

COMPUTATIONALLY OPTIMIZED BROADLY REACTIVE ANTIGENS FOR H3N2, H2N2, AND B INFLUENZA VIRUSES

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of U.S. Provisional Application No. 61/596,014, filed February 7, 2012, which is herein incorporated by reference in its entirety.

FIELD

10 This disclosure concerns optimized influenza hemagglutinin proteins that elicit broadly reactive immune responses to H3N2, H2N2 or B influenza viruses, and their use as vaccines.

BACKGROUND

15 Influenza virus is a member of *Orthomyxoviridae* family. There are three subtypes of influenza viruses, designated influenza A, influenza B, and influenza C. The influenza virion contains a segmented negative-sense RNA genome, which encodes the following proteins: hemagglutinin (HA), neuraminidase (NA), matrix (M1), proton ion-channel protein (M2), nucleoprotein (NP), polymerase basic
20 protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), and nonstructural protein 2 (NS2). The HA, NA, M1, and M2 are membrane associated, whereas NP, PB1, PB2, PA, and NS2 are nucleocapsid associated proteins. The M1 protein is the most abundant protein in influenza particles. The
25 HA and NA proteins are envelope glycoproteins, responsible for virus attachment and penetration of the viral particles into the cell, and the sources of the major immunodominant epitopes for virus neutralization and protective immunity. Both
HA and NA proteins are considered the most important components for prophylactic influenza vaccines.

Each year, seasonal influenza causes over 300,000 hospitalizations and
30 36,000 deaths in the U.S. alone (Simonsen *et al.*, *Lancet Infect Dis* 7:658-66, 2007). The emergence of the novel H1N1 influenza virus in 2009 demonstrated how

quickly a new influenza pandemic can sweep across the world. H3N2 influenza strains can infect both birds and mammals and H3N2 influenza is becoming increasingly more abundant in seasonal influenza. H2N2 is another influenza subtype that has previously caused human pandemics. An H2N2 strain caused the
5 1957 pandemic in Asia. Influenza B virus is another type of influenza that infects humans and represents a significant cause of seasonal flu, but is not known to cause pandemics due to its limited host range (humans and seals).

There are currently two influenza vaccine approaches licensed in the United States – the inactivated, split vaccine and the live-attenuated virus vaccine. The
10 inactivated vaccines can efficiently induce humoral immune responses but generally only poor cellular immune responses. Live virus vaccines cannot be administered to immunocompromised or pregnant patients due to their increased risk of infection. Thus, a need exists for a broadly protective influenza virus vaccine.

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SUMMARY

Disclosed herein is the generation of computationally-optimized H2N2, H3N2 and B influenza HA polypeptides for eliciting a broadly reactive immune response to influenza virus. The optimized HA polypeptides were developed through a series of HA protein alignments, and subsequent generation of consensus
20 sequences based on selected H2N2, H3N2 and B influenza virus isolates.

Provided herein are recombinant influenza HA polypeptides having an optimized amino acid sequence for eliciting a broadly reactive immune response against H2N2, H3N2 or B influenza. In some embodiments, the HA polypeptide comprises an amino acid sequence at least 99.6% identical to SEQ ID NO: 1, at least
25 99.4% identical to SEQ ID NO: 2, at least 99.7% identical to SEQ ID NO: 4, at least 99.6% identical to SEQ ID NO: 5, at least 98.8% identical to SEQ ID NO: 6, at least 99.7% identical to SEQ ID NO: 8, at least 98.4% identical to SEQ ID NO: 9, at least 97.8% identical to SEQ ID NO: 10 or at least 98.9% identical to SEQ ID NO: 11. In some embodiments, the amino acid sequence of the polypeptide comprises no more
30 than 2 amino acid substitutions relative to SEQ ID NO: 1; no more than 3 amino acid substitutions relative to SEQ ID NO: 2; no more than 1 amino acid substitution

relative to SEQ ID NO: 4; no more than 2 amino acid substitutions relative to SEQ ID NO: 5; no more than 7 amino acid substitutions relative to SEQ ID NO: 6; no more than 10 amino acid substitutions relative to SEQ ID NO: 8; no more than 9 amino acid substitutions relative to SEQ ID NO: 9; no more than 10 amino acid substitutions relative to SEQ ID NO: 10; or no more than 6 amino acid substitutions relative to SEQ ID NO: 11. In some embodiments, the influenza HA polypeptide lacks the N-terminal methionine residue.

In one embodiment, there is provided a recombinant influenza hemagglutinin (HA) polypeptide, comprising: (i) an amino acid sequence at least 97.7% identical to residues 2-566 of SEQ ID NO: 8; (ii) an amino acid sequence at least 98.4% identical to residues 2-566 of SEQ ID NO: 9; (iii) an amino acid sequence at least 97.8% identical to residues 2-566 of SEQ ID NO: 10; or (iv) an amino acid sequence at least 98.9% identical to residues 2-566 of SEQ ID NO: 11.

Isolated nucleic acid molecules and vectors encoding the recombinant HA polypeptides are also provided by the present disclosure. Further provided are isolated cells comprising such vectors.

Also provided are influenza virus-like particles (VLPs) and fusion proteins comprising the optimized HA polypeptides disclosed herein.

Further provided are compositions that include the optimized influenza HA polypeptides, fusion proteins or VLPs disclosed herein in a pharmaceutically acceptable carrier. Methods of eliciting an immune response against influenza virus in a subject by administering the disclosed compositions, fusion proteins or VLPs are also provided by the present disclosure.

Also provided are methods of immunizing a subject against influenza virus by administering to the subject a composition comprising a VLP that contains an optimized HA polypeptide.

In another aspect, there is provided a use of the influenza HA polypeptide disclosed herein, the VLP disclosed herein, the fusion protein disclosed herein or the composition disclosed herein in the manufacture of a medicament for eliciting an immune response to influenza virus in a subject.

In another aspect, there is provided a use of a composition comprising the VLP disclosed herein and pharmaceutically acceptable carrier in the manufacture of a medicament for immunizing a subject against influenza virus.

The foregoing and other objects, features, and advantages of the invention will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

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BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic summarizing the process for generating a H2N2 influenza COBRA HA sequence according to Method 2 as described in Example 1.

FIG. 2 is a schematic summarizing the process for generating a H2N2 influenza COBRA HA sequence according to Method 3 as described in Example 1.

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FIG. 3 is a schematic summarizing the process for generating an influenza B COBRA HA sequence according to Method 2 as described in Example 2.

FIG. 4 is a schematic summarizing the process for generating an influenza B COBRA HA sequence according to Method 3 as described in Example 2.

5 **FIG. 5** is a schematic summarizing the process for generating a H3N2 influenza COBRA HA sequence according to Method 1 as described in Example 3.

FIG. 6 is a schematic summarizing the process for generating a H3N2 influenza COBRA HA sequence according to Method 2 as described in Example 3.

FIG. 7 is a schematic summarizing the process for generating a H3N2
10 influenza COBRA HA sequence according to Method 3 as described in Example 3.

FIG. 8 is a schematic summarizing the process for generating a H3N2 influenza COBRA HA sequence according to Method 4 as described in Example 3.

SEQUENCE LISTING

15 The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is
20 submitted as an ASCII text file, created on January 30, 2013, 53.7 KB, which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NOs: 1-4 are COBRA amino acid sequences for H2N2 influenza HA.

SEQ ID NOs: 5-7 are COBRA amino acid sequences for influenza B HA.

25 **SEQ ID NOs: 8-11** are COBRA amino acid sequences for H3N2 influenza HA.

DETAILED DESCRIPTION

I. Abbreviations

30 COBRA: computationally optimized broadly reactive antigen
HA: hemagglutinin

HAI: hemagglutination inhibition

HRP: horseradish peroxidase

M1: matrix protein 1

NA: neuraminidase

5 PFU: plaque form unit

VLP: virus-like particle

II. Terms and Methods

Unless otherwise noted, technical terms are used according to conventional
10 usage. Definitions of common terms in molecular biology may be found in
Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-
854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published
by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers
(ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*,
15 published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the disclosure, the
following explanations of specific terms are provided:

Adjuvant: A substance or vehicle that non-specifically enhances the
immune response to an antigen. Adjuvants can include a suspension of minerals
20 (alum, aluminum hydroxide, or phosphate) on which antigen is adsorbed; or water-
in-oil emulsion in which antigen solution is emulsified in mineral oil (for example,
Freund's incomplete adjuvant), sometimes with the inclusion of killed mycobacteria
(Freund's complete adjuvant) to further enhance antigenicity. Immunostimulatory
oligonucleotides (such as those including a CpG motif) can also be used as adjuvants
25 (for example, see U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371;
6,239,116; 6,339,068; 6,406,705; and 6,429,199). Adjuvants also include biological
molecules, such as costimulatory molecules. Exemplary biological adjuvants
include IL-2, RANTES, GM-CSF, TNF- α , IFN- γ , G-CSF, LFA-3, CD72, B7-1, B7-
2, OX-40L and 41 BBL.

30 **Administer:** As used herein, administering a composition to a subject means
to give, apply or bring the composition into contact with the subject. Administration

can be accomplished by any of a number of routes, such as, for example, topical, oral, subcutaneous, intramuscular, intraperitoneal, intravenous, intrathecal and intradermal.

Antibody: An immunoglobulin molecule produced by B lymphoid cells with a specific amino acid sequence. Antibodies are evoked in humans or other animals by a specific antigen (immunogen). Antibodies are characterized by reacting specifically with the antigen in some demonstrable way, antibody and antigen each being defined in terms of the other. "Eliciting an antibody response" refers to the ability of an antigen or other molecule to induce the production of antibodies.

Antigen: A compound, composition, or substance that can stimulate the production of antibodies or a T-cell response in an animal, including compositions that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous immunogens. *In some embodiments of the disclosed compositions and methods, the antigen is an influenza HA protein.*

Codon-optimized: A "codon-optimized" nucleic acid refers to a nucleic acid sequence that has been altered such that the codons are optimal for expression in a particular system (such as a particular species or group of species). For example, a nucleic acid sequence can be optimized for expression in mammalian cells. Codon optimization does not alter the amino acid sequence of the encoded protein.

Fusion protein: A protein generated by expression of a nucleic acid sequence engineered from nucleic acid sequences encoding at least a portion of two different (heterologous) proteins. To create a fusion protein, the nucleic acid sequences must be in the same reading frame and contain no internal stop codons. For example, a fusion protein can include an influenza HA fused to a heterologous protein.

Hemagglutinin (HA): An influenza virus surface glycoprotein. HA mediates binding of the virus particle to a host cells and subsequent entry of the virus into the host cell. The nucleotide and amino acid sequences of numerous

influenza HA proteins are known in the art and are publically available, such as through the NCBI Influenza Virus Resource database (Bao *et al.*, *J Virol* 82:596-601, 2008). HA (along with NA) is one of the two major influenza virus antigenic determinants.

5 **Immune response:** A response of a cell of the immune system, such as a B-cell, T-cell, macrophage or polymorphonucleocyte, to a stimulus such as an antigen or vaccine. An immune response can include any cell of the body involved in a host defense response, including for example, an epithelial cell that secretes an interferon or a cytokine. An immune response includes, but is not limited to, an innate
10 immune response or inflammation. As used herein, a protective immune response refers to an immune response that protects a subject from infection (prevents infection or prevents the development of disease associated with infection). Methods of measuring immune responses are well known in the art and include, for example, measuring proliferation and/or activity of lymphocytes (such as B or T
15 cells), secretion of cytokines or chemokines, inflammation, antibody production and the like.

Immunogen: A compound, composition, or substance which is capable, under appropriate conditions, of stimulating an immune response, such as the production of antibodies or a T-cell response in an animal, including compositions
20 that are injected or absorbed into an animal. As used herein, an “immunogenic composition” is a composition comprising an immunogen (such as an HA polypeptide).

Immunize: To render a subject protected from an infectious disease, such as by vaccination.

25 **Influenza virus:** A segmented negative-strand RNA virus that belongs to the *Orthomyxoviridae* family. There are three types of Influenza viruses, A, B and C. Influenza A viruses infect a wide variety of birds and mammals, including humans, horses, marine mammals, pigs, ferrets, and chickens. In animals, most influenza A viruses cause mild localized infections of the respiratory and intestinal
30 tract. However, highly pathogenic influenza A strains, such as H5N1, cause systemic infections in poultry in which mortality may reach 100%. In 2009, H1N1

influenza was the most common cause of human influenza. A new strain of swine-origin H1N1 emerged in 2009 and was declared pandemic by the World Health Organization. This strain was referred to as "swine flu." H1N1 influenza A viruses were also responsible for the Spanish flu pandemic in 1918, the Fort Dix outbreak in 5 1976, and the Russian flu epidemic in 1977-1978. H3N2, H2N2 and influenza B viruses also infect humans and are causative agents of seasonal influenza.

Isolated: An "isolated" biological component (such as a nucleic acid, protein or virus) has been substantially separated or purified away from other biological components (such as cell debris, or other proteins or nucleic acids). Biological 10 components that have been "isolated" include those components purified by standard purification methods. The term also embraces recombinant nucleic acids, proteins or viruses (or VLPs), as well as chemically synthesized nucleic acids or peptides.

Linker: One or more amino acids that serve as a spacer between two polypeptides of a fusion protein.

15 **Matrix (M1) protein:** An influenza virus structural protein found within the viral envelope. M1 is thought to function in assembly and budding.

Neuraminidase (NA): An influenza virus membrane glycoprotein. NA is involved in the destruction of the cellular receptor for the viral HA by cleaving terminal sialic acid residues from carbohydrate moieties on the surfaces of infected 20 cells. NA also cleaves sialic acid residues from viral proteins, preventing aggregation of viruses. NA (along with HA) is one of the two major influenza virus antigenic determinants.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional 25 relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

Optimized influenza HA protein: As used herein, "optimized influenza HA 30 protein" refers to an HA protein consensus sequence generated by sequence alignments of H2N2, H3N2 or B influenza viruses isolates (as described in Examples 1-3 below).

Nucleotide sequences encoding optimized HA proteins can be further optimized for expression in mammalian cells via codon-optimization and RNA optimization (such as to increase RNA stability). Optimized influenza HA proteins disclosed herein (and set forth herein as SEQ ID NOs: 1-11) are also referred to as “COBRA” (computationally-
5 optimized broadly reactive antigen) sequences. Optimized HA polypeptides are designed to elicit broadly reactive immune responses in a subject. In the context of the present disclosure, “broadly reactive” means the protein sequence elicits an immune response in a subject that is sufficient to inhibit, neutralize or prevent infection of a broad range of influenza viruses (such as most or all influenza viruses within a specific subtype). In
10 some instances, the optimized influenza HA protein is capable of eliciting an immune response, such as a protective immune response, against most or all H3N2 influenza virus isolates, most or all H2N2 influenza virus isolates, or most or all influenza B virus isolates.

Outbreak: As used herein, an influenza virus “outbreak” refers to a collection of
15 virus isolates from within a single country in a given year.

Pharmaceutically acceptable vehicles: The pharmaceutically acceptable carriers (vehicles) useful in this disclosure are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical
20 delivery of one or more therapeutic compositions, such as one or more influenza vaccines, and additional pharmaceutical agents.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically
25 acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical
30 compositions to be administered can contain minor amounts of non-toxic auxiliary

substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Polypeptide: A polymer in which the monomers are amino acid residues which are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used. The terms “polypeptide” or “protein” as used herein are intended to encompass any amino acid sequence and include modified sequences such as glycoproteins. The term “polypeptide” is specifically intended to cover naturally occurring proteins, as well as those which are recombinantly or synthetically produced. The term “residue” or “amino acid residue” includes reference to an amino acid that is incorporated into a protein, polypeptide, or peptide.

Conservative amino acid substitutions are those substitutions that, when made, least interfere with the properties of the original protein, that is, the structure and especially the function of the protein is conserved and not significantly changed by such substitutions. Examples of conservative substitutions are shown below.

	Original Residue	Conservative Substitutions
20	Ala	Ser
	Arg	Lys
	Asn	Gln, His
	Asp	Glu
	Cys	Ser
	Gln	Asn
25	Glu	Asp
	His	Asn; Gln
	Ile	Leu, Val
	Leu	Ile; Val
	Lys	Arg; Gln; Glu
30	Met	Leu; Ile
	Phe	Met; Leu; Tyr
	Ser	Thr
	Thr	Ser
	Trp	Tyr
35	Tyr	Trp; Phe
	Val	Ile; Leu

Conservative substitutions generally maintain (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

5 The substitutions which in general are expected to produce the greatest changes in protein properties will be non-conservative, for instance changes in which (a) a hydrophilic residue, for example, seryl or threonyl, is substituted for (or by) a hydrophobic residue, for example, leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a
10 residue having an electropositive side chain, for example, lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, for example, glutamyl or aspartyl; or (d) a residue having a bulky side chain, for example, phenylalanine, is substituted for (or by) one not having a side chain, for example, glycine.

Preventing, treating or ameliorating a disease: “Preventing” a disease
15 refers to inhibiting the full development of a disease. “Treating” refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop. “Ameliorating” refers to the reduction in the number or severity of signs or symptoms of a disease.

Promoter: A promoter is an array of nucleic acid control sequences which
20 direct transcription of a nucleic acid. A promoter includes necessary nucleic acid sequences near the start site of transcription. A promoter also optionally includes distal enhancer or repressor elements. A “constitutive promoter” is a promoter that is continuously active and is not subject to regulation by external signals or molecules. In contrast, the activity of an “inducible promoter” is regulated by an
25 external signal or molecule (for example, a transcription factor). In some embodiments herein, the promoter is a CMV promoter.

Purified: The term “purified” does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified peptide, protein, virus, VLP or other active compound is one that is isolated in whole or in part from
30 naturally associated proteins and other contaminants. In certain embodiments, the term “substantially purified” refers to a peptide, protein, virus, VLP or other active

compound that has been isolated from a cell, cell culture medium, or other crude preparation and subjected to fractionation to remove various components of the initial preparation, such as proteins, cellular debris, and other components.

Recombinant: A recombinant nucleic acid, protein, virus or VLP is one that has
5 a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or by the artificial manipulation of isolated segments of nucleic acids, for example, by genetic engineering techniques.

Sequence identity: The similarity between amino acid or nucleic acid sequences
10 is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are. Homologs or variants of a given gene or protein will possess a relatively high degree of sequence identity when aligned using standard methods.

15 Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith and Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman and Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85:2444, 1988; Higgins and Sharp, *Gene* 73:237-244, 1988; Higgins and Sharp, *CABIOS* 5:151-153, 1989; Corpet *et al.*, *Nucleic*
20 *Acids Research* 16:10881-10890, 1988; and Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85:2444, 1988. Altschul *et al.*, *Nature Genet.* 6:119-129, 1994.

The NCBI Basic Local Alignment Search Tool (BLASTTM) (Altschul *et al.*, *J. Mol. Biol.* 215:403-410, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the
25 Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx.

Subject: Living multi-cellular vertebrate organisms, a category that includes both human and non-human mammals, such as non-human primates.

Therapeutically effective amount: A quantity of a specified agent
30 sufficient to achieve a desired effect in a subject being treated with that agent. For example, this may be the amount of an influenza virus vaccine useful for eliciting an

immune response in a subject and/or for preventing infection or disease caused by influenza virus. Ideally, in the context of the present disclosure, a therapeutically effective amount of an influenza vaccine is an amount sufficient to increase resistance to, prevent, ameliorate, and/or treat infection caused by influenza virus in a subject without causing a substantial cytotoxic effect in the subject. The effective amount of an influenza vaccine useful for increasing resistance to, preventing, ameliorating, and/or treating infection in a subject will be dependent on, for example, the subject being treated, the manner of administration of the therapeutic composition and other factors.

10 **Transformed:** A transformed cell is a cell into which has been introduced a nucleic acid molecule by molecular biology techniques. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including transfection with viral vectors, transformation with plasmid vectors, and introduction of naked DNA by
15 electroporation, lipofection, and particle gun acceleration.

Vaccine: A preparation of immunogenic material capable of stimulating an immune response, administered for the prevention, amelioration, or treatment of disease, such as an infectious disease. The immunogenic material may include, for example, attenuated or killed microorganisms (such as attenuated viruses), or
20 antigenic proteins (including VLPs), peptides or DNA derived from them. Vaccines may elicit both prophylactic (preventative) and therapeutic responses. Methods of administration vary according to the vaccine, but may include inoculation, ingestion, inhalation or other forms of administration. Inoculations can be delivered by any of a number of routes, including parenteral, such as intravenous, subcutaneous or
25 intramuscular. Vaccines may be administered with an adjuvant to boost the immune response.

Vector: A vector is a nucleic acid molecule allowing insertion of foreign nucleic acid without disrupting the ability of the vector to replicate and/or integrate in a host cell. A vector can include nucleic acid sequences that permit it to replicate
30 in a host cell, such as an origin of replication. An insertional vector is capable of inserting itself into a host nucleic acid. A vector can also include one or more

selectable marker genes and other genetic elements. An expression vector is a vector that contains the necessary regulatory sequences to allow transcription and translation of an inserted gene or genes. In some embodiments of the present disclosure, the vector encodes an influenza HA, NA or M1 protein. In some
5 embodiments, the vector is the pTR600 expression vector (U.S. Patent Application Publication No. 2002/0106798; Ross *et al.*, *Nat Immunol.* 1(2):102-103, 2000; Green *et al.*, *Vaccine* 20:242-248, 2001).

Virus-like particle (VLP): Virus particles made up of one or more viral structural proteins, but lacking the viral genome. Because VLPs lack a viral
10 genome, they are non-infectious. In addition, VLPs can often be produced by heterologous expression and can be easily purified. Most VLPs comprise at least a viral core protein that drives budding and release of particles from a host cell. One example of such a core protein is influenza M1. In some embodiments herein, an influenza VLP comprises the HA, NA and/or M1 proteins. Influenza VLPs can be
15 produced by transfection of host cells with plasmids encoding the HA and NA proteins, and optionally the M1 protein. After incubation of the transfected cells for an appropriate time to allow for protein expression (such as for approximately 72 hours), VLPs can be isolated from cell culture supernatants. Example 5 provides an exemplary protocol for purifying influenza VLPs from cell supernatants. In this
20 example, VLPs are isolated by low speed centrifugation (to remove cell debris), vacuum filtration and ultracentrifugation through 20% glycerol. Other methods of producing influenza VLPs are known in the art (see, for example, U.S. Patent Application Publication Nos. 2006/0263804; 2008/0031895; 2010/0166769; and 2010/0239610).

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Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. "Comprising A or B"
30 means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for

nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

III. Overview of Several Embodiments

Disclosed herein is the generation of computationally-optimized H2N2, H3N2 and B influenza HA polypeptides for eliciting a broadly reactive immune response to influenza virus. The optimized HA polypeptides were developed through a series of HA protein alignments, and subsequent generation of consensus sequences based on selected H2N2, H3N2 and B influenza virus isolates. The methods used to generate the optimized HA consensus sequences are described in Examples 1-3, and shown in FIGS. 1-7. The amino acid sequences of 10 specific HA polypeptides are set forth herein as SEQ ID NOs: 1-11.

Provided herein are recombinant influenza HA polypeptides having an optimized amino acid sequence for eliciting a broadly reactive immune response against H2N2, H3N2 or B influenza. In some embodiments, the HA polypeptide comprises: an amino acid sequence at least 99.6% identical to residues 2-562 of SEQ ID NO: 1; an amino acid sequence at least 99.4% identical to residues 2-562 of SEQ ID NO: 2; an amino acid sequence comprising residues 2-562 of SEQ ID NO: 3; an amino acid sequence at least 99.7% identical to residues 2-562 of SEQ ID NO: 4; an amino acid sequence at least 99.6% identical to residues 2-584 of SEQ ID NO: 5; an amino acid sequence at least 98.8%, at least 99% or at least 99.5% identical to residues 2-585 of SEQ ID NO: 6; an amino acid sequence comprising residues 2-585 of SEQ ID NO: 7; an amino acid sequence at least 97.7%, at least 98%, at least 98.5%, at least 99% or at least 99.5% identical to residues 2-566 of SEQ ID NO: 8; an amino acid sequence at least 98.4%, at least 99% or at least 99.5% identical to

residues 2-566 of SEQ ID NO: 9; an amino acid sequence at least 97.8%, at least 98%, at least 98.5%, at least 99% or at least 99.5% identical to residues 2-566 of SEQ ID NO: 10; an amino acid sequence at least 98.9%, at least 99% or at least 99.5% identical to residues 2-566 of SEQ ID NO: 11.

5 In specific examples, the amino acid sequence of the HA polypeptide comprises or consists of the amino acid sequence of residues 2-562 of SEQ ID NO: 1, residues 2-562 of SEQ ID NO: 2, residues 2-562 of SEQ ID NO: 3, residues 2-562 of SEQ ID NO: 4, residues 2-584 of SEQ ID NO: 5, residues 2-585 of SEQ ID NO: 6, residues 2-585 of SEQ ID NO: 7, residues 2-566 of SEQ ID NO: 8, residues
10 2-566 of SEQ ID NO: 9 residues 2-566 of SEQ ID NO: 10 or residues 2-566 of SEQ ID NO: 11.

 In some embodiments, the influenza HA polypeptide comprises: an amino acid sequence at least 99.6% identical to SEQ ID NO: 1; an amino acid sequence at least 99.4% identical to SEQ ID NO: 2; the amino acid sequence of SEQ ID NO: 3;
15 an amino acid sequence at least 99.7% identical to SEQ ID NO: 4; an amino acid sequence at least 99.6% identical to SEQ ID NO: 5; an amino acid sequence at least 98.8%, at least 99% or at least 99.5% identical to SEQ ID NO: 6; the amino acid sequence of SEQ ID NO: 7; an amino acid sequence at least 99.7% identical to SEQ ID NO: 8; an amino acid sequence at least 98.4%, at least 99% or at least 99.5%
20 identical to SEQ ID NO: 9; an amino acid sequence at least 97.8%, at least 98%, at least 98.5%, at least 99% or at least 99.5% identical to SEQ ID NO: 10; or an amino acid sequence at least 98.9%, at least 99% or at least 99.5% identical to SEQ ID NO: 11.

 In some examples, the amino acid sequence of the HA polypeptide is more
25 identical than the percentages identities described above. In other examples, the amino acid sequence comprises or consists of the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10 or SEQ ID NO: 11.

30 In some embodiments, the HA polypeptide comprises: no more than 2 amino acid substitutions relative to SEQ ID NO: 1; no more than 3 amino acid substitutions

relative to SEQ ID NO: 2; no more than 1 amino acid substitution relative to SEQ ID NO: 4; no more than 2 amino acid substitutions relative to SEQ ID NO: 5; no more than 7 amino acid substitutions relative to SEQ ID NO: 6; no more than 10 amino acid substitutions relative to SEQ ID NO: 8; no more than 9 amino acid substitutions relative to SEQ ID NO: 9; no more than 10 amino acid substitutions relative to SEQ ID NO: 10; or no more than 6 amino acid substitutions relative to SEQ ID NO: 11. In some examples, the amino acid substitution is a conservative substitution. In some examples, the amino acid substitution is a non-conservative substitution. In other examples, the number of substitutions relative to the identified sequences is less than the number of substitutions identified above.

Further provided are isolated nucleic acid molecules encoding a recombinant influenza HA polypeptide disclosed herein. In some embodiments, the nucleic acid molecule is codon-optimized for expression in mammalian cells. The nucleic acid molecule is optionally further optimized for RNA stability.

Vectors comprising the nucleic acid molecules encoding recombinant HA polypeptides are also provided by the present disclosure. The vector can be any suitable vector for expression of the HA polypeptide, such as a mammalian expression vector. In particular examples, the vector is the pTR600 expression vector (U.S. Patent Application Publication No. 2002/0106798, herein incorporated by reference; Ross *et al.*, *Nat Immunol.* 1(2):102-103, 2000; Green *et al.*, *Vaccine* 20:242-248, 2001).

In some examples, the vector includes a promoter operably linked to the nucleic acid sequence encoding the HA polypeptide. In particular examples, the promoter is a CMV promoter.

Also provided are isolated cells comprising the disclosed vectors. In some cases, the cell is any suitable cell type for production and expression of VLPs, such as a mammalian cell.

Further provided are influenza VLPs comprising an optimized HA polypeptide disclosed herein. The influenza VLPs can further include any additional influenza proteins necessary to form the virus particle. In some embodiments, the

influenza VLPs further include influenza neuraminidase (NA) protein, influenza matrix (M1) protein, or both.

Also provided are influenza VLPs comprising an influenza HA polypeptide disclosed herein, produced by transfecting a host cell with a vector encoding the HA
5 polypeptide, a vector encoding an influenza NA protein and a vector encoding an influenza M1 protein under conditions sufficient to allow for expression of the HA, M1 and NA proteins.

Fusion proteins comprising an optimized influenza HA polypeptide are further provided by the present disclosure.

10 Also provided herein are compositions comprising an optimized influenza HA protein as disclosed herein, or a fusion protein or VLP comprising the optimized influenza HA protein. In some embodiments, the compositions further comprise a pharmaceutically acceptable carrier and/or an adjuvant. For example, the adjuvant can be alum, Freund's complete adjuvant, a biological adjuvant or
15 immunostimulatory oligonucleotides (such as CpG oligonucleotides).

Further provided is a method of eliciting an immune response to influenza virus in a subject by administering an optimized influenza HA protein, a fusion protein comprising an optimized influenza HA, VLPs containing an optimized influenza HA, or compositions thereof, as disclosed herein. In some embodiments,
20 the influenza virus is an H3N2, H2N2 or B influenza virus. In some embodiments, the HA protein, HA fusion protein or VLP can be administered using any suitable route of administration, such as, for example, intramuscular, intranasal or oral. In some embodiments, the HA protein, fusion protein or VLP is administered as a composition further comprising a pharmaceutically acceptable carrier and/or an
25 adjuvant. For example, the adjuvant can be alum, Freund's complete adjuvant, a biological adjuvant or immunostimulatory oligonucleotides (such as CpG oligonucleotides).

Also provided is a method of immunizing a subject against influenza virus by administering to the subject VLPs containing an optimized influenza HA protein
30 disclosed herein, or administering a composition thereof. In some embodiments of the method, the composition further comprises a pharmaceutically acceptable carrier

and/ or an adjuvant. For example, the adjuvant can be alum, Freund's complete adjuvant, a biological adjuvant or immunostimulatory oligonucleotides (such as CpG oligonucleotides). In some embodiments, the VLPs (or compositions thereof) are administered intramuscularly.

5 In some embodiments of the methods of eliciting an immune response or immunizing a subject, the subject is administered about 1 to about 25 µg of the VLPs containing an optimized HA protein. In particular examples, the subject is administered about 5 to about 20 µg of the VLPs, or about 10 to about 15µg of the VLPs. In one specific non-limiting example, the subject is administered about 15 µg
10 of the VLPs. However, one of skill in the art is capable of determining a therapeutically effective amount (for example an amount that provides protection against H3N3, H2N2 or B influenza virus infection) of VLPs to administer to a subject.

15 IV. Influenza

Influenza viruses are segmented negative-strand RNA viruses that belong to the *Orthomyxoviridae* family. There are three types of Influenza viruses, A, B and C. Influenza A viruses infect a wide variety of birds and mammals, including humans, horses, marine mammals, pigs, ferrets, and chickens. In animals, most
20 influenza A viruses cause mild localized infections of the respiratory and intestinal tract. However, highly pathogenic influenza A strains, such as H5N1, cause systemic infections in poultry in which mortality may reach 100%. Animals infected with influenza A often act as a reservoir for the influenza viruses and certain subtypes have been shown to cross the species barrier to humans.

25 Influenza A viruses can be classified into subtypes based on allelic variations in antigenic regions of two genes that encode surface glycoproteins, namely, hemagglutinin (HA) and neuraminidase (NA) which are required for viral attachment and cellular release. Currently, sixteen subtypes of HA (H1-H16) and nine NA (N1-N9) antigenic variants are known for influenza A virus. Previously,
30 only three subtypes were known to circulate in humans (H1N1, H1N2, and H3N2). However, in recent years, the pathogenic H5N1 subtype of avian influenza A has

been reported to cross the species barrier and infect humans as documented in Hong Kong in 1997 and 2003, leading to the death of several patients.

Influenza B viruses cause the same spectrum of disease as influenza A viruses. However, influenza B viruses do not cause pandemics, which is likely the consequence of the limited host range of this virus (humans and seals), which limits the generation of new strains by reassortment. Influenza B viruses cause significant morbidity; in the US in 2008, approximately one-third of all laboratory confirmed cases of influenza were caused by influenza B. Thus, the current seasonal trivalent influenza vaccine contains an influenza B component.

In 2009, H1N1 influenza was the most common cause of human influenza. A new strain of swine-origin H1N1 emerged in 2009 and was declared pandemic by the World Health Organization. This strain was referred to as "swine flu." H1N1 influenza A viruses were also responsible for the Spanish flu pandemic in 1918, the Fort Dix outbreak in 1976, and the Russian flu epidemic in 1977-1978.

The influenza virus segmented genome contains eight negative-sense RNA (nsRNA) gene segments (PB2, PB1, PA, NP, M, NS, HA and NA) that encode at least ten polypeptides, including RNA-directed RNA polymerase proteins (PB2, PB1 and PA), nucleoprotein (NP), neuraminidase (NA), hemagglutinin (subunits HA1 and HA2), the matrix proteins (M1 and M2) and the non-structural proteins (NS1 and NS2) (Krug *et al.*, In "The Influenza Viruses," R. M. Krug, ed., Plenum Press, N.Y., 1989, pp. 89 152).

Influenza virus' ability to cause widespread disease is due to its ability to evade the immune system by undergoing antigenic change, which is believed to occur when a host is infected simultaneously with both an animal influenza virus and a human influenza virus. During mutation and reassortment in the host, the virus may incorporate an HA and/or NA surface protein gene from another virus into its genome, thereby producing a new influenza subtype and evading the immune system.

HA is a viral surface glycoprotein generally comprising approximately 560 amino acids and representing 25% of the total virus protein. It is responsible for adhesion of the viral particle to, and its penetration into, a host cell in the early

stages of infection. Cleavage of the virus HA0 precursor into the HA1 and HA2 sub-fragments is a necessary step in order for the virus to infect a cell. Thus, cleavage is required in order to convert new virus particles in a host cell into virions capable of infecting new cells. Cleavage is known to occur during transport of the integral HA0 membrane protein from the endoplasmic reticulum of the infected cell to the plasma membrane. In the course of transport, hemagglutinin undergoes a series of co- and post-translational modifications including proteolytic cleavage of the precursor HA into the amino-terminal fragment HA1 and the carboxy terminal HA2. One of the primary difficulties in growing influenza strains in primary tissue culture or established cell lines arises from the requirement for proteolytic cleavage activation of the influenza hemagglutinin in the host cell.

Although it is known that an uncleaved HA can mediate attachment of the virus to its neuraminic acid-containing receptors on a cell surface, it is not capable of the next step in the infectious cycle, which is fusion. It has been reported that exposure of the hydrophobic amino terminus of HA2 by cleavage is required so that it can be inserted into the target cell, thereby forming a bridge between virus and target cell membrane. This process is followed by fusion of the two membranes and entry of the virus into the target cell.

Proteolytic activation of HA involves cleavage at an arginine residue by a trypsin-like endoprotease, which is often an intracellular enzyme that is calcium dependent and has a neutral pH optimum. Since the activating proteases are cellular enzymes, the infected cell type determines whether the HA is cleaved. The HA of the mammalian influenza viruses and the nonpathogenic avian influenza viruses are susceptible to proteolytic cleavage only in a restricted number of cell types. On the other hand, HA of pathogenic avian viruses among the H5 and H7 subtypes are cleaved by proteases present in a broad range of different host cells. Thus, there are differences in host range resulting from differences in hemagglutinin cleavability which are correlated with the pathogenic properties of the virus.

Neuraminidase (NA) is a second membrane glycoprotein of the influenza viruses. The presence of viral NA has been shown to be important for generating a multi-faceted protective immune response against an infecting virus. For most

influenza A viruses, NA is 413 amino acids in length, and is encoded by a gene of 1413 nucleotides. Nine different NA subtypes have been identified in influenza viruses (N1, N2, N3, N4, N5, N6, N7, N8 and N9), all of which have been found among wild birds. NA is involved in the destruction of the cellular receptor for the viral HA by cleaving terminal neuraminic acid (also called sialic acid) residues from carbohydrate moieties on the surfaces of infected cells. NA also cleaves sialic acid residues from viral proteins, preventing aggregation of viruses. Using this mechanism, it is hypothesized that NA facilitates release of viral progeny by preventing newly formed viral particles from accumulating along the cell membrane, as well as by promoting transportation of the virus through the mucus present on the mucosal surface. NA is an important antigenic determinant that is subject to antigenic variation.

In addition to the surface proteins HA and NA, influenza virus comprises six additional internal genes, which give rise to eight different proteins, including polymerase genes PB1, PB2 and PA, matrix proteins M1 and M2, nucleoprotein (NP), and non-structural proteins NS1 and NS2 (Horimoto *et al.*, *Clin Microbiol Rev.* 14(1):129-149, 2001).

In order to be packaged into progeny virions, viral RNA is transported from the nucleus as a ribonucleoprotein (RNP) complex composed of the three influenza virus polymerase proteins, the nucleoprotein (NP), and the viral RNA, in association with the influenza virus matrix 1 (M1) protein and nuclear export protein (Marsh *et al.*, *J Virol*, 82:2295-2304, 2008). The M1 protein that lies within the envelope is thought to function in assembly and budding. A limited number of M2 proteins are integrated into the virions (Zebedee, *J. Virol.* 62:2762-2772, 1988). They form tetramers having H⁺ ion channel activity, and when activated by the low pH in endosomes, acidify the inside of the virion, facilitating its uncoating (Pinto *et al.*, *Cell* 69:517-528, 1992). Amantadine is an anti-influenza drug that prevents viral infection by interfering with M2 ion channel activity, thus inhibiting virus uncoating.

NS1, a nonstructural protein, has multiple functions, including regulation of splicing and nuclear export of cellular mRNAs as well as stimulation of translation.

The major function of NS1 seems to be to counteract the interferon activity of the host, since an NS1 knockout virus was viable although it grew less efficiently than the parent virus in interferon-nondefective cells (Garcia-Sastre, *Virology* 252:324-330, 1998).

5 NS2 has been detected in virus particles (Richardson *et al.*, *Arch. Virol.* 116:69-80, 1991; Yasuda *et al.*, *Virology* 196:249-255, 1993). The average number of NS2 proteins in a virus particle was estimated to be 130-200 molecules. An *in vitro* binding assay shows direct protein-protein contact between M1 and NS2. NS2-M1 complexes have also been detected by immunoprecipitation in virus-
10 infected cell lysates. The NS2 protein is thought to play a role in the export of RNP from the nucleus through interaction with M1 protein (Ward *et al.*, *Arch. Virol.* 140:2067-2073, 1995).

VI. Influenza VLPs and Administration Thereof

15 Influenza VLPs comprising an optimized HA (such as the HA having the amino acid sequence set forth as any one of SEQ ID NOs: 1-11) are provided herein. The influenza VLPs are generally made up of the HA, NA and M1 proteins. The production of influenza VLPs has been described in the art and is within the abilities of one of ordinary skill in the art. For example, influenza VLPs can be produced by
20 transfection of host cells with plasmids encoding the HA, NA and M1 proteins. After incubation of the transfected cells for an appropriate time to allow for protein expression (such as for approximately 72 hours), VLPs can be isolated from cell culture supernatants. Example 5 below provides an exemplary protocol for purifying influenza VLPs from cell supernatants. In this example, VLPs are
25 isolated by low speed centrifugation (to remove cell debris), vacuum filtration and ultracentrifugation through 20% glycerol.

 The influenza VLPs disclosed herein can be used as influenza vaccines to elicit a protective immune response against H3N2 and H2N2 influenza viruses, as well as influenza B viruses.

30 Influenza VLPs, or compositions thereof, can be administered to a subject by any of the routes normally used for introducing recombinant virus into a subject.

Methods of administration include, but are not limited to, intradermal, intramuscular, intraperitoneal, parenteral, intravenous, subcutaneous, vaginal, rectal, intranasal, inhalation or oral. Parenteral administration, such as subcutaneous, intravenous or intramuscular administration, is generally achieved by injection.

- 5 Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Administration can be systemic or local.

- 10 Influenza VLPs, or compositions thereof, are administered in any suitable manner, such as with pharmaceutically acceptable carriers. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of
15 pharmaceutical compositions of the present disclosure.

- Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water,
20 alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be
25 present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

- Some of the compositions may potentially be administered as a pharmaceutically acceptable acid- or base-addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric
30 acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid,

oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

5 Administration can be accomplished by single or multiple doses. The dose administered to a subject in the context of the present disclosure should be sufficient to induce a beneficial therapeutic response in a subject over time, or to inhibit or prevent H2N2, H3N2 and/or B influenza virus infection. The dose required will vary from subject to subject depending on the species, age, weight and general
10 condition of the subject, the severity of the infection being treated, the particular composition being used and its mode of administration. An appropriate dose can be determined by one of ordinary skill in the art using only routine experimentation.

Provided herein are pharmaceutical compositions which include a therapeutically effective amount of the influenza VLPs alone or in combination with
15 a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The carrier and composition can be sterile, and the formulation suits the mode of administration. The composition can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The
20 composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, and
25 magnesium carbonate. Any of the common pharmaceutical carriers, such as sterile saline solution or sesame oil, can be used. The medium can also contain conventional pharmaceutical adjunct materials such as, for example, pharmaceutically acceptable salts to adjust the osmotic pressure, buffers, preservatives and the like. Other media that can be used with the compositions and
30 methods provided herein are normal saline and sesame oil.

The influenza VLPs described herein can be administered alone or in combination with other therapeutic agents to enhance antigenicity. For example, the influenza VLPs can be administered with an adjuvant, such as Freund incomplete adjuvant or Freund's complete adjuvant.

5 Optionally, one or more cytokines, such as IL-2, IL-6, IL-12, RANTES, GM-CSF, TNF- α , or IFN- γ , one or more growth factors, such as GM-CSF or G-CSF; one or more molecules such as OX-40L or 41 BBL, or combinations of these molecules, can be used as biological adjuvants (see, for example, Salgaller *et al.*, 1998, *J. Surg. Oncol.* 68(2):122-38; Lotze *et al.*, 2000, *Cancer J. Sci. Am.* 6(Suppl 1):S61-6; Cao *et al.*, 1998, *Stem Cells* 16(Suppl 1):251-60; Kuiper *et al.*, 2000, *Adv. Exp. Med. Biol.* 465:381-90). These molecules can be administered systemically (or
10 locally) to the host.

A number of means for inducing cellular responses, both *in vitro* and *in vivo*, are known. Lipids have been identified as agents capable of assisting in priming
15 CTL *in vivo* against various antigens. For example, as described in U.S. Patent No. 5,662,907, palmitic acid residues can be attached to the alpha and epsilon amino groups of a lysine residue and then linked (for example, via one or more linking residues, such as glycine, glycine-glycine, serine, serine-serine, or the like) to an immunogenic peptide. The lipidated peptide can then be injected directly in a
20 micellar form, incorporated in a liposome, or emulsified in an adjuvant. As another example, *E. coli* lipoproteins, such as tripalmitoyl-S-glycerylcysteinylserine can be used to prime tumor specific CTL when covalently attached to an appropriate peptide (see, Deres *et al.*, *Nature* 342:561, 1989). Further, as the induction of neutralizing antibodies can also be primed with the same molecule conjugated to a
25 peptide which displays an appropriate epitope, two compositions can be combined to elicit both humoral and cell-mediated responses where that is deemed desirable.

Although administration of VLPs containing a COBRA HA protein is exemplified herein, one of skill in the art would understand that it is also possible to administer the influenza HA protein itself (in the absence of a viral particle) or as a
30 fusion protein to elicit an immune response in a subject. In some embodiments, a fragment of the HA protein is administered such as the HA1 or HA2 sub-fragment.

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the disclosure to the particular features or embodiments described.

5

EXAMPLES

Example 1: Generation of H2N2 Influenza COBRA Sequences

This example describes the generation of four H2N2 influenza HA COBRA sequences using four different methods.

10 To generate the H2N2 influenza HA COBRA sequence according to Method 1, an HA consensus sequence was generated using 59 H2N2 strains isolated from 1957-1968. The COBRA sequence generated according to Method 1 is shown below and is set forth herein as SEQ ID NO: 1:

15 MAIYLLILLFTAVRGDQICIGYHANNSTEKVDITLERNVTVTTHAKDILEKTHN
GKLCKLNGIPPLELGDCSIAGWLLGNPECDRLLRVPEWSYIMEKENPRDGLC
YPGSFNDYEELKHLLSSVKHFEKVKILPKDRWTQHHTTTGGSRAVSGNPSP
FRNMVWLTKKGSNYPVAKGSYNNTSGEQMLIHWGVHHPNDETEQRTLYQN
VGTYVSVSTSTLNKRSTPEIATRPKVNLGSRMEFSWTLTDMWDITINFESTG
20 NLIAPYGFYKISKRGSSGIMKTEGTLGNCETKCQTPLGAINTTLPFHNVHPLTI
GECPKYVKSEKLVLATGLRNVLPQIESRGLFGAIAAGFIEGGWQGMVDGWYG
YHHSNDQSGSYAADKESTQKAFDGITNKVNSVIEKMNTQFEAVGKEFSNLE
RRLENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVR
MQLRDNVKELGNGCFEFYHKCDDECMNSVKNGTYDYPKYEEESKLNREI
KGVKLSSMGVYQILAIYATVAGSLSLAIMMAGISFWMCNGLSLQCRICI
25

In Method 2, two layers of consensus sequences were generated (see FIG. 1). In the first layer, four individual consensus sequences were generated using (1) 21 strains isolated in 1957, (2) 12 strains isolated from 1958-1960, (3) 8 strains isolated from 1961-1964 and (4) 18 strains isolated from 1965-1968. In the second layer, a
30 final consensus sequence was produced using the four individual consensus sequences generated in the first layer. The COBRA sequence generated according to Method 2 is shown below and is set forth herein as SEQ ID NO: 2:

35 MAIYLLILLFTAVRGDQICIGYHANNSTEKVDITLERNVTVTTHAKDILEKTHN
GKLCKLNGIPPLELGDCSIAGWLLGNPECDRLLSVPEWSYIMEKENPRYSLC
YPGSFNDYEELKHLLSSVKHFEKVKILPKDRWTQHHTTTGGSRAVSGNPSP
FRNMVWLTKKGSNYPVAKGSYNNTSGEQMLIHWGVHHPNDETEQRTLYQN

VGTYYVSVSTSTLNKRSTPDIATRPKVNGLGSRMEFSWTLLDMWDTINFESTG
 NLIAPEYGFKISKRGSSGIMKTEGTLGNCETKCQTPLGAINTTLPFHNVHPLTI
 GECPKYVKSEKLVLATGLRNV PQIESRGLFGAIA GFIEGGWQGMVDGWYG
 YHHSNDQSGSYAADKESTQKAFDGITNKNVSVIEKMNTQFEAVGKEFSNLE
 5 KRLLENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVR
 MQLRDNVKELGNGCFEFYHKCDDECMNSVKNGTYDYPKYEEESKLNREI
 KGVKLSSMGVYQILAIYATVAGSLSLAIMMAGISFWMCSNGSLQCRICI

In Method 3, two layers of consensus sequences were generated using 98
 10 human and avian H2N2 isolates (see FIG. 2). In the first layer, six individual
 consensus sequences were generated using (1) 21 strains isolated in 1957, (2) 12
 strains isolated from 1958-1960, (3) 8 strains isolated from 1961-1964, (4) 18 strains
 isolated from 1965-1968, (5) 1 strain isolated in 2005, and (6) 38 avian strains
 isolated from 1961-2007. In the second layer, a final consensus sequence was
 15 produced using the six individual consensus sequences generated in the first layer.
 The COBRA sequence generated according to Method 3 is shown below and is set
 forth herein as SEQ ID NO: 3:

MAIYLLILLFTAVRGDQICIGYHANNSTEKVDITLERNVTVTTHAKDILEKTHN
 GKLCKLNGIPPELGDCSIAGWLLGNPECDRLLSVPEWSYIMEKENPRDGLC
 20 YPGSFNDYEELKHLLSSVKHFEKVKILPKDRWTQH TTTGGSRACAVSGNPSF
 FRNMVWLTKKGSNYPVAKGSYNNTSGEQMLIHWGVHHPNDETEQRTLYQN
 VGTYYVSVGTSTLNKRSTPEIATRPKVNGLGSRMEFSWTLLDMWDTINFEST
 GNLIAPEYGFKISKRGSSGIMKTEGTLGNCETKCQTPLGAINTTLPFHNVHPL
 TIGECPKYVKSEKLVLATGLRNV PQIESRGLFGAIA GFIEGGWQGMVDGWY
 25 GYHHSNDQSGSYAADKESTQKAFDGITNKNVSVIEKMNTQFEAVGKEFSNLE
 ERLLENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKV
 RMQLRDNVKELGNGCFEFYHKCDDECMNSVKNGTYDYPKYEEESKLNREI
 EIKGVKLSSMGVYQILAIYATVAGSLSLAIMMAGISFWMCSNGSLQCRICI

30 In Method 4, a single consensus sequence was generated using 98 human
 and avian H2N2 strains isolated from 1957-2007. The COBRA sequence generated
 according to Method 4 is shown below and is set forth herein as SEQ ID NO: 4:

MAIYLLILLFTAVRGDQICIGYHANNSTEKVDITLERNVTVTTHAKDILEKTHN
 GKLCKLNGIPPELGDCSIAGWLLGNPECDRLLSVPEWSYIMEKENPRDGLC
 35 YPGSFNDYEELKHLLSSVKHFEKVKILPKDRWTQH TTTGGSRACAVSGNPSF
 FRNMVWLTKKGSNYPVAKGSYNNTSGEQMLIHWGVHHPNDEAEQRTLYQN
 VGTYYVSVGTSTLNKRSTPEIATRPKVNGLGSRMEFSWTLLDMWDTINFEST
 GNLIAPEYGFKISKRGSSGIMKTEGTLGNCETKCQTPLGAINTTLPFHNVHPL
 TIGECPKYVKSEKLVLATGLRNV PQIESRGLFGAIA GFIEGGWQGMVDGWY

GYHHSNDQSGYAADKESTQKAFDGITNKVNSVIEKMNTQFEAVGKEFSNL
 ERRLENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKV
 RMQLRDNVKELGNGCFEFYHKCDDECMNSVKNGTYDYPKYEEESKLNRN
 EIKGVKLSSMGVYQILAIYATVAGSLSLAIMMAGISFWMCNGLSLQCRICI

5

Example 2: Generation of Influenza B COBRA Sequences

This example describes the generation of three influenza B HA COBRA sequences using three different methods.

To generate the influenza B HA COBRA sequence according to Method 1,
 10 an HA consensus sequence was generated using 318 influenza B strains isolated from 1940-2011. The COBRA sequence generated according to Method 1 is shown below and is set forth herein as SEQ ID NO: 5:

MKAIIVLLMVVTSNADRICTGITSSNSPHVVKTATQGEVNVTVGVIPLTTTPTK
 SHFANLKGTKTRGKLCPNCLNCTDLDAVALGRPMC VGTTTPSAKASILHEVRP
 15 VTSGCFPIIMHDRTKIRQLPNLLRGYENIRLSTQNVINA EKAPGGPYRLGTSGS
 CPNVTSRSGFFATMAWAVPRDNNKTATNPLTVEVPYICTKGEDQITVWGFH
 SDNKTQMKNLYGDSNPQKFTSSANGVTTHYVSQIGGFNPQTEDGGLPQSGR
 IVVDYMVQKPGKTGTIVYQRGVLLPQKVWCASGRSKVIKGSPLIGEADCL
 HEKYGGLNKS KPYTGEHAKAIGNCPIWVKTP LKLANGTKYRPPAKLLKER
 20 GFFGAIA GFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITK
 NLNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDL RADTISSQIELAVLLS
 NEGIINSEDEHLLALERKLKKMLGPSAVDIGNGCFETKHKCNQTCLDRIAAG
 TFNAGEFSLPTFDSL NITAASLND DGLDNHTILLYYSTAASSLAVTL MIAIFIV
 YMVSRDNVSCSICL

25

In Method 2, two layers of consensus sequences were generated using 318 influenza B isolates (see FIG. 3). In the first layer, ten individual consensus sequences were generated using (1) 15 strains isolated from 1940-1986, (2) 3 strains isolated in 1987, (3) 5 strains isolated from 1988-1989, (4) 17 strains isolated from
 30 1990-1992, (5) 61 strains isolated from 1993-1998, (6) 52 strains isolated from 1999-2000, (7) 39 strains isolated from 2002-2003, (8) 29 strains isolated from 2004-2005; (9) 36 strains isolated from 2006-2007, and (10) 61 strains isolated from 2008-2011. In the second layer, a final consensus sequence was produced using the ten individual consensus sequences generated in the first layer. The COBRA
 35 sequence generated according to Method 2 is shown below and is set forth herein as SEQ ID NO: 6:

MKAIIVLLMVVTSNADRICTGITSSNSPHVVKTATQGEVNVTVGVIPLTTTPTK
 SHFANLKGTKTRGKLCPKCLNCTDLVALGRPKCMGTIPSAKASILHEVRPV
 TSGCFPIMHDRTKIRQLPNLLRGYENIRLSTHNVINAEEKAPGGPYRIGTSGSC
 PNVTNGNGFFATMAWAVPKNDNNKTATNPLTVEVPYICTEGEDQITVWGF
 5 HSDNETQMKKLYGDSKPQKFTSSANGVTTHYVSQIGGFPNQTEDGGLPQSG
 RIVVDYMVQKSGKTGTIVYQRGILLPQKVWCASGRSKVIKGSPLIGEADCL
 HEKYGGLNKSHPYYTGEHAKAIGNCPIWVKTPLKLANGTKYRPPAKLLKER
 GFFGAIAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITK
 NLNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDLRLADTISSQIELAVLLS
 10 NEGIINSEDEHLLALERKLKKMLGPSAVDIGNGCFETKHKCNQTCLDRIAAG
 TFNAGEFSLPTFDSLNTAASLNDDGLDNHTILLYYSTAASSLAVTLMIAIFIV
 YMVSRDNVSCSICL

In Method 3, two layers of consensus sequences were generated using 318
 15 influenza B isolates (see FIG. 4). In the first layer, five individual consensus
 sequences were generated using (1) 52 strains isolated from 1999-2001, (2) 39
 strains isolated from 2002-2003, (3) 29 strains isolated from 2004-2005, (4) 36
 strains isolated from 2006-2007 and (5) 61 strains isolated from 2008-2011. In the
 second layer, a final consensus sequence was produced using the five individual
 20 consensus sequences generated in the first layer. The COBRA sequence generated
 according to Method 3 is shown below and is set forth herein as SEQ ID NO: 7:

MKAIIVLLMVVTSNADRICTGITSSNSPHVVKTATQGEVNVTVGVIPLTTTPTK
 SHFANLKGTKTRGKLCPKCLNCTDLVALGRPKCTGNIPSAKVSIHEVRPV
 TSGCFPIMHDRTKIRQLPNLLRGYEHIRLSTHNVINAEEKAPGGPYKIGTSGSC
 25 PNVTNGNGFFATMAWAVPKNDNNKTATNPLTIEVPYICTEGEDQITVWGFH
 SDNETQMAKLYGDSKPQKFTSSANGVTTHYVSQIGGFPNQTEDGGLPQSGR
 IVVDYMVQKSGKTGTITYQRGILLPQKVWCASGRSKVIKGSPLIGEADCLH
 EKYGGLNKSHPYYTGEHAKAIGNCPIWVKTPLKLANGTKYRPPAKLLKERG
 FFGAIAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKN
 30 LNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDLRLADTISSQIELAVLLSN
 EGIINSEDEHLLALERKLKKMLGPSAVEIGNGCFETKHKCNQTCLDRIAAGT
 FDAGEFSLPTFDSLNTAASLNDDGLDNHTILLYYSTAASSLAVTLMIAIFVV
 YMVSRDNVSCSICL

35 Example 3: Generation of H3N2 Influenza COBRA Sequences

This example describes the generation of four H3N2 influenza HA COBRA
 sequences using four different methods.

To generate the H3N2 influenza HA COBRA sequence according to Method
 1 (decade consensus sequence), two layers of consensus sequences were generated

using 935 H3N2 isolates (see FIG. 5). In the first layer, four individual consensus sequences were generated using (1) 61 strains isolated from 168-1979; (2) 33 strains isolated from 1980-1990; (3) 288 strains isolated from 1991-1999; and (4) 553 strains isolated from 2000-2011. In the second layer, a final consensus sequence was produced using the four individual consensus sequences generated in the first layer. The H3N2 COBRA sequence generated according to Method 1 is shown below and is set forth herein as SEQ ID NO: 8:

MKTHIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTLVKTITNDQIEVT
 NATELVQSSSTGRICDSPHRILDGENCTLIDALLGDPHCDGFGQNKKWDLFVE
 10 RSKAYSNCPYDVPDYASLRSLVASSGTLEFINESFNWTGVTQNGGSSACK
 RGSVNSFFSRLNWLTKLKYKYPALNVTMPNNDKFDKLYIWGVVHHPSTDQD
 QTSLYVQASGRVTVSTKRSQQTVIPNIGSRPWVRGLSSRISYWTIVKPGDILL
 INSTGNLIAPRGYFKIRSGKSSIMRSDAPIGTCNSECTPNGSIPNDKPFQNVNR
 ITYGACPRYVKQNTLKLATGMRNVPEKQTRGIFGAIAGFIENGWEGMVDG
 15 WYGFRHQNSEGTGQAADLKSTQAAIDQINGKLNRLIEKTNEKFHQIEKEFSE
 VEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSMNKLFETR
 KQLRENAEDMGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIK
 GVELKSGYKDWILWISFAISCFLLCVLLGFIMWACQKGNIRCNICI

20 In Method 2 (antigenic era consensus), two layers of consensus sequences were generated using 909 H3N2 isolates (see FIG. 6). In the first layer, 12 individual consensus sequences were generated using (1) 23 strains isolated from 1968-1972; (2) 8 strains isolated from 1973-1974; (3) 21 strains isolated from 1975-1978; (4) 9 strains isolated from 1979-1981; (5) 19 strains isolated from 1982-1985; (6) 14 strains isolated from 1986-1988; (7) 13 strains isolated from 1989-1992; (8) 275 strains isolated from 1993-1998; (9) 294 strains isolated from 1999-2003; (10) 57 strains isolated in 2004; (11) 80 strains isolated from 2005-2006; and (12) 96 strains isolated from 2007-2008. In the second layer, a final consensus sequence was produced using the 12 individual consensus sequences generated in the first layer. The H3N2 COBRA sequence generated according to Method 2 is shown below and is set forth herein as SEQ ID NO: 9:

MKTHIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTLVKTITNDQIEVT
 NATELVQSSSTGRICDSPHRILDGKNCTLIDALLGDPHCDGFGQNKKWDLFVE
 35 RSKAFSNCPYDVPDYASLRSLVASSGTLEFINESFNWTGVTQNGGSYACK
 RGSVNSFFSRLNWLHKSESKYPALNVTMPNNDKFDKLYIWGVVHHPSTDQE
 QTSLYVQASGRVTVSTKRSQQTVIPNIGSRPWVRGLSSRISYWTIVKPGDILL

INSTGNLIAPRGYFKIRTGKSSIMRSDAPIGTCSSSECITPNGSIPNDKPFQNVNK
 ITYGACPRYVKQNTLKLATGMRNVPEKQTRGIFGAIAGFIENGWEGMVDG
 WYGFRHQNSEGTGQAADLKSTQAAIDQINGKLNRLIEKTNEKFHQIEKEFSE
 VEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSMNKLFKTR
 5 KQLRENAEDMGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIK
 GVELKSGYKDWILWISFAISCFLLCVLLGFIMWACQKGNIRCNICI

In Method 3 (1968-2011), two layers of consensus sequences were generated using 935 H3N2 isolates (see FIG. 7). In the first layer, 13 individual consensus sequences were generated using (1) 23 strains isolated from 1968-1972; (2) 8 strains isolated from 1973-1974; (3) 21 strains isolated from 1975-1978; (4) 9 strains isolated from 1979-1981; (5) 19 strains isolated from 1982-1985; (6) 14 strains isolated from 1986-1988; (7) 13 strains isolated from 1989-1992; (8) 275 strains isolated from 1993-1998; (9) 294 strains isolated from 1999-2003; (10) 57 strains isolated in 2004; (11) 80 strains isolated from 2005-2006; (12) 96 strains isolated from 2007-2008; and (13) 26 strains isolated from 2009-2011. In the second layer, a final consensus sequence was produced using the 13 individual consensus sequences generated in the first layer. The H3N2 COBRA sequence generated according to Method 3 is shown below and is set forth herein as SEQ ID NO: 10:

20 MKTIIALS YILCLVFAQKLPGNDNSTATLCLGHHAVPNGTLVKTITNDQIEVT
 NATELVQSSSTGRICDSPHRILDGKNCTLIDALLGDPHCDGFGQNKKWDLFVE
 RSKAYSNCPYDVPDYASLRSLVASSGTLEFINESFNWTGVTQNGGSYACK
 RGSNNSFFSRLNWLTKSESKYPALNVTMPNNEKFDKLYIWGVHHPSTDKEQ
 TSLYVQASGRVTVSTKRSQQTVIPNIGSRPWVRGLSSRSIYWTIVKPGDILLI
 25 NSTGNLIAPRGYFKIRTGKSSIMRSDAPIGTCNSECITPNGSIPNDKPFQNVNRI
 TYGACPRYVKQNTLKLATGMRNVPEKQTRGIFGAIAGFIENGWEGMVDGW
 YGFRHQNSEGTGQAADLKSTQAAIDQINGKLNRLIEKTNEKFHQIEKEFSEV
 EGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSMNKLFKTRK
 QLRENAEDMGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIKG
 30 VELKSGYKDWILWISFAISCFLLCVLLGFIMWACQKGNIRCNICI

In Method 4 (1968-2004), two layers of consensus sequences were generated using 733 H3N2 isolates (see FIG. 8). In the first layer, 10 individual consensus sequences were generated using (1) 23 strains isolated from 1968-1972; (2) 8 strains isolated from 1973-1974; (3) 21 strains isolated from 1975-1978; (4) 9 strains isolated from 1979-1981; (5) 19 strains isolated from 1982-1985; (6) 14 strains isolated from 1986-1988; (7) 13 strains isolated from 1989-1992; (8) 275 strains

isolated from 1993-1998; (9) 294 strains isolated from 1999-2003; and (10) 57 strains isolated in 2004. In the second layer, a final consensus sequence was produced using the 10 individual consensus sequences generated in the first layer. The H3N2 COBRA sequence generated according to Method 4 is shown below and is set forth herein as SEQ ID NO: 11:

MKTIIALSYIFCLVFAQKLPGNDNSTATLCLGHHAVPNGTLVKTTITNDQIEVT
 NATELVQSSSTGRICDSPHRILDGKNCTLIDALLGDPHCDGFGQNEKWDLFVE
 RSKAFSNCYPYDVPDYASLRSLVASSGTLEFINEGFNWTGVTQNGGSYACK
 RGSVNSFFSRLNWLHKSESKYPVLNVTMPNNDKFDKLYIWGVHHPSTDKE
 10 QTSLYVQASGRVTVSTKRSQQTVIPNIGSRPWVRGLSSRISYWTIVKPGDILL
 INSTGNLIAPRGYFKIRTGKSSIMRSDAPIGTCSSECITPNGSIPNDKPFQNVNK
 ITYGACPRYVKQNTLKLATGMRNVPEKQTRGIFGAIAGFIENGWEGMVDG
 WYGFRHQNSEGTGQAADLKSTQAAIDQINGKLNRLIEKTNEKFHQIEKEFSE
 VEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTIDSEMKNLFEKTR
 15 KQLRENAEDMGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIK
 GVELKSGYKDWILWISFAISCFLLCVLLGFIMWACQKGNIRCNICI

Example 4: Codon-optimized COBRA gene sequences

The COBRA amino acid sequences disclosed herein can be reverse translated and optimized for expression in mammalian cells, including codon usage and RNA optimization (GeneArt; Regensburg, Germany). The optimized nucleic acid sequences can be inserted into an appropriate expression vector, such as the pTR600 expression vector (U.S. Patent Application Publication No. 2002/0106798; Ross *et al.*, *Nat Immunol.* 1(2):102-103, 2000; Green *et al.*, *Vaccine* 20:242-248, 2001). Expression vectors encoding the codon-optimized COBRA gene sequences can be used, for example, to generate VLPs containing the COBRA HA.

Example 5: Preparation of and immunization with influenza VLPs

The following methods can be used to produce and characterize influenza VLPs comprising a COBRA HA. Exemplary methods for immunization of mice, ferrets and macaques are also described below (see also, Giles and Ross, *Vaccine* 29(16):3043-3054, 2011).

Vaccine Preparation

293T cells are transiently transfected with plasmids expressing M1, NA and an optimized HA, and incubated for 72 hours at 37°C. The M1, NA and HA coding sequences can be codon-optimized for expression in mammalian cells. Supernatants are collected and cell debris is removed by low speed centrifugation followed by vacuum filtration through a 0.22 µm sterile filter. VLPs are purified via ultracentrifugation (100,000 x g through 20% glycerol, weight per volume) for 4 hours at 4°C. The pellets are subsequently resuspended in PBS pH 7.2 and stored in single use aliquots at -80°C until use. Total protein concentration is determined by Micro BCA™ Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA).

Dose determination

HA specific content can be determined by western blot and densitometry. Purified recombinant COBRA HA and purified VLPs are prepared in standard total protein amounts and are electrophoresed on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The blot is probed with mouse polyclonal antisera from influenza infected mice and the HA-antibody complexes are detected using a goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Southern Biotech; Birmingham, AL, USA). HRP is detected by chemiluminescent substrate (Pierce Biotechnology; Rockford IL, USA) and exposed to X-ray film (ThermoFisher; Pittsburgh, PA, USA). Density of bands is determined using ImageJ software (NIH). Density of recombinant HA bands is used to calculate a standard curve and the density of the purified VLPs is interpolated using the results from the recombinant HA.

Mouse studies

BALB/c mice (*Mus musculus*, females, 6-8 weeks old) can be purchased from Harlan Sprague Dawley (Indianapolis, IN, USA). Mice are housed in microisolator units and allowed free access to food and water and are cared for under USDA guidelines for laboratory animals. Mice are vaccinated with one of

three doses of purified COBRA HA VLPs (1.5 µg, 0.3 µg or 0.06 µg), based upon HA content from a densitometry assay, via intramuscular injection at week 0 and then boosted with the same dose at week 3. Vaccines at each dose are formulated with alum adjuvant (Imject Alum, Pierce Biotechnology; Rockford, IL, USA), CpG oligonucleotides, or vehicle alone. Fourteen to twenty-one days after each vaccination, blood is collected from anesthetized mice via the retro-orbital plexus and transferred to a microfuge tube. Tubes are centrifuged and sera is removed and frozen at $-80 \pm 5^{\circ}\text{C}$. Hemagglutination inhibition (HAI) serum antibody titer for each vaccine group is determined at week 5 using representative reassortant viruses or COBRA HA VLPs.

Three weeks after final vaccination, mice are challenged intranasally with a pathogenic influenza virus (such as a pathogenic H2N2, H3N2 or B influenza virus isolate) in a volume of 50µl. After infection, mice are monitored daily for weight loss, disease signs and death for 14 days after infection. Individual body weights, sickness scores (Toapanta and Ross, *Respiratory Research* 10(1):112, 2009) and death are recorded for each group on each day after inoculation.

Ferret studies

Fitch ferrets (*Mustela putorius furo*, female, 6-12-months of age), influenza naïve and de-scented, can be purchased from Marshall Farms (Sayre, PA, USA). Ferrets are pair housed in stainless steel cages (Shor-line, Kansas City, KS, USA) containing Sani-chips Laboratory Animal Bedding (P.J. Murphy Forest Products, Montville, NJ, USA). Ferrets are provided with Teklad Global Ferret Diet (Harlan Teklad, Madison, WI, USA) and fresh water *ad libitum*. The COBRA HA VLPs are diluted in PBS, pH 7.2 to achieve final concentration. Ferrets are vaccinated with one of two doses of purified COBRA VLPs (15 µg, 3 µg), based upon HA content as determined by densitometry assay, via intramuscular injection in the quadriceps muscle in a volume of 0.25 ml at week 0 and then boosted with the same dose at week 3. Vaccines are stored at -80°C prior to use and formulated with alum adjuvant (Imject Alum; Pierce Biotechnology, Rockford, IL, USA) immediately prior to use. Animals are monitored for adverse events including weight loss,

temperature, decrease in activity, nasal discharge, sneezing and diarrhea weekly during the vaccination regimen. Prior to vaccination, animals are confirmed by HAI assay to be seronegative for circulating influenza A and influenza B viruses.

Fourteen to twenty-one days after each vaccination, blood is collected from
5 anesthetized ferrets via the anterior vena cava and transferred to a microfuge tube. Tubes are centrifuged and sera is removed and frozen at $-80 \pm 5^{\circ}\text{C}$. HAI serum antibody titer for each vaccine group is determined at week 5 using representative reassortant viruses or COBRA HA VLPs.

Three weeks after final vaccination, ferrets are challenged intranasally with a
10 pathogenic influenza virus (such as a pathogenic H2N2, H3N2 or B influenza virus isolate) in a volume of 1 ml. After infection, ferrets are monitored daily for weight loss, disease signs and death for 14 days after infection. Individual body weights, sickness scores, and death are recorded for each group on each day after inoculation. Nasal washes are performed by instilling 3 ml of PBS into the nares of anesthetized
15 ferrets each day for 7 days after inoculation. Washes are collected and stored at -80°C until use.

Primate immunizations

Cynomolgus macaques (*Macaca fascicularis*, male, 3-5 years old) can be
20 purchased from Harlan Sprague Dawley (Indianapolis, IN, USA). Macaques are vaccinated with purified COBRA HA VLPs (15 μg), based upon HA content from a densitometry assay, via intramuscular injection at week 0 and then boosted with the same dose at weeks 3 and 6. Vaccines are formulated with alum adjuvant (Imject Alum, Pierce Biotechnology; Rockford, IL, USA) immediately prior to use.
25 Twenty-one days after each vaccination, blood is collected from anesthetized macaques via the femoral vein and transferred to a serum separator tube. Tubes are allowed to activate clotting followed by centrifugation and sera is removed and frozen at $-80 \pm 5^{\circ}\text{C}$. End point IgG titers and HAI serum antibody titer for each vaccine group is determined at week 5 using representative reassortant viruses or
30 COBRA HA VLPs.

Three weeks after final vaccination, macaques are challenged by intranasal, intratracheal, and orbital inoculation with a pathogenic influenza virus (such as a pathogenic H2N2, H3N2 or B influenza virus isolate) in a volume of 1 ml. After infection, macaques are monitored daily for weight loss, disease signs and death for
5 5 days after infection. Individual body weights, sickness scores and death are recorded for each group on each day after inoculation.

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated
10 embodiments are only preferred examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

CLAIMS

1. A recombinant influenza hemagglutinin (HA) polypeptide, comprising:
 - (i) an amino acid sequence at least 97.7% identical to residues 2-566 of SEQ ID NO: 8;
(
 - (ii) an amino acid sequence at least 98.4% identical to residues 2-566 of SEQ ID NO: 9;
 - (iii) an amino acid sequence at least 97.8% identical to residues 2-566 of SEQ ID NO: 10; or
 - (iv) an amino acid sequence at least 98.9% identical to residues 2-566 of SEQ ID NO: 11.

2. The influenza HA polypeptide of claim 1, comprising:
 - (i) an amino acid sequence at least 97.7% identical to SEQ ID NO: 8;

- (ii) an amino acid sequence at least 98.4% identical to SEQ ID NO: 9;
- (iii) an amino acid sequence at least 97.8% identical to SEQ ID NO: 10; or
- (iv) an amino acid sequence at least 98.9% identical to SEQ ID NO: 11.

3. The influenza HA polypeptide of claim 1 or claim 2, wherein the amino acid sequence of the polypeptide comprises:

- (i) no more than 10 amino acid substitutions relative to SEQ ID NO: 8;
- (ii) no more than 9 amino acid substitutions relative to SEQ ID NO: 9;
- (iii) no more than 10 amino acid substitutions relative to SEQ ID NO: 10; or
- (iv) no more than 6 amino acid substitutions relative to SEQ ID NO: 11.

4. The influenza HA polypeptide of claim 1, comprising the amino acid sequence of residues 2-566 of SEQ ID NO: 8, residues 2-566 of SEQ ID NO: 9, residues 2-566 of SEQ ID NO: 10 or residues 2-566 of SEQ ID NO: 11.

5. The influenza HA polypeptide of claim 1, consisting of the amino acid sequence of residues 2-566 of SEQ ID NO: 8,

residues 2-566 of SEQ ID NO: 9, residues 2-566 of SEQ ID NO: 10 or residues 2-566 of SEQ ID NO: 11.

6. The influenza HA polypeptide of claim 2, comprising the amino acid sequence of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10 or SEQ ID NO: 11.

7. The influenza HA polypeptide of claim 2, consisting of the amino acid sequence of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10 or SEQ ID NO: 11.

8. An isolated nucleic acid molecule encoding the influenza HA polypeptide of any one of claims 1-7.

9. The isolated nucleic acid molecule of claim 8, wherein the nucleic acid molecule is codon-optimized for expression in mammalian cells.

10. A vector comprising the nucleic acid molecule of claim 8 or claim 9.

11. The vector of claim 10, further comprising a promoter operably linked to the nucleic acid sequence encoding the influenza HA polypeptide.

12. An isolated cell comprising the vector of claim 10 or claim 11.

13. An influenza virus-like particle (VLP) comprising the influenza HA polypeptide of any one of claims 1-7.

14. The influenza VLP of claim 13, further comprising an influenza neuraminidase (NA) protein, an influenza matrix (M1) protein, or both.

15. An influenza VLP comprising the influenza HA polypeptide of any one of claims 1-7, produced by transfecting a host cell with a vector encoding the HA polypeptide, a vector encoding an influenza NA protein and a vector encoding an influenza M1 protein under conditions sufficient to allow for expression of the HA, M1 and NA proteins.

16. A fusion protein comprising the influenza HA polypeptide of any one of claims 1-7.

17. A composition comprising the influenza HA polypeptide of any one of claims 1-7, the VLP of any one of claims 13-15 or the fusion protein of claim 16, and a pharmaceutically acceptable carrier.

18. Use of the influenza HA polypeptide of any one of claims 1-7, the VLP of any one of claims 13-15, the fusion protein of claim 16 or the composition of claim 17 in the manufacture of a medicament for eliciting an immune response to influenza virus in a subject.

19. Use of a composition comprising the VLP of any one of claims 13-15 and pharmaceutically acceptable carrier in the manufacture of a medicament for immunizing a subject against influenza virus.

20. The use of claim 19, wherein the composition further comprises an adjuvant.

21. The use of claim 19 or claim 20, wherein the composition is to be administered intramuscularly.

22. The use of any one of claims 19-21, wherein the composition comprises about 1 to about 25 µg of the VLP.

23. The use of claim 22, wherein the composition comprises about 15 µg of the VLP.