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(54) Title: IMMUNOGENIC COMPOSITIONS COMPRISING POLYNUCLEOTIDES ENCODING MATRIX PROTEIN 2 AND METHODS OF USE

(57) Abstract: The disclosure encompasses M2 polypeptides, polynucleotides, variants, and immunogenic fragments thereof. M2 polypeptides and/or polynucleotides are useful in immunogenic compositions. The immunogenic compositions of the disclosure can be administered alone or in combination with other agents useful in the control of influenza infection.



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**IMMUNOGENIC COMPOSITIONS COMPRISING POLYNUCLEOTIDES
ENCODING MATRIX PROTEIN 2 AND METHODS OF USE**

This application is being filed on 27 March 2007, as a PCT International Patent
5 application in the name of The Government of the United States as represented by the
Secretary, Department of Health and Human Services, applicant for the designation of all
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Julia Ann Misplon, and Chia-Yun Lo, all citizens of the U.S., applicants for the designation
of the US only, and claims priority to U.S. Provisional Patent Application No. 60/786,152,
10 filed March 27, 2006.

**Statement of Rights to Disclosure Made Under
Federally Sponsored Research and Development**

15 The work performed during the development of this disclosure utilized support from
the Food and Drug Administration and the National Vaccine Program Office. The United
States government has certain rights in the disclosure.

Background of the Disclosure

20 Influenza virus infection is a major public health problem. Birds, for example, can be
infected by influenza A viruses of 16 hemagglutinin (HA) and 9 neuraminidase (NA)
subtypes. (Luke and Subbarao, 2006, Emerg. Infect. Dis., 12:66-72). Infected birds can
serve as a reservoir for influenza viruses, from which novel influenza subtypes can be
introduced into human populations and cause pandemics. The outbreaks of avian influenza
25 viruses in humans (H7N7 in The Netherlands in 2003, H5N1 in Hong Kong in 1997 and
more widely in Southeast Asia since 2003, and H9N2 in China in 1999 and in Hong Kong in
2003) have raised concerns that these subtypes, and others, may have the potential to cause
pandemics.

Although inactivated virus vaccines have been reported to be 60-80% effective
30 against matched influenza strains, vaccination coverage is a problem worldwide.
Vaccination based on eliciting neutralizing antibodies that are specific to subtype and strain
requires accurate prediction of the viral strains that will circulate during the influenza season
and leaves little time for vaccine preparation. This strategy also does not provide protection
against unexpected strains, outbreaks such as the H5N1 avian influenza outbreak in Hong
35 Kong in 1997 and the current outbreak in Southeast Asia, or pandemics. A rapidly
developing pandemic would shorten the time frame to identify the viral strain and prepare
an antigenically matched vaccine, with antigenic changes continuing meanwhile. Moreover,
the need to vaccinate an entirely naïve population would exacerbate vaccine production and

supply issues. Even with the strains that commonly cause infection, difficulties and delays in the production of an adequate vaccine supply have occurred in some years.

Vaccines using conserved components of influenza A virus can induce protection against many influenza A strains. Animal studies have shown partial heterosubtype specific immunity: exposure to influenza A virus of one subtype can partially protect against challenge infection with influenza A of a different subtype (Schulman et al., J. Bacteriol., 89:170-174 (1965)). The mechanisms of heterosubtypic immunity are not completely understood, but are believed to include T cell immunity, in particular CD8+ cytotoxic T cells (CTL) and CD4+ T cells, as well as antibodies to conserved epitopes (reviewed in Epstein, Expert Rev. Anti-Infect. Therapy, 1(4):627-638 (2003)). Heterosubtypic immunity has occasionally been reported in humans, but its effectiveness and duration are unknown.

Vaccines based on heterosubtypic protection would not require knowing the identity of strains that would circulate during the coming influenza season and could avoid hurried manufacturing in response to outbreaks. It has been reported that immunization with DNA constructs expressing conserved influenza A nucleoprotein (NP) or NP and matrix (M) can induce antibody and T cell responses and protect against H3N2 heterosubtypic challenge (Ulmer et al, Science, 1993, 259:1745-1749; Epstein et al, 2000, Intl. Immunol., 12: 91-101). H5N1 viruses from the 1997 human outbreak in Hong Kong represent a demanding test for an influenza vaccine because of their virulence and rapid kinetics of infection. Limited protection against an H5N1 strain was achieved by DNA vaccination with NP and M against some challenges (Epstein et al, 2002, Emer. Infect. Dis., 8:796-801). The M2 antigen has been explored as a vaccine candidate using peptide-carrier conjugates, protein vaccines, and expression by DNA and other vaccine constructs. However, none of the previously presented work has documented protection against H5N1 influenza challenge.

A great deal of effort is being directed to the design and testing of influenza compositions that offer broad protection against a number of influenza subtypes, including H5N1. Effective vaccines for influenza are still needed.

Summary

In one aspect, the disclosure includes an immunogenic composition comprising at least one expression vector comprising at least one polynucleotide encoding an M2 polypeptide comprising the amino acid sequence of

MSLLTEVETX₁₀X₁₁X₁₂NX₁₄WX₁₆CRCX₂₀X₂₁SSD,

wherein X₁₀, X₁₁, X₁₂, X₁₄, X₁₆, X₂₀, and X₂₁ can be any amino acid, preferably a naturally occurring amino acid. In some embodiments, X₁₀ is P or L; X₁₁ is E or G; X₁₂ is R or K; X₁₄ is E or G; X₁₆ is G or E; X₂₀ is N, S or R; and X₂₁ is G or D in combination with a carrier. In some embodiments, X₁₀ is a proline.

In some embodiments, the expression vector is a plasmid vector or a viral vector. In some embodiments, an immunogenic composition comprises a plasmid vector comprising a polynucleotide encoding an M2 polypeptide, and another immunogenic composition comprises a viral vector comprising a polynucleotide encoding an M2 polypeptide, wherein
5 the immunogenic compositions encode the same M2 polypeptide or different M2 polypeptides. In other embodiments, the immunogenic composition can comprise an M2 polypeptide composition. The M2 polypeptide composition may be used in combination with one or more of the immunogenic compositions comprising a plasmid or viral vector. The immunogenic compositions can encode the same M2 polypeptide or different M2
10 polypeptides and the M2 polypeptide composition can include the same or different M2 polypeptides encoded by the immunogenic compositions.

In some embodiments, the polypeptide can be a naturally occurring M2 polypeptide or a fragment of M2 polypeptide such as an extracellular domain or immunogenic fragment. In other embodiments, the polypeptide is an M2 consensus sequence. In some
15 embodiments, the polynucleotide does not encode at least one other influenza protein.

One aspect of the invention comprises an immunogenic composition comprising expression vector, comprising at least one polynucleotide encoding an M2 polypeptide having at least 80% amino acid sequence identity to a polypeptide comprising the amino acid sequence of SEQ ID NO:10 and/or that of SEQ ID NO:1, wherein said polynucleotide
20 upon uptake of the vector by a suitable host cell is expressed by the cell, in combination with a carrier. Another embodiment comprises an immunogenic composition comprising a viral or plasmid expression vector, comprising at least one polynucleotide encoding an M2 polypeptide having at least 80% amino acid sequence identity to a polypeptide comprising the amino acid sequence of SEQ ID NO:1 and/or SEQ ID NO:10, wherein said
25 polynucleotide upon uptake of the vector by a suitable host cell is expressed by the cell, in combination with a carrier.

In some embodiments of the immunogenic composition, the M2 polypeptide has at least 80% sequence identity to the amino acid sequence of SEQ ID NO:10 and 90% sequence identity to the amino acid sequence of SEQ ID NO:1. In some embodiments the
30 M2 polypeptide does not include the amino acid sequence of SEQ ID NO:10. In some cases, the M2 polypeptide is from an A/H1N1 isolate or strain or A/H5N1 isolate or strain. In a specific embodiment, M2 polypeptide comprises the amino acid sequence of SEQ ID NO:1.

In some embodiments, the immunogenic composition can comprise a plurality of polynucleotides or a single polynucleotide encoding at least two M2 polypeptides, wherein
35 each polynucleotide encodes an M2 polypeptide from a different influenza A subtype. In some embodiments, the M2 polypeptide is an immunogenic fragment, preferably including at least one T cell epitope. In some embodiments, the immunogenic composition may

include an M2 polypeptide or peptide from one or more subtypes. A variety of immunogenic compositions may be used in an immunization for example, including immunizing with polynucleotides and boosting with polypeptides or peptides or immunizing with polypeptides and boosting with polynucleotides. In other embodiments, the
5 immunogenic compositions of the disclosure can be combined with other subunit or heat killed vaccines or polynucleotides or polypeptides encoding other influenza proteins.

In some embodiments, the immunogenic composition as described herein can comprise at least one adjuvant or immunomodulator or may be combined with a carrier, liposomes, nanofibers or with other particles for delivery. Such adjuvants may include
10 ganglioside receptor-binding toxins (cholera toxin, LT enterotoxin, their B subunits and mutants); surface immunoglobulin binding complex CTA1-DD; TLR4 binding lipopolysaccharide; TLR2-binding muramyl dipeptide; mannose receptor-binding mannan; dectin-1-binding ss 1,3/1,6 glucans; TLR9-binding CpG-oligodeoxynucleotides; cytokines and chemokines; antigen-presenting cell targeting ISCOMATRIX and ISCOM. In addition,
15 adjuvants able to prime the mucosal immune system following a systemic immunization, include 25(OH)2D3, cholera toxin, CTA1-DD alone or in combination with ISCOM. In some embodiments, the adjuvant may be encoded or expressed by the expression vector used herein.

Another aspect of the disclosure provides a method or use for inhibiting influenza A
20 infection in a subject, comprising administering to the subject an immunogenic composition comprising an expression vector comprising at least one polynucleotide encoding an M2 polypeptide having at least 80% amino acid sequence identity to the polypeptide comprising the amino acid sequence of SEQ ID NO:1 and/or SEQ ID NO:10, and boosting the subject with a second expression vector comprising at least one polynucleotide encoding an M2
25 polypeptide having at least 80% amino acid sequence identity to the polypeptide comprising the amino acid sequence of SEQ ID NO:1 and/or SEQ ID NO:10. In some embodiments, the immunogenic composition is a plasmid vector and the second expression vector is a viral vector.

In some embodiments of the methods as described herein, the plasmid expression
30 vector and the viral vector each comprise a polynucleotide encoding M2 polypeptide from A/H1N1 or A/H5N1. In other embodiments, the plasmid expression vector and the viral vector each comprise a plurality of polynucleotides encoding at least two M2 polypeptides, wherein each polynucleotide encodes an M2 polypeptide from a different influenza A subtype. In some embodiments, the M2 polypeptide encoded by the plasmid expression
35 vector and the viral vector have the same amino acid sequence, such as that of SEQ ID NO:1. In some embodiments, the M2 polypeptide is an immunogenic fragment, preferably including at least one T cell epitope. In some embodiments, the T cell epitope comprises

amino acids 2 to 24 of the M2 protein. In other embodiments, the immunogenic compositions of the disclosure can be combined with other subunit or heat killed vaccines or polynucleotides or polypeptides encoding other influenza proteins.

In other embodiments, the methods comprise administering an M2 polypeptide or immunogenic fragment thereof or a plurality of polypeptides comprising at least two M2 polypeptides, wherein each M2 polypeptide has the sequence of a different influenza A subtype. In some embodiments, the M2 polypeptide comprises the amino acid sequence of SEQ ID NO:1. In some embodiments, the M2 polypeptide is an immunogenic fragment, preferably including at least one T cell epitope. In some embodiments, the T cell epitope comprises amino acids 2 to 24 of an M2 protein. In other embodiments, the immunogenic compositions of the disclosure can be combined with other subunit or heat killed vaccines or polynucleotides or polypeptides encoding other influenza proteins.

The methods as described herein provide protective immunity against a homologous, heterosubtypic, or mismatched influenza virus isolate or strain in a variety of animals. The animals include birds or mammals such as pigs, mice, monkeys or humans. For example, when the M2 polypeptide of the immunogenic composition and the viral vector has the same sequence as M2 protein from a H1N1 isolate or strain, protection against the heterosubtypic influenza H5N1 is provided.

The disclosure also includes kits useful in the methods of the disclosure. In some embodiments, the disclosure includes a kit comprising

- (a) an immunogenic composition comprising a plasmid expression vector, comprising at least one polynucleotide encoding an M2 polypeptide having at least 80% amino acid sequence identity to a polypeptide comprising the amino acid sequence of SEQ ID NO:1 or SEQ IDNO:10;
- (b) a viral vector comprising at least one polynucleotide encoding an M2 polypeptide having at least 80% amino acid sequence identity to a polypeptide comprising the amino acid sequence of SEQ ID NO:1 or SEQ IDNO:10; and optionally
- (c) instructions for administering to a human or animal the immunogenic composition and boosting the human or animal with the viral vector. In another embodiment, the plasmid expression vector and the viral vector each comprise a polynucleotide encoding M2 polypeptide from A/H1N1 or A/H5N1.

The kit may further comprise one or more M2 polypeptides or immunogenic fragments thereof. In other embodiments, the kit may comprise one or more adjuvants or cytokines. Such adjuvants may include ganglioside receptor-binding toxins (cholera toxin, LT enterotoxin, their B subunits and mutants); surface immunoglobulin binding complex CTA1-DD; TLR4 binding lipopolysaccharide; TLR2-binding muramyl dipeptide; mannose receptor-binding mannan; dectin-1-binding ss 1,3/1,6 glucans; TLR9-binding CpG-

oligodeoxynucleotides; cytokines and chemokines; antigen-presenting cell targeting ISCOMATRIX and ISCOM. In addition, adjuvants able to prime the mucosal immune system following a systemic immunization, include 25(OH)2D3, cholera toxin, CTA1-DD alone or in combination with ISCOM. In some embodiments, the adjuvant may be encoded
 5 or expressed by the expression vector used herein.

Brief Description of the Figures

Figures 1A-1C show testing of sera from mice immunized with KLH (open squares) or KLH conjugated to the extracellular portion of an M2 consensus sequence (M2e-con; filled squares), extracellular portion of an M2 from A/FM-MA (M2e-FM; diamonds), or
 10 extracellular portion of an M2 from A/HK/156 (M2e-H5; triangles) on ELISA plates coated with synthetic M2e-PR8 (Fig. 1A), M2e-FM (Fig. 1B), or M2e-H5 (Fig. 1C) peptides. See Table 3 for sequences.

Figures 2A and 2B show the morbidity of mice immunized with KLH (open squares) or the three KLH-M2e conjugates M2e-con (filled squares), M2e-FM (diamonds), or M2e-H5 (triangles) and challenged with a lethal dose of A/PR/8 (Fig. 2A) or A/FM-MA (Fig. 2B).

Figures 3A and 3B show the mortality of mice immunized with KLH (open squares) or the three KLH-M2e conjugates M2e-con (filled squares), M2e-FM (diamonds), or M2e-H5 (triangles) and challenged with a lethal dose of A/PR/8 (10 LD₅₀; Fig. 3A) or A/FM-MA (10 LD₅₀; Fig. 3B).

Figure 4 shows the mortality of mice immunized three times at 2 week intervals with a plasmid DNA expression vector encoding full-length M2 polypeptide similar to A/PR/8 with a change from glycine to aspartic acid at residue 21 (SEQ ID NO:39; M2 DNA; filled squares), the entire M gene from A/PR/8 encoding both M1 and M2 (A/M-PR/8 DNA; triangles), or B/NP control DNA (open squares) and challenged with a lethal dose of A/PR/8 (7 LD₅₀).

Figures 5A and 5B show the mortality of mice immunized three times at 2 week intervals with M2 DNA (filled squares), A/M-A/PR/8 DNA (diamonds), or B/NP control DNA (open squares) challenged with a lethal dose (7 LD₅₀) of heterosubtypic virus (A/Phil, Fig. 5A) or mismatched H1N1 virus (A/FM-MA, Fig. 5B).

Figure 6 shows the mortality of mice immunized three times at 2 week intervals with A/M1-A/PR/8 DNA (triangles), M2 DNA (filled squares), A/M-A/PR/8 DNA (diamonds), A/M1-A/PR/8 DNA + M2 DNA (asterisks), or B/NP DNA (open squares)
 35 challenged with a lethal dose of A/PR/8 (7 LD₅₀).

Figures 7A-7C show testing of sera from mice prime-boost immunized with M2 DNA and M2 Adenovirus (M2-Ad) (circles), B/NP DNA and B/NP-Ad (diamonds), B/NP

DNA and M2 Ad (filled squares), or M2 DNA and B/NP-Ad (triangles) on ELISA plates coated with synthetic M2e-A/PR/8 (Fig. 7A), M2e-FM (Fig. 7B), or M2e-H5 (Fig. 7C) peptides. The mice were primed 3 times with DNA at 2 week intervals and boosted with the adenovirus vector 2 weeks after the last DNA prime. The adenoviral vector encoded full-length M2 polypeptide with a sequence similar to A/PR/8 including a change from glycine to aspartic acid at residue 21 (SEQ Id NO:39; M2 Ad).

Figure 8 Role of T and B cell immunity in M2-specific protective immunity. (a) Mice (n=9 per group) were immunized with M2-DNA or B/NP-DNA and boosted with matched Ad as described in Methods. Three weeks after Ad boost, M2-DNA groups were acutely depleted with mAbs to CD4⁺ or CD8⁺ or both, or given control mAb SFR3-DR5 as described in Methods. Mice were challenged with 1.5×10^4 LD₅₀ of A/PR/8. Compared to the SFR control, survival differed significantly for mice depleted of both T cell subsets (p<0.001, log-rank) although leaving some protection significantly differed from the B/NP control (p<0.001, log-rank). (b) Mice (n=10 per group) were immunized as in (a). 5 months after Ad boost, spleen cells were isolated from immune mice, fractionated into T cell and non-T cell populations and assayed for IFN γ producing cells by ELISPOT assay as described in Methods. (c and d) Serum collected from immune mice was passively transferred into naïve BALB/c mice i.p. (n=8 per group). The recipients were challenged with 10 LD₅₀ of A/PR/8 and monitored for survival (c) and weight loss (d). Survival of mice given A/PR/8 immune serum, M2-DNA+M2-Ad-immune serum or M2e-H5(HK)/KLH-immune serum was significantly better than mice given with B/NP-DNA+B/NP-Ad-immune serum (p<0.001, log rank). For weight loss, p<0.003 at day 8 and day 10, M2 prime-boost differs from B/NP prime-boost.

Figures 9A and 9B show the mortality of mice prime-boost immunized with B/NP-DNA+B/NP-Ad (circles) or M2 DNA+M2 Ad (triangles), and challenged with a lethal dose of mismatched H1N1 virus A/FM-MA, 10 LD₅₀ (Fig 9A) or A/PR/8 virus (7 LD₅₀; Fig 9B). The mice were primed once with DNA and boosted with the adenovirus vector 2 weeks later.

Figures 10A and 10B show the morbidity of mice prime-boost immunized with A/NP-PR/8 DNA and A/NP-PR/8 Ad (diamonds), M2 DNA and M2 Ad (squares), or B/NP-DNA and BNP-Ad (triangles) and challenged with a sublethal dose of A/HK/156 (Fig. 10A) or a lethal dose of A/SP/83 (Fig. 10B). The mice were primed 3 times with DNA at 2 week intervals and boosted with the adenovirus vector 2 weeks after the last DNA prime.

Figure 11A shows the mortality of mice prime-boost immunized with A/NP-PR/8 DNA and A/NP-PR/8 Ad (squares), M2 DNA and M2 Ad (triangles), or B/NP-DNA and BNP-Ad (circles) and challenged with a lethal dose of A/SP/83. Figure 11B shows the lung virus titers of additional prime-boost immunized mice 5 days post-challenge with A/SP/83

(A/NP-PR/8 DNA and A/NP-PR/8 Ad (shaded), M2 DNA and M2 Ad (white), B/NP-DNA and BNP-Ad (black)). The mice were primed 3 times with DNA at 2 week intervals and boosted with the adenovirus vector 2 weeks after the last DNA prime.

Figure 12 shows that the T cell response in mice prime-boost immunized with M2-DNA+M2-Ad (open bars) to peptide fragments (peptides M2-1 and M2-2 as shown in Table 7) from M2 is greater than the T-cell response to the same peptides in mice with immunity induced by A/PR/8 infection (filled bars).

Figure 13 shows the Eli spot results from splenocytes harvested from non-immunized mice previously challenged with A/PR/8 (A) or influenza naïve mice immunized with M2-DNA (3 times)+M2-Ad (B). Peptides used were 18-mers overlapping by 12 amino acids spanning the complete amino acid sequence of M2-A/PR/8 as shown in Table 7. Peptides were pooled in groups of two.

Detailed Description

Influenza A viruses each contain eight segments of single stranded RNA with negative polarity. The influenza A genome encodes eleven polypeptides. Segments 1-3 encode three polypeptides, making up an RNA-dependent RNA polymerase. Segment 1 encodes the polymerase complex protein PB2. The remaining polymerase proteins PB1 and PA are encoded by segment 2 and segment 3, respectively. In addition, segment 2 of some influenza strains encodes a small protein, PB1-F2, produced from an alternative reading frame within the PB1 coding region. Segment 4 encodes the hemagglutinin (HA) surface glycoprotein involved in cell attachment and entry during infection. Segment 5 encodes the nucleocapsid nucleoprotein (NP) polypeptide, the major structural component that associates with viral RNA. Segment 6 encodes a neuraminidase (NA) envelope glycoprotein. Segment 7 encodes two matrix proteins, designated M1 and M2, which are translated from differentially spliced mRNAs. Segment 8 encodes NS1 and NS2, two nonstructural proteins, which are translated from alternatively spliced mRNA variants.

Type A influenza strains are described by a nomenclature system that includes the geographic site of isolation, identification number, year of isolation, and the subtype of HA and NA, in parentheses, for example, A/Hong Kong/156/97 (H5N1). If the virus infects non-humans, the host species is included before the geographical site, for example, A/Chicken/Hong Kong/G9/97 (H9N2).

The terms "M2 polypeptide" or "M2 protein" or "M2" are used interchangeably and encompass both naturally occurring matrix protein 2 (M2) of an influenza A virus and M2 variants. The term "M2e" refers to the extracellular portion of M2. In an embodiment, the extracellular region of the M2 (M2e) corresponds to about the first 24 amino acids of the N-

terminal end of the M2 (Fischer et al., 2002, *Biochem. Biophys. Acta.*, 1561:27-45; Zhong et al., 1998, *FEBS Lett.*, 434:265-71).

The terms "naturally occurring M2 polypeptide" or "naturally occurring M2" are used interchangeably and encompass polypeptides that have the same amino acid sequence as a polypeptide obtained from nature from an influenza A virus or cell infected with influenza A. The terms "naturally occurring M2 polypeptide" or "naturally occurring M2" specifically encompasses any of the naturally occurring forms of the polypeptides, including mature forms. Naturally occurring variants include secreted forms, alternatively spliced forms, and those naturally occurring variants from other influenza A strains or isolates that differ in sequence from a reference sequence for a particular M2 polypeptide. In an embodiment, the reference sequence comprises an amino acid sequence of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:40. Naturally occurring M2 can be isolated or purified from nature, prepared recombinantly or synthetically.

"M2 polypeptide variant" or "M2 variant" refers to an M2 polypeptide that differs in amino acid sequence from a particular M2 polypeptide reference sequence. In an embodiment, the M2 polypeptide reference sequence comprises an amino acid sequence of SEQ ID NO:1 or SEQ ID NO:10. "M2 variant polypeptides" or "M2 variants" specifically encompasses modifications of the reference sequence, and naturally occurring M2 polypeptide variants. When the variant is a naturally occurring M2 polypeptide variant of the reference sequence, the variant is designated "a naturally occurring M2 variant." The variants may include deletions and additions of amino acids, as well as amino acid substitutions as described herein.

An M2 variant has at least about any number of % sequence identity from 70% to 100 % sequence identity to a full-length mature M2 polypeptide reference sequence. AN M2 variant has at least about 70% sequence identity, more preferably at least about 75% sequence identity, more preferably at least about 80% sequence identity, more preferably at least about 85% sequence identity, more preferably at least about 90% sequence identity, more preferably at least about 95% sequence identity and even 100% sequence identity to an M2e polypeptide reference sequence such as that of SEQ ID NO:10 or full-length mature M2 polypeptide reference sequence, such as that of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:40.

The disclosure also includes variants of nucleic acid molecules encoding M2 or M2e polypeptides. In one embodiment, the disclosure includes polynucleotides encoding a polypeptide having at least about any number of sequence identity from 70% to 100% sequence identity to the reference polypeptide for M2, more preferably about 70% sequence identity, more preferably about 75% sequence identity, more preferably about 80% sequence identity, more preferably about 85% sequence identity, more preferably about

90% sequence identity, more preferably about 95% sequence identity, and even up to 100% sequence identity to a reference M2, such as that having an amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or reference M2e, such as that having an amino acid sequence of SEQ ID NO:10.

5 As used herein, the term "M2 consensus" or "M2 consensus sequence" refers to a consensus sequence for the N-terminal extracellular domain of M2 polypeptide from influenza A subtypes H2N1, H1N2, and H3N2 (Neirynck, et al., Nature Medicine, 5:1157-1163, 1999). The term "M2e-con" or "M2e consensus" refers to a consensus sequence of the extracellular portion of M2. In an embodiment, M2e-con comprises an amino acid
10 sequence of SEQ ID NO:10. Other consensus sequences may be derived from aligning M2 peptide sequences and identifying a consensus sequence, for example, for a particular subtype.

 The term "heterosubtype" or "heterosubtypic" refers to a polynucleotide, polypeptide, virus strain or virus isolate that has the sequence of or is obtained from a
15 different subtype with respect to hemagglutinin and/or neuraminidase than a reference polynucleotide, polypeptide, virus strain or virus isolate. In some embodiments, the reference virus isolate or strain has the subtype H1N1 and the heterosubtypic virus isolate or strain is H5N1.

 The term "homologous subtype" refers to a polynucleotide, polypeptide, virus strain
20 or virus isolate that has the sequence of or is obtained from the same subtype with respect to hemagglutinin and/or neuraminidase as a reference polynucleotide, polypeptide, virus strain or virus isolate. In some embodiments, the reference virus isolate or strain has the subtype H1N1 and a homologous virus isolate or strain is a different H1N1 strain or isolate.

 The term "mismatched" refers to a polynucleotide, polypeptide, virus strain or virus
25 isolate that has the sequence of or is obtained from the same subtype class with respect to hemagglutinin and/or neuraminidase as a reference polynucleotide, polypeptide, virus strain or virus isolate but differs in the sequence of one more polypeptides. In some embodiments, the polypeptide of one viral isolate or strain differs by at least 80 %, more preferably 85%, and most preferably at least 90%. In some embodiments, the reference virus isolate or strain
30 has the subtype H1N1 and a sequence of M2 protein of SEQ ID NO:1 and a mismatched isolate or strain is a different H1N1 strain or isolate having an M2 protein comprising the amino acid sequence of SEQ ID NO:3.

 The term "isolated" refers to a biological material, such as a virus, a nucleic acid or a polypeptide, which is substantially free from components that normally accompany or
35 interact with it in its naturally occurring environment. The isolated biological material optionally comprises additional material not found with the biological material in its natural environment. For example, if the material is in its natural environment, such as a cell, the

material can have been placed at a location in the cell, such as in a genome or genetic element, not native to such material found in that environment. For example, a naturally occurring nucleic acid, such as a coding sequence, a promoter, or enhancer, becomes isolated if it is introduced by non-naturally occurring means to a locus of the genome (e.g., a
5 vector, such as a plasmid or virus vector, or amplicon) not native to that nucleic acid. An isolated virus, for example, is in an environment, such as a cell culture system, or purified from cell culture, other than the native environment of wild-type virus, such as the nasopharynx of an infected individual.

The term "isolated," when used to describe the various polypeptides disclosed
10 herein, means a polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Preferably, the isolated polypeptide is free of association with at least one component with which it is naturally associated. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide and may include enzymes, and other
15 proteinaceous or non-proteinaceous solutes. An isolated polypeptide includes polypeptide in situ within recombinant cells, since at least one component of the M2 polypeptide natural environment will not be present. Ordinarily, however, an isolated polypeptide will be prepared by at least one purification step.

An "isolated" nucleic acid molecule encoding an M2 polypeptide or M2e
20 polypeptide is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the M2-encoding nucleic acid. Preferably, the isolated nucleic acid is free of association with all components with which it is naturally associated. An isolated M2-encoding nucleic acid molecule is other than in the form or setting in which it is found in
25 nature. Isolated nucleic acid molecules therefore are distinguished from the M2-encoding nucleic acid molecule as it exists in natural cells or virus. In an embodiment, the nucleic acid molecule comprises a nucleic acid sequence of SEQ ID NO:9 or SEQ ID NO:39.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences
30 that are suitable include, for example, a promoter, and optionally an enhancer sequence.

A nucleic acid sequence is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is
35 operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being

linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

5 The term "vector" refers to the means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include plasmids, viruses, bacteriophages, pro-viruses, phagemids, transposons, artificial chromosomes, and the like, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA
10 polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that is not autonomously replicating. Preferred vectors as described herein are plasmids.

 An "expression vector" is a vector, such as a plasmid that is capable of promoting
15 expression, as well as replication of a nucleic acid incorporated therein. Typically, the nucleic acid to be expressed is "operably linked" to a promoter and/or enhancer, and is subject to transcription regulatory control by the promoter and/or enhancer.

 The term "host cell" means a cell that contains a heterologous nucleic acid, such as a vector, and supports the replication and/or expression of the nucleic acid. Host cells can be
20 prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, avian or mammalian cells, including human cells. Exemplary host cells can include, but are not limited to Vero (African green monkey kidney) cells, BHK (baby hamster kidney) cells, primary chick kidney (PCK) cells, MDCK (Madin-Darby Canine Kidney), 293 cells, and COS cells.

25 An "immunogenic effective amount" of an M2 or M2e polypeptide or polynucleotide refers to an amount of a polypeptide or polynucleotide that is capable of inducing an immune response in an animal. The immune response may be determined by measuring a T or B cell response. Levels of induced immunity can be monitored, for example, by measuring amounts of neutralizing secretory and/or serum antibodies, by
30 plaque neutralization, complement fixation, enzyme-linked immunosorbent, microneutralization assay, or assays for T cell function. Typically, the induction of an immune response is indicated by the detection of antibodies specific for an M2 or M2e polypeptide.

 As used herein, the term "immunogenic fragment thereof" refers to a fragment an
35 M2 or M2e polypeptide that is of a sufficient size to elicit an immune response in an animal. Typically, immunogenic fragments are at least 8 amino acids long and may include up to the full-length polypeptide. In some embodiments, an immunogenic fragment is about 9 amino

acids, an immunogenic fragment is about 10 amino acids, 15 amino acids, 30 amino acids, or 45 amino acids. The immunogenic fragment is capable of stimulating an antibody or T cell response specific for at least one M2 or M2e polypeptide. The sequence of immunogenic fragments can be readily predicted using available programs such as

- 5 Epiredict. The immune response includes both a T and B cell response. In some cases, the immune response is identified by the ability of the fragment to elicit antibodies or to stimulate a T cell response.

- A “protective immune response” against influenza virus refers to an immune response exhibited by an animal that is protective against disease when the animal is
- 10 subsequently exposed to and/or infected with such influenza virus. In some instances, the influenza virus can still cause infection, but the infection is less than serious in non-immune controls. A protective immune response can be characterized by % decrease in morbidity, % increase in survival, and/or a decrease in viral load. Typically, the protective immune response results in detectable levels of host-engendered serum and secretory antibodies that
- 15 are capable of reacting with antigens from virus of the same strain and/or subgroup and in some cases, also of a different, non-vaccine strain and/or subgroup in vitro and in vivo.

Peptide and protein sequences defined herein are represented by one-letter symbols for amino acid residues as follows:

	A	alanine	L	leucine
20	R	arginine	K	lysine
	N	asparagine	M	methionine
	D	aspartic acid	F	phenylalanine
	C	cysteine	P	proline
	Q	glutamine	S	serine
25	E	glutamic acid	T	threonine
	G	glycine	W	tryptophan
	H	histidine	Y	tyrosine
	I	isoleucine	V	valine

- “Percent (%) amino acid sequence identity” with respect to the M2 or M2e
- 30 polypeptide sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in an M2 or M2e polypeptide reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of
- 35 determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, clustal V (DNASTAR) or Megalign (DNASTAR) software.

Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. Alignments of M2 from different influenza A subtypes and strains can be found and performed using the Influenza Sequence Database (Macken et al. in
 5 Options for the Control of Influenza IV. A.D.M.E. Osterhaus, N. Cox & A.W. Hampson (Eds.) Amsterdam: Elsevier Science, 2001, 103-106) at the Los Alamos website (<http://www-flu-lanl-gov>).

For purposes herein, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be
 10 phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

$$100 \text{ times the fraction } X/Y$$

where X is the number of amino acid residues scored as identical matches by the
 15 sequence alignment program in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. In an embodiment, the B amino acid sequence is that of SEQ ID NO:1 or SEQ ID
 20 NO:10.

"Percent (%) nucleic acid sequence identity" with respect to the M2 polypeptide-encoding nucleic acid sequences identified herein is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in a reference M2
 polypeptide-encoding nucleic acid sequence, after aligning the sequences and introducing
 25 gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, or Megalign (DNASTAR) software. Alignments of M2 nucleic acids from different influenza A subtypes and strains can be
 30 found and performed using the Influenza Sequence Database (Macken et al. in Options for the Control of Influenza IV. A.D.M.E. Osterhaus, N. Cox & A.W. Hampson (Eds.) Amsterdam: Elsevier Science, 2001, 103-106) at the Los Alamos website (<http://www-flu-lanl-gov>). Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-
 35 length of the sequences being compared.

For purposes herein, the % nucleic acid sequence identity of a given nucleic acid sequence C to, with, or against a given nucleic acid sequence D (which can alternatively be

phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid sequence identity to, with, or against a given nucleic acid sequence D) is calculated as follows:

$$100 \text{ times the fraction } W/Z$$

- 5 where W is the number of nucleotides scored as identical matches by the sequence alignment program in that program's alignment of C and D, and where Z is the total number of nucleotides in D. It will be appreciated that where the length of nucleic acid sequence C is not equal to the length of nucleic acid sequence D, the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C.

- 10 "Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured DNA to reanneal when
15 complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization
20 reactions, see Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995).

- "Stringent conditions" or "high stringency conditions", as defined herein, may be identified by those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at
25 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium
30 pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SW (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C.

- "Moderately stringent conditions" may be identified as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Press, 1989,
35 and include the use of washing solution and hybridization conditions (e.g., temperature, ionic strength and % SDS) less stringent than those described above. An example of

moderately stringent conditions is overnight incubation at 37°C in a solution comprising: 20% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1 x SSC at about 37-50°C.

- 5 The skilled artisan will recognize how to adjust the temperature, ionic strength, in etc. as necessary to accommodate factors such as probe length and the like.

As used herein "recombinant" refers to a nucleic acid molecule that has been isolated and/or altered by the human hands. Typically a DNA sequence encoding a polypeptide is isolated and combined with other control sequences in a vector. The other
10 control sequences may be those that are found in the naturally occurring gene or others. The vector provides for introduction into host cells, amplification of the nucleic acid sequence and expression of the nucleic acid sequence.

I. Immunogenic Compositions

The present invention is directed to immunogenic compositions and methods for
15 priming or enhancing the immune response of an animal to influenza A antigens. The present invention provides an immunogenic composition containing at least one polynucleotide encoding an M2 polypeptide from an influenza A subtype. The M2 can be naturally occurring or variant, full length or an immunogenic fragment thereof. One example of a suitable immunogenic fragment is the extracellular portion of M2 (M2e). In
20 an embodiment, the extracellular region of the M2 (M2e) corresponds to about the first 24 amino acids of the N-terminal end of the M2. A single polynucleotide may encode more than a single M2 or M2e polypeptide or a combination of M2 and/or M2e polypeptides from different influenza A subtypes or strains. In some embodiments, the polynucleotide encodes one or more M2 polypeptides from a naturally occurring H1N1 or H5N1 virus. In some
25 embodiments, a polynucleotide encodes a M2 polypeptide that has at least 80% sequence identity to a polypeptide comprising the amino acid sequence of SEQ ID NO:10 and/or comprising the amino acid sequence of SEQ ID NO:1. In some embodiments, a polynucleotide encodes a M2 polypeptide that has at least 80% sequence identity to a polypeptide comprising the amino acid sequence of SEQ ID NO:10 and/or 90% sequence
30 identity to an M2 polypeptide comprising the amino acid sequence of SEQ ID NO:1. In some embodiments, the M2 polypeptide does not include the amino acid sequence of SEQ ID NO:10.

Amino acid and nucleic acid sequences for influenza A M2 polypeptides are known in the art and can be found, for example, using GenBank (www.ncbi.nlm.gov) or the
35 Influenza Sequence Database at the Los Alamos website (<http://www.flu-lanl.gov>). Examples of M2 amino acid and nucleic acid sequences are shown in Table 1 and Table 2. M2 amino acid and nucleic acid sequences of influenza strains or isolates may have been

passed at different places or times and therefore the sequence of any particular strain may vary from other strains. However, such sequences are readily obtainable using methods known to those of skill in the art.

TABLE 1

Strain	Abbrev.	Subtype	M2 Amino Acid Sequence	GenBank Access. No.	SEQ ID NO:
A/Puerto Rico/8/34 M2 with a G to D change at position 21	M2 DNA or M2 Ad	H1N1	MSLLTEVETPIRNEWGCR CNDSSDPLAIAANIIGILHL ILWILDRLFFKCIYRRFKY GLKGGPSTEGVPKSMREE YRKEQQSAVDADDGHFV SIELE		1
A/Puerto Rico/8/34	A/PR/8	H1N1	MSLLTEVETPIRNEWGCR CNGSSDPLAIAANIIGILHL ILWILDRLFFKCIYRRFKY GLKGGPSTEGVPKSMREE YRKEQQSAVDADDGHFV SIELE	NP_040979	2
A/Fort Monmouth h/1/47-MA	FM-MA	H1N1	MSLLTEVETPTKNEWECR CNDSSDPLVVAASIIGILH LILWILDRLFFKCIYRLF HGLKRGSTEGVPESMRE EYRKEQQSAVDADDMHF VNIELE	AAB39916	3
A/HK/1/68	HK/68	H3N2	MSLLTEVETPIRNEWGCR CNDSSDPLVVAASIIGILH LILWILNRLFFKCIYRFFE HGLKRGSTEGVPESMREE YRKEQQSAVDADDSHFV SIELE	AAK51749	4
A/HK/483/97	HK/483	H5N1	MSLLTEVETLTRNGWGC RCDSSDPLVVAASIIGILH LILWILDRLFFKCIYRRFK YGLKRGSTEGVPESMRE EYRQEQQNAVDVDDGHF VNIELE	AAF74335	5
A/HK/156/97	HK/156	H5N1	MSLLTEVETLTRNGWGC RCDSSDPLVVAASIIGILH LILWILDRLFFKCIYRRFK YGLKRGSTEGVPESMRE EYRQEQQNAVDVDDGHF VNIELE	AAC32091	6
A/Thailand/SP83/04	SP-83	H5N1	MSLLTEVETPTRNEWECR CSDSSDPIVVAANIIGILHL ILWILDRLFFKCIYRRFKY GLKRGPATAGVPESMREE YRQEQQSAVDVDDGHFV NIELE	AAV32647	7
A/HK/1073/97	HK/1073	H9N2	MSLLTEVETLTRNGWECK CSDSSDPLVVAASIIGILHL ILWILDRLFFKCIYRRFKY GLKRGSTEGVPESMREE YRQEQQNAVDVDDGHFV NIELE	CAC04082	8

A/PR/8 modified sequence			MSLLTEVETPIRNEWGCR CNDSSDPLAIAANIIGILHL TLLILDRLFFKCIYRRFKY GLKGGPSTEGVPKSMREE YRKEQQSAVDADDGHFV SIELE		40
A/PR/8 modified sequence			MSLLTEVETPIRNEWGCR CNDSSDPLAIAANIIGILHL TLWILDRLFFKCIYRRFKY GLKGGPSTEGVPKSMREE YRKE QQSAVDADDGHFVSIELE		41

TABLE 2

Strain	M2 Nucleic Acid Sequence	GenBank Access. No.	SEQ ID NO:
A/PR/8	atg agt ctt cta acc gag gtc gaa acg cct atc aga aac gaa tgg ggg tgc aga tgc aac ggt tca agt gat cct ctc gct att gcc gca aat atc att ggg atc ttg cac ttg ata ttg tgg att ctt gat cgt ctt ttt ttc aaa tgc att tac cgt cgc ttt aaa tac gga ctg aaa gga ggg cct tct acg gaa gga gtg cca aag tct atg agg gaa gaa tat cga aag gaa cag cag agt gct gtg gat gct gac gat ggt cat ttt gtc agc ata gag ctg gag	NC_002016 Winter,G. and Fields,S., Nucleic Acids Res. 8 (9), 1965-1974 (1980)	9
A/PR/8 with aa 21 modified from Gly to Asp	ATG AGT CTT CTA ACC GAG GTC GAA ACG CCT ATC AGA AAC GAA TGG GGG TGC AGA TGC AAC <u>GAT</u> TCA AGT GAT CCT CTC GCT ATT GCC GCA AAT ATC ATT GGG ATC TTG CAC TTG ACA TTG TGG ATT CTT GAT CGT CTT TTT TTC AAA TGC ATT TAC CGT CGC TTT AAA TAC GGA CTG AAA GGA GGG CCT TCT ACG GAA GGA GTG CCA AAG TCT ATG AGG GAA GAA TAT CGA AAG GAA CAG CAG AGT GCT GTG GAT GCT GAC GAT GGT CAT TTT GTC AGC ATA GAG CTC GAG		39

- Amino acid sequences for M2e are known in the art and can be found using
- 5 GenBank and the Influenza Sequence Database as described herein. Examples of M2e amino acid sequences are shown in Table 3.

TABLE 3

Strain	Subtype	M2e Amino Acid Sequence	SEQ ID NO:
Immunizing M2e domain		MSLLTEVETPIRNEWGCRCNDSSD	10
A/PR/8	H1N1	MSLLTEVETPIRNEWGCRCNGSSD	11
FM-MA	H1N1	MSLLTEVETPTKNEWECRCNDSSD	12
HK/68	H3N2	MSLLTEVETPIRNEWGCRCNDSSD	13
HK/483	H5N1	MSLLTEVETLTRNGWGCRCSDSSD	14
HK/156	H5N1	MSLLTEVETLTRNGWGCRCSDSSD	15
Thailand/SP/83	H5N1	MSLLTEVETPTRNEWECRCSDSSD	16
NL/219/03	H7N7	MSLLTEVETPTRNGWECKCNDSSD	17
HK/1073/97	H9N2	MSLLTEVETLTRNGWECKCRDSSD	18
A/CK/TH/04	H5N1	MSLLTEVETPTRNEWECRCSDSSD	42
A/CK/NY/95	H7N2	MSLLTEVETPTRNGWECKCSDSSD	43
Con P to L	H1N1	MSLLTEVETLIRNEWGCRCNDSSD	44

The underlined amino acids in Table 3 show the where the amino acids vary in the extracellular domain of M2 as compared to the reference A/PR/8 (SEQ ID NO:11). In a specific embodiment, the extracellular domain of the M2 polypeptide expressed by the nucleic acid constructs has the same amino acid sequence of a consensus sequence having the amino acid sequence of SEQ ID NO:10.

In some embodiments, the disclosure includes an immunogenic composition comprising an expression vector or a polypeptide comprising at least one polynucleotide encoding an M2 polypeptide or a polypeptide comprising the amino acid sequence of

MSLLTEVETX₁₀X₁₁X₁₂NX₁₄WX₁₆CRCX₂₀X₂₁SSD (SEQ ID No:45),

wherein X₁₀, X₁₁, X₁₂, X₁₄, X₁₆, X₂₀, and X₂₁ can be any amino acid, preferably a naturally occurring amino acid. In some embodiments, X₁₀ is P or L; X₁₁ is E or G; X₁₂ is R or K; X₁₄ is E or G; X₁₆ is G or E; X₂₀ is N, S or R; and X₂₁ is G or D in combination with a carrier. In some embodiments, X₁₀ is a proline. The immunogenic composition of claim 1, wherein the M2 polypeptide comprises the amino acid sequence selected from the group consisting of SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:42, and SEQ ID NO:43.

In other embodiments, the peptide comprises the amino acid sequence:

VETX₄X₅RNX₈WX₁₀CX₁₂ (SEQ ID NO:19), LTEVETX₇X₈ (SEQ ID NO:48), LTEVETPX₈ (SEQ ID NO:46), or VETPX₅X₆NX₈W (SEQ ID NO:47), wherein X is any amino acid. In

some embodiments, the peptide comprises an amino acid sequence of

VETX₄X₅RNX₈WX₁₀CX₁₂, wherein X₄ is P or L, X₅ is E or G; X₈ is E or G; X₁₀ is G or E;

and X₁₂ is N, S or R; in combination with a carrier. In some embodiments, the peptide comprises an amino acid sequence of LTEVETX₇X₈ (SEQ ID NO:48), wherein X₇ is P or L, and X₈ is E or G, in combination with a carrier. In some embodiments, a peptide

comprises an amino acid sequence of LTEVETPX₈ (SEQ ID NO:46), wherein X₈ is E or G, in combination with a carrier. In some embodiments, a peptide comprises an amino acid sequence of VETPX₅X₆NX₈W (SEQ ID NO:47), wherein X₅ is E or G; X₆ is R or K, X₈ is E or G; in combination with a carrier.

5 In some embodiments, the expression vector is a plasmid vector or a viral vector. In some embodiments, the polypeptide can be a naturally occurring M2 polypeptide or a fragment of M2 polypeptide such as an extracellular domain or immunogenic fragment. In other embodiments, the polypeptide is an M2 consensus sequence. In some embodiments, the polynucleotide does not encode at least one other influenza protein.

10 In an embodiment, the polynucleotide encodes an M2 polypeptide having at least about 70% sequence identity, at least about 75% sequence identity, at least about 80% sequence identity, at least about 85% sequence identity, at least about 90% sequence identity, at least about 95% sequence identity, or at least about 100% sequence identity to a reference M2 sequence. In an embodiment, the reference sequence is SEQ ID NO:1 or SEQ
15 ID NO:40. In an embodiment, the polynucleotide encodes an M2 variant polypeptide having at least about 70% sequence identity, at least about 75% sequence identity, at least about 80% sequence identity, at least about 85% sequence identity, at least about 90% sequence identity, at least about 95% sequence identity, or at least about 100% sequence identity to a full-length mature M2 reference sequence or the extracellular domain of M2. In an
20 embodiment, the reference sequence is SEQ ID NO:1 or SEQ ID NO:10. Preferably, the M2 variants generate antibodies that cross react with heterologous M2 polypeptides and/or provide protective immunity.

In an embodiment, the polynucleotide encodes an M2e polypeptide having at least about 70% sequence identity, at least about 75% sequence identity, at least about 80%
25 sequence identity, at least about 85% sequence identity, at least about 90% sequence identity, at least about 95% sequence identity, or at least about 100% sequence identity to a reference M2e sequence. In an embodiment, the reference sequence is SEQ ID NO:10.

M2 variants include naturally occurring variants having the sequence of M2 polypeptide isolated from nature from different influenza A subtypes and/or strains.

30 Variations in the naturally occurring full-length M2 polypeptides described herein, can also be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Patent No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the M2 polypeptide that results in a change in the amino acid sequence of the M2 polypeptide as compared with
35 a naturally occurring M2 polypeptide.

Guidance in determining which amino acid residue may be inserted, substituted or deleted without adversely affecting the desired activity may be found by comparing the

sequence of the M2 polypeptide with that of homologous known protein molecules and minimizing the number of amino acid sequence changes made in regions of high homology. For example, when full length M2 proteins are aligned from several different isolates or strains, amino acid positions corresponding to positions 10, 11, 12, 14, 16, 19, 20, or 21 in the extracellular domain can have varied amino acids; amino acid positions corresponding to positions 51, 54, or 56 can have varied amino acids, and/or amino acid positions corresponding to 84, 88, or 95 can have varied amino acids. In some embodiments, variants may include amino acid substitution so that the amino acid sequence of the extracellular domain of M2 corresponds to that of a different subtype. (See Table 3 for examples) For example, positions 10, 11, 14, 16 and 20 may have amino acids L, T, G, and S respectively as compared to P, I, E, G and N of the M2e domain of SEQ ID NO:11. In addition, solvent accessible residues may be determined using standard methodologies and may include residues having about 25% or greater solvent accessibility such as amino acid residues 2, 5, 6, 8, 9, 11-14, 18, and 20-24. Amino acids in solvent accessible positions may be varied without disrupting structure.

Functional domains can also be identified in those M2 polypeptides that have homology to known polypeptides. For example, certain positions in the extracellular domain show more variability than others. These positions can be identified using sequence alignments and changes made to those amino acid positions showing high variability (e.g. 3 or more different amino acids in that position when a number of sequences are aligned). See Table 3. In some embodiments, the first nine amino acids are not varied. The sequences of these functional domains can be compared and aligned to other known sequences that may be provided at the Los Alamos website or GenBank, and locations of amino acid positions for substitutions can be identified as those positions that show a high degree of variability in amino acids, i.e., at least 3 different amino acids are found at that position when different sequences are aligned and compared or have a lower percentage of sequence identity i.e., less than 90% sequence identity. When sequences are aligned, the positions that show variability can either have conservative amino acid substitutions or non-conservative amino acid substitutions. If the position has conservative amino acid substitutions, that would indicate that the amino acid substituted at that position should be of the same type as those observed to be at that position in naturally occurring proteins. For examples of such substitutions, see Table 4.

In some embodiments, the polynucleotide encodes an immunogenic fragment of an M2 protein including at least 10 amino acids, more preferably 12 amino acids, more preferably 18 amino acids, more preferably 20 amino acids, more preferably 24 amino acids, and most preferably about 30 contiguous amino acids. In some embodiments, the fragment comprises at least one T cell epitope. In some embodiments, a T cell epitope comprises the

amino acid sequence of amino acids 2 to 24 of the M2 protein. In some embodiments, the fragment comprises an amino acid sequence of VETX₄X₅RNX₈WX₁₀CX₁₂, LTEVETX₇X₈ (SEQ ID NO:48), LTEVETPX₈ (SEQ ID NO:46) or VETPX₅X₆NX₈W (SEQ ID NO:47), wherein X is any amino acid. In some embodiments, X is not proline or cysteine. In some

5 embodiments, a peptide comprises an amino acid sequence of VETX₄X₅RNX₈WX₁₀CX₁₂, wherein X₄ is P or L, X₅ is E or G; X₈ is E or G; X₁₀ is G or E; and X₁₂ is N, S or R; in combination with a carrier. In some embodiments, a peptide comprises an amino acid sequence of LTEVETX₇X₈ (SEQ ID NO:48), wherein X₇ is P or L, and X₈ is E or G, in combination with a carrier. In some embodiments, a peptide comprises an amino acid

10 sequence of LTEVETPX₈ (SEQ ID NO:46), wherein X₈ is E or G, in combination with a carrier. In some embodiments, a peptide comprises an amino acid sequence of VETPX₅X₆NX₈W (SEQ ID NO:47), wherein X₅ is E or G; X₆ is R or K, X₈ is E or G; in combination with a carrier.

In a specific embodiment, the fragment comprises amino acids 1-18, 1-24, 2-24, 7 -

15 18 or 7-24 of the extracellular domain of an M2 protein, such as SEQ ID NO:10. In other embodiments, the fragment comprises at least the peptide VETPIRNEWGCR (SEQ ID NO:20) and is a peptide of about 15, 16, 17, 18, 19, 20, 21, 22, or 23 amino acids. In some embodiments the peptide comprising VETPIRNEWGCR (SEQ ID NO:20) excludes the extracellular domain consensus sequence (SEQ ID NO:10) or the full length M2 sequence.

20 Amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with a serine, i.e., conservative amino acid replacements. Insertions or deletions may optionally be in the range of about 1 to 5 amino acids. The variation allowed may be determined by systematically making insertions, deletions or substitutions of amino acids in

25 the sequence and testing the resulting variants for activity exhibited by the full-length or mature naturally occurring sequence. Preferably, M2 variants have the biological activity of the source molecule or are bound by anti-M2 antibodies.

In particular embodiments, conservative substitutions of interest are shown in Table 4 under the heading of preferred substitutions.

30

TABLE 4

<u>Original Residue</u>	<u>Exemplary Substitutions</u>	<u>Preferred Substitutions</u>
Ala (A)	val; leu; ile	Val
Arg (R)	lys; gln; asn	Lys
Asn (N)	gln; his; lys; arg	Gln
Asp (D)	glu	Glu
Cys (C)	ser	Ser
Gln (Q)	asn	Asn
Glu (E)	asp	Asp
Gly (G)	pro; ala	Ala
His (H)	asn; gln; lys; arg	Arg
Ile (I)	leu; val; met; ala; phe; norleucine	
Leu (L)	norleucine; ile; val; met; ala; phe	Ile
Lys (K)	arg; gln; asn	Arg
Met (M)	leu; phe; ile	Leu
Phe (F)	leu; val; ile; ala; type	Leu
Pro (P)	ala	Ala
Ser (S)	thr	Thr
Thr (T)	ser	Ser
Trp (W)	tyr; phe	Tyr
Tyr (Y)	trp; phe; thr; ser	Phe
Val (V)	ile; leu; met; phe; ala; norleucine	Leu

The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the M2 polypeptide variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant [Cunningham and Wells, Science, 244: 1081-1085 (1989)]. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)].

In some embodiments, the polynucleotide can encode one or more M2 polypeptides or immunogenic fragments thereof and one or more variable influenza components, one or more conserved influenza components, or a combination thereof. In some embodiments, the

polynucleotide can encode M2 proteins from a variety of influenza A strains of different subtypes. In other embodiments the polynucleotide encodes one or more M2 polypeptides from a H1N1 virus isolate. In some embodiments, the polynucleotide does not encode the consensus M2 polypeptide having the sequence of SEQ ID NO:10 but rather an M2 polypeptide that has the sequence of a naturally occurring isolate. In some embodiments, the polynucleotide encodes an M2 polypeptide that comprises at least 90% sequence identity to SEQ ID NO:1 or SEQ ID NO:40.

Examples of other influenza components include hemagglutinin (HA), neuraminidase (NA), and immunogenic fragments thereof. Examples of conserved influenza components include matrix protein 1 (M1), nucleoprotein (NP) acidic polymerase (PA), basic polymerase 1 (PB1), basic polymerase 2 (PB2), nonstructural protein 1 (NS1), nonstructural protein 2 (NS2), and immunogenic fragments thereof. In some embodiments, the same polynucleotide encoding one or more M2 proteins does not encode a nucleoprotein or M1 protein either as individual proteins or as fusions to M2. In other embodiments, the same polynucleotide does not encode matrix protein 1 (M1), nucleoprotein (NP) acidic polymerase (PA), basic polymerase 1 (PB1), basic polymerase 2 (PB2), nonstructural protein 1 (NS1), or nonstructural protein 2 (NS2). Influenza amino acid and nucleic acid sequences for these variable and conserved influenza components are known in the art and can be found, for example, using GenBank (www.ncbi.nlm.gov) or the Influenza Sequence Database at the Los Alamos website (<http://www.flu-lanl.gov>).

In some embodiments, the immunogenic compositions of the disclosure can be combined with other influenza vaccines, such as heat killed or subunit vaccines.

In some embodiments, the immunogenic compositions of the invention comprise an immunogenic effective amount of M2-encoding polynucleotide. An immunogenic effective amount is an amount of polynucleotide that induces an immune response to the encoded polypeptide when administered to a host, for example an animal. In an embodiment, the polynucleotides are incorporated into host cells in vivo and an immunogenic effective amount of the encoded M2 polypeptide or fragment thereof is produced in vivo. The actual amount of the immunogenic composition may vary depending on the animal to be immunized, the route of administration and adjuvants. Immunogenic dosages can be determined by those of skill in the art. The immune response can be humoral, cellular, or both. Generally, the immune response inhibits the influenza viral levels in the immunized host compared to influenza viral levels in non-immunized hosts. The immunogenic composition optionally includes a pharmaceutically acceptable excipient or carrier.

An embodiment provides an immunogenic composition according to the present disclosure also including immunomodulators such as cytokines or chemokines. In some embodiments, a nucleic acid encodes the immunomodulator or adjuvant.

Immunomodulators refers to substances that potentiate an immune response including, but not limited to cytokines and chemokines. Examples of cytokines include but are not limited to IL-2, IL-15, IL-12, or GM-CSF.

5 An embodiment provides an immunogenic composition further comprising an adjuvant. Such adjuvants may include ganglioside receptor-binding toxins (cholera toxin, LT enterotoxin, their B subunits and mutants); surface immunoglobulin binding complex CTA1-DD; TLR4 binding lipopolysaccharide; TLR2-binding muramyl dipeptide; mannose receptor-binding mannan; dectin-1-binding ss 1,3/1,6 glucans; TLR9-binding CpG-oligodeoxynucleotides; cytokines and chemokines; antigen-presenting cell targeting
10 ISCOMATRIX and ISCOM. Adjuvants such as lipids (fatty acids, phospholipids, Freund's incomplete adjuvant in particular), Vaxfectin, polaxomer, anionic copolymers, CpG units, etc. may be added to the composition. In addition, adjuvants able to prime the mucosal immune system following a systemic immunization, include 25(OH)2D3, cholera toxin, CTA1-DD alone or in combination with ISCOM. In some embodiments, the adjuvant may
15 be encoded or expressed by the expression vector used herein.

An embodiment provides an immunogenic composition comprising at least one naked DNA or a naked RNA encoding at least one polypeptide according to the disclosure. Naked DNA or RNA is DNA or RNA that does not have proteins or lipids associated with it.

20 In certain embodiments, the immunogenic composition comprises at least one recombinant vector or DNA comprising a nucleic acid sequence encoding M2. Examples of vectors include, but are not limited to, recombinant viral vectors, such as poxvirus, vaccinia virus, lenti virus, or adenovirus, and plasmids. Typically a plasmid contains an origin of replication that is functional in bacterial host cells, e.g., *Escherichia coli*, and selectable
25 markers for detecting bacterial host cells containing the plasmid. Plasmids of the present invention may include genetic elements as described herein arranged such that an inserted coding sequence can be transcribed and translated in eukaryotic cells. In certain embodiments described herein, a plasmid is a closed circular DNA molecule.

Examples of plasmids that can be used in the present invention include expression
30 vector VR1012 or VR10551 (Vical, San Diego, CA). These vectors are built on a modified pUC18 background (see Yanisch et al., 1985, *Gene*, 33:103-119), and contain a kanamycin resistance gene, the human cytomegalovirus immediate early promoter/enhancer and intron A, and the bovine growth hormone transcription termination signal, and a polylinker for inserting foreign genes (see Hartikka et al., 1996, *Hum. Gene Ther.*, 7:1205-1217). Other
35 commercially available eukaryotic expression vectors can be used in the present invention, including, but not limited to plasmids pcDNA3, pHCMV/Zeo, pCR3.1, pEF1/His, pIND/GS, pRc/HCMV2, pSV40/Zeo2, pTRACER-HCMV, pUB6/V5-His, pVAX1,

pVAX200, and pZeoSV2 (Invitrogen, San Diego, Calif.), plasmid pCI (Promega, Madison, Wis.) and plasmid pDNA-VACC (Nature Tech. Corp., Lincoln, NE).

In an embodiment, the immunogenic composition includes a plasmid that comprises a nucleic acid sequence encoding at least one M2 polypeptide or immunogenic fragment thereof from an influenza A virus under the transcriptional control of a promoter region active in a variety of cells. In an embodiment, the promoter region is a human cytomegalovirus (CMV) promoter. In an embodiment, the plasmid is pVR1012. The M2 can be naturally occurring, variant, or an immunogenic fragment thereof. In an embodiment, the plasmid encodes an M2 polypeptide having at least 80% sequence identity with a polypeptide comprising the amino acid sequence of SEQ ID NO: 1 and/or SEQ ID NO:10. In some embodiments, the M2 protein or extracellular domain is that of a H1N1 strain, preferably A/PR/8. In another embodiment, the plasmid encodes an immunogenic fragment of M2 polypeptide having at least 90% sequence identity with an amino acid sequence of SEQ ID NO: 10.

To permit selection of plasmid-containing bacteria during the production process, the plasmid may also contain an antibiotic resistance gene with a bacterial origin of replication. DNA is generally less costly to produce than peptide or protein, and is chemically stable under a variety of conditions. DNA is generally administered intramuscularly, using either a needle and syringe or a needle-free injector, or intranasally.

The M2 polypeptide, or fragment thereof, may be expressed in a modified form, such as a fusion protein, and may include secretion signals and/or additional heterologous functional regions. For example, a region of additional amino acids may be added to the N-terminus or C-terminus of the polypeptide to facilitate detection or purification, improve immunogenicity, improve half-life, or improve persistence in the host cell during, for example, purification or subsequent handling and storage. Examples of additional amino acids include peptide tags that may be added to the polypeptide to facilitate detection and/or purification. Such peptide tags include, but are not limited to, His, HA, Avi, biotin, c-Myc, VSV-G, HSV, V5, or FLAGTM. Examples of a polypeptide that can enhance immunogenicity include bovine serum albumin, and/or keyhole lymphocyte hemocyanin (KLH). Examples of molecules that improve half-life include polyethylene glycol.

In some embodiments, the immunogenic composition may include an M2 polypeptide or fragment thereof as described herein.

The immunogenic compositions of the invention can also include a carrier. Carriers include pharmaceutically acceptable carriers, excipients, or stabilizers which are nontoxic to the cell or animal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other

organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates
5 including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™ polyethylene glycol (PEG), and PLURONICS™.

The immunogenic compositions of the invention can be in the form of sterile injectable preparations, such as sterile injectable aqueous or oleaginous suspensions. For
10 administration as injectable solutions or suspensions, the immunogenic compositions can be formulated according to techniques well-known in the art, using suitable dispersing or wetting and suspending agents, such as sterile oils, including synthetic mono- or diglycerides, and fatty acids, including oleic acid.

A. Polynucleotides

15 A second aspect of the disclosure relates to polynucleotides encoding M2 polypeptides, recombinant vectors, and host cells containing the recombinant vectors, as well as methods of making such vectors and host cells by recombinant methods. The polynucleotides encoding M2 or M2 variants are useful as immunogenic compositions or to produce M2 polypeptides.

20 The M2 polynucleotides of the disclosure may be synthesized or prepared by techniques well known in the art. See, for example, Creighton, *Proteins: Structures and Molecular Principles*, W. H. Freeman & Co., New York, NY (1983). Nucleotide sequences encoding the M2 polypeptides of the disclosure may be synthesized, and/or cloned, and expressed according to techniques well known to those of ordinary skill in the art. See, for
25 example, Sambrook, et al., *Molecular Cloning, A Laboratory Manual*, Vols. 1-3, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989). In some embodiments, the polynucleotide sequences will be codon optimized for a particular recipient using standard methodologies. For example, the DNA construct encoding the M2 protein can be codon optimized for expression in humans.

30 The polynucleotides may be produced by standard recombinant methods known in the art, such as polymerase chain reaction (PCR) or reverse transcriptase PCR (Sambrook, et al., 1989, *Molecular Cloning, A Laboratory Manual*, Vols. 1-3, Cold Spring Harbor Press, Cold Spring Harbor, NY), or the DNA can be synthesized and optimized for expression in bacteria or eukaryotic cells. Primers can be prepared using the polynucleotide sequences
35 provided, for example, in Tables 1-3 or that are available in publicly available databases. The polynucleotide constructs may be assembled from polymerase chain reaction cassettes sequentially cloned into a vector containing a selectable marker for propagation in a host.

Such markers include but are not limited to dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline, ampicillin, or kanamycin resistance genes for culturing in *E. coli* and other bacteria.

Representative examples of appropriate hosts include, but are not limited to,
5 bacterial cells such as *E. coli*, *Streptomyces* and *Salmonella typhimurium*, fungal cells such as yeast; insect cells such as *Drosophila* S2 and *Spodoptera Sf9*, animal cells such as CHO, COS, and Bowes melanoma cells, and plant cells. Appropriate culture medium and conditions for the above-described host cells are known in the art.

The polynucleotide should be operably linked to an appropriate promoter, such as
10 CMV. Other suitable promoters are known in the art. The expression constructs may further contain sites for transcription initiation, transcription termination, and a ribosome binding site for translation. The coding portion of the mature polypeptide expressed by the constructs preferably includes a translation initiating codon at the beginning and a termination codon (UAA, UGA, or UAG) appropriately positioned at the end of the
15 polypeptide to be translated.

Introduction of the recombinant vector into the host cell can be effected by injection, by mucosal administration such as by the intranasal route, or by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, or other methods. Such methods are
20 described in standard laboratory manuals such as Sambrook, et al., 1989, *Molecular Cloning, A Laboratory Manual*, Vols. 1-3, Cold Spring Harbor Press, Cold Spring Harbor, NY or Davis et al., 1986, *Basic Methods in Molecular Biology*. Commercial transfection reagents, such as Lipofectamine (Invitrogen, Carlsbad, CA), Effectene (Qiagen, Valencia, CA) and FuGENE 6™ (Roche Diagnostics, Indianapolis, IN), are also available.

25 The M2 polypeptide can be recovered and purified from recombinant cell cultures by methods known in the art, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, and lectin chromatography.

30 Naturally occurring polynucleotides encoding variants of M2 polypeptides can be isolated from cloning out viral isolates from infected individuals at various times post infection. Such polynucleotides can be obtained using primers for amplifying polynucleotide encoding M2. Such polynucleotides or polypeptides may be utilized in the immunogenic compositions described herein.

35 The disclosure also includes variants of nucleic acid molecules encoding M2 polypeptides. In some embodiments, the disclosure includes polynucleotides having at least about 70% sequence identity, more preferably about 75% sequence identity, more

preferably about 80% sequence identity, more preferably about 85% sequence identity, more preferably about 90% sequence identity, more preferably about 95% sequence identity, and even up to 100% sequence identity to a polynucleotide sequence encoding an M2 polypeptide having an amino acid sequence of SEQ ID NO: 1 and/or SEQ ID NO:10. In some embodiments, the polynucleotide variants encode an M2 polypeptide that generates antibodies in an animal that can bind to or cross react with a heterologous M2 polypeptide.

In an embodiment, the polynucleotide encodes an M2 polypeptide having at least about 70% sequence identity, at least about 75% sequence identity, at least about 80% sequence identity, at least about 85% sequence identity, at least about 90% sequence identity, at least about 95% sequence identity, or at least about 100% sequence identity to a reference M2 sequence. In an embodiment, the reference sequence is SEQ ID NO:1 or SEQ ID NO:40. In an embodiment, the polynucleotide encodes an M2 variant polypeptide having at least about 70% sequence identity, at least about 75% sequence identity, at least about 80% sequence identity, at least about 85% sequence identity, at least about 90% sequence identity, at least about 95% sequence identity, or at least about 100% sequence identity to a full-length mature M2 reference sequence or the extracellular domain of M2. In an embodiment, the reference sequence is SEQ ID NO:1 or SEQ ID NO:10. Preferably, the M2 variants generate antibodies that cross react with heterologous M2 polypeptides and/or provide protective immunity.

In an embodiment, the polynucleotide encodes an M2e polypeptide having at least about 70% sequence identity, at least about 75% sequence identity, at least about 80% sequence identity, at least about 85% sequence identity, at least about 90% sequence identity, at least about 95% sequence identity, or at least about 100% sequence identity to a reference M2e sequence. In an embodiment, the reference sequence is SEQ ID NO:10.

The disclosure also includes polynucleotides encoding immunogenic fragments of M2 polypeptides. The fragments can be used to generate antibodies that specifically bind to an M2 or M2e polypeptide. Immunogenic fragments are at least 8 amino acids in length, more preferably 8-50 amino acids, more preferably at least 10 amino acids, and more preferably at least 20 amino acids up to a full-length polypeptide. Immunogenic fragments can be predicted by analyzing the primary amino acid sequence of an M2 polypeptide using commercially available services such as EpiPredict or Epitope informatics or publicly available programs such as are available.

In some embodiments, the disclosure includes an immunogenic composition comprising an expression vector or a polypeptide comprising at least one polynucleotide encoding an M2 polypeptide or a polypeptide comprising the amino acid sequence of

MSLLTEVETX₁₀X₁₁X₁₂NX₁₄WX₁₆CRCX₂₀X₂₁SSD (SEQ ID No:45),

wherein X_{10} , X_{11} , X_{12} , X_{14} , X_{16} , X_{20} , and X_{21} can be any amino acid, preferably a naturally occurring amino acid. In some embodiments, X_{10} is P or L; X_{11} is E or G; X_{12} is R or K; X_{14} is E or G; X_{16} is G or E; X_{20} is N, S or R; and X_{21} is G or D in combination with a carrier. In some embodiments, X_{10} is a proline. The immunogenic composition of claim 1, wherein the

5 M2 polypeptide comprises the amino acid sequence selected from the group consisting of SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:42, and SEQ ID NO:43.

In other embodiments, the peptide comprises the amino acid sequence:

- 10 VETX₄X₅RNX₈WX₁₀CX₁₂ (SEQ ID NO:19), LTEVETX₇X₈ (SEQ ID NO:48), LTEVETPX₈ (SEQ ID NO:46), or VETPX₅X₆NX₈W (SEQ ID NO:47), wherein X is any amino acid. In some embodiments, a peptide comprises an amino acid sequence of VETX₄X₅RNX₈WX₁₀CX₁₂, wherein X₄ is P or L, X₅ is E or G; X₈ is E or G; X₁₀ is G or E; and X₁₂ is N, S or R; in combination with a carrier. In some embodiments, a peptide
- 15 comprises an amino acid sequence of LTEVETX₇X₈ (SEQ ID NO:48), wherein X₇ is P or L, and X₈ is E or G, in combination with a carrier. In some embodiments, a peptide comprises an amino acid sequence of LTEVETPX₈ (SEQ ID NO:46), wherein X₈ is E or G, in combination with a carrier. In some embodiments, a peptide comprises an amino acid sequence of VETPX₅X₆NX₈W (SEQ ID NO:47), wherein X₅ is E or G; X₆ is R or K, X₈ is
- 20 E or G; in combination with a carrier.

In some embodiments, amino acid positions are varied that are about 25% or greater solvent accessible. Methods for predicting epitopes and/or solvent accessible residues are known to those of skill in the art and are available. For example, amino acid positions 10, 11, 12, 14, and 20-24 have about 25% or greater solvent accessibility. In a specific

25 embodiment, the fragment comprises amino acids 1-18, 2-24, 1-24, 7-18 or 7-24 of the extracellular domain of an M2 protein, such as SEQ ID NO:10. In other embodiments, the fragment comprises at least the peptide VETPIRNEWGCR (SEQ ID NO:20) and is a peptide of about 15, 16, 17, 18, 19, 20, 21, 22, or 23 amino acids. In some embodiments the peptide comprising VETPIRNEWGCR (SEQ ID NO:20) does not include the extracellular

30 domain consensus sequence (SEQ ID NO:10) or the full length M2 polypeptide.

Polynucleotides encoding M2 polypeptide fragments may be prepared by any of a number of conventional techniques. Polynucleotides encoding desired peptide fragments may be obtained by digesting the DNA with suitable restriction enzymes and isolating the desired fragment. Yet another suitable technique involves isolating and amplifying a DNA

35 fragment encoding a desired polypeptide fragment, by polymerase chain reaction (PCR). Oligonucleotides that define the desired termini of the DNA fragment are employed as the 5' and 3' primers in the PCR. Preferably, M2 polypeptide fragments share at least one

biological and/or immunological activity with M2 polypeptide comprising an amino acid sequence of SEQ ID NO:10.

Vectors that are useful for expression of the polynucleotides of the disclosure include plasmid vectors as well as viral vectors. Examples of such vectors are described
5 herein.

The nucleic acids disclosed herein are useful in immunogenic compositions and for producing M2 polypeptides as described herein.

B. Polypeptides

10 Another aspect of the disclosure relates to M2 polypeptides. The M2 polypeptides are useful as immunogenic compositions, especially for use in combination with the polynucleotides encoding M2 polypeptides.

The M2 polypeptides of the disclosure may be synthesized or prepared by techniques well known in the art. For recombinant production, nucleotide sequences
15 encoding the M2 polypeptides of the disclosure may be synthesized, and/or cloned, and expressed according to techniques well known to those of ordinary skill in the art. See, for example, Sambrook, et al., Molecular Cloning, A Laboratory Manual, Vols. 1-3, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989). In some embodiments, the polynucleotide sequences will be codon optimized for a particular recipient using standard
20 methodologies. For example, the DNA construct encoding the M2 protein can be codon optimized for expression in humans.

The polynucleotides encoding the polypeptides may be produced by standard recombinant methods known in the art, such as polymerase chain reaction (PCR) or reverse transcriptase PCR (Sambrook, et al., 1989, Molecular Cloning, A Laboratory Manual, Vols.
25 1-3, Cold Spring Harbor Press, Cold Spring Harbor, NY), or the DNA can be synthesized and optimized for expression in bacteria or eukaryotic cells. Primers can be prepared using the polynucleotide sequences provided, for example, in Tables 1-3 or that are available in publicly available databases. The polynucleotide constructs may be assembled from polymerase chain reaction cassettes sequentially cloned into a vector containing a selectable
30 marker for propagation in a host. Such markers include but are not limited to dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline, ampicillin, or kanamycin resistance genes for culturing in *E. coli* and other bacteria.

Representative examples of appropriate hosts include, but are not limited to, bacterial cells such as *E. coli*, *Streptomyces* and *Salmonella typhimurium*, fungal cells such as
35 yeast; insect cells such as *Drosophila* S2 and *Spodoptera Sf9*, animal cells such as CHO, COS, and Bowes melanoma cells, and plant cells. Appropriate culture medium and conditions for the above-described host cells are known in the art.

The M2 polypeptide can be recovered and purified from recombinant cell cultures by methods known in the art, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, and lectin chromatography.

Naturally occurring M2 polypeptides can be isolated from cloning out viral isolates from infected individuals at various times post infection. Such polypeptides can be obtained using primers for amplifying polynucleotide encoding M2. Such polypeptides may be utilized in the immunogenic compositions described herein.

In some embodiments, the M2 polypeptide composition includes one or more M2 polypeptides or immunogenic fragments thereof and one or more variable influenza components, one or more conserved influenza components, or a combination thereof. In some embodiments, the composition includes M2 proteins from a variety of influenza A strains of different subtypes. In other embodiments the composition comprises one or more M2 polypeptides from a H1N1 virus isolate. In some embodiments, the composition does not include the consensus M2 polypeptide having the sequence of SEQ ID NO:10 but rather an M2 polypeptide that has the sequence of a naturally occurring isolate. In some embodiments, the composition comprises a M2 polypeptide that comprises at least 80% sequence identity to SEQ ID NO:1 or SEQ ID NO:10.

In some embodiments, the disclosure includes an immunogenic composition comprising an M2 polypeptide or a polypeptide comprising the amino acid sequence of MSLLETVETX₁₀X₁₁X₁₂NX₁₄WX₁₆CRCX₂₀X₂₁SSD (SEQ ID No:45), wherein X₁₀, X₁₁, X₁₂, X₁₄, X₁₆, X₂₀, and X₂₁ can be any amino acid, preferably a naturally occurring amino acid. In some embodiments, X₁₀ is P or L; X₁₁ is E or G; X₁₂ is R or K; X₁₄ is E or G; X₁₆ is G or E; X₂₀ is N, S or R; and X₂₁ is G or D in combination with a carrier. In some embodiments, X₁₀ is a proline. The immunogenic composition of claim 1, wherein the M2 polypeptide comprises the amino acid sequence selected from the group consisting of SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:42, and SEQ ID NO:43.

In other embodiments, the peptide comprises the amino acid sequence: VETX₄X₅RNX₈WX₁₀CX₁₂ (SEQ ID NO:19), LTEVETX₇X₈ (SEQ ID NO:48), LTEVETPX₈ (SEQ ID NO:46), or VETPX₅X₆NX₈W (SEQ ID NO:47), wherein X is any amino acid. In some embodiments, a peptide comprises an amino acid sequence of VETX₄X₅RNX₈WX₁₀CX₁₂, wherein X₄ is P or L, X₅ is E or G; X₈ is E or G; X₁₀ is G or E; and X₁₂ is N, S or R; in combination with a carrier. In some embodiments, a peptide comprises an amino acid sequence of LTEVETX₇X₈ (SEQ ID NO:48), wherein X₇ is P or

L, and X_8 is E or G, in combination with a carrier. In some embodiments, a peptide comprises an amino acid sequence of LTEVETPX₈ (SEQ ID NO:46), wherein X_8 is E or G, in combination with a carrier. In some embodiments, a peptide comprises an amino acid sequence of VETPX₅X₆NX₈W (SEQ ID NO:47), wherein X_5 is E or G; X_6 is R or K, X_8 is E or G; in combination with a carrier.

An M2 polypeptide variant has at least about any number % from 70% to about 100% sequence identity to a reference M2 polypeptide such as a full-length mature M2 polypeptide reference sequence or the extracellular domain of M2. In an embodiment, the polynucleotide encodes an M2 variant polypeptide having at least about 70% sequence identity, at least about 75% sequence identity, at least about 80% sequence identity, at least about 85% sequence identity, at least about 90% sequence identity, at least about 95% sequence identity, or at least about 100% sequence identity to a full-length mature M2 reference sequence or the extracellular domain of M2. In an embodiment, the reference sequence is SEQ ID NO:1 or SEQ ID NO:10. Preferably, the M2 variants generate antibodies that cross react with heterologous M2 polypeptides and/or provide protective immunity.

M2 variants include naturally occurring variants having the sequence of M2 polypeptide isolated from nature from different influenza A subtypes and/or strains. Variations in the naturally occurring full-length M2 polypeptides described herein, can also be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Patent No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the M2 polypeptide that results in a change in the amino acid sequence of the M2 polypeptide as compared with a naturally occurring M2 polypeptide.

Guidance in determining which amino acid residue may be inserted, substituted or deleted without adversely affecting the desired activity may be found by comparing the sequence of the M2 polypeptide with that of homologous known protein molecules and minimizing the number of amino acid sequence changes made in regions of high homology. For example, when full length M2 proteins are aligned from several different isolates or strains, amino acid positions corresponding to positions 10, 11, 12, 14, 16, 19, 20, or 21 in the extracellular domain can have varied amino acids; amino acid positions corresponding to positions 51, 54, or 56 can have varied amino acids, and/or amino acid positions corresponding to 84, 88, or 95 can have varied amino acids. In some embodiments, variants may include amino acid substitution so that the amino acid sequence of the extracellular domain of M2 corresponds to that of a different subtype. (See Table 3 for examples) For example, positions 10, 11, 14, 16 and 20 may have amino acids L, T, G, and S respectively as compared to P, I, E, G and N of the M2e domain of SEQ ID NO:11. In addition solvent

accessible residues may be determined using standard methodologies and may include residue 2, 5, 6, 8, 9, 11-14, 18, and 20-24. Amino acids in solvent accessible positions may be varied without disrupting structure.

Functional domains can also be identified in those M2 polypeptides that have
5 homology to known polypeptides. For example, certain positions in the extracellular domain show more variability than others. These positions can be identified using sequence alignments and changes made to those amino acid positions showing high variability (e.g. 3 or more different amino acids in that position when a number of sequences are aligned). See Table 3. In some embodiments, the first nine amino acids are not varied. The sequences of
10 these functional domains can be compared and aligned to other known sequences that may be provided at the Los Alamos website or GenBank, and locations of amino acid positions for substitutions can be identified as those positions that show a high degree of variability in amino acids, i.e., at least 3 different amino acids are found at that position when different sequences are aligned and compared or have a lower percentage of sequence identity i.e.,
15 less than 90% sequence identity. When sequences are aligned, the positions that show variability can either have conservative amino acid substitutions or non-conservative amino acid substitutions. If the position has conservative amino acid substitutions, that would indicate that the amino acid substituted at that position should be of the same type as those observed to be at that position in naturally occurring proteins. For examples of such
20 substitutions, see Table 4.

Amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with a serine, i.e., conservative amino acid replacements. Insertions or deletions may optionally be in the range of about 1 to 5 amino acids. The variation allowed may be
25 determined by systematically making insertions, deletions or substitutions of amino acids in the sequence and testing the resulting variants for activity exhibited by the full-length or mature naturally occurring sequence. Preferably, M2 variants have the biological activity of the source molecule or are bound by anti-M2 antibodies.

The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed
30 mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the M2
35 polypeptide variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are

relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant [Cunningham and Wells, Science, 244: 1081-1085 (1989)].

- 5 Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)].

- In some embodiments, the polypeptide comprises an immunogenic fragment of an M2 protein including at least 10 amino acids, more preferably 12 amino acids, more
10 preferably 18 amino acids, more preferably 20 amino acids, more preferably 24 amino acids, and most preferably about 30 contiguous amino acids. In some embodiments, the fragment comprises at least one T cell epitope. In some embodiments, a T cell epitope comprises the amino acid sequence of amino acids 2 to 24 of the M2 protein. In some embodiments, the fragment comprises an amino acid sequence of VETX₄X₅RNX₈WX₁₀CX₁₂, LTEVETPX₈
15 (SEQ ID NO:46) or VETPX₅X₆NX₈W (SEQ ID NO:47), wherein X is any amino acid. In some embodiments, X is not proline or cysteine. In some embodiments, a peptide comprises an amino acid sequence of VETX₄X₅RNX₈WX₁₀CX₁₂, wherein X₄ is P or L, X₅ is E or G; X₈ is E or G; X₁₀ is G or E; and X₁₂ is N, S or R; in combination with a carrier. In some embodiments, a peptide comprises an amino acid sequence of LTEVETPX₈ (SEQ ID
20 NO:46), wherein X₈ is E or G, in combination with a carrier. In some embodiments, a peptide comprises an amino acid sequence of VETPX₅X₆NX₈W (SEQ ID NO:47), wherein X₅ is E or G; X₆ is R or K, X₈ is E or G; in combination with a carrier.

- In a specific embodiment, the fragment comprises amino acids 1-18, 1-24, 2-24, 7 - 18 or 7-24 of the extracellular domain of an M2 protein, such as SEQ ID NO:10. In other
25 embodiments, the fragment comprises at least the peptide VETPIRNEWGCR (SEQ ID NO:20) and is a peptide of about 15, 16, 17, 18, 19, 20, 21, 22, or 23 amino acids. In some embodiments the peptide comprising VETPIRNEWGCR (SEQ ID NO:20) excludes the extracellular domain consensus sequence (SEQ ID NO:10) or the full length M2 sequence.

- The M2 polypeptide, or fragment thereof, may be in a modified form, such as a
30 fusion protein, and may include secretion signals and/or additional heterologous functional regions. For example, a region of additional amino acids may be added to the N-terminus or C-terminus of the polypeptide to facilitate detection or purification, improve immunogenicity, improve half-life, or improve persistence in the host cell during, for example, purification or subsequent handling and storage. Examples of additional amino
35 acids include peptide tags that may be added to the polypeptide to facilitate detection and/or purification. Such peptide tags include, but are not limited to, His, HA, Avi, biotin, c-Myc, VSV-G, HSV, V5, or FLAG™. Examples of a polypeptide that can enhance

immunogenicity include bovine serum albumin, and/or keyhole lymphocyte hemocyanin (KLH). Examples of molecules that improve half-life include polyethylene glycol.

One or more M2 polypeptides or immunogenic fragments thereof may be utilized in the compositions or methods of the invention. An immunogenic composition may further
5 comprise an adjuvant or a cytokine as described herein.

C. Animal models

A variety of animal models are available for testing of any of the immunogenic compositions described herein. For example, well-established models include mice, poultry, ferrets, pigs, guinea pigs, or non-human primates. An animal model that provides for an
10 immune response and has a response to challenge with infectious virus is suitable for testing of the immunogenic compositions.

Mouse models systems are available and in some embodiments, include challenge with mouse adapted influenza strains. The mouse model system includes immunizing the mice with an M2 polypeptide or fragment thereof and/or a polynucleotide encoding an M2
15 polypeptide or fragment thereof. After the mice are immunized, the mice are challenged with an influenza virus strain and evidence of infection can be determined by viral titers in tissues including the respiratory tract or in the case of systemic infection, other tissues as well, and/or by weight loss and/ or death. Suitable mice include BALB/c mice, as well as any of the commercially available mice such as knockout mice and mice that have a human
20 immune system.

Another model system for influenza infection is ferrets. Ferrets are naturally susceptible to infection with human influenza viruses, as well as avian, equine, and swine influenza viruses. Influenza virus infection in ferrets can be detected by detecting viral titers, and/or weight loss, fever, and respiratory symptoms such as nasal discharge. Other
25 symptoms may be detected in ferrets having a systemic infection including neurological symptoms, diarrhea, and lethargy.

II. Uses and Methods

The present disclosure is also directed to uses and methods for immunizing an
30 animal, including a human, other mammal, or bird, with the immunogenic compositions of the invention to inhibit, control, or prevent influenza infection.

In an embodiment, the method comprises administering to an animal an immunogenic effective amount of an immunogenic composition. An immunogenic effective amount is an amount of polynucleotide or other vector that induces an immune
35 response to the encoded polypeptide when administered to a host, for example an animal. In an embodiment, the animal is a human, pig, horse, birds including domestic birds, or other animals, especially those used in animal models such as mouse, rat, ferret, or non-human

primate. In an embodiment, the polynucleotides are incorporated into host cells in vivo and an immunogenic effective amount of the encoded M2 polypeptide or fragment thereof is produced in vivo. The actual amount of the immunogenic composition may vary depending on the animal to be immunized, the route of administration and adjuvants.

5 Immunogenic dosages can be determined by those of skill in the art. The immune response may be indicated by T and/or B cell responses. Typically, the immune response is detected by the presence of antibodies that specifically bind to a particular M2 or M2e polypeptide. Methods of detecting antibodies to M2 polypeptides are known to those of skill in the art and include such assays as ELISA assays, western blot assays, and
10 competition assays. Methods of detecting T cell responses include ELISPOT assays, ICS assays, and cytotoxicity assays. The particular region of the M2 protein that is stimulating a T cell or antibody response can be mapped using peptide scans of the M2 protein.

In some embodiments, the immunogenic composition administered to an animal includes a polynucleotide or polynucleotides encoding one or more M2 polypeptides or
15 immunogenic fragments thereof and one or more of variable influenza components, one or more conserved influenza component, or a combination thereof. In some embodiments, the polynucleotide encodes one or more full length M2 proteins from a H1N1 virus or immunogenic fragment thereof. In an embodiment, the variable influenza component is HA, NA, an immunogenic fragment thereof, or combination thereof. In an embodiment, the
20 conserved influenza component is M1, NP, PA, PB1, PB2, NS1, NS2, an immunogenic fragment thereof or combination thereof. In some embodiments, the same polynucleotide does not encode an influenza component such as M1 and/ or NP. In other embodiments, the polynucleotide does not encode an influenza component selected from the group consisting of M1, NP, PA, PB1, PB2, NS1, NS2, an immunogenic fragment thereof and combinations
25 thereof.

In an embodiment, an animal is immunized with an immunogenic composition of the invention and then boosted one or more times with the immunogenic composition. In an embodiment, the animal is boosted about 2 to about 4 weeks after the initial administration of the immunogenic composition. If the animal is to be boosted more than once, there is
30 about a 2 to 12 week interval between boosts. In an embodiment, the animal is boosted at about 12 weeks and about 36 weeks after the initial administration of the immunogenic composition. In another embodiment, the animal is a mouse and the mouse is boosted 3 times at 2 week intervals. In yet another embodiment, the animal is a primate and the primate is boosted 1 month and 6 months after the initial administration of the immunogenic
35 composition. The dose used to boost the immune response can include one more cytokines, chemokines, or immunomodulators not present in the priming dose of the immunogenic composition.

The methods of the invention also include prime-boost immunization methods utilizing the immunogenic compositions of the invention. Providing M2 in different forms in the prime and boost maximizes the immune response to the M2. In some embodiment, an animal is primed with a polynucleotide encoding an M2 polypeptide in one vector. The animal may be primed 1 to 8 times. Typically there is a 1, 2, or 3 week interval between administrations. In an embodiment, the animal is primed 3 times at 2 week intervals. The primed animal is then boosted with an M2 polypeptide or polynucleotide encoding an M2 polypeptide in a second vector that is different from the first vector. In an embodiment, the animal is boosted with the second vector at least 2 weeks after the last dose of the first viral vector. In an embodiment, the animal is boosted with the second vector at 4 weeks after the last dose of the first viral vector. In another embodiment, the animal is primed with a polynucleotide encoding an M2 polypeptide in one vector at 0, 4 and 26 weeks, and then boosted with an M2 polypeptide or polynucleotide encoding an M2 polypeptide on a second vector that is different than the first vector at 46 and 66 weeks. The dose used to boost the immune response can include one more cytokines, chemokines, immunomodulators, or influenza antigens not present in the priming dose.

In some embodiments, the second vector is a viral vector comprising a polynucleotide encoding at least one M2 or M2e polypeptide or a combination of M2 and/or M2e polypeptides from different influenza A subtypes or strains. Viral delivery vectors are known and commercially available. Examples of viral vectors include, but are not limited to, recombinant poxvirus including vaccinia virus, lentivirus, or adenovirus. In an embodiment, the viral vector is adenovirus type 5. Examples of commercially available viral delivery vectors include, but are not limited to, VIRAPOWERTM lentivirus expression system, VIRAPOWERTM adenovirus expression system (Invitrogen, Carlsbad, CA), and ADENO-X adenovirus expression system (Clontech, Mountain View, CA).

The methods of the invention also include methods for protecting an animal against a lethal influenza challenge. In some embodiments, the method of the disclosure provides for protective immunity against an infection with virus of the same subtype (whether or not mismatched for M2 sequence), and against heterosubtypic virus. In an embodiment, the influenza is a highly pathogenic H5N1. In an embodiment, the influenza is A/Thailand/SP83/04 or A/Honk Kong/156/97.

The method of the invention includes immunizing the animal as described herein with a plasmid vector containing a polynucleotide encoding an M2 polypeptide having at least about 80% sequence identity with an amino acid sequence of SEQ ID NO:1 and/or SEQ ID NO:10 and then boosting the animal as described herein with a viral vector containing a polynucleotide encoding an M2 polypeptide having at least about 80% sequence identity with an amino acid sequence of SEQ ID NO:1 and/or SEQ ID NO:10. In

an embodiment, the amino acid sequence comprises SEQ ID NO:1. In an embodiment, the plasmid vector is pVR1012. In an embodiment, the viral delivery vector is adenovirus type 5. In some embodiments, the M2 polypeptide in the plasmid and viral vector have the same amino acid sequence and in other embodiments the M2 proteins have different sequences.

5 A method for inhibiting influenza A infection comprises administering to the subject an immunogenic composition as described herein, and boosting the subject with an M2 polypeptide composition, wherein the M2 polypeptide has at least 80% amino acid sequence identity to SEQ ID NO:1 or SEQ IDNO:10. In some embodiments, the immunogenic composition comprises a plasmid expression vector or viral expression vector
10 or both. In some embodiments, the immunogenic composition encodes an M2 polypeptide from A/H1N1 or A/H5N1. In other embodiments, expression vector comprises a polynucleotide encoding an M2 polypeptide having an amino acid sequence of SEQ ID NO:1 and the M2 polypeptide composition comprises an M2 polypeptide having the amino acid sequence of SEQ ID NO:1. In some cases, the M2 polypeptide composition comprises
15 at least two M2 polypeptides, wherein each polypeptide is an M2 polypeptide from a different influenza A subtype. Optionally, the M2 polypeptide encoded by the expression vector and the M2 polypeptide in the M2 polypeptide composition have the same amino acid sequence. The methods may also involve administering at least one adjuvant or immunomodulator as described herein.

20 The methods of the invention can be used to immunize birds to prevent the spread of avian influenza. In an embodiment, the avian influenza is H5N1. In an embodiment, the birds are domesticated poultry.

Any mode of administration can be used in the methods of the inventions so long as the mode results in the expression of the desired peptide or protein, in the desired tissue, in
25 an amount sufficient to generate an immune response to influenza A and/or to generate a prophylactically or therapeutically effective immune response to influenza A in an animal. The immunogenic compositions of the invention can be administered by intramuscular (i.m.), subcutaneous (s.c.), or intrapulmonary route in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants, or vehicles. Other
30 suitable routes of administration include, but are not limited to intratracheal, transdermal, intraocular, intranasal, inhalation, intracavity, and intravenous (i.v.) administration. Transdermal delivery includes, but is not limited to intradermal, transdermal, and transmucosal administration. Intracavity administration includes, but is not limited to administration into oral or nasal cavities. The immunogenic compositions can be coated
35 onto particles or nanofibers for delivery or formulated in liposomes.

Administration modes of the present invention include needle injection; catheter infusion; biolistic injectors; particle accelerators such as, for example, "gene guns" or

pneumatic "needleless" injectors such as Med-E-Jet (Vahlsing et al., 1994, J. Immunol. Methods, 171:11-22), Pigjet (Schrijver et al., 1997, Vaccine, 15:1908-1916), Biojector (Davis et al., 1994, Vaccine, 12:1503-1509; Gramzinski et al., 1998, Mol. Med., 4: 109-118), AdvantaJet (Linmayer et al., 1986, Diabetes Care, 9:294-297), or Medi-jector (Martins and Roedl, 1979, Occup. Med., 21:821-824); gelfoam sponge depots; other commercially available depot materials such as, for example, hydrogels, osmotic pumps, oral or suppositorial solid (tablet or pill) pharmaceutical formulations, topical skin creams, and decanting, polynucleotide coated suture (Qin, Y., et al., 1999, Life Sci., 65: 2193-2203), or topical applications during surgery. Certain modes of administration are intramuscular needle-based injection and pulmonary application via catheter infusion. Energy-assisted plasmid delivery (EAPD) methods may also be employed to administer the compositions of the invention. One such method involves the application of brief electrical pulses to injected tissues, a procedure commonly known as electroporation. See generally Mir et al., 1999, Proc. Natl. Acad. Sci USA, 96:4262-7; Hartikka et al., 2001, Mol. Ther., 4:407-15; Mathiesen, 1999, Gene Ther., 6:508-14; Rizzuto et al., 2000, Hum. Gen. Ther. 11:1891-900.

The present disclosure is also directed to kits for practicing the methods of the invention. In some embodiments, the kit includes a plasmid expression vector of the invention, a viral vector of the invention, and instructions for priming an animal (including human) with the plasmid expression vector and boosting the animal with the viral vector. In an embodiment, the kit includes a plasmid expression vector comprising at least one polynucleotide encoding an M2 polypeptide having at least about 80% sequence identity with an amino acid sequence of SEQ ID NO:1 and/or SEQ ID NO:10 or an immunogenic fragment thereof, a viral delivery vector comprising at least one polynucleotide encoding an M2 polypeptide having at least about 80% sequence identity with an amino acid sequence of SEQ ID NO:1 and/or SEQ ID NO:10 or an immunogenic fragment thereof, and instructions for priming an animal with the plasmid expression vector and boosting the animal with the viral delivery vector.

In some embodiments, the kit comprises a plasmid expression vector and a viral vector each comprising a polynucleotide encoding an M2 polypeptide from A/H1N1 or A/H5N1. For example, the plasmid expression vector and the viral vector may each comprise a polynucleotide encoding an M2 polypeptide having an amino acid sequence of SEQ ID NO:1 or the plasmid expression vector and the viral vector each comprise a plurality of polynucleotides encoding at least two M2 polypeptides, wherein each polynucleotide encodes an M2 polypeptide from a different influenza A subtype. In some embodiments, the kit may further comprise an M2 polypeptide composition. In some cases, the M2 polypeptide of the M2 polypeptide composition and the M2 polypeptide encoded by the plasmid and viral expression vector have the same sequence. In some embodiments, the

kit may further comprise at least one adjuvant or immunomodulator. The adjuvant or immunomodulator can be encoded by a polynucleotide. In a specific embodiment, the adjuvant is CTA1-DD alone or in combination with ISCOM.

5 The following examples are provided for illustrative purposes only, and are in no way intended to limit the scope of the present disclosure.

EXAMPLES

Example 1

10 Immunization of Mice with the Extracellular Portion of M2 Protects Against Homologous and Heterologous Influenza A Challenge

The extracellular portion of M2 (M2e) is highly conserved, particularly among H1, H2, and H3 viruses. There are differences, however, in M2e of H5, H7, and H9 isolates that could negate the universality of M2e-specific immunity (see antibody results in Fan et al.,
15 2004, Vaccine, 22:2993-3003). See, for example, Table 3 supra. To determine the potential for M2e-mediated protection against influenza, we tested the breadth of protection conferred by immunization with an M2e consensus sequence (M2e-con; SEQ ID NO:10).

Methods

Peptide Synthesis, Purification and Characterization

20 Synthetic M2e peptides were synthesized at the 0.1 mmole synthesis scale on Applied Biosystems peptide synthesizer model 433 (Foster City, California) (1. Merrifield, R.B.: Solid Phase Peptide Synthesis, in Gutte B., editor. Peptides Synthesis, Structure and Applications, San Diego: Academic Press 1995, p.93) with 9-fluorenylmethoxycarbonyl (Fmoc) chemistry mediated by 2-[1-H-Benzotriazole-1-yl]-1-13.3-tetramethyluronium
25 hexafluorophosphate/1-hydroxybenzotriazole activation (HBTU/HOBt). Amino acids were purchased from Applied Biosystems and from Anaspec (San Jose, California). One millimole of each residue was used for single coupling. Two millimoles of amino acids were required for double coupling. The following side chain protection groups were used: Lys (Boc), Ser (tBu), Glu (OtBu), His (Trt), Asp (OtBu), Asn (Trt), Arg (Pbf), Gln (Trt), Trp
30 (Boc), Cys (Trt), Tyr (OtBu) and Thr (tBu).

Peptide cleavage

Following final deprotection of the N-terminal amino acid by piperidine, peptides were cleaved for approximately 3 – 3 and 1/2 hours with a cocktail containing TFA, water, triisopropylsilane and phenol (95%:1.25:1.5:2.25) and 250 mg DTT per 10 ml of cocktail.
35 Following cleavage, peptides were precipitated and washed with methyl tert-butyl ether (MTBE). Peptide pellets were resuspended in 0.1% TFA or 10-30% acetic acid and were either subjected immediately to RP-HPLC purification or were lyophilized for long-term storage.

Purification of peptides

Peptides were purified by RP-HPLC using Waters DeltaPak C18 column (19 mm x 300 mm, 15 microns, 300 Angstroms pore size, 5 μ M particle size) and a linear gradient of 0.1% TFA/water and 0.1% TFA/acetonitrile. Fractions containing the desired peptide products were evaporated to remove acetonitrile prior to lyophilization for long-term storage (refrigerated at 4°C or frozen at -20°C).

Peptide Characterization

The identity of peptide products was established by matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis (MALDI-TOF MS). Purity was determined by N-terminal amino acid sequence analysis.

Mass Spectrometry Analysis

Mass spectrometry analysis was used to identify and confirm the molecular mass of the product. Mass spectrometry was conducted on a Voyager DE-RP MALDI-TOF mass spectrometer (PE Biosystems, Foster City, CA, USA), using alpha cyano-4-hydroxy cinnamic acid in 0.1% TFA, 50% acetonitrile. All mass spectrometry analyzes were conducted in the positive ion mode.

N-terminal amino acid sequence analysis

The N-terminal sequence and purity of the peptide product was determined by Edman sequencing on Applied Biosystems Model 494A peptide/protein sequanator, Foster City, CA, USA, using the manufacturer's software.

Conjugation to KLH

Peptides were conjugated to maleimide-activated KLH (Pierce) via cysteine residues, and then dialyzed into PBS.

Table 5 shows the amino acid sequence of the synthetic M2e peptides.

Strain	Abbrev.	Subtype	M2e Sequence	SEQ ID NO:
Consensus	M2e-con		MSLLTEVETPIRNEWGCRCNDSSD	10
A/PR/8	M2e-PR8	H1N1	MSLLTEVETPIRNEWGCRCNGSSD	11
A/FM-MA	M2e-FM	H1N1	MSLLTEVETPTKNEWECRCNDSSD	12
A/HK/483	M2e-H5	H5N1	MSLLTEVETLTRNGWGCRCSDSSD	14

Immunization

Female BALB/cAnNCR mice from the Division of Cancer Treatment, National Cancer Institute, Frederick, Maryland, were immunized i.p. with M2e-con, M2e-FM, or M2e-H5 peptide conjugated to KLH, 40 μ g in complete Freund's adjuvant (emulsified 1:1 with antigen in PBS), total volume of 200 μ l. Three weeks later, the mice were boosted i.p. with M2e-con, M2e-FM, or M2e-H5 peptide respectively conjugated to KLH, 40 μ g in incomplete Freund's Adjuvant, total volume of 200 μ l.

ELISA for antibodies

The mice were bled 13 days post-immunization and the sera were analyzed for anti-influenza antibodies by ELISA on plates coated with synthetic M2e-PR8, M2e-FM, or M2e-H5 peptides.

5 **Challenge**

Influenza virus strain A/PR/8 was propagated in the allantoic cavity of embryonated hen eggs at 34°C for 48-72 hours. Influenza strain A/FM-MA was propagated in BALB/c mice by intranasal administration followed by harvesting of lungs at day 4, homogenization, and centrifugation. The supernatant was aliquoted for use as a viral stock. Mice were
10 challenged intranasally with virus in 50 µl per mouse in phosphate buffered saline (PBS, pH 7.0) under anesthesia with isoflurane. The mice were monitored for body weight and mortality until all animals had succumbed to infection or were recovering body weight.

Statistical Analysis

All statistical analysis was done with SigmaStat Software v3.11 (Systat Software, Point Richmond, CA). Weight loss was compared using one-way ANOVA statistical
15 analysis, followed by pairwise multiple comparison using the Holm-Sidak method. Comparison of survival was done using Log-Rank test.

Results

A/FM-MA (H1N1) provides a model of challenge with a virus that has a similar
20 amount of divergence in the extracellular portion of M2 as H5N1, but unlike H5N1 does not require BSL-3⁺ containment. Immunization of mice with peptide extracellular domains M2e-consensus, M2e-FM (H1N1) or M2e-H5 conjugated to KLH induced specific IgG responses to M2e-PR8 (H1N1)(Fig. 1A), M2e-FM (H1N1)(Fig. 1B), and M2e-H5 (Fig. 1C) respectively. Immunization with M2e-con, M2e-FM, or M2e-H5 primed potent, subtype
25 cross-reactive antibody responses in the mice. Sera from mice immunized with M2e-con crossreacted with M2e-PR/8, M2-FM, and M2e-H5. Sera from mice immunized with M2e-FM crossreacted with M2e-PR/8 and M2-H5. Sera from mice immunized with M2e-H5 crossreacted with M2e-PR/8, M2-FM, and M2-H5 peptides. Immunization with M2e-con primed a cross-reactive antibody response to M2e-H5 that was substantially similar in
30 potency to the antibody response to M2-H5 primed by immunization with M2e-H5 (Fig. 1C).

To determine whether immunization with M2e-con (filled squares), M2e-FM (filled diamonds), or M2e-H5 (filled triangles) protected the mice against infection, mice were
35 challenged with sublethal and lethal doses of the same virus (in the case of mice immunized with M2e-FM; virus A/FM-MA) or mismatched virus (virus A/PR/8 or A/FM-MA in all other cases) (Figs. 2A, 2B, 3A and 3B). Mice immunized with M2e-con, M2e-FM, or M2e-H5 had only minor weight loss after challenge with H1N1 strain A/PR/8 or A/FM-MA,

while mice immunized with KLH control lost weight dramatically after challenge with A/PR/8 (Fig. 2A). One hundred percent of mice immunized with M2e-con or M2e-FM and almost 90% of mice immunized with M2e-H5 survived the A/PR/8 challenge dose that was lethal to almost 60% of the KLH control mice (Fig. 3A).

5 Mice immunized with M2e-con or M2e-FM lost only about 10% of their body weight after challenge with A/FM-MA and were almost fully recovered 20 days post-challenge (Fig. 2B). Mice immunized with M2e-H5 had significant weight loss at 6 and 8 days post challenge but had only minor weight loss at 13, 15, and 20 days post challenge (Fig. 2B). Mice immunized with KLH as a control had significant weight loss at 6 and 8
10 days post-challenge, and only about 20% of them survived the A/FM-MA challenge dose. The progressive regaining of weight starting at day 11 in KLH control mice (Figure 2B) represents recovery of the few survivors. 100% of mice immunized with M2e-con or M2e-FM and almost 80% of mice immunized with M2e-H5 survived the A/FM-MA challenge dose that was lethal to almost 80% of the control mice (Fig. 3B).

15

Example 2

Immunization of Mice with a Plasmid DNA Expression Vector Encoding M2 Confers Protection Against Lethal Challenge with Homosubtypic and Heterosubtypic Influenza A Virus

20

To determine the potential for M2-mediated protection against different subtypes of influenza, we tested immunity induced by a plasmid DNA expression vector encoding full length M2 from one influenza A subtype against challenge with a different subtype.

Vectors

25

Plasmid DNA expression constructs comprising a polynucleotide encoding full length M2 from influenza A H1N1 were constructed as follows. pCR3-M2 was subcloned from the vector pCR3-M previously derived from A/PR/8 virus by RT-PCR (Huang, et al., Virology 287: 405-416, 2001). To modify the sequence to the more widely shared M2e sequence, two DNA fragments, with 72 overlapping nucleotides, covering the full-length
30 M2 cDNA sequence (SEQ ID NO:39) from influenza virus strain A/PR/8/34 (PR/8, H1N1), with Kozak sequence at its 5' end, were subcloned from pCR3-M2 (pCr3 vector backbone purchased from Invitrogen, Carlsbad, CA) by PCR using the following two sets of primers:

Forward primer 1 (SEQ ID NO:21)

AAGGAAAAAAGCGGCCGCCACCATGAGTCTTCTAACCGAG

35

Forward primer 2(SEQ ID NO:22)

GCAACGATTCAAGTGATCCTCTCGCTATTGCCGCAAATATCATTGG

Reverse primer 1 (SEQ ID NO:23)

GGAAGATCTTTACTCGAGCTCTATGCTGACAAAATGACC

Reverse primer 2 (SEQ ID NO:24)

GAATCCACAATATCAAGTGCAAGATCCCAATGATATTTGCGGC

The PR8/M2 fragment was subsequently cloned into the NotI/BglII site of plasmid VR1012 (Vical, San Diego, CA), a plasmid DNA expression vector for direct injection into skeletal muscle (Hartikka et al., 1996, Hum. Gene Ther., 7:1205-1217).

The nucleotide sequence of the PR8/M2 insert in plasmid VR1012-M2 was confirmed by restriction digestion and agarose gel electrophoresis. This vector will be referred to as M2-DNA in the examples that follow. The sequence of the M2 polypeptide encoded by the nucleic acid sequence referred to as M2 DNA (SEQ ID NO:39) herein comprises an M2 polypeptide similar to that of strain A/PR/8 including a change at amino acid position 21 from a glycine to aspartic acid. (See Table 1; SEQ ID NO:40)

Plasmid DNA vectors expressing B/NP (B/NP-DNA), M1 DNA, or A/PR8/M (A/M-DNA) were constructed as described in Epstein et al., 2000, Int. Immunol., 12:91-101 and Epstein et al., 2002, Emerg. Infect. Dis., 8:796-801.

Bulk preparations of all plasmids were prepared by DNA Technologies (Gaithersburg, MD) by fermentation in Escherichia coli and two rounds of CsCl purification. Quality control testing of the bulk preparations of plasmids by restriction digestion and agarose gel electrophoresis confirmed plasmid size and purity. In order to avoid non-specific polyclonal effects in vivo, all lots of plasmid DNA were tested for endotoxin using the LAL assay. Endotoxin levels were less than 1 EU/100µg dose.

Immunization

Female BALB/cAnNCR mice from the Division of Cancer Treatment, National Cancer Institute, Frederick, Maryland, were immunized intramuscularly in the quadriceps with 50 µg pVR1012-M2, pVR1012-M1, pVR1012-B/NP, or pVR1012-A/M in low endotoxin PBS (AccuGENE, Cambrex, East Rutherford, NJ) to each leg. Three doses of the plasmid expression vectors were given 2 weeks apart. Negative control mice were immunized as described above with pVR1012-B/NP plus B/NP-Ad.

Challenge

Influenza virus strain A/PR/8 was propagated in the allantoic cavity of embryonated hen eggs at 34°C for 48-72 hours. Influenza strain FM-MA was propagated in BALB/c mice by intranasal administration followed by harvesting of lungs at day 4, homogenization, and centrifugation. The supernatant was aliquoted for use as a viral stock. Influenza virus A/Philippines/2/82/X-79 (X-79), an A/H3N2 reassortant, (abbreviated A/Phil) was propagated in mice as described for FM-MA virus. Mice were challenged intranasally with virus in 50 µl per phosphate buffered saline (PBS, pH 7.0) under anesthesia with isoflurane. The mice were monitored for body weight and mortality until all animals had succumbed to infection or were recovering body weight.

Results

Immunization with DNA encoding M2 provided protection against homosubtypic influenza challenge. One hundred percent of mice immunized with M2 DNA (filled squares) survived a challenge dose of A/PR/8 (7 LD₅₀) that was lethal to 100% of the control mice (mice immunized with B/NP-DNA; open squares) (Fig. 4). M-A/PR/8 DNA was partially protective against homologous influenza challenge. Sixty percent of mice immunized with M-A/PR/8 DNA (triangles) survived the challenge dose of A/PR/8 that was lethal to 100% of control mice (Fig. 4).

Immunization with DNA encoding M2 from an H1N1 strain protected against challenge with a divergent H1N1 virus and an H3N2 virus. One hundred percent of mice immunized with M2 DNA (filled squares) survived a challenge dose of heterosubtypic A/Phil (7 LD₅₀) that was lethal to approximately 40% of the control mice (mice immunized with B/NP-DNA; open squares) (Fig. 5A). Approximately 90 percent of mice immunized with M-A/PR/8 DNA (triangles) survived the challenge dose of A/Phil (Fig. 5A).

Approximately 60% of mice immunized with M2 DNA survived the challenge dose of A/FM-MA (7 LD₅₀) that was lethal to approximately 90% of control mice (Fig. 5B). Note that A/FM-MA virus has an M2e amino acid sequence divergent from that of A/PR/8 and that encoded by the M2 DNA construct. (See Table 3) M-A/PR/8 DNA could not protect against this more virulent challenge.

Immunization with M-DNA (diamonds), M1-DNA (triangles), M2-DNA (filled squares), and M1+M2 DNA (asterisks) revealed that M2 is the protective component of matrix immunization in BALB/c mice (Fig. 6). One hundred percent of mice immunized with M2-DNA or M1+M2-DNA and 90% of mice immunized with M-DNA survived a challenge with homosubtypic A/PR/8 (7 LD₅₀) (Fig. 6). In contrast, only 20% of mice immunized with M1-DNA survived the challenge dose. These results show that M2 is the protective component of matrix immunization.

Example 3

DNA Prime-Recombinant Adenoviral Boost Immunization to M2 Confers Protection Against Lethal Challenge with Influenza A Viruses With Varying Degrees of M2e divergence

To enhance the potency of potential M2-mediated protection against different subtypes of influenza, we tested an immunization regimen that included first immunizing animals with a DNA construct encoding full-length M2 from influenza A followed by a boost with an adenoviral vector also encoding full length M2 from influenza A.

Methods

Vectors

Plasmid DNA expression constructs comprising a polynucleotide encoding full length M2 similar to that of A/Puerto Rico/8/34 including a change at amino acid position 21 from glycine to aspartic acid were constructed as in Example 2 and are referred to as M2DNA. Plasmid DNA vectors expressing B/NP (B/NP-DNA) were constructed as described in Epstein et al., 2002, *Emerg. Infect. Dis.*, 8:796-801.

Bulk preparations of all plasmids were prepared by DNA Technologies (Gaithersburg, MD) by fermentation in *Escherichia coli* and two rounds of CsCl purification. Quality control testing of the bulk preparations of plasmids by restriction digestion and agarose gel electrophoresis confirmed plasmid size and purity. In order to avoid non-specific polyclonal effects in vivo, all lots of plasmid DNA were tested for endotoxin using the LAL assay. Endotoxin levels were less than 1 EU/100µg dose.

Replication incompetent adenovirus expressing full-length A/PR8/M2 modified by a single change at position 21 from glycine to aspartic acid (M2-Ad) was constructed using the Gateway cloning and ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. In brief, the A/PR8/M2 cDNA from pVR1012-M2 was cloned by PCR into the pENTR/D-TOPO Gateway vector, followed by transfer into the pAd/CMV/V5-DEST Adenoviral Gateway vector by LR clonase reaction. Integrity and proper insertion of the cloned A/PR8/M2 cDNA was confirmed by sequencing. M2-Ad was generated by transfection of 293A cells with the pAd/CMV-M2. M2 expression was confirmed by immunohistochemical staining of M2-Ad-infected MDCK cells with M2-specific polyclonal sera (data not shown). Adenoviral vectors expressing A/NP (A/NP-Ad) and B/NP (B/NP-Ad) were used as positive and negative controls, and have been reported previously (Epstein et al., 2005, *Vaccine*, 23:5404-5410). Viruses were amplified, purified twice through a CsCl gradient, and stored in PBS plus 13% glycerol at -20°C.

High titered stocks of M2-Ad, A/NP-Ad and B/NP-Ad were prepared and tested for replication competent adenovirus (RCA) by ViraQuest, Inc (North Liberty, IA). Adenovirus stocks were stored in 3% sucrose/PBS at a concentration of $1-2 \times 10^{12}$ particles/ml and confirmed as RCA-negative.

Immunization

Female BALB/cAnNCR mice from the Division of Cancer Treatment, National Cancer Institute, Frederick, Maryland, were immunized intramuscularly in the quadriceps muscle of each leg with 50 µg M2-DNA in low endotoxin PBS (AccuGENE, Cambrex, East Rutherford, NJ). Three doses of the plasmid expression vectors were given 2 weeks apart. The mice were boosted with 10^{10} particles/mouse M2-Ad two weeks after the last dose of

M2-DNA. Positive and negative control mice were immunized as described above with or A/NP-DNA + A/NP-Ad, or B/NP-DNA + B/NP-Ad, respectively.

ELISA for antibodies

The mice were bled 10-14 days post-immunization and the sera were analyzed for anti-M2 antibodies by ELISA on plates coated with synthetic M2e-PR/8, M2e-FM, or M2e-H5 peptides as described in Example 1.

Challenge

Influenza viruses were propagated in the allantoic cavity of embryonated hen eggs at 34°C for 48-72 hours. Mice were challenged intranasally with virus in 50 μ l per phosphate buffered saline (PBS, pH 7.0) under anesthesia with isoflurane, or with ketamine in the experiment of Fig. 8. The mice were monitored for body weight and mortality until all animals had succumbed to infection or were recovering body weight.

In vivo depletion

The following monoclonal antibodies were used for in vivo depletion of CD4 and CD8 in mice: GK1.5 specific for mouse CD4; 2.43 specific for mouse CD8; and SFR3-DR5 specific for human leukocyte antigen (used as a negative control). The monoclonal antibodies were prepared and purified from tissue culture supernatants by the National Cell Culture Center (NCCC), Minneapolis, MN. All doses were 1 mg/mouse. Anti-CD4, CD8, and SFR3-DR5 were injected 3 days before challenge. For subsequent depletions, all antibodies given to a group were injected together every 6 days until mice recovered.

Statistical Analysis.

All statistical analysis was done with SigmaStat Software v3.11 (Systat Software, Point Richmond, CA). Weight loss was compared using one-way ANOVA statistical analysis, followed by pairwise multiple comparison using the Holm-Sidak method. Comparison of survival was done using Log-Rank test.

Results

Immunization of mice with M2 DNA + M2 Ad induced serum IgG responses specific for M2e-PR/8 (Fig. 7A). Sera from mice immunized with M2-DNA3x+M2-Ad (circles) had significantly greater reactivity with M2e-PR/8 than did sera from mice immunized with B/NP-DNA3x+B/NP-Ad (diamonds) B/NP-DNA3x+M2-Ad (squares) or M2-DNA3x+B/NP-Ad (triangles) (Fig. 7A).

Immunization with M2 DNA + M2 Ad also induced antibodies cross-reactive with M2e-FM (Fig. 7B) and with M2e-H5 (Fig. 7C). Crossreactivity with M2e-H5, however, was significantly less than with M2e-PR8 or M2e-FM (Fig. 7C).

Testing of sera from animals immunized with KLH, con-M2-KLH (SEQ ID NO:10) M2FM-KLH (SEQ ID NO:12), M2HK-KLH (SEQ ID NO:14); con-M2DNA plus

Ad;B/NP DNA plus Ad for binding to a variety of M2 peptide sequences was conducted. The results are shown in Table 6.

Table 6. Antibody titer (od>0.2) M2e peptides

		Peptide sequence	KLH	con-M2-KLH	M2FM-KLH	M2HK-KLH	con-M2 DNA+Ad	B/NP-DNA+Ad
Con	H1N1	MSLLTEVETPIRNE WGCRCDSSD	80	>20480	>20480	5120	5120	<20
PR/8	H1N1	MSLLTEVETPIRNE WGCRCDSSD	20	5120	5120	1280	1280	<20
FM-MA	H1N1	MSLLTEVETPTKN EWECRCNDSSD	<20	5120	20480	5120	1280	<20
A/HK/156/97	H5N1	MSLLTEVETLTRN GWGCRCDSSD	<20	1280	5120	5120	80	80
A/CK/TH/04	H5N1	MSLLTEVETPTRN EWECRCSDSSD	<20	5120	5120	5120	5120	<20
A/CK/NY/95	H7N2	MSLLTEVETPTRN GWECKCDSSD	<20	20480	20480	5120	320	<20
Con P to L	H1N1	MSLLTEVETLIRNE WGCRCDSSD	<20	320	1280	1280	<20	<20

The results show that immunization of animals with KLH conjugates of con-M2, M2FM, or M2HK resulted in antibodies that react with M2 peptides that differ from the immunizing peptide including M2 peptides from H5N1 and H7N2 strains. Immunization with a DNA construct encoding full length consensus M2 also resulted in antibodies that react not only with the consensus but also with M2 peptides from several other strains including H5N1.

In vivo depletion of T cells during the challenge phase was used to analyze the immune mechanism responsible for M2-DNA induced protection. The protection induced by immunization with M2 DNA+M2 Ad was found to require T cell responses. One hundred percent of mice immunized with M2-DNA+M2-Ad (filled triangles) survived a challenge dose of A/PR/8 (7 LD₅₀) that was lethal to 100% of mice immunized with B/NP DNA +B/N-Ad (filled circles)(Fig. 8a). Depletion of immune cells with monoclonal antibody to CD4 (x's) or CD8 (open triangles) had no effect on survival (Fig. 8a). However, depletion of all CD4/CD8 T cells by administration of monoclonal antibodies to both CD4 and CD8 abrogated protection. Only 20% of mice treated with monoclonal antibodies to CD4 and CD8 (filled squares) survived the challenge dose (Fig. 8a).

To determine whether the immunization protected against a mismatched challenge, mice immunized with M2 DNA 1x + M2 Ad were challenged with A/PR/8 (H1N1, one amino acid difference in the M2e sequence compared to the vaccine constructs) or FM-MA (H1N1, three amino acid differences in the M2e sequence compared to the vaccine constructs) 2 weeks after the adenoviral boost. The A/PR/8 challenge dose (7 LD₅₀) was lethal to about 80% of mice immunized with B/NP-DNA 1x + B/NP-Ad by 11 days post challenge (Fig. 9B), while 100% of the mice immunized with M2 DNA 1x + M2 Ad

survived. The FM-MA challenge dose (10 LD₅₀) was lethal to 100% of the mice immunized with B/NP-DNA 1x + B/NP-Ad by 8 days post challenge (Fig. 9A), while 75 percent of mice immunized with M2 DNA 1x + M2 Ad survived. Since FM-MA has an M2e sequence quite divergent from the immunizing M2 sequence, the protection against FM-MA infection suggested that there might also be protection against H5N1.

Example 4

DNA Prime-Recombinant Adenoviral Boost Immunization to M2 Confers Protection Against Lethal Challenge with Highly Pathogenic H5N1 Virus

To determine whether prime-boost immunization with M2 DNA and M2 Ad protects mice against heterosubtypic challenge with highly pathogenic influenza viruses of subtype A/H5N1, immunized mice were challenged with a sublethal dose of A/HK/156/97 (abbreviated HK/156) or a lethal dose of A/Thailand/SP83/04 (abbreviated SP/83).

Methods

M2DNA and M2 Ad were made as described in Example 3. Plasmid DNA vectors expressing A/NP (A/NP-DNA) were constructed as described in Epstein et al., 2000, Int. Immunol., 12:91-101, and B/NP-DNA as described in Epstein et al., 2002, Emerg. Infect. Dis., 8:796-801.

Adenoviral vectors expressing A/NP (A/NP-Ad) and B/NP (B/NP-Ad) were used as positive and negative controls, respectively, and have been reported previously (Epstein et al., 2005, Vaccine, 23:5404-5410). Viruses were amplified, purified twice through a CsCl gradient, and stored in PBS plus 13% glycerol at -20°C.

HK/156 and SP/83 viruses were propagated in the allantoic cavity of embryonated hen eggs at 37°C for 24 hours. Female BALB/cAnNCR mice were immunized and challenged as described in Example 3.

Virus titers

Influenza virus titers were quantitated from lungs of the challenged mice. Lungs from challenged mice were harvested, homogenized in 1 ml of sterile PBS, clarified by centrifugation, and titrated for virus infectivity by egg infectious dose (EID₅₀) assay as previously described in Epstein et al., 2002, Emerg. Infect. Dis., 8:796-801. Briefly, lung homogenates were titrated in 10-day-old embryonated eggs in 10-fold steps from initial dilutions of 1:10, and positive eggs identified by hemagglutination using allantoic fluid. Values are expressed as log₁₀ EID₅₀/ml ± SEM. The limit of virus detection is 1.2 log₁₀ EID₅₀/ml.

ELISPOT

Enzyme-linked immunospot assays (ELISPOT) for IFN- γ secreting cells were performed on splenocytes harvested from non-immunized mice previously challenged with A/PR/8 as described above (PR/8 immune) or influenza naïve mice immunized with M2-DNA (3 times)+M2-Ad. The ELISPOT assay was performed as described in Sambhara et al., 1998, Cell. Immuno., 187:13-18. Briefly, spleens were removed from the mice after 6.5 months, gently homogenized to a single-cell suspension, erythrocytes were lysed, and then the splenocytes were washed with medium and stimulated at 37°C throughout the culture period with peptide pools. Peptides used were 18-mers overlapping by 12 amino acids spanning the complete amino acid sequence of M2-A/PR/8. See Table 7. Elispot assays were also conducted with M2-1, M2-2, and NP 147.

TABLE 7

M2 Peptide		SEQ ID NO:
M2-1	MSLLTEVETPIRNEWGCR	25
M2-2	VETPIRNEWGCRCNGSSD	26
M2-3	NEWGCRCNGSSDPLAIAA	27
M2-4	CNGSSDPLAIAANIIGIL	28
M2-5	PLAIAANIIGILHLTLWI	29
M2-6	NIIGILHLTLWLDRLFFK	30
M2-7	HLTLWLDRLFFKCIYRRF	31
M2-8	LDRLFFKCIYRRFKYGLKG	32
M2-9	KCIYRRFKYGLKGGPSTEG	33
M2-10	FKYGLKGGPSTEGVPKSMR	34
M2-11	GGPSTEGVPKSMREEYRKE	35
M2-12	GVPKSMREEYRKEQQSAVD	36
M2-13	REEYRKEQQSAVDADDGHF	37
M2-14	EQQSAVDADDGHFVSIELE	38

Millipore ELISPOT IP plates were coated with 50 μ l of HBSS containing 5 μ g/ml of anti-IFN γ monoclonal antibody AN18 overnight at 4°C. After washing, the membrane was blocked with medium containing 10% FBS for 60-90 minutes at room temperature. Spleens were aseptically removed from euthanized mice, a single cell suspension prepared, and red blood cells lysed. Two dilutions (2-fold) of splenocytes were added to wells starting at 250,000 cells/well in a volume of 50 μ l. Indicated peptides were added at a final concentration of 1 μ g/ml. After incubation for 48 hours at 37°C, bound IFN γ was detected by the addition of 50 μ l of biotinylated monoclonal antibody R4-6A2 at 1 μ g/ml. Spots were developed using alkaline phosphatase-labeled streptavidin and BCIP/NBT substrate. Counts were obtained using an AID ELISpot plate reader.

Statistical Analysis

Lung virus titers were compared using one-way ANOVA statistical analysis on log-transformed data, followed by pairwise multiple comparison using the Holm-Sidak method. Weight loss was compared using one-way ANOVA statistical analysis, followed by pairwise multiple comparison using the Holm-Sidak method. Comparison of survival was done using Log-Rank test. All statistical analysis was done with SigmaStat Software v3.11 (Systat Software, Point Richmond, CA).

Results

Mice prime-boost immunized with M2 DNA 3X + M2 Ad or A/NP-A/PR/8 DNA 3X + A/NP-A/PR/8 Ad were given a sublethal challenge dose of HK/156 (four amino acid differences compared to the M2e sequence of the vaccine constructs). Mice immunized with M2 DNA+M2 Ad (squares) or A/NP-A/PR/8 DNA+A/NP-A/PR/8 Ad (diamonds) had only minor weight loss after challenge with A/HK/156 and rapidly recovered (Fig. 10A), while those given B/NP DNA 1x + B/NP-Ad (triangles) lost approximately 15% of their body and regained the weight as they recovered (Fig. 10A).

Additional groups of mice were given a lethal challenge dose of A/Thailand/SP83/04(SP-83) (three amino acid differences compared to the M2e sequence of the vaccine constructs). Eighty percent of mice prime-boost immunized with M2 DNA and M2 Ad (triangles) and 100% of mice immunized with A/NP-A/PR/8 DNA+A/NP-A/PR/8 Ad (squares) survived challenge with a dose of SP-83 lethal to 100% of the control mice (mice immunized with B/NP DNA + B/NP-Ad; circles) by 11 days post challenge (Fig. 11A). Mice immunized with M2 DNA+M2-Ad (squares) or A/NP-DNA+A/NP-Ad (diamonds) lost weight, but regained the weight as they recovered (Fig. 10B). Control mice (triangles) lost approximately 25% of their weight and all eventually succumbed to SP-83 infection (Fig. 10B). M2 DNA+M2-Ad prime-boost immunization significantly reduced SP83 virus titers in the lungs compared to B/NP DNA + B/NP-Ad controls (Fig. 11B), as did A/NP-DNA+A/NP-Ad.

Antibody reactivity in sera from another group of mice prime-boost immunized with M2-DNA+M2-Ad gave significantly less binding to M2e-H5 than to M2e-PR8 or M2e-FM (Fig. 7). Nevertheless, 80% of mice prime-boost immunized with M2-DNA+M2-Ad survived a lethal challenge dose of highly pathogenic SP83 (Fig. 11A) and had significantly reduced SP83 virus titers in the lungs compared to B/NP DNA + B/NP-Ad controls (Fig. 11B).

Prime-boost immunization with M2-DNA+M2-Ad activated T cells in an antigen-specific manner (Fig. 12). Splenocytes from A/PR/8-immune mice and mice immunized with M2-DNA+M2-Ad generated an IFN- γ response when stimulated with peptide M2-1 or peptide M2-2. A greater number of T-cells were activated by the M2 peptides in the cells

from the prime-boost immunized mice than the PR/8-immune mice (Fig. 12). Peptide NP147-155 (NP147), the dominant epitope recognized by CD8⁺ T cells of BALB/c mice, was used as a control. Results with NP147 show that the cells from the A/PR/8-immune mice are well immunized and highly responsive, though they respond poorly to M2 peptides. Results with NP147 also demonstrate the specificity of the response in the M2-DNA+M2-Ad immune mice, since their cells do not respond to the NP peptide.

Spleen cells were isolated from mice immunized with M2-DNA+M2-Ad immune mice 5 months after Ad boost, fractionated into T cell and non-T cell populations and assayed for IFN γ producing cells by ELISPOT assay as described in Methods. The results are shown in Figure 8B. Responses of intact spleen cells and isolated T cells to an M2 peptide having the amino acids sequence of residues 2-24 (PR/8) were observed.

The Elispot results for the peptide scan using the peptides of Table 7 are shown in Figure 13. Splenocytes from A/PR/8-immune mice and mice immunized with M2-DNA+M2-Ad generated an IFN- γ response when stimulated with a peptide pool comprising peptides 1 and 2 that correspond to the extracellular domain. A smaller response was also seen in both animals to a pool of peptides 7 and 8.

Serum collected from immune mice was passively transferred into naïve BALB/c mice i.p. (n=8 per group). The recipients were challenged with 10 LD₅₀ of A/PR/8 and monitored for survival (Figure 8c) and weight loss (Figure 8d). Survival of mice given A/PR/8 immune serum, M2-DNA+M2-Ad-immune serum or M2e-H5(HK)/KLH-immune serum was significantly better than of mice given B/NP-DNA+B/NP-Ad-immune serum ($p<0.001$, log rank). For weight loss, $p<0.003$ at day 8 and day 10, M2 prime-boost differs from B/NP prime-boost.

These results indicate that immune T cells are required for protection against high virus challenge dose (T cell depletion data), but that serum antibody is sufficient for protection against challenge with a lower virus dose (passive serum transfer data).

It should be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. It should also be noted that the term "or" is generally employed in its sense including "and/or" unless the content clearly dictates otherwise.

All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this disclosure pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference.

The disclosure has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the disclosure.

WHAT IS CLAIMED IS:

1. An immunogenic composition comprising at least one expression vector comprising at least one polynucleotide encoding an M2 polypeptide comprising the amino acid sequence of
MSLLTEVETX₁₀X₁₁X₁₂NX₁₄WX₁₆CRCX₂₀X₂₁SSD (SEQ ID NO:45),
wherein X₁₀, X₁₁, X₁₂, X₁₄, X₁₆, X₂₀, and X₂₁ is any amino acid; in combination with a carrier.
2. The immunogenic composition of claim 1, wherein X₁₀ is P or L; X₁₁ is I or T; X₁₂ is R or K; X₁₄ is E or G; X₁₆ is G or E; X₂₀ is N, S or R; and X₂₁ is G or D; in combination with a carrier.
3. The immunogenic composition of claim 1, wherein the M2 polypeptide comprises the amino acid sequence selected from the group consisting of SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:42, and SEQ ID NO:43.
4. The immunogenic composition of any of claims 1 to 3, wherein the M2 polypeptide comprises the amino acid sequence of SEQ ID NO:10.
5. An immunogenic composition comprising an expression vector comprising at least one polynucleotide encoding an M2 polypeptide comprising the amino acid sequence of VETX₄X₅RNX₈WX₁₀CX₁₂ (SEQ ID NO:19), LTEVETX₇X₈ (SEQ ID NO:48), LTEVETPX₈ (SEQ ID NO:46), or VETPX₅X₆NX₈W (SEQ ID NO:47), wherein X is any amino acid.
6. The immunogenic composition of claim 5, wherein the M2 polypeptide comprises the amino acid sequence of VETX₄X₅RNX₈WX₁₀CX₁₂, wherein X₄ is P or L, X₅ is E or G; X₈ is E or G; X₁₀ is G or E; and X₁₂ is N, S or R.
7. The immunogenic composition of claim 5, wherein the M2 polypeptide comprises the amino acid sequence of LTEVETX₇X₈ (SEQ ID NO:48), and wherein X₇ is P or L, and X₈ is E or G.

8. The immunogenic composition of claim 5, wherein the M2 polypeptide comprising the amino acid sequence of LTEVETPX₈ (SEQ ID NO:46), and wherein X₈ is E or G.
9. The immunogenic composition of claim 5, wherein the M2 polypeptide comprises the amino acid sequence of VETPX₅X₆NX₈W (SEQ ID NO:47), and wherein X₅ is E or G; X₆ is R or K, X₈ is E or G.
10. An immunogenic composition comprising an expression vector comprising at least one polynucleotide encoding an M2 polypeptide having at least 80% amino acid sequence identity to the polypeptide comprising the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:10 in combination with a carrier.
11. The immunogenic composition of any of claims 1 to 10, wherein the polynucleotide comprises a promoter.
12. The immunogenic composition of any of claims 1 to 10, wherein the expression vector is a plasmid or a viral expression vector.
13. The immunogenic composition of claim 12, wherein the plasmid comprises pVR1012.
14. The immunogenic composition of any of claims 1 to 13, wherein the M2 polypeptide is from an A/H1N1 isolate or strain or A/H5N1 isolate or strain.
15. The immunogenic composition of claim 14, wherein the M2 polypeptide is from A/PR/8/34 isolate.
16. The immunogenic composition of any of claims 1 to 13, wherein the M2 polypeptide comprises an amino acid sequence of SEQ ID NO:1.
17. The immunogenic composition of any of claim 1 to 16, wherein a first immunogenic composition comprises a plasmid vector comprising a polynucleotide encoding an M2 polypeptide, and a second immunogenic composition comprises a viral vector comprising a polynucleotide encoding an M2 polypeptide, wherein the first and second immunogenic composition encode the same M2 polypeptide or different M2 polypeptides.

18. The immunogenic composition of any of claims 1 to 16, comprising a plurality of polynucleotides encoding at least two M2 polypeptides, wherein each polynucleotide encodes an M2 polypeptide from a different influenza A subtype.

19. The immunogenic composition of any of claims 1 to 18, wherein a third immunogenic composition comprises an M2 polypeptide composition, wherein the M2 polypeptide has at least 80% amino acid sequence identity to SEQ ID NO:1 or SEQ IDNO:10.

20. The immunogenic composition of any of claims 1 to 19, further comprising at least one adjuvant or immunomodulator.

21. The immunogenic composition of claim 20, wherein the adjuvant or immunomodulator is encoded by the polynucleotide.

22. The immunogenic composition of claim 20, wherein the adjuvant is CTA1-DD alone or in combination with ISCOM.

23. A method for inhibiting influenza A infection in a subject, comprising administering to the subject an immunogenic composition of any of claims 1 to 22, and boosting the subject with a second expression vector comprising at least one polynucleotide encoding an M2 polypeptide having at least 80% amino acid sequence identity to SEQ ID NO:1 or SEQ IDNO:10.

24. The method of claim 23, wherein the immunogenic composition comprises a plasmid expression vector and the second expression vector is a viral expression vector.

25. The method of claims 23 or 24, wherein the immunogenic composition and the second expression vector each comprise a polynucleotide encoding M2 polypeptide from A/H1N1 or A/H5N1.

26. The method of claim 25, wherein the viral vector comprises a polynucleotide encoding M2 polypeptide from A/PR/8/34.

27. The method of claim 24 or 25, wherein the plasmid expression vector and the viral vector each comprise a polynucleotide encoding an M2 polypeptide having an amino acid sequence of SEQ ID NO:1.

28. The method of claim 24, wherein the plasmid expression vector and the viral vector each comprise a plurality of polynucleotides encoding at least two M2 polypeptides, wherein each polynucleotide encodes an M2 polypeptide from a different influenza A subtype.

29. The method of any of claims 23 to 28, wherein the viral vector is an adenovirus.

30. The method of any of claims 23 to 29, wherein the M2 polypeptide encoded by the plasmid expression vector and the viral vector have the same amino acid sequence.

31. The method of claim 30, wherein the M2 polypeptide has the sequence of A/H1N1 or A/H5N1 isolate.

32. The method of any of claim 23 to 31, wherein the administration of the immunogenic composition and viral vector provides protective immunity against a homologous, heterosubtypic, or mismatched influenza virus isolate or strain.

33. The method of claim 32, wherein the M2 polypeptide of the immunogenic composition and the viral vector has the same sequence as M2 protein from a H1N1 isolate or strain and the heterosubtypic influenza is A/H5N1.

34. The method of any of claims 23 to 33, wherein the subject is a domestic bird, ferret, wild bird, or mammal.

35. The method of any of claims 23 to 33, wherein the mammal is a human.

36. A method for inhibiting influenza A infection in a subject, comprising administering to the subject an immunogenic composition of any of claims 1 to 18, and boosting the subject with an M2 polypeptide composition, wherein the M2 polypeptide has at least 80% amino acid sequence identity to SEQ ID NO:1 or SEQ IDNO:10.

37. The method of claim 36, wherein the immunogenic composition comprises a plasmid expression vector or viral expression vector.

38. The method of claims 36 or 37, wherein the immunogenic composition encodes an M2 polypeptide from A/H1N1 or A/H5N1.

39. The method of claim 37 or 38, wherein the viral vector comprises a polynucleotide encoding M2 polypeptide from A/PR/8/34.

40. The method of any of claims 36 to 39, wherein the expression vector comprise a polynucleotide encoding an M2 polypeptide having an amino acid sequence of SEQ ID NO:1 and the M2 polypeptide composition comprises an M2 polypeptide having the amino acid sequence of SEQ ID NO:1.

41. The method of any of claim 36 to 38, wherein the expression vector comprises a plurality of polynucleotides encoding at least two M2 polypeptides, wherein each polynucleotide encodes an M2 polypeptide from a different influenza A subtype.

42. The method of any of claim 36 to 39, wherein the M2 polypeptide composition comprises at least two M2 polypeptides, wherein each polypeptide is an M2 polypeptide from a different influenza A subtype.

43. The method of any of claims 36 to 42, wherein the viral vector is an adenovirus.

44. The method of claim 43, wherein the adenovirus is adenovirus type 5.

45. The method of any of claims 36 to 44, wherein the M2 polypeptide encoded by the expression vector and the M2 polypeptide in the M2 composition have the same amino acid sequence.

46. The method of claim 45, wherein the M2 polypeptide has the sequence of A/H1N1 or A/H5N1 isolate.

47. The method of any of claims 36 to 46, wherein the administration of the immunogenic composition and M2 polypeptide composition provides protective immunity against a homologous, heterosubtypic, or mismatched influenza virus isolate or strain.

48. The method of claim 47, wherein the M2 polypeptide of the immunogenic composition and M2 polypeptide composition has the same sequence as an M2 protein from a H1N1 isolate or strain and the heterosubtypic influenza is A/H5N1.

49. The method of any of claims 36 to 48, wherein the subject is a domestic bird, ferret, wild bird, or mammal.

50. The method of claim 49, wherein the mammal is a human.

51. The method of any of claims 23 to 50, further comprising administering at least one adjuvant or immunomodulator.

52. The method of claim 51, wherein the adjuvant or immunomodulator is encoded by a polynucleotide.

53. The method of claim 51 or 52, wherein the adjuvant is CTA1-DD alone or in combination with ISCOM.

54. A kit, comprising:

(a) an immunogenic composition comprising a plasmid expression vector, comprising at least one polynucleotide encoding a M2 polypeptide having at least 80% amino acid sequence identity to a polypeptide comprising the amino acid sequence of SEQ ID NO:1 or SEQ IDNO:10;

(b) a viral vector comprising at least one polynucleotide encoding an M2 polypeptide having at least 80% amino acid sequence identity to a polypeptide comprising the amino acid sequence of SEQ ID NO:1 or SEQ IDNO:10; and optionally

(c) instructions for administering to an animal the immunogenic composition and boosting the animal with the viral vector.

55. The kit of claim 54, wherein the plasmid expression vector and the viral vector each comprise a polynucleotide encoding M2 polypeptide from A/H1N1 or A/H5N1.

56. The kit of claim 55, wherein the viral vector comprises a polynucleotide encoding M2 polypeptide from A/PR/8/34.

57. The kit of any of claims 54 to 56, wherein the plasmid expression vector and the viral vector each comprise a polynucleotide encoding an M2 polypeptide having an amino acid sequence of SEQ ID NO:1.

58. The kit of any of claims 54 to 57, wherein the plasmid expression vector and the viral vector each comprise a plurality of polynucleotides encoding at least two M2

polypeptides, wherein each polynucleotide encodes an M2 polypeptide from a different influenza A subtype.

- 59. The kit of any of claims 54 to 58, wherein the viral vector is an adenovirus.
- 60. The kit of claim 59, wherein the adenovirus is adenovirus type 5.
- 61. The kit of any of claims 54 to 60, wherein the M2 polypeptide encoded by the plasmid expression vector and the viral vector have the same amino acid sequence.
- 62. The kit of any of claim 54 to 61, further comprising an M2 polypeptide composition.
- 63. The kit of claim 62, wherein the M2 polypeptide of the M2 polypeptide composition and the M2 polypeptide encoded by the plasmid and viral expression vector have the same sequence.
- 64. The kit of any of claims 54 to 63, further comprising administering at least one adjuvant or immunomodulator.
- 65. The kit of claim 64, wherein the adjuvant or immunomodulator is encoded by a polynucleotide.
- 66. The kit of claim 64 or 65, wherein the adjuvant is CTA1-DD alone or in combination with ISCOM.
- 67. Use of an immunogenic composition of any of claims 1 to 18 in the preparation of a medicament for inhibiting influenza infection in a subject.
- 68. The use of claim 67, wherein the composition comprises a plasmid or a viral expression vector comprising at least one polynucleotide encoding an M2 polypeptide having at least 80% amino acid sequence identity to the polypeptide comprising the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:10.
- 69. The use of claims 67 or 68, wherein the polynucleotide encodes an M2 polypeptide having the sequence of SEQ ID NO:1 or SEQ ID NO:10.

70. The use of any of claims 67 to 69, wherein the immunogenic composition further comprises an M2 polypeptide composition.

71. The use of claim 70, wherein the M2 polypeptide of the M2 polypeptide composition and the M2 polypeptide encoded by the plasmid and viral expression vector have the same sequence.

72. The use of any of claims 67 to 71, wherein the immunogenic composition comprises at least one adjuvant or immunomodulator.

73. The use of claim 72, wherein the adjuvant or immunomodulator is encoded by a polynucleotide.

74. The use of claim 72 or 73, wherein the adjuvant is CTA1-DD alone or in combination with ISCOM.

75. The use of any of claims 67 to 74, wherein the subject is a domestic bird, wild bird, or mammal.

76. The use of any of claims 67 to 74, wherein the subject is a human.

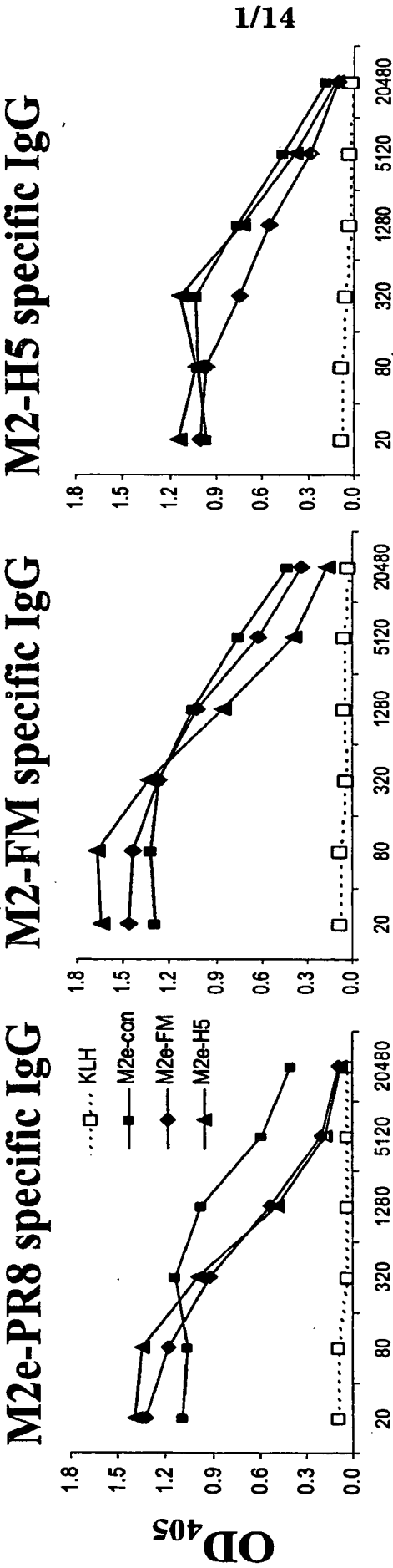
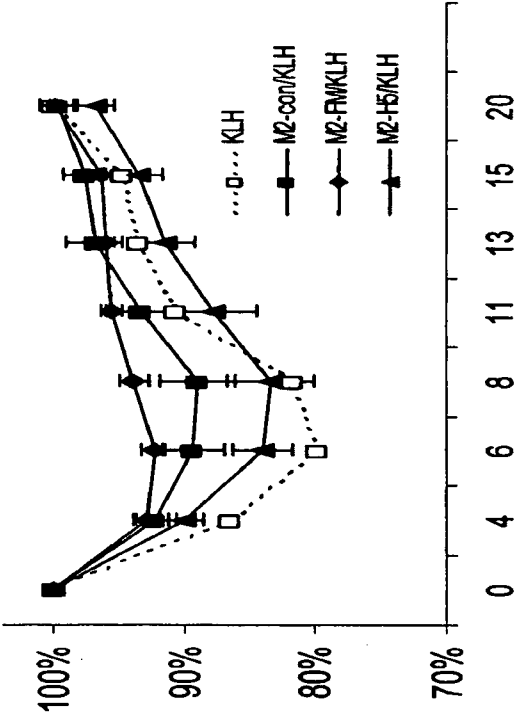


Fig. 1A

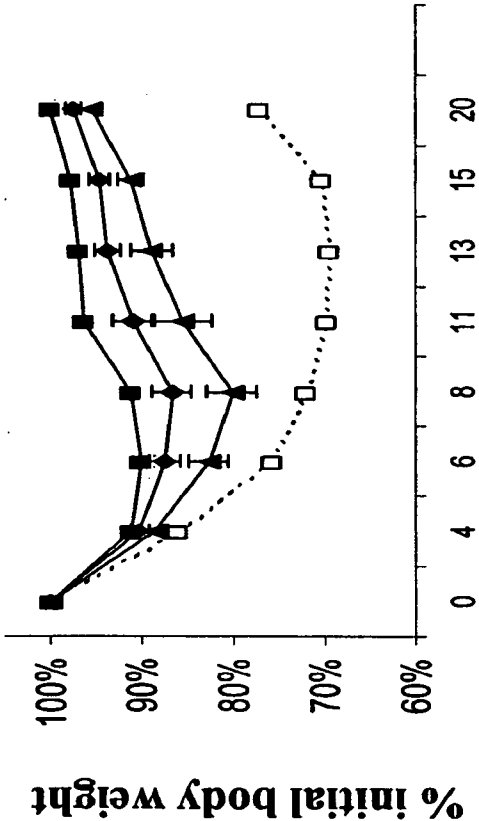
Fig. 1B

Fig. 1C

A/FM-MA



A/PR/8



Day post-challenge

Fig. 2B

Fig. 2A

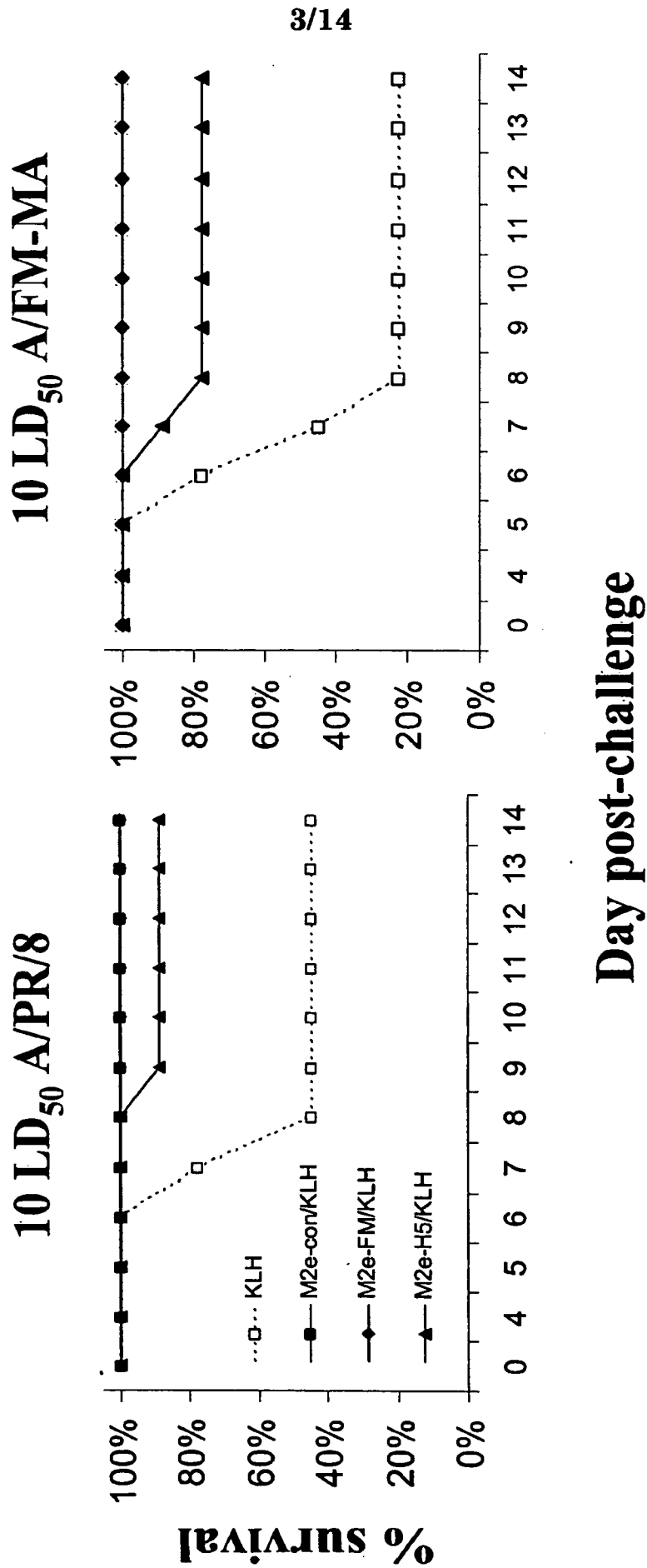


Fig. 3B

Fig. 3A

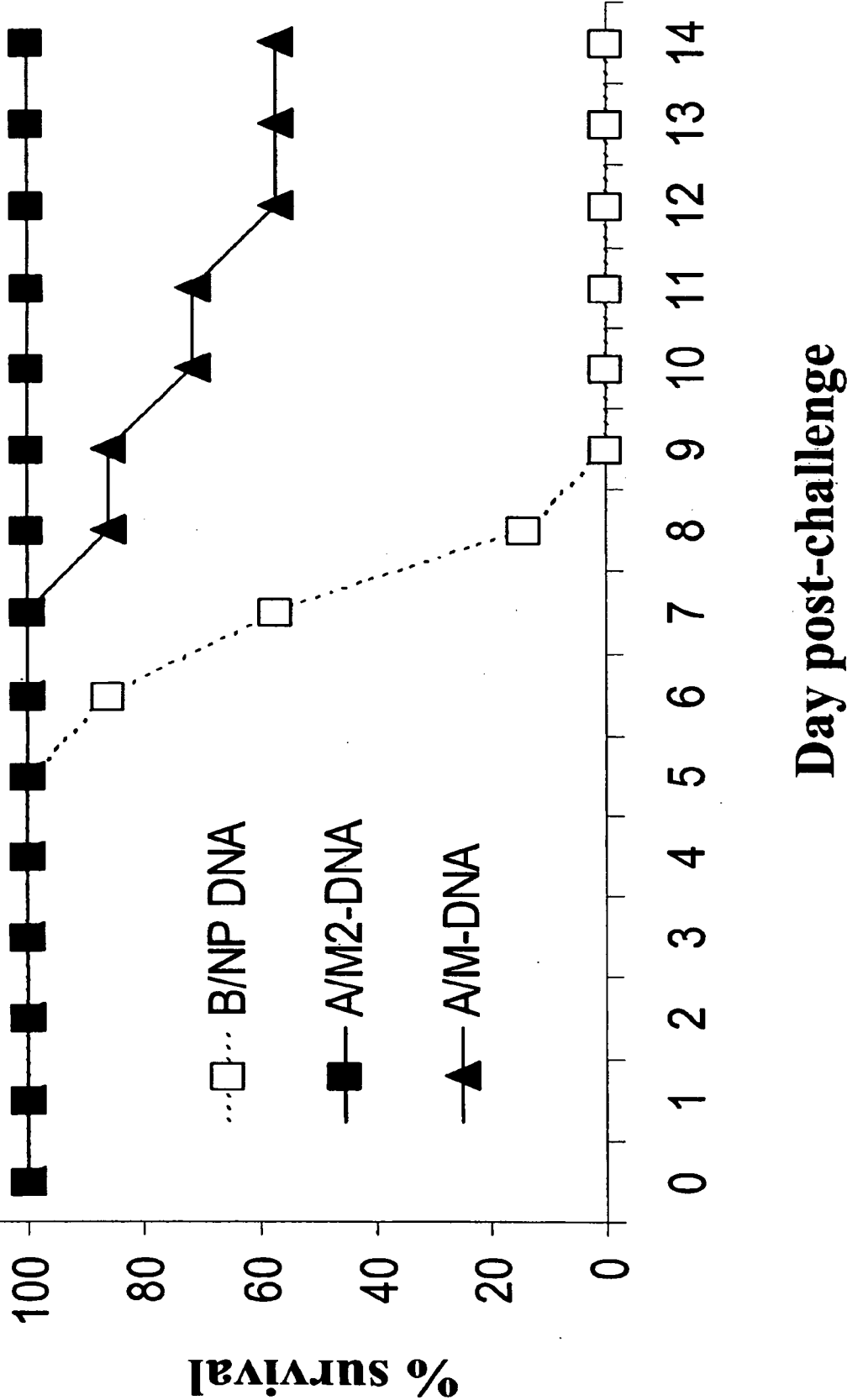


Fig. 4

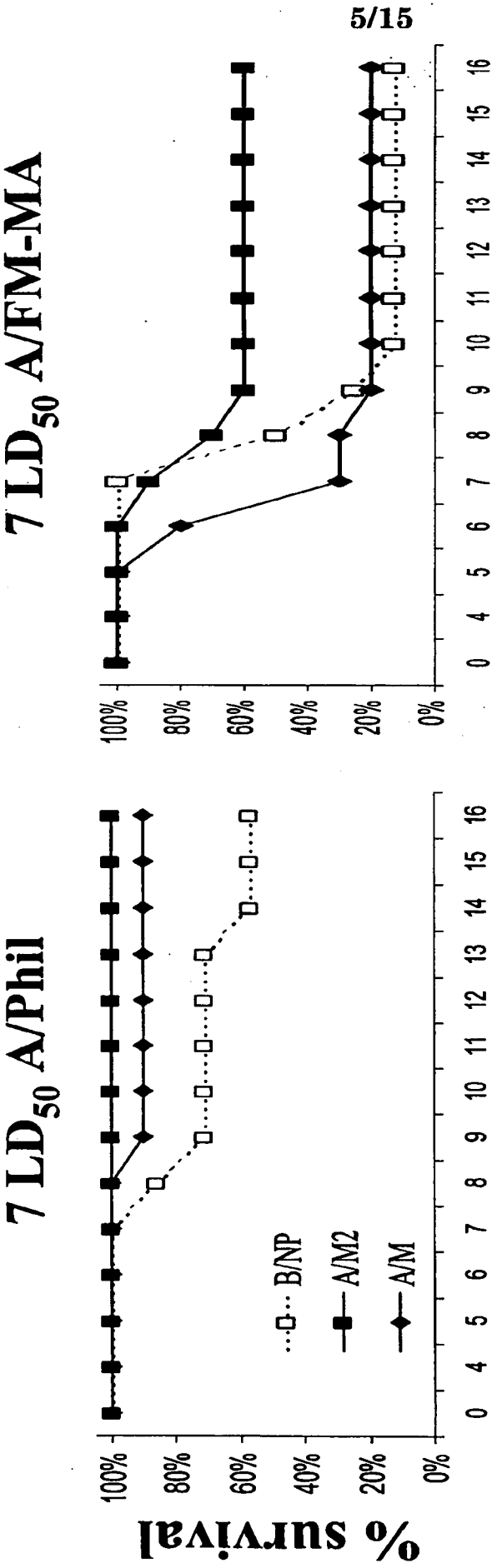


Fig. 5A

Fig. 5B

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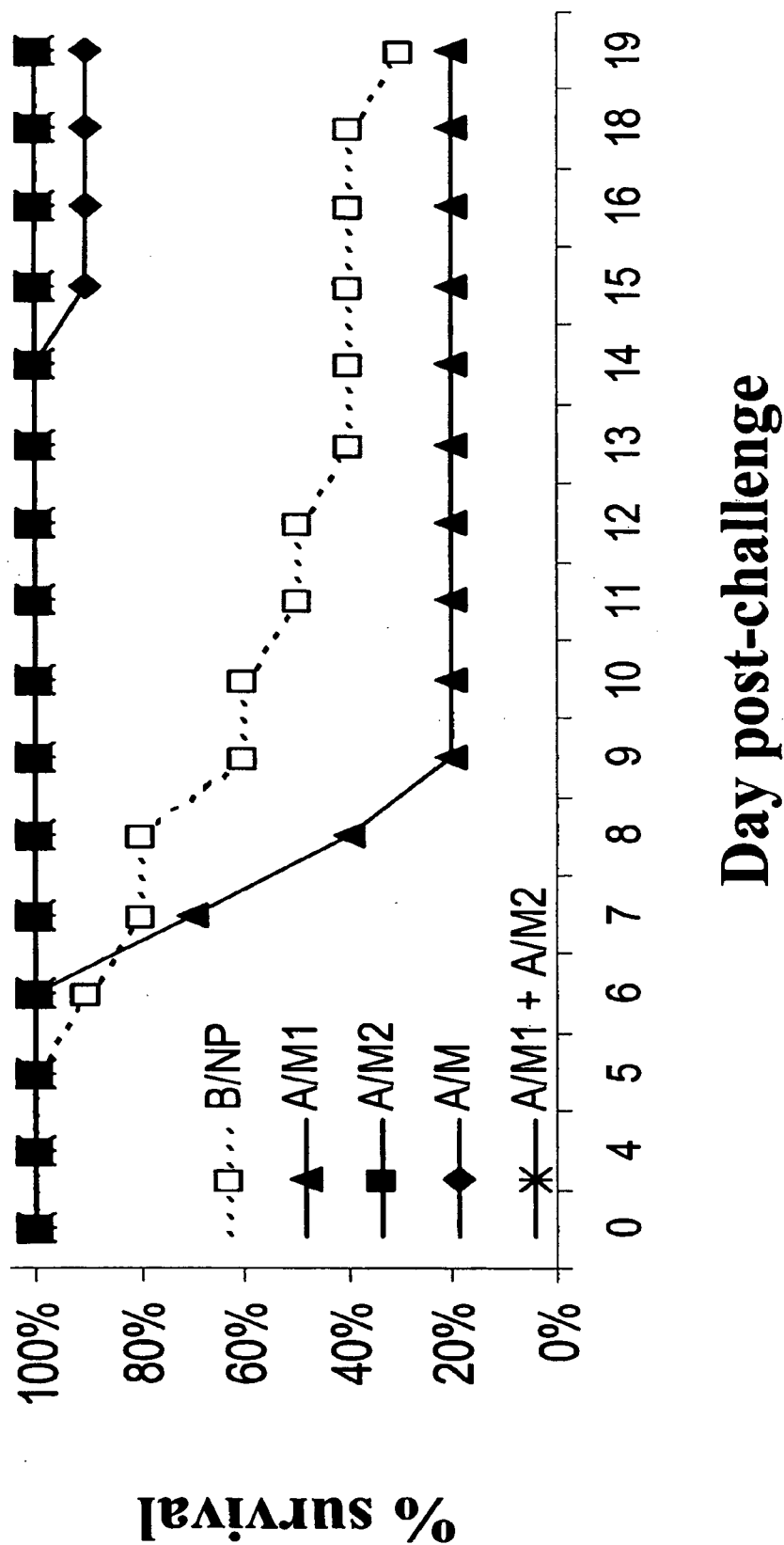
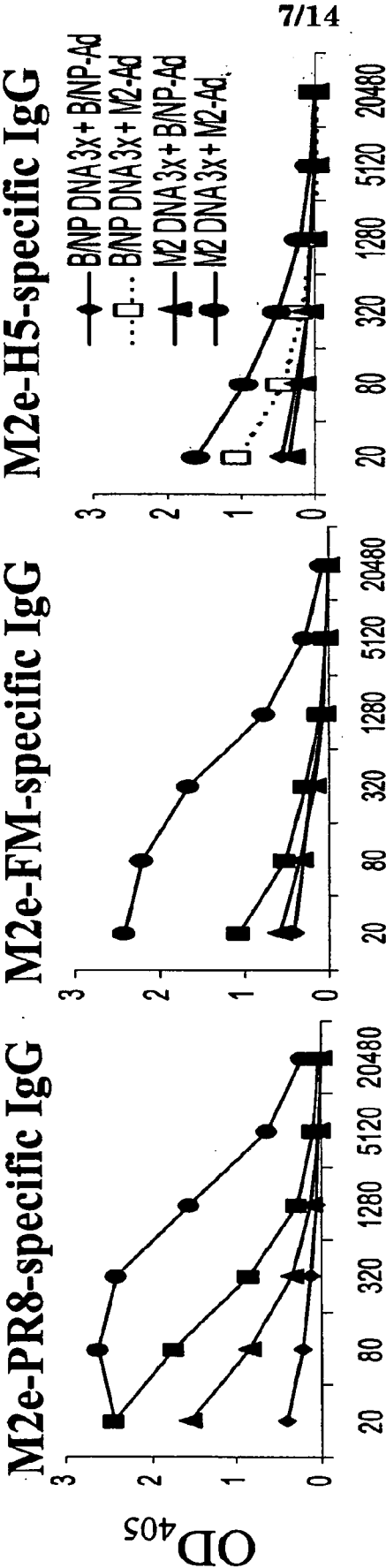


Fig. 6



Serum Dilution ⁻¹

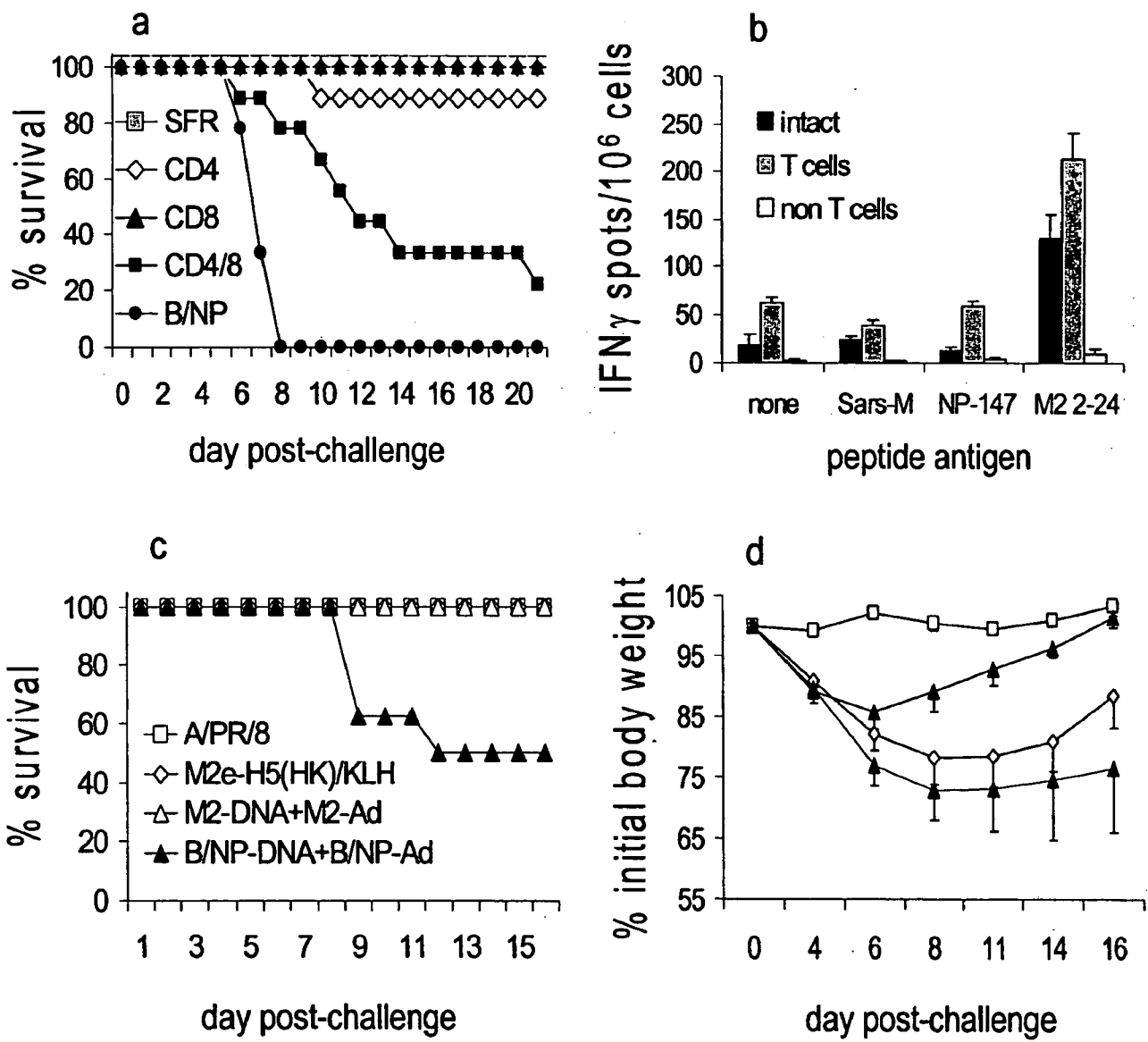
Fig. 7A

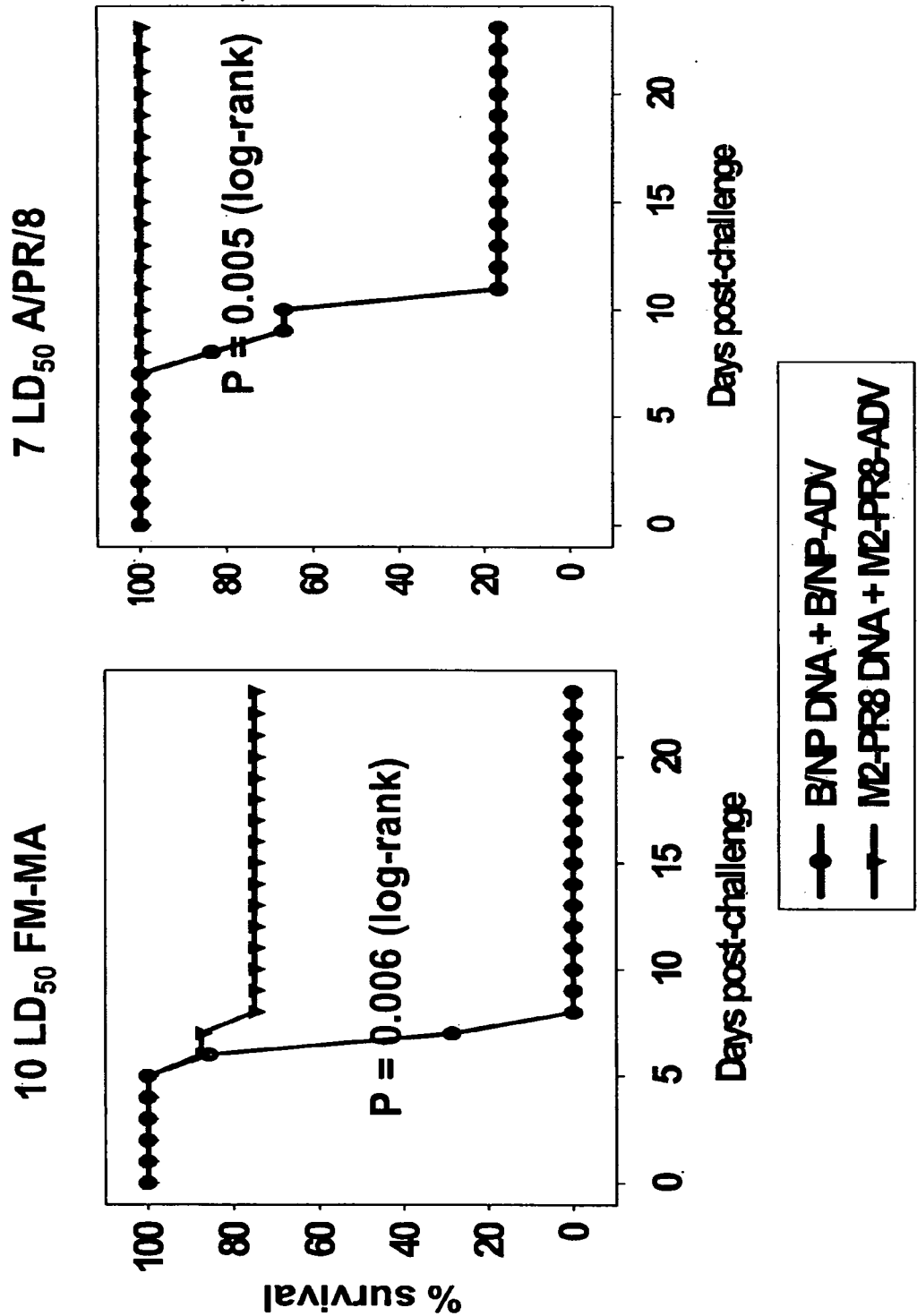
Fig. 7B

Fig. 7C

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Fig. 8





1x DNA + ADV boost, PR/8 sequence of M2

Fig. 9A

Fig. 9B

HK/156

SP/83

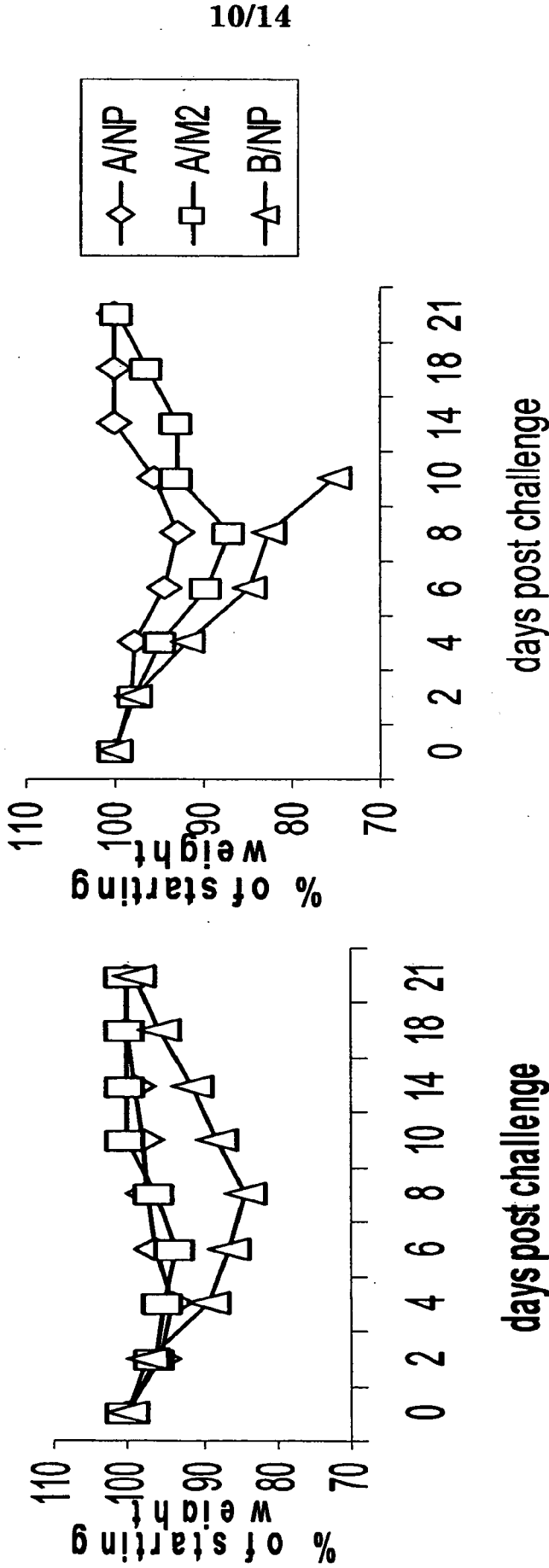


Fig. 10A

Fig. 10B

Challenge with A/Thailand/SP83/04

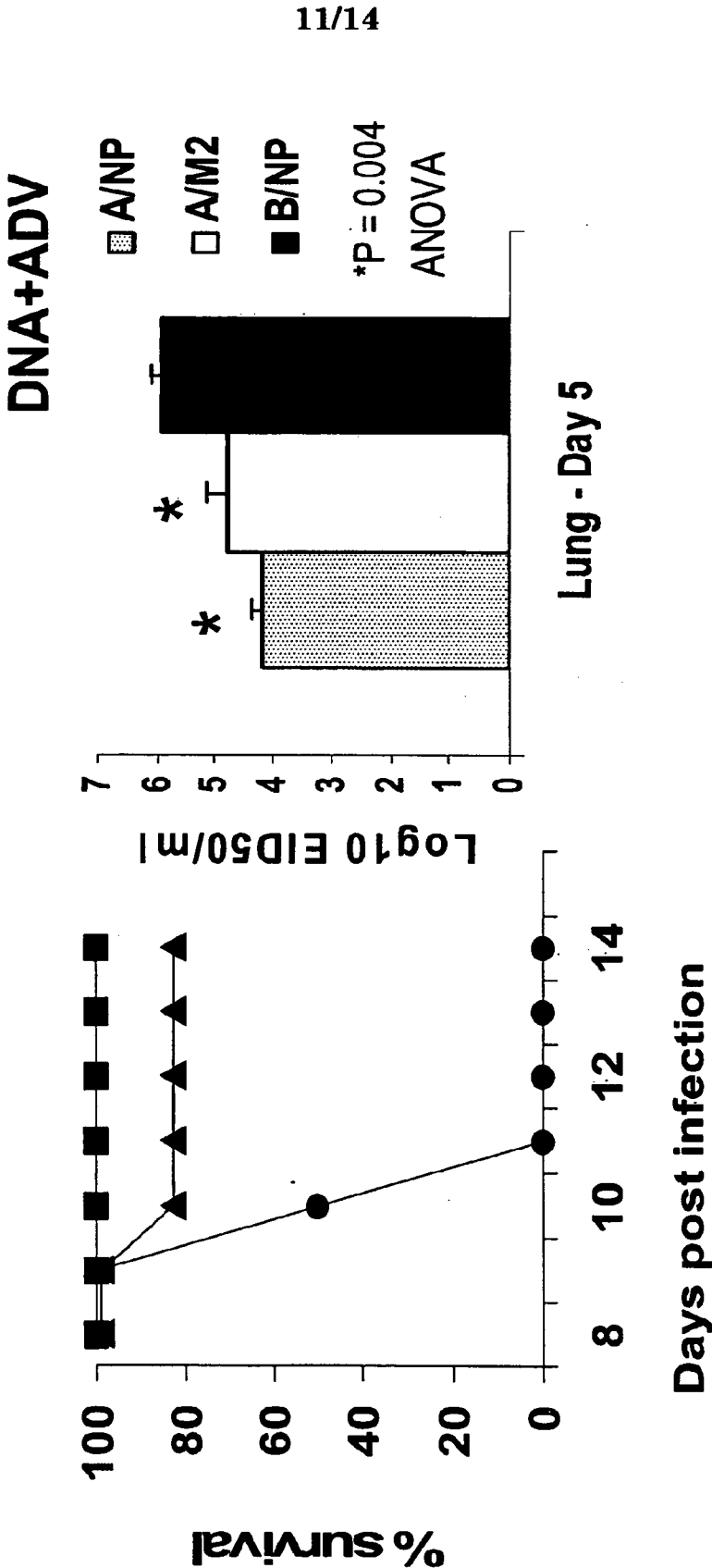


Fig. 11A

Fig. 11B

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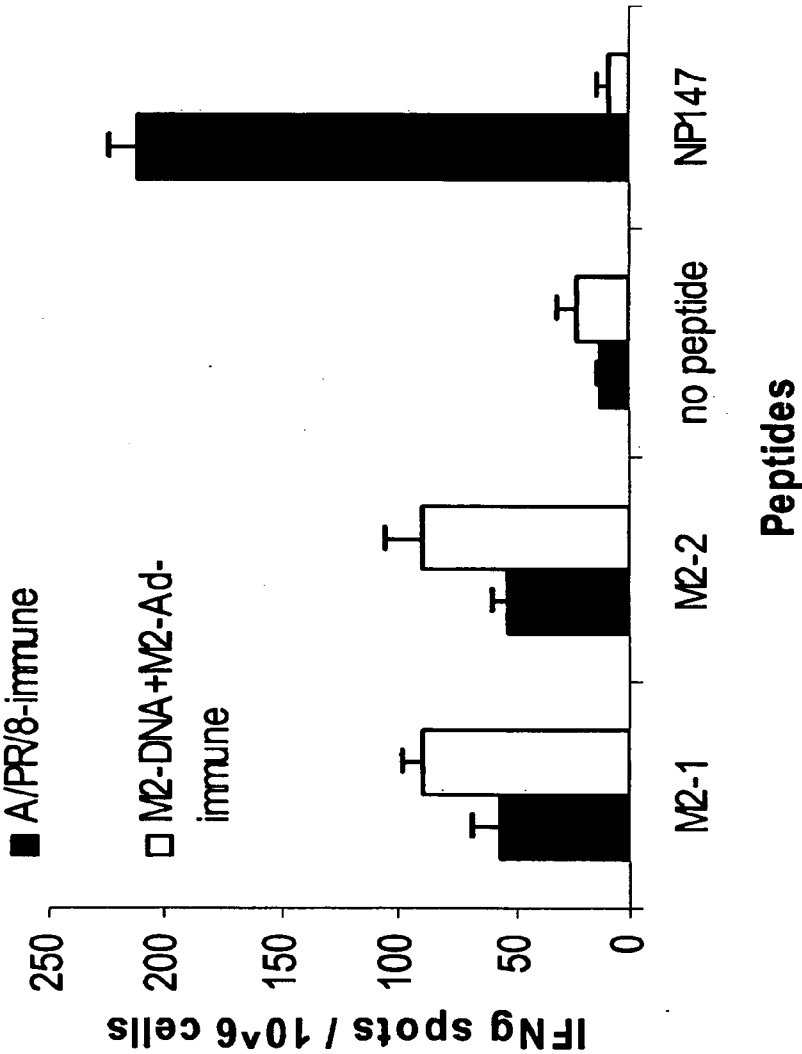
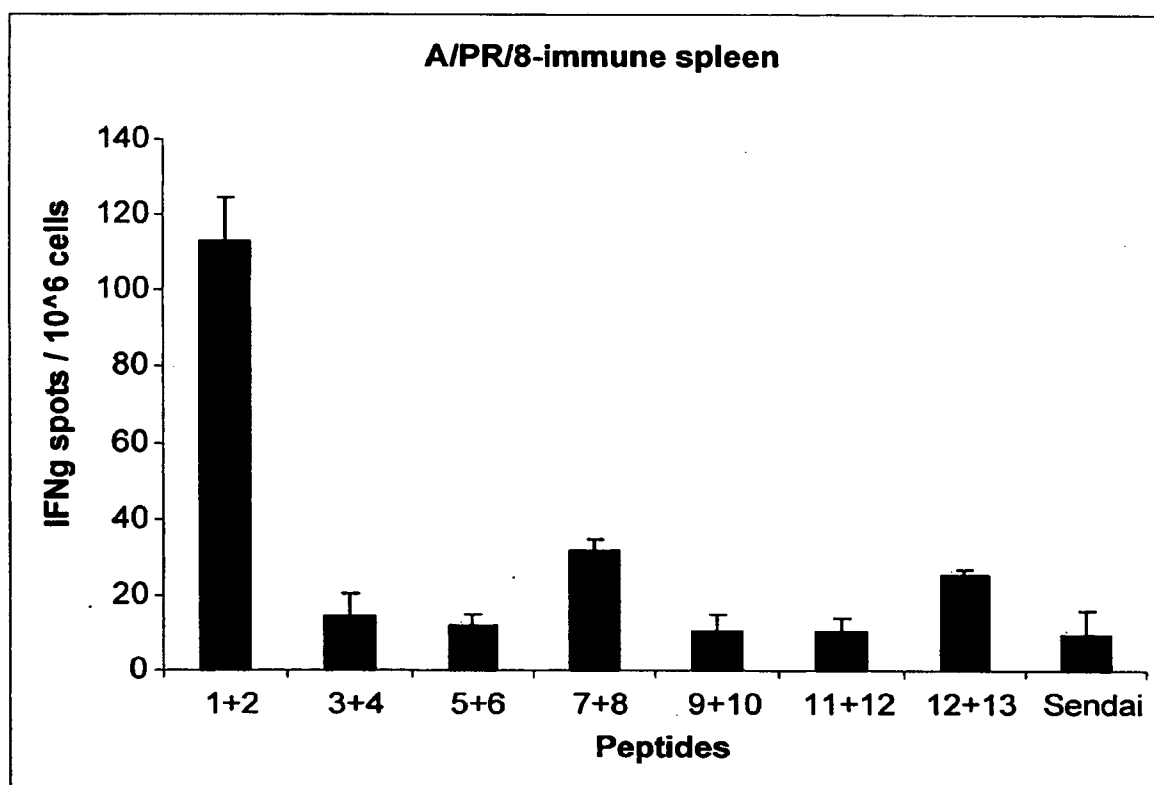


Fig. 12

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Fig. 13A



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Fig. 13B

