, and 0.1% bromophenol blue). Lysates were heated at 100°C for 5 minutes and then separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane (Whatman, Inc.). To detect HA-based proteins in VLP preparations, pelleted VLPs were suspended in phosphate buffered saline (PBS), lysed through 1:1 mixing with 2x protein loading

buffer, boiled for 5 minutes, separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were then probed with mAb 12D1 or 3951 antiserum at a 1:2000 dilutions.

[00387] **Flow Cytometric Analysis**

[00388] To assess levels of HA-based proteins at the cell surface, 293T cells were transfected with 1 μg of the appropriate plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 24 hours post-transfection, cells were trypsinized and resuspended in PBS containing 2% FBS prior to staining with 3951 antiserum at a 1/250 dilution or mAb 12D1 at a 1/200 dilution. Stained cells were enumerated on a Beckman Coulter Cytomics FC 500 flow cytometer and results were analyzed using Flow Jo software.

[00389] **Generation of Virus Like Particles**

[00390] For the production of virus like particles, $6x10^6$ 293T cells were seeded into a 10 cm dish in 8 ml of DMEM with 10% FBS. While still in suspension, cells were transfected with Lipofectamine 2000 (Invitrogen) combined with the desired plasmid DNAs at a 4:1 ratio and as per the manufacturer's instructions. The amounts of plasmid DNA used were as follows: for Gag-only VLPs, 7.5 μg pGagEGFP was transfected; for gag + PR8 4G VLPs, 4.5 μg of pGagEGFP plus 4.5 μg of pCAGGS PR8 4G was used; for Gag + HK68 4G VLPs, 4.5 μg of pGagEGFP plus 4.5 μg of pCAGGS HK68 4G was used; and for Gag + PR8 HA + PR8 NA VLPs, 3 μg of pGagEGFP was combined with 3 μg each of pDZ PR8 HA and pDZ PR8 NA. At 6 hours post-transfection, medium was changed to fresh DMEM containing 10% FBS or to Opti-MEM (Gibco) supplemented with 3% bovine serum albumin (Sigma) and 10 μg/ml TPCK-treated trypsin (Sigma). VLPs were harvested at 28 hours post-transfection by layering clarified cell culture supernatant over a cushion of 30% sucrose in NTE buffer (1M sodium chloride, 0.1 M Tris, 0.01 M EDTA, pH 7.4) and centrifuging for 2 h at 4°C and 25,000 RPM in an SW28 rotor (Beckman). Pellets were resuspended in PBS and HA protein content was assessed by Western blotting in parallel with serial dilutions of a known amount of gradient purified PR8 or HK68 virus (prepared as described in Palese, P., and J. L. Schulman. 1976. Differences in RNA patterns of influenza A viruses. J Virol 17:876-84). VLPs shown in Figure 13 were produced in the presence of exogenous trypsin, while those used to boost mice were produced without the addition of trypsin.

[00391] **Mouse Vaccine-Challenge Experiment**

[00392] Female, 6 to 8-week-old C57BL/6 mice (Charles River Laboratories) were initially vaccinated by intramuscular administration of plasmid DNA followed immediately by the application of electrical stimulation (TriGrid Delivery System, Ichor Medical Systems (Luxembourg, A., C. F. Evans, and D. Hannaman. 2007. Electroporation-based DNA immunisation: translation to the clinic. Expert Opin Biol Ther 7:1647-64; Luxembourg, A., D. Hannaman, E. Nolan, B. Ellefsen, G. Nakamura, L. Chau, O. Tellez, S. Little, and R. Bernard. 2008. Potentiation of an anthrax DNA vaccine with electroporation. Vaccine 26:5216-22)). The spacing of the TriGrid electrode array is 2.5 mm, and the electrical field is applied at an amplitude of 250 V/cm of electrode spacing for six pulses totaling 40 msec duration applied over a 400 msec interval. Each DNA vaccination comprised 37.5 μg of pGagEGFP alone or in combination with 75 μg of pDZ PR8 HA, pCAGGS PR8 4G or pCAGGS HK68 4G. Three weeks later a DNA boost was performed following the same procedure. Five weeks after the second dose of DNA was administered, the mice were boosted a second time with VLPs. For the HA-containing VLPs, HA content was normalized such that each mouse received approximately 150 ng HA protein. Prior to intraperitoneal administration, VLP suspensions were combined in a 1:1 ratio with complete Freund's adjuvant (Pierce) and emulsified by multiple passes through two linked syringes. Mice were challenged three weeks following the VLP boost via intranasal inoculation of 2 MLD50 (50% mouse lethal dose) of PR8 virus in a total volume of 50 μl PBS. Body weight was monitored daily and mice losing greater than 25% of their initial weight were sacrificed and scored as dead.

[00393] **Serological Assays**

[00394] Sera were collected from mice immediately prior to challenge and at 21 days post-challenge. To remove non-specific inhibitors of hemagglutination, trypsin-heat-periodate treatment was performed as described previously (Lowen, A. C., J. Steel, S. Mubareka, E. Carnero, A. Garcia-Sastre, and P. Palese. 2009. Blocking inter-host transmission of influenza virus by vaccination in the guinea pig model. J Virol. 83: 2803-18). For hemagglutination inhibition assays, sera from each vaccination group were pooled; for ELISA, sera collected from individual mice were evaluated separately. HI assays were carried out as described previously (Lowen, A. C., J. Steel, S. Mubareka, E. Carnero, A. Garcia-Sastre, and P. Palese. 2009. Blocking inter-host transmission of influenza virus by vaccination in the guinea pig model. J Virol. 83: 2803-18). For ELISA, 96-well plates (Co-Star) were coated with 0.25 μg per well of PR8 virus or with

0.1 μg per well of purified recombinant HA protein in PBS. PR8 virus was prepared from allantoic fluid by concentration through a 30% sucrose cushion as previously described (Lowen, A. C., J. Steel, S. Mubareka, E. Carnero, A. Garcia-Sastre, and P. Palese. 2009. Blocking inter-host transmission of influenza virus by vaccination in the guinea pig model. J Virol. 83: 2803-18). The recombinant HA proteins of the A/California/04/2009, A/Viet Nam/1203/2004, and A/Singapore/1/1957 viruses were obtained from BEI Resources; the A/Hong Kong/1/1968 HA was the generous gift of Ian Wilson; and the A/New Caledonia/20/99 HA was purchased from Feldan-Bio. Five-fold serial dilutions of anti-sera were incubated on the plates and, after extensive washing, bound antibody was detected with an alkaline phosphatase linked anti-mouse IgG antibody (Caltag) and PNPP substrate (Sigma). In each assay a rabbit immune serum raised against whole PR8 virus was included as a positive control, and serum obtained from a naïve C57BL/6 mouse was included as a negative control.

6.2.2 Results

[00395] **Design and Construction of Headless HA Constructs**

[00396] The goal was to generate an immunogen consisting of the complete HA2 polypeptide plus the regions of HA1 contributing to the stalk region, but lacking the globular head domain of HA1. With this aim in mind, the existence of a conserved disulfide bond linking cysteines 52 and 277 (H3 numbering) of HA1 was noted. The loop flanked by these two cysteines comprises the bulk of the globular head domain, while the N-terminal 51 and the C-terminal 52 amino acids of HA1 extend downward from the cysteine bridge and contribute to the stalk region. Due to the proximity of cysteines 52 and 277 in the three-dimensional structure of HA (Stevens, J., A. L. Corper, C. F. Basler, J. K. Taubenberger, P. Palese, and I. A. Wilson. 2004. Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. Science 303:1866-70 and Figure 10), it was predicted that replacement of the intervening loop with a short linker peptide would not disrupt the folding of the remainder of the molecule. Based on this principle, a panel of headless HA constructs was designed (Figure 10).

[00397] First, sequences encoding linker peptides of two glycines (2G), four glycines (4G) or a proline and a glycine (PG) were inserted into the open reading frames of the A/Puerto Rico/8/1934 (H1N1) (PR8) and the A/Hong Kong/1968 (H3N2) (HK68) hemagglutinins in place of the respective nucleotide sequences encoding amino acids 53

to 276. These three linker peptides were selected to have a range of flexibilities, with 4G predicted to be the most flexible and PG the most rigid. To test whether insertion of a linker in the absence of a disulfide bond at this position would yield a more stable product, three additional constructs in the context of the PR8 HA were designed: sequences encoding one, two or three glycines were inserted in place of the sequences encoding amino acids 52 to 277 (that is, both the cysteines and the connecting loop were replaced). Based on the hypothesis that glycosylation may improve trafficking through the Golgi, the insertion of a glycosylation site (NAS) in place of amino acids 52 to 277 in both the PR8 and the HK68 backgrounds was also tested. Finally, a series of three constructs were made in each of the PR8 and the HK68 HAs in which existing wild-type amino acids were directly linked: amino acid 51 to 278, 51 to 279, or 50 to 280. Constructs were made in the context of an H1 (representative of group 1) and an H3 (representative of group 2) HA since the activity of neutralizing antibodies targeting the stalk region appears to be limited to HA subtypes within the same major phylogenetic group (Ekiert, D. C., G. Bhabha, M. A. Elsliger, R. H. Friesen, M. Jongeneelen, M. Throsby, J. Goudsmit, and I. A. Wilson. 2009. Antibody recognition of a highly conserved influenza virus epitope. Science 324:246-51; Kashyap, A. K., J. Steel, A. F. Oner, M. A. Dillon, R. E. Swale, K. M. Wall, K. J. Perry, A. Faynboym, M. Ilhan, M. Horowitz, L. Horowitz, P. Palese, R. R. Bhatt, and R. A. Lerner. 2008. Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. Proc Natl Acad Sci U S A 105:5986-91; Okuno, Y., Y. Isegawa, F. Sasao, and S. Ueda. 1993. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. J Virol 67:2552-8; and Sui, J., W. C. Hwang, S. Perez, G. Wei, D. Aird, L. M. Chen, E. Santelli, B. Stec, G. Cadwell, M. Ali, H. Wan, A. Murakami, A. Yammanuru, T. Han, N. J. Cox, L. A. Bankston, R. O. Donis, R. C. Liddington, and W. A. Marasco. 2009. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat Struct Mol Biol 16:265-73).

[00398] These constructs are summarized in the Table 6 below.

Table 6: Summary of Constructs

Name	HA1 N-terminal Stem Segment	Linker	HA1 C-terminal Stem Segment	HA2 Domain
PR8-2G	SEQ ID NO:34	Gly-Gly	SEQ ID NO:50	SEQ ID NO:66
PR8-4G	SEQ ID NO:34	Gly-Gly-Gly-Gly	SEQ ID NO:50	SEQ ID NO:66
PR8-PG	SEQ ID NO:34	Pro-Gly	SEQ ID NO:50	SEQ ID NO:66
PR8-No Cys-1G	SEQ ID NO:177	Gly	SEQ ID NO:226	SEQ ID NO:66
PR8-No Cys 2G	SEQ ID NO:177	Gly-Gly	SEQ ID NO:226	SEQ ID NO:66
PR8-No Cys 3G	SEQ ID NO:177	Gly-Gly-Gly	SEQ ID NO:226	SEQ ID NO:66
PR8-No Cys	SEQ ID NO:177	direct bond	SEQ ID NO:226	SEQ ID NO:66
PR8-No Cys Δ1	SEQ ID NO:178	direct bond	SEQ ID NO:227	SEQ ID NO:66
PR8-No Cys Δ3	SEQ ID NO:179	direct bond	SEQ ID NO:228	SEQ ID NO:66
PR8-No Cys NAS	SEQ ID NO:177	Asn-Ala-Ser	SEQ ID NO:226	SEQ ID NO:66
PR8-CON-A	SEQ ID NO:309	Gly-Gly-Gly-Gly	SEQ ID NO:310	SEQ ID NO:66
HK68-2G	SEQ ID NO:36	Gly-Gly	SEQ ID NO:52	SEQ ID NO:68
HK68-4G	SEQ ID NO:36	Gly-Gly-Gly-Gly	SEQ ID NO:52	SEQ ID NO:68
HK68-PG	SEQ ID NO:36	Pro-Gly	SEQ ID NO:52	SEQ ID NO:68
HK68-No Cys	SEQ ID NO:183	direct bond	SEQ ID NO:232	SEQ ID NO:68
HK68-No Cys Δ1	SEQ ID NO:184	direct bond	SEQ ID NO:233	SEQ ID NO:68
HK68-No Cys Δ3	SEQ ID NO:185	direct bond	SEQ ID NO:234	SEQ ID NO:68
HK68-No Cys NAS	SEQ ID NO:183	Asn-Ala-Ser	SEQ ID NO:232	SEQ ID NO:68
HK68-CON-A	SEQ ID NO:308	Gly-Gly-Gly-Gly	SEQ ID NO:52	SEQ ID NO:68

[00399] **Expression of Headless HA Constructs in Transfected Cell Cultures**

[00400] As a preliminary test of protein integrity and stability, levels of the headless HA constructs expressed in transiently transfected cells were assessed by Western blotting. As shown in Figure 11A, headless HA constructs based on the PR8 HA protein were expressed to levels comparable to the corresponding full length protein at 24 hours post-transfection. Within the panel of constructs tested, those which retained cys 52 and 277 and carried the linker peptides 2G, 4G or PG exhibited the highest steady state levels. In the context of the HK68 HA (Figure 11B) similar results were seen: all HK68 headless HAs tested were detected using an antibody specific to the stalk domain, and those carrying the 2G, 4G or PG linker between cys 52 and cys 277 were the most abundant. For both HK68 and PR8, the least abundant constructs were those with the direct linkage between amino acids 50 and 280 and those with the inserted glycosylation site (Figure 11).

[00401] To test whether the headless HA constructs were also being transported to the cell surface, FACS analysis of transiently transfected 293T cells was performed following surface staining with HA2-specific antibodies. Only the 2G, 4G and PG constructs, which showed high levels by Western blotting, were tested in this assay. As shown in Figure 12, the three PR8 and the three HK68 based constructs were detected, indicating that transport through the Golgi to the cell surface was not disrupted by the removal of the globular head domain. No marked differences among the three linker bridges were noted in either the Western blotting or FACS based assays. The constructs carrying the 4G linker bridge were selected for further characterization.

[00402] **Incorporation of Headless HA into Viral Like Particles**

[00403] As a further test of the functionality of the headless HA molecules, their ability to bud from the cell surface to produce virus like particles (VLP) was assessed. While transient expression of the headless HA constructs alone in 293T cells was not found to result in VLP production, co-transfection with an HIV Gag-based construct did lead to the production of headless HA-containing particles. Specifically, when either the PR8 or HK68 4G headless HA constructs was co-expressed in 293T cells with a Gag-EGFP (enhanced green fluorescent protein) fusion protein, particles capable of sedimenting through a 30% sucrose cushion and containing headless HA proteins were released into the cell culture medium (Figure 13). Similar results were obtained in the presence (as in Figure 13) or absence of exogenous trypsin. Unlike the full-length HA protein, and as expected based on the lack of a globular head domain, the release of headless HA containing particles was not found to be dependent on the presence of neuraminidase activity.

[00404] **Vaccination with the PR8 Headless HA Provides Protection Against Homologous Challenge in Mice**

[00405] The potential of vaccination with a headless HA construct to induce a protective immune response was evaluated in the mouse model. A three-dose vaccine regimen was followed in which mice received plasmid DNA on days 0 and 21 and VLP preparations delivered with Freund's adjuvant on day 56. Each DNA vaccine comprised pGagEGFP alone or in combination with a protein expression vector encoding the full length PR8 HA, the PR8 4G headless HA or the HK68 4G headless HA and was administered intramuscularly with electroporation. For a final boost, VLP preparations with an HA content of 150 ng (or an equivalent amount of Gag-only VLP) were combined with Freund's complete adjuvant and administered intraperitoneally to each

mouse. On day 77, mice were challenged intranasally with PR8 virus and then monitored daily for morbidity and mortality for 10 days. In the Gag-only vaccinated group, three out of four mice lost >25% of their initial body weight and were therefore scored as dead and the fourth animal was seen to lose 15% body weight. By contrast, all mice vaccinated with the PR8 4G headless HA survived and experienced a maximum, on average, of only 6% weight loss (Figure 14).

[00406] Vaccination of Mice with the PR8 Headless HA Elicits Cross-Reactive Anti-Sera

[00407] The reactivity of serum collected from vaccinated mice against influenza virus HA proteins was assessed by hemagglutination inhibition (HAI) assay and ELISA. As expected based on the absence of a globular head domain in the vaccine constructs, the pooled sera from mice immunized with Gag alone, the HK68 4G headless HA or the PR8 4G headless HA did not show HAI activity against PR8 virus prior to challenge. In contrast, pre-challenge sera obtained from mice that received the full length PR8 HA vaccine, as well as all post-challenge sera, were strongly reactive against PR8 virus in the HAI assay (Table 7).

[00408] Table 7. Lack of Hemagglutination Inhibition Activity in Immune Sera of Headless HA Vaccinated Mice.

	Fold-Increase Over Gag-Only Pre-Challenge Serum	
Vaccine	Pre-Challenge	Post-Challenge
Gag-only	-	8
HK68 4G headless HA plus Gag	1	8
PR8 4G headless HA plus Gag	1	8
PR8 full length HA plus Gag	≥128	≥128

[00409] By ELISA, pre-challenge sera were tested against a panel of HA substrates in order to evaluate the breadth of reactivity (Figure 15). Against concentrated PR8 virion (Figure 15A), Gag-only and HK68 4G anti-sera showed only a low level of background activity at the lowest dilution (1:50), while sera from the PR8 full length HA vaccinated animals gave a positive signal at a 1:6250 dilution. Antisera against the PR8 4G headless HA were less potent than those against the full length HA, but reacted positively at a 1:50 dilution. When tested against recombinant HA proteins derived from a recent seasonal H1N1 (A/New Caledonia/20/1999; Figure 12B) and a 2009

pandemic H1N1 (A/California/04/2009; Figure 15C) influenza virus, Gag-only and HK68 4G anti-sera were negative, while sera from mice that received the full length PR8 HA were either negative or showed a low level of reactivity. On these heterologous H1 substrates, the PR8 4G anti-sera showed the highest level of reactivity, with the sera from two of the five mice in particular demonstrating high titers. Similar results were seen with recombinant HA proteins of the H2 and H5 subtypes: against the A/Singapore/1/1957 (H2N2) and A/Viet Nam/1203/2004 (H5N1) HAs, sera derived from PR8 4G vaccinated mice showed moderate to high activity, while sera from the remaining groups (including the full length HA) were largely negative (Figures 15D and 15E). Finally, against the H3 subtype HA of A/Hong Kong/1/1968 (H3N2) only the HK68 4G anti-sera produced a positive signal (Figure 15F). Thus, overall, sera obtained from mice vaccinated with the headless PR8 HA showed greater activity against heterologous strains than did sera from full length PR8 HA vaccinated animals. While serum titers of PR8 4G vaccinated mice appeared to be higher against the heterologous HA proteins than against the homologous PR8 virus, a direct comparison should not be made due to differing substrates used (purified HA versus whole virus). Within the PR8 4G group, the sera from two mice in particular consistently showed relatively high titers by ELISA. These serological findings correlated with the protection data in that these same two mice were fully protected from disease while their three remaining counterparts each exhibited some weight loss after challenge.

6.2.3 Conclusion

[00410] This example describes influenza hemagglutinin (HA) stem domain polypeptides ("headless HA constructs") which lack the highly immunogenic globular head of the HA protein and are thereby designed to present the conserved HA stalk region to immune cells. These headless HA constructs can be stably expressed in mammalian cells and targeted to the cell surface in a similar manner to full length HA polypeptides. Immunization of mice with a PR8-based HA stem domain polypeptide in plasmid DNA and VLP formats provided full protection against death and partial protection from disease following a lethal homologous challenge.

[00411] Serological analysis revealed that the PR8 4G influenza headless HA construct, but not the full-length PR8 HA vaccine, induced antibodies which are cross-reactive among group 1 HA subtypes. This finding suggests that the globular head domain of an intact HA molecule inhibits recognition of the stem region by immune

cells, either through steric shielding or due to the immune dominance of the membrane distal portion of the protein. These data furthermore suggest that vaccination with an headless HA construct can lead to protection against divergent influenza strains.

6.3 EXAMPLE 3: CHALLENGE WITH HETEROLOGOUS VIRUSES

[00412] The data described in Example 2 above shows that mice vaccinated with a PR8 headless HA construct are protected against challenge with PR8 virus (that is, protected against homologous challenge). These data indicate that an influenza virus hemagglutinin stem domain polypeptide (sometimes referred to herein as a "headless HA") is sufficiently immunogenic to act as a vaccine but do not provide information on the breadth of protection achieved. To test whether an influenza virus hemagglutinin stem domain polypeptide can elicit an immune response which will protect against challenge with a range of heterologous viruses, mice will be vaccinated through intraperitoneal injection of 5 μg of a purified influenza virus hemagglutinin stem domain polypeptide or, as a control, 5 μg of full length HA in the context of whole inactivated influenza virus preparations. In both cases, the vaccine will be combined with MF-59 adjuvant prior to administration. At three weeks post-vaccination, groups of 8 mice will be challenged by intranasal inoculation with 10 MLD50 (50% mouse lethal dose) of the virus strains identified in Table 8. Mice will be monitored daily up to 14 days post-challenge for changes in body weight and death. The influenza virus hemagglutinin stem domain polypeptide vaccines are expected to provide superior protection from death and disease following heterologous virus challenges compared to the conventional whole inactivated virus vaccines.

[00413] Table 8. Summary of Challenge Experiments

Vaccine	Challenge Virus	Mouse Model[a]
PR8 4G headless HA	A/Puerto Rico/8/1934 (H1N1)	C57BL/6
	A/Netherlands/602/2009 (novel H1N1)	DBA-2
	A/Viet Nam/1203/2004 (H5N1)[b]	C57BL/6
PR8 virus	A/Puerto Rico/8/1934 (H1N1)	C57BL/6
	A/Netherlands/602/2009 (novel H1N1)	DBA-2
	A/Viet Nam/1203/2004 (H5N1)	C57BL/6
HK68 4G headless HA	X31 (H3N2)	DBA-2
	A/Rhea/North Carolina 39482/1993 (H7N1)	DBA-2

X31[c] virus	X31 (H3N2)	DBA-2
	A/Rhea/North Carolina 39482/1993 (H7N1)	DBA-2

[a] The strain of inbred mouse to be used is based on the lethality of the challenge viruses. Virus strains that are less pathogenic to mice must be used in the more susceptible DBA-2 model.

[b] Rather than the wild-type virus, a reassortant virus carrying the HA and NA genes of A/Viet Nam/1203/04 and the remaining six genes from PR8 virus will be used. In addition, the multibasic cleavage site in the HA segment of this virus is mutated to a low pathogenic form. These changes do not affect the antigenicity of the HA protein.

[c] X31 is a mouse adapted virus carrying the HA and NA genes of A/Hong Kong/1/1968 (H3N2) virus and the remaining six genes from PR8.

[00414] 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. A polypeptide comprising:

a. an influenza hemagglutinin HA1 domain that comprises an HA1 N-terminal stem segment covalently linked to a linker of 1 to 50 heterologous residues that is in turn covalently linked to an HA1 C-terminal stem segment; said HA1 domain in tertiary or quaternary association with

b. an influenza hemagglutinin HA2 domain.

2. The polypeptide of claim 1, wherein the HA1 domains contact the HA2 domain.

3. The polypeptide of claim 1 wherein the HA1 C-terminal stem segment is covalently linked to the HA2 domain.

4. The polypeptide of claim 1 that has a tertiary or quaternary structure having 0-5 Å RMS deviation from the tertiary or quaternary structure of the corresponding polypeptide of 1RUZ.

5. The polypeptide of claim 1 that selectively binds neutralizing antiserum capable of binding an influenza hemagglutinin .

6. The polypeptide of claim 1 that lacks an influenza globular domain.

7. The polypeptide of claim 1, wherein the amino acid sequences of the HA1 domains are at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to the amino acid sequences of the corresponding domains of an HA1 from an H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16 influenza A.

8. The polypeptide of claim 1, wherein the amino acid sequence of the HA2 domain is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to the amino acid sequence of an HA2 from an H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16 influenza A.

9. The polypeptide of claim 1, wherein the amino acid sequence of the HA2 domain is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to the amino acid sequence of an HA2 from an H3 influenza A.

10. The polypeptide of claim 1, wherein the amino acid sequence of the HA2 domain is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to the amino acid sequence of an HA2 from an H1 influenza A.

11. The polypeptide of claim 1, wherein the amino acid sequence of the HA1 N-terminal stem segment consists of a sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to residues 1-52 of an HA1 from an H3 influenza A.

12. The polypeptide of claim 1, wherein the amino acid sequence of the HA1 C-terminal stem segment consists of a sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to residues 277-328 of an HA1 from an H3 influenza A.

13. The polypeptide of claim 1, wherein the amino acid sequence of the HA1 N-terminal stem segment consists of a sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to residues 1-44 of an HA1 from an H1 influenza A.

14. The polypeptide of claim 1, wherein the amino acid sequence of the HA1 C-terminal stem segment consists of a sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to residues 291-326 of an HA1 from an H1 influenza A.

15. The polypeptide of claim 1, wherein the amino acid sequence of the HA2 domain is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to the amino acid sequence of an HA2 from an influenza B.

16. The polypeptide of claim 1, wherein the amino acid sequences of the HA1 domains are at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to the amino acid sequences of the corresponding domains of an HA1 from influenza B.

17. The polypeptide of claim 1, wherein the amino acid sequence of the HA1 N-terminal stem segment consists of a sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to residues 1-80 of an HA1 from an influenza B.

18. The polypeptide of claim 1, wherein the amino acid sequence of the HA1 C-terminal stem segment consists of a sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to residues 277-344 of an HA1 from an influenza B.

19. The polypeptide of claim 1, wherein the amino acid sequence of the HA1 N-terminal stem segment consists of a sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to residues 1-50 of an HA1 from an influenza B.

20. The polypeptide of claim 1, wherein the amino acid sequence of the HA1 C-terminal stem segment consists of a sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to residues 277-344 of an HA1 from an influenza B.

21. The polypeptide of claim 1, wherein the amino acid sequence of the HA1 N-terminal stem segment consists of a sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to residues 1-66 of an HA1 from an influenza B.

22. The polypeptide of claim 1, wherein the amino acid sequence of the HA1 C-terminal stem segment consists of a sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96% or 98% identical to residues 271-344 of an HA1 from an influenza B.

23. The polypeptide of claim 1, wherein the HA1 N-terminal stem segment comprises a cysteine residue covalently linked to a cysteine residue of the HA1 C-terminal stem segment via a disulfide bridge.

24. The polypeptide of claim 1, wherein the HA1 N-terminal stem segment comprises the amino acid sequence A_{17}-A_{18}-$(Xaa)_n$-A_{38} (SEQ ID NO:146),

wherein

A_{17} is Y or H;

A_{18} is H, L, or Q;

$(Xaa)_n$ represents a sequence of 18-20 amino acid residues; and

A_{38} is H, S, Q, T or N.

25. The polypeptide of claim 1, wherein the HA1 C-terminal stem segment comprises the amino acid sequence A_{291}-A_{292}, wherein

A_{291} is T, S, N, D, P or K; and

A_{292} is L, M, K or R.

26. The polypeptide of claim 1, wherein the HA2 domain comprises the amino acid sequence A_{18}-A_{19}-A_{20}-A_{21}, wherein

A_{18} is V or I;

A_{19} is D, N or A;

A_{20} is G, and

A_{21} is W.

27. The polypeptide of claim 1, wherein the HA2 domain comprises the amino acid sequence A_{38}-A_{39}-A_{40}-A_{41}-A_{42}-A_{43}-A_{44}-A_{45}-A_{46}-A_{47}-A_{48}-A_{49}-A_{50}-A_{51}-A_{52}-A_{53}-A_{54}-A_{55}-A_{56} (SEQ ID NO:149), wherein

A_{38} is K, Q, R, L or Y;

A_{39} is any amino acid residue;

A_{40} is any amino acid residue;

A_{41} is T;

A_{42} is Q;

A_{43} is any amino acid residue;

A_{44} is A;

A_{45} is I;

A_{46} is D;

A_{47} is any amino acid residue;

A_{48} is I, V or M;

A_{49} is T, Q or N;

A_{50} is any amino acid residue;

A_{51} is K;

A_{52} is V or L;

A_{53} is N;

A_{54} is any amino acid residue;

A_{55} is V, I or L; and

A_{56} is V or I.

28. The polypeptide of claim 1, wherein said linker is of 1 to 40, 1 to 30 residues, 1 to 20 residues, 1 to 10 residues, 1 to 5 residues, 1 to 4 residues, 1 to 3 residues, 1 to 2 residues or 1 residue.

29. The polypeptide of claim 1, wherein said linker is selected from the group consisting of GG, PG, GGG, GGGG, GGGGG, ITPNGSIPNDKPFQNVNKITYGA, NAS and a direct bond.

30. A nucleic acid encoding the polypeptide of any one of claims 1 to 29.

31. A cell expressing the nucleic acid of claim 30.

32. A virus comprising a genome engineered to express the nucleic acid of claim 30.

33. A virus comprising the polypeptide of any one of claims 1 to 29.

34. The virus of claim 32 or 33, wherein the virus is an influenza virus.

35. The virus of claim 34 which is an influenza A virus.

36. The virus of claim 34 which is an influenza B virus.

37. The virus of claim 32 or 33, wherein the virus is a Newcastle disease virus (NDV), a vaccinia virus, an adenovirus, an adeno-associated virus (AAV), or a retrovirus.

38. The virus of claim 33 which is inactivated or split.

39. The virus of claim 34 which is inactivated or split.

40. A viral-like particle comprising the polypeptide of any one of claims 1 to 29.

41. An immunogenic composition comprising the polypeptide of any one of claims 1 to 29.

42. An immunogenic composition comprising the virus of claim 32 and a pharmaceutically acceptable carrier.

43. An immunogenic composition comprising the virus of clam 33 and a pharmaceutically acceptable carrier.

44. An immunogenic composition comprising the virus of claim 34.

45. An immunogenic composition comprising the virus of claim 38 and a pharmaceutically acceptable carrier.

46. An immunogenic composition comprising the virus of claim 39 and a pharmaceutically acceptable carrier.

47. The immunogenic composition of claim 45 further comprising an adjuvant.

48. The immunogenic composition of claim 46 further comprising an adjuvant.

49. An immunogenic composition comprising the viral-like particle of claim 40 and a pharmaceutically acceptable carrier.

50. A method of immunizing a subject comprising administering to the subject an effective amount of the immunogenic composition of claim 41.

51. A method of immunizing a subject comprising administering to the subject an effective amount of the immunogenic composition of claim 43.

52. A method of immunizing a subject comprising administering to the subject an effective amount of the immunogenic composition of claim 44.

53. A method of immunizing a subject comprising administering to the subject an effective amount of the immunogenic composition of claim 45.

54. A method of immunizing a subject comprising administering to the subject an effective amount of the immunogenic composition of claim 46.

55. A method of immunizing a subject comprising administering to the subject an effective amount of the immunogenic composition of claim 47.

56. A method of immunizing a subject comprising administering to the subject an effective amount of the immunogenic composition of claim 48.

57. A method of immunizing a subject comprising administering to the subject an effective amount of the immunogenic composition of claim 49.

58. The method of claim 50, wherein the subject is a human.

59. The method of claim 51, wherein the subject is a human.

60. The method of claim 52, wherein the subject is a human.

61. The method of claim 53, wherein the subject is a human.

62. The method of claim 54, wherein the subject is a human.

63. The method of claim 55, wherein the subject is a human.

64. The method of claim 56, wherein the subject is a human.

65. The method of claim 57, wherein the subject is a human.

66. The method of claim 51, wherein the immunogenic composition is administered intramuscularly or intranasally to the subject.

67. A method of preventing an influenza virus disease comprising administering to a subject an effective amount of the immunogenic composition of claim 41.

68. A method of treating an influenza virus infection or an influenza virus disease comprising administering to a subject an effective amount of the immunogenic composition of claim 41.

69. A method of preventing an influenza virus disease comprising administering to a subject an effective amount of the immunogenic composition of claim 44.

70. A method of treating an influenza virus infection or an influenza virus disease comprising administering to a subject an effective amount of the immunogenic composition of claim 44.

71. The method of claim 67, wherein the subject is a human.

72. The method of claim 68, wherein the subject is a human.

73. The method of claim 69, wherein the subject is a human.

74. The method of claim 70, wherein the subject is a human.

```
                    ▼(Mature residue 1)
H1   MKANLLVLLCALA---------AADADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLE
H2   --MAIIYLILLFT---------AVRGDQICIGYHSNNSTEKVDTILERNVTVTHAQNILE
H3   MKTIIALSYIFCLALGQDLPGNDNSTATLCLGHHAVPNGTLVKTITDDQIEVTNATELVQ
H4   MLSIVILFLLIAENS----SQNYTGNPVICMGHHAVANGTMVKTLADDQVEVVTAQELVE
H5   -MERIVLLLAIVS---------LVKSDQICIGYHANKSTKQVDTIMEKNVTVTHAQDILE
H6   -MIAIIVVAILAT---------AGRSDKICIGYHANNSTTQIDTILEKNVTVTHSVELLE
H7   MNTQILVFALVAVIPTN--------ADKICLGHHAVSNGTKVNTLTERGVEVVNATETVE
H8   --MEKFIAIAT-LAS-------TNAYDRICIGYQSNNSTDTVNTLIEQNVPVTQTMELVE
H9   -METKAIIAALLMVT-------AANADKICIGYQSTNSTETVDTLTESNVPVTHTKELLH
H10  MYKVVVIIALLGAVKG---------LDRICLGHHAVANGTIVKTLTNEQEEVTNATETVE
H11  -MEKTLLFAAIFL---------CVKADEICIGYLSNNSTDKVDTIIENNVTVTSSVELVE
H12  --MEKFIILSTVLAA-------SFAYDKICIGYQTNNSTETVNTLSEQNVPVTQVEELVH
H13  MALNVIATLTLIS-V-------CVHADRICVGYLSTNSSERVDTLLENGVPVTSSIDLIE
H14  MIALILVALALSHTAYSQITNGTTGNPIICLGHHAVENGTSVKTLTDNHVEVVSAKELVE
H15  MNTQIIVILVLGLSMVK--------SDKICLGHHAVANGTKVNTLTERGVEVVNATETVE
H16  MMIKVLYFLIIVLGR-------YSKADKICIGYLSNNSSDTVDTLTENGVPVTSSVDLVE
                    ▲(Mature resdidue 1)

           ▼(Residue Ap)
H1   DSHNGKLCRLKGIAPLQLGKCNIAGWLLGNPECDPLLPVRSWSYIVETPNSENGICYPGD
H2   KTHNGKLCKLNGIPPLELGDCSIAGWLLGNPECDRLLTVPEWSYIMEKENPRNGLCYPGS
H3   SSSTGKICNN-PHRILDGIDCTLIDALLGDPHCDVFQN-ETWDLFVERSKAFS-NCYPYD
H4   SQNLPELCPS-PLRLVDGQTCDIINGALGSPGCDHLNG-AEWDVFIERPNAVD-TCYPFD
H5   RTHNGKLCSLNGVKPLILRDCSVAGWLLGNPMCDEFLNLPEWLYIVEKDNPINSLCYPGD
H6   NQKEERFCKILKKAPLDLKGCTIEGWILGNPQCDLLLGDQSWSYIVERPTAQNGICYPGV
H7   RTNIPKICSK-GKRTTDLGQCGLLGTITGPPQCDQFLE-FSADLIIERREGND-VCYPGK
H8   TEKHPAYCNTDLGAPLELRDCKIEAVIYGNPKCDIHLKDQGWSYIVERPSAPEGMCYPGS
H9   TEHNGMLCATDLGHPLILDTCTIEGLIYGNPSCDILLGGKEWSYIVERSSAVNGMCYPGN
H10  STNLNKLCMK-GRSYKDLGNCHPVGMLIGTPVCDPHLT-GTWDTLIERENAIA-HCYPGA
H11  TEHTGSFCSINGKQPISLGDCSFAGWILGNPMCDELIGKTSWSYIVEKPNPTNGICYPGT
H12  RGIDPILCGTELGSPLVLDDCSLEGLILGNPKCDLYLNGREWSYIVERPKEMEGVCYPGS
H13  TNHTGTYCSLNGVSPVHLGDCSFEGWIVGNPACTSNFGIREWSYLIEDPAAPHGLCYPGE
H14  TNHTDELCPS-PLKLVDGQDCHLINGALGSPGCDRLQD-TTWDVFIERPTAVD-TCYPFD
H15  ITGIDKVCTK-GKKAVDLGSCGILGTIIGPPQCDLHLE-FKADLIIERRNSSD-ICYPGR
H16  TNHTGTYCSLNGISPIHLGDCSFEGWIVGNPSCATNINIREWSYLIEDPNAPNKFCYPGE
           ▲(Residue Ap)

H1   FIDYEELREQLSSVSSFERFEIFPKESSWPNHNTNGV-TAACSHE-GKSSFYRNLLWLTE
H2   FNDYEELKHLLSSVTHFEKVKILPK-DRWTQHTTTGG-SRACAVS-GNPSFFRNMVWLTK
H3   VPDYASLRSLVAS-S--GTLEFITEGFTW-TGVTQNGGSNACKRGPG-NGFFSRLNWLTK
H4   VPEYQSLRSILAN-N--GKFEFIAEEFQW-NTVKQNGKSGACKRANV-DDFFNRLNWLVK
H5   FNDYEELKYLLSSTNHFEKIRIIPR-SSWSNHDASSGVSSACPYI-GRSSFLRNVVWLIK
H6   LNEVEELKALIGSGERVERFEMFPK-STWTGVDTSSGVTRACPYN-SGSSFYRNLLWIIK
H7   FVNEEALRQILRG-S--GGIDKETMGFTY-SGIRTNGTTSACRRSG--SSFYAEMEWLLS
H8   VENLEELRFVFSSAASYKRIRLFDY-SRWNVTRS--GTSKACNASTGGQSFYRSINWLTK
H9   VENLEELRSLFSSAKSYKRIQIFPD-KTWNVTYS--GTSRACSN-----SFYRSMRWLTH
H10  TINEEALRQKIME-S--GGISKMSTGFTYGSSITSAGTTKACMRNGG-DSFYAELKWLVS
H11  LESEEELRLKFSGVLEFNKFEVFTS-NGWGAVNSGVGVTAACKFG-GSNSFFRNMVWLIH
H12  IENQEELRSLFSSIKKYERVKMFDF-TKWNVTYT--GTSKACNNTSNQGSFYRSMRWLTL
H13  LNNNGELRHLFSGIRSFSRTELIPP-TSWGEVLD--GTTSACRDNTGTNSFYRNLVWFIK
H14  VPDYQSLRSILAS-S--GSLEFIAEQFTW-NGVKVDGSSSACLRGGR-NSFFSRLNWLTK
H15  FTNEEALRQIIRE-S--GGIDKESMGFRY-SGIRTDGATSACKRTV--SSFYSEMKWLSS
H16  LDNNGELRHLFSGVNSFSRTELINP-SKWGNVLD--GVTASCLDR-GASSFYRNLVWIVK


H1   -K-EGSYPKLKNSYVNKKGKEVLVLWGIHHPPNSKEQQNLYQNENAYVSVVTSNYNRRFT
H2   -K-GSNYPIAKGSYNNTSGEQMLIIWGVHHPNDETEQRTLYQNVGTYVSIGTSTLNKRSI
H3   S--GSTYPVLNVTMPNNDNFDKLYIWGVHHPSTNQEQTSLYVQESGRVTVSTRRSQQSII
H4   SD-GNAYPLQNLTKINNGDYARLYIWGVHHPSTSTEQTNLYKNNPGRVTVSTKTSQTSVV
H5   -K-NNTYPTIKRSYNNTNQEDLLILWGIHHPNDAAEQTKLYQNPTTYVSVGTSTLNQRSI
H6   TK-SAAYSVIKGAYNNTGNQPILYFWGVHHPPDTNEQNTLYGSGDRYVRMGTESMNFAKS
H7   NTDNASFPQMTKSYKNTRRESALIVWGIHHSGSTTEQTKLYGSGNKLITVGSSKYHQSFV
H8   KE-PDTYDFNEGAYVNNEDGDIIFLWGIHHPPDTKEQTTLYKNANTLSSVTTNTINRSFQ
```

FIG. 1A

```
H9    K--SNSYPFQNAHYTNNERENILFMWGIHHPPTDTEQTDLYKNADTTTSVTTEDINRTFK
H10   KTKGQNFPQTTNTYRNTDTAEHLIIWGIHHPSSTQEKNDLYGTQSLSISVESSTYQNNFV
H11   -Q-SGTYPVIKRTFNNTKGRDVLIVWGIHHPATLTEHQDLYKKDSSYVAVGSETYNRRFT
H12   K--SGQFPVQTDEYKNTRDSDIVFTWAIHHPPTSDEQVKLYKNPDTLSSVTTVEINRSFK
H13   -K-NTRYPVISKTYNNTTGRDVLVLWGIHHPVSVDETKTLYVNSDPYTLVSTKSWSEKYK
H14   AT-NGNYGPINVTKENTGSYVRLYLWGVHHPSSDNEQTDLYKVATGRVTVSTRSDQISIV
H15   SMNNQVFPQLNQTYRNTRKEPALIVWGVHHSSSLDEQNKLYGTGNKLITVGSSKYQQSFS
H16   -K-DEKYPVIKGDYNNTTGRDVLVLWGIHHPDTETTATNLYVNKNPYTLVSTKEWSKRYE

H1    PEIAERPKVRDQAGRMNYYWTLLKPGDTIIFEANGNLIAPMYAFALSRGFG--------S
H2    PVIATRPKVNGQGGRMEFSWTILDIWDTINFESTGNLIAPEYGFRISKRGS--------S
H3    PNIGSRPWVRGQSSRISIYWTIVKPGDVLVINSNGNLIAPRGYFKMRTG---------KS
H4    PDIGSRPLVRGQSGRVSFYWTIVEPGDLIVFNTIGNLIAPRGHYKLNNQK--------KS
H5    PEIATRPKVNGQSGRMEFFWTILKPNDAINFESNGNFIAPRYAYKIVKKGD--------S
H6    PEIAARPAVNGQRGRIDYYWSILKPGETLNVESNGNLIAPWYAFRFVSTSNK-------G
H7    PSPGTRPQINGQSGRIDFHWLILDPNDTVTFSFNGAFIAPNRASFLR----------GKS
H8    PNIGPRPLVRGQQGRMDYYWGILKRGETLKIRTNGNLIAPEFGYLLKGESYG-------R
H9    PVIGPRPLVNGQQGRIDYYWSVLKPGQTLRIRSNGNLIAPWYGHVLTGESHG-------R
H10   PVVGARPQVNGQSGRIDFHWTLVQPGDNITFSDNGGLIAPSRVSKLT----------GRD
H11   PEINTRPRVNGQAGRMTFYWKIVKPGESITFESNGAFLAPRYAFEIVSVGN--------G
H12   PNIGPRPLVRGQQGRMDYYWAVLKPGQTVKIQTNGNLIAPEYGHLITGKSHG-------R
H13   LETGVRPGYNGQRSWMKIYWSLIHPGEMITFESNGGFLAPRYGYIIEEYGK--------G
H14   PNIGSRPRVRNQSGRISIYWTLVNPGDSIIFNSIGNLIAPRGHYKISKST--------KS
H15   PSPGARPKVNGQAGRIDFHWMLLDPGDTVTFTFNGAFIAPDRATFLRSNAPSGIEYNGKS
H16   LEIGTRIG-DGQRSWMKLYWHLMHPGERIMFESNGGLIAPRYGYIIEKYGT--------G

                ▼(Residue Aq)
H1    GIITSNASMHE-CNTKCQTPLGAINSSLPYQNIHPVTIGECPKYVRSAKLRMVTGLRNNP
H2    GIMKTEGTLEN-CETKCQTPLGAINTTLPFHNVHPLTIGECPKYVKSERLVLATGLRNVP
H3    SIMSSDAPIDT-CISECITPNGSIPNDKPFQNVNKITYGACPKYVKQNTLKLATGMRNVP
H4    TILNTAIPIGS-CVSKCHTDKGSLSTTKPFQNISRIAVGDCPRYVKQGSLKLATGMRNIP
H5    AIMKSGLAYGN-CDTKCQTPVGEINSSMPFHNIHPHTIGECPKYVKSDRLVLATGLRNVP
H6    AVFKSNLPIEN-CDATCQTVAGVLRTNKTFQNVSPLWIGECPKYVKSESLRLATGLRNVP
H7    MGIQSDVQVDANCEGECYHSGGTITSRLPFQNINSRAVGKCPRYVKQESLLLATGMKNVP
H8    IIQNEDIPIGN-CNTKCQTYAGAINSSKPFQNASRHYMGECPKYVKKASLRLAVGLRNTP
H9    ILKT-DLNNGN-CVVQCQTEKGGLNTTLPFHNISKYAFGNCPKYVGVKSLKLPVGLRNVP
H10   LGIQSEALIDNSCESKCFWRGGSINTKLPFQNLSPRTVGQCPKYVNQRSLLLATGMRNVP
H11   KLFRSELNIES-CSTKCQTEIGGINTNKSFHNVHRNTIGDCPKYVNVKSLKLATGPRNVP
H12   ILKN-NLPMGQ-CVTECQLNEGVMNTSKPFQNTSKHYIGKCPKYIPSGSLKLAIGLRNVP
H13   RIFQSRIRMSR-CNTKCQTSVGGINTNRTFQNIDKNALGDCPKYIKSGQLKLATGLRNVP
H14   TVLKSDKRIGS-CTSPCLTDKGSIQSDKPFQNVSRIAIGNCPKYVKQGSLMLATGMRNIP
H15   LGIQSDAQIDESCEGECFYSGGTINSPLPFQNIDSRAVGKCPRYVKQSSLPLALGMKNVP
H16   RIFQSGVRMAR-CNTKCQTSLGGINTNKTFQNIERNALGDCPKYIKSGQLKLATGLRNVP
                ▲(Residue Aq)

              ▼(HA2 domain starts)
H1    ----SIQSRGLFGAIAGFIEGGWTGMIDGWYGYHHQNEQGSGYAADQKSTQNAINGITNK
H2    ----QIESRGLFGAIAGFIEGGWQGMIDGWYGYHHSNDQGSGYAADKESTQKAIDGITNR
H3    ----EKQTRGLFGAIAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGK
H4    ----EKASRGLFGAIAGFIENGWQGLIDGWYGFRHQNAEGTGTAADLKSTQAAIDQINGK
H5    ----QRKKRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGITNK
H6    ----QIETRGLFGAIAGFIEGGWTGMIDGWYGYHHENSQGSGYAADRESTQKAVDGITNK
H7    EPSKKRKKRGLFGAIAGFIENGWEGLVDGWYGFRHQNAQGEGTAADYKSTQSAIDQITGK
H8    ----SVEPRGLFGAIAGFIEGGWSGMIDGWYGFHHSNSEGTGMAADQKSTQEAIDKITNK
H9    ----AVSSRGLFGAIAGFIEGGWPGLVAGWYGFQHSNDQGVGMAADKGSTQKAIDKITSK
H10   ---EVVQGRGLFGAIAGFIENGWEGMVDGWYGFRHQNAQGTGQAADYKSTQAAIDQITGK
H11   ----AIASRGLFGAIAGFIEGGWPGLINGWYGFQHRDEEGTGIAADKESTQKAIDQITSK
H12   ----QVQDRGLFGAIAGFIEGGWPGLVAGWYGFQHQNAEGTGIAADRDSTQRAIDNMQNK
H13   ----AISNRGLFGAIAGFIEGGWPGLINGWYGFQHQNEQGTGIAADKESTQKAIDQITTK
H14   ----GKQAKGLFGAIAGFIENGWQGLIDGWYGFRHQNAEGTGTAADLKSTQAAIDQINGK
H15   ---EKIRTRGLFGAIAGFIENGWEGLIDGWYGFRHQNAQGQGTAADYKSTQAAIDQITGK
H16   ----SIGERGLFGAIAGFIEGGWPGLINGWYGFQHQNEQGTGIAADKASTQKAINEITTK
              ▲(HA2 domain starts)
```

FIG. 1B

```
H1   VNTVIEKMNIQFTAVGKEFNKLEKRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFH
H2   VNSVIEKMNTQFEAVGKEFSNLEKRLENLNKKMEDGFLDVWTYNAELLVLMENERTLDFH
H3   LNRVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLT
H4   LNRLIEKTNDKYHQIEKEFEQVEGRIQDLENYVEDTKIDLWSYNAELLVALENQHTIDVT
H5   VNSIIDKMNTRFEAVGKEFNNLERRVENLNKKMEDGFLDVWTYNVELLVLMENERTLDFH
H6   VNSIIDKMNTQFEAVDHEFSNLERRIDNLNKRMEDGFLDVWTYNAELLVLLENERTLDLH
H7   LNRLIEKTNQQFELIDNEFTEVEKQIGNLINWTKDSITEVWSYNAELIVAMENQHTIDLA
H8   VNNIVDKMNREFEVVNHEFSEVEKRINMINDKIDDQIEDLWAYNAELLVLLENQKTLDEH
H9   VNNIIDKMNKQYEVIDHEFNELEARLNMINNKIDDQIQDIWAYNAELLVLLENQKTLDEH
H10  LNRLIEKTNTEFESIESEFSETEHQIGNVINWTKDSITDIWTYNAELLVAMENQHTIDMA
H11  VNNIVDRMNTNFESVQHEFSEIEERINQLSKHVDDSVVDIWSYNAQLLVLLENEKTLDLH
H12  LNNVIDKMNKQFEVVNHEFSEVESRINMINSKIDDQITDIWAYNAELLVLLENQKTLDEH
H13  INNIIDKMNGNYDSIRGEFNQVEKRINMLADRIDDAVTDIWSYNAKLLVLLENDKTLDMH
H14  LNRLIEKTNEKYHQIEKEFEQVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDVT
H15  LNRLIEKTNKQFELIDNEFTEVEQQIGNVINWTRDSLTEIWSYNAELLVAMENQHTIDLA
H16  INNIIEKMNGNYDSIRGEFNQVEKRINMLADRVDDAVTDIWSYNAKLLVLLENDRTLDLH

H1   DSNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCDNECMESVRNGTYDYPKYSEESKLNRE
H2   DSNVKNLYDRVRMQLRDNAKELGNGCFEFYHKCDDECMNSVKNGTYDYPKYEEESKLNRN
H3   DSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIRNGTYDHDVYRDEALNNRF
H4   DSEMNKLFERVRRQLRENAEDKGNGCFEIFHKCDNNCIESIRNGTYDHDIYRDEAINNRF
H5   DSNVNNLYDKVRLQLKDNARELGNGCFEFYHKCDNECMESVRNGTYDYPQYSEEARLNRE
H6   DANVKNLYERVKSQLRDNAMILGNGCFEFWHKCDDECMESVKNGTYDYPKYQDESKLNRQ
H7   DSEMNRLYERVRKQLRENAEEDGTGCFEIFHKCDDDCMASIRNNTYDHSKYREEAMQNRI
H8   DSNVKNLFDEVKRRLSANAIDAGNGCFDILHKCDNECMETIKNGTYDHKEYEEEAKLERS
H9   DANVNNLYNKVKRALGSNAVEDGNGCFELYHKCDDQCMETIRNGTYDRQKYQEESRLERQ
H10  DSEMLNLYERVRKQLRQNAEEDGKGCFEIYHTCDDSCMESIRNNTYDHSQYREEALLNRL
H11  DSNVRNLHEKVRRMLKDNAKDEGNGCFTFYHKCDNKCIERVRNGTYDHKEFEEESKINRQ
H12  DANVRNLHDRVRRVLRENAIDTGDGCFEILHKCDNNCMDTIRNGTYNHKEYEEESKIERQ
H13  DANVKNLHEQVRRELKDNAIDEGNGCFELLHKCNDSCMETIRNGTYDHTEYAEESKLKRQ
H14  DSEMNKLFERVRRQLRENAEDQGNGCFEIFHQCDNNCIESIRNGTYDHNIYRDEAINNRI
H15  DSEMNKLYERVRRQLRENAEEDGTGCFEIFHRCDDQCMESIRNNTYNHTEYRQEALQNRI
H16  DANVRNLHDQVKRALKSNAIDEGDGCFNLLHKCNDSCMETIRNGTYNHEDYREESQLKRQ

H1   KVDGVKLESMG-IYQILAIYSTVASSLVLLVSLGAISFWMCSNGSLQCRICI
H2   EIKGVKLSNMG-VYQILAIYATVAGSLSLAIMIAGISLWMCSNGSLQCRICI
H3   QIKGVELKSGY--KDWILWISFAISCFLLCVVLLGFIMWACQRGNIRCNICI
H4   QIQGVKLTQGY--KDIILWISFSISCFLLVALLLAFILWACQNGNIRCQICI
H5   EISGVKLESMG-VYQILSIYSTVASSLALAIMIAGLSFWMCSNGSLQCRICI
H6   EIESVKLESLG-VYQILAIYSTVSSSLVLVGLIIAVGLWMCSNGSMQCRICI
H7   QIDPVKLSSGY--KDVILWFSFGASCFLLLAIAMGLVFICVKNGNMRCTICI
H8   KINGVKLEENT-TYKILSIYSTVAASLCLAILIAGGLILGMQNGSCRCMFCI
H9   KIEGVKLESEG-TYKILTIYSTVASSLVLAMGFAAFLFWAMSNGSCRCNICI
H10  NINPVKLSSGY--KDIILWFSFGESCFVLLAVVMGLVFFCLKNGNMRCTICI
H11  EIEGVKLDSSGNVYKILSIYSCIASSLVLAALIMGFMFWACSNGSCRCTICI
H12  KVNGVKLEENS-TYKILSIYSSVASSLVLLLMIIGGFIFGCQNGNVRCTFCI
H13  EIDGIKLKSEDNVYKALSIYSCIASSVVLVGLILSFIMWACSSGNCRFNVCI
H14  KINPVTLTMGY--KDIILWISFSMSCFVFVALILGFVLWACQNGNIRCQICI
H15  MINPVKLSSGY--KDVILWFSFGASCVMLLAIAMGLIFMCVKNGNLRCTICI
H16  EIEGIKLKTEDNVYKVLSIYSCIASSIVLVGLILAFIMWACSNGSCRFNVCI
```

FIG. 1C

```
H1   MK--ANLLVLLCALAAADAD-------TICIGYHANNSTDTVDTVLEKNVTVTHSVNLLE
H3   MKTIIALSYIFCLALGQDLPGNDNSTATLCLGHHAVPNGTLVKTITDDQIEVTNATELVQ
HB   MK---AIIVILMVVTSNADR--------ICTGITSSNSPHVVKTATQGEVNVTGVIPLTT

            ▼(Cys52,HA1)
H1   DSHNGKLCRLKG-----IAPLQLGKCNIAGWLLGNPECDPLLPVRSWSYIVETPNSENG-
H3   SSSTGKICNNP------HRILDGIDCTLIDALLGDPHCD-VFQNETWDLFVERSKAFS--
HB   TPTKSHFANLKGTETRGKLCPKCLNCTDLDVALGRPKCTGKIPSARVSILHEVRPVTSGC
                     ▲(Arg50,B-HA1)  ▲(Ala66,B-HA1)▲(Arg80,B-HA1)

H1   ICYPGDFIDYEELREQLSSVSSF--ERFEIFPKESSWPNHNTNGVTAACS-HEGKSSFYR
H3   NCYPYDVPDYASLRSLVASSG----T-LEFITEGFTWTGVTQNGGSNACK-RGPGNGFFS
HB   FPIMHDRTKIRQLPNLLRGYEHIRLSTHNVINAENAPGGPYKIGTSGSCPNITNGNGFFA


H1   NLLWLTEKE------GSYPKLKNSYVNKKGKEVLVLWGIHHPPNSKEQQNLYQNENAYVS
H3   RLNWLTKSG------STYPVLNVTMPNNDNFDKLYIWGVHHPSTNQEQTSLYVQESGRVT
HB   TMAWAVPKNDKNKTATNPLTIEVPYICTEGEDQITVWGFHSDSETQMAKLYGDSKPQKFT


H1   VVTSNYNRRFTPEIAERPKVRD-----QAGRMNYYWTLLKPGDTIIFEANGNLIAPMYAF
H3   VSTRRSQQSIIPNIGSRPWVRG-----QSSRISIYWTIVKPGDVLVINSNGNLIAPRGYF
HB   SSANGVTTHYVSQIGGFPNQTEDGGLPQSGRIVVDYMVQKSGKTGTITYQRGILLPQKVW
                                                          ▲(Trp271)

H1   ALSRGFGSGIITSNASMHECNTKCQTPLGAINSSLPYQNIHPVTIGECPKYVRSAKLRMV
H3   KMRTGKSS-IMSSDAPIDTCISECITPNGSIPNDKPFQNVNKITYGACPKYVKQNTLKLA
HB   CASGRSKV-IKGSLPLIGEADCLHEKYGGLNKSKPYYTGEHAKAIGNCPIWVKTP-LKLA
          ▲(Ser277,B-HA1)

                     ▼(HA2 domain starts)
H1   TGLRNNP---SIQSRGLFGAIAGFIEGGWTGMIDGWYGYHHQNEQGSGYAADQKSTQNAI
H3   TGMRNVP---EKQTRGLFGAIAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAI
HB   NGTKYRPPAKLLKERGFFGAIAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAI
                     ▲(B-HA2 domain starts)

H1   NGITNKVNTVIEKMNIQFTAVGKEFNKLEKRMENLNKKVDDGFLDIWTYNAELLVLLENE
H3   DQINGKLNRVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQ
HB   NKITKNLNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDLRADTISSQIELAVLLSNE


H1   RTLDFHDSNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCDNECMESVRNGTYDYPKYSEE
H3   HTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIRNGTYDHDVYRDE
HB   GIINSEDEHLLALERKLKKMLGPSAVEIGNGCFETKHKCNQTCLDRIAAGTFDAGEFSLP


H1   SKLNR-EKVDGVKLESMGIYQILAIYSTVASSLVLLVSLGAISFWMCSNGSLQCRICI
H3   ALNNR-FQIKGVELKSGYKDWILWISFAISCFLLCVVLLGFI-MWACQRGNIRCNICI
HB   TFDSLNITAASLNDDGLDNHTILLYYSTAASSLAVTLMIAIFVVYMVSRDNVSCSICL
```

FIG. 2

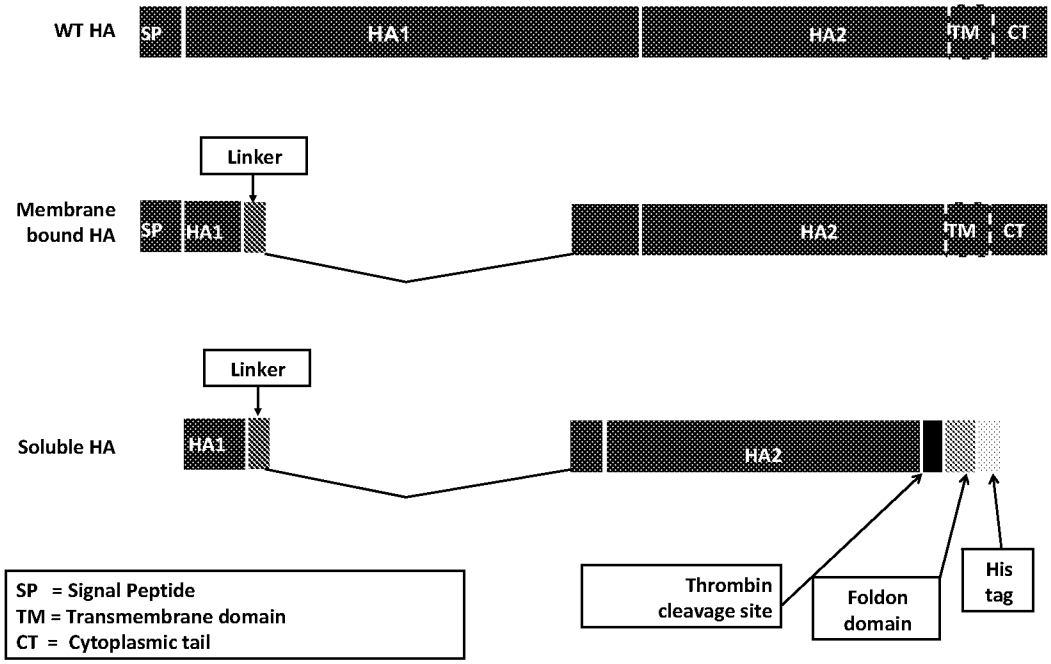
WT HA
SP
HA1
HA2
TM
CT
Linker
Membrane bound HA
SP
HA1
HA2
TM
CT
Linker
Soluble HA
HA1
HA2
SP = Signal Peptide
TM = Transmembrane domain
CT = Cytoplasmic tail
Thrombin cleavage site
Foldon domain
His tag

FIG. 3

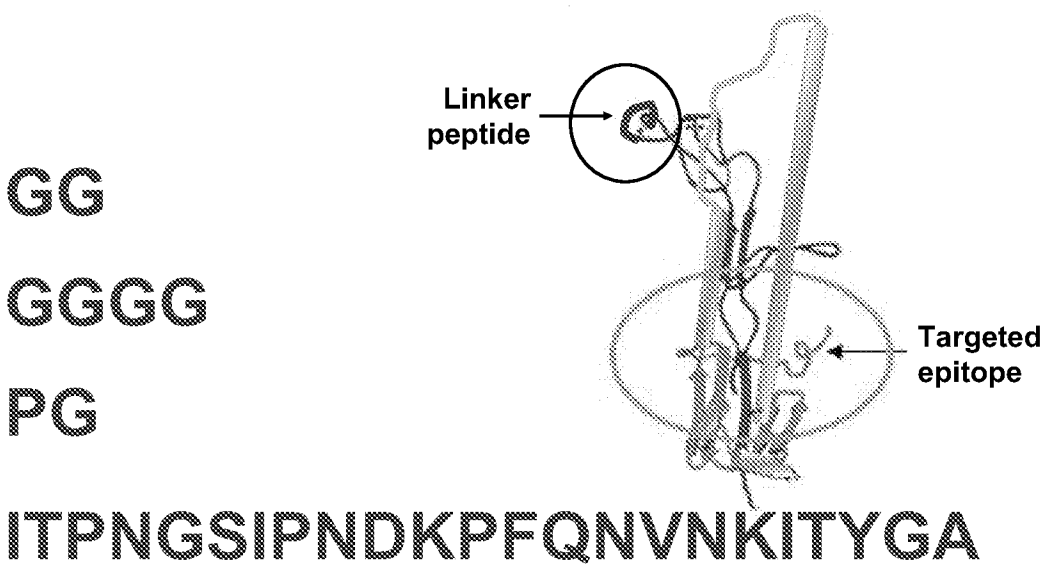
Linker peptide
Targeted epitope
GG
GGGG
PG
ITPNGSIPNDKPFQNVNKITYGA

FIG. 4

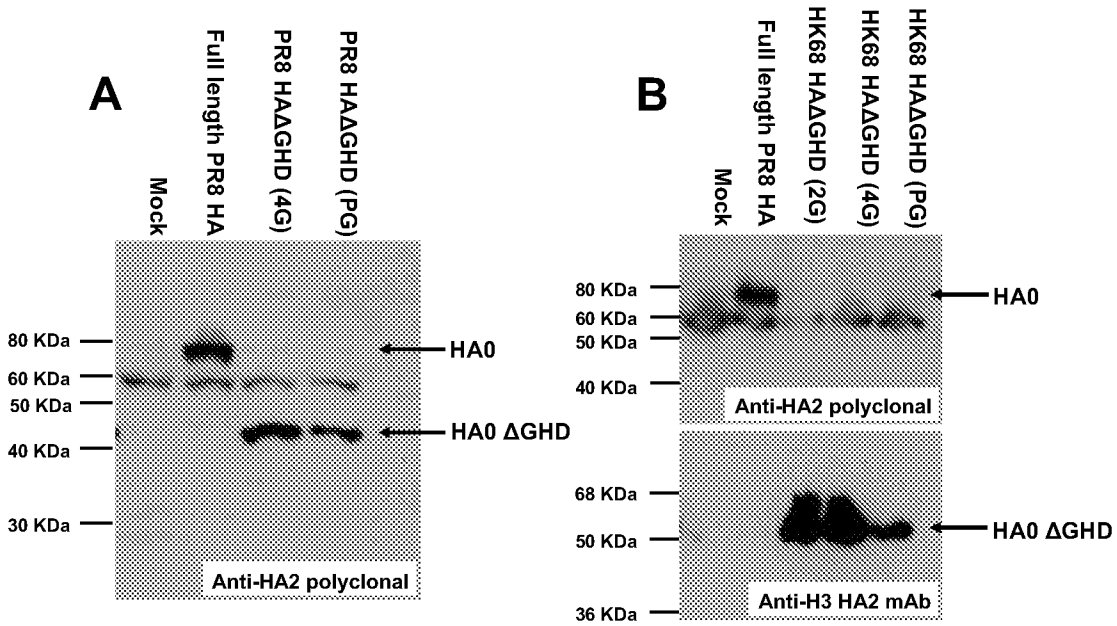
A
Mock
Full length PR8 HA
PR8 HAΔGHD (4G)
PR8 HAΔGHD (PG)
80 KDa
60 KDa
50 KDa
40 KDa
30 KDa
HA0
HA0 ΔGHD
Anti-HA2 polyclonal
B
Mock
Full length PR8 HA
HK68 HAΔGHD (2G)
HK68 HAΔGHD (4G)
HK68 HAΔGHD (PG)
80 KDa
60 KDa
50 KDa
40 KDa
HA0
Anti-HA2 polyclonal
68 KDa
50 KDa
36 KDa
HA0 ΔGHD
Anti-H3 HA2 mAb

FIG. 5

```
  1 AGCAAAAGCA GGGGAAAATA AAAACAACCA AAATGAAGGC AAACCTACTG
                                      M  K  A  N  L  L

 51 GTCCTGTTAA GTGCACTTGC AGCTGCAGAT GCAGACACAA TATGTATAGG
    V  L  L  S  A  L  A  A  A  D  A  D  T  I  C  I  G

101 CTACCATGCG AACAATTCAA CCGACACTGT TGACACAGTA CTCGAGAAGA
    Y  H  A  N  N  S  T  D  T  V  D  T  V  L  E  K  N

151 ATGTGACAGT GACACACTCT GTTAACCTGC TCGAAGACAG CCACAACGGA
    V  T  V  T  H  S  V  N  L  L  E  D  S  H  N  G

201 AAACTATGTG GAGGCTGTAA CACGAAGTGT CAAACACCCC TGGGAGCTAT
    K  L  C  G  G  C  N  T  K  C  Q  T  P  L  G  A  I

251 AAACAGCAGT CTCCCTTACC AGAATATACA CCCAGTCACA ATAGGAGAGT
    N  S  S  L  P  Y  Q  N  I  H  P  V  T  I  G  E  C

301 GCCCAAAATA CGTCAGGAGT GCCAAATTGA GGATGGTTAC AGGACTAAGG
    P  K  Y  V  R  S  A  K  L  R  M  V  T  G  L  R

351 AACACTCCGT CCATTCAATC CAGAGGTCTA TTTGGAGCCA TTGCCGGTTT
    N  T  P  S  I  Q  S  R  G  L  F  G  A  I  A  G  F

401 TATTGAAGGG GGATGGACTG GAATGATAGA TGGATGGTAT GGTTATCATC
    I  E  G  G  W  T  G  M  I  D  G  W  Y  G  Y  H  H

451 ATCAGAATGA ACAGGGATCA GGCTATGCAG CGGATCAAAA AAGCACACAA
    Q  N  E  Q  G  S  G  Y  A  A  D  Q  K  S  T  Q

501 AATGCCATTA ACGGGATTAC AAACAAGGTG AACACTGTTA TCGAGAAAAT
    N  A  I  N  G  I  T  N  K  V  N  T  V  I  E  K  M

551 GAACATTCAA TTCACAGCTG TGGGTAAAGA ATTCAACAAA TTAGAAAAAA
    N  I  Q  F  T  A  V  G  K  E  F  N  K  L  E  K  R

601 GGATGGAAAA TTTAAATAAA AAAGTTGATG ATGGATTTCT GGACATTTGG
    M  E  N  L  N  K  K  V  D  D  G  F  L  D  I  W

651 ACATATAATG CAGAATTGTT AGTTCTACTG GAAAATGAAA GGACTCTGGA
    T  Y  N  A  E  L  L  V  L  L  E  N  E  R  T  L  D

701 TTTCCATGAC TCAAATGTGA AGAATCTGTA TGAGAAAGTA AAAAGCCAAT
    F  H  D  S  N  V  K  N  L  Y  E  K  V  K  S  Q  L

751 TAAAGAATAA TGCCAAAGAA ATCGGAAATG GATGTTTTGA GTTCTACCAC
    K  N  N  A  K  E  I  G  N  G  C  F  E  F  Y  H
```

FIG. 6A

```
801 AAGTGTGACA ATGAATGCAT GGAAAGTGTA AGAAATGGGA CTTATGATTA
     K  C  D  N   E  C  M   E  S  V   R  N  G  T   Y  D  Y

851 TCCCAAATAT TCAGAAGAGT CAAAGTTGAA CAGGGAAAAG GTAGATGGAG
     P  K  Y   S  E  E  S   K  L  N   R  E  K   V  D  G  V

901 TGAAATTGGA ATCAATGGGG ATCTATCAGA TTCTGGCGAT CTACTCAACT
     K  L  E   S  M  G   I  Y  Q  I   L  A  I   Y  S  T

951 GTCGCCAGTT CACTGGTGCT TTTGGTCTCC CTGGGGGCAA TCAGTTTCTG
     V  A  S  S   L  V  L   L  V  S   L  G  A  I   S  F  W

1001 GATGTGTTCT AATGGATCTT TGCAGTGCAG AATATGCATC TGAGATTAGA
      M  C  S   N  G  S  L   Q  C  R   I  C  I   *

1051 ATTTCAGAAA TATGAGGAAA AACACCCTTG TTTCTACT
```

FIG. 6B

```
  1 AGCAAAAGCA GGGGAAAATA AAAACAACCA AAATGAAGGC AAACCTACTG
                                      M  K  A  N  L  L

 51 GTCCTGTTAA GTGCACTTGC AGCTGCAGAT GCAGACACAA TATGTATAGG
    V  L  L  S  A  L  A  A  A  D  A  D  T  I  C  I  G

101 CTACCATGCG AACAATTCAA CCGACACTGT TGACACAGTA CTCGAGAAGA
    Y  H  A  N  N  S  T  D  T  V  D  T  V  L  E  K  N

151 ATGTGACAGT GACACACTCT GTTAACCTGC TCGAAGACAG CCACAACGGA
    V  T  V  T  H  S  V  N  L  L  E  D  S  H  N  G

201 AAACTATGTG GAGGCGGAGG TTGTAACACG AAGTGTCAAA CACCCCTGGG
    K  L  C  G  G  G  G  C  N  T  K  C  Q  T  P  L  G

251 AGCTATAAAC AGCAGTCTCC CTTACCAGAA TATACACCCA GTCACAATAG
    A  I  N  S  S  L  P  Y  Q  N  I  H  P  V  T  I  G

301 GAGAGTGCCC AAAATACGTC AGGAGTGCCA AATTGAGGAT GGTTACAGGA
    E  C  P  K  Y  V  R  S  A  K  L  R  M  V  T  G

351 CTAAGGAACA CTCCGTCCAT TCAATCCAGA GGTCTATTTG GAGCCATTGC
    L  R  N  T  P  S  I  Q  S  R  G  L  F  G  A  I  A

401 CGGTTTTATT GAAGGGGGAT GGACTGGAAT GATAGATGGA TGGTATGGTT
    G  F  I  E  G  G  W  T  G  M  I  D  G  W  Y  G  Y

451 ATCATCATCA GAATGAACAG GGATCAGGCT ATGCAGCGGA TCAAAAAAGC
    H  H  Q  N  E  Q  G  S  G  Y  A  A  D  Q  K  S

501 ACACAAAATG CCATTAACGG GATTACAAAC AAGGTGAACA CTGTTATCGA
    T  Q  N  A  I  N  G  I  T  N  K  V  N  T  V  I  E

551 GAAAATGAAC ATTCAATTCA CAGCTGTGGG TAAAGAATTC AACAAATTAG
    K  M  N  I  Q  F  T  A  V  G  K  E  F  N  K  L  E

601 AAAAAAGGAT GGAAAATTTA AATAAAAAAG TTGATGATGG ATTTCTGGAC
    K  R  M  E  N  L  N  K  K  V  D  D  G  F  L  D

651 ATTTGGACAT ATAATGCAGA ATTGTTAGTT CTACTGGAAA ATGAAAGGAC
    I  W  T  Y  N  A  E  L  L  V  L  L  E  N  E  R  T

701 TCTGGATTTC CATGACTCAA ATGTGAAGAA TCTGTATGAG AAAGTAAAAA
    L  D  F  H  D  S  N  V  K  N  L  Y  E  K  V  K  S

751 GCCAATTAAA GAATAATGCC AAAGAAATCG GAAATGGATG TTTTGAGTTC
    Q  L  K  N  N  A  K  E  I  G  N  G  C  F  E  F
```

FIG. 7A

```
 801 TACCACAAGT GTGACAATGA ATGCATGGAA AGTGTAAGAA ATGGGACTTA
     Y  H  K  C   D  N  E   C  M  E   S  V  R  N   G  T  Y

 851 TGATTATCCC AAATATTCAG AAGAGTCAAA GTTGAACAGG GAAAAGGTAG
      D  Y  P   K  Y  S  E   E  S  K   L  N  R   E  K  V  D

 901 ATGGAGTGAA ATTGGAATCA ATGGGGATCT ATCAGATTCT GGCGATCTAC
       G  V  K   L  E  S   M  G  I  Y   Q  I  L   A  I  Y

 951 TCAACTGTCG CCAGTTCACT GGTGCTTTTG GTCTCCCTGG GGGCAATCAG
     S  T  V  A   S  S  L   V  L  L   V  S  L  G   A  I  S

1001 TTTCTGGATG TGTTCTAATG GATCTTTGCA GTGCAGAATA TGCATCTGAG
      F  W  M   C  S  N  G   S  L  Q   C  R  I   C  I  *

1051 ATTAGAATTT CAGAAATATG AGGAAAAACA CCCTTGTTTC TACT
```

FIG. 7B

```
  1 AGCAAAAGCA GGGGAAAATA AAAACAACCA AAATGAAGGC AAACCTACTG
                                      M  K  A  N  L  L

 51 GTCCTGTTAA GTGCACTTGC AGCTGCAGAT GCAGACACAA TATGTATAGG
    V  L  L  S  A  L  A  A  A  D  A  D  T  I  C  I  G

101 CTACCATGCG AACAATTCAA CCGACACTGT TGACACAGTA CTCGAGAAGA
     Y  H  A  N  N  S  T  D  T  V  D  T  V  L  E  K  N

151 ATGTGACAGT GACACACTCT GTTAACCTGC TCGAAGACAG CCACAACGGA
     V  T  V  T  H  S  V  N  L  L  E  D  S  H  N  G

201 AAACTATGTC CAGGCTGTAA CACGAAGTGT CAAACACCCC TGGGAGCTAT
    K  L  C  P  G  C  N  T  K  C  Q  T  P  L  G  A  I

251 AAACAGCAGT CTCCCTTACC AGAATATACA CCCAGTCACA ATAGGAGAGT
     N  S  S  L  P  Y  Q  N  I  H  P  V  T  I  G  E  C

301 GCCCAAAATA CGTCAGGAGT GCCAAATTGA GGATGGTTAC AGGACTAAGG
     P  K  Y  V  R  S  A  K  L  R  M  V  T  G  L  R

351 AACACTCCGT CCATTCAATC CAGAGGTCTA TTTGGAGCCA TTGCCGGTTT
    N  T  P  S  I  Q  S  R  G  L  F  G  A  I  A  G  F

401 TATTGAAGGG GGATGGACTG GAATGATAGA TGGATGGTAT GGTTATCATC
     I  E  G  G  W  T  G  M  I  D  G  W  Y  G  Y  H  H

451 ATCAGAATGA ACAGGGATCA GGCTATGCAG CGGATCAAAA AAGCACACAA
     Q  N  E  Q  G  S  G  Y  A  A  D  Q  K  S  T  Q

501 AATGCCATTA ACGGGATTAC AAACAAGGTG AACACTGTTA TCGAGAAAAT
    N  A  I  N  G  I  T  N  K  V  N  T  V  I  E  K  M

551 GAACATTCAA TTCACAGCTG TGGGTAAAGA ATTCAACAAA TTAGAAAAAA
     N  I  Q  F  T  A  V  G  K  E  F  N  K  L  E  K  R

601 GGATGGAAAA TTTAAATAAA AAAGTTGATG ATGGATTTCT GGACATTTGG
     M  E  N  L  N  K  K  V  D  D  G  F  L  D  I  W

651 ACATATAATG CAGAATTGTT AGTTCTACTG GAAAATGAAA GGACTCTGGA
    T  Y  N  A  E  L  L  V  L  L  E  N  E  R  T  L  D

701 TTTCCATGAC TCAAATGTGA AGAATCTGTA TGAGAAAGTA AAAAGCCAAT
     F  H  D  S  N  V  K  N  L  Y  E  K  V  K  S  Q  L

751 TAAAGAATAA TGCCAAAGAA ATCGGAAATG GATGTTTTGA GTTCTACCAC
     K  N  N  A  K  E  I  G  N  G  C  F  E  F  Y  H
```

FIG. 8A

```
 801 AAGTGTGACA ATGAATGCAT GGAAAGTGTA AGAAATGGGA CTTATGATTA
     K  C  D  N   E  C  M   E  S  V   R  N  G  T   Y  D  Y

 851 TCCCAAATAT TCAGAAGAGT CAAAGTTGAA CAGGGAAAAG GTAGATGGAG
      P  K  Y   S  E  E  S   K  L  N   R  E  K   V  D  G  V

 901 TGAAATTGGA ATCAATGGGG ATCTATCAGA TTCTGGCGAT CTACTCAACT
       K  L  E   S  M  G   I  Y  Q  I   L  A  I   Y  S  T

 951 GTCGCCAGTT CACTGGTGCT TTTGGTCTCC CTGGGGGCAA TCAGTTTCTG
     V  A  S  S   L  V  L   L  V  S   L  G  A  I   S  F  W

1001 GATGTGTTCT AATGGATCTT TGCAGTGCAG AATATGCATC TGAGATTAGA
      M  C  S   N  G  S  L   Q  C  R   I  C  I   *

1051 ATTTCAGAAA TATGAGGAAA AACACCCTTG TTTCTACT
```

FIG. 8B

```
  1 ATGGACACAA TATGTATAGG CTACCATGCG AACAATTCAA CCGACACTGT
    M  D  T  I   C  I  G   Y  H  A   N  N  S  T   D  T  V

 51 TGACACAGTA CTCGAGAAGA ATGTGACAGT GACACACTCT GTTAACCTGC
     D  T  V   L  E  K  N   V  T  V   T  H  S   V  N  L  L

101 TCGAAGACAG CCACAACGGA AAACTATGTG GAGGCGGAGG TTGTAACACG
      E  D  S   H  N  G   K  L  C  G   G  G  G   C  N  T

151 AAGTGTCAAA CACCCCTGGG AGCTATAAAC AGCAGTCTCC CTTACCAGAA
    K  C  Q  T   P  L  G   A  I  N   S  S  L  P   Y  Q  N

201 TATACACCCA GTCACAATAG GAGAGTGCCC AAAATACGTC AGGAGTGCCA
     I  H  P   V  T  I  G   E  C  P   K  Y  V   R  S  A  K

251 AATTGAGGAT GGTTACAGGA CTAAGGAACA CTCCGTCCAT TCAATCCAGA
      L  R  M   V  T  G   L  R  N  T   P  S  I   Q  S  R

301 GGTCTATTTG GAGCCATTGC CGGTTTTATT GAAGGGGGAT GGACTGGAAT
    G  L  F  G   A  I  A   G  F  I   E  G  G  W   T  G  M

351 GATAGATGGA TGGTATGGTT ATCATCATCA GAATGAACAG GGATCAGGCT
     I  D  G   W  Y  G  Y   H  H  Q   N  E  Q   G  S  G  Y

401 ATGCAGCGGA TCAAAAAAGC ACACAAAATG CCATTAACGG GATTACAAAC
      A  A  D   Q  K  S   T  Q  N  A   I  N  G   I  T  N

451 AAGGTGAACA CTGTTATCGA GAAAATGAAC ATTCAATTCA CAGCTGTGGG
    K  V  N  T   V  I  E   K  M  N   I  Q  F  T   A  V  G

501 TAAAGAATTC AACAAATTAG AAAAAAGGAT GGAAAATTTA AATAAAAAAG
     K  E  F   N  K  L  E   K  R  M   E  N  L   N  K  K  V

551 TTGATGATGG ATTTCTGGAC ATTTGGACAT ATAATGCAGA ATTGTTAGTT
      D  D  G   F  L  D   I  W  T  Y   N  A  E   L  L  V

601 CTACTGGAAA ATGAAAGGAC TCTGGATTTC CATGACTCAA ATGTGAAGAA
    L  L  E  N   E  R  T   L  D  F   H  D  S  N   V  K  N

651 TCTGTATGAG AAAGTAAAAA GCCAATTAAA GAATAATGCC AAAGAAATCG
     L  Y  E   K  V  K  S   Q  L  K   N  N  A   K  E  I  G

701 GAAATGGATG TTTTGAGTTC TACCACAAGT GTGACAATGA ATGCATGGAA
      N  G  C   F  E  F   Y  H  K  C   D  N  E   C  M  E

751 AGTGTAAGAA ATGGGACTTA TGATTATCCC AAATATTCAG AAGAGTCAAA
    S  V  R  N   G  T  Y   D  Y  P   K  Y  S  E   E  S  K
```

FIG. 9A

```
801 GTTGAACAGG GAAAAGGTAG ATGGAGTGCG TTCTCTGGTT CCGCGTGGTT
      L  N  R   E  K  V  D   G  V  R   S  L  V   P  R  G  S

851 CTCCGGGTTC TGGTTACATC CCGGAAGCTC CGCGTGACGG TCAGGCTTAC
      P  G  S   G  Y  I   P  E  A  P   R  D  G   Q  A  Y

901 GTTCGTAAAG ACGGTGAATG GGTTCTGCTG TCTACCTTCC TGCACCACCA
    V  R  K  D   G  E  W   V  L  L   S  T  F  L   H  H  H

951 CCACCACCAC TGA
      H  H  H   *
```

FIG. 9B

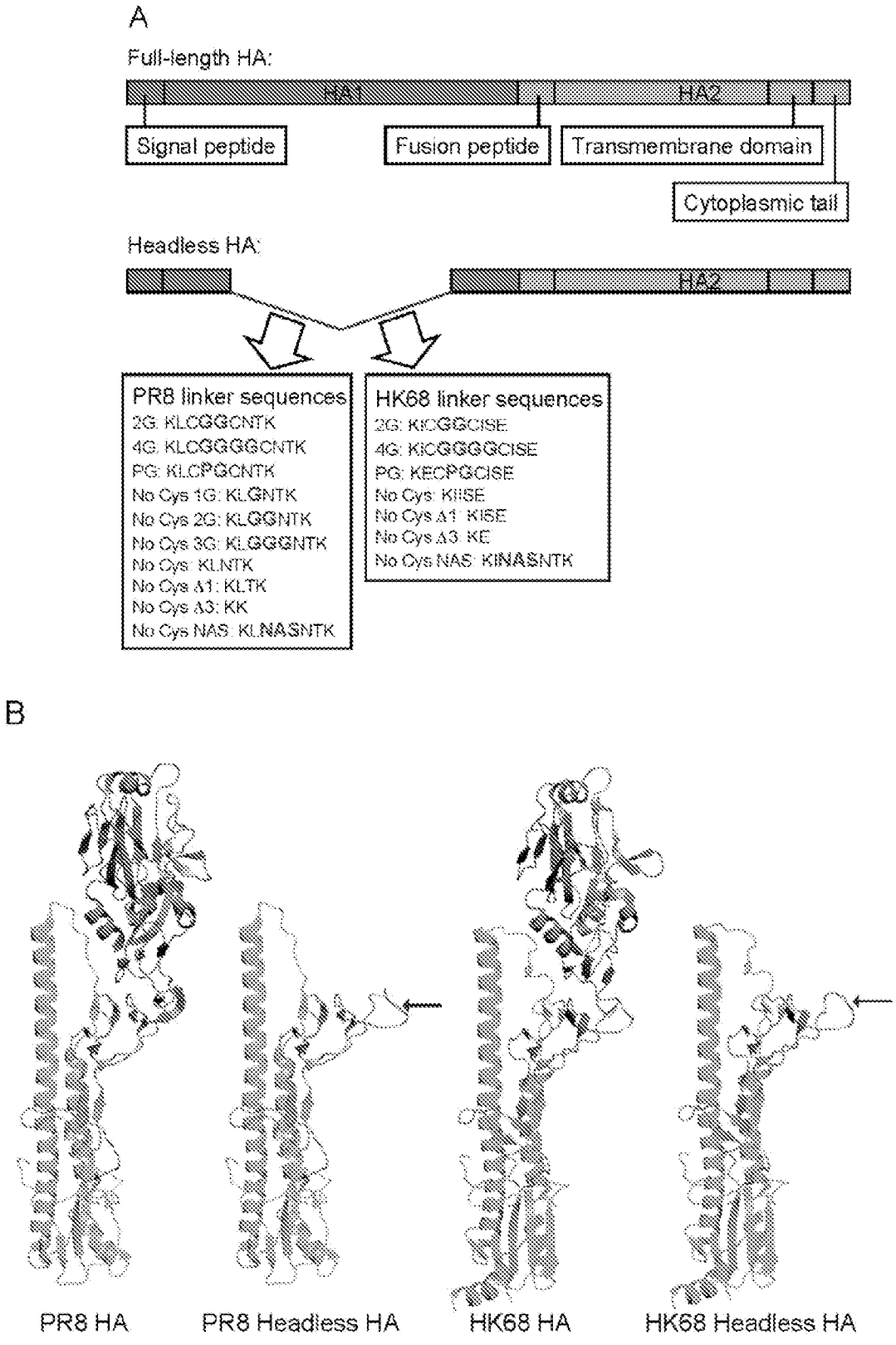
A
Full-length HA:
HA1
HA2
Signal peptide
Fusion peptide
Transmembrane domain
Cytoplasmic tail
Headless HA:
HA2
PR8 linker sequences
2G: KLCGGCNTK
4G: KLCGGGGCNTK
PG: KLCPGCNTK
No Cys 1G: KLGNTK
No Cys 2G: KLGGNTK
No Cys 3G: KLGGGNTK
No Cys: KLNTK
No Cys Δ1: KLTK
No Cys Δ3: KK
No Cys NAS: KLNASNTK
HK68 linker sequences
2G: KICGGCISE
4G: KICGGGGCISE
PG: KECPGCISE
No Cys: KIISE
No Cys Δ1: KISE
No Cys Δ3: KE
No Cys NAS: KINASNTK
B
PR8 HA
PR8 Headless HA
HK68 HA
HK68 Headless HA

FIG. 10A – 10B

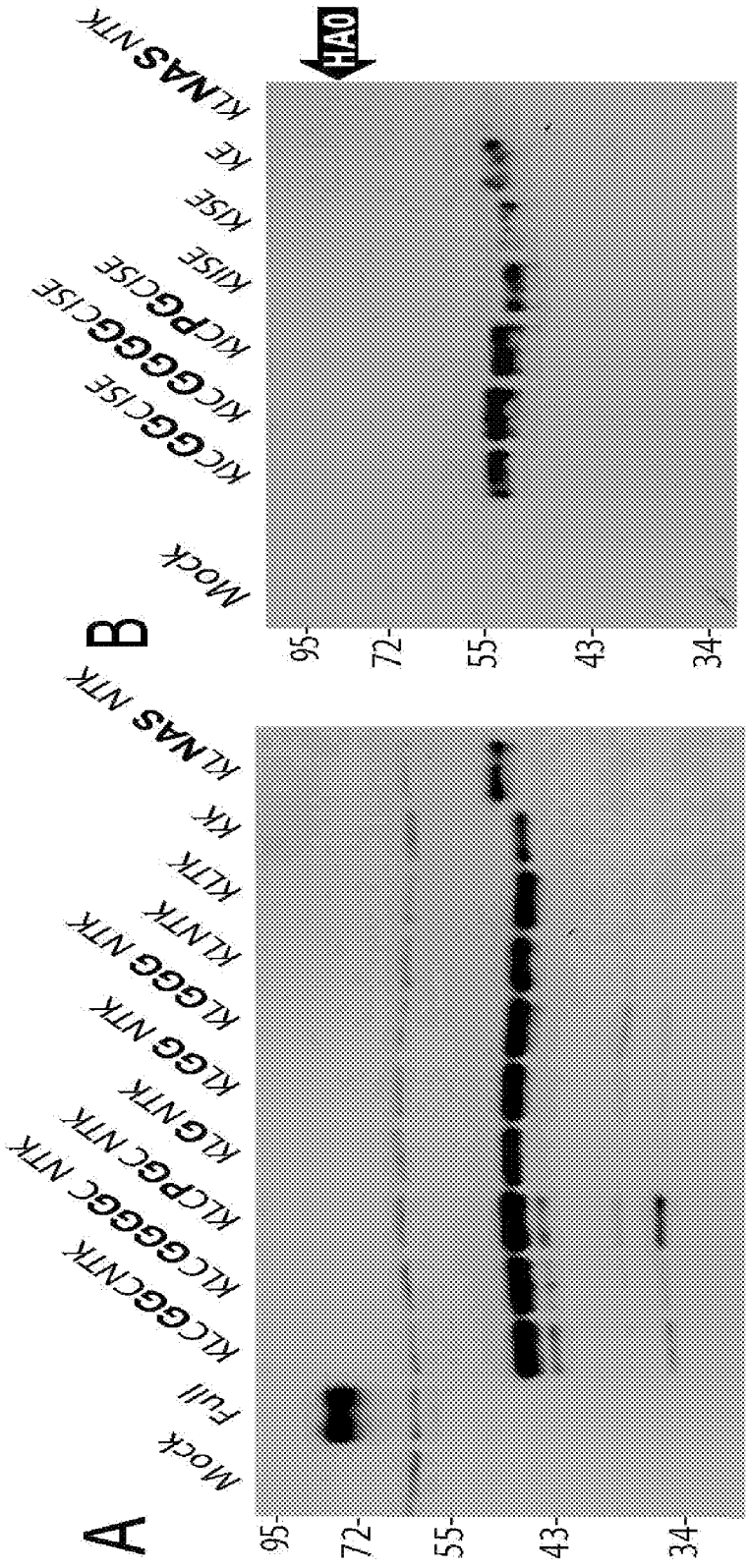
A
Mock
Full
KLCGGCNTK
KLCGGGGC NTK
KLCPGC NTK
KLG NTK
KLGG NTK
KLGGG NTK
KLNTK
KLTK
KK
KLNAS NTK
95-
72-
55-
43-
34-
B
Mock
KlCGGCISE
KlCGGGGCISE
KlCPGCISE
KlSE
KlSE
KE
KLNAS NTK
HA0
95-
72-
55-
43-
34-

FIG. 11A – 11B

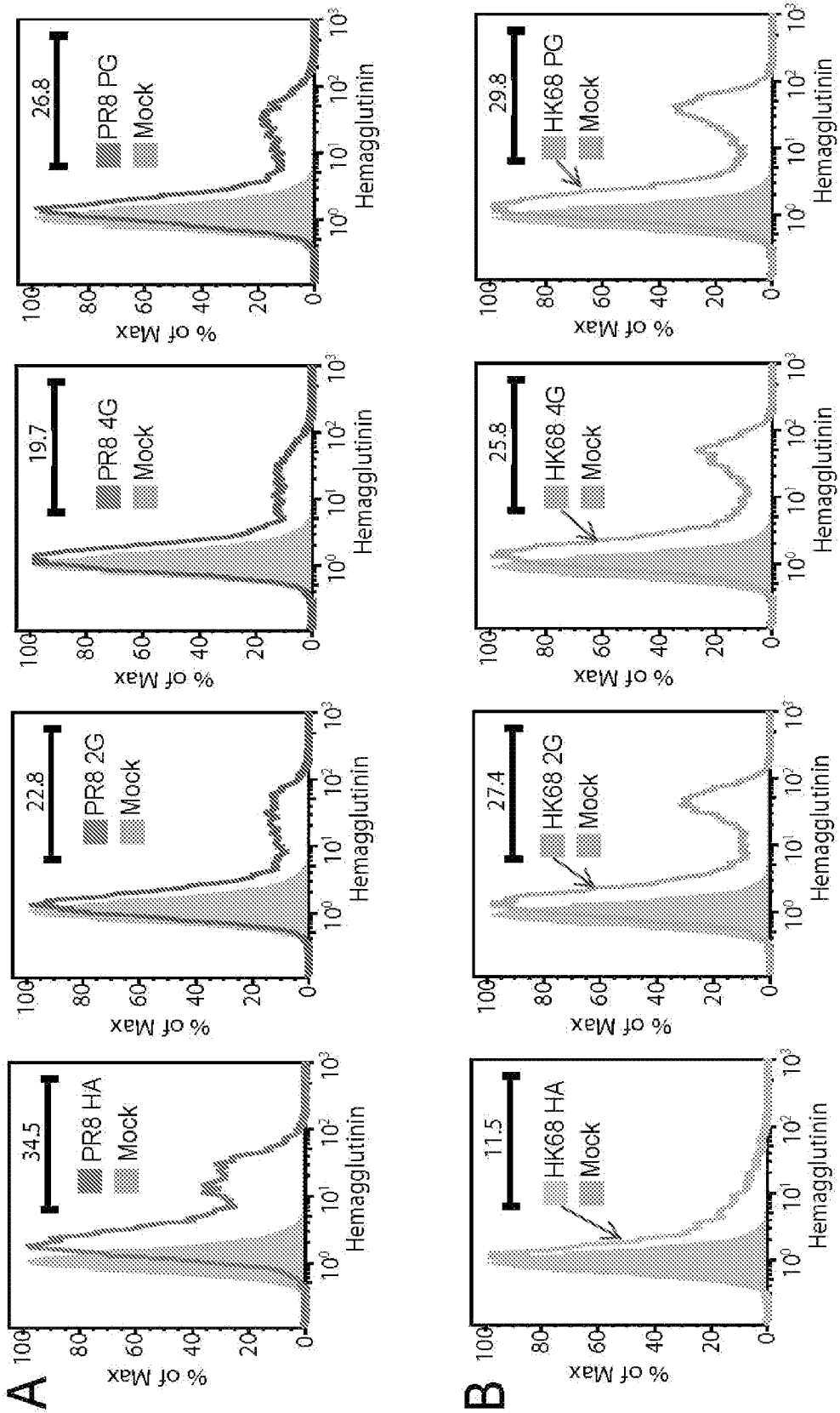
A
B
% of Max
Hemagglutinin
34.5
PR8 HA
Mock
22.8
PR8 2G
Mock
19.7
PR8 4G
Mock
26.8
PR8 PG
Mock
11.5
HK68 HA
Mock
27.4
HK68 2G
Mock
25.8
HK68 4G
Mock
29.8
HK68 PG
Mock

FIG. 12A – 12B

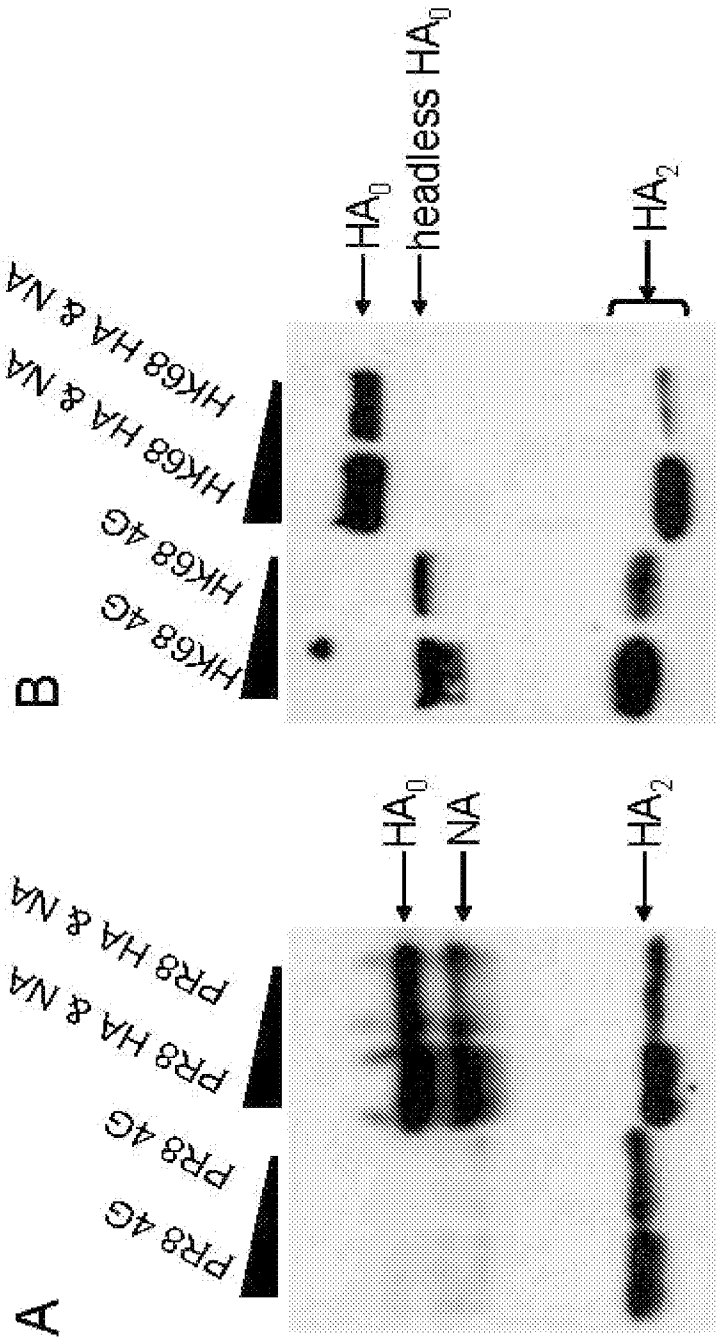
A
PR8 4G
PR8 4G
PR8 HA & NA
PR8 HA & NA
HA0
NA
HA2
B
HK68 4G
HK68 4G
HK68 HA & NA
HK68 HA & NA
HA0
headless HA0
HA2

FIG. 13A – 13B

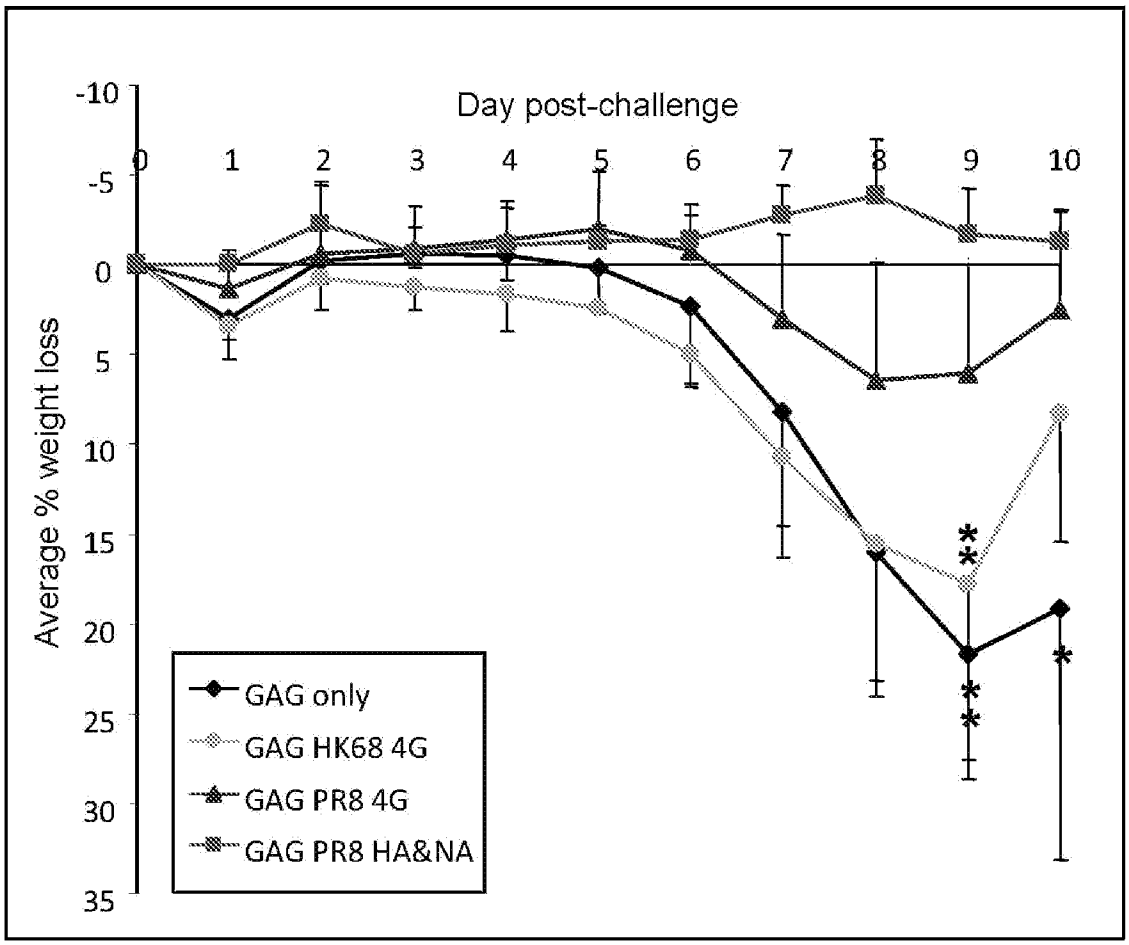
Day post-challenge
Average % weight loss
-10
-5
0
5
10
15
20
25
30
35
0
1
2
3
4
5
6
7
8
9
10
GAG only
GAG HK68 4G
GAG PR8 4G
GAG PR8 HA&NA

FIG. 14

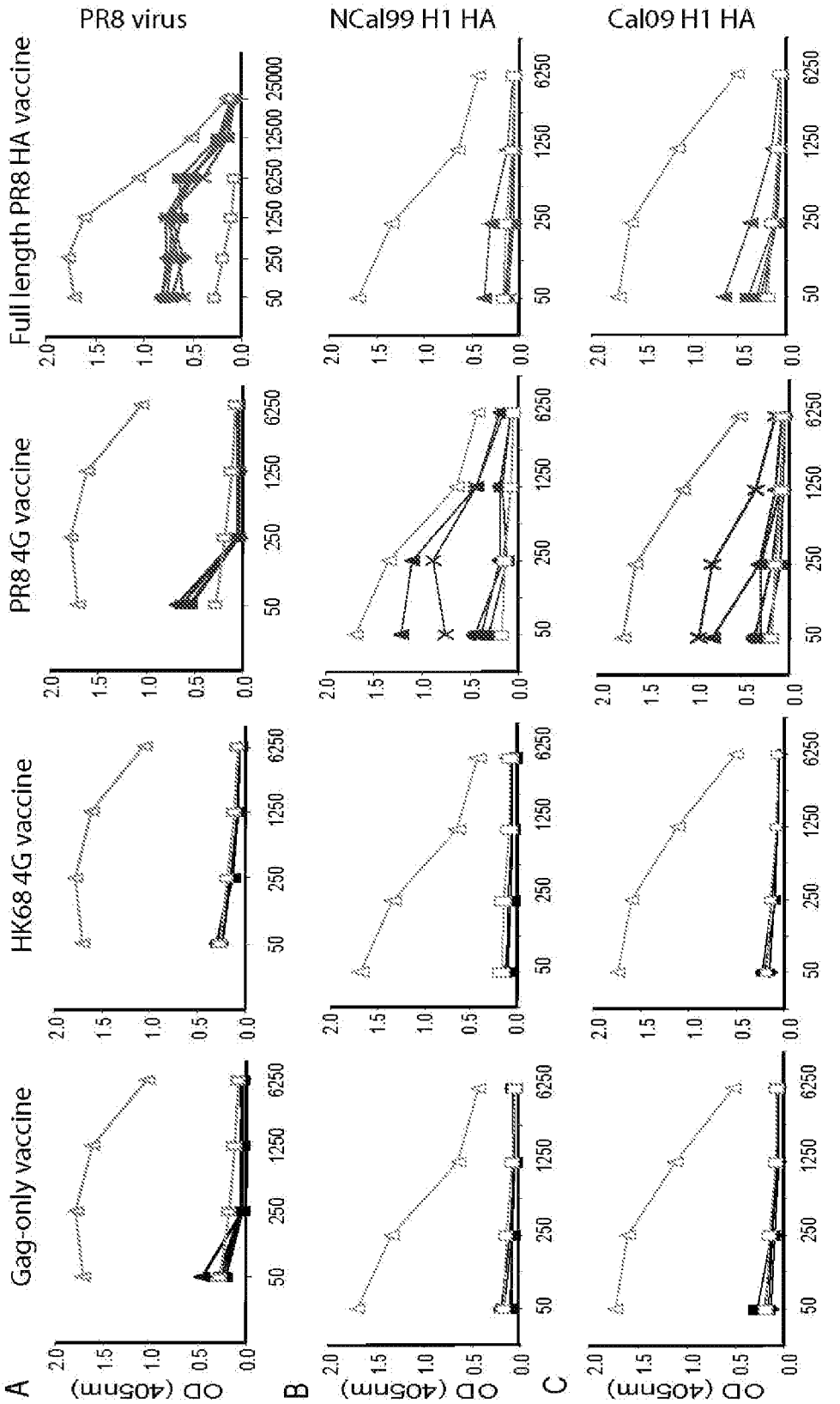
A
B
C
Gag-only vaccine
HK68 4G vaccine
PR8 4G vaccine
Full length PR8 HA vaccine
PR8 virus
NCal99 H1 HA
Cal09 H1 HA
OD (405nm)
2.0
1.5
1.0
0.5
0.0
50
250
1250
6250
12500
25000

FIG. 15A – 15C

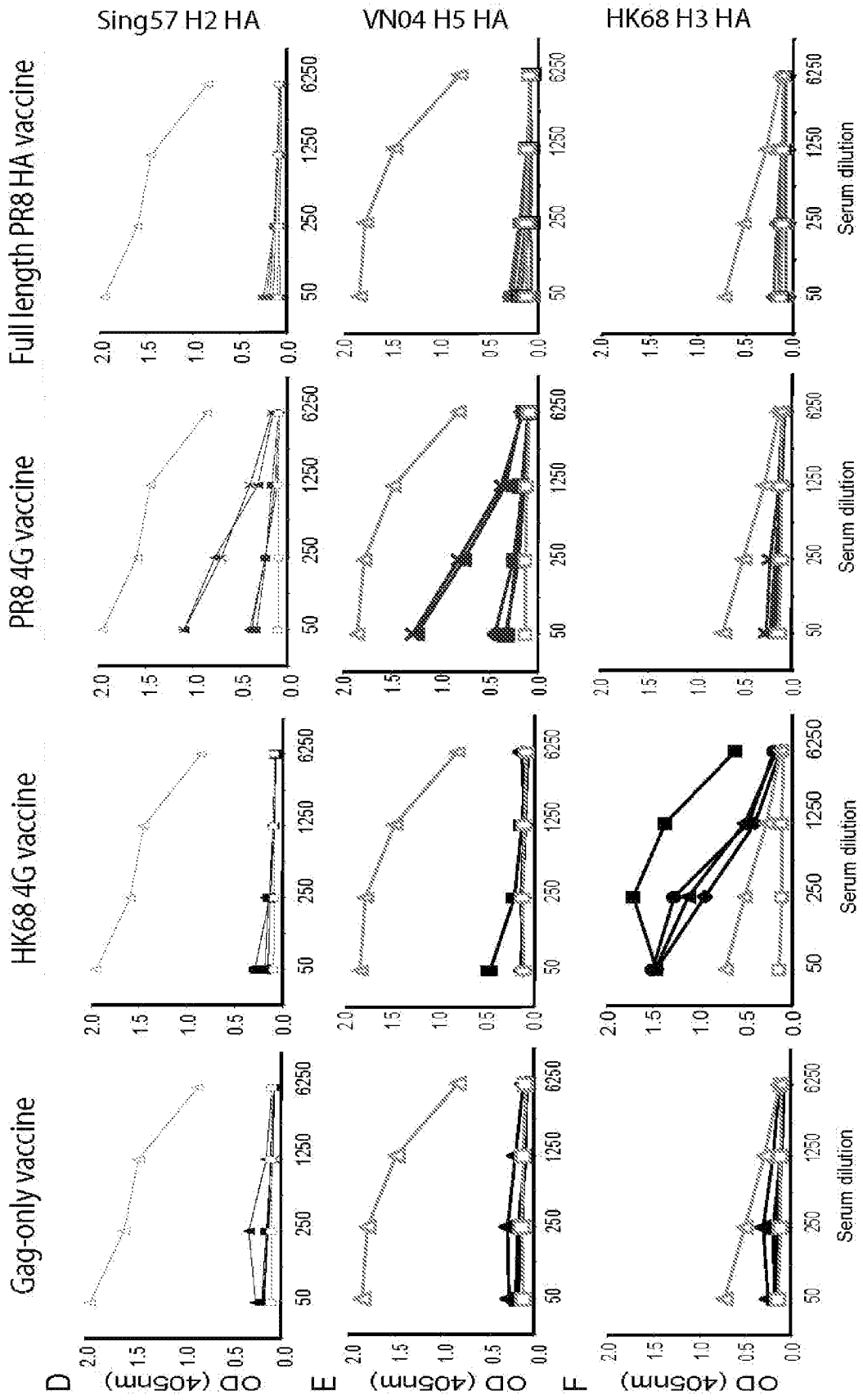
Sing57 H2 HA
VN04 H5 HA
HK68 H3 HA
Full length PR8 HA vaccine
PR8 4G vaccine
HK68 4G vaccine
Gag-only vaccine
D
E
F
OD (405nm)
Serum dilution
2.0
1.5
1.0
0.5
0.0
50
250
1250
6250

FIG. 15D – 15F

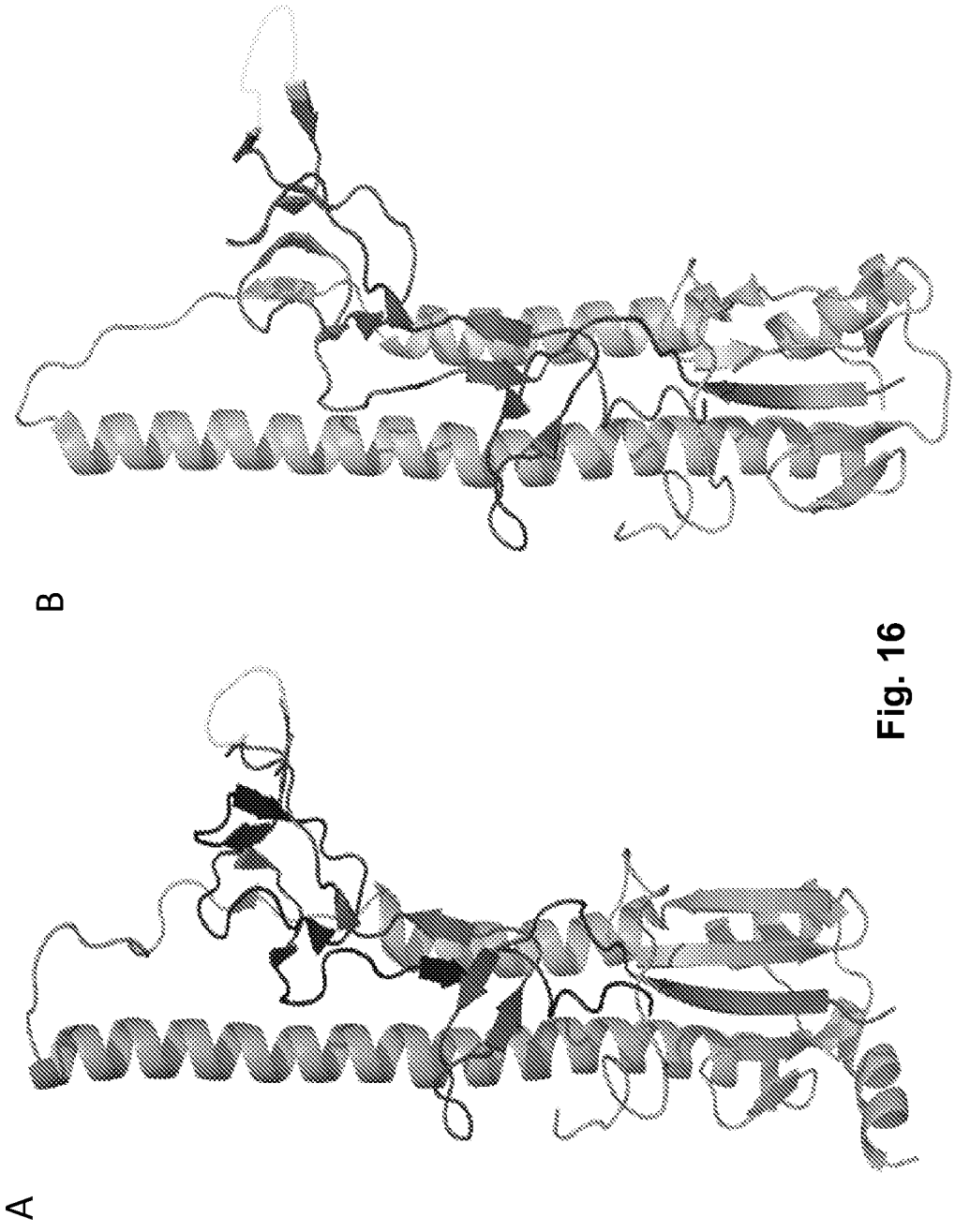

Fig. 16

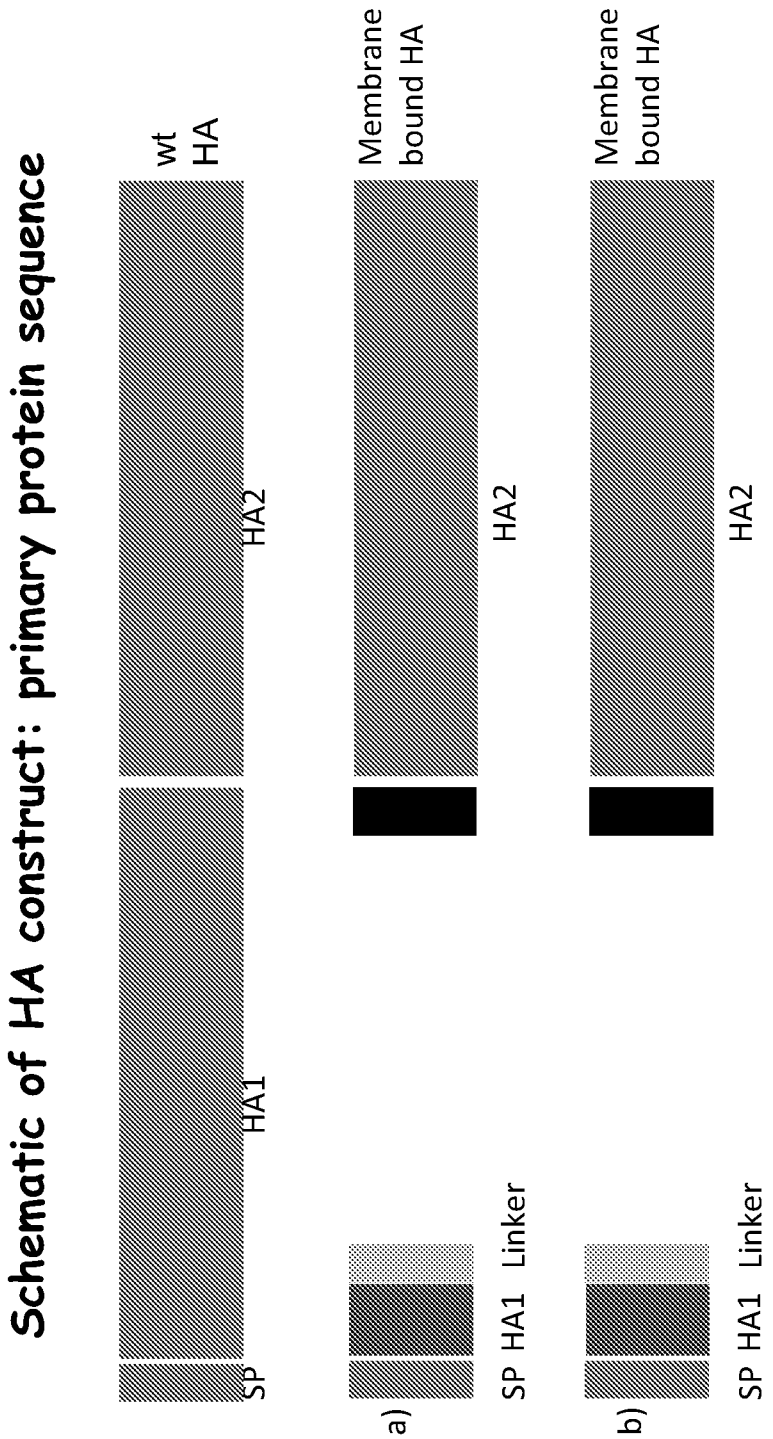
Schematic of HA construct: primary protein sequence
wt HA
SP
HA1
HA2
Membrane bound HA
a)
SP HA1 Linker
HA2
Membrane bound HA
b)
SP HA1 Linker
HA2

Fig. 17

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/029202

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/145 C07K14/11
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GERHARD W ET AL: "Prospects for universal influenza virus vaccine" EMERGING INFECTIOUS DISEASES, EID, ATLANTA, GA, US, vol. 12, no. 4, 1 April 2006 (2006-04-01), pages 569-574, XP009087881 ISSN: 1080-6040 page 571 - page 572	1-74

-/--

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 August 2010	24/08/2010

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

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HORVATH A ET AL: "HEMAGGLUTININ-BASED MULTIPEPTIDE CONSTRUCT ELICITS ENHANCED PROTECTIVE IMMUNE RESPONSE IN MICE AGAINST INFLUENZA A VIRUS INFECTION" IMMUNOLOGY LETTERS, ELSEVIER BV, NL LNKD-DOI:10.1016/S0165-2478(97)00137-5, vol. 60, no. 2/03, 1 February 1998 (1998-02-01), pages 127-136, XP000917227 ISSN: 0165-2478 the whole document	1-74
Y	BIANCHI ELISABETTA ET AL: "Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US LNKD-DOI:10.1128/JVI.79.12.7380-7388.2005, vol. 79, no. 12, 1 June 2005 (2005-06-01), pages 7380-7388, XP002445847 ISSN: 0022-538X the whole document	1-74
Y	MOK H ET AL: "Enhancement of the CD8<+> T cell response to a subdominant epitope of respiratory syncytial virus by deletion of an immunodominant epitope" VACCINE, ELSEVIER LTD, GB LNKD-DOI:10.1016/J.VACCINE.2008.07.012, vol. 26, no. 37, 2 September 2008 (2008-09-02), pages 4775-4782, XP023980037 ISSN: 0264-410X [retrieved on 2008-07-26] the whole document	1-74
X	DATABASE Geneseq [Online] 15 November 2007 (2007-11-15), "Influenza A virus hemagglutinin protein, H1PR8." XP002595511 retrieved from EBI accession no. GSP:AJG95109 Database accession no. AJG95109 * abstract; compound	1-5, 30-35, 41,42
A	LANDRY SAMUEL J: "Three-dimensional structure determines the pattern of CD4+ T-cell epitope dominance in influenza virus hemagglutinin." JOURNAL OF VIROLOGY FEB 2008 LNKD-PUBMED:18057238, vol. 82, no. 3, February 2008 (2008-02), pages 1238-1248, XP002595512 ISSN: 1098-5514 the whole document	1-74

3